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Cover Letter

22-January-2018

Dr. W. Baumeister
Editor-in-Chief
Biochemical and Biophysical Research Communications

Dear Editor:

I wish to submit a Short Communication for publication in Biochemical and Biophysical Research Communications, titled "Optimal route of diphtheria toxin administration to eliminate native nephron progenitor cells in vivo for kidney regeneration." The paper was coauthored by Shohei Fukunaga, Shuichiro Yamanaka, Toshinari Fujimoto, Susumu Tajiri, Taketo Uchiyama, Kei Matsumoto, Takafumi Ito, and Kazuaki Tanabe.

We previously developed a method for in vitro nephron progenitor cell replacement based on the diphtheria toxin receptor system to facilitate organ regeneration. To enable the application of this method in vivo, we characterized the effects of diphtheria toxin on fetal mouse kidneys and optimized the route of administration and dose. In particular, we showed that the intra-amniotic injection of diphtheria toxin reduces kidney volume, decreases glomeruli, decreases differentiation, and was sufficient for nephron progenitor cell elimination. This method has applications for kidney regeneration and kidney research.

Owing to organ shortages, methods for organ regeneration have the potential to transform clinical approaches to the treatment of a variety of diseases. Accordingly, we believe that this paper has important practical implications and will be of interest to the readership of your journal

This manuscript has not been published or presented elsewhere in part or in entirety and is not under consideration by another journal. The study design was approved by the appropriate ethics review board. We have read and understood your journal's policies, and we believe that neither the manuscript nor the study violates any of these. There are no conflicts of interest to declare.

Thank you for your consideration. I look forward to hearing from you.

Sincerely,

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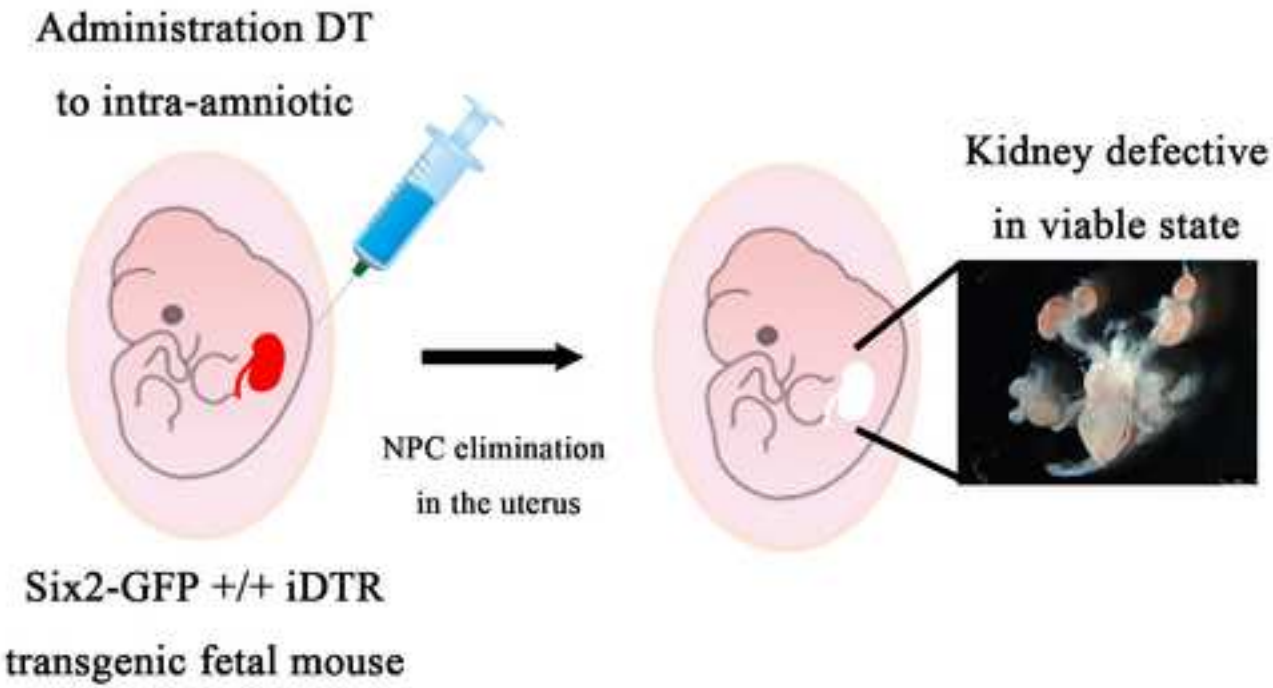
Highlights

- A method for in vivo nephron progenitor cell replacement was developed.
- Cell replacement via diphtheria toxin receptor was achieved in fetal mice.
- Intra-amniotic diphtheria toxin injection reduces glomeruli and eliminates NPCs.
- Intra-peritoneal is insufficient for NPC elimination in fetal mouse kidneys.

Graphical Abstract



Future Plan

- Kidney regeneration
- Analysis kidney development
- Analysis onset mechnism of CAKUT
- Disease model (Nephron number decrease model)

Title

Optimal route of diphtheria toxin administration to eliminate native nephron progenitor cells in vivo for kidney regeneration

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Abstract

To address the lack of organs for transplantation, we previously developed a method for organ regeneration in which nephron progenitor cell (NPC) replacement is performed via the diphtheria toxin receptor (DTR) system. In transgenic mice with NPC-specific expression of DTR, NPCs were eliminated by DT and replaced with NPCs lacking the DTR with the ability to differentiate into nephrons. However, this method has only been verified in vitro. For applications to natural models, such as animal fetuses, it is necessary to determine the optimal administration route and dose of DT. In this study, two DT administration routes (intra-peritoneal and intra-amniotic injection) were evaluated in fetal mice. The fetus was delivered by caesarean section at E18.5, and the fetal mouse kidney and RNA expression were evaluated. Additionally, the effect of the DT dose (25, 5, 0.5, and 0.05 ng/fetus-body) was studied. Intra-amniotic injection of DT led to a reduction in kidney volume, loss of glomeruli, and decreased differentiation marker expression. The intra-peritoneal route was not sufficient for NPC elimination. By establishing that intra-amniotic injection is the optimal administration route for DT, these results will facilitate studies of kidney regeneration in vivo. In addition, this method might be useful for analysis of kidney development at various time points by deleting NPCs during development.

Keywords: kidney regeneration, nephron progenitor cell, administration route, diphtheria toxin receptor

1. Introduction

Owing to the increase in patients with chronic kidney disease and those needing dialysis, the lack of organs for transplantation is a serious problem. There are many approaches to address this lack of organs [1]. Research in regenerative medicine has made remarkable progress; for example, iPS cells can differentiate into nephron progenitor cells (NPCs) and ureteric bud cells in vitro [2-3]. NPCs have attracted attention as cell sources for kidney regeneration [4].

We previously reported kidney regeneration from NPCs ex vivo and developed a host NPC elimination strategy using the iDTR system. The diphtheria toxin receptor (DTR) is expressed in Six2-positive NPCs; we eliminated host NPCs by diphtheria toxin (DT) administration. Using this method, 100% replacement of host NPCs with donor NPCs was possible by simultaneously eliminating host NPCs and transplanting donor NPCs [5]. This kidney regeneration strategy has two important features: (1) the nephron genesis area of a xenogeneic host is used [6-7] and (2) donor NPCs form host NPCs using the progenitor replacement system.

In our previous study, we evaluated the method ex vivo; in the progenitor replacement system, DT was added to fetal kidney culture medium and NPCs were replaced. We have also used an in vivo strategy to generate a functional kidney (Neo Kidney) de novo using an organogenic niche method

that uses the inherent developmental system of an immunocompromised xenogeneic host [6-11]. However, the natural kidney environment is considered important for kidney regeneration. Hence, it is necessary to investigate the progenitor replacement system using living fetuses in the womb, but such analyses using the iDTR system are lacking. DT is a very large molecule; therefore, it likely cannot pass through the blood-placental barrier by intra-peritoneal DT administration, a common route.

Establishment of a method for elimination of NPCs in fetuses in the uterus, without embryonic lethality, is necessary for the success of our kidney regeneration strategy. Therefore, we investigated (1) the optimal route of DT administration and (2) optimal DT dose for NPC elimination. We successfully established a living NPC elimination model by optimized intra-amniotic DT injection, without embryonic lethality.

2. Materials and Methods

2.1. Mouse maintenance and experiments

Mouse experiments were performed according to the Guidelines for the Proper Conduct of Animal Experiments of the Science Council of Japan (2006) and were approved by the institutional Animal Care and Use Committee of the Jikei University School of Medicine (Protocol number 2017-051). All efforts were made to minimize animal suffering.

C57BL6/NCrSlc mice were purchased from SLC Japan (Shizuoka, Japan). C57BL/6-Gt (ROSA) 26Sor [tm1(HBEGF)Awai]/J mice (iDTR) were purchased from Jackson Laboratory (Bar Harbor, ME, USA) [12]. Six2-GFP-Cre transgenic mice (Six2-mice) were gifted by McMahon [27]. Six2-mice were crossed with iDTR mice to obtain bigenic offspring (Six2-GFP +/+ iDTR-mice).

Genomic DNA was obtained from tail biopsies, and genotyping was performed using the following primers: for iDTR (wt product ~620 bp, iDTR product ~240 bp), 5′-aaagtcgctctgagttgttat-3′ (RosaFA), 5′-ggagcgggagaaatggatatg-3′ (RosaRA), and 5′- catcaaggaaaccctggactactg-3′ (SpliAcB); for Cre (~410 bp), 5′-ctaatcgccatcttccagcagg-3′ (F) and 5′-aggtgtagagaaggcacttagc-3′ (R).

2.2. Reagents

Diphtheria toxin (DT: 50 ng/μL; Wako, Osaka, Japan) was stored at -80°C until use. Freshly thawed DT stock solution was diluted in phosphate-buffered saline (PBS) and injected via the intra-peritoneal or intra-amniotic route.

2.3. Diphtheria toxin injection

Two pregnant female mice were injected intraperitoneally (IP) at E13.5 with 300 ng DT in 300 μL PBS. Additionally, for intra-amniotic injection, four pregnant female mice were anesthetized, a laparotomy was performed, and the uterus was delivered through the incision. Each embryo was

microinjected with 25, 5, 0.5, or 0.05 ng/fetus-body DT diluted in 50 μL PBS. The uterus was placed back into the abdominal cavity, and the incision was closed. Embryos were allowed to develop in situ until the indicated stages.

2.4. Kidney volume

The long axis (L) and width (W) were recorded using a stereomicroscope. Kidneys were approximated to spheroids and the kidney volume was calculated using the spheroid calculation method (Kidney volume (mL) = $\pi/6 \times L \times W^2$).

2.5. Histology and immunohistochemistry

For paraffin histology, fetal kidneys were fixed in 10% neutral-buffered formalin and stained with hematoxylin–eosin. For the immunohistochemical analysis, fetal kidneys were fixed in 4% formaldehyde, embedded in optimal cutting temperature compound, and cryosectioned at a thickness of 8 μm. Immunostaining was performed manually. Prior to immunostaining, frozen sections were warmed at room temperature, air-dried, and fixed to the slide using precooled acetone (-20°C) for 10 min, followed by rinsing with PBS. After blocking for 1 h at room temperature, the sections were incubated overnight at 4°C with primary antibodies. Sections were rinsed with PBS and incubated with secondary antibodies conjugated with AlexaFluor 488, 546, and 647 at room temperature for 1 h. Nuclei were stained with DAPI (S36938; Thermo Fisher Scientific, Waltham, MA, USA). The primary antibodies were as follows: rabbit anti-Six2 (11562-AP; Proteintech, Rosemont, IL, USA), rat anti- Cytokeratin-8 (TROMA-IC; Developmental Studies Hybridoma Bank, Iowa City, IA, USA), rabbit anti-Calbindin (C7354; SIGMA, Tokyo, Japan), goat anti-GATA3 (AF2605; R&D, Minneapolis, MN, USA), fluorescein-labeled Lotus Tetragonolobus Lectin (LTL) (FL1321; Vector Laboratories, Burlingame, CA, USA), rabbit anti-Podocin (ab50339; Abcam, Cambridge, UK), and mouse anti-GFP (MBL048-3; MBL, Nagoya, Japan).

2.6. Glomerular Number

Glomeruli were counted in the hematoxylin-eosin-stained E18.5 fetal mouse kidney and the sectioned area was determined. Next, the glomerular number was divided by the area of the section to obtain the glomerular number per unit area (pieces/mm^2). The number of glomeruli were counted by two different researchers.

2.7. Quantitative PCR

Total RNA was isolated from whole kidney using the RNeasy Micro Kit (Qiagen, Hilden, Germany), and cDNA was synthesized from 20 ng RNA using the PrimeScript™ RT Reagent Kit with gDNA Eraser (Takara, Shiga, Japan). Real-time PCR was performed using TaqMan® Master

Mix and TaqMan® Assay (Six2; Mm03003557_s1, Podocin; Mm01292252_m1, Calbindin 1; Mm00488647_m1; Thermo Fisher) with the Rotor-Gene Q (Qiagen). The housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) was used to normalize expression levels (Mm99999915_g1).

2.8. Electron microscopy

Specimens were fixed with a 2.5% glutaraldehyde and 2% paraformaldehyde mixture in 0.1 M phosphate buffer overnight at 4°C and then post-fixed with 1% osmium tetroxide in the same buffer at 4°C for 2 h. Specimens were dehydrated using a graded ethanol series, placed in propylene oxide, and subsequently embedded in Epok 812 (Oken, Tokyo, Japan). Ultrathin sections were prepared using a diamond knife, stained with a uranium acetate and lead citrate solution, and observed using an H-7500 electron microscope (Hitachi, Tokyo, Japan) at an accelerating voltage of 80 kV.

2.9. Statistics

Statistical analyses were performed with GraphPad Prism (version 7.0, GraphPad Software, CA, USA). P-values were determined using unpaired, two-tailed Student's t-tests. $P < 0.05$ was considered to be a statistically significant difference.

3. Results

3.1. Establishment of the optimal DT administration route

We investigated the route of DT administration for fetal NPC elimination in the uterus. First, we administered 300 ng/mother (approximately 15 μg/kg) DT to the mother's abdominal cavity by IP injection. In the IP group, the fetal kidney exhibited a slightly reduced volume (4.61 ± 0.2912 vs. 3.456 ± 0.2039 mL, $P = 0.0049$) (Fig. 1-C); however, the number of glomeruli per unit area did not differ between groups (18.89 ± 2.103 vs. 24.14 ± 2.508, $P = 0.142$) (Fig. 1-D), and sufficient NPC elimination was not observed.

Next, we directly administered DT at 25 ng/fetus-body by the intra-amniotic route at the E13.5 stage. For intra-amniotic injection, the fetal kidney exhibited a reduced volume (3.699 ± 0.244 vs. 0.6069 ± 0.1365 mL, $P < 0.0001$) (Fig. 1-G), number of glomeruli per unit area decreased by 94%, and we obtained sufficient NPC elimination (15.6 ± 2.114 vs. 0.9596 ± 0.5876, $P = 0.0006$) (Fig. 1-H).

3.2. Histological analysis

We performed a histological analysis of the fetal kidneys of Six2-GFP +/+ iDTR transgenic mice (Six2-GFP +/+ iDTR) and Six2-GFP -/+ iDTR transgenic mice (Six2-GFP -/+ iDTR). The Six2-GFP +/+ iDTR fetal kidney was defective and the glomeruli were almost entirely eliminated (Fig. 2). Some glomeruli remained; however, based on electron microscopy, the remaining glomeruli were

not structurally normal (Supplementary Fig. 1). In Six2-GFP +/+ iDTR mice, Six2-positive cells and cells positive for podocin, a marker of mature glomeruli, decreased compared to those of Six2-GFP -/+ iDTR mice. The ureteric bud (Cytokeratin-8 [CK8], Calbindin 1-positive cells) had complex branching in Six2-GFP -/+ iDTR, but less branching in Six2-GFP +/+ iDTR (Fig. 3).

3.3. mRNA expression

We compared mRNA expression levels between Six2-GFP +/+ iDTR and Six2-GFP -/+ iDTR fetal kidneys. The relative expression level of *Six2* in Six2-GFP +/+ iDTR fetal kidneys was lower than that in Six2-GFP -/+ iDTR fetal kidneys (1 ± 0.09111 vs. 0.15 ± 0.0331, $P = 0.0001$). In addition, *podocin*, a marker of mature glomeruli, and *Calbindin* levels were lower in a Six2-GFP +/+ iDTR fetus kidney than in a Six2-GFP -/+ iDTR fetal kidney (Fig. 3).

3.4. Optimization of the DT dose for NPC elimination

We investigated the appropriate dose of DT. We administered 25, 5, 0.5, and 0.05 ng/fetus-body DT by intra-amniotic delivery. At 5 ng/fetus-body DT or greater, fetal kidney volume and number of glomeruli per unit area decreased. NPC elimination was lower for 5 ng/fetus-body DT than 25 ng/fetus-body DT. Next, we compared the effects of each DT dose and found that kidney volume and glomeruli per unit area were inversely related to the DT dose (Fig. 4).

4. Discussion

The iDTR system was designed to enable the ablation of many different cell populations using a Cre-inducible DTR transgene, iDTR, and Cre-expressing mouse strains [12]. The iDTR system can be applied to organ regeneration. We obtained 100% replacement of host NPCs with donor NPCs in vivo by simultaneously eliminating host NPCs and transplanting donor NPCs at the nephrogenic zone. This method was used for kidney regeneration by NPC replacement using the iDTR system. Other studies have reported that the iDTR system is applicable to the liver, kidney, and pancreas [13-15]. However, despite numerous studies of the iDTR system in adult animals, few have examined its application in animal fetuses. There are no reports of NPC elimination by DT administration to the mother.

In this study, we initially administered DT to the abdominal cavity of the mother by IP injection. IP is a standard administration route for cell elimination using the iDTR system. In iDTR adult models, the DT dose differs among studies. In the TRECK (toxin receptor-mediated cell knockout) adult model, which is also a model of DTR-mediated ablation, DT is effective at greater than 0.5 μg/kg [16]. Therefore, we administered 300 ng/mother (approximately 15 μg/kg) DT to the abdominal cavity. After IP injection, the fetal kidney volume was decreased by 25%, but the glomerular number per unit area did not decrease and sufficient NPC elimination was not observed. DT has a large molecular weight (58342 Da); therefore, it is difficult for DT to pass the chorioallantoic placenta [17]. Even though a high DT dose was administered to the mother, sufficient DT was not delivered

to the fetus. A small amount of DT eliminated some, but not all, NPCs and therefore did not reduce glomeruli. Thus, for the IP route, the number of glomeruli per unit area did not decrease, despite reduction in the fetal kidney volume. Additionally, even in wild-type mice, when 500 μg/kg DT or greater is administered, mice die due to cytotoxicity by nonspecific uptake [18]; therefore, it is assumed that complete NPC elimination by the IP administration of DT is difficult. Similarly, for intravenous administration, DT must pass through the placenta to exert its effects, and it is assumed that complete NPC elimination is difficult. We evaluated the intra-amniotic injection method, i.e., direct injection of DT into the amniotic fluid. Intra-amniotic injection has been applied to create a mouse model of intrauterine infection and preterm delivery [19] and a model of fetal cardiomyopathy [20] and had been used for investigations of the effects of antibacterial agents against infections in utero [21, 22]. We evaluated the direct amniotic administration of 25 ng/fetus-body DT at E13.5. After intra-amniotic injection, fetal kidney volume was reduced by 84%, the number of glomeruli per unit area decreased by 94%, and sufficient NPC elimination was obtained (Fig. 1). Six2-positive cells nearly completely disappeared based on immunohistochemical staining. Based on GFP expression, the remaining Six2-positive cells did not express the DTR because the Cre-LoxP system was ineffective (Supplementary Fig. 2).

Additionally, an RT-PCR analysis showed that the expression of calbindin 1, which is expressed in the ureteric bud epithelium, decreased by 65%. By a process of mutual induction between the ureteric bud and metanephric mesenchyme, the ureteric bud undergoes a number of iterative dichotomous branching events to form the urinary collecting system [23-25]. The *Six2* gene is involved in this process; therefore, it is assumed that UB branching was suppressed by NPC elimination.

We further optimized the dose of DT by the intra-amniotic administration of 25, 5, 0.5, or 0.05 ng/fetus-body DT, and observed an effect for 5 ng/fetus-body DT or greater (Supplemental Fig. 3). NPC elimination was less extensive for 5 ng/fetus-body DT than 25 ng/fetus-body DT, and administration of 25 ng/fetus-body DT was necessary for sufficient NPC elimination (Fig. 4). In addition, a dose-dependent effect of DT on NPC elimination was observed (Fig. 4). This result is consistent with previous in vitro analyses (5). It is interesting that the injection of 5 ng/fetus-body and 25 ng/fetus-body DT reduced the number of glomeruli per unit area and the fetus was alive, suggesting that it can be used as a potential model of decreased nephron counts.

Although we did not examine the precise effects of DT on other organs, there were no apparent fetal abnormalities, and the fetuses were living, indicating a lack of lethal injury (Supplemental Fig. 4). In addition, we did not transplant NPCs after elimination of host NPCs; therefore, it is not clear whether this method can be applied to kidney regeneration in the maternal environment (i.e., in the uterus). Further studies are underway; for example, we are investigating donor NPC transplantation to this NPC elimination model as well as kidney regeneration from NPCs, as evaluated in previous

in vitro studies.

This study is the first to report that DT eliminates NPCs in the viable state using the iDTR mouse model. Establishment of NPC elimination in the uterus using the iDTR system is very important for establishing a new kidney regeneration method using NPCs. Furthermore, this method can also be applied to eliminate cells expressing other genes of interest, thereby helping to analyze the mechanisms underlying the development of congenital anomalies of the kidney and urinary tract [26] by the elimination of specific cells at an arbitrary developmental stage. In addition, this model, in which Six2-positive NPCs are eliminated, can be used to analyze the effects of nephron reductions in mice. This method for NPC elimination in the mother's womb using the iDTR system can not only be applied to kidney regeneration, but also to various disease models and embryology research.

Conflict of interest

The authors declare no competing financial interest.

Acknowledgements

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Figure Captions

Fig.1 Effects of the intra-peritoneal administration and intra-amniotic injection of DT
(A, B, E, F) Images show fetus kidneys dissected at E18.5 (Scale bar, 1 mm). (A, E) Images of Six2-GFP -/+ iDTR transgenic mouse kidneys. (E, F) Images of Six2-GFP +/+ iDTR transgenic mouse kidneys. Yellow arrow marks the mouse kidney. After intra-peritoneal administration, the fetal kidney was slightly smaller (A: n = 6 Six2-GFP -/+ iDTR transgenic mouse kidneys, B: n = 10 Six2-GFP +/+ iDTR transgenic mouse kidneys, C: n = 6 Six2-GFP -/+ iDTR transgenic mouse kidneys, n = 5 Six2-GFP +/+ iDTR transgenic mouse kidneys), but the number of glomeruli pre unit area did not decrease (D: n = 8 Six2-GFP -/+ iDTR transgenic mouse kidneys, n=5 Six2-GFP +/+ iDTR transgenic mouse kidneys). After intra-amniotic injection, the fetal kidney was smaller (E: n = 18 Six2-GFP -/+ iDTR transgenic mouse kidneys, F: n = 24 Six2-GFP +/+ iDTR transgenic mouse kidneys, G: n = 18 Six2-GFP -/+ iDTR transgenic mouse kidneys, n = 24 Six2-GFP +/+ iDTR transgenic mouse kidneys), the numbers of glomeruli pre unit decreased, and sufficient NPC elimination was obtained (H: n = 6 Six2-GFP -/+ iDTR transgenic mouse kidneys, 4 Six2-GFP +/+ iDTR transgenic mouse kidneys). **P < 0.005, ***P < 0.001, ****P < 0.0001, ns: not significant. Error bars in bar plots represent standard errors of the mean (SEM). DT: Diphtheria toxin, DTR: Diphtheria toxin receptor, GFP: Green fluorescent protein.

Figure 2. Histological analysis

(A, B) Images of fetus kidneys dissected at E18.5 revealed that Six2-GFP +/+ iDTR transgenic mouse kidneys (A) were markedly smaller than those of Six2-GFP -/+ iDTR transgenic mice (B) (A, B; Scale bar, 1 mm). (C–F) Hematoxylin and eosin staining showed that Six2-GFP +/+ iDTR fetus kidneys (C) were defective compared to those of Six2-GFP -/+ iDTR transgenic mice (D). (C, D; Scale bar, 500 μm). (E, F) In Six2-GFP -/+ iDTR transgenic mice (E), many glomeruli remained. In contrast, those of Six2-GFP +/+ iDTR transgenic mice (F) were almost entirely eliminated (E, F; Scale bar, 50 μm). DTR: Diphtheria Toxin Receptor, GFP: Green fluorescent protein.

Figure 3. Immunohistochemistry and mRNA expression

(A) Confocal immunofluorescence imaging of the kidney with anti-six2 (red), anti-Cytokeratin-8 (white). Nuclei were stained with DAPI (blue). In Six2-GFP +/+ iDTR kidneys, Six2-positive cells (red) decreased markedly compared to Six2-GFP -/+ iDTR kidneys. (B) Confocal immunofluorescence imaging of the kidney with anti-Podocin (green), anti-Cytokeratin-8 (white), and anti-GATA3 (red). Nuclei were stained with DAPI (blue). In Six2-GFP +/+ iDTR kidneys, podocin (a marker of mature glomeruli)-positive cells decreased markedly compared to Six2-GFP -/+ iDTR kidneys. (C) Confocal immunofluorescence imaging of the kidney with anti-GATA3 (red), anti-Calbindin 1 (white), and fluorescein-labeled Lotus Tetragonolobus Lectin (green). Nuclei were stained with DAPI (blue). The ureteric bud (Calbindin 1-positive cells) had complex branching in Six2-GFP -/+ iDTR, but branching was less extensive in Six2-GFP +/+ iDTR. (A; upper row; scale bar, 100 μm, lower column; scale bar, 50 μm) (B; scale bar, 100 μm) (C; scale bar, 100 μm) (D) The expression level of Six2 in a Six2-GFP +/+ iDTR fetus kidney was lower than that in a Six2-GFP -/+ iDTR fetus kidney (n = 4 Six2-GFP +/+ iDTR transgenic mouse kidneys treated by intra-peritoneal DT injection, 4 Six2-GFP +/+ iDTR transgenic mouse kidneys treated by intra-amniotic DT injection). In addition, podocin, which is a marker of mature glomeruli, and Calbindin levels were lower in a Six2-GFP +/+ iDTR fetus kidney than in the Six2-GFP -/+ iDTR fetus kidney. Data were normalized to the levels in glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*). **P < 0.05, ****P < 0.0001, ns: not significant. Error bars in bar plots represent standard errors of the mean (SEM). DT: Diphtheria toxin, DTR: Diphtheria Toxin Receptor, GFP: Green fluorescent protein. CK8: Cytokeratin-8. LTL: Lotus Tetragonolobus Lectin

Figure 4. Investigation of the DT dose.

We compared the effects of various DT doses administered by intra-amniotic injection, and found that the kidney volume (n = 4 Six2-GFP +/+ iDTR transgenic mouse kidneys treated with 0.05 ng/fetus-body DT, 4 that were treated 0.5 ng/fetus-body DT, 6 that were treated with 5 ng/fetus-body DT, and 24 that were treated with 25 ng/fetus-body DT) and the number of glomeruli per unit (n = 4 Six2-GFP +/+ iDTR transgenic mouse kidneys treated with 0.05 ng/fetus-body DT, 4 that were

treated with 0.5 ng/fetus-body DT, 6 that were treated with 5 ng/fetus-body DT, 4 that were treated with 25 ng/fetus-body DT) were inversely related to the DT dose. **P < 0.05, ****P < 0.0001, ns: not significant. Error bars in bar plots represent standard errors of the mean (SEM). DT: Diphtheria toxin, DTR: Diphtheria Toxin Receptor.

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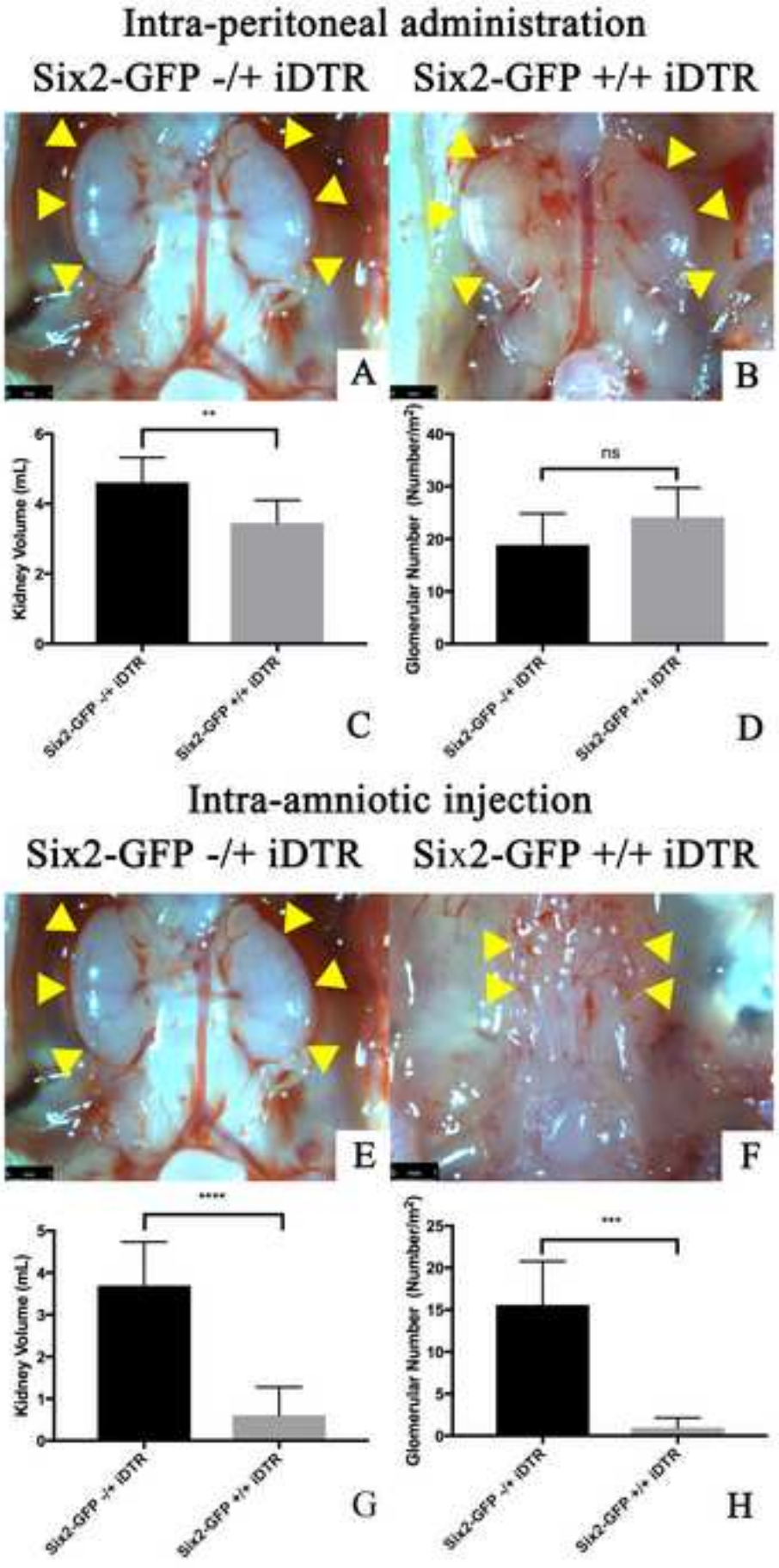

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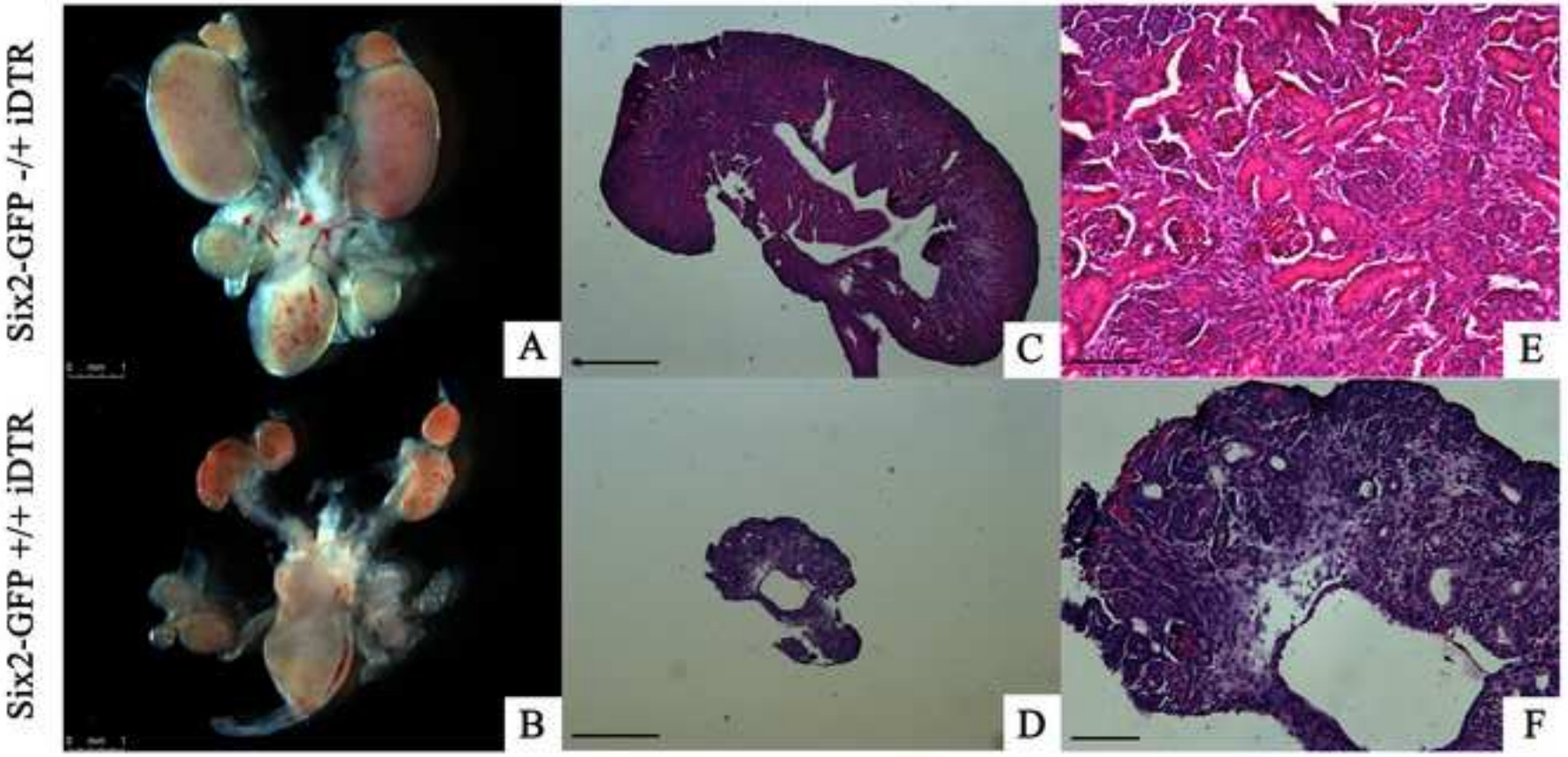

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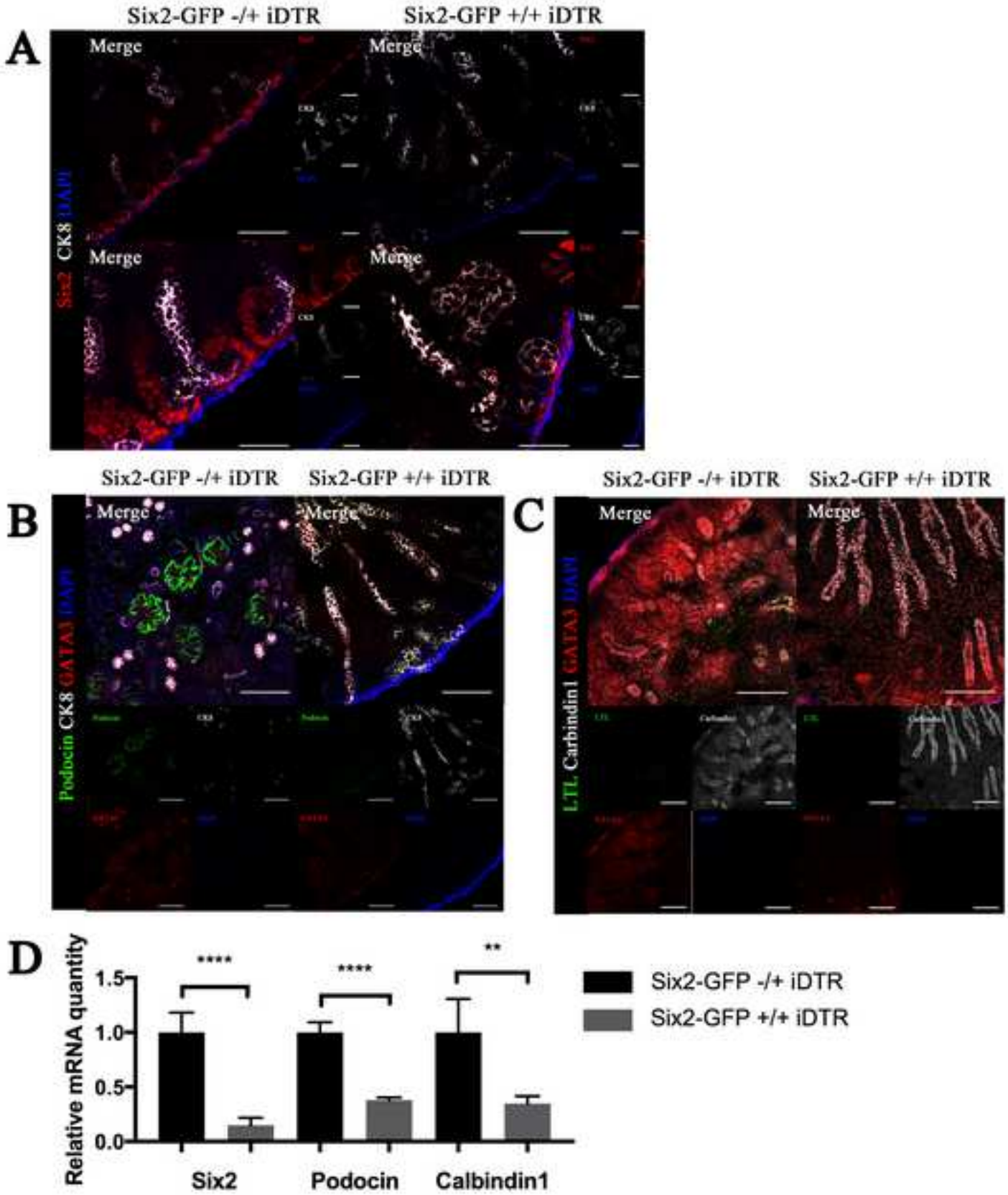

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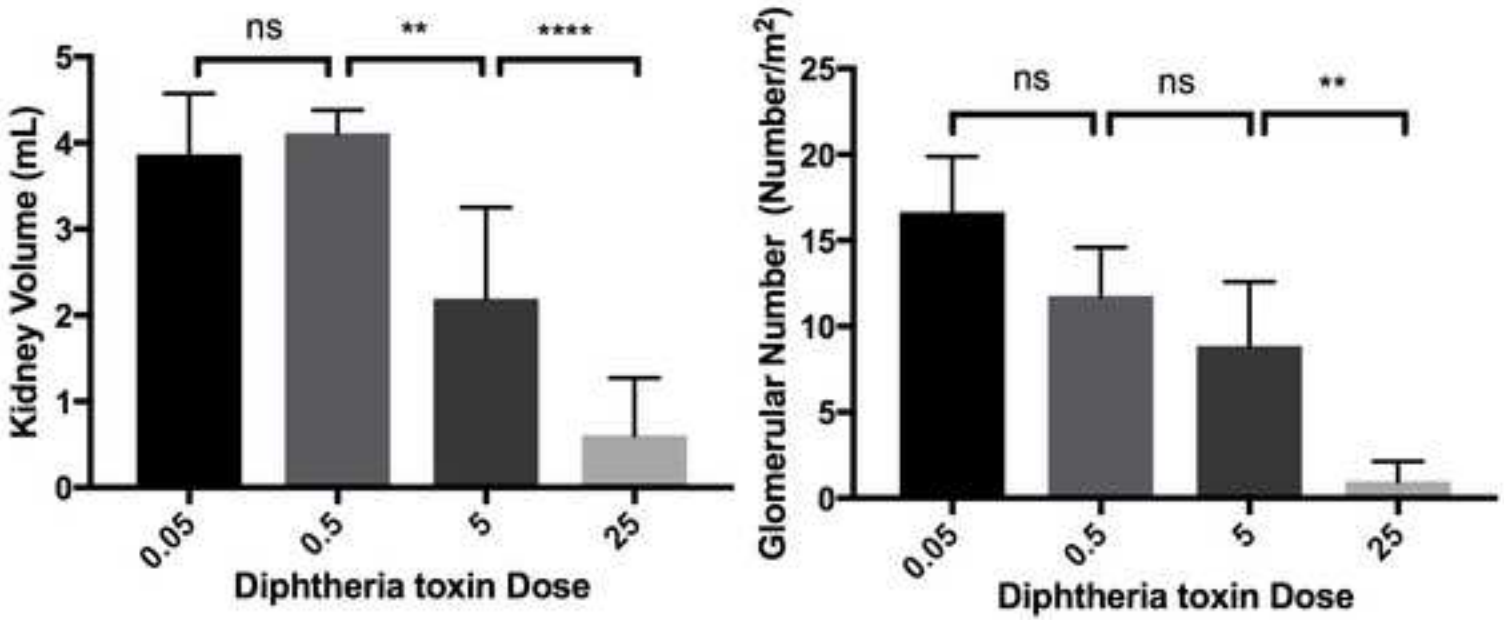

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