Renal Cells from Spermatogonial Germline Stem Cells Protect against Kidney Injury

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ABSTRACT
Spermatogonial stem cells reside in specific niches within seminiferous tubules and continuously generate differentiating daughter cells for production of spermatozoa. Although spermatogonial stem cells are unipotent, these cells are able to spontaneously convert to germline cell–derived pluripotent stem cells (GPSCs) in vitro. GPSCs have many properties of embryonic stem cells and are highly plastic, but their therapeutic potential in tissue regeneration has not been fully explored. Using a novel renal epithelial differentiation protocol, we obtained GPSC-derived tubular-like cells (GTCs) that were functional in vitro, as demonstrated through transepithelial electrical resistance analysis. In mice, GTCs injected after ischemic renal injury homed to the renal parenchyma, and GTC-treated mice showed reduced renal oxidative stress, tubular apoptosis, and cortical damage and upregulated tubular expression of the antioxidant enzyme hemeoxygenase-1. Six weeks after ischemic injury, kidneys of GTC-treated mice had less fibrosis and inflammatory infiltrate than kidneys of vehicle-treated mice. In conclusion, we show that GPSCs can be differentiated into functionally active renal tubular-like cells that therapeutically prevent chronic ischemic damage in vivo, introducing the potential utility of GPSCs in regenerative cell therapy.


AKI is a common complication characterized by a rapid reduction in kidney function that results in failure to maintain fluid. AKI is a potentially reversible disease. However, some patients recover incompletely from AKI, and these patients either continue undergoing dialysis or progress to CKD. Moreover, development of CKD can lead to ESRD. One of the main causes of AKI is ischemia/reperfusion injury (IRI). IRI is a pathologic condition characterized by an initial restriction of blood supply followed by the subsequent restoration of perfusion and concomitant reoxygenation that is frequently associated with an exacerbation of tissue injury and a strong inflammatory response. The cells within the renal parenchyma that suffer the most damage upon kidney ischemia are the proximal tubular cells. This is likely due to the presence of a brush border on these cells that increases cell surface area and sensitizes them to damage.

The recent discovery of the ability to reprogram adult cells into pluripotent stem cells (iPSCs) has profound therapeutic implications for diseases involving tissue damage and degeneration. Derivation of pluripotent stem cells from an adult source avoids several ethical concerns of obtaining such cells from embryos. However, efficient generation of iPSCs typically requires transduction of cells with reprogramming factors, including potent proto-oncogenes that can limit their therapeutic uses. The existence of inherent epigenetic differences between iPSCs and regular embryonic stem cells (ESCs) can adversely affect iPSCs functionality.

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We explored alternative sources for therapeutically relevant adult pluripotent stem cells, in particular, from germ cells of the testis. Spermatogonial stem cells (SSCs) from the mouse can be cultured for the long term in vitro while maintaining stem cell potential. Under specific culture conditions, the unipotent SSCs spontaneously convert at low frequency into pluripotent embryonic-like stem cells, known as germline cell–derived pluripotent stem cells (GPSCs). This conversion occurs spontaneously without transduction of reprogramming factors, and thus GPSCs can be a valuable adult source of pluripotent stem cells for therapy.

GPSCs share many features with ESCs and show great plasticity, being able to differentiate in vitro into functional cardiomyocytes, neurons, hematopoietic cells, hepatocytes, and vascular cells, although the therapeutic potential of GPSCs in tissue regeneration has not been extensively studied. It was also demonstrated that the unipotent SSCs were able to transdifferentiate into renal cells once injected directly in the parenchyma of kidney. In addition, studies performed on ESCs and iPSCs have provided evidence of expression of markers associated with early stages of embryonic kidney development.

Here we demonstrate that GPSCs can differentiate into functional renal tubular–like cells in vitro. We also tested the functional capabilities of these tubular–like cells derived from GPSCs (GTCs) in treatment of an in vivo model of kidney ischemia and demonstrate that they protect against both acute and chronic kidney damage.

RESULTS

GPSCs Differentiate into Renal Tubular Cells In Vitro

GPSCs were obtained as previously described. To induce differentiation of GPSCs into renal tubular cells, embryoid bodies (EBs) were formed by static culture and a new, specific protocol was designed to induce renal tubular differentiation (Figure 1). EBs were assessed at various differentiation days for the expression of genes involved in kidney development. The genes were analyzed by real-time PCR and immunofluorescence, as listed: brachyury and goosecoid as markers of mesodermal layer; vimentin as marker of mesenchymal–derived cells; cadherin-16/kidney-specific protein (KSP), Tamm-Horsfall protein (THP), and mineralocorticoid receptor as markers of tubular epithelial cells, and podocalyxin, nephrin, Wt1, and aquaporin-2 as markers of podocytes, glomerular cells, and collecting duct cells, respectively. After 6 days in suspension, EBs start to develop the mesodermal layer, as confirmed by the expression of brachyury (Figure 2A) and goosecoid. The expression of goosecoid dropped at day 14 (Figure 2C), whereas brachyury was expressed until day 35 (Figure 2B). Vimentin (Figure 2D) was highly expressed from day 14 and remained stable. Because vimentin is usually not expressed in differentiated tubular cells, the stable expression of this marker (Figure 2, D and H) in our culture highlights that these cells are not fully differentiated and that they are closer to progenitor cells. At day 21, we detected the expression of cadherin-16/KSP by real-time PCR (Figure 2F) and immunofluorescence analysis (Figure 2I). Cadherin-16 is expressed specifically in renal tubular cells, and in vivo its expression starts at the S–shaped body stage, around E14.5 in mice. THP, the most abundant protein secreted in the urine, was detected by immunofluorescence (Figure 2J) starting from day 28. Mineralocorticoid receptor was expressed consistently from day 21 (Figure 2E). During differentiation, GPSCs underwent a process of tubulogenesis stimulated by collagen type IV, a component of the renal basal membrane, that was used to coat the culture plates. Epidermal growth factor (EGF) is another key factor involved in this process. The EGF added to the culture medium not only was able to stimulate cell proliferation but was also crucial for formation of tubular–like structures. Tubular–like structures started to appear at day 21 (Figure 2G). Similar structures were formed by baby mouse kidney epithelial cells in tridimensional culture.

Furthermore, we investigated by real-time PCR analysis the expression of podocalyxin, nephrin, Wt1, and aquaporin-2. These markers were expressed only within the first two weeks of culture and dropped after the treatment of EBs with the conditioned medium (Supplemental Figure 1), with the exception of aquaporin-2, which was undetectable (data not shown). Thus, we demonstrated that GTCs express only markers specific for renal tubular cells.

To obtain a pure population of tubular–like cells, we isolated KSP+ cells from EBs at day 35 of differentiation. The cells were sorted, taking advantage of the magnetic-activated cell sorting (MACS) method. The KSP+ cells strongly expressed KSP (Figure 2L), mineralocorticoid receptor (Supplemental Figure 2A) but not oct4, Wt1, goosecoid, and podocalyxin (Supplemental Figure 2, B–E). KSP+ cells expressed vimentin at a very high level (Figure 2D) from day 14 and remained stable. Because vimentin is usually not expressed in differentiated tubular cells, the stable expression of this marker (Figure 2, D and H) in our culture highlights that these cells are not fully differentiated and that they are closer to progenitor cells. At day 21, we detected the expression of cadherin-16/KSP by real-time PCR (Figure 2F) and immunofluorescence analysis (Figure 2I). Cadherin-16 is expressed specifically in renal tubular cells, and in vivo its expression starts at the S–shaped body stage, around E14.5 in mice. THP, the most abundant protein secreted in the urine, was detected by immunofluorescence (Figure 2J) starting from day 28. Mineralocorticoid receptor was expressed consistently from day 21 (Figure 2E). During differentiation, GPSCs underwent a process of tubulogenesis stimulated by collagen type IV, a component of the renal basal membrane, that was used to coat the culture plates. Epidermal growth factor (EGF) is another key factor involved in this process. The EGF added to the culture medium not only was able to stimulate cell proliferation but was also crucial for formation of tubular–like structures. Tubular–like structures started to appear at day 21 (Figure 2G). Similar structures were formed by baby mouse kidney epithelial cells in tridimensional culture.

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low level (Figure 2K), indicating that this fraction of cells represents the most differentiated cells in EBs. Two days after MACS isolation, the KSP+ cells started to dedifferentiate, as demonstrated by the re-expression of vimentin. This highlights the importance of the EB environment in supporting the differentiation process of tubular-like cells.

Transepithelial Electrical Resistance Measurement Confirms That GTCs Are Functional Epithelial Cells

To assess functionality of the GTCs, we measured transepithelial electrical resistance (TEER), which indicates the presence of tight junctions that are typical structures of epithelial cells.27–29 Similar results were obtained from three independent experiments. Tubular-like structures (G) appear spontaneously after 21 days in culture (original magnification $\times 100$). We assessed the expression of vimentin (H), KSP (I), and THP (J) through immunofluorescence staining. Nuclei were counterstained in DAPI. (Original magnification: THP $\times 400$, vimentin/KSP $\times 630$.) Finally, we evaluated the expression pattern of KSP+ cells. After 2 days in the absence of EB environment, cells start to dedifferentiate, as demonstrated by the decrease of cadherin-16/KSP (L) and the increase of vimentin (K) expression. (KSP+ d0: RNA was extracted immediately after isolation; KSP+ d2: RNA was extracted 2 days after cells isolation.) Data are presented as mean ± SEM.

Figure 2. GTCs show a renal tubular epithelial phenotype. RNA was extracted at different time points, and real-time PCR analysis (A–F) was performed. The mesodermal marker Brachyury began to be expressed after 6 days in suspension (A) and decreased during the culture (B). Goosecoid, another mesodermal marker, is undetectable starting from day 21 (C). Vimentin, a marker of mesenchymal-derived cells, is stably expressed for the whole duration of the culture (D). Mineralocorticoid receptor (E) and cadherin-16/KSP (F) are expressed consistently from day 21 until day 35 of the EB culture. Each column refers to three independent samples. Tubular-like structures (G) appear spontaneously after 21 days in culture (original magnification $\times 100$). We assessed the expression of vimentin (H), KSP (I), and THP (J) through immunofluorescence staining. Nuclei were counterstained in DAPI. (Original magnification: THP $\times 400$, vimentin/KSP $\times 630$.) Finally, we evaluated the expression pattern of KSP+ cells. After 2 days in the absence of EB environment, cells start to dedifferentiate, as demonstrated by the decrease of cadherin-16/KSP (L) and the increase of vimentin (K) expression. (KSP+ d0: RNA was extracted immediately after isolation; KSP+ d2: RNA was extracted 2 days after cells isolation.) Data are presented as mean ± SEM.
injected under left renal capsule with $1 \times 10^6$ GTCs or undifferentiated GPSCs, as positive control. After 6 weeks, all kidneys injected with undifferentiated GPSCs showed prominent teratomas (Figure 4A), visible by eye inspection. The analysis of hematoxylin and eosin-stained sections showed the presence of structures derived from mesodermal, ectodermal, and endodermal layers (data not shown). On the contrary, kidneys injected with GTCs did not show teratoma formation (Figure 4B).

GTCs Protect against AKI
To investigate whether GTCs could protect against AKI, we chose a model of IRI based on unilateral nephrectomy followed by left kidney ischemia. After damage, we injected GTCs in female mice through the tail vein. GTCs labeled with Vybrant CFDA SE Cell Tracer Kit (CFSE) (Molecular Probes, Leiden, The Netherlands) were detected in renal parenchyma 48 hours after injection, showing their capacity to home injured kidney (Figure 5). The homing ability of GTCs was confirmed by in situ analysis of Y chromosome. GTCs positive for Y chromosome were detected in renal parenchyma 2 days after injection (Figure 6B) and represented 1.5% of the total number of nuclei. Six weeks after IRI, the number of $Y^+$ cells, mostly located in the tubular structures (Figure 6C), was 2% of total number of nuclei. This percentage was slightly higher than that of $Y^+$ cells after 48 hours (Supplemental Figure 4E). Double staining for Y chromosome and BrdU 48 hours after IRI revealed that although $Y^+$ cells were able to proliferate, the number of BrdU$^+$/Y$^+$ was lower than the total number of proliferating cells (Supplemental Figure 4, C and D).

Ischemized mice injected with GTCs showed significant reduction in BUN ($P<0.01$) and creatinine ($P<0.05$) levels compared with control mice injected with PBS (Figure 7A).

Histologic analysis of IRI kidneys revealed a marked reduction of tissue damage in GTC-injected mice with respect to that of PBS-injected mice (Figure 7B). The number of CASTs (hyaline material inside the tubular lumen that block and impair the tubular functionality) and necrotic tubules in the renal cortex of mice treated with GTCs was significantly lower ($P<0.001$) compared with PBS-injected mice (Figure 7C). Moreover, apoptotic cells were reduced in IRI mice treated with GTCs ($P<0.05$), suggesting an antiapoptotic effect of GTCs on tubular cells (Figure 7G). In association with this result, we found that the number of tubules expressing hemeoxygenase-1 (HO-1), a well known protective enzyme that exerts an activity against...
oxidative stress,31 was increased in GTC-treated mice (Figure 7F) compared with mice injected with PBS (P<0.01), and this result was also confirmed by real-time PCR analysis (P<0.05) (Supplemental Figure 5A). Cell proliferation and CD18+ infiltration did not show any difference in GTCs or PBS injected mice (Supplemental Figure 5, B and C).

To evaluate the contribution of GTCs to the repair process, undifferentiated GPSCs or KSP+ cells 4 days after MACS isolation were injected after IRI. Injection of GPSCs did not affect renal functionality or HO-1 expression (Supplemental Figure 6), whereas KSP+ cells up-regulated HO-1 (Supplemental Figure 7) albeit less markedly versus differentiated GTCs.

Taken together, these results demonstrate that GTCs can protect against AKI caused by ischemic damage.

GTCs Protect against CKD
It is known that AKI can trigger CKD.32,33 To test the possibility that the treatment with GTCs could protect against chronic disease progression, we evaluated kidney functionality and histology in mice injected with GTCs 6 weeks after IRI-AKI. We scored the percentage of mice in the groups of GTC/PBS-injected mice that developed chronic renal damage. The scoring of CKD highlighted that GTC-injected mice were less prone to develop a chronic disease (Figure 8A).

To confirm the damaging of left kidney after ischemic insult, we analyzed BUN and creatinine levels after 48 hours (data not shown). Six weeks after IRI, BUN and creatinine levels were decreased in both groups compared with levels measured 48 hours after IRI. We did not find any significant difference between mice treated with GTCs and those injected with PBS alone (Figure 8, C and D). In fact, it is known that BUN and creatinine levels are normal until 60% of total kidney function is lost. On the other hand, measurement of cystatin C, a more accurate marker of renal functionality, revealed that serum cystatin C was significantly lower (P<0.01) in mice treated with GTCs than those injected with PBS alone (Figure 8E). As expected, the relative kidney weight did not differ significantly between ischemized mice treated

Figure 5. GTCs are able to reach the injured kidney and migrate in the renal parenchyma. GTCs labeled with CFSE are present in renal parenchyma 48 hours after ischemia. CFSE+ cells are found to be localized among the tubules. No signal is detected in PBS-injected mice. *Tubules. CFSE+ cells are revealed with a secondary antibody Alexa 565; nuclei are counterstained with DAPI. (Original magnification ×630.)
with GTCs or with PBS.\textsuperscript{34} Whereas the relative kidney weight was significantly decreased in ischemized mice injected with PBS, GTC-injected mice showed a reduction of cortical damage (B), as shown by periodic acid-Schiff staining (original magnification, $\times 200$) compared with PBS-injected mice. GTC-treated mice also showed a lower incidence of tubular necrosis (C) than PBS-injected mice; the left chart indicates incidence of CAST formation, and the right chart represents the total number of necrotic tubules (tubules undergoing necrosis plus CAST). HO-1 is upregulated in GTC-treated mice compared with PBS-injected mice (D), and apoptotic ratio is decreased (E) (original magnification: HO-1, $\times 100$; TUNEL staining, $\times 630$). The lower panel represents the quantification of HO-1$^+$ (F) tubules and apoptotic cells (G). Data are presented as mean $\pm$ SEM. (GTC-injected mice, $n=8$; PBS-injected mice, $n=8$.)

**DISCUSSION**

Adult stem cells have great plasticity, providing an opportunity for the treatment of a wide range of diseases,\textsuperscript{38–40} representing an alternative to ESCs in regenerative cell therapy. In particular,
GPSCs have aroused a growing interest in the recent years. Murine GPSCs resemble ESCs in colony morphology, in their ability to form all the three germ layers and teratomas in vivo. In this study, we demonstrate the differentiation of murine GPSCs, in vitro, into renal tubular-like cells and their ability, in vivo, to restore kidney function after AKI. After 6 days in culture, EBs start to express mesodermal genes. Then, as a result of the treatment with the conditioned medium, a small fraction of EBs shifts toward a more differentiated state, expressing genes such as KSP and mineralocorticoid receptor. KSP is a cadherin expressed exclusively in renal tubular epithelial cells. The number of cells expressing KSP protein is about 10% of the total cell population (data not shown). More specific differentiating factors in the medium are needed to increase the number of KSP+ cells.

GTCs stably express vimentin-like immature progenitor tubular cells. Vimentin, normally not present in the adult renal tubular cells, starts to be expressed at a high level within the first days after severe injury, during the repair process. In this process, renal tubular cells recapitulate gene expression patterns typical of the developing nephron. This observation may have important implications in the use of GTCs in kidney repair.

To test the functionality of the GTCs in vivo, we chose the renal IRI as a model of renal failure. After damage induction, we injected GTCs at day 35 of differentiation into the tail vein of female mice. In situ analysis of Y chromosome on the renal parenchyma of female mice demonstrates that transplanted male GTCs are able to reach the injured kidney and migrate in the renal parenchyma. Y+ cells are present in renal parenchyma 48 hours after ischemia, mostly in the interstitial area or inside the glomeruli. After 6 weeks, cells positive for the Y chromosome persisted in tubules, indicating a long-term engraftment of these cells in renal parenchyma. Y+ cells are present in renal parenchyma 48 hours after ischemia, mostly in the interstitial area or inside the glomeruli. After 6 weeks, cells positive for the Y chromosome persisted in tubules, indicating a long-term engraftment of these cells in renal parenchyma. We found that the percentage of engrafted cells is increased 6 weeks after IRI compared with 48 hours after IRI. Although 48-hour IRI mice display a small amount of double-positive staining for both Y chromosome and BrdU, we could not detect any adjacent Y+ cells or Y+/BrdU+ cells 6 weeks after IRI. These results indicate that GTC proliferation is not a prominent event involved in kidney repair.

Renal ischemic injury permanently damages peritubular capillaries, causing hypoxia, which may be involved in the progression of CKD after AKI. HO-1 is an inducible enzyme

Figure 8. Mice injected with GTCs are protected against CKD development. The score (A) shows that ischemized mice injected with PBS are more prone to develop a chronic disease. The relative kidney weight (B) does not differ between mice treated with GTCs and those treated with PBS, but it is decreased in mice injected only with PBS compared with the control nonischemized mice. The relative percentage of kidney weight is evaluated on the total body weight of the mice. BUN (C) and creatinine (D) analysis shows no difference between mice injected or not injected with GTCs 6 weeks after ischemia. BUN returns to normal level whereas creatinine remains slightly higher. Cystatin C (E) is downregulated in mice injected with GTCs compared with mice injected with PBS (PBS-injected mice, n=10; GTC-injected mice, n=10; nonischemized mice, n=4). Data are presented as mean ± SEM.
that protects against oxidative stress, regulating cellular respiration and oxygen availability, and its upregulation is linked to a decrease in the apoptotic ratio. The expression of HO-1 is always correlated with a better prognosis after ischemic damage. We found that mice injected with GTCs showed a strong upregulation of HO-1 2 days after renal damage induction. This finding suggests that HO-1 upregulation induced by GTCs could be involved in kidney protection from IRI. A high level of expression of HO-1 after ischemic insult is related to less impairment of renal parenchyma and fewer apoptotic cells because of the ant apoptotic role of HO-1. Our results support the hypothesis that the upregulation of HO-1 in mouse kidneys treated with GTCs may be the mechanism involved in functional kidney protection. Interestingly, the intravenous injection of KSP+ cells helped protect against AKI, resulting in an intermediate phenotype compared with that of GPSCs and differentiated GTCs. This result confirmed that the effect is specific because the injection of undifferentiated GPSCs cannot protect kidneys against IRI.

After a single kidney ischemic event, patients can develop CKD. Chronic damage is accompanied by loss of kidney weight, tubular dilation, and persistent inflammation that lead to the release of profibrogenic factors. Usually the kidney is able to restore normal functionality, and the main challenge is to prevent fibrosis progression. Six weeks after ischemia, fibrosis, together with tubular dilation, glomerular sclerosis, and tubular atrophy, was almost absent in the renal parenchyma of mice injected with GTCs. These factors are important hallmarks of chronic disease, and their absence confirm the role of GTCs in protecting kidney from CKD progression.

We did not detect any differences in kidney weight between ischemized mice injected or not injected with GTCs. The explanation could reside in the strong compensative hypertrophic stimulus consequence of the presence of a single kidney, as dictated by our IRI model.

In conclusion, these data show that GTCs can be derived from GPSCs and that they are functionally active in vivo for the repair of renal damage in a murine model of IRI. These findings open the possibility to repair damage in acute renal disease, with the advantage of using GPSCs directly isolated from patients. Furthermore, because of the high plasticity displayed by GPSCs, the hope is that these cells could be applied to other diseases.

Figure 9. Mice injected with GTCs do not show tubular dilation. Representative hematoxylin and eosin staining (A) of kidney cortical sections of ischemized mice 6 weeks after ischemia and of a healthy mouse (original magnification, ×200). The charts below show the quantification of tubular dilation (B and C). In particular, the amount of microcysts and cysts in mice injected with GTCs is similar to that in nonischemized healthy mice (PBS-injected mice, n=10; GTC-injected mice, n=10; nonischemized mice, n=4). Data are presented as mean ± SEM.
**CONCISE METHODS**

**Culture of Undifferentiated GPSCs and EB Formation**

GPSCs were maintained in DMEM (Invitrogen), 15% FBS (HyClone FBS characterized; Thermo Scientific), with 1000 U leukemia inhibitory factor per liter. EB formation was induced by seeding 500 cells/50 μl in an ultra-low-attachment 96-well plate (Corning, Lowell, MA). EBs were maintained in 1:1 DMEM/F12:RPMI (Invitrogen) containing 0.01 mM nonessential amino acid (Gibco), 1 mM sodium pyruvate (Gibco), 100 U/ml penicillin (Gibco), 100 μg/ml streptomycin (Gibco), 0.1% BSA fraction V (Gibco), 0.05 mM 2-β mercaptoethanol (Sigma-Aldrich) (basal medium composition), and 10% FBS for 6 days in suspension, allowing mesoderm induction; medium was added every 2 days.

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**Figure 10.** GTCs injection in mice prevent renal fibrosis. The upper panel (A) shows three representative pictures of fibrotic tissue in ischemized mice and healthy nonischemized mice, stained with picrosirius red. The lower panel (C) shows, for the same mice, the presence of infiltrating CD18⁺ cells and quantification of infiltrating cells per field (D). (Original magnification: picrosirius red, ×40; CD18⁺, ×100.) The quantification of fibrogenesis (B) is performed through MetaMorph software, and results are expressed as the mean ratio of the stained area to the total tissue area. Evaluation of renal fibrotic area on Masson trichrome staining (G) confirms the result obtained with picrosirius red staining. Glomerular sclerosis (E) and tubular atrophy are also evaluated (F). The number of sclerotic glomeruli and atrophic tubules is significantly decreased in the ischemized mice injected with GTCs compared with PBS-injected mice. (PBS-injected mice, n=10; GTC-injected mice, n=10; nonischemized mice, n=4.) Data are presented as mean ± SEM. CD18⁺ is the marker for all the infiltrating cells.
**Renal Tubular Differentiation of GPSCs In Vitro**

After mesoderm induction, EBs were plated on collagen 10 μg/cm² (collagen type IV from human placenta; Sigma-Aldrich). EBs were cultured from day 6 to day 16 in 1:1 DMEM/F12:RPMI (Invitrogen) containing the basal medium components, a "nephrogenic cocktail" of fibroblast growth factor-2 (50 ng/ml; Voden), TGF-β, leukemia inhibitory factor (20 ng/ml; PeProtech), and low serum concentration (1%). Starting from day 16, EBs were stimulated with a conditioned medium collected from primary renal tubular cells cultured as described below. Several factors were added to the medium: 1% FBS, EGF (10 ng/ml; PeProtech); a cocktail of insulin, transferrin, and sodium selenite (50 ng/ml; Sigma-Alrich); hydrocortisone (36 μg/μl; Sigma-Alrich); and full cell line--derived neurotrophic factor (50 ng/μl; PeProtech); 33; retinoic acid (10^{-7} M; Sigma-Alrich); glucose (0.15 g; Sigma-Alrich); and all components of the basal medium (Figure 1).

**Culture of Mouse Primary Renal Tubular Cells**

Primary renal tubular cells were isolated under sterile conditions from kidneys of 129sv mice (8 weeks old) by a modification of previously described methods. Renal cortex was sliced with surgical scalpel and then passed in three different filters to achieve tubular cells isolation (50 μm mesh, 70 μm, 40 μm). Primary renal tubular cells were maintained in DMEM to which 15% serum was added. When the cells reached 60%–70% of confluence, they were rinsed with PBS and then starved for 24 hours in DMEM containing only 0.1% BSA. The conditioned medium was collected from primary renal tubular cells cultured as described methods. Renal cortex was sliced with surgical scalpel and then passed in three different filters to achieve tubular cells isolation (50 μm mesh, 70 μm, 40 μm). Primary renal tubular cells were maintained in DMEM to which 15% serum was added. When the cells reached 60%–70% of confluence, they were rinsed with PBS and then starved for 24 hours in DMEM containing only 0.1% BSA. The conditioned medium was collected from primary renal tubular cells cultured as described methods. The conditioned medium was collected 24 hours after starvation and centrifuged at 3000 rpm for 10 minutes to remove the debris. Two collections were performed for each plate.

**Analysis of mRNA Expression**

Total RNA was extracted from EBs at days 2, 4, 6, 7, 14, 21, 28, and 35 according to the manufacturer’s instruction (PureLink RNA Mini KIt; Invitrogen). Primary renal tubules and kidneys were used as positive controls.

For the analysis of HO-1 mRNA, total RNA was extracted from kidneys 48 hours after ischemia, according to manufacturer’s instruction (TRI Reagent; Ambion). cDNA was synthesized starting from 500 ng of RNA, as previously described. Quantitative real-time-PCR was performed using primers listed in Table 1.

**Immunofluorescence Staining**

EBs were fixed with 4% paraformaldehyde for 10 minutes; all the primary antibodies were diluted in 1% BSA (Sigma-Alrich) and incubated for 1 hour at room temperature. The following antibodies were used: goat polyclonal anti-KSP (caderin-16; Santa Cruz Biotechnology), rabbit polyclonal anti-THP (Uromodulin; Santa Cruz Biotechnology), goat polyclonal antivimentin (Santa Cruz Biotechnology), rabbit polyclonal anti–ZO-1 (Invitrogen). Nuclei were counterstained with DAPI (Sigma-Alrich).

**Isolation of KSP+ Cells**

KSP+ cells were isolated from EB culture using MACS (Miltenyi Biotec). The cell suspension was incubated with KSP (caderin-16; Santa Cruz Biotechnology) primary antibody, 1 μl of antibody/10^6 cells, then washed in buffer. Two-cell suspensions were obtained: the labeled fraction (KSP+ cells) and the unlabeled fraction (KSP− cells).

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**Table 1. Primers used for PCR**

<table>
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<tr>
<th>Transcript</th>
<th>Sequence (5’-3’)</th>
<th>Bases</th>
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<tbody>
<tr>
<td>Brachyury</td>
<td>GAGCTTGGGTTAGGTTA</td>
<td>20</td>
</tr>
<tr>
<td>Cadherin-16/KSP</td>
<td>CAGCCCACTCACTGTTCTTA</td>
<td>20</td>
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<td>Nephrin</td>
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<tr>
<td>Vimentin</td>
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<td>20</td>
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<tr>
<td>Wt1</td>
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</tr>
<tr>
<td>Mineralocorticoid receptor</td>
<td>TTTCCTGGAATCTCATCAATATGC</td>
<td>25</td>
</tr>
<tr>
<td>Neprhin</td>
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<tr>
<td>Podocalyxin</td>
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<td>Mineralocorticoid receptor</td>
<td>TTTCCTGGAATCTCATCAATATGC</td>
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**In Vitro Functional Analysis**

KSP+ and KSP− sorted cells fractions, undifferentiated GPSCs, and primary renal tubules were plated on 24-transwell (polycarbonate membrane; Corning). TEER analysis was performed 7 days after cells plating using an STX2 electrode (EVOM2; World Precision Instruments, Inc.); this allowed the formation of tight junction. Electrical resistance can be derived continuously and measured with an ohmmeter. TEER values were expressed in ohm × cm².

**Production of Biotinylated Probe for In Situ Analysis of Y Chromosome**

The probe was synthesized starting from a bacterial artificial chromosome (RP24-418C24; Children’s Hospital Oakland Research Institute) that cover a specific telomeric region of Y chromosome, the RBMY gene. A biotinylated probe was produced by using the random priming method to insert biotinylated deoxyuridine triphosphates (dUTPs) (BioPrime DNA Labeling System; Invitrogen). Probes were purified by passing through purification columns (Illustra Microspin G-50 Columns; GE Healthcare Life Science). In situ analysis was performed on paraffin-embedded sections (3.5 μm). Antigens were retrieved using a SPOT-Light Tissue Pretreatment kit (Invitrogen). Sections were dehydrated at 70°C for 5 minutes and then hybridized at 37°C for 19 hours. The biotinylated dUTPs were revealed with Cy3-conjugated streptavidin (Jackson ImmunoResearch Laboratories, Inc.). We scored the percentage of cells capable of kidney homing by counting 10 sections 10 μm apart. The number of Y+ chromosomes was calculated on the total number of nuclei.

**Animal Model**

Studies were conducted in accordance with National Institutes of Health Guide for Care and Use of Laboratory Animals. All experiments were performed with 8-week-old female 129sv/C57; mice were allowed free access to water and standard mouse chow. Renal IRI was...
modified from methods previously described.\textsuperscript{37,38} In brief, mice were anesthetized by intraperitoneal injection with Zoletil 100 (Virbac), 2 mg/kg body weight. The left renal artery and vein were clamped for 40 minutes with a nontraumatic microaneurysm clamp, and right nephrectomy was performed. During the period of ischemia, body temperature was maintained at 37°C by placing the mice on a warming pad. After clamp removal, reperfusion was confirmed by visual inspection. To test the effect of GTCs, mice were divided in two groups. In the treated group \((n=8)\), soon after IRI, \(2.5 \times 10^6\) GTCs, at day 35 of differentiation, were infused intravenously through the tail vein. In the untreated group \((n=8)\), mice were given only vehicle. Both groups were euthanized 2 days after IRI. In the chronic damage model, the same experimental procedure was performed but mice \((n=10\) treated/untreated group) were euthanized 6 weeks after ischemia.

**Identification of CFSE-Labeled Cells**

To detect GTCs in renal parenchyma, cells were labeled before injection with CFSE. The cells were injected after IRI in mice tails. Two days after injection, mice were euthanized and kidneys were embedded in optimal cutting temperature compound (VWR DBH Prolabo) and snap-frozen in liquid nitrogen. Tissue slides were stained with an anti-fluorescein/Oregon green polyclonal antibody (Molecular Probes) to detect CFSE-positive cells. DAPI (Sigma-Aldrich) was used to stain nuclei.

**Renal Morphology and Tubular Dilation/Cystic Index**

For all histologic analyses, kidneys were fixed overnight in 10% formalin and then embedded in paraffin. Renal histology was evaluated by staining kidney sections with periodic acid-Schiff reagents according to the manufacturer’s instructions (Bio-Optica). Luminal hyaline CAST and tubular necrosis were assessed in 20 non-overlapping fields using a 20\(\times\) objective (Olympus BH2 RFCA). For tubular dilation/cyst formation, sections were stained with hematoxylin and eosin (Bio-Optica). Numbers of CAST and tubular profiles showing necrosis were recorded in a blinded fashion. Tubular dilation/cyst quantification was performed using a screen with dots distant one from another by 13.625 microns. The degree of tubular dilation was defined by the number of dots in the lumen, following previously suggested criteria.\textsuperscript{59}

In the chronic damage experiment, kidney sections (3.5 \(\mu\)m) were stained with picrosiris red (Sigma-Aldrich) to assess the amount of fibrosis.\textsuperscript{60} The quantification of fibrosis extension was evaluated with MetaMorph software (Molecular Devices, LLC); results were expressed as the mean ratio of the stained area to the total tissue area. Glomerular sclerosis was defined as the presence of dense abundant deposition of periodic acid Schiff–positive material at the glomerular tuft, with occlusion of capillary loops and segmental hyalinization in 100 consecutives glomeruli, by determining the percentage of glomeruli exhibiting sclerotic lesion. Masson trichrome (Bio-Optica) staining was performed according to manufacturer’s instruction to evaluate tubular atrophy and interstitial fibrosis. The number of atrophic tubules were evaluated in 10 nonoverlapping fields using a 20\(\times\) objective (Olympus BH2 RFCA). The quantification of fibrosis was evaluated as the extension of fibrotic area to the total tissue area with MetaMorph software. The scoring of CKD was carried out by a blinded observer through a semiquantitative evaluation, based on arbitrary score on Masson trichrome sections. The score ranged from 0 to 3+ as follows: 0: no changes; 1+: damage to <25% of the interstitial area; 2+: damage to 25%–50% of the interstitial area; and 3+: damage to >50% of the interstitial area. Kidney and mice were weighed 6 weeks after ischemia; the relative percentage of kidney weight was evaluated on the weight of mice total body.

**In Vivo Apoptosis, Proliferation, Cell Infiltration, and HO-1 Expression**

To determine the degree of infiltrating cells and HO-1 expression, renal sections of 3.5 \(\mu\)m were analyzed. Anti-CD18 antibody (BMA Biomedicals; clone YTS 213.1) was used as a marker of inflammatory infiltrates, and anti-HO-1 antibody (Enzo Life Sciences) was used to evaluate the level of HO-1 expression.

To evaluate the proliferation rate, mice were injected intraperitoneally with BrdU (BD Pharmigen), 100 mg/kg body weight, 24 hours after IRI (or 24 hours before euthanasia in the case of CKD) and 50 mg/kg body weight 36 hours after IRI (12 hours before euthanasia for CKD experiment).\textsuperscript{61} Kidney sections were deparaffinized and boiled for 15 minutes in 6 mM sodium citrate buffer (pH, 6.0) for HO-1 and BrdU staining (DAKO; clone Bu20a). Antigen retrieval for CD18 staining was accomplished through digestion with 0.005% trypsin for 30 minutes. Nonspecific binding and endogenous peroxidase activity were blocked using 3% hydrogen peroxidase.

Apoptosis was measured by terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling performed on paraffin-embedded tissue sections using the fluorescein-based In Situ Cell Death Detection kit (Roche) according to the manufacturer’s instruction. BrdU\(^T\) and CD18\(^T\) cells were scored by counting the number of positive cells per field in 20 randomly chosen sections of kidney cortex using a 20\(\times\) objective (Olympus BH2 RFCA). TUNEL\(^T\) cells were scored in 10 randomly chosen sections of kidney using 40\(\times\) objective (ApoTome, Zeiss). The number of BrdU\(^T\) cells and TUNEL\(^T\) cells was normalized on the number of total nuclei.

**Renal Functionality Assessment**

To assess renal functionality, serum was collected. Blood samples were incubated at 37°C for 30 minutes to allow blood clotting. Serum was then isolated from the supernatant by centrifugation of blood samples (3000 rpm, 10 minutes). Creatinine level was measured using a colorimetric assay based on the Jaffe reaction (Quantichrom Creatinine Assay; BioAssay Systems). BUN was measured by direct quantification of serum urea with a colorimetric assay kit according to the instruction protocol (Quantichrom Urea Assay; BioAssay Systems).

Cystatin C level was assayed by ELISA according to the manufacturer’s instruction (Abcam).

**Teratoma Formation Assay**

To assess teratoma formation, after 42 days of differentiation in culture, GTCs were injected under renal capsule. A total of \(1 \times 10^6\) GTCs or undifferentiated GPSCs, both from two independent cultures, were resuspended in 100 \(\mu\)l of PBS. The 129sv/C57 mice were anesthetized by an intraperitoneal injection with zoletil 100 (Virbac), 2 mg/kg body weight, and cell suspensions were injected under the left renal capsule. Mice were euthanized after 6 weeks, and the left kidneys were removed to check teratoma formation. Right
Statistical Analyses
Values were reported as the mean ± SEM. Statistical analyses were performed by using the two-tailed t test (\(*P<0.05\); \(**P<0.01; \***P<0.001\)) for the graphs comparing only two variables. For analysis of more than two categories, statistical significance was calculated with one-way ANOVA and Bonferroni post-tests (\(*P<0.05\); \(**P<0.01; \***P<0.001\)). All analyses were performed with PRISM5 (GraphPad Software, Inc., La Jolla, CA).

ACKNOWLEDGMENTS
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DISCLOSURES
None.

REFERENCES
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10.登录

Testis Stem Cells in Renal Damage

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This article contains supplemental material online at http://jasn.asnjournals.org/lookup/suppl/doi:10.1681/ASN.2013040367/-/DCSupplemental.
SUPPLEMENTARY MATERIALS

Supplementary methods

Isolation of KSP⁺ cells

KSP⁺ positive cells were isolated from EBs culture using magnetic activated cells separation (MACS) (miltenyi Biotec. 1989). The cell suspension was incubated in buffer (phosphate buffered saline supplemented with 0.5% BSA, Sigma) containing KSP primary antibody (SantaCruz, diluted 1:50), 1 µl of diluted antibody/10⁶ cells for 25 minutes at 4°C, then washed in buffer. The cell pellet was resuspended in buffer containing secondary biotinylated anti-goat (DAKO, diluted 1:200) for 25 minutes at 4°C, then washed in buffer. Finally, the cell pellet was resuspended in buffer with streptavidin microbeads (diluted 1:10), then washed. The supernatant was discarded and the cell pellet resuspended in buffer and loaded onto the magnetic separation (MS) column which was placed in a magnetic field. Unlabeled cells were washed though the column (3x with buffer). The column was then removed from the magnetic field and washed with buffer to elute the labelled cells. Two cell suspensions were obtained: the labelled cells (KSP⁺ cells) and the non-labelled cells (KSP⁻ cells).

GPSCs and KSP⁺ cells injection after renal IRI

Studies were conducted in accordance with National Institute of Health Guide for Care and Use of Laboratory Animals. All experiments were performed with 8-week-old female 129sv/C57; mice were allowed free access to water and standard mouse chow. The experimental protocol was the same of the previous experiments and the mice were sacrificed 48h after IRI. We injected immediately after IRI, 2.5x10⁵ undifferentiated GPSCs (N=6) or KSP⁺ cells (N=6) recovered from MACS sorting 4 days after isolation, through tail vein. In both experiments, control mice were only given vehicle (N=4).
**Tubular Dilation(TD)/cystic index**

Ten cortical grid fields (original magnification x200) for 2 serial sections of each kidney were evaluated. The TD/cystic quantification was performed using a screen with dots distant one from another by 13.625 micron. The measures were obtained by counting the number of dots located in the tubular lumen. The degree of TD was defined by the number of dots in the lumen, following previously suggested criteria: (1) TDI, including only one dot; (2) TDII (Tubular Dilation type II), two dots; (3) MCs, three to nine dots; and (4) cysts, ten dots or more.

**Proliferation of GTCs Y<sup>+</sup> cells**

To evaluate the proliferative potential of GTCs-injected Y<sup>+</sup> cells, we performed BrdU staining after *in situ* analysis of Y chromosome on renal sections. The primary antibody (DAKO, clone Bu20a) was diluted in 1% Bovine Serum Albumin (BSA, Sigma) and incubated for 1h at room temperature.
Figure S1. Characterization of GPSCs derived tubular-like cells. RNA was extracted at different time points and RealTime-PCR analysis was performed. Podocalyxin is a marker of the podocytes, a type of cell that compose the glomerulus, and its expression drops after day 14 (A). The same trend is showed by nephrin (B) and Wt1 (C), another two markers of glomerular cells. Each column refers to 3 independent samples (N=3).
Figure S2. KSP\(^+\) cells fraction collected from MACS were tested for the expression of various differentiation markers. KSP\(^+\) cells express mineralcorticoid receptor (A) that start to decrease two days from MACS separation. This fraction of cells do not express podocalyxin (B), Wt-1 (C), oct4 (D) and goosecoid (E). (KSP\(^+\) d0: RNA was extracted immediately after isolation, KSP\(^+\) d2: RNA was extracted 2 days after cells isolation).
Figure S3. The pictures refer to immunofluorescence staining for ZO-1 of NT fraction, KSP⁺ fraction and primary tubules. DNA is counterstained with DAPI. Original magnification x630. NT: undifferentiated GSPCs, KSP⁺: cells recovered from MACS sorting. (ZO-1: Zonula Occludens protein-1; DAPI: 4,6 diamidino-2-phenylindole).
Figure S4. Proliferation of Y⁺ cells 48h post IRI. Picture (A) show a Y⁺/BrdU⁻ cell in the tubular interstitium, picture (B) represents a negative control of the same mouse stained for BrdU alone. Pictures (C) and (D) display two areas characterized by the presence of Y⁺/BrdU⁺ cells in the renal parenchyma. Pictures C and D highlight that the number of Y⁺ proliferating cells is lower compared to the endogenous proliferating ones. Original magnification x1000. BrdU⁺ cells are revealed with a secondary antibody alexa 565 (green signal), red dots represent the Y chromosome, nuclei are counterstained with DAPI. The chart below (E) shows the quantification of Y⁺ cells in renal parenchyma 48h and six weeks after IRI. (acute damage, N=8; chronic damage, N=10).
**Figure S5.** (A) RealTime-PCR analysis 48h post IRI that show up-regulation of HO-1 in mice injected with GTCs compared to mice injected with PBS. No difference is detected between ischemized mice injected or not with GTCs 48h post ischemia concerning the CD18+ cells (B) and the proliferation rate (C). (GTCs-injected mice, N=8; PBS-injected mice, n=8).
Figure S6. GPSCs injection after AKI. Representative H&E of PBS-injected mice (A) and GPSCs-injected mice (B) (Original magnification x100). The level of BUN and creatinine (C) in the blood do not differ significantly between the two groups of mice. Real-Time PCR analysis shows that GPSCs are not able to up-regulate HO-1 (D), as confirmed by the counting of HO-1+ tubules (E). No statistically relevant difference is detected in the number of inflammatory infiltrates (F), CAST and tubular necrosis (H). The incidence of apoptosis (G) is not affected by GPSCs injection. (GPSC-injected mice, N=6; PBS-injected mice, N=4).
Supplementary figure 7

A

B

C

D

E

F

G

H

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Supplementary figure 7

A

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C

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Figure S7. Protection of kidney 48h post ischemia by KSP+ cells. Representative H&E staining of IRI-PBS mice (A) and IRI-KSP+ cells mice (B) (Original magnification x100). BUN but not creatinine (C) in the blood of mice injected with KSP+ cells is significantly lower compared to mice injected with PBS. RealTime-PCR analysis (D) shows up-regulation of HO-1, confirmed by HO-1 staining (E), in renal parenchyma of mice injected with KSP+ cells compared to control mice. Number of apoptotic cells (F), CAST and tubular necrosis (G), is significantly down-regulated in mice injected with KSP+ cells compared to PBS-injected mice. No difference is found in the number of inflammatory infiltrates (H). Pictures (I) show a representative in situ analysis for Y chromosome. (*) Indicate the tubules. (Original magnification x1000, nuclei are counterstained with DAPI). (KSP-injected mice, N=6; PBS injected mice, N=4).