J. Microbiol. Biotechnol. (2005), 15(1), 105–111

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Renal Precursor Cell Transplantation Using Biodegradable Polymer Scaffolds

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Received: March 18, 2004
Accepted: October 25, 2004

Abstract End-stage renal disease is a fatal and devastating disease that is caused by progressive and irreversible loss of functioning nephrons in the kidney. Dialysis and renal transplantation are the common treatments at present, but these treatments have severe limitations. The present study investigated the possibility of reconstructing renal tissues by transplantation of renal precursor cells to replace the current treatments for end-stage renal disease. Embryonic renal precursor cells, freshly isolated from metanephroi of rat fetus at day 15 post-gestation, were seeded on biodegradable polymer scaffolds and transplanted into peritoneal cavities of athymic mice for three weeks. Histologic sections stained with hematoxylin & eosin and periodic acid-Schiff revealed the formation of primitive glomeruli, tubules, and blood vessels, suggesting the potential of embryonic renal precursor cells to reconstitute renal tissues. Immunohistochemical staining for proliferating cell nuclear antigen, a marker of proliferating cells, showed intensive nuclear expression in the regenerated renal structures, suggesting renal tissue reconstitution by transplanted embryonic renal precursor cells. This study demonstrates the reconstitution of renal tissue *in vivo* by transplanting renal precursor cells with biodegradable polymer scaffolds, which could be utilized as a new method for partial or full restoration of renal structure and function in the treatment of end-stage renal disease.

Key words: Tissue engineering, renal precursor cell, biodegradable polymer scaffold

End-stage renal disease (ESRD) is a devastating disease that is caused by progressive and irreversible loss of functioning nephrons. Dialysis and renal transplantation are the common treatments of ESRD at present. Although these treatments obviously reduce the morbidity and mortality of patients, the treatments have several limitations. Dialysis can substantially reduce the morbidity and extend the life-times of patients, but it is only a temporal treatment that cannot restore the whole renal functions and often brings severe complications. Renal transplantation can restore the whole renal functions, but the organ supply is limited, while the number of patients waiting for renal transplantation is increasing [2, 6, 10, 23]. In addition, renal transplantation has the risk of allograft failure and requires life-long immunosuppression. The poor quality of life and morbidity of the patients who are receiving dialysis or waiting for renal transplantation necessitate the development of alternative treatments.

There have been efforts directed towards the development of intracorporeal or extracorporeal bioartificial renal units as a potential treatment for ESRD. A bioartificial renal tubule device has been developed by culturing renal proximal tubule cells on the inner surface of hollow fibers [19]. Recently, it was demonstrated that the combination of a synthetic filtration device and a renal tubule cell therapy in an extracorporeal perfusion circuit replaces certain physiologic functions of the kidney in uremic dogs [8]. However, the application of these extracorporeal devices may be best reserved for temporary situations rather than a permanent solution.

Tissue engineering approaches have also been proposed as an alternative method for ESRD treatment [7]. Tissue engineering offers the possibility of creating functional new tissues that may replace lost or malfunctioning organs or tissues [16]. Therefore, this approach could provide an alternative to organ and tissue transplantation therapies, both of which suffer from a severe limitation of supply

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[11-13, 15, 18]. Transplantation of renal cells or segments has been investigated to develop a new therapy for renal failure. In a study, therapeutically cloned renal cells were cultured *in vitro*, seeded on polymer scaffolds, and transplanted into the nuclear donor animals. The transplanted cells regenerated glomerulus-like and tubule-like structures and excreted urine-like fluid *in vivo* [17]. In another study, transplantation of kidney precursor tissues (metanephroi) dissected from early human and porcine embryos resulted in renal structure formation and dilute urine excretion [5].

In the present study, we investigated the possibility of regenerating renal tissues *in vivo* by transplanting embryonic renal precursor cells, using three-dimensional, biodegradable polymer scaffolds. Embryonic renal precursor cells were isolated from rat metanephroi, seeded onto polyglycolic acid (PGA) scaffolds, and transplanted into peritoneal spaces of athymic mice. Three weeks after transplantation, newly-formed renal tissues were examined histologically and immunohistochemically.

MATERIALS AND METHODS

Isolation of Renal Precursor Cells

Renal precursor cells were isolated from rat fetus at day 15 (E15) of gestation. In brief, embryonic kidneys (metanephroi) were surgically dissected from fetus of Sprague-Dawley rats (SLC, Tokyo, Japan). Isolated metanephroi were washed with cold Hank's balanced salt solution (Gibco BRL, Grand Island, NY, U.S.A.) containing 10 mM N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid (Gibco BRL) in sterile conditions. The metanephroi were transferred to cold Dulbecco's modified Eagle's medium (DMEM, Gibco BRL) containing 10% (v/v) fetal bovine serum (FBS, Gibco BRL) and antibiotics (100 units/ml penicillin, and 0.1 mg/ml streptomycin, Gibco BRL). Transferred metanephroi were minced well into small pieces with a scalpel and digested in 1 mg/ml collagenase/dispase (Roche, Indianapolis, IN, U.S.A.) solution at 37°C for 30 min. Isolated cells were strained through a 40 μm nylon mesh (Cell Strainer, Becton Dickinson and Company, Franklin Lakes, NJ, U.S.A.) to remove tissue fragments. Strained cells were collected by centrifugation and used for cell seeding.

PGA Scaffolds

Biodegradable, non-woven meshes fabricated from PGA fibers (12 μm in diameter, Albani International Inc., Mansfield, MA, U.S.A.) were utilized as three-dimensional scaffolds for renal tissue reconstitution. Prior to use, the scaffolds were cut into squares (5×5 mm^2, 2 mm in thickness), sterilized with ethanol, and washed with sterile distilled water.

Cell Attachment to PGA Scaffolds

Embryonic renal precursor cells (6.2×10^5 cells) were isolated from 10 fetus at E15 and suspended in 0.3 ml of medium [DMEM containing 10% (v/v) FBS, 100 units/ml penicillin, and 0.1 mg/ml streptomycin]. The cell suspension of 0.05 ml was seeded onto each PGA scaffold. The cell-seeded scaffolds were incubated in a humidified 5% CO_2 atmosphere to assure cell attachment to PGA scaffolds for 30 min prior to transplantation.

SEM Analyses

Prior to transplantation, scanning electron microscopic (SEM) analyses were performed to confirm cell attachment on the PGA scaffold. Cell-seeded scaffolds were fixed in 1% (v/v) glutaraldehyde (Sigma) and 10% (v/v) buffered formalin (Merck, Darmstadt, Germany) for 30 min and 24 h, respectively. Fixed specimens were dehydrated with a series of graded ethanol, dried, mounted on aluminum supports, and coated with platinum by a Sputter Coater (Cressington Scientific Instruments Inc., Cranberry, PA, U.S.A.). A scanning electron microscope (JEOL, Tokyo, Japan) was operated at 5 kV to image the samples.

Transplantation

The cell-seeded scaffolds (n=4) were transplanted into peritoneal cavities of 4 athymic mice (BALB/c-nu, 7 week-old, female, SLC, Tokyo, Japan) for three weeks. After mice were anesthetized with an intramuscular administration of ketamin hydrochloride (50 mg/kg, Yuhan Co., Seoul, Korea) and xylazine hydrochloride (5 mg/kg, Bayer Korea Ltd., Seoul, Korea), a midline laparotomy was performed. The cell-seeded scaffolds were sutured onto peritoneal cavity walls of mice, and the wounds were closed by using 5-0 suture (Vicryl, Ethicon, Somerville, NJ, U.S.A.). The mice were maintained under defined flora conditions in sterile microisolator cages. Animal experiments were carried out according to the Guide for Care and Use of Experimental Animals of National Institutes of Health.

Histological and Immunohistochemical Analyses

For histological analyses, implants and normal rat kidneys were retrieved, fixed in 10% (v/v) buffered formalin, dehydrated with a graded ethanol, and embedded in paraffin. Five-micrometer-thick tissue sections were processed for hematoxylin & eosin (H&E) staining and periodic acid-Schiff (PAS) staining.

For immunohistochemical analyses, 5 μm-thick sections were stained by using antibodies against proliferating cell nuclear antigen (PCNA, DAKO, Carpinteria, CA, U.S.A.). The staining signals were developed using the streptavidin biotin universal detection system (UltraTech HRP, Immunotech, Marseille, France) and 3,3'-diaminobenzidine tetrahydrochloride (DAB) substrate solution (Vector Laboratories, Burlingame,

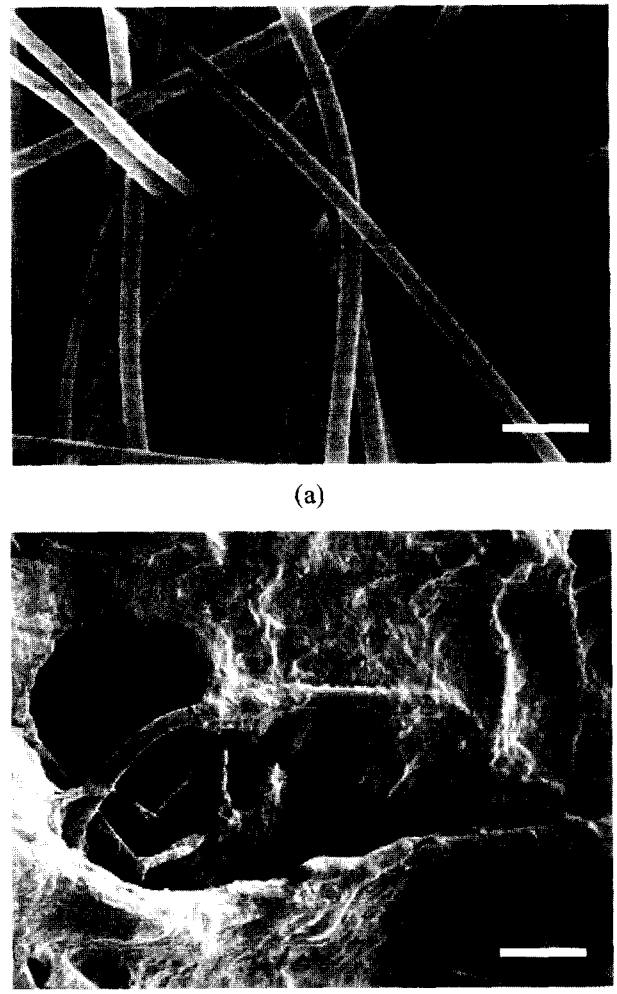
CA, U.S.A.). Mayer's hematoxylin (ScyTec, Logan, UT, U.S.A.) was used for counterstaining.

RESULTS AND DISCUSSION

Embryonic renal precursor cells were isolated (Fig. 1), seeded onto three-dimensional polymer scaffolds, and transplanted to the peritoneal cavity of athymic mice. The seeding of renal precursor cells onto three-dimensional scaffolds resulted in the formation of three-dimensional cell-polymer constructs. PGA meshes served as a porous scaffold, which provides surfaces for cell adhesion and three-dimensional space for renal tissue formation upon transplantation (Fig. 2a). One day after seeding, SEM examination of cell-seeded scaffolds revealed that the cells adhered well on the polymer scaffolds (Fig. 2b).

Transplantation of cells seeded onto three-dimensional scaffolds resulted in the formation of three-dimensional tissues (Fig. 3). Transplanted renal precursor cells on polymer scaffolds reconstituted primitive renal structures, including renal corpuscles and tubules, and induced vascularization. Histological examination by H&E staining of the retrieved transplants showed early kidney structures with comma- or S-shaped bodies and maturing nephrons, including developing primitive tubules and primitive glomeruli with vascular tufts (Figs. 4a, 4b). Primitive glomerular capillary tufts were surrounded with Bowmans capsule, being composed of flat epithelial cells and basal lamina (Fig. 4c). Various stages of fetal and developing glomerular structures were appreciated. Small vascular branches seem to be connected to arteries within adjacent interstitial tissue and showed abundant red blood cells (RBCs) (Figs. 4b, 4c). The prominent visceral epithelial layer characteristic of fetal glomeruli was noted (Fig. 4c). On PAS staining, the glomerular basement membrane and mesangial matrix were well visualized in the regenerated renal tissues (Fig. 5b). However, the PAS staining was less intense and the basement membrane-like materials of mesangium were less abundant than mature normal glomeruli (Fig. 5c) that stained strongly positive for PAS.

Fig. 1. Light micrograph of embryonic renal precursor cells isolated from rat fetus at day 15 of gestation.
The scale bar indicates 50 μm.



(a)

(b)

Fig. 2. SEM photographs of (a) a porous mesh fabricated from biodegradable PGA fibers, which allows for cell adhesion and subsequent three-dimensional tissue formation, and (b) embryonic renal precursor cell-seeded scaffold.
Cells attached well onto the polymer scaffolds. The scale bars indicate 50 μm.

Histological examination of the transplants revealed the regeneration of primitive tubular structures consisting of tubular epithelium and basement membranes supporting the tubular epithelium (Figs. 4a, 4c). The reconstituted tubules had clearly defined lumen from which the brush border was absent, and the lumens were lined by low cuboidal cells having pale eosinophilic cytoplasm (Fig. 4a). The brush border of microvilli was not evident in the luminal surface of the primitive proximal tubules (Fig. 4c), indicating

Fig. 3. Gross view of a renal tissue formed by transplantation of embryonic renal precursor cell-seeded scaffold into peritoneal cavities of athymic mice for 3 weeks.
The scales are in millimeter.

less differentiated primitive tubules. The tubular basement membranes of the developing tubules were well recognized on PAS staining (Figs. 5a, 5b). PAS-positive brush borders were not observed in the lumen of the primitive tubules (Fig. 5b), whereas those in mature normal proximal tubules were strongly PAS-positive (Fig. 5c).

Extensive vascularization was observed, which is important for the survival, growth, and development of the transplanted cells and for integration between the transplanted cells and the host body. Capillaries, arterioles, and large vessels were observed within the regenerated three-dimensional renal constructs (Fig. 4a). A network of capillaries surrounding the proximal tubules was observed, and the vascular structures were connected to glomerular capillary tufts containing RBCs (Figs. 4b, 4c).

Immunohistochemical analyses by using antibody against PCNA, a marker of proliferating cells, confirmed the intensive proliferating activity of transplanted embryonic renal precursor

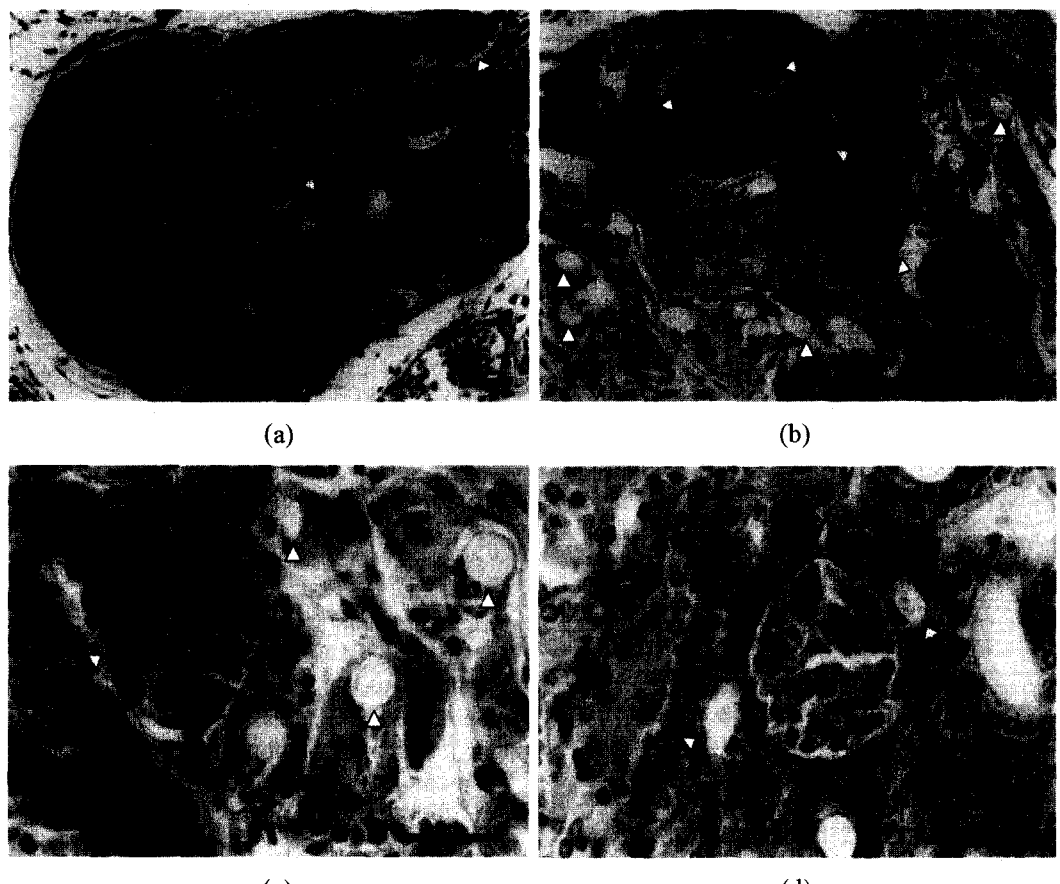


Fig. 4. Histological sections (H&E staining) of (a, b, c) the regenerated renal tissues 3 weeks after transplantation showed various stages of early nephrons with primitive tubules (closed arrows), glomeruli (open arrows), and neovessels (closed arrowheads). PGA fibers (open arrowheads) were present in the transplants. In the capillaries, RBCs (asterisks) were abundantly observed. The prominent visceral epithelial layer (dotted line) was noted in primitive glomeruli. (d) The native rat kidney structures with matured proximal (closed arrows), distal tubules (open arrows), and glomeruli (closed arrowhead).
The scale bars indicate 100 μm.

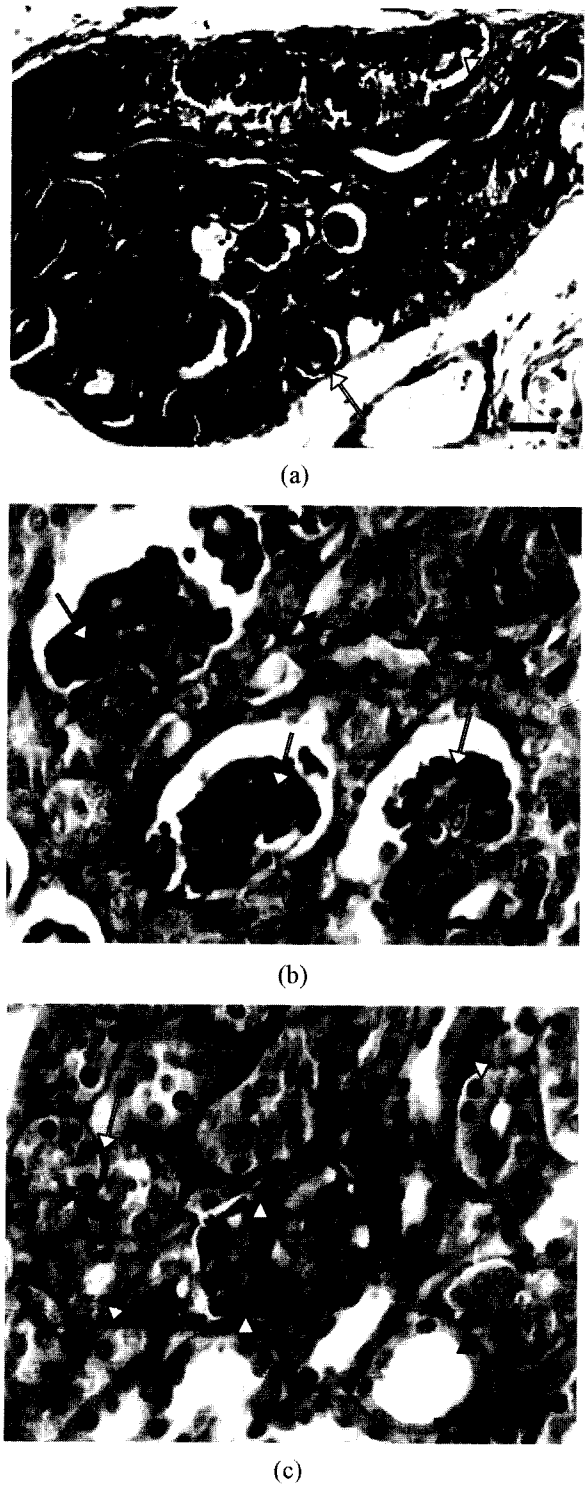


Fig. 5. (a, b) Histological sections (PAS staining) of the regenerated renal tissues 3 weeks after transplantation showed less intense PAS-positive basement membranes from primitive tubules (closed arrows), and glomeruli (open arrows) in transplants. (c) In sections of native rat kidney, PAS-positive brush borders (closed arrows) and basement membranes of proximal tubules (open arrows), and distal tubules (closed arrowheads) were observed. The mesangium (open arrowheads) of glomeruli were also stained positive for PAS. The scale bars indicate 100 μm.

cells in the transplants. Positive staining for PCNA was abundantly observed in S-shaped nephrogenic vesicle and tubules (Figs. 6a, 6b), whereas PCNA-positive cells were very rarely seen in normal kidney tissues as a control (Fig. 6c). This intensive proliferation activity would demonstrate the embryonic stem cell characteristics of the renal precursor cells.

There were foreign body giant cells surrounding the remaining PGA fibers (Fig. 4c). PGA fibers were degrading, but still present in the transplants at three weeks. PGA is a biodegradable and biocompatible polymer that is widely being used in tissue engineering. The inflammatory reaction for PGA fibers was observed, but no inflammatory reactions were observed for the transplanted cells [22].

Instead of differentiated adult renal cells, embryonic renal precursor cells were utilized as a cell source in the present study, because renal precursor cells isolated from embryonic kidney could be more effective for renal tissue reconstitution by cell transplantation than already differentiated renal cells. Since kidney is composed of at least 26 terminally differentiated cells of various types and has extremely complex structures and functions [1], the kidney is one of the most difficult organs to reconstruct by using tissue engineering techniques [2]. Therefore, it may not be feasible to regenerate renal tissues *in vivo* by transplanting various types of terminally differentiated kidney cells. Mesenchyme of metanephroi, an embryonic kidney, contains embryonic renal stem cells that can differentiate into epithelial cells, endothelial cells, myofibroblasts, and smooth muscle cells [20]. Thus, it would be advantageous to use renal precursor cells with the characteristics of embryonic renal stem cells, which can differentiate into whole types of renal cells for renal tissue reconstitution.

There have been several studies to develop kidney by transplanting metanephroi [3, 4, 5, 9, 14, 21, 24]. They studied the feasibility of transplanting murine, porcine, and human metanephroi into murine hosts and showed the transplants connected to the host's urine system and functioning. However, these studies aimed for the transplantation of xenogenic or allogenic kidney precursor tissues as organ transplantations, which may not solve the problems of current treatments of ESRD.

In summary, we report herein the *in vivo* reconstitution of renal tissues by transplanting renal precursor cells isolated from embryonic kidneys on three-dimensional, biodegradable polymer scaffolds. The renal tissue reconstructs contained vascularized structures of primitive glomerular and tubular structures with a high proliferation activity. These results demonstrate the possibility of using tissue engineering technique for renal tissue reconstruction as a potential method to replace the current treatments for ESRD. However, further studies are necessary to assess the clinical potential of this approach. Studies on the functions of the reconstituted renal tissues are necessary to obtain successful regeneration

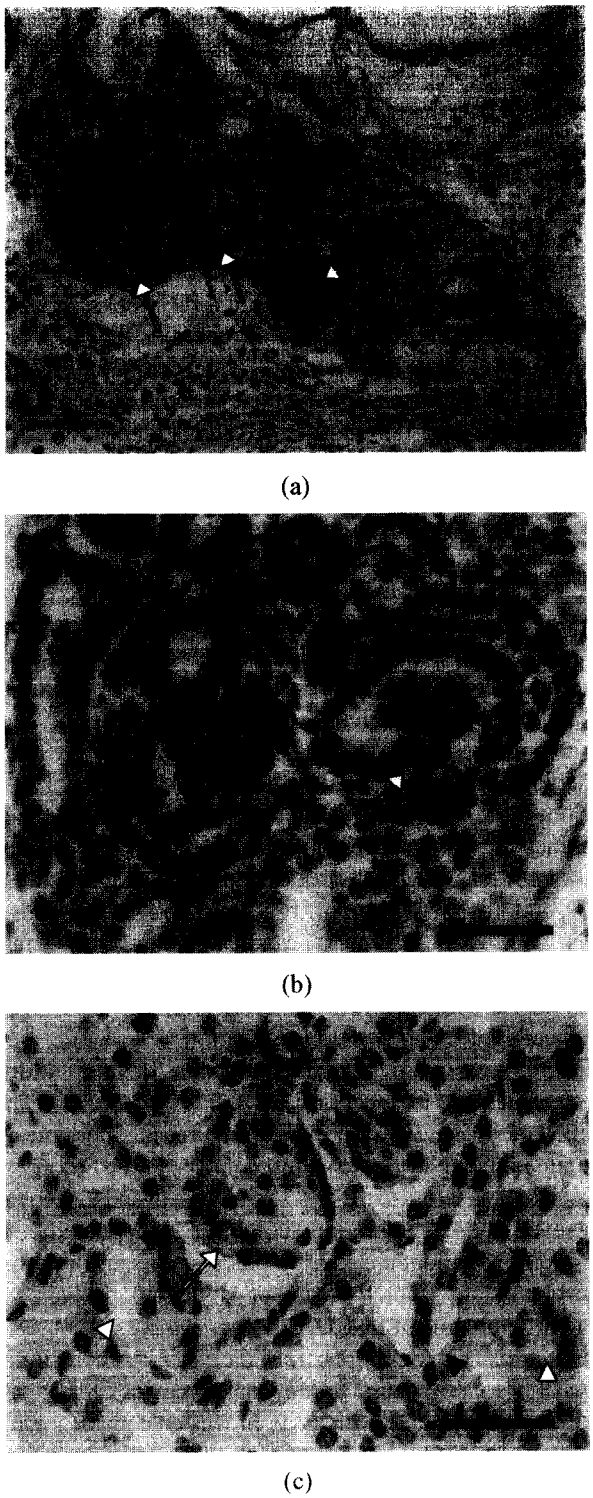


Fig. 6. Immunohistochemical staining against PCNA, a marker of proliferating cells, identified the proliferating cells.
(a, b) Many PCNA-positive nuclei were observed in the developing primitive tubules (closed arrows) and glomeruli (open arrows) in the transplants, whereas (c) PCNA-positive cells were rarely observed in normal kidney tissues at glomeruli (open arrow), distal tubules (closed arrowheads), and proximal tubules (open arrowheads). The scale bars indicate 100 μm.

of functional renal units. Implantation of embryonic renal precursor cell-polymer constructs in animal models with renal failure would be critical to evaluate the therapeutic potential of this approach. In this study, renal tissue regeneration by cell transplantation was performed in immunodeficient animal models. Embryonic renal precursor cell transplantation experiments need to be performed in animal models with fully immunocompetent systems, since immunological response may affect renal tissue regeneration and function. The effects of immunosuppressive treatments also remain to be investigated. After additional studies, this method could be utilized as an alternative treatment of ESRD that can partially or fully restore the renal structures and functions and avoid organ shortages and undesirable characteristics of the current treatments.

Acknowledgment

This work was supported by a grant (No. R01-2001-000-00491-0) from the Basic Research Program of the Korea Science & Engineering Foundation.

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