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Pathfinder Cells Provide A Novel Therapeutic Intervention For Acute Kidney Injury

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Abstract

Pathfinder cells (PCs) are a novel class of adult-derived cells that facilitate functional repair of host tissue. We used rat PCs to demonstrate that they enable the functional mitigation of ischemia reperfusion (I/R) injury in a mouse model of renal damage. Female C57BL/6 mice were subjected to 30 min of renal ischemia and treated with intravenous (i.v.) injection of saline (control) or male rat pancreas-derived PCs in blinded experimentation. Kidney function was assessed 14 days after treatment by measuring serum creatinine (SC) levels. Kidney tissue was assessed by immunohistochemistry (IHC) for markers of cellular damage, proliferation, and senescence (TUNEL, Ki67, p16^{ink4a}, p21). Fluorescence *in situ* hybridization (FISH) was performed to determine the presence of any rat (*i.e.*, pathfinder) cells in the mouse tissue. PC-treated animals demonstrated superior renal function at day 14 post-I/R, in comparison to saline-treated controls, as measured by SC levels (0.13 mg/dL vs. 0.23 mg/dL, $p < 0.001$). PC-treated kidney tissue expressed significantly lower levels of p16^{ink4a} in comparison to the control group ($p = 0.009$). FISH analysis demonstrated that the overwhelming majority of repaired kidney tissue was mouse in origin. Rat PCs were only detected at a frequency of 0.02%. These data confirm that PCs have the ability to mitigate functional damage to kidney tissue following I/R injury. Kidneys of PC-treated animals showed evidence of improved function and reduced expression of damage markers. The PCs appear to act in a paracrine fashion, stimulating the host tissue to recover functionally, rather than by differentiating into renal cells. This study demonstrates that pancreatic-derived PCs from the adult rat can enable functional repair of renal damage in mice. It validates the use of PCs to regenerate damaged tissues and also offers a novel therapeutic intervention for repair of solid organ damage *in situ*.

Introduction

ACUTE KIDNEY INJURY (AKI) is a condition of varied aetiology associated with high rates of mortality and morbidity. Clinically, AKI is characterized by a rapid deterioration in kidney function resulting in a failure to maintain fluid, electrolyte, and acid–base homeostasis (Renal Association Guidelines¹). Recent figures suggest that approximately 5%–20% of critically ill patients experience an episode of AKI during the course of their illness.¹ Following cardiac surgery, up to 30% of patients can develop AKI.² Whereas the overall incidence of AKI following cardiac surgery may remain low, the associated mortality is high. Data have shown that an increase of only 0.3 mg/dL in serum creatinine levels is an independent predictor of morbidity and

mortality in patients undergoing cardiac surgery.³ AKI is generally the consequence of an ischemic, or toxic, insult that results in damage to the renal proximal tubule cells. Renal ischemia/reperfusion (I/R) injury is frequently associated with AKI and is particularly pertinent in transplantation (renal replacement therapy). I/R injury can have a detrimental effect on organ function post-transplant, resulting in delayed graft function, acute rejection, and late graft dysfunction.

Despite this, there is no gold standard treatment for repairing and restoring kidney function. Renal replacement therapy (RRT) is currently the best treatment modality for those with end-stage renal disease (ESRD). Renal dialysis is a common first line of defense, but it is unsatisfactory in terms of outcome (only 10.5% of patients survive 10 years on

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dialysis), patient quality of life, and cost. Transplantation, while life saving, is compromised by the ongoing critical shortfall in available donor organs and episodes of organ rejection and dysfunction. Renal disease is a growing medical problem, with high levels of associated morbidity and mortality. Currently, there are 1:2,000 people within the European Union and 1:1,000 in the United States, with chronic kidney disease (CKD) who would benefit from a transplant. In the United States and European Union over 100,000 patients are awaiting a kidney transplant, but only 20,700 transplants are performed annually.

A preferred treatment option would be to initiate repair of kidney damage as early as possible, and hence prevent, or at least postpone, progression to ESRD or organ dysfunction post-transplant. This is particularly pertinent to the nature of molecular deterioration in the damaged organ, as either a consequence of disease or pathology following RRT. In each instance, the organ shows accelerated bioaging, with an accumulation of senescent cells, correlated with increased CDKN2A (p16^{ink4a}) expression.⁴⁻⁶ One hypothesis suggests that replacement of such cells is required to enhance function and regenerate the organ. Precedence for this hypothesis exists in a recent report that indicates that the removal of p16^{ink4a}-expressing cells from tissues delays the onset of age-related pathologies.⁷

Cellular therapy is thus an attractive option to regenerate damaged kidneys *in situ*. However, the kidney is a particularly morphologically complex organ, requiring approximately 26 terminally differentiated cell types to be organized into a highly ordered, spatially organized structure.⁸⁻¹⁰ Consequently, the proliferative potential of the kidney is low in comparison to other tissues. Despite this, the kidney does have the capacity for self-regeneration and repair, with the ability to recover from insults such as acute tubular necrosis.¹¹

Current candidate cellular therapies to tackle kidney injury are all in their infancy. These have included bone marrow stem cells (BMSCs) and multi-potent stromal cells/mesenchymal stem cells (MSCs). BMSCs, although a promising candidate for treating renal disease, have produced equivocal results. Unfractionated BMSCs have been shown to differentiate into endothelial and mesangial cells in a model of progressive glomerulosclerosis.¹² There is further evidence that they can also differentiate into tubular epithelial cells and podocytes.¹¹ However, the use of unfractionated BMSCs produces very low rates of tubular cell replacement and limited functional improvement.^{8,13}

Several studies using MSCs have indicated an improvement in renal structure and increased tubular cell proliferation, accompanied by a concomitant recovery in renal function, as measured by lower blood urea nitrogen (BUN) levels.¹⁴ This has also been substantiated in a study of glycerol-induced acute renal failure (ARF).¹⁵ MSCs have been demonstrated to produce essential growth factors, such as vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), and insulin-like growth factor-1 (IGF-1), which are associated with enhanced proliferation, improved survival, and decreased apoptosis.^{8,16} In addition, these factors have been shown to have a specific role in kidney function and repair.¹¹ The release of these factors by MSCs may therefore promote proliferation and survival of tubular epithelial cells, while minimizing apoptosis. Recently, adipose-derived MSCs have been shown to protect

rat kidneys following I/R injury via reduction of oxidative stress and suppression of the inflammatory response, while concomitantly promoting angiogenesis. Transcription levels of genes encoding anti-inflammatory, anti-oxidative, and anti-apoptotic biomarkers were higher in rats treated with adipose-derived MSCs following I/R injury than in controls.¹⁷ The hypothesis that MSCs may promote repair by reducing the levels of oxidative stress following insult is further supported by a report indicating that MSC treatment reduced levels of superoxide dismutase and glutathione peroxidase following I/R injury in rats.¹⁸

A further candidate cell type for renal repair comprises adult parietal epithelial multipotent progenitors (APEMPs), which have been identified in the Bowman capsule of the adult kidney.¹⁹ These are reported to display multi-differentiation potential and self-renewal capacity. Mice treated with APEMPs have improved recovery from ARF, as measured by lowered BUN levels, with smaller areas of necrotic kidney tissue, suggesting that APEMPs have the ability to repair tubular damage.²⁰

An alternative cell-based approach to treat kidney injury entails the use of pathfinder cells (PCs).^{21,22} PCs have been demonstrated to initiate repair of host tissue across a species barrier in the pancreas.²² In a mouse streptozotocin-induced diabetes model, both rat and human PCs have been demonstrated to induce regeneration of the damaged host (mouse) pancreas²² in a paracrine fashion and to restore long-term normal glycemia. No immunosuppression was administered with either rat or human PC treatments, as preliminary experiments were consistent with the PC cells being immunologically null. PC cell administration in the presence of 20 mg/kg cyclosporin did not affect the capacity of PC cells to reduce blood glucose levels.²²

Therefore, we have hypothesized that PCs may similarly be able to facilitate repair in other organs. Given the increasing incidence of kidney disease, the lack of treatment options and suitable cell therapies, we considered the kidney to be an attractive organ for testing the capacity of the PCs to stimulate regeneration. We undertook this experiment with a view to achieving solely a therapeutic end point, namely restoration of normal kidney function in treated animals. To test this hypothesis we used a well-established renal ischemia reperfusion injury mouse model²³ to determine if following an I/R injury, rat PCs could facilitate restoration of normal tissue architecture in damaged kidneys and critically restore renal function.

Materials and Methods

Isolation, maintenance, and characterization of pancreas-derived PCs

Pancreatic ductal tissue was isolated from 12-month-old Albino Swiss (Glasgow) rats. Tissue was micro-dissected and minced, prior to seeding in CMRL-1066 medium (Invitrogen, Paisley, UK). The PCs emerged as a confluent monolayer after approximately 5 weeks in culture. These were then harvested and washed in phosphate-buffered saline (PBS). PCs were maintained in culture in 20 mL of CMRL-1066 medium supplemented with 10% fetal bovine serum (FBS; Sigma, Poole, UK), 2 mM glutamate, 1.25 mg/mL amphotericin B, and 100 U/mL penicillin, 100 µg/mL streptomycin (all Invitrogen, Paisley, UK) in T75 culture

flasks with 0.2- μm filter caps (Corning, UK) at 37°C in a 5% CO₂ atmosphere.

PCs were comprised of a mixed CD90 cytotype, positive for expression of c-met, nestin, CD147, CD44, CD49f, and CD71 and negative for CD31, CD34, CD45, CD105, CD73, and c-kit expression.²¹

Renal ischemia model

For this model, female C57BL/6 mice were used, and the experimental details are described fully in Hochegger et al.²³ In this instance, animals underwent 30 min of renal ischemia, applied via renal clamping.

PC administration

Adult rat pancreas derived PCs were administered intravenously (i.v.) via the tail vein to subject mice ($n=14$) following an ischemic period of 30 min. Within this group there were three different treatment regimens. The first group (group A, $n=5$) had 1.5×10^6 PCs (in a volume of 500 μL of saline) injected 24 h after surgery. A second injection of 1.5×10^6 PCs was administered to the mice 1 week later. The second group (group B, $n=5$) received two injections of 1.5×10^6 PCs, 24 h after surgery. The final treatment group (group C, $n=4$) was treated with 1.5×10^6 PCs immediately after surgery. They received a second injection of 1.5×10^6 cells 1 week later.

Controls comprised a group that had no surgery performed on them (untreated healthy controls $n=6$) and animals that were subjected to an ischemic event, but were left untreated (saline controls, ischemia, no treatment, $n=6$). These animals were injected with 500 μL of saline into their tail vein 24 h after surgery.

Assessment of renal function

Blood samples were taken from mice 14 days post-ischemic event, and serum creatinine levels were measured using a creatinine autoanalyzer (Beckman Coulter, Fullerton, CA) to derive a measure of renal function.

Assessment of histological injury

Kidney tissue was fixed in buffered 4% formalin and then embedded in paraffin wax. The kidneys were sectioned at 5 μm and stained with Periodic Acid Schiff (PAS) prior to examination by light microscopy.

Immunohistochemistry

Kidneys from PC-treated and control mice were removed, snap-frozen in liquid nitrogen, and embedded in Tissuetek OCT compound. Both immunohistochemistry (IHC) and histoscore analysis were performed blindly on the tissue sections, i.e., the treatment group was unknown. Fixed frozen sections were rehydrated in PBS for 5 min. Endogenous peroxidase was quenched by incubating sections in 3% hydrogen peroxide (H₂O₂) for 10 min. Blocking was performed by incubating sections in 20% goat serum in Tris-buffered saline (TBS) for 1 h at 25°C. Tissue sections were incubated with 2 $\mu\text{g}/\text{mL}$ p21 Ab (Clone C-19, Santa Cruz Biotechnology, Inc), 8 $\mu\text{g}/\text{mL}$ p16^{ink4a} Ab (Clone M-156, Santa Cruz Biotechnology, Inc), or 20 $\mu\text{g}/\text{mL}$ Ki67 (Abcam) in Antibody Diluent (Dako) overnight

at 4°C. In each run, negative and positive controls were included. Signal was visualized using a goat anti-rabbit secondary antibody (Dako, 1:200) for 1 h at 25°C followed by 3, 3'-diaminobenzidine (DAB; Vector Laboratories).

Terminal deoxynucleotidyl transferase dUTP nick end-labeling assay (TUNEL)

Apoptotic cells were identified within the tissue using the ApopTag Plus Peroxidase *In Situ* Apoptosis Detection Kit (Chemicon) following the manufacturer's instructions. Briefly, fixed frozen sections were re-hydrated in PBS for 5 min. Slides were then incubated with 20 $\mu\text{g}/\text{mL}$ Proteinase K solution for 15 min at 25°C, treated with 3% H₂O₂ for 5 min, followed by equilibrium buffer for 10 min. Slides were then incubated with terminal deoxynucleotidyl transferase (TdT) for 1 h before being washed and then treated with anti-digoxigenin for 30 min. Signal was visualized using DAB.

Histoscore analysis

To assess the expression of the various senescence- and apoptosis-related markers in the kidney tissue, a semi-quantitative weighted histoscore method was employed as described previously.^{24,25} Briefly the intensity of cytoplasmic and nuclear staining was categorized as negative (0), weak (1), moderate (2), and strong (3), and the percentage of cells within each category was estimated. The histoscore was calculated using the following formula: Histoscore = 0 × % negative cells + 1 × % weakly stained cells + 2 × % moderately stained cells + 3 × % cells stained strongly. The histoscore ranged from a minimum of zero to a maximum of 300. To assess for Ki67 staining nuclei, the percentages of positive and negative nuclei were determined. Cytoplasmic and nuclear staining for p21, p16^{ink4a}, Ki67, and TdT dUTP nick end-labeling assay (TUNEL) were scored blindly by two independent scorers.

Fluorescence in situ hybridization

To track the transplanted cells, we used a xenogeneic model in which male rat PCs were given to female C57BL/6 mice; this allowed us to distinguish rat PCs from the host mouse cells via the detection of the rat Y chromosome. Frozen sections were rehydrated in PBS, then pretreated with 1 M sodium thiocyanate at 80°C for 20 min. Tissue digestion was performed by treating sections with pepsin for 25 min at 37°C. Slides were then treated with 0.2% glycine for 5 min, then washed in TBS and incubated with 4% paraformaldehyde for 5 min. A probe to detect rat Y chromosome (Rat YCy3/12FITC, Cambio) was used. Before use, the probe was denatured at 65°C for 10 min. The tissue was denatured in denaturation solution at 65°C for 1.5 min, and then incubated in ice-cold ethanol for 2 min. Tissues were then dehydrated through a series of graded alcohols, before 10 μL of probe was added to each section. Probe and tissue were hybridized for 10 min at 80°C and then overnight at 42°C. Slides were then washed in post-hybridization wash buffer for 5 min at room temperature, followed by a wash at 72°C for 2 min. Slides were allowed to air dry before being mounted onto coverslips using VECTASHIELD (Vector). The percentage of PCs present within the treated mice kidneys was then calculated, by determining the total

number of cells and the number of Rat Y/12-positive cells present within the tissue.

Statistical analysis

t-Tests were performed to establish any differences in the serum creatinine levels and expression levels of the senescent and apoptotic immunohistochemical markers for the various treatment groups.

Results

PCs stimulate improved kidney function following I/R injury

We observed significant improvement in kidney function in PC-treated animals versus saline-treated controls, as measured by serum creatinine (SC) levels 14 days after the I/R injury. PC-treated groups A (mean SC=0.13 mg/dL), B (0.13 mg/dL), and C (0.14 mg/dL) all had significantly lower SC levels than the saline-treated control group (0.23 mg/dL) ($p=0.005$, $p=0.002$, $p=0.01$, respectively; Fig. 1). There was no significant difference between the three PC-treated groups and the untreated control (no ischemia) mice. However, the saline-treated group of mice had significantly higher SC levels than the untreated mice (0.23 vs. 0.10 mg/dL, $p<0.001$). Despite the different PC doses and treatment times, there was no significant difference in the SC levels of the three different PC-treated groups.

PCs facilitate repair of kidney tissue damage following I/R injury

Assessment of the expression of p16^{ink4a} was used to provide a molecular correlate of renal function, as determined by SC levels. We and others have previously demonstrated that p16^{ink4a} is a superior marker for the assessment of renal function.^{4,5} p16^{ink4a} expression was significantly lower in the kidneys of PC-treated mice than those mice treated with saline ($p=0.009$; Fig. 2A, 2B). No significant difference in the expression levels of p16^{ink4a} was observed between the untreated mice and the PC-treated mice (Fig. 2A,B). These observations are consistent with kidneys of PC-treated animals having less damage and hence fewer senescent cells present than the saline-treated animals. PCs thus appear to facilitate tissue repair in the kidney.

Expression of p21 and TUNEL did not differ between the PC-treated animals and the two control groups of mice 14 days after ischemia (Fig. 2A,B). This result is expected as changes in p21 expression and apoptosis are known to occur earlier than the 14-day time point in this model.

Determination of Ki67 was undertaken to assess the extent of ongoing cellular proliferation at the end of the experiment (day 14). No significant difference in the Ki67 levels was observed between untreated healthy controls and PC-treated animals. Ischemic controls (saline treatment) expressed higher levels of Ki67 at day 14 than untreated animals ($p=0.029$; Fig. 2A, 2C), suggesting continued proliferation in

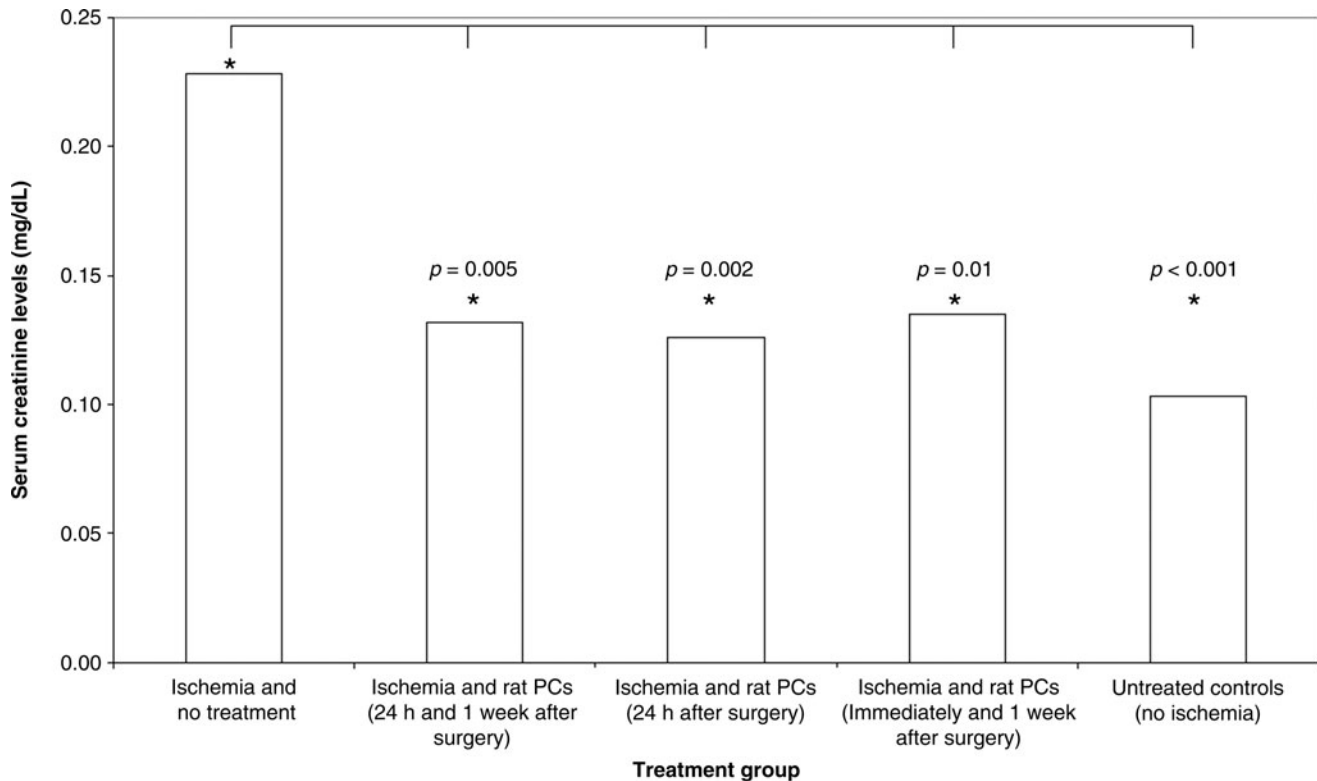


FIG. 1. Rat pathfinder cell (PC) treatment improves renal function in mice subject to ischemia. Histogram depicting serum creatinine (SC) levels as a measure of renal function at the end point of the experiment ($t=14$ days), in untreated controls ($n=6$), the different ischemia and PC-treated animals ($n=5$, $n=5$, $n=4$) and ischemia and no treatment (saline) controls ($n=6$). The serum creatinine levels were higher in ischemia and no treatment animals in comparison to the three PC-treated groups ($p=0.005$, $p=0.002$, and $p=0.01$) and the untreated control ($p<0.001$). There were no significant differences in serum creatinine levels between the three PC-treated groups or between the PC-treated groups and untreated controls. *Statistically significant difference $p<0.05$.

the ischemic animals to compensate for the absence of external stimuli to repair the damaged kidneys.

The findings that PC treatment facilitates repair of kidney damage induced by ischemia is further supported by histological analysis of the kidneys (Fig. 3). Analysis of kidneys stained with PAS indicates that PC-treated animals displayed normal histology and renal tissue morphology.

Repair and restoration of kidney function is due to PC-stimulated host tissue regeneration rather than PC differentiation

To establish whether the observed repair of the ischemic damaged kidneys was a result of host tissue regeneration, PC differentiation, or a combination of both, FISH experiments

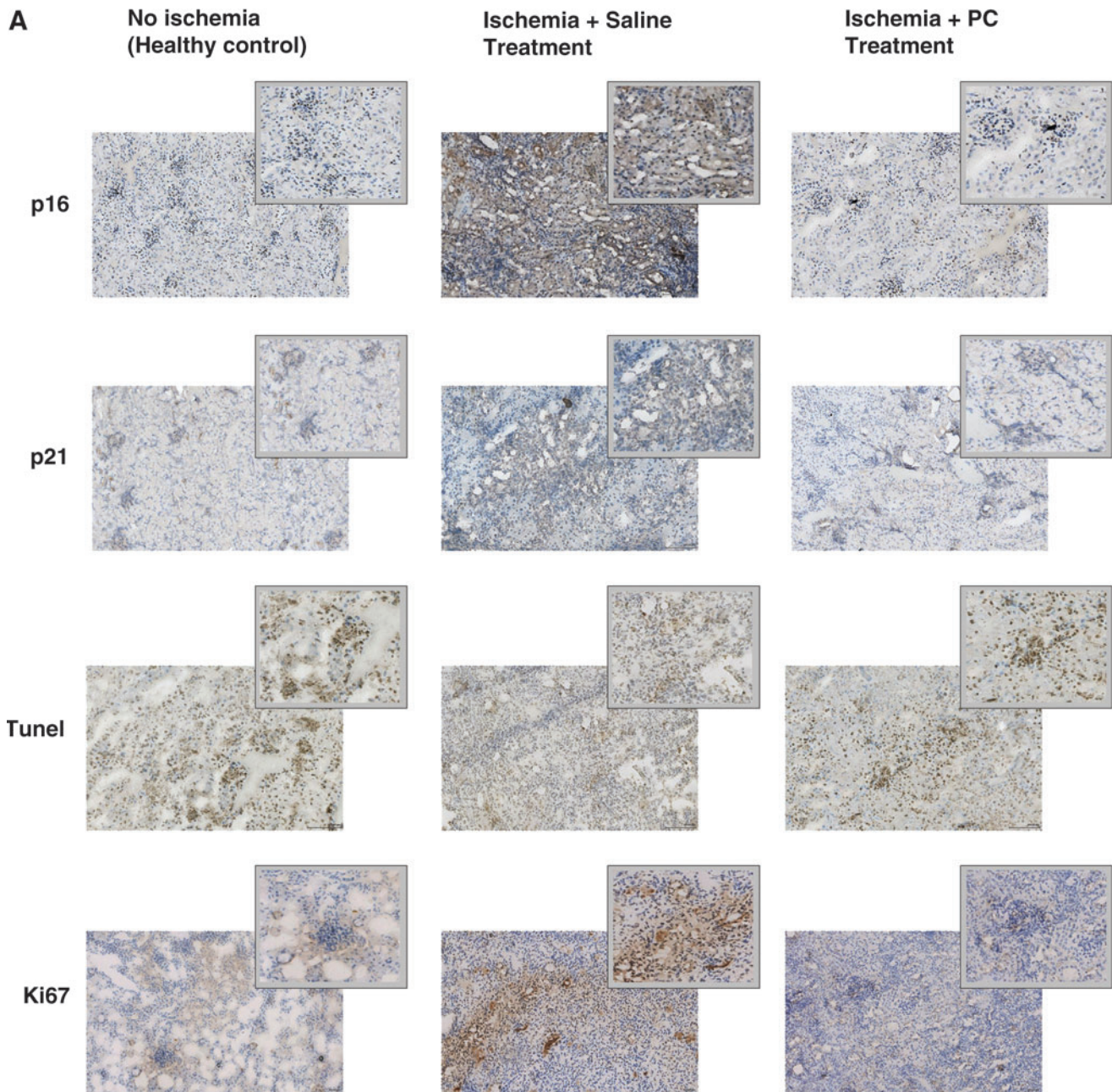


FIG. 2. (A) Assessment of cellular bioaging in pathfinder cell (PC)-treated animals and controls following ischemia. Representative pictures of p16^{ink4A}, p21, terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL), and Ki67 IHC staining in kidney tissue from untreated controls, ischemia and saline-treated animals, and ischemia and PC-treated animals. (B) Histogram depicting average histoscores for p16^{ink4A}, p21, and TUNEL expression in untreated controls, ischemia and PC-treated animals, and ischemia and no treatment (saline) controls. There was significantly less p16^{ink4A} expression detected in the kidneys of PC-treated animals post-ischemia/reperfusion (I/R) event in comparison to those from saline-treated ischemic controls ($p=0.009$). (C) Histogram depicting the average percentage of Ki67-positive nuclei in untreated (healthy) controls, ischemia and PC-treated animals, and ischemia and no treatment (saline) controls. *Statistically significant difference $p<0.05$.

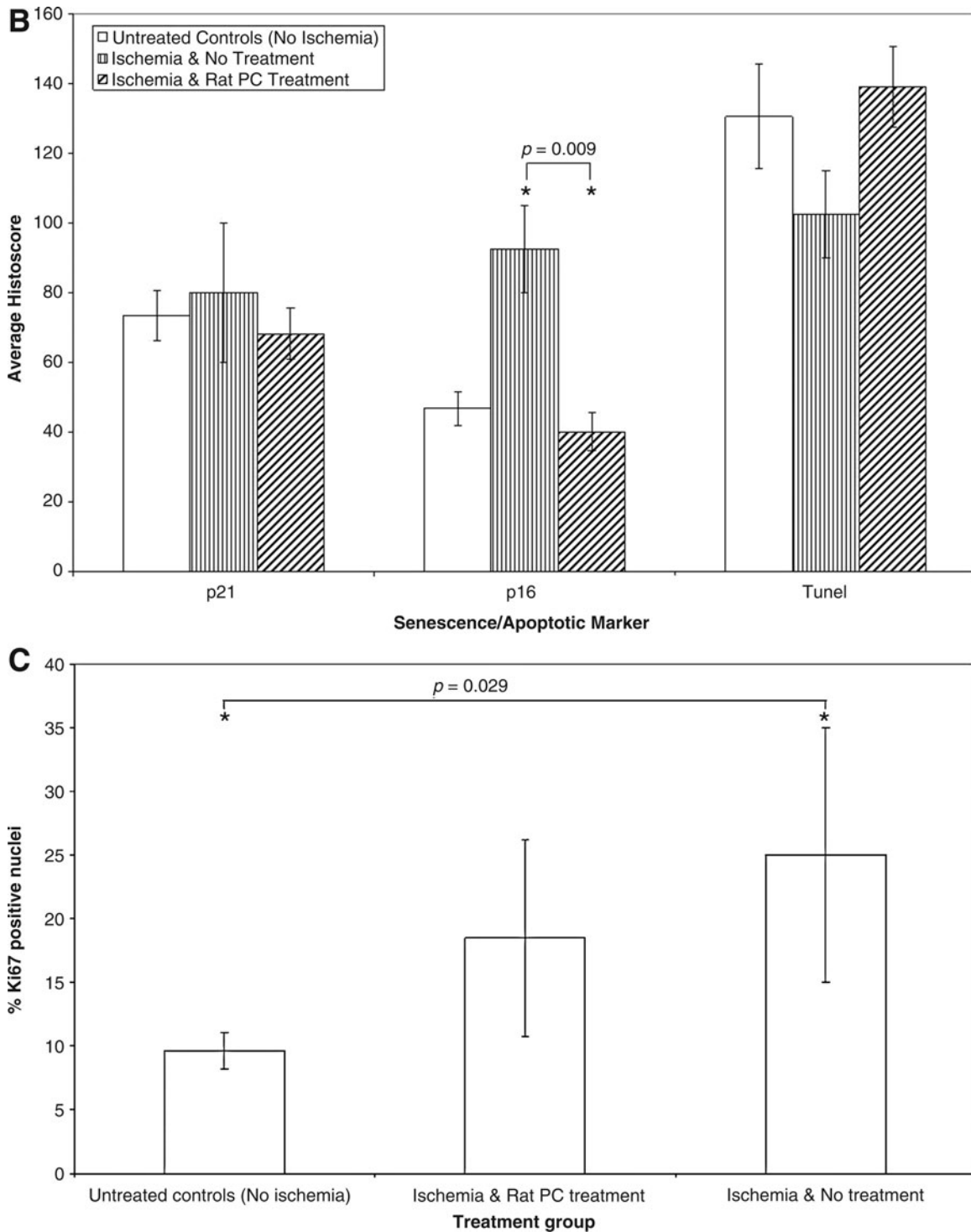


FIG. 2. Continued.

were performed to detect the presence of any male rat cells (PCs or PC-derived) within the kidneys of the female C57Bl/6 mice.

Mice from each PC-treated group were randomly selected for FISH analysis (5 kidney tissue sections per animal). Detection rate of the xenogeneic signal was low in all PC-treated animals (0.02%). These observations are consistent

with the enablement of regeneration of host (mouse) kidney tissue by rat PCs (Fig. 4), as opposed to direct participation in rebuilding renal tissue. As we have no information about the frequency of PC division, we do not know if these signals are from surviving original cells or from progeny of these cells. Consequently, we do not make any statements concerning survival of the original cells.

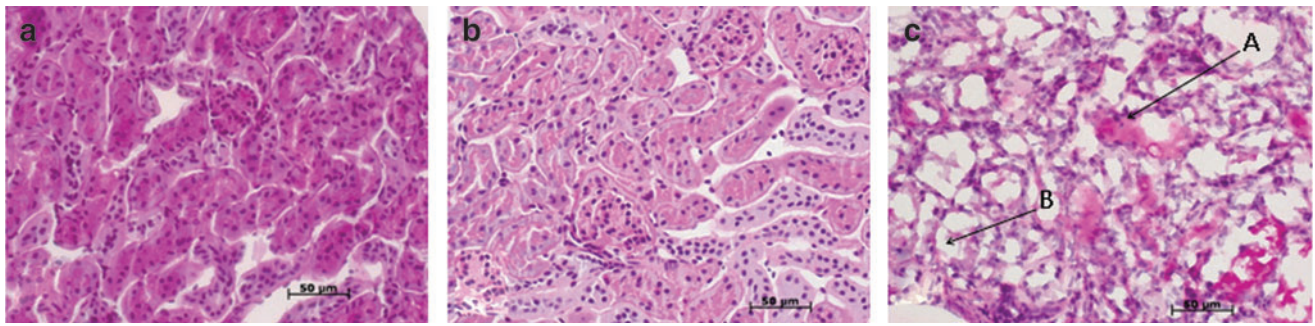


FIG. 3. Histological examination of kidneys from pathfinder cell (PC)-treated mice and controls. Light microscopy showing Periodic Acid Schiff (PAS) staining of representative cortical areas from untreated control kidney (a), ischemic kidney treated with PCs (b), and untreated ischemic kidney (c) Arrows in c indicate increased casts and brush border loss.

Discussion

We have demonstrated that PCs derived from the adult rat pancreas facilitate repair of damaged mouse kidneys and restoration of kidney function following severe ischemic insult. Repaired kidneys appear histologically normal and display reduced biological age compared to untreated ischemic kidneys, as determined by $p16^{\text{ink4a}}$ expression levels in the repaired organ. Significantly, the repaired tissue was overwhelmingly mouse (host) in origin. Rat cells were detected at a maximal frequency of less than 0.02% in treated animals. This is consistent with previous observations in streptozotocin model of mouse diabetes.²² To our knowledge, this is the first demonstration of an adult cell type derived from pancreatic tissue fulfilling such a role and the first demonstration of this in a xenogenic setting.

PC-treated animals were shown to have significantly lower serum creatinine levels than saline-treated controls after I/R injury. Fourteen days post-ischemia, serum creatinine levels were restored to within a normal range ($SC < 0.15 \text{ mg/dL}$). This restoration of function was coincident with restoration of normal kidney histology and a decrease in the number of senescent and damaged cells present within

the kidney tissue of PC-treated animals in comparison to saline-treated control mice.

Saline-treated controls exhibited a higher number of cells expressing $p16^{\text{ink4a}}$ than those observed in PC-treated animals, consistent with DNA damage induced by ischemia. Previously, it has been shown that ischemic injuries up-regulate expression of cyclin-dependent kinase inhibitors such as p21 and $p16^{\text{ink4a}}$.^{23,26–28}

Additionally, $p16^{\text{ink4a}}$ has been identified as a biomarker for pre-transplant prediction of renal function post-transplant. Higher levels of $p16^{\text{ink4a}}$ expression are associated with poorer function as measured by serum creatinine and urine protein-to-creatinine ratio (UPCR) levels at 6 months and 1 year post-transplant.^{29,30} This correlates with the findings from this study that control mice that presented with higher serum creatinine levels also exhibited higher expression levels of $p16^{\text{ink4a}}$.

Within the time course of this experiment, we would have expected to see an initial rise in p21, peaking by day 7, then reducing to control levels by day 14, with a correlated increase in $p16^{\text{ink4a}}$ expression from day 7, peaking at day 14.²⁸ Our observations on p21 expression at day 14 post-ischemia are consistent with this, because we observed no significant

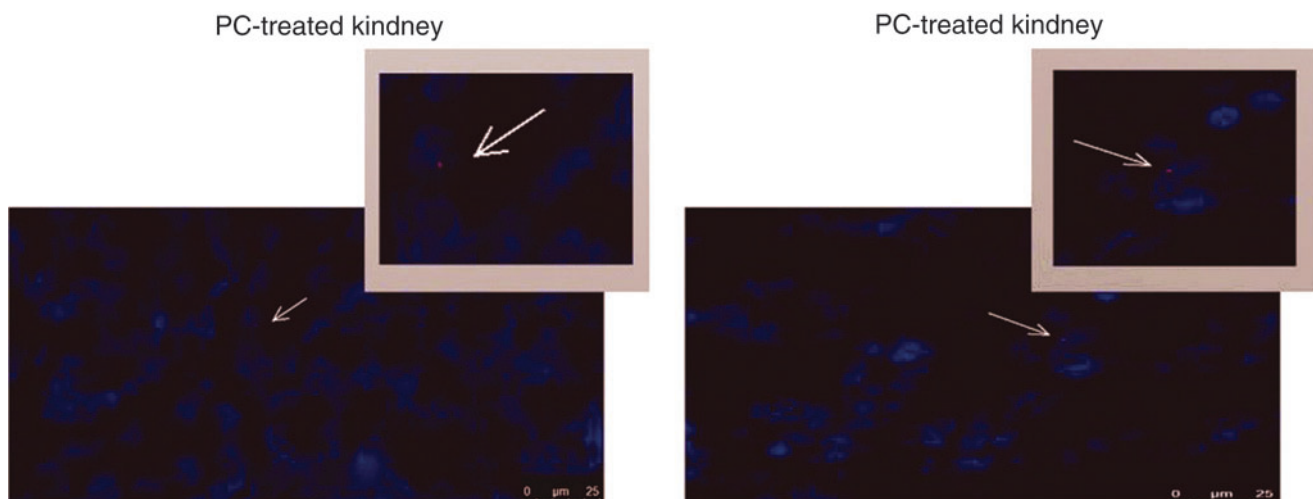


FIG. 4. Fluorescence *in situ* hybridization (FISH) detection for the rat Y chromosome, corresponding to pathfinder cells (PCs) or PC-derived cells, in the kidneys of mice subject to renal ischemia and treated with rat PCs. White arrows indicate detection of the rat Y chromosome. Scale bar, 25 μm .

differences between the PC-treated group and controls. We also observed no difference in the levels of apoptotic cells, as measured by the TUNEL assay, between PC and control animals. As for p21 expression, this is because apoptosis in this model would have finished before day 14 when the mice were taken for analysis.²⁸

The mechanism by which PCs contribute to the repair and regeneration process remains to be determined. There are at least five possible modes of action that may be considered in this respect. First, the decreased levels of p16^{ink4a} observed in PC-treated animals may indicate that PCs have solely a protective role against ischemic AKI. Second, the cells are recruited to the damaged kidney and differentiate into the required renal cell types. Third, the injected cells fuse with existing host cells to repair any damage. Fourth, the cells release paracrine/endocrine signals that stimulate repair processes without actually becoming engrafted into the host tissue. A fifth possibility is that the PCs stimulate endogenous stem cells present within the host kidney, or a differentiated cell type, to regenerate kidney tissue. These latter two options may not necessarily be mutually exclusive and either or both may be extant in the repair process.

In considering the first possible mechanism, while this is a formal possibility, it is not in keeping with previous data on pancreatic repair, where xenogenic PCs were responsible for a paracrine mediated developmental recapitulation of islets,²² nor with repair of ischaemic cardiac damage (McGlynn et al., in preparation). It is not however, mutually exclusive from other possible mechanisms.

Options two and three seem unlikely from our results and those of other studies to be the main modes of action undertaken by PCs. If the PCs were to be recruited to the damaged organ, engrafted within the tissue, and subsequently differentiated into renal epithelial cells, it would be expected that the detection rate of these cells, or their progeny, within the repaired kidney would be relatively high. In our xenogenic model, the detection rate for the male rat PCs was very low (0.02%). The overwhelming majority of cells present within the kidney were of host origin. Given the low frequency of PCs present, it seems unlikely that they operate by differentiating and populating the kidney or by forming cell fusions with the host cells. This is in agreement with findings from our previous diabetes mouse model that indicated that the overwhelming majority of the regenerated islet tissue was host in origin.²² Therefore, it is likely that cells already present within the kidney are stimulated to regenerate by the presence of the PCs. Whether these cells are differentiated renal cells or renal stem/progenitor cells remains to be determined. At least one report has suggested that differentiated tubular epithelial cells that survive an ischemic event are responsible for regenerating almost all of the new tubular epithelial cells.³¹ The rationale for this is that there is a large reserve of these cells in the G₁ phase that enables them to elicit a proliferative response when activated.³¹⁻³³

More recently, it has been reported that the repair of renal proximal tubules is facilitated by self-duplication of differentiated epithelial cells and not by intra-tubular progenitor cells, as previously suggested. This same study also reports that the cells most likely to be involved in the repair process are the result of dedifferentiation/redifferentiation events.³⁴ It has also been reported that repair of damaged renal epithelial cells is initiated when the tubular epithelial cells begin

to proliferate and that this is characterized by the detection of mesenchymal markers, usually only detectable during nephrogenesis. It has been hypothesized that following injury tubular cells may undergo an epithelial-to-mesenchymal transition^{31,35} to enable repair. This proposed reprogramming of the tubular cells is interesting and parallels our previous findings from the use of PCs to treat diabetic mice.²² In the regenerated pancreata of PC-treated mice, we detected the Insulin (Ins) I transcript, which suggested that developmental reprogramming had occurred as part of the repair process.²² Ins I is normally only produced during embryonic development in the mouse. Such developmental recapitulation is again in keeping with other observations in the field.

It remains to be determined if PCs act solely by paracrine signaling to enable any effect in the ischemic kidney and, or, whether this process incorporates dedifferentiation/redifferentiation of terminal renal cell types. Evidence from the use of MSCs *in vivo* suggests that they function in repairing renal damage not by differentiating themselves but by inhibiting pro-inflammatory cytokines and stimulating anti-inflammatory cytokines.⁸ This hypothesis is supported by the observation of up-regulation of inflammatory responses following organ I/R injury, coincident with an increase in cellular apoptosis, reactive oxygen species generation, and mitochondrial damage.¹⁷

A further consideration for the mechanism of PC-mediated renal repair is the involvement of a PC micro-secretome. The release of MSC-derived microvesicles (MV) and the associated transfer of MV mRNA has already been demonstrated to activate the proliferation of tubular epithelial cells and to protect against both acute and chronic kidney injury induced by I/R injury.^{36,37} The release of these secretory factors by the transplanted cells and not the engraftment and differentiation of the cells is consistent with a paracrine mediated repair of the kidney. Investigation of the factors secreted by the PCs is ongoing. The data presented here and in our previous study²² have offered possible mechanisms that may explain the action of PCs. However, their definitive mode of action remains to be determined. Further work is currently ongoing to ascertain how PCs stimulate the repair and regeneration processes in damaged tissue.

This study has clearly demonstrated that pancreatic-derived PCs enable functional recovery in mouse kidneys following an ischemic event. Our observations replicate those observed in an streptozotocin-induced diabetes model, whereby PCs repaired the pancreatic islets to such an extent that the effects of the diabetes were reversed and normal glycemia was restored.²² Consequently, we believe the use of PCs is neither restricted to their tissue, nor species of origin and that their use may be applicable to a wide range of tissue damage. As such, they appear to have great translational potential.

Author Disclosure Statement

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P.G.S. acts as a CSA for Pathfinder LLC.

R.W.D. acts as a CSO for Pathfinder LLC.

References

1. Lewington A, Kanagasundaram S. Clinical Practice Guidelines—Acute Kidney Injury. 5th Edition of the Renal Association Clinical Practice Guidelines 2011. Available at

- <http://www.renal.org/clinical/guidelinessection/guidelines.aspx/>. Accessed October 17, 2012.
2. Rosner MH, Okusa MD. Acute kidney injury associated with cardiac surgery. *Clin J Am Soc Nephrol* 2006;1:19–32.
 3. Thakar CV. Acute kidney injury after cardiac surgery. *US Nephrol* 2008;3:46–50.
 4. McGlynn LM, Stevenson K, Lamb K, Zino S, Brown M, Prina A, Kingsmore D, Shiels PG. Cellular senescence in pre-transplant renal biopsies predicts postoperative organ function. *Aging Cell* 2009;8:45–51.
 5. Koppelstaetter C, Schratzberger G, Perco P, Hofer J, Mark W, Ollinger R, Oberbauer R, Schwarz C, Mitterbauer C, Kainz A, Karkoszka H, Wiecek A, Mayer B, Mayer G. Markers of cellular senescence in zero hour biopsies predict outcome in renal transplantation. *Aging Cell* 2008;7:491–497.
 6. Transplantation—Experimental. *Nephrol Dialysis Transplant* 2006;21:iv259–iv262.
 7. Baker DJ, Wijshake T, Tchkonja T, LeBrasseur NK, Childs BG, van de Sluis B, Kirkland JL, van Deursen JM. Clearance of p16Ink4a-positive senescent cells delays ageing-associated disorders. *Nature* 2011;479:232–236.
 8. Sagrinati C, Ronconi E, Lazzeri E, Lasagni L, Romagnani P. Stem-cell approaches for kidney repair: choosing the right cells. *Trends Mol Med* 2008;14:277–285.
 9. Little MH. Regrow or repair: Potential regenerative therapies for the kidney. *J Am Soc Nephrol* 2006;17:2390–2401.
 10. Al Awqati Q, Oliver JA. Stem cells in the kidney. *Kidney Int* 2002;61:387–395.
 11. Iwatani H, Imai E. Kidney repair using stem cells: Myth or reality as a therapeutic option? *J Nephrol* 2010;23:143–146.
 12. Li B, Morioka T, Uchiyama M, Oite T. Bone marrow cell infusion ameliorates progressive glomerulosclerosis in an experimental rat model. *Kidney Int* 2006;69:323–330.
 13. Duffield JS, Park KM, Hsiao LL, Kelley VR, Scadden DT, Ichimura T, Bonventre JV. Restoration of tubular epithelial cells during repair of the postischemic kidney occurs independently of bone marrow-derived stem cells. *J Clin Invest* 2005;115:1743–1755.
 14. Imberti B, Morigi M, Tomasoni S, Rota C, Corna D, Longaretti L, Rottoli D, Valsecchi F, Benigni A, Wang J, Abbate M, Zoja C, Remuzzi G. Insulin-like growth factor-1 sustains stem cell mediated renal repair. *J Am Soc Nephrol* 2007;18:2921–2928.
 15. Herrera MB, Bussolati B, Bruno S, Fonsato V, Romanazzi GM, Camussi G. Mesenchymal stem cells contribute to the renal repair of acute tubular epithelial injury. *Int J Mol Med* 2004;14:1035–1041.
 16. Asanuma H, Meldrum DR, Meldrum KK. Therapeutic applications of mesenchymal stem cells to repair kidney injury. *J Urol* 2010;184:26–33.
 17. Chen YT, Sun CK, Lin YC, Chang LT, Chen YL, Tsai TH, Chung SY, Chua S, Kao YH, Yen CH, Shao PL, Chang KC, Leu S, Yip HK. Adipose-derived mesenchymal stem cell protects kidneys against ischemia-reperfusion injury through suppressing oxidative stress and inflammatory reaction. *J Translat Med* 2011;9:51.
 18. Zhuo W, Liao L, Xu T, Wu W, Yang S, Tan J. Mesenchymal stem cells ameliorate ischemia-reperfusion-induced renal dysfunction by improving the antioxidant/oxidant balance in the ischemic kidney. *Urol Int* 2011;86:191–196.
 19. Sagrinati C, Netti GS, Mazzinghi B, Lazzeri E, Liotta F, Frosali F, Ronconi E, Meini C, Gacci M, Squecco R, Carini M, Gesualdo L, Francini F, Maggi E, Annunziato F, Lasagni L, Serio M, Romagnani S, Romagnani P. Isolation and characterization of multipotent progenitor cells from the Bowman's capsule of adult human kidneys. *J Am Soc Nephrol* 2006;17:2443–2456.
 20. Mazzinghi B, Ronconi E, Lazzeri E, Sagrinati C, Ballerini L, Angelotti ML, Parente E, Mancina R, Netti GS, Becherucci F, Gacci M, Carini M, Gesualdo L, Rotondi M, Maggi E, Lasagni L, Serio M, Romagnani S, Romagnani P. Essential but differential role for CXCR4 and CXCR7 in the therapeutic homing of human renal progenitor cells. *J Exp Med* 2008;205:479–490.
 21. Stevenson K, McGlynn L, Hodge M, McLinden H, George WD, Davies RW, Shiels PG. Isolation, characterization, and differentiation of Thy1.1-sorted pancreatic adult progenitor cell populations. *Stem Cells Dev* 2009;18:1389–1398.
 22. Stevenson K, Chen DX, MacIntyre A, McGlynn LM, Montague P, Charif R, Subramaniam M, George WD, Payne AP, Davies RW, Dorling A, Shiels PG. Pancreatic-derived pathfinder cells enable regeneration of critically damaged adult pancreatic tissue and completely reverse streptozotocin-induced diabetes. *Rejuvenation Res* 2011;14:163–171.
 23. Hoehgegger K, Koppelstaetter C, Tagwerker A, Huber JM, Heining D, Mayer G, Rosenkranz AR. p21 and mTERT are novel markers for determining different ischemic time periods in renal ischemia-reperfusion injury. *Am J Physiol Renal Physiol* 2007;292:F762–F768.
 24. Kirkegaard T, Edwards J, Tovey S, McGlynn LM, Krishna SN, Mukherjee R, Tam L, Munro AF, Dunne B, Bartlett JMS. Observer variation in immunohistochemical analysis of protein expression, time for a change? *Histopathology* 2006;48:787–794.
 25. McGlynn LM, Kirkegaard T, Edwards J, Tovey S, Cameron D, Twelves C, Bartlett JM, Cooke TG. Ras/Raf-1/MAPK pathway mediates response to tamoxifen but not chemotherapy in breast cancer patients. *Clin Cancer Res* 2009;15:1487–1495.
 26. Megyesi J, Udvarhelyi N, Safirstein RL, Price PM. The p53-independent activation of transcription of p21(WAF1/CIP1/SDI1) after acute renal failure. *Am J Physiol-Renal Physiol* 1996;271:F1211–F1216.
 27. Kieran NE, Doran PP, Connolly SB, Greenan MC, Higgins DF, Leonard M, Godson C, Taylor CT, Henger A, Kretzler M, Burne MJ, Rabb H, Brady HR. Modification of the transcriptional response to renal ischemia/reperfusion injury by lipoxin analog. *Kidney Int* 2003;64:480–492.
 28. Joosten SA, van Ham V, Nolan CE, Borrias MC, Jardine AG, Shiels PG, van Kooten C, Paul LC. Telomere shortening and cellular senescence in a model of chronic renal allograft rejection. *Am J Pathol* 2003;162:1305–1312.
 29. McGlynn LM, Stevenson K, Lamb K, Zino S, Brown M, Prina A, Kingsmore D, Shiels PG. Cellular senescence in pre-transplant renal biopsies predicts postoperative organ function. *Aging Cell* 2009;8:45–51.
 30. Koppelstaetter C, Schratzberger G, Perco P, Hofer J, Mark W, Ollinger R, Oberbauer R, Schwarz C, Mitterbauer C, Kainz A, Karkoszka H, Wiecek A, Mayer B, Mayer G. Markers of cellular senescence in zero hour biopsies predict outcome in renal transplantation. *Aging Cell* 2008;7:491–497.
 31. Anglani F, Ceol M, Mezzabotta F, Torregrossa R, Tiralongo E, Tosetto E, Del Prete D, D'Angelo A. The renal stem cell system in kidney repair and regeneration. *Front Biosci* 2008;13:6395–6405.
 32. Vogetseder A, Palan T, Bacic D, Kaissling B, Le Hir M. Generation of proximal tubular cells in the healthy kidney

- occurs by division of differentiated cells. *Nephrol Dialysis Transplant* 2006;21:554–555.
33. Vogetseder A, Picard N, Gaspert A, Walch M, Kaissling B, Le Hir M. Proliferation capacity of the renal proximal tubule involves the bulk of differentiated epithelial cells. *Am J Physiol-Cell Physiol* 2008;294:C22–C28.
 34. Humphreys BD, Czerniak S, DiRocco DP, Hasnain W, Cheema R, Bonventre JV. Repair of injured proximal tubule does not involve specialized progenitors. *Proc Natl Acad Sci* 2011;108:9226–9231.
 35. Humphreys BD, Valerius MT, Kobayashi A, Mugford JW, Soeung S, Duffield JS, McMahon AP, Bonventre JV. Intrinsic epithelial cells repair the kidney after injury. *Cell Stem Cell* 2008;2:284–291.
 36. Gatti S, Bruno S, Deregibus MC, Sordi A, Cantaluppi V, Tetta C, Camussi G. Microvesicles derived from human adult mesenchymal stem cells protect against ischaemia-reperfusion-induced acute and chronic kidney injury. *Nephrol Dialysis Transplant* 2011;26:1474–1483.
 37. Bonventre JV. Microvesicles from mesenchymal stromal cells protect against acute kidney injury. *J Am Soc Nephrol* 2009;20:927–928.

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