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TISSUE ENGINEERING AND REGENERATIVE MEDICINE

In Situ Tissue Regeneration of Renal Tissue Induced by Collagen Hydrogel Injection

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Key Words. Tissue-specific stem cells • Renal progenitors • Regeneration • Collagen • Renal failure

ABSTRACT

Host stem/progenitor cells can be mobilized and recruited to a target location using biomaterials, and these cells may be used for in situ tissue regeneration. The objective of this study was to investigate whether host biologic resources could be used to regenerate renal tissue in situ. Collagen hydrogel was injected into the kidneys of normal mice, and rat kidneys that had sustained ischemia/reperfusion injury. After injection, the kidneys of both animal models were examined up to 4 weeks for host tissue response. The infiltrating host cells present within the injection regions expressed renal stem/progenitor cell markers, PAX-2, CD24, and CD133, as well as mesenchymal stem cell marker, CD44. The regenerated renal structures were identified by immunohistochemistry for renal cell specific markers, including synaptopodin and CD31 for glomeruli and cytokeratin and neprilysin for tubules. Quantitatively, the number of glomeruli found in the injected regions was significantly higher when compared to normal regions of renal cortex. This phenomenon occurred in normal and ischemic injured kidneys. Furthermore, the renal function after ischemia/reperfusion injury was recovered after collagen hydrogel injection. These results demonstrate that introduction of biomaterials into the kidney is able to facilitate the regeneration of glomerular and tubular structures in normal and injured kidneys. Such an approach has the potential to become a simple and effective treatment for patients with renal failure. STEM CELLS TRANSLATIONAL MEDICINE *2018;7:241–250*

SIGNIFICANCE STATEMENT

Current treatment options for acute/chronic renal failures are limited to dialysis or renal transplantation. Dialysis can extend the survival for many patients, and renal transplantation can only restore the normal renal function. However, renal transplantation is severely limited by a donor supply and association with immunosuppressive complications and graft failure. The strategy of in situ renal regeneration is to utilize the host renal progenitor cells or adjacent stem cells to target the renal injury sites by biomaterials. The presence of an underlying regenerative mechanism in the form of tissue-specific stem/progenitor cells can suggest that there may be a potential therapeutic opportunity to bias the host response toward repair of renal injury.

INTRODUCTION

Chronic renal disease is a common condition that elevates the risk of renal failure, cardiovascular disease, and other complications [1]. The prevalence of chronic renal failure has continuously increased in the U.S. in the last decade [2]. The current standard treatment options for renal failure are dialysis and organ transplantation, both of which are associated with limitations. Dialysis, usually performed three times per week, impairs the patient's quality of life and does not replace renal functions such as synthesis of erythropoietin and calcitriol [3]. Transplantation, on the other hand, can replace all renal functions, but the rising occurrence of end-stage renal disease in the U.S. continues to outpace the rate of organ donation, as reflected by the fact that the waiting list continues to grow by 3,000–4,000 people per year [4]. Additionally, long-term outcomes of kidney transplantation remain unsatisfactory, mainly because of chronic immune rejection and complications associated with immunosuppressive medications [5, 6]. As a result, development of alternate therapies for renal failure is critically needed.

Cell-based approaches using tissue engineering and regenerative medicine techniques have offered new therapeutic opportunities for treating various pathologic conditions [7, 8]. During the past decade, a number of cell-based approaches using renal cells and/or biomaterials has been attempted to replace or restore renal functions [9–20]. These approaches usually require the selection of an appropriate cell source that can be

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isolated and expanded in large quantities in vitro, while maintaining cellular phenotypic and functional characteristics. However, the kidney is an extremely complex organ, which consists of at least 26 terminally differentiated cell types, including tubular epithelial cells, interstitial cells, glomerular cells, vascular cells, and so on [21]. To overcome these limitations, we have previously demonstrated that host cells expressing various stem cell markers could be mobilized into an implanted biomaterial, and these cells were able to differentiate into multiple cell lineages if appropriate culture conditions are provided [22–24]. This suggests that it may be possible to control tissue morphogenesis in vivo by providing the appropriate cues to infiltrating multipotent stem cells or tissue-specific progenitor cells. This novel strategy could lead to the production of functional tissues in situ, which is in contrast to conventional cell-based strategies that focus on in vitro manipulation of cells [25].

Several researchers have reported the existence of renal stem/progenitor cells, which can contribute to regeneration and repair in the kidney [26–37]. Several genes expressed during embryonic development are downregulated in mature kidney tissue, but are expressed again during recovery after renal injury. One such factor is paired box gene 2 (PAX-2) [36], which belongs to a family of transcription factors, and is required for development and proliferation of renal tubules [32]. Renal progenitor cells expressing CD24, CD133, and PAX-2 have been identified at the tubular and glomerular levels and can regenerate tubular cells in an animal model of acute renal failure [26, 31, 35, 37]. In addition, bone marrow-derived mesenchymal stem cells (MSCs) represent a potential source of cells that can regenerate renal tubules [27, 34]. The presence of an underlying regenerative mechanism in the form of tissue-specific stem and progenitor cells suggests that there might be a potential opportunity to bias the host response toward repair of renal injury. We hypothesized that these host stem cells or progenitors could be mobilized and recruited into target locations using an injectable or implantable biomaterial system. In the present study, we explored this hypothesis by introducing a biomaterial (collagen hydrogel) into normal kidneys and kidneys damaged by ischemia/reperfusion. We evaluated histological and functional changes after introduction of the biomaterial in order to determine whether renal tissue could be regenerated in situ.

Materials and Methods

Hydrogel Preparation

Rat tail type I collagen solution was obtained from BD Biosciences (San Jose, CA). Collagen hydrogels were prepared on ice prior to injection. Briefly, collagen solution (0.2% wt/vol) was neutralized by adding 1N NaOH solution to give a final pH of 7.4. In this study, neutralized collagen hydrogel as an injectable biomaterial was selected by testing with various hydrogel biomaterials (Supporting Information Fig. S1). Upon injection into the kidney, the collagen hydrogels thermo-gelled as they reached 37°C. All chemicals were obtained from Sigma-Aldrich Co. (St Louis, MO) and used as received unless stated otherwise.

Normal Mouse Model

All animal procedures were performed in accordance with a protocol approved by Wake Forest University Institutional Animal Care and Use Committee. CD1 mice (age, 6–8 weeks) were purchased from Charles River Laboratories Inc. (Wilmington, MA). Under anesthesia using isoflurane, the kidneys were accessed through a dorsal incision and then collagen hydrogels were injected into kidneys. Mice were divided into three experimental groups ($n = 20$ animals per group). The following treatments were administered via three injections into both kidneys using a 22-gauge needle: (a) collagen hydrogel (0.2% wt/vol, 50 μl/kidney), (b) saline (0.9% NaCl, 50 μl/kidney, Hospira, Inc, Lake Forest, IL), and (c) needle stick only. The injections with 3 mm depth and 30° angle were in the upper and lower poles of the kidney as well as the central region of the kidney. Sham operation served as a control ($n = 3$). The kidneys were harvested at 1, 2, 3, and 4 weeks after injection for analyses.

GFP Bone Marrow Chimeric Mouse Model

In order to identify the migration of bone marrow cells to the kidneys, donor bone marrow cells from transgenic C57Bl/6 GFP mice (C57Bl/6-Tg[UBC-GFP]30Scha/J, The Jackson Lab., Bar Harbor, ME) were isolated from femurs by flushing the marrow using a 1 ml syringe fitted with a 22-gauge needle. Repeated gently aspirations with the syringe was performed to prepare a single cell suspension of marrow cells. C57Bl/6 recipient mice were irradiated with 950 cGy using a Co^{60} irradiator 18–24 hours prior to bone marrow transplantation. Irradiated recipient C57Bl/6 mice were injected with 1×10^7 C57Bl/6 GFP bone marrow cells in a volume of 0.2 ml via the lateral tail vein. At 2 weeks post-transplantation, engraftment of the GFP bone marrow stem cells was monitored by obtaining a peripheral blood sample and determining the percent of circulating GFP positive nucleated cells using flow cytometry. Recipient mice expressing greater that 50% GFP positive circulating cells were designated as chimeric GFP mice and were then selected to be included in the experimental groups ($n = 5$). The injections of collagen hydrogel were performed as described above.

Renal Ischemia/Reperfusion Rat Model

Sixty Lewis rats (6–8 weeks, weight approximately 200 g, Charles River Laboratories Inc.) were anesthetized with an intraperitoneal injection of sodium pentobarbital (Nembutal, Ovation Pharmaceuticals, Inc., Deerfield, IL) at an initial dose of 50 mg/kg. If necessary, anesthesia was maintained using a second 25 mg/kg dose. The kidneys were exposed through a 3 cm midline abdominal incision. The bilateral renal pedicles were isolated. Each renal artery and vein were occluded with non-traumatic clamps (Micro-serrefine curved 6 mm, Fine Science Tools Inc. Foster, CA) for 60 minutes. At the end of this time period, the clamps were released to allow renal reperfusion. The abdominal wall was closed in two layers. The post-surgical pain was managed with buprenorphine (0.05 mg/kg subcutaneously, Reckitt Benckiser Pharmaceuticals, Richmond, VA). At 2 weeks after ischemia/reperfusion surgery, three injections of the neutralized collagen hydrogel were applied to the kidneys using a 20-gauge needle ($n = 10$ animals per time point). Three injections (5 mm depth and 30° angle) of the collagen hydrogel (0.2% wt/vol, 400 μl/kidney) were in the upper and lower poles of the kidney as well as the central region of the kidney using 20-gauge needle. Sham operation ($n = 10$ animals per time point) and saline injection (400 μl/kidney, $n = 10$ animals per time point) groups served as controls as described above. After collagen injection, the kidneys were harvested at 2 and 4 weeks for analyses.

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Histology and Immunohistochemistry

The retrieved kidneys were fixed in 4% paraformaldehyde and embedded in paraffin. Paraffin-embedded kidneys were cut into 5-μm sections. The sections were deparaffinized and rehydrated in a routine manner. Some sections were deparaffinized and stained with standard H&E for morphological evaluation.

For immunohistochemistry, the sections were immersed in 3% hydrogen peroxide in methanol for 30 minutes at room temperature to quench endogenous peroxidase activity. Target retrieval solution (Dako, Carpinteria, CA) was used to expose antigens in the samples. All slides were blocked in 10% serum (from the species in which the appropriate second antibody was raised) for 40 minutes. Sections were incubated with the primary antibodies for 60 minutes at room temperature. The sections were analyzed using the following antibodies for renal stem/progenitor cell-specific antigens: polyclonal rabbit anti-PAX-2 (5 μg/ml, Invitrogen, Carlsbad, CA), polyclonal rabbit anti-CD24 (1:50, Santa Cruz Biotechnology, Inc., Santa Cruz, CA), rat anti-mouse CD44 (1:20, BD Biosciences), and polyclonal rabbit anti-CD133 (1:200, Abcam, Cambridge, MA). Next, the sections washed and incubated with the biotinylated secondary antibody (Vector Laboratories, Burlingame, CA) for 30 minutes. The sections were then incubated with peroxidase-conjugated streptavidin for 30 minutes at room temperature. Detection of peroxidase was performed using either 3,3′-diaminobenzidine or Nova-RED (Vector Laboratories). Finally, the sections were counterstained with haematoxylin for 1 minute. Negative controls were incubated with 3% serum without primary antibody.

The infiltrating host cells into the injection region were examined by immunohistochemical staining for bromodeoxyuridine (BrdU, Sigma-Aldrich) and proliferating cell nuclear antigen (PCNA, Dako). For BrdU labeling, BrdU (100 mg/kg) was injected intraperitoneally into normal CD1 mice daily for 2 weeks. Quantitative analysis of PCNA-positive cells was performed by counting the positive nuclei in injured and normal regions from five randomly selected fields under a light microscope at magnification ×200.

For the GFP bone marrow chimeric mouse model, GFP+/PAX-2+ cells, GFP+/CD44+ cells, or PAX-2+/CD44+ cells were determined by double-immunofluorescence. Alexa Fluor 488 (1:300, goat anti-mouse IgG, Life Technologies) and Alexa Fluor 594 (1:300, goat anti-rabbit IgG, Life Technology) were used to visualize the double-stained cells in the injection region. Quantitative analysis of the numbers of GFP+/PAX-2+ cells, GFP+/CD44+ cells, and PAX-2+/CD44+ cells were counted, respectively. The percentage of expressed cells was calculated by dividing the total number of the antibody-stained cells by the total number of DAPI-stained cells.

The regenerated renal structures were identified by immunohistochemistry with cell specific markers: mouse anti-human CD31 (Dako), mouse anti-rat synaptopodin (Fitzgerald industries International, Concord, MA), cytokeratin (Dako), and polyclonal rabbit anti-neprilysin (1:100, Millipore, Billerica, MA). To quantify the effects of the injection of collagen gel, we counted and quantified the number of glomeruli (per mm^2) in the entire kidney in the injected and normal animals, respectively, by the 20 serial sections of the entire kidney (per animal, $n = 6$).

Functional Testing

Blood samples for creatinine determination were collected. Briefly, blood samples were collected from rat tail artery at weekly intervals from 1 week before ischemia/reperfusion injury until 2 weeks after surgery for all the animals. Serum creatinine was measured using an automatic modular analyzer (Synchron CX5 delta, Beckman Coulter Inc., Brea, CA).

Statistical Analysis

Data from the number of PCNA-positive cells and glomeruli and from the blood serum testing were analyzed by single-factor analysis of variance. A $p < .05$ was considered significant.

Results

Characterization of Infiltrating Host Cells and Renal Tissue Regeneration in Normal Mouse Kidney

To assess the levels of host cellular infiltration and determine whether these cells could contribute to renal tissue regeneration, neutralized collagen hydrogels (0.2% wt/vol) were injected into the kidneys of normal CD1 mice. Saline injection and needle stick without injection of collagen hydrogel were also performed as controls (Supporting Information Fig. S2). At 2 weeks after injection, each kidney contained inflammatory and fibroblastic cells in the injected regions (Fig. 1A). However, a higher number of regenerated glomerular-like structures was seen in the collagen injected group as compared to the other groups. To detect host cells that had infiltrated into the injection region of the kidney, we examined the localization of cells positive for PCNA, which is expressed particularly in the early G1 and S phases of the cell cycle, and is a marker for proliferating cells. Kidneys injected with collagen contained a large PCNA-positive cell population and showed progressive renal tissue formation in the injected regions over time (Fig. 1B). The number of PCNA-positive cells in the injected regions was significantly higher than in normal regions ($p < .01$, Fig. 1C). This indicates that host cells are able to migrate from other areas into the injected regions. Interestingly, it seems that these host cells (PCNA-positive) contribute to the formation of renal structures, as evidenced by the high number of glomeruli that were observed in the injected regions in the collagen hydrogel treated animals.

H&E stained image showed the injected and normal region (Fig. 2A), and the regenerated renal structures in the injected region were identified by immunohistochemistry with renal cell specific markers (Fig. 2B–2H). The glomerular-like structures expressed synaptopodin (Fig. 2B) and CD31 (Fig. 2C). In addition, the regenerated glomerular-like structure in the injected regions were confirmed by detecting PCNA expression (Fig. 2D). In fact, the number of glomeruli found in the collagen hydrogel injected regions was significantly higher as compared to native kidney tissue regions and the other groups ($p < .05$, Fig. 2F). Also, the tubular-like structure expressed cytokeratin (Fig. 2G) in the injection region. To localize and follow the cells that infiltrated into the collagen biomaterial in normal mouse kidneys, thymidine analog BrdU was injected intraperitoneally daily for 2 weeks, and then the kidneys were removed and stained with an anti-BrdU antibody. BrdU-positive cells were localized in both the glomeruli (Fig. 2E) and tubules (Fig. 2H).

To determine if the cells that infiltrated into the collagen biomaterial contain host renal stem/progenitor cells, immunohistochemistry for PAX-2, CD24, and CD44 was performed. The infiltrating host cells present within the injected regions of these kidneys expressed both renal stem/progenitor cell markers, PAX-2 and CD24, as well as the MSC marker, CD44. We observed the

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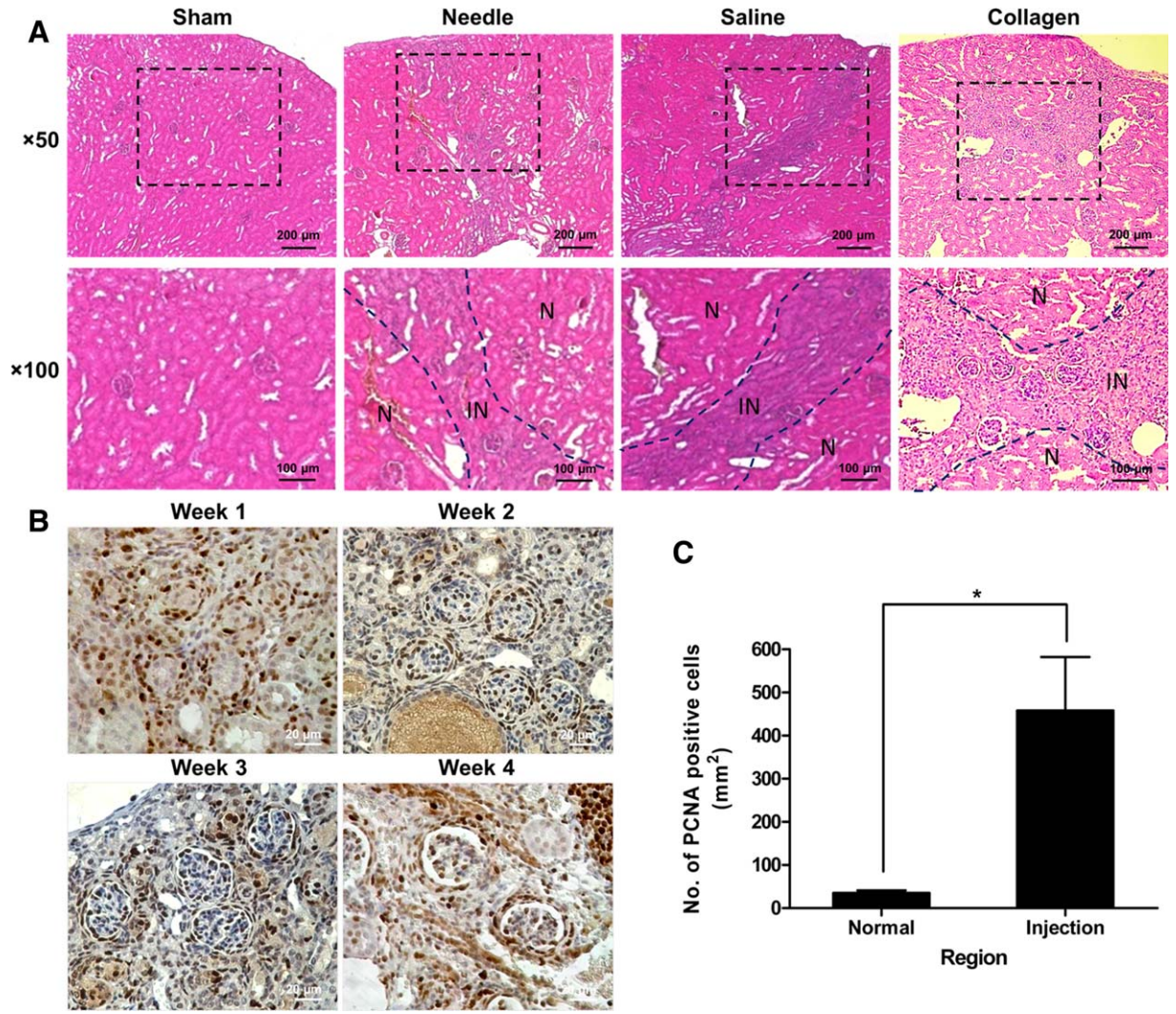

Figure 1. Histological evaluation of all experimental groups after 2 weeks injection. **(A)**: H&E staining of sham control, needle, saline, and collagen (×50: scale bar = 200 μm, ×100: scale bar = 100 μm). **(B)**: PCNA-positive cells migrating to the injection regions of the normal CD1 mouse kidneys at 1, 2, 3, and 4 weeks after collagen injection (×400, scale bar = 20 μm). **(C)**: Number of PCNA-positive cells in the normal and injection regions after 2-week collagen injection (*, $p < .01$). Abbreviation: PCNA, proliferating cell nuclear antigen.

presence of CD44-positive cells (Fig. 2I–2K) and CD133-positive cells (Fig. 2L–2N) in the injured regions after collagen hydrogel injection. These cells were localized within the tubular area and at the glomerular level within the parietal layer of Bowman's capsule. However, we did not observe cells expressing CD44 or CD133 in the normal regions (uninjured) of the same kidney.

GFP Bone Marrow Chimeric Mouse Model

To determine the origin of the infiltrating cells in the kidney tissue, GFP-expressing bone marrow chimeric mouse model was created. Injection of collagen hydrogel into the kidney induced the migration of bone marrow cells to the kidney as evidenced by the presence of GFP positive cells in the injection region. After 2 weeks of collagen hydrogel injection, PAX-2 positive cells were significantly increased at the site of collagen injection when compared to the normal region (Fig. 3A). The PAX-2 positive cells appear to be associated with the collecting ducts within the kidney. PAX-2 is expressed during embryological development. The presence of PAX-2 positive cells suggests that early progenitor cells may have been activated by the injected collagen hydrogels. At least 10% of the PAX-2 positive cells were also GFP positive suggesting that these cells originated from the bone marrow (Fig. 3B). CD44 positive cells are also present at the injection region (Fig. 3C). CD44 is a receptor for hyaluronic acid and is expressed on some stem cell populations including bone marrow hematopoietic stem cells and bone marrow-derived MSCs [30]. There is a small population of GFP and CD44 positive cells suggesting that this population of cells originated from the bone marrow (Fig. 3D). Very few of these cells expressed both CD44 and PAX-2, indicating that these markers identify two distinct cell populations within the kidney (Fig. 3E, 3F).

Renal Ischemia/Reperfusion Rat Model

To determine whether infiltrated cells form renal structures in injured kidney, the rat model of renal ischemia and reperfusion was created and observed for least 2 weeks after injury. The ischemia/reperfusion-injured kidneys showed tubular dilation and brush border loss (Fig. 4B) as well as intratubular cast formation

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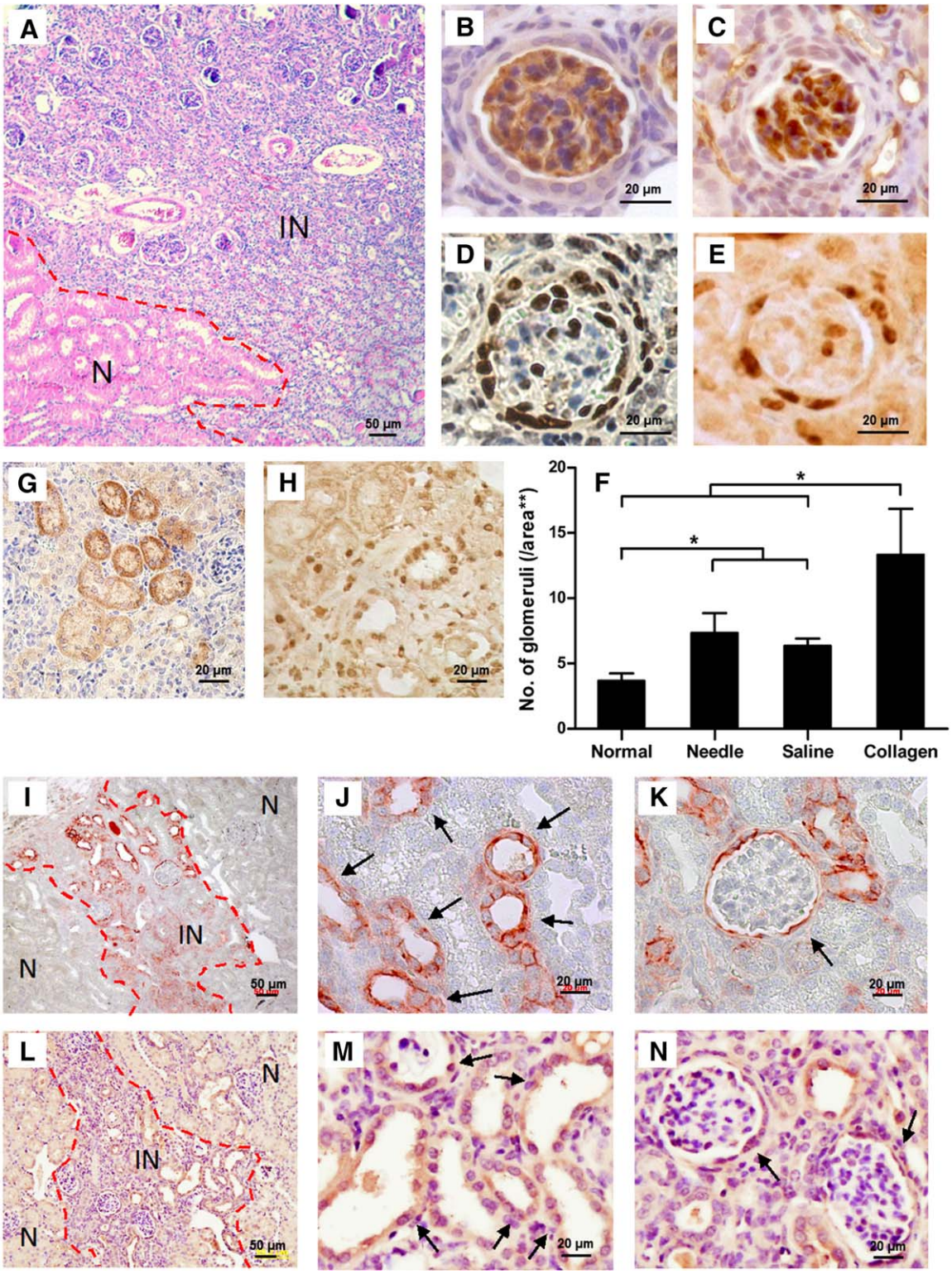

Figure 2. Regenerated renal structures in the injection regions of the normal CD1 mouse kidneys at 2 weeks after injection. **(A)**: The dashed line separates injection region from host tissue (H&E, scale bar = 50 μm). Identification of regenerated glomerular-like structure; **(B)** synaptopodin, **(C)** CD31, **(D)** proliferating cell nuclear antigen, **(E)** bromodeoxyuridine (BrdU) expression, and **(F)** quantitative analysis of number of glomeruli per area in the normal CD1 mouse kidneys after injection (*, $p < .05$). (B–E, **G**, **H**) scale bar = 20 μm. Tubular-like structure; (G) cytokeratin and (H) BrdU expression. **(I–K)**: CD44 and **(L–N)** CD133 expression of the infiltrating cells in the injection regions of the normal CD1 mouse kidneys. Immunohistochemistry shows expression of both CD44 and CD133 in the Bowman's capsule and tubuli at 1 week after injection. No observation of CD44 and CD133 expression in the normal regions in the same kidneys. (I, L) scale bar = 50 μm, (J, K, M, N) scale bar = 20 μm. Arrows indicate the positive expression of CD44 and CD133. Abbreviations: IN, injection region; N, normal region.

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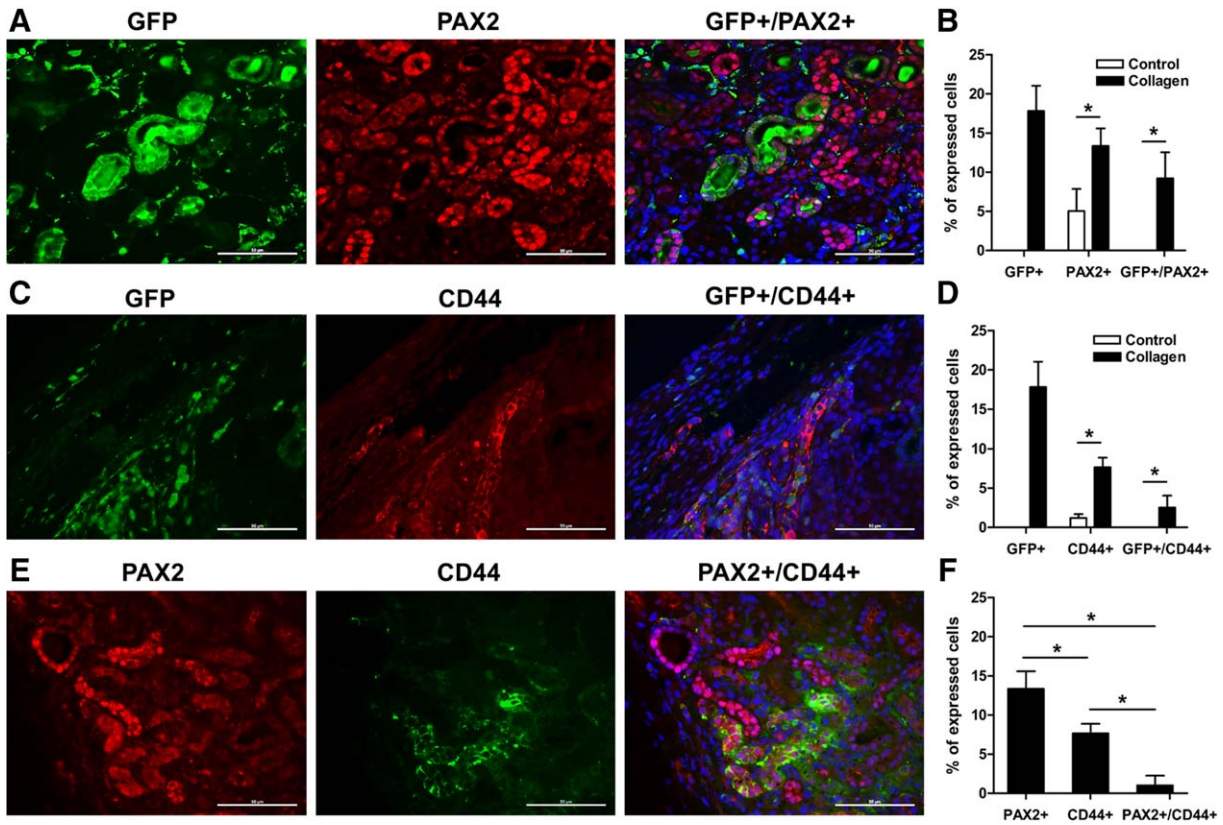

Figure 3. Immunofluorescent analysis of GFP expressing cells in the injection region at 2 weeks after collagen injection; **(A)** GFP+/PAX-2+ and **(B)** numbers of GFP+ cells and/or PAX-2+ cells, **(C)** GFP+/CD44+ and **(D)** numbers of GFP+ cells and/or CD44+ cells, and **(E)** PAX-2+/CD44+ and **(F)** numbers of PAX-2+ cells and/or CD44+ cells (*, $p < .05$). Scale bar = 50 μm.

and degeneration of tubular architecture. Some tubular structures became edematous and necrotic. The number of glomeruli decreased in the injured kidneys, and some swelled and developed sclerosis. After ischemia/reperfusion injury was confirmed, the collagen hydrogels were injected directly into the injured kidneys. The injected regions were readily identified, as most showed the presence of increased interstitial leukocytes and other infiltrating cells (Fig. 4C).

Regenerated glomerular-like (Fig. 4D) and tubular-like (Fig. 4H) structures were observed at 2 weeks after the collagen hydrogel injection. To characterize the phenotype of the regenerated structures, we examined the expression of the renal cell markers, synaptopodin (Fig. 4E) and CD31 (Fig. 4F). In addition, the tubular structure stained positive for neprilysin (Fig. 4I). After ischemic injury, the number of glomeruli in the native tissue was significantly decreased ($7.9 \pm 0.35/mm^2$, $4.02 \pm 0.18/mm^2$, and $3.29 \pm 0.186/mm^2$; normal kidney, 2 weeks, and 4 weeks after surgery, respectively; $p < .0001$). However, in the collagen hydrogel injection group, the number of glomeruli was significantly increased in the injected regions compared to native regions with ischemic injury ($11.44 \pm 0.72/mm^2$ vs. $4.02 \pm 0.18/mm^2$; $12.08 \pm 1.2/mm^2$ vs. $3.29 \pm 0.186/mm^2$; 2 weeks and 4 weeks; $p < .01$). Interestingly, the density of the glomeruli was higher within the collagen biomaterial than in normal kidney (Fig. 4G).

To identify the host cells present in the injected regions, immunohistochemistry for specific renal stem/progenitor cell markers was performed (Fig. 5). PCNA-positive cells in the injected regions were increased (Fig. 5A). Immunohistochemistry of the collagen injection regions from ischemic kidneys revealed that CD24, a marker of the renal embryonic progenitor cells, was expressed. In the injection regions, we observed the presence of CD24-positive cells of the interstitial and tubular structures (Fig. 5B). The CD44-positive (Fig. 5C) and PAX-2-positive (Fig. 5D) cells were identified not only in the interstitial and tubular structure, but also at the glomerular level within the parietal layer of Bowman's capsule in the injection area (Fig. 5E). These cells were rarely found in the native kidney tissue outside the injection regions.

To determine whether the regenerated glomeruli and tubules could contribute to functional recovery of the kidney, blood samples were analyzed for creatinine levels. At 2 weeks after renal ischemia and reperfusion, the blood serum creatinine level was maximized. One week after the collagen injection, the renal function improved in all experimental groups (blood serum creatinine level; 1.26 ± 0.29 mg/dl in the saline group and 0.76 ± 0.1 mg/dl in the collagen group). Although there was no significant difference in the study groups (p value = .8), there was a trend indicating that collagen injection led to the improvement in renal function when compared with the sham group (Fig. 5F).

Discussion

Previous studies demonstrate that host cell infiltrates into a biomaterial implant are not entirely comprised of inflammatory and fibroblast-like cells, and that the normal inflammatory process can be altered by incorporating agents that influence

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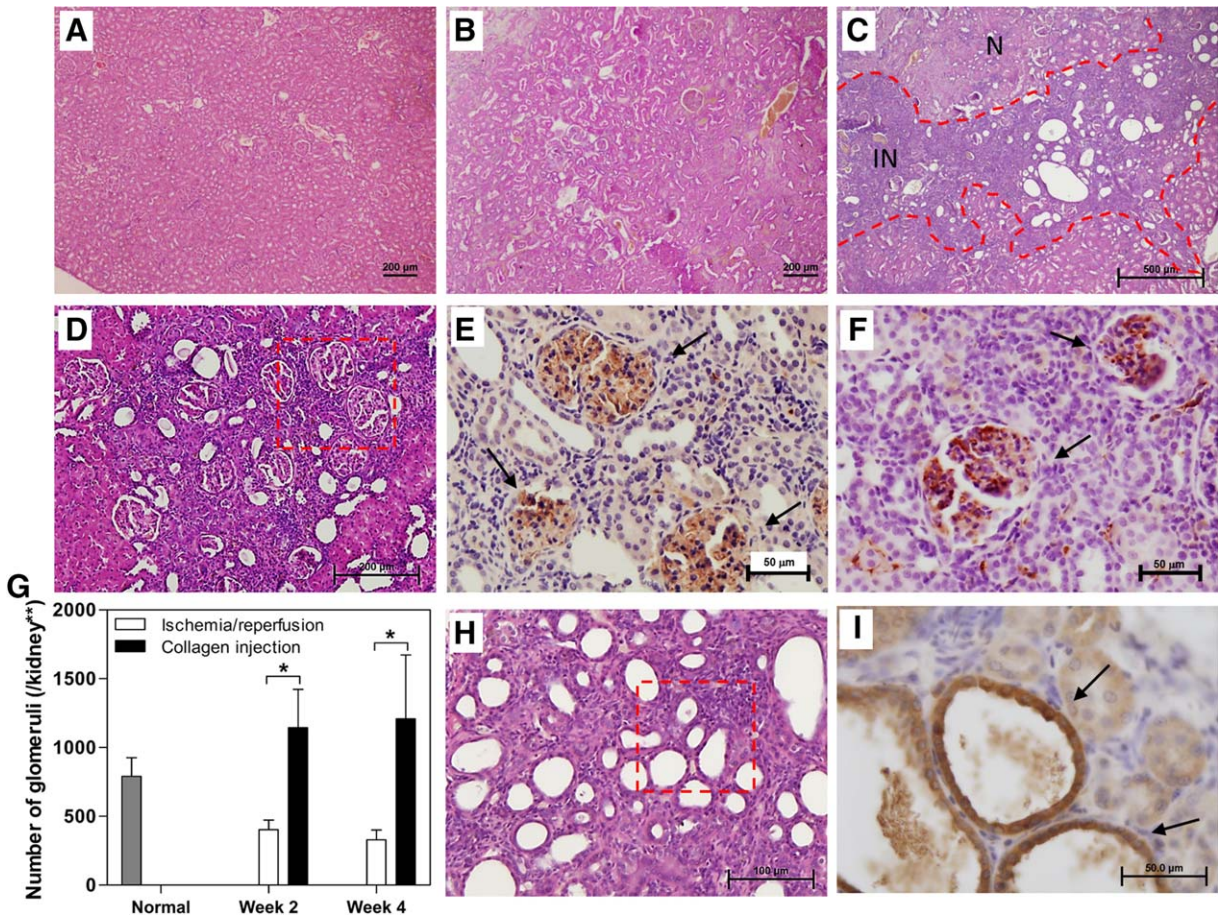

Figure 4. Renal ischemia/reperfusion rat model at 2 weeks after injection; **(A)** normal (H&E, scale bar = 200 μm), **(B)** ischemic injured (H&E, scale bar = 200 μm), and **(C)** collagen-injection regions of rat kidneys (H&E, scale bar = 500 μm). Identification of regenerated renal structures containing glomerular-like structure; **(D)** H&E (scale bar = 200 μm), **(E)** synaptopodin (scale bar = 50 μm), **(F)** CD31 (scale bar = 50 μm), and **(G)** quantitative analysis of number of glomeruli in the whole kidney of the renal ischemia/reperfusion rats; number of glomeruli per kidney (*, $p < .05$, **20 serial sections of the entire kidney), and tubular-like structure; **(H)** H&E (scale bar = 100 μm) and **(I)** neprilysin (scale bar = 50 μm) in the injection regions of the renal ischemia/reperfusion rats. Arrows indicate the positive expression of specific antibody. Abbreviations: IN, injection region; N, normal region.

microenvironmental cues [22]. We showed that infiltrating cells are capable of differentiating into multiple cell lineages if appropriate conditions are provided. These results suggest that it is possible to recruit a predominance of cells with multi-lineage potential into a specific biomaterial system. Therefore, it may be feasible to enrich the infiltrate with such cell types and control their fate, provided proper substrate-mediated signaling can be imparted into the biomaterial [23, 24].

Various types of host cells can be utilized for in situ renal tissue regeneration. Some research groups have recently reported the existence of renal stem/progenitor cells in the kidney. Bussolati et al. demonstrated the presence of a resident population of stem cells expressing CD133 and PAX-2 markers in adult normal human kidney and suggested that these cells were capable of expansion and, potentially, self-renewal [26]. Sagrinati et al. isolated and characterized multipotent progenitor cells that expressed CD24 and CD133 from the Bowman's capsule of adult human kidneys [35]. These cells could be induced to generate mature, functional, tubular cells with phenotypic features of proximal and/or distal tubules. Furthermore, Herrera et al. demonstrated that the mechanisms underlying the migration and homing of CD44 expressing MSCs to injured renal tissue [29, 30]. It would seem that these cells are the key to the underlying regenerative machinery [38–40].

In the present study, we investigated the possibility of using the body's biologic and environmental resources in situ for renal tissue regeneration. As an initial step, we examined whether injectable biomaterials could facilitate recruitment of host renal stem/progenitor cells that could participate in the renal regenerative process. We used a simple approach to address this concept by using collagen hydrogel as an injectable biomaterial. Collagen is known as one of the most abundant extracellular matrix (ECM) in the body [41]. Collagen plays a major role in the formation of tissues and organs, and is involved in various functional properties of cells. Moreover, collagen hydrogel is flowable, suggesting the possibility of an easily injectable, biocompatible material [42–44].

After collagen hydrogel injection, we found that a population of host cells expressing PAX-2, CD24, CD133, and CD44 was able to infiltrate the injection regions of both normal mice and rats with renal ischemia/reperfusion injury. Moreover, it seems that these cells contribute to the regeneration of renal tissue structures. The CD44-positive cells were localized within the tubular

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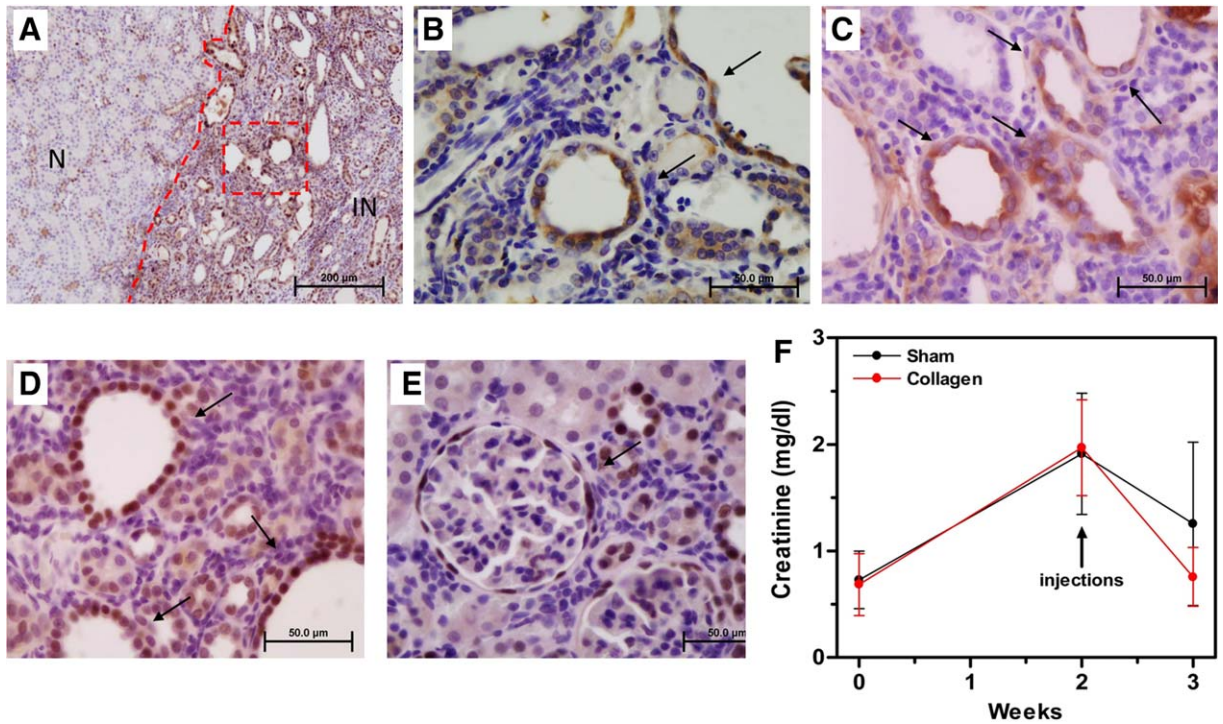

Figure 5. Identification of the infiltrating cells in the injection regions of the renal ischemia/reperfusion rats at 2 weeks after injection; **(A)** proliferating cell nuclear antigen (scale bar = 200 μm), **(B)** CD24 (scale bar = 50 μm), **(C)** CD44 (scale bar = 50 μm), and **(D, E)** PAX-2 (scale bar = 50 μm) expression. Arrows indicate the positive expression of specific antibody. **(F)** Blood serum analysis of creatinine levels for functional evaluation of the ischemic injured kidneys. Abbreviations: IN, injection region; N, normal region.

area and at the glomerular level within the parietal layer of Bowman's capsule in the injection regions. These cells proliferate and eventually re-differentiate into typical renal cells during the regenerative process. Many researchers have reported that the renal stem cells may activate in the entire injured region of the kidney. Interestingly, in our present study, the renal stem/progenitor cells were localized to the injured regions.

In the ischemic injured kidney of rat, we found PAX-2^+, $CD24^+$, and $CD133^+$ cells as well as $CD44^+$ cells. These cells proliferated, differentiated, and formed renal structures, including those with glomerular-like and tubular-like morphologies. However, a small population of these cells was also observed in adjacent normal regions. During the recovery after renal ischemia, these cells might activate in larger regions [30, 45, 46].

The present study shows that recruitment of host renal stem/progenitor cells within the injured kidney facilitated the structural and functional recovery. Although the mechanism of this phenomenon is unclear, it may be due to the host response to the injury as well as biological microenvironment (flexible space) and mechanical stimulus (pressure) created by the administration of collagen hydrogel (Supporting Information Fig. S3). Additionally, collagen hydrogel, being the major ECM in the kidney, may have promoted repair, reduce cell death locally and diminishes post-injury glomeruli loss. The host cells may respond to local environmental stimulation with differentiation into glomerular and tubular cells in situ. Furthermore, it is now known that there are somatic stem cells that mobilize to remote damaged tissue sites, where they differentiate into required lineages and participate in tissue/organ repair and regeneration [47–49]. However, the mechanisms of stem cell migration to injured tissue and cell differentiation into glomerular and tubular cells are still unclear. In order to determine whether the bone marrow cells could contribute to renal regeneration, bone marrow reconstitution using chimeric GFP-expressing cells was performed after whole-body irradiation. Of interest, it has been found that the GFP-expressing cells were localized in only the injection region, suggesting that BM-derived cells also in the regenerative process. Further evidence of the renal regeneration by the collagen injection was also observed from the presence of GFP^+/PAX-2^+ and GFP^+/$CD44^+$ cells in the injection region.

We have shown the influence of renal tissue regeneration due to host response activated by the collagen injection. This can also include inflammatory cells, such as lymphocytes, macrophages (dendritic cells), and neutrophils [50]. Cytokines such as IL-6 and IL-22 may be involved in this response induced tissue regeneration by activation of local stem cell/progenitor cells [51]. It may be possible that after hydrogel injection, a proliferative response involving immune cells and migrations of host cells into the injected region to contribute to the regenerative process. More detailed mechanistic studies including the role of inflammation are planned for the future experiments. Obviously, understanding the activation, recruitment, and differentiation of the renal stem/progenitor cells that accelerate in situ tissue regeneration will assist the development of novel therapeutics for the treatment of renal failure.

Conclusion

This study demonstrates that the introduction of injectable collagen hydrogel into kidneys is able to facilitate recruitment of host

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renal stem cells or progenitors that contribute to the in situ regeneration of renal glomerular and tubular structures. These results suggest that it is possible to recruit a predominance of cells with renal regenerative potential into a scaffolding system. This simple approach may become a preferred treatment for patients with renal failure.

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Author Contributions

S.J.L., A.A., and J.J.Y.: conception and design; S.J.L., H.-J.W., T.-H.K., J.S.C., and G.K.: collection and/or assembly of data; S.J.L., H.-J.W., T.-H.K., J.S.C., G.K., and J.D.J.: data analysis and interpretation; S.J.L., H.-J.W., and J.D.J.: manuscript writing; S.J.L., A.A., and J.J.Y.: final approval of manuscript; A.A. and J.J.Y.: financial support.

Disclosure of Potential Conflicts of Interest

The authors indicted no potential conflicts of interest.

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