A Simple Way to Generate Cell-Specific Knockdown Mice by Double Transfection of Cre/LoxP Lentiviral Vector

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A Simple Way to Generate Cell-Specific Knockdown Mice by Double Transfection of Cre/LoxP Lentiviral Vector

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ABSTRACT

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Background: In case of eternal gene manipulation, there can be limits in figuring out functions of genes that are temporarily activated at diseases. To overcome such problems, cell-specific and tissue-specific gene technology was recently developed and has been greatly helpful for understanding functions of genes of persons suffering from various diseases. It is one of organism-specific gene expression manipulation systems widely utilized for conditional gene knockout in selective cell or tissue by Cre, a site-specific recombinase. To develop gene manipulation animals, Cre genetically modified animals are

established through fertilization and crossbreeding after cell-specific Cre genes are injected to embryonic stem cells. However, there are limits to use it in terms of time and money. On the other hand, lentivirus-based gene delivery system, gene has incorporated into host chromosomes and continuously expressed for a long time, therefore, genes can be effectively conveyed to already differentiated and non-proliferating cells such as nerve cells.

Purpose: In this study, I invented a simple way to knockdown a specific gene in a cell-specific pattern in adult mice by lentivirus-assisted transfer of shRNA.

Methods: In vitro, for lentiviral transfection, 4×10^5 TU of the lentivirus suspension containing LV-Hoxb7 Cre and/or LV-shAQP3 was added to cultured Mouse renal collecting duct cells and mouse mesangial cells. After 48 hr, the media were changed to routine culture media. At 72 hr after the media change, cells were harvested. In vivo experiments, there were three models to check efficiency of each virus. First, LV-Hoxb7 Cre is injected into the loxP-EGFP mice to check efficiency of the Hoxb7 promoter. These mice were divided into two groups, and were injected with PBS (Con) or LV-Hoxb7 Cre. For the second animal experiments, Hoxb7 Cre transgenic mice were used. They were classified into three groups based on what was injected with PBS (Con), LV-loxP scr, or LV-loxP shAQP3. For the third animal experiments, male

C57BL6/J mice were assigned to one of the four groups, and were injected with PBS (Con), LV-Hoxb7 Cre, LV-loxP shAQP3, or LV-Hoxb7 Cre + LV-loxP shAQP3. All animals were given the selected treatment on days 0, 4, and 7 via hydrodynamic tail vein injection as described previously. For C57BL6/J mice treated with consecutive injection of LV-Hoxb7 Cre and LV-loxP shAQP3, LV-shAQP3 was introduced on days 3, 7, and 10 after the final injection of LV-Hoxb7 Cre. Briefly, a volume of 1 ml of PBS alone or PBS containing 4×10^8 TU of lentivirus were injected to mice via the tail vein within 10 sec. Mice were sacrificed at 6-week after the first injection of PBS or lentivirus. EGFP, mCherry, and AQP3 protein expression in renal collecting duct cells, the kidney were evaluated by Western blot analysis, immunofluoresence staining, and AQP3 mRNA expression by real-time PCR.

Results: In vitro, LV-Hoxb7 Cre, which contained Hoxb7 promoter, worked only in collecting duct cells due to the presence of Hoxb7 in collecting duct cells but not in mesangial cells. Furthermore, combined infection of collecting duct cells with LV-Hoxb7 Cre and LV-loxP shAQP3 significantly inhibited the protein expression of AQP3 along with the disappearance of EGFP protein expression, suggesting that LV-Hoxb7 Cre and LV-loxP shAQP3 used in the present study worked together effectively.

In vivo, both EGFP and mCherry protein were completely expressed at the

same site, in kidney collecting duct cells of loxP-EGFP mice, where Hoxb7 promoter was working, but not in the liver of LoxP-EGFP + LV-Hoxb7 Cre mice. The protein expression of EGFP in the kidney was observed in Hoxb7 Cre mice injected with LV-loxP scr or LV-loxP shAQP3. Furthermore, AQP3 protein and mRNA expression were significantly reduced in Hoxb7 Cre mice injected with LV-loxP shAQP3 compared to inject with PBS and LV-loxP scr. In addition, even though urine volume was significantly increased, urine osmolality was significantly decreased by 44.4% in Hoxb7 Cre + LV-loxP shAQP3 mice compared to the other groups. In contrast, there was no significant change in the protein expression of AQP3 in the colon and trachea by LV-loxP scr or LV-loxP shAQP3 injection in spite of demonstrable expression of EGFP protein. Finally, I tried to generate AQP3 knockout mice in C57BL/6J mice by consecutive injection of LV-Hoxb7 Cre and LV-loxP shAQP3. When LV-Hoxb7 Cre was introduced in these mice, the expression of mCherry protein was found only in the kidney collecting duct cells. Moreover, injection of LV-loxP shAQP3 resulted in an increase in EGFP protein expression in all kidney cells except for collecting duct cells. However, there were no significant differences in the protein and mRNA expression of AQP3 in C57BL/6J mice injected with LV-Hoxb7 Cre or LV-loxP shAQP3 alone. In contrast, when consecutive injection of LV-Hoxb7 Cre and LV-loxP shAQP3 was performed in these mice, AQP3 protein and mRNA expression were significantly reduced. Even though urine

volume was significantly increased, urine osmolality was significantly decreased in LV-Hoxb7 Cre and LV-loxP shAQP3 injected mice compared to the other groups. Meanwhile, the protein expression of EGFP was also observed in the colon and trachea of C57BL/6J mice by the administration of LV-loxP shAQP3 with or without LV-Hoxb7 Cre, but AQP3 protein expression was not changed by these treatment.

Conclusions: These findings suggest that double transduction of Cre- and loxP-based lentivirus can be a simple way to generate cell-specific knockdown mice, and this method may also be applicable to other species.

Key words: Cre/loxP system, lentiviral vector, renal collecting duct cell, Hoxb7, aquaporin-3

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I. INTRODUCTION

Upregulation or downregulation of certain genes is associated with the development and progression of most diseases. Therefore, genetically engineered mice with a gene overexpression or knockout have been used to elucidate the precise role of a specific gene in the pathogenesis of a disease[1].

Specifically, regarding a downregulated gene, transgenic mice generated by conventional knockout techniques using oocytes or embryonic stem (ES) cells have been the most commonly used approach, but these methods have some limitations: persistent impact of the deleted gene throughout mice ontogeny with a possibility to result in a lethal phenotype[2] or an unexpected

compensatory increase in other genes[3], affection of all cell types, and cost- and time-consuming procedure.

To overcome some of these problems[4], a number of modern technologies to manipulate cell- or tissue-specific genes have been developed and been remarkably helpful for figuring out the definite function of genes in each cell or tissue during the disease process. Among these, the Cre-recombinant-loxP system has been widely used[5,6]. It is one of organism-specific gene expression manipulation systems widely utilized for conditional gene knockout in selective cell or tissue by Cre, a site-specific recombinase[5-8]. Gene disruption appears by target gene flanked by the loxP rank when bacteriophage P1 Cre recombinase recognizes the loxP region[5-7]. Furthermore, this system is operative to generate conditional knockout mice in an inducible manner by using the tamoxifen- or tetracycline-based system[9-11]. Nevertheless an advance in genetic engineering, there are still several constraints such as the need of time to breed mice, a relatively low success rates, and a difficulty in applying to other species. In addition, a huge amount of money, about 100-200 thousand dollars according to the report of the National Institute of Health (NIH), are expected to be spent for implanting a cell-specific Cre gene to ES cells to develop a Cre recombinase animal and then to establish Cre gene recombinant animal through fertilization and crossbreeding.

In this study, I invented a simple way to knockdown a specific gene in a cell-specific pattern in adult mice by lentivirus-assisted transfer of short hairpin RNA (shRNA). First, a group of collecting duct-specific aquaporin-3 (AQP3) knockdown mice was generated by injecting loxP-AQP3 shRNA expressing lentivirus (LV-loxP shAQP3) to adult homeobox B7 (Hoxb7) Cre transgenic mice, which expressed the Cre recombinase only in the collecting ducts under the control of the mouse Hoxb7 enhancer and promoter. Second, another group of collecting duct-specific AOP3 knockdown mice was generated by consecutive injection of Hoxb7-Cre expressing lentivirus (LV-Hoxb7 Cre) and LV-loxP shAQP3 in adult C57BL6/J mice.

II. MATERIALS AND METHODS

1. Generation of loxP-AQP3 shRNA expressing lentivirus and Hoxb7-Cre expressing lentivirus

According to the mouse cDNA sequence of AQP3, a small interfering RNA (siRNA) target sites were selected using the siRNA Selection Web Server (http://jura.wi.mit.edu/bioc/siRNA). In addition, a scrambled sequence was created as a control. The annealed oligonucleotides were inserted into the XhoI-HpaI sites of the pSico lentiviral vector[12] (Addgene, Cambridge, MA, USA). The PCR-positive samples were sequenced and named pSico-AQP3 shRNA and scrambled (scr) (Table 1). The Hoxb7 promoter was also inserted into the XbaII-NheI sites of the Puro-Cre empty vector[13] (Addgene) (Figure 1).

Table 1. Sequences of AQP3shRNA and AQP3shRNA-scambled

		Sequence
AQP3 shRNA	Sense oligo	5'-T**GCCATCGTTGACCCTTATA**TTCAAGAGA**TATAAGGGTCAACGATGGC**TTTTTTC
	antisense oligo	5'-TCGAGAAAAAA**GCCATCGTTGACCCTTATA**TCTCTTGAA**TATAAGGGTCAACGATGGC**A
Scramble	Sense oligo	5'-T**GCGTATGAGTAGTGCGCAGAT**TTCAAGAGA**ATCTGCGCACTACTCATACGC**TTTTTTC
	antisense oligo	5'-T**GCGTATGAGTAGTGCGCAGAT**TCTCTTGAA**ATCTGCGCACTACTCATACGC**A

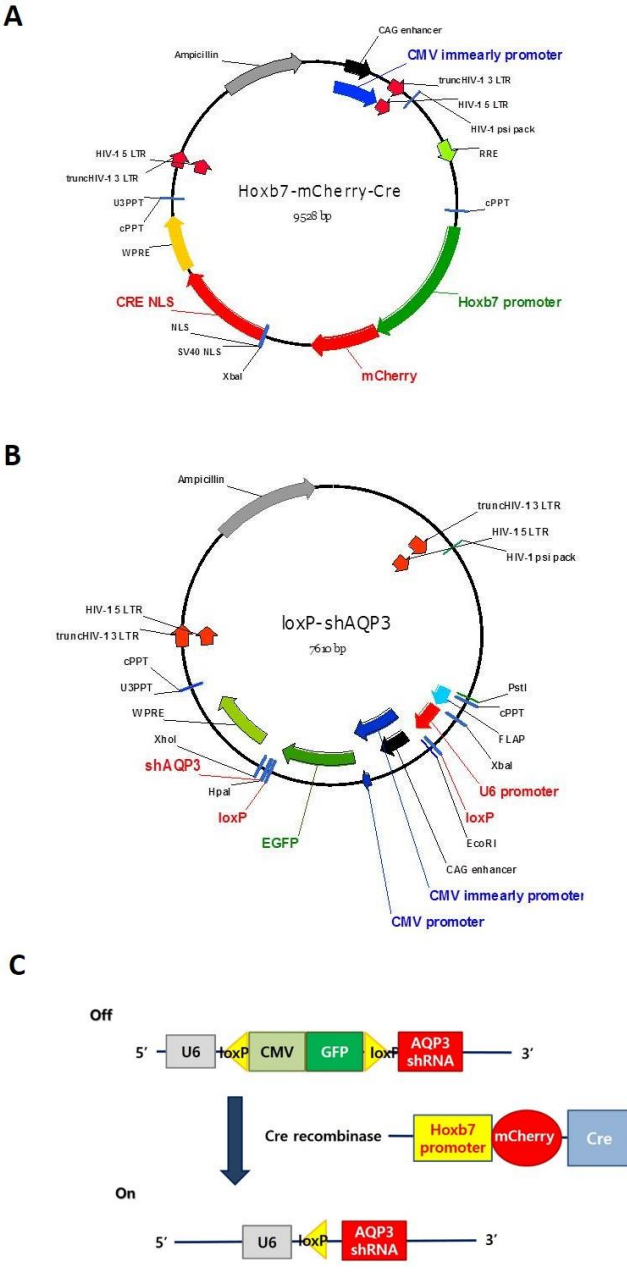


Figure 1. Lentiviral vector designs. (A) Hoxb7-mCherry-Cre lentiviral vector and (B) pSico-loxP-GFP-shAQP3 lentiviral vector. (C) The mechanism of Cre/loxP system.

2. Lentivirus production

Lentivirus were produced as previously described[14]. Human embryonic kidney (HEK) 293FT cells (Invitrogen, Carlsbad, CA, USA) were transfected with the expression vector and two helper plasmids, namely packaging plasmid pCMV Δ8.9 and vesicular stomatitis virus G protein plasmid, at 1, 7.5, and 5.5 μg of DNA per 150 mm plate, using the calcium phosphate method. After 72 hr, supernatants from four plates were pooled, centrifuged at 780 g for 5 min, and filtered through a 0.45-μm pore size filter. Next, centrifugation was performed at 83,000 g for 1.5 hr, and the resulting pellet was resuspended in 100 μl of phosphate-buffered saline (PBS). Lentivirus titers were determined by transfecting HEK 293FT cells with a dilution series of the viral suspension, and lentivirus samples with a titer of 4×10^8 transfection units/ml (TU) were stored at -80℃.

3. Animal experiments

All animal study protocols were approved by the Committee for the Care and Use of Laboratory Animals at Yonsei University College of Medicine in Seoul, Korea, and carried out in accordance with the Principles of Laboratory Animal Care (NIH Publication no. 85-23, revised 1985).

For the first animal experiments, loxP-EGFP mice[15] were purchased from Jackson Laboratories (Bar Harbor, ME, USA). They possess loxP sites on either

side of a membrane-targeted tdTomato (mT) cassette and express strong red fluorescence in all tissues and cell types. Tail or whole body epifluorescence is sufficient to identify mT/mG homozygotes. When bred to Cre recombinase expressing mice, the offsprings have the mT cassette deleted in the Cre expressing tissue(s), resulting in the expression of the membrane-targeted EGFP (mG) cassette located just downstream. These mice were divided into two groups, and were injected with PBS (Con) or LV-Hoxb7 Cre.

For the second animal experiments, Hoxb7 Cre transgenic mice[16] (Jackson Laboratories) were used. They were classified into three groups based on what was injected; PBS (Con), LV-loxP scr, or LV-loxP shAQP3.

For the third animal experiments, male C57BL6/J mice (Jackson Laboratories) weighing 24-26 g were assigned to one of the five groups, and were injected with PBS (Con), LV-Hoxb7 Cre, LV-loxP shAQP3, LV-Hoxb7 Cre + LV-loxP scr, or LV-Hoxb7 Cre + LV-loxP shAQP3.

All animals were given the selected treatment on days 0, 4, and 7 via hydrodynamic tail vein injection as described previously[17]. For C57BL6/J mice treated with consecutive injection of LV-Hoxb7 Cre and LV-loxP shAQP3, LV-shAQP3 was introduced on days 3, 7, and 10 after the final injection of LV-Hoxb7 Cre. Briefly, a volume of 1 ml of PBS alone or PBS containing 4×10^8 TU of lentivirus were injected to mice via the tail vein within 10 sec. Mice were killed at 6-week after the first injection of PBS or lentivirus.

Mice were housed in a temperature-controlled room and were given free access to water and standard laboratory chow during the study period.

4. Cell culture

Mouse renal collecting duct cells were purchased from American Type Culture Collection (ATCC) (Manassas, VA, USA) (CRL-2038) and maintained in a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and Ham's F12 medium with 2.5 mM L-glutamine, 15 mM HEPES, 0.5 mM sodium pyruvate, and 1.2 g/L sodium bicarbonate supplemented with 0.005 mM dexamethasone and 5% fetal bovine serum (FBS) (Invitrogen). Mouse mesangial cells from an SV40 transgenic mouse (MES-13) were also purchased from ATCC and maintained in DMEM containing 5% FBS. The cells were grown at 37℃ in humidified 5% CO_2 in air.

For lentiviral transfection, 4×10^5 TU of the lentivirus suspension containing LV-Hoxb7 Cre and/or LV-shAQP3 was added to cultured cells. After 48 hr, the media were changed to routine culture media. At 72 hr after the media change, cells were harvested. The doses LV-Hoxb7 Cre and/or LV-shAQP3 used in the experiments were determined based on the results of preliminary experiments.

5. Total RNA extraction

Total RNA was extracted from whole kidney, colon, and trachea as previously

described[18]. Briefly, a piece of the whole kidney was snap-frozen in liquid nitrogen, pulverized with a mortar and pestle while frozen, and suspended in 100 μl of RNA STAT-60 reagent (Tel-Test, Friendswood, TX, USA), which was lysed by freezing and thawing three times. Another 700 μl of RNA STAT-60 reagent was then added, and the mixture was vortexed and stored for 5 min at room temperature. Next, 160 μl of chloroform was added and the mixture was shaken vigorously for 30 sec. After 3 min, the mixture was centrifuged at 12,000 g for 15 min at 4℃ and the upper aqueous phase containing the extracted RNA was transferred to a new tube. RNA was precipitated from the aqueous phase by adding 400 μl of isopropanol, and then pelleted by centrifugation at 12,000 g for 30 min at 4℃. The RNA precipitate was washed with 70% ice-cold ethanol, dried using a Speed Vac, and dissolved in DEPC-treated distilled water. RNA yield and quality were assessed based on spectrophotometric measurements at wavelengths of 260 and 280 nm. Total RNAs from the colon and trachea, and cultured cells were extracted similarly.

6. Reverse transcription

First strand cDNA was made by using a Boehringer Mannheim cDNA synthesis kit (Boehringer Mannheim GmbH, Mannheim, Germany). Two micrograms of total RNA extracted from tissues and cultured cells were reverse transcribed using 10 μM random hexanucleotide primer, 1 mM dNTP, 8 mM

$MgCl_2$, 30 mM KCl, 50 mM Tris·HCl, pH 8.5, 0.2 mM dithiothreithol, 25 U RNAse inhibitor, and 40 U AMV reverse transcriptase. The mixture was incubated at 30℃ for 10 min and 42℃ for 1 hr, followed by inactivation of the enzyme at 99℃ for 5 min.

7. Real-time PCR

The primers used for aquaporin-3 (AQP3) and 18s amplification were as follows: AQP 3 sense 5′-AGCCCTGGATCAAGCTGCCC-3′, antisense 5′-TTGGCA AAGGCCCAGATTG-3′; and 18s sense 5′-AGTCCCTGCCCT TTGTACACA-3′, antisense 5′- GATCCGAGGGCCTCACTAAAC-3′. cDNAs from 25 ng RNA of tissues and cultured cells per reaction tube were used for amplification.

Using the ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, CA), PCR was performed with a total volume of 20 μl in each well, containing 10 μl of SYBR Green PCR Master Mix (Applied Biosystems), 5 μl of cDNA, and 5 pM sense and antisense primers. Primer concentrations were determined by preliminary experiments that analyzed the optimal concentrations of each primer. The PCR conditions were as follows: 35 cycles of denaturation at 94.5℃ for 30 sec, annealing at 60℃ for 30 sec, and extension at 72℃ for 1 min. Initial heating at 95℃ for 9 min and final extension at 72℃ for 7 min were performed for all PCR reactions. Each sample

was run in triplicate in separate tubes and a control without cDNA was also run in parallel with each assay. After real-time PCR, the temperature was increased from 60 to 95℃ at a rate of 2℃/min to construct a melting curve. The cDNA content of each specimen was determined using a comparative C_T method with $2^{-\Delta\Delta CT}$. The results are given as relative expression of AQP3 normalized to the expression of 18s rRNA and expressed in arbitrary units. Signals from C tissues and cells were assigned a relative value of 1.0. In pilot experiments, PCR products revealed a single band on agarose gels.

8. Western blot analysis

Pieces of tissues and harvested cultured cells were lysed in SDS sample buffer [2% SDS, 10 mM Tris·HCl, pH 6.8, 10% (vol/vol) glycerol], treated with Laemmli sample buffer, heated at 100℃ for 5 min, and electrophoresed in an 12% acrylamide denaturing SDS-polyacrylamide gel. Proteins were then transferred to a Hybond-ECL membrane using a Hoeffer semidry blotting apparatus (Hoeffer Instruments, San Francisco, CA), and the membrane was then incubated in blocking buffer A (1× PBS, 0.1% Tween 20, and 5% nonfat milk) for 1 hr at room temperature, followed by an overnight incubation at 4℃ in a 1:1,000 dilution of polyclonal antibodies to AQP3, EGFP (Abcam, Cambridge, MA, USA), mCherry (GeneCopoeia, Germantown, MD, USA), or β-actin (Sigma-Aldrich, St. Louis, MO, USA). The membrane was then washed

once for 15 min and twice for 5 min in 1× PBS with 0.1% Tween 20. Next, the membrane was incubated in buffer A containing a 1:2,000 dilution of horseradish peroxidase-linked donkey anti-goat IgG (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). The washes were repeated, and the membrane was developed with a chemiluminescent agent (ECL; Amersham Life Science, Inc., Arlington Heights, IL, USA). The band densities were measured using TINA image software (Raytest, Straubenhardt, Germany), and the changes in optical densities in the treated groups relative to C tissues or cells were used for analysis.

9. Immunofluoresence staining

For double-immunofluorescence staining, 4-μm frozen sections of the kidney, colon, and trachea were used. Slides were fixed in acetone for 10 min at 4℃, air-dried for 10 min, blocked with 10% donkey serum for 20 min, and incubated with a 1:1,000 dilution monoclonal antibody to EGFP (Abcam) for 3 hrs at room temperature. After washing, Cy2 (green)-conjugated anti-mouse IgG antibody (Research Diagnostics, Flanders, NJ, USA) was added for 60 min. A 1:500 dilution of monoclonal rabbit anti-mCherry or polyclonal anti-AQP3 (Abcam) was then applied, followed by Cy3 (red)-conjugated anti-rabbit IgG antibody or Cy2 (green)-conjugated anti-rabbit IgG antibody.

10. Urine volume and osmolality

One or two days before killing mice, they were put in metabolic cages, and 24-hr urine was collected. Urine volume was measured and osmolality was determined using a vapor pressure osmometer (5100C; Wescor, Logan, UT, USA).

11. Statistical analysis

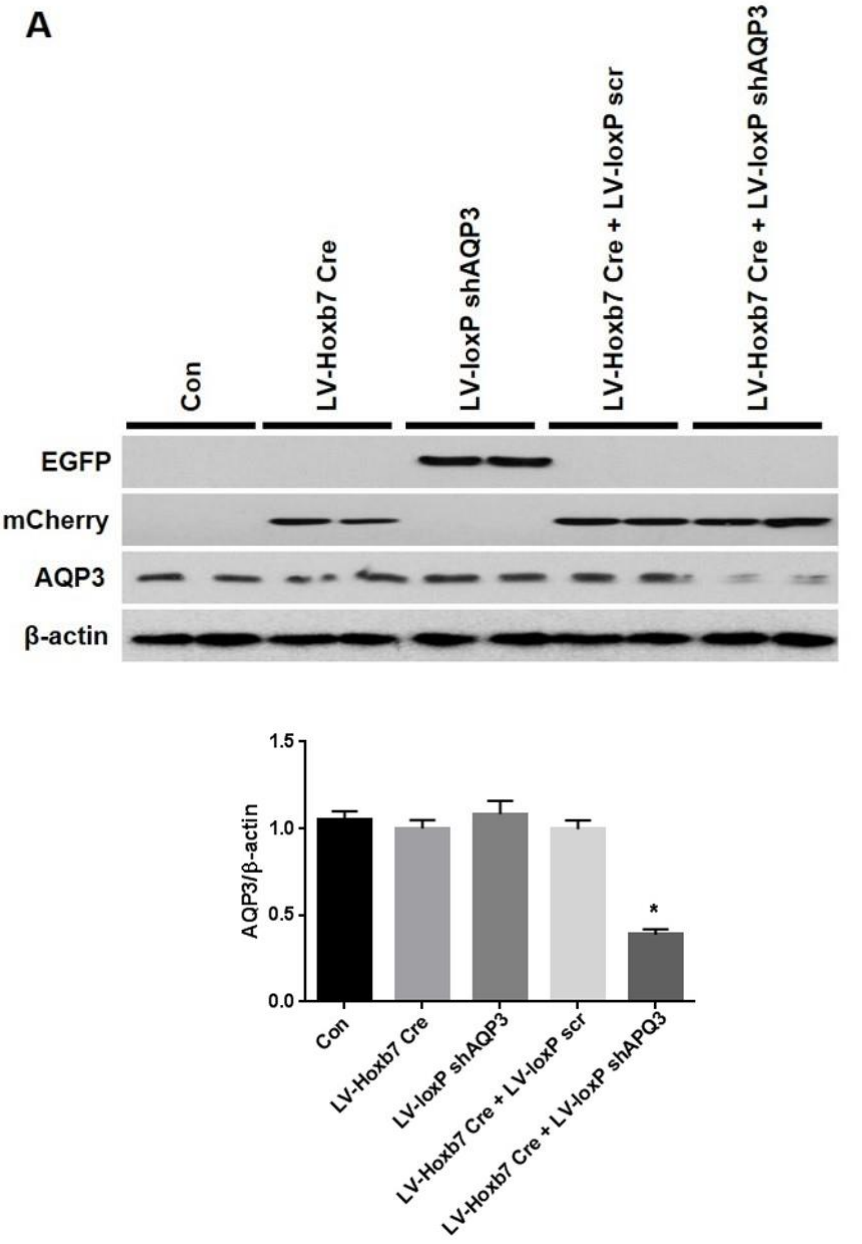
All values are expressed as means ± standard errors of the mean (SEM). Statistical analyses were performed using the Statistical Package for Social Sciences for Windows Ver. 11.0 (SPSS, Inc., Chicago, IL, USA). Results were analyzed using one-way ANOVA with a *post hoc* Bonferonni's test for multiple comparisons. P values less than 0.05 were considered statistically significant.

III. RESULTS

1. Gene-silencing efficiency

To verify whether LV-Hoxb7 Cre and LV-loxP shAQP3 generated for the current study can effectively silence AQP3 gene in mice, I first performed in vitro study using kidney collecting duct and mesangial cells. Effective transection of LV-Hoxb7 Cre and LV-loxP shAQP3 into cultured cells was confirmed by checking the protein expression of each virus-taq, mCherry and EGFP, respectively. As seen in Figure 2A, the protein expression of mCherry was demonstrated in cultured collecting duct cells treated with LV-Hoxb7 Cre or LV-Hoxb7 Cre + LV-loxP scr, while EGFP protein expression was observed in collecting duct cells exposed to LV-loxP shAQP3. In these cells, however, there was no significant change in the expression of AQP3 protein. Meanwhile, concomitant treatment LV-Hoxb7 Cre and LV-loxP shAQP3 significantly reduced the protein expression of AQP3 along with the presence of mCherry but not EGFP protein expression. The mRNA expression showed a similar pattern to the expression of each protein (Fig. 2B). In contrast to collecting duct cells, the protein expression of EGFP was found in mesangial cells treated with LV-Hoxb7 Cre, LV-Hoxb7 Cre + LV-loxP scr, or LV-Hoxb7 Cre + LV-loxP shAQP3, while there was no mCherry protein expression in these cells. Moreover, the expression of AQP3 protein was comparable among the groups (Fig. 2C). These findings implied that LV-Hoxb7 Cre, which contained Hoxb7 promoter, worked

only in collecting duct cells due to the presence of Hoxb7 in collecting duct cells but not in mesangial cells. Furthermore, combined infection of collecting duct cells with LV-Hoxb7 Cre and LV-loxP shAQP3 significantly inhibited the protein expression of AQP3 along with the disappearance of EGFP protein expression, suggesting that LV-Hoxb7 Cre and LV-loxP shAQP3 used in the present study worked together effectively.


A
Con
LV-Hoxb7 Cre
LV-loxP shAQP3
LV-Hoxb7 Cre + LV-loxP scr
LV-Hoxb7 Cre + LV-loxP shAQP3
EGFP
mCherry
AQP3
β-actin
AQP3/β-actin
1.5
1.0
0.5
0.0
*
Con
LV-Hoxb7 Cre
LV-loxP shAQP3
LV-Hoxb7 Cre + LV-loxP scr
LV-Hoxb7 Cre + LV-loxP shAPQ3

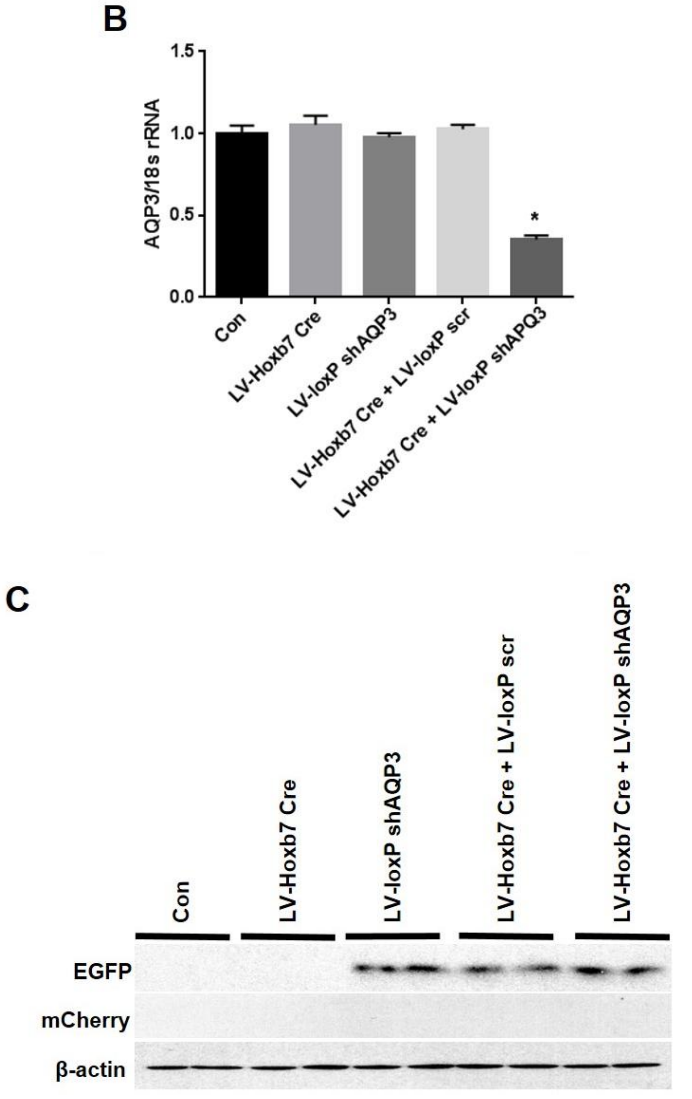


Figure 2. A representative Western blot of EGFP, mCherry, and β-actin in collecting duct cells (A) and mesangial cells (C) treated with PBS (Con), LV-Hoxb7 Cre, LV-loxP shAQP3, LV-Hoxb7 Cre + LV-loxP scr, or LV-Hobx7 Cre + LV-loxP shAQP3 (representative of four blots). (B) Real-time PCR for AQP3 in these five groups.

*; $p < 0.0001$ vs. Con

2. In vivo transduction efficiency

Next, animal experiments were performed to test in vivo transduction efficiency. Hydrodynamic tail vein injection of PBS (Con), LV-Hoxb7 Cre, LV-loxP scr, or LV-loxP shAQP3 was done to twelve C57BL/6J mice thrice with a 3-4 days interval (n=4 per group). Semi-nested PCR revealed that EGFP expression was the highest in the liver and spleen, kidney at 6-week after the first injection, but mCherry expression was found only in the kidney (Fig. 3A). In addition, immunofluorescence staining demonstrated that EGFP protein was significantly expressed in all tissues of LV-injected mice but not in those of PBS-injected mice at 6-week. The expression of mCherry protein was found only in the kidney collecting duct cells (Fig. 3B).

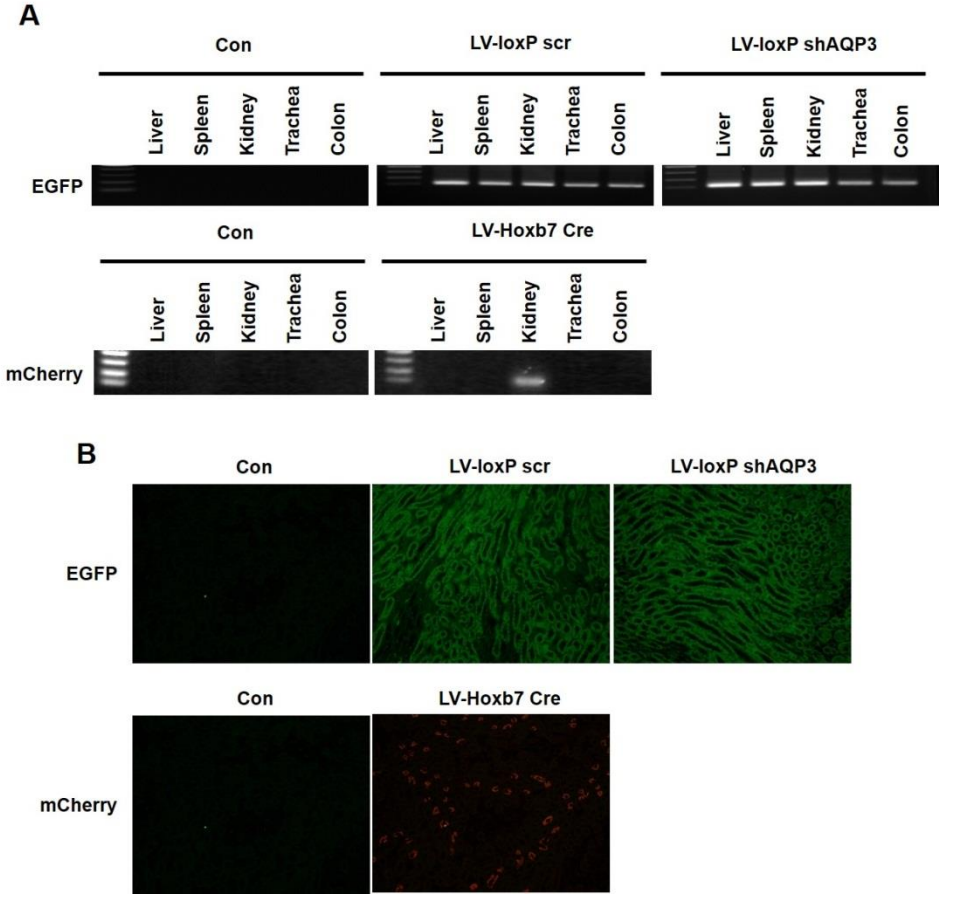


Figure 3. Distribution and transduction efficiencies of hydrodynamically injected lentiviral vectors. (A) A representative semi-nested PCR for EGFP and mCherry. EGFP expression was observed in the liver, spleen, kidney, colon, and trachea, but mCherry expression was found only in the kidney. (B) A representative immunofluorescence staining for EGFP and mCherry. EGFP protein was strongly expressed in the kidney of lentivirus-injected mice at 6-week after the first lentiviral injection. In contrast, mCherry protein was expressed only in the kidney collecting duct cells (x 200).

3. Animal data of loxP-EGFP mice injected with LV-Hoxb7 Cre

loxP-EGFP mice, which express EGFP in the presence of Cre recombinase, were treated with LV-Hoxb7 Cre via hydrodynamic tail vein injection. As seen in Figure 4A, both EGFP and mCherry protein expression were found only in the kidney, where Hoxb7 promoter was working, but not in the liver of loxP-EGFP + LV-Hoxb7 Cre mice. Immunofluorescence staining also confirmed that EGFP and mCherry protein were completely expressed at the same site, kidney collecting duct cells (Fig. 4B).

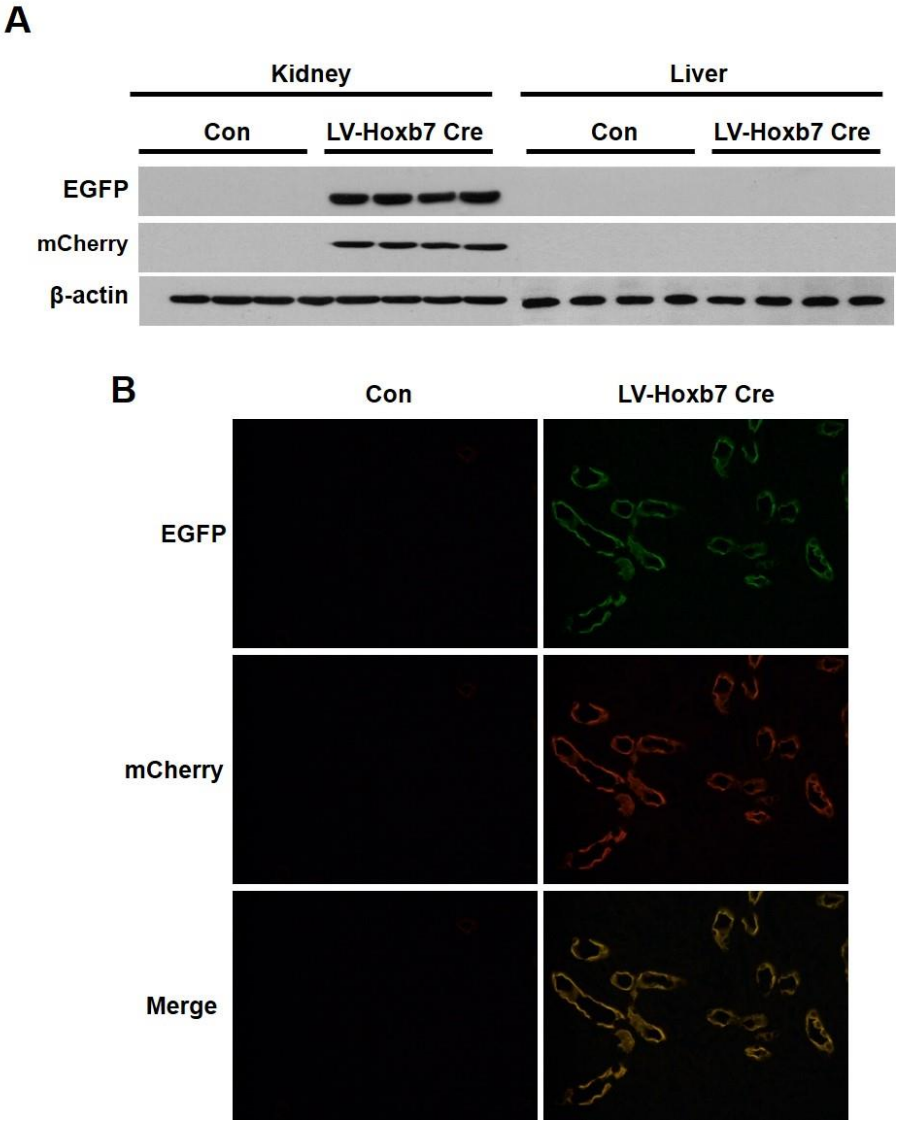
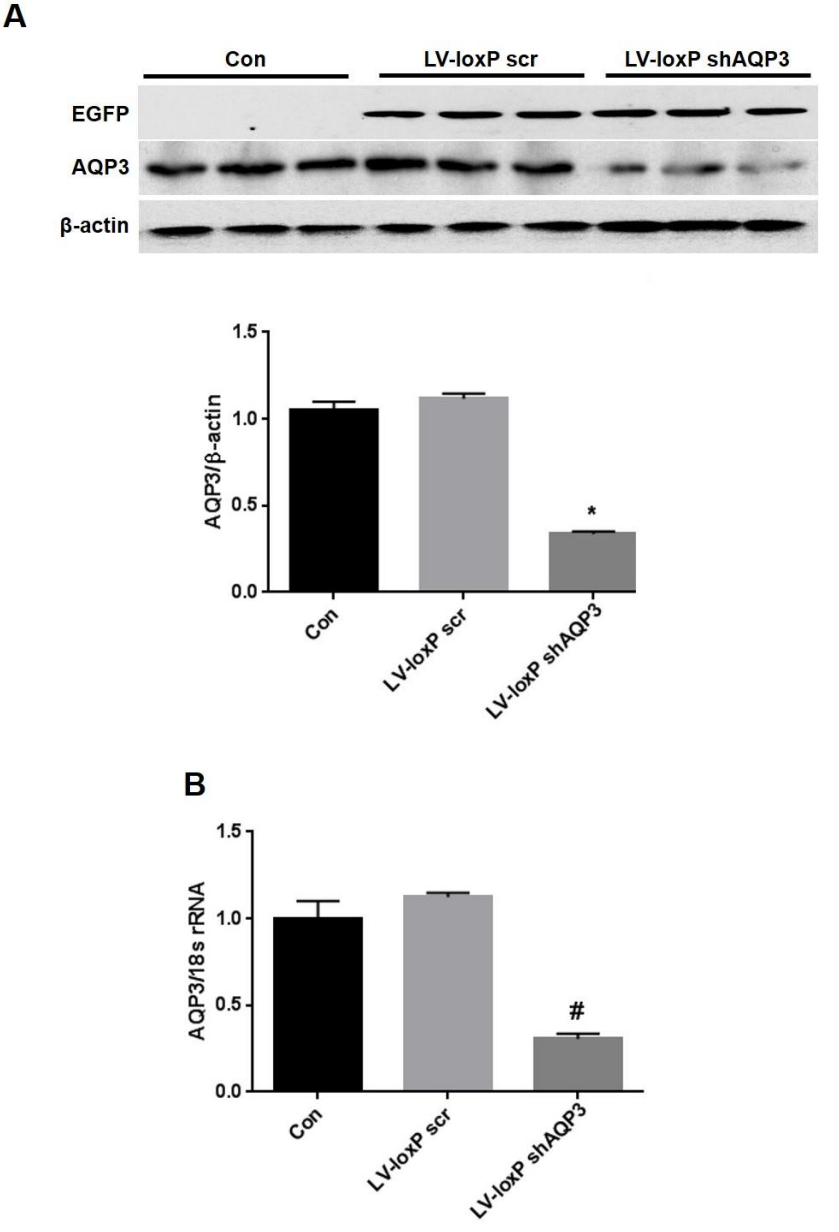


Figure 4. EGFP and mCherry protein expression in loxP-EGFP mice injected with LV-Hoxb7 Cre. (A) Western blot analysis revealed that both EGFP and mCherry protein expression were found only in the kidney, but not in the liver of LoxP-EGFP + LV-Hoxb7 Cre mice. (B) Both EGFP and mCherry protein, assessed by immunofluorescence staining, were completely expressed at the same site, kidney collecting duct cells (x 400).

4. Animal data of Hoxb7 Cre mice injected with LV-loxP shAQP3

Hoxb7 Cre mice, which express Cre recombinase only in kidney collecting duct cells, were administered with LV-loxP shAQP3 via hydrodynamic tail vein injection. As seen in Figure 5A, the protein expression of EGFP in the kidney was observed in Hoxb7 Cre mice injected with LV-loxP scr or LV-loxP shAQP3. Furthermore, AQP3 protein and mRNA expression, assessed by Western blot, immunofluorescence staining and nested real-time PCR, were significantly reduced in Hoxb7 Cre + LV-loxP shAQP3 mice compared to Hoxb7 Cre + PBS and Hoxb7 Cre + LV-loxP scr mice (Fig. 5A-5C). In addition, while urine volume was significantly increased, urine osmolality was significantly decreased by 44.4% in Hoxb7 Cre + LV-loxP shAQP3 mice compared to the other groups (Fig. 5D).

In contrast, there was no significant change in the protein expression of AQP3 in the colon and trachea by LV-loxP scr or LV-loxP shAQP3 injection in spite of demonstrable expression of EGFP protein, denoting that LV-loxP shAQP3 had no effects on AQP3 protein expression in the tissues where Hoxb7 protein did not exist (Fig. 6).


A
Con
LV-loxP scr
LV-loxP shAQP3
EGFP
AQP3
β-actin
AQP3/β-actin
1.5
1.0
0.5
0.0
*
Con
LV-loxP scr
LV-loxP shAQP3
B
AQP3/18s rRNA
1.5
1.0
0.5
0.0
#
Con
LV-loxP scr
LV-loxP shAQP3

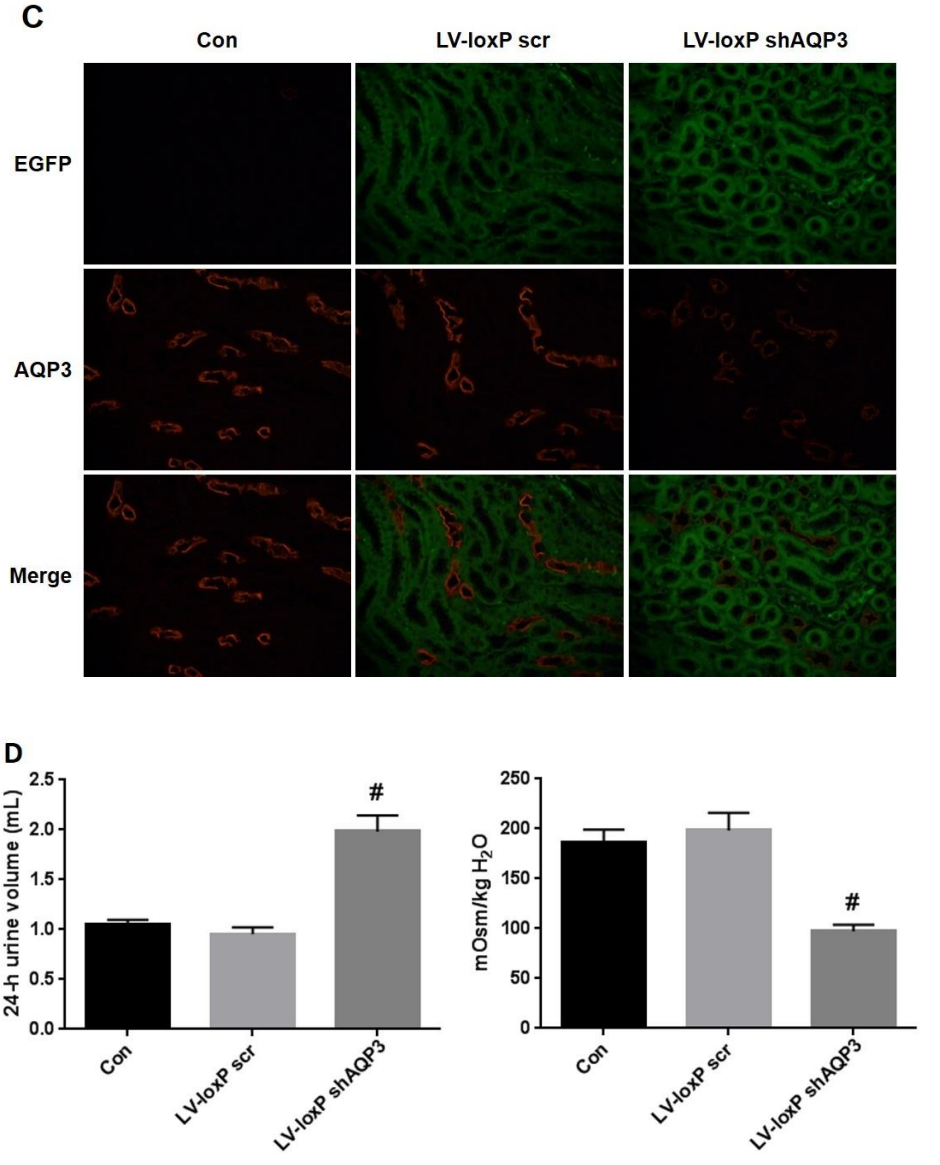


Figure 5. EGFP and AQP3 expression in the kidney, and urine volume and osmolality in Hoxb7 Cre mice injected with LV-loxP shAQP3. (A) Western blot analysis revealed that the protein expression of EGFP in the kidney was observed in

Hoxb7 Cre mice injected with LV-loxP scr or LV-loxP shAQP3. In addition, AQP3 protein expression was significantly reduced in Hoxb7 Cre + LV-loxP shAQP3 mice compared to Hoxb7 Cre + PBS and Hoxb7 Cre + LV-loxP scr mice. (B) AQP3 mRNA expression, assessed by nested real-time PCR, was also significantly reduced in Hoxb7 Cre + LV-loxP shAQP3 mice. (C) Double-immunofluorescence staining showed that EGFP protein (green) expression within the kidney was observed in all cells except for the collecting duct cells in Hoxb7 Cre mice injected with LV-loxP scr or LV-loxP shAQP3. Moreover, the expression of AQP3 protein (red) was significantly decreased in the collecting duct cells in LV-loxP shAQP3-injected mice, while LV-loxP scr had no effect on the protein expression of AQP3 (x 400). (D) While urine volume was significantly increased, urine osmolality was significantly decreased by 44.4% in Hoxb7 Cre + LV-loxP shAQP3 mice compared to the other groups.

#; $p < 0.001$ vs. Con, *; $p < 0.0001$ vs. Con

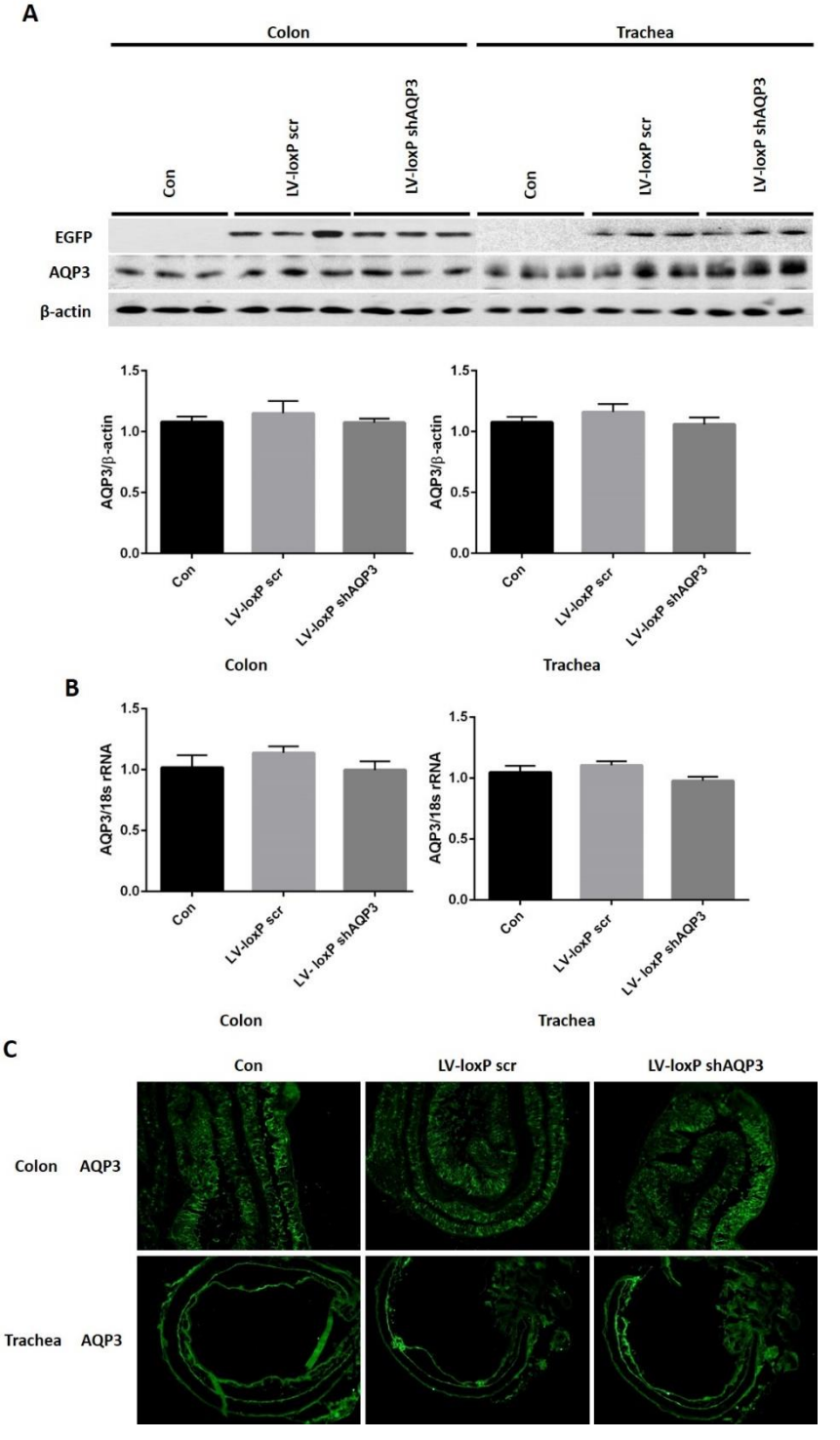
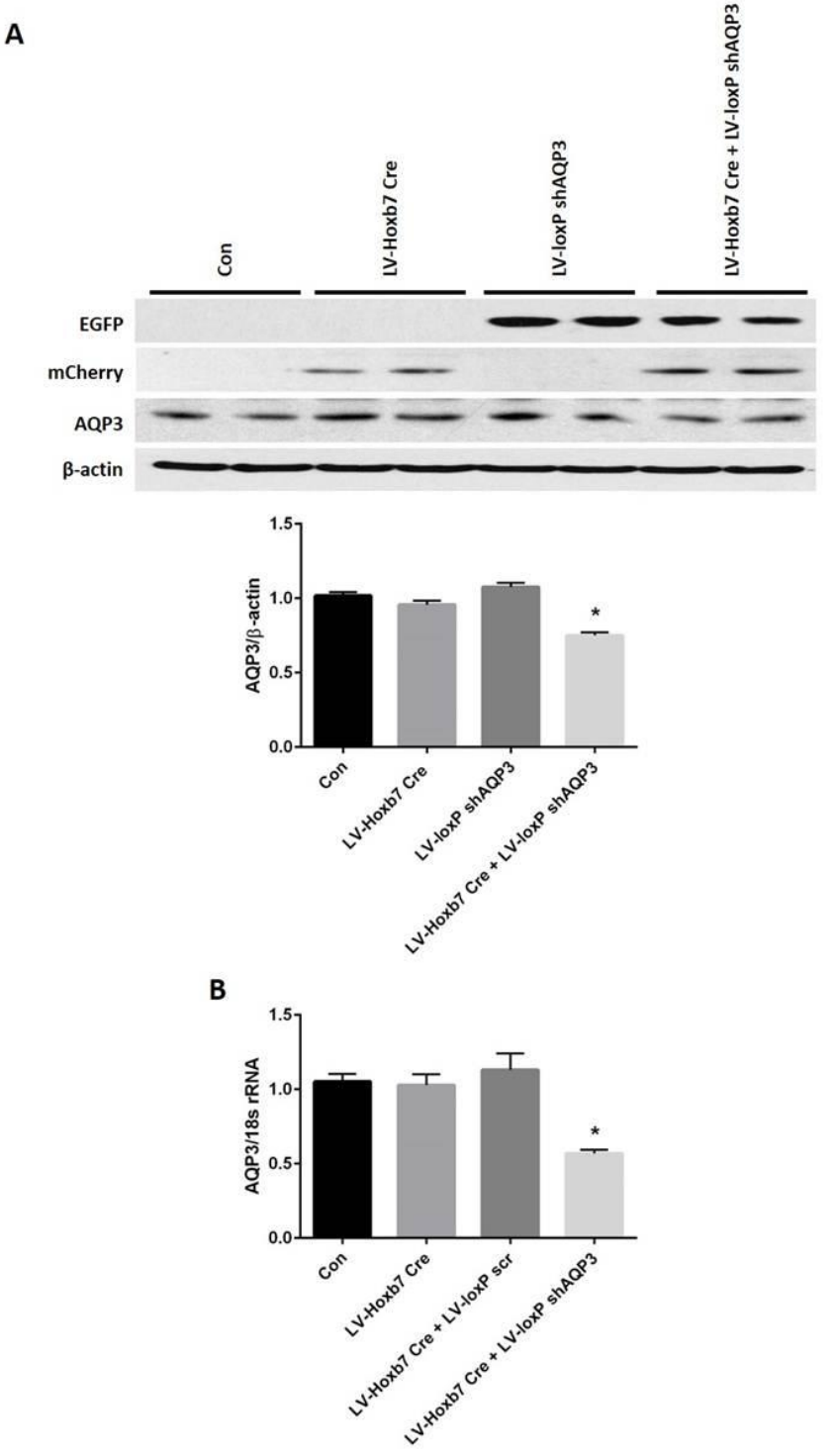

A
Colon
Trachea
Con
LV-loxP scr
LV-loxP shAQP3
Con
LV-loxP scr
LV-loxP shAQP3
EGFP
AQP3
β-actin
AQP3/β-actin
1.5
1.0
0.5
0.0
Con
LV-loxP scr
LV-loxP shAQP3
Colon
Trachea
B
AQP3/18s rRNA
Colon
Trachea
C
Con
LV-loxP scr
LV-loxP shAQP3
Colon AQP3
Trachea AQP3

Figure 6. EGFP and AQP3 expression in the colon and trachea of Hoxb7 Cre mice injected with LV-loxP shAQP3. There were no significant changes in AQP3 protein and mRNA expression in the colon and trachea of Hoxb7 Cre mice injected with LV-loxP scr or LV-loxP shAQP3 in spite of demonstrable expression of EGFP protein.

5. Animal data of C57BL/6J mice treated with consecutive injection of LV-Hoxb7 Cre and LV-loxP shAQP3

Finally, I tried to generate AQP3 knockout mice in C57BL/6J mice by consecutive injection of LV-Hoxb7 Cre and LV-loxP shAQP3. When LV-Hoxb7 Cre was introduced in these mice, the expression of mCherry protein was found only in the kidney collecting duct cells. Moreover, injection of LV-loxP shAQP3 resulted in an increase in EGFP protein expression in all kidney cells except for collecting duct cells. However, there were no significant differences in the protein and mRNA expression of AQP3, urine volume, and urine osmolality in C57BL/6J mice injected with LV-Hoxb7 Cre or LV-loxP shAQP3 alone (Fig. 7). In contrast, when consecutive injection of LV-Hoxb7 Cre and LV-loxP AQP3 was performed in these mice, AQP3 protein and mRNA expression were significantly reduced along with a significant increase in urine volume and a significant decrease in urine osmolality (Fig. 7).

Meanwhile, the protein expression of EGFP was also observed in the colon and trachea of C57BL/6J mice by the administration of LV-loxP shAQP3 with or without LV-Hoxb7 Cre, but AQP3 protein expression was not changed by these treatment (Fig. 8).


A
Con
LV-Hoxb7 Cre
LV-loxP shAQP3
LV-Hoxb7 Cre + LV-loxP shAQP3
EGFP
mCherry
AQP3
β-actin
AQP3/β-actin
1.5
1.0
0.5
0.0
*
Con
LV-Hoxb7 Cre
LV-loxP shAQP3
LV-Hoxb7 Cre + LV-loxP shAQP3
B
AQP3/18s rRNA
1.5
1.0
0.5
0.0
*
Con
LV-Hoxb7 Cre
LV-Hoxb7 Cre + LV-loxP scr
LV-Hoxb7 Cre + LV-loxP shAQP3

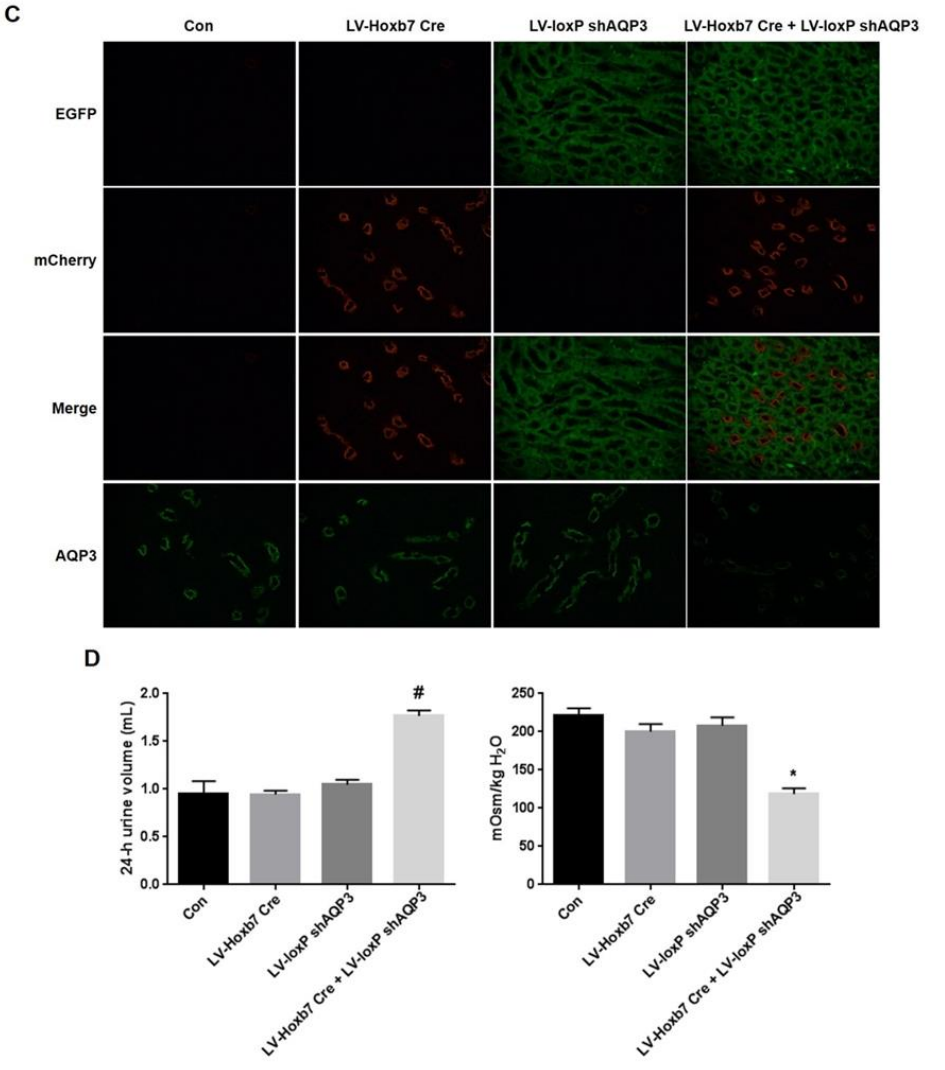
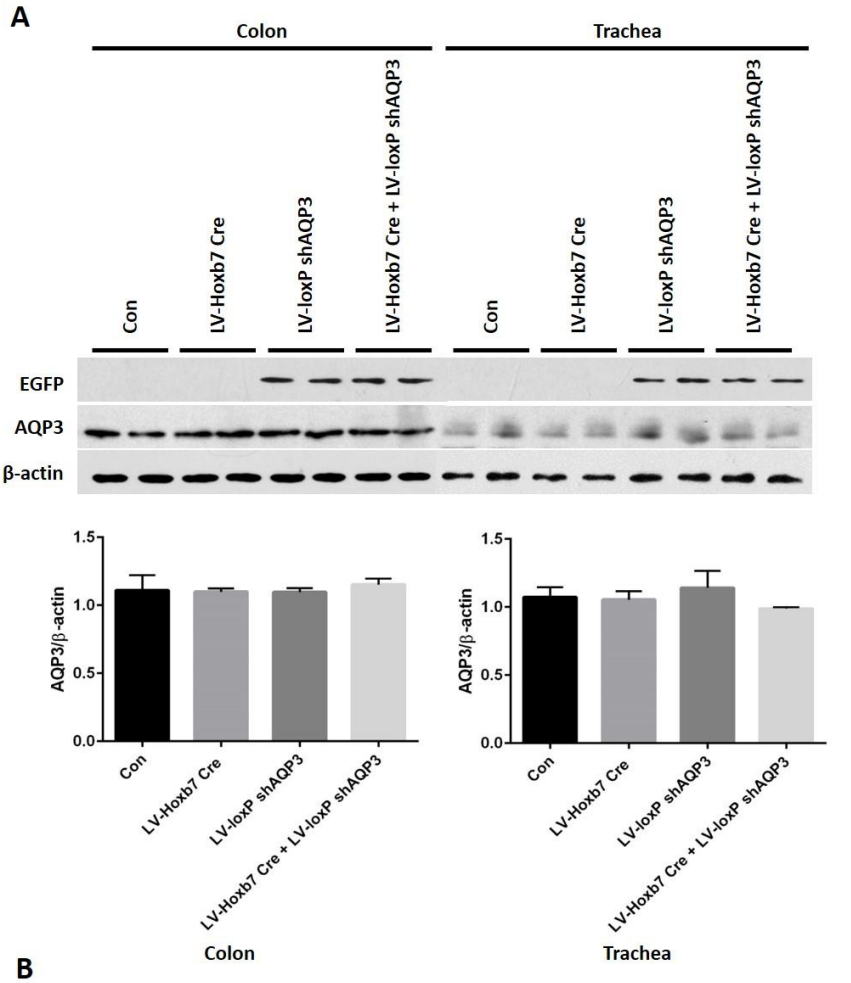
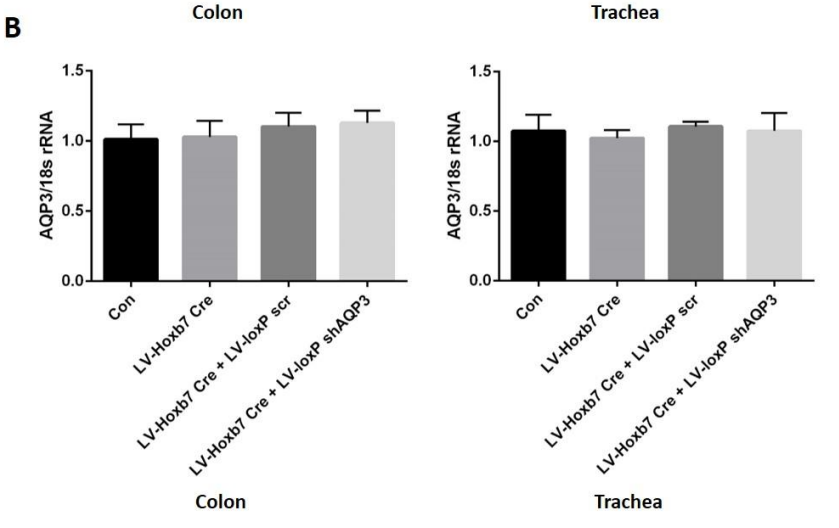


Figure 7. EGFP, mCherry, and AQP3 expression in the kidney, and urine volume and osmolality in C57BL/6J mice treated with consecutive injection of LV-Hoxb7 Cre and LV-loxP shAQP3. (A) Western blot analysis revealed that the protein expression of EGFP in the kidney was observed in C57BL/6J injected with LV-loxP shAQP3 or LV-Hoxb7 Cre + LV-loxP shAQP3, while the expression of mCherry protein was found in the kidney of LV-Hoxb7 Cre or LV-Hoxb7 Cre + LV-loxP shAQP3 mice. In addition, AQP3 protein expression was significantly reduced in C57BL/6J mice injected with LV-Hoxb7 Cre + LV-loxP shAQP3 compared to mice treated with LV-

Hoxb7 Cre or LV-loxP shAQP3 alone. (B) AQP3 mRNA expression, assessed by nested real-time PCR, was also significantly reduced in Hoxb7 Cre + LV-loxP shAQP3 mice. (C) Double-immunofluorescence staining showed that EGFP protein (green) expression within the kidney was observed in C57BL/6J injected with LV-loxP shAQP3 or LV-Hoxb7 Cre + LV-loxP shAQP3 in all cells except for the collecting duct cells, while the expression of mCherry protein was found only in the kidney collecting duct cells of LV-Hoxb7 Cre or LV-Hoxb7 Cre + LV-loxP shAQP3 mice. Moreover, the expression of AQP3 protein was significantly decreased in the collecting duct cells in C57BL/6J mice injected with LV-Hoxb7 Cre + LV-loxP shAQP3 (x 400). (D) While urine volume was significantly increased, urine osmolality was significantly decreased in LV-Hoxb7 Cre + LV-loxP shAQP3 mice compared to the other groups.

#; $p < 0.001$ vs. Con, *; $p < 0.0001$ vs. Con


A
Colon
Trachea
Con
LV-Hoxb7 Cre
LV-loxP shAQP3
LV-Hoxb7 Cre + LV-loxP shAQP3
EGFP
AQP3
β-actin
AQP3/β-actin
1.5
1.0
0.5
0.0
Con
LV-Hoxb7 Cre
LV-loxP shAQP3
LV-Hoxb7 Cre + LV-loxP shAQP3
Colon
Trachea


B
AQP3/18s rRNA
1.5
1.0
0.5
0.0
Con
LV-Hoxb7 Cre
LV-Hoxb7 Cre + LV-loxP scr
LV-Hoxb7 Cre + LV-loxP shAQP3
Colon
Trachea

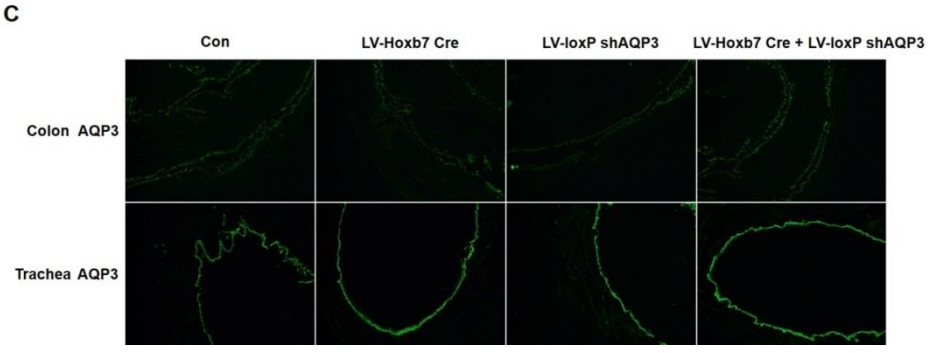


Figure 8. EGFP and AQP3 expression in the colon and trachea of C57BL/6J mice treated with consecutive injection of LV-Hoxb7 Cre and LV-loxP shAQP3. There were no significant changes in AQP3 protein and mRNA expression in the colon and trachea of C57BL/6J mice injected with LV-Hoxb7 Cre and LV-loxP shAQP3 in spite of demonstrable expression of EGFP protein.

IV. DISCUSSION

In this study, injection of LV-loxP shAQP3 to Hoxb7 Cre mice significantly reduced AQP3 expression along with a significant increase in urine volume and a significant decrease in urine osmolality. Similar findings were observed in C57BL/6J mice with a consecutive introduction of LV-Hoxb7 Cre and LV-loxP shAQP3. In contrast, there were no changes in AQP3 expression in the colon and trachea of these mice. These findings suggest that double transduction of Cre- and loxP-based lentivirus can be a simple way to generate cell-specific knockdown mice, and this method may also be applicable to other species.

Since the first transgenic mice were generated in 1980[19-22], genetically engineered transgenic and knockout mice have become exceptionally valuable experimental models to assess the role of a specific gene in not only the development and organogenesis but also the pathogenesis of a certain disease[1,23,24]. However, conventional transgenic and knockout techniques had some limitations. Specifically, conventional knockout method using oocytes or ES cells can result in an embryonic or perinatal phenotype if the manipulated gene is essential for the development or organogenesis. Sterile mice can also be generated. In addition, since the function of the gene of interest can be disparate in diverse tissues and cells as well as at different developmental stages, deletion of this gene in the whole body throughout mice ontogeny can make it hard or impossible to understand its precise function in a specific tissue. Moreover,

compensatory overexpression of other genes can be accompanied.

To overcome these limitations, the second generation of genetically engineer knockout mice was developed. Of these mice, namely conditional knockout mice, tissue-specific knockout mice were generated using a tissue-specific promoter and site-specific recombinase systems (SSR) such as Cre-loxP, Flp-FRT, and ΦC31 systems. Among these SSR, the Cre-loxP system has been the most widely used system both in vitro and in vivo[25-29], Furthermore, a combination of this Cre-loxP system with inducible systems, such as tetracycline[9,30-32] and tamoxifen-inducible systems[33], made it possible to generate tissue- and stage-specific conditional knockout mice. Nevertheless, this procedure is still difficult and time-consuming. It needs to spend several years of work, and sometimes in vain. In addition, the cost for making a transgenic animal have reduced with advances in biotechnology, it still needs a huge amount of money. Moreover, this technique is not easily applicable to other species. Therefore, an easier and simpler way to generate tissue- or cell-specific knockout mice at any developmental stage is keenly necessary. In the current study, renal collecting duct cell-specific AQP3 knockdown mice was simply generated mainly based on the Cre-loxP system. Furthermore, based on the preliminary results showing that injection of LV-Hoxb7 Cre to loxP-EGFP mice via tail vein thrice at 3-4 days intervals resulted in significantly higher transfection efficiency compared to single or twice injection (data not shown), it

is inferred that the magnitude of AQP3 knockdown in the collecting tubule can be regulated or even knockout can be accomplished by modifying the number of times and the dose of LV-shAQP3 injection.

For AQP3 shRNA delivery, lentiviral vector was used in the present study. Various viruses such as retrovirus, adenovirus, and lentivirus, and plasmid vectors that are DNA itself, have been used for gene transfer. Among these, lentivirus-based gene delivery system has several advantages; genes are incorporated into host chromosomes and continuously expressed for a long time, therefore able to maintain long-term treatment efficiency[34]. In addition, genes can be effectively conveyed to differentiated and non-dividing cells such as nerve cells, while ordinary retrovirus does not[35-37]. Recently, moreover, transgenic animals were successfully generated by infecting siRNA or shRNA into zygotes or ES cells via lentivirus-based vector[38-41]. Collectively, lentivirus has been widely employed to manipulate gene in mammalian cells both in vitro and in vivo. The results of my study also showed that lentivirus-based delivery of Hoxb7 Cre and/or AQP3 shRNA was effectively incorporated in renal collecting duct cells, and the transfer effect lasted for up to 3 months (data not shown).

On the other hand, previous studies have demonstrated several undesirable effects of shRNA transduction, including induction of interferon response, off-target gene effects, and hepatotoxicity with increased mortality. Regarding

hepatotoxicity, Grimm et al[42] found a significantly high incidence of mortality due to a dose-dependent liver injury, which was attributed to the saturation of nuclear karyopherin exportin-5 and downregulation of liver-derived microRNAs (miRNAs). Based on these findings, they suggested this risk can be minimized by optimizing shRNA dose and sequence. In this study, LV-AQP3 shRNA were injected three times, but the dose of one injection was 4×10^8 TU, which was determined based on the results of our previous study. It was significantly less compared to the previous study by Grimm et al[42]. Furthermore, the size of shRNA was 19-mer, the same size associated with the least toxicity in that study. In addition, the shRNA used in the current study was design to exert its effect only in the kidney. These may be the reason why I did not observe any serious side effects of in vivo shRNA transduction. Nevertheless, the possibility of similar consequences of oversaturated shRNA in the kidney as in the liver cannot be completely excluded.

To deliver gene or shRNA in a specific organ, the route of administration is an important issue. Specifically for the kidney, even though several invasive delivery techniques[43-46]; via renal artery, renal vein, and ureter, and direct parenchymal injection, have been developed and used in small rodents, hydrodynamic tail vein[47] delivery has recently been regarded as a simple and effective method for gene transfer into these animals. A previous study by Kobayashi et al[48-50] showed that intravenous injection of pU6-stem21, a model

of siRNA-expressing vector, by the hydrodynamics-based procedure resulted in significant suppression of the target transgene expression in the liver, kidney, and lung. Another previous study also demonstrated that hydrodynamic tail vein delivery of gene successfully changed the levels of transgene expression in the kidney and lung, even though their expression levels were approximately one-sixth to one-fifth of those in the liver. Based on these findings, a simple hydrodynamic tail vein injection of lentiviral vector was performed in the present study. Instead the number of injection time was increased to delivery more effectively to the kidney. As a result, LV-shAQP3 delivery, assessed by EGFP protein expression, was successful and sufficient in not only the kidney but also the colon and trachea.

Because of my main interest in kidney disease, the gene of interest selected to knockdown in this study was AQP3. The gene of interest was assumed to be the best if the presenting phenotype with deleted gene can be easily recognized by a simple test or assessment of blood or urine even before killing the mice. At the first time, therefore, AQP1 was selected for the candidate gene to be deleted. However, since the distribution of AQP1 was exclusively confined to the kidney (with scanty expression in the testis)[51], it was difficult to discriminate collecting duct cell-specific knockdown from non-specific knockdown. Accordingly, AQP3 was finally chosen based on the fact that AQP3 knockout mice also had polyuria and impairment of urinary concentrating[52] ability as AQP1 knockout

mice and that AQP3 was widely expressed in the body[53], including the colon, trachea, brain[54], and eye[55]. The results of the current study revealed that LV-shAQP3 injection to Hoxb7 Cre mice and consecutive injection of LV-Hoxb7 Cre and LV-shAQP3 knocked down AQP3 expression in the collecting duct cells of the kidney along with a significant increase in urine volume and a significant decrease in urine osmolality, while there was no changes in the expression of AQP3 in the colon and trachea, suggesting that this method successfully generated and could be a simple way to generate cell-specific knockdown mice.

V. CONCLUSION

In this study, introduction of LV-loxP shAQP3 to Hoxb7 Cre mice or consecutive injection of LV-Hoxb7 Cre and LV-loxP shAQP3 in C57BL/6J mice significantly reduced AQP3 protein and mRNA expression along with significant changes in urine volume and osmolality. In contrast, there were no changes in AQP3 protein and mRNA expression in the colon and trachea of these mice. These findings suggest that double transduction of Cre- and loxP-based lentivirus can be a simple way to generate cell-specific knockdown mice, and this method may also be applicable to other species.

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ABSTRACT (IN KOREAN)

Cre/loxP lentiviral vector 이중 형질주입을 이용한 세포-특이적 녹다운 마우스의 제작

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배경: 유전자의 생체 내 기능을 정확하게 규명하기 위해서는 특정 유전자 조작을 통하여 개발된 형질전환 동물의 이용이 유용하다. 그러나, 생명 유지에 필수적인 유전자를 조작하였을 때 실험 동물이 사망할 수 있을 뿐만 아니라, 영구적인 전신적 유전자 조작에 따른 모든 조직에서의 영향과 다른 유전자의 활성화 또는 불활성화, 그리고 성인에서 발생하는 질병에서의 유전자의 기능을 규명하는 것이 제한적이라는 등의 문제점이 있을 수 있다. 최근에는 이러한 문제의 극복을 위하여 조직-특이적, 세포-특이적 유전자 조작 기술이 개발되어 질환에 따른 각각의 조직 및 세포에서의 유전자 기능을

규명하는데 큰 도움이 되고 있다. 이러한 세포-특이적 유전자 조작의 방법으로는 Cre/loxP 시스템과 Tet-on/off 시스템들이 이용되고 있으나, 이들 방법은 embryonic stem cell에 세포-특이적 Cre 유전자와 loxP 유전자를 주입한 후 수정과 교배 과정을 거쳐 유전자 재조합 동물을 제작하게 되기 때문에 시간적뿐만 아니라 경제적인 문제로 이용이 제한적이라는 단점이 있다. 또한, 이들 방법 역시 위에서 언급하였던 문제점들을 완벽하게 해결해 주지는 못하고 있다.

목적: 본 연구에서는 Cre/loxP 시스템과 lentiviral vector를 이용하여 조건부 시기에, 그리고 특이 세포 및 조직에서만 유전자가 조작될 수 있는 동물 모델 시스템을 개발하고자 한다.

방법: 생체 외 실험으로는 집합관 세포와 메산지움 세포를 Hoxb7-Cre lentivirus (LV-Hoxb7-Cre)와 아쿠아포린-3 (AQP3)에 대한 shRNA를 포함한 loxP-shAQP3 lentivirus (LV-loxP shAQP3)로 처리하고 72시간 후 각 군에서 세포를 수집하였다. 생체 내 실험으로는 각각의 바이러스의 효과를 확인하기 위하여 3가지의 동물 모델을 대상으로 실험하였다. 첫째로, loxP-EGFP 마우스에 LV-Hoxb7 Cre를 주입하여 Hoxb7 프로모터의 효과를 확인하였다. 둘째로, Hoxb7 Cre 마우스에

LV-loxP scr과 LV-loxP shAQP3를 주입하여 loxP-shAQP3의 효과를 확인하였다. 셋째로, C57BL6/J 마우스에 LV-Hoxb7 Cre와 LV-loxP shAQP3를 각각 투여한 군들과 LV-Hoxb7 Cre + LV-loxP shAQP3 동시에 투여한 군으로 나누어 그 효과를 관찰하였다. 모든 동물들은 5일 간격으로 3회에 걸쳐 1ml의 phosphate-buffered saline 또는 lentivirus를 꼬리정맥 내로 주사하였다. 마지막 주사 후 4주째에 24시간 소변을 채취하고 동물을 희생하여 조직을 채취하였다. 각각의 세포와 마우스의 조직 내에서 EGFP, mCherry, 그리고 AQP3의 단백 발현은 Western blot과 면역형광염색방법을 이용하여 분석하였고, AQP3의 mRNA 발현은 Real-time PCR로 평가하였다. 24시간 동안 채취한 동물의 소변 양과 삼투질 농도도 측정하였다.

결과: 메산지움 배양세포를 각각의 바이러스로 처리하였을 때 LV-loxP shAQP3 처리군에서는 EGFP의 발현이 확인되었으나, LV-Hoxb7 Cre 처리군에서는 mCherry의 발현이 확인되지 않았다. 반면, 집합관 배양세포에서는 LV-loxP shAQP3 처리군에서의 EGFP와 LV-Hoxb7 Cre 군에서의 mCherry 모두가 발현되었으며, LV-Hoxb7 Cre + LV-loxP shAQP3 처리군에서만 AQP3의 발현 감소를 확인함으로써 Hoxb7 프로모터가 신장 집합관 세포에서만 작동함과 동시에 생체 외

실험에서 shAQP3의 효과를 규명하였다. 생체 내 첫 번째 실험에서는 EGFP-loxP 마우스에 LV-Hoxb7 Cre를 투여한 군에서 mCherry와 EGFP가 동일한 부분, 즉 신장 집합관 세포에서 발현되는 것을 관찰함으로써 마우스 생체 내에서 Hoxb7 프로모터의 작동을 확인하였다. 두 번째 실험인 Hoxb7 Cre 형질전환 마우스를 이용한 실험에서는 LV-loxP shAQP3를 투여한 군에서의 AQP3의 mRNA와 단백 발현이 대조군 및 LV-loxP scr 투여군에 비하여 의미있게 감소됨을 확인하였다. 또한, LV-loxP shAQP3 투여군에서 24시간 소변양은 의의있게 증가되었으며, 24시간 소변 내 삼투질 농도는 유의하게 감소되었다. 이와는 반대로, 대장과 기도 조직 내에서의 AQP3 발현은 각 군간에 차이가 없었다. 마지막으로, C57BL/6J 마우스를 이용한 실험에서는 LV-loxP shAQP3를 투여한 군에서는 신장 집합관 세포를 포함한 모든 세포에서 EGFP가 발현되었던 반면, LV-Hoxb7 Cre를 투여한 군에서는 신장 집합관 세포에서만 mCherry가 발현되었다. LV-Hoxb7 Cre + LV-loxP shAQP3를 동시에 투여한 군에서는 신장 집합관 세포에서만 mCherry가 발현되었으며, 신장 집합관 세포를 제외한 모든 세포에서 EGFP 발현이 확인되었고, 신장 집합관 세포에서의 AQP3 단백 발현 역시 대조군을 비롯한 다른 군들과 비교하였을 때 의미있게 감소되었다. 또한, LV-Hoxb7 Cre + LV-loxP shAQP3 군에서의

24시간 소변 양은 다른 군에 비하여 의의있게 증가되었으며, 24시간 소변 내 삼투질 농도는 유의하게 감소되었다. 그러나, AQP3가 발현되는 다른 장기인 대장과 기도 조직에서는 EGFP만이 발현되고 mCherry가 발현되지 않았으며, AQP3의 발현도 각 군간에 차이가 없었다.

결론: Lentiviral vector를 기반으로 한 Cre/loxP 시스템을 이용할 경우 매우 간단하게 세포-특이적 녹다운 마우스 모델을 제작할 수 있을 것으로 생각되며, 이러한 방법은 다른 종에도 적용이 가능할 것으로 사료된다.

핵심되는 말: Cre/loxP 시스템, lentiviral vector, 신장 집합관 세포, Hoxb7, 아쿠아포린-3