




ORIGINAL RESEARCH

Transplantation of Apoptosis-Resistant Endothelial Progenitor Cells Improves Renal Function in Diabetic Kidney Disease

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BACKGROUND: Diabetic kidney disease is associated with glomerulosclerosis and poor renal perfusion. Increased capillary formation and improved perfusion may help to halt or reverse the injury. Transplanting apoptosis-resistant p53-silenced endothelial progenitor cells (p53sh-EPCs) may help improve vascularization and renal perfusion and could be more beneficial than another stem cell such as the mouse mesenchymal stromal cell (mMSC).

METHODS AND RESULTS: Hyperglycemia and proteinuria were confirmed at 8 to 10 weeks in streptozotocin-induced type1 diabetic C57Bl/6 mice, followed by transplantation of 0.3 million p53sh-EPCs, Null-EPCs (control), or mMSC under each kidney capsule. Urine was collected weekly for creatinine and protein levels. Blood pressure was measured by direct arterial cannulation and renal perfusion was measured by renal ultrasound. The kidneys were harvested for histology and mRNA expression. Reduction of protein/creatinine (AUC) was observed in p53sh-EPC-transplanted mice more than null-EPC (1.8-fold, $P=0.03$) or null-mMSC (1.6-fold, $P=0.04$, $n=4$) transplanted mice. Markers for angiogenesis, such as endothelial nitric oxide synthase (1.7-fold, $P=0.06$), were upregulated post p53sh-EPC transplantation compared with null EPC. However, vascular endothelial growth factor-A expression was reduced (7-fold, $P=0.0004$) in mMSC-transplanted mice, compared with p53sh-EPC-transplanted mice. Isolectin-B4 staining of kidney section showed improvement of glomerular sclerosis when p53sh-EPC was transplanted, compared with null-EPC or mMSC. In addition, mean and peak renal blood velocity (1.3-fold, $P=0.01$, 1.4-fold, $P=0.001$, respectively) were increased in p53sh-EPC-transplanted mice, relative to null-EPC transplanted mice.

CONCLUSIONS: Apoptosis-resistant p53sh EPC transplantation could be beneficial in the treatment of diabetic kidney disease by decreasing proteinuria, and improving renal perfusion and glomerular architecture.

Key Words: diabetes (kidney) ■ diabetes mellitus ■ endothelial progenitor cells

The rapidly growing prevalence of diabetes mellitus is becoming a major public health problem worldwide. The International Diabetes Federation data from 2019 showed that 463 million people were living with diabetes mellitus globally, and it is predicted that the number of patients with the diagnosis of diabetes mellitus will be 700 million by 2045.¹ The major contributors to the increasing prevalence of type 2 diabetes mellitus in the United States include an aging population with increasing incidence of overweight and obesity.²⁻⁵ Complications of diabetes mellitus include multiple vascular diseases, such as cardiovascular

disease, hypertension, nephropathy, neuropathy, peripheral vascular disease, and retinopathy.⁶⁻⁹ Diabetes mellitus, hypertension, or both cause 80% of end-stage renal disease, globally. Based on the report from the United States and the United Kingdom, 40% of patients with diabetes mellitus develop chronic kidney disease, as a consequence of diabetes mellitus.¹⁰ In addition, endothelial dysfunction plays a dominant role to develop end-stage renal disease.¹¹

In diabetes mellitus, the hyperglycemic environment disrupts the function and homeostasis of the endothelium. Damaged endothelium compromises

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CLINICAL PERSPECTIVE

What Is New?

- In this study we have found that transplantation of modified endothelial cells could be a promising cell therapy for diabetic kidney disease.

What Are the Clinical Implications?

- Transplantation of apoptosis-resistant endothelial progenitor cells could repair renal injury caused by hyperglycemia.

Nonstandard Abbreviations and Acronyms

CM	conditioned media
DKD	diabetic kidney disease
EPC	endothelial progenitor cells
mMSC	mouse mesenchymal stromal cell
p53sh-EPCs	p53-silenced endothelial progenitor cells

vascular smooth muscle function, leading to further endothelial dysfunction. In addition, endothelial dysfunction is a key mediator of oxidative stress, inflammation, and atherosclerosis.¹²⁻¹⁵ Hence, poor function of the endothelium causes ischemic injury and affects various organs, including the kidneys, thereby causing chronic kidney disease.^{16,17} Therefore, improvement of persistent ischemic injury that is known to occur in both type 1 and type 2 diabetes mellitus is important. Improving renal perfusion should help treat and ameliorate diabetic kidney disease (DKD). Available treatments of DKD include glycemic control, hypertension control, improvement of lipid profile, and healthy lifestyle modification.^{18,19} However, it is important to investigate a therapy that can halt and preferably reverse ongoing renal damage caused by poor renal perfusion and ischemia because of the hyperglycemic environment. There are only a few published studies that showed a positive effect of mesenchymal stromal cells (MSCs) through their paracrine property in diabetic nephropathy.²⁰⁻²² However, endothelial-to-mesenchymal transformation is known to occur in DKD,²³ which has been associated with progression and worsening of DKD. Therefore, a concern remains that transplanting MSCs can promote endothelial-to-mesenchymal transition²³ by an MSC-paracrine effect.

In DKD, the primary goal is to increase vascularization or reperfusion. Therefore, an endothelial lineage stem or progenitor cell therapy such as endothelial

progenitor cells (EPCs), in an ischemic diabetic kidney, may be a more appropriate and useful therapy than MSC therapy, even though MSC therapy may be more convenient.

EPCs belong to the hematopoietic stem cell lineage and help to repair damaged ischemic tissue. It is well established that EPCs release growth factors and cytokines that promote repair in damaged tissue.^{24,25} EPCs also help angiogenesis and vascular repair.^{26,27} Studies showed that transplantation of EPCs helps to improve ischemia and promote angiogenesis, but the literature on the use of endothelial lineage cell therapy in nephropathy is lacking. Previously, we demonstrated that EPCs are susceptible to apoptosis in a hyperglycemic environment, and apoptosis significantly increases within 48 hours of high glucose exposure. We also demonstrated that prevention of apoptosis by silencing p53 or p21 pro-apoptotic genes increases survival of EPCs in a hyperglycemic environment and can be used to treat peripheral vascular disease in a diabetic mouse model.²⁷ In this study, we investigated whether renal-specific delivery of p53-silenced mouse EPCs improves diabetic nephropathy.

In our experiment model, we used p53sh EPC, null EPCs, and compared the modified EPC therapy to MSC therapy because MSC therapy can be an alternative cell therapy modality, as mentioned earlier.

METHODS

We state that the data, methods used in the analysis, and materials used to conduct the research will be made available to any researcher for purposes of reproducing the results or replicating the procedure, from the corresponding author upon reasonable request.

Ex Vivo Adenoviral Gene Transduction

Ad-human-P53 (TP53)-shRNA (Vector Biolabs) was used to silence p53 and Ad-scrambled-null-shRNA was used as control. Adenovirus was expanded and titered by using HEK-293 cells (Lonza). After isolation of EPCs from peripheral blood mononuclear cells, they were cultured in EGM2 media (Lonza) media for 5 days. The cells were transduced by virus at 100 multiplicity of infection and kept in endothelial cell culture media for another 4 to 5 days before transplantation under the kidney capsule of the mouse.

Conditioned Media for Scratch Test In Vitro Experiment

To collect conditioned media (CM), mouse EPCs were cultured in exosome-free endothelial cell culture

media, grown in supplement-free culture media for 5 days, following transduction with Ad-mp53sh or Ad-null, as the case may be. The collected supernatant media were concentrated 10-fold to obtain the CM.

Endothelial Cell Scratch Test or Wound Healing Test, In Vitro

Mouse endothelial cells were cultured in a wound-healing plate for 24 hours, following the manufacturer's instructions. CM obtained from null-EPC and p53sh-EPC were added 1:20 in separate wells, and exosome-free culture media were added in separate wells, as controls. After 24 hours, the cells were stained with a stain provided by the manufacturer and an image was taken under a microscope.

Animals

Adult male (4–6-weeks old), wild-type (WT) mice (C57/BL6) were purchased from The Jackson Laboratory. The mice were acclimated in their cages for at least 3 days before any procedure. A 12-hour artificial light–dark cycle and 21°C room temperature were maintained. Normal diet (Harlan Teklab, Global 19% Protein Extruded Rodent Diet, catalog # 2019S) was provided, ad libitum. All animal procedures were approved by Institutional Animal Care and Use Committee (IACUC), The George Washington University, Washington, DC.

WT (C57BL/6) mice were used for EPC harvest from peripheral whole blood and the same mouse strains were used to transplant p53sh EPCs, postinduction of hyperglycemia.

Streptozotocin-Induced Hyperglycemia

Type 1 diabetes mellitus or β -cell-depleted hyperglycemia was induced in C57BL/6 mice by the intraperitoneal injection of streptozotocin. Tail vein blood glucose level was tested (Contour Next One blood glucose meter, Bayer Inc.) after 6 hours of fasting before streptozotocin injection. Streptozotocin (40 mg/kg body weight) was intraperitoneally injected daily for 5 consecutive days. Two to 4 weeks poststreptozotocin injection, mouse tail vein blood glucose was rechecked to verify consistent hyperglycemia (>250 mg/dL). Blood glucose was rechecked to confirm hyperglycemia, before transplanting the EPCs.

Subcapsular Bolus Injection

Mice were given an injection of the analgesic Buprenex (0.1 mg per kg body weight 1 hour) before the start of surgery. They were then anesthetized by pentobarbital sodium (0.1 mg/kg body weight) before the procedure. The abdominal area was shaved

and made aseptic, using alternating swabs of 70% ethanol and iodine, 3 times. Body temperature was maintained using a water-circulating heating pad and monitored via a rectal thermometer. The mice were placed in a supine position and the 4 legs were taped down. An abdominal surgical drape was then placed over the mice. The abdominal cavity was opened by a midline abdominal incision and the contents were displaced and wrapped in sterile gauze, immersed in sterile saline solution. A small portion of the kidney capsule was gently lifted from the kidney using thumb forceps. The cultured EPCs (0.3 million of p53-silenced or/and Ad-null mouse EPCs [resuspended in 100 μ L EGM-2 media] and 0.15 million of mouse MSC [mMSC]) were injected into the subcapsular space using a tuberculin syringe fitted with a 34G needle. EPC was harvested following the method used by Kundu et al.²⁷ A drop of surgical glue was placed over the puncture site as the needle was retracted to seal in the infused solution and prevent backflow. During the surgical procedure, vital signs were monitored every 5 minutes. The displaced abdominal organs were then replaced and the muscle was sutured using nonabsorbable 5-0 suture. The skin layer was sutured using 4-0 to 6-0 monofilament suture.

Femoral Artery Cannulation (for Measurement of Blood Pressure)

Blood pressure was monitored from the aorta via a cannulated femoral artery, before surgery and on week 4 postsurgery, as previously described.²⁸ mice. Buprenex at a dose of 0.1 mg per kg body weight was injected twice a day for 2 days to relieve postoperative pain. Saline was also administered, subcutaneously, to maintain hydration.

Ultrasound Imaging by Visual-Sonics for Renal Perfusion Measurement

Ultrasound imaging of the kidney was performed using the Vevo 3100 ultrasound system and analyzed by Vevo lab analysis software. The mice were anesthetized by isoflurane followed by removal of hair from the back. Blood flow of the renal artery of both kidneys was detected by ultrasound at week 4 posttransplantation.

Protein and Creatinine Concentration Measurements

Urine was collected by keeping the mice in the metabolic cages for 24 hours before transplantation (baseline) and at week 1, week 2, week 3, and week 4, posttransplantation of EPCs. Protein concentration was measured by using a Randox Inc kit and quantified

by a Monza Inc kit. Creatinine concentration was also measured by using Randox and quantified by a Monza Inc kit.

RNA Extraction, cDNA Synthesis, and Gene Expression of Right and Left Kidney

The mouse right and left kidneys were flash frozen in liquid nitrogen after harvest at day 28 postsurgery and kept at -80°C . The frozen kidney was then homogenized and total RNA was isolated, using RNeasy Lipid Tissue Mini Kit (Qiagen). T100 Thermal Cycler (Bio-Rad Hercules, CA) was used to convert mRNA to cDNA, using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). The genes of interest were analyzed by CFX96 Real-Time qPCR System (Bio-Rad), using TaqMan Universal Master Mix II (Applied Biosystems, Foster City, CA). The expression of a particular gene was normalized to house-keeping genes 18S/GAPDH.

Immunohistochemistry

Immunohistochemistry was performed using H&E staining. Vascularization was quantified using isolectin beta 4 expression. Isolectin beta 4 antibody was diluted at a 1:200 ratio (Abcam) and horseradish-conjugated anti-Rat ImmPress (RTU) was used as a secondary antibody followed by 3,3-diaminobenzidine staining. The tissue section was counterstained with hematoxylin.

Statistical Analysis

Results were analyzed by using a multiple-comparison *t* test, where $P < 0.05$ was considered significant. Every statistical analysis was done based on 4 biological repetitions for in vivo experiments and 2 biological repetitions for in vitro experiments.

RESULTS

Renal Function Assessed by Protein:Creatinine Ratio

In this study, one of the main outcome measures was assessment of renal function. Protein and creatinine concentrations were measured in urine collected for 24 hours from mice in metabolic cages. Area under the curve of urine protein/creatinine ratio showed significant reduction that is greater when transplanted with p53sh-EPC compared with null-EPC and mMSC, 1.8-fold ($P = 0.03$) and 1.6-fold ($P = 0.04$), respectively, $n = 4$ in each group (Figure 1).

Gene Expression

Another key outcome measurement of this study was the expression of genes associated with angiogenesis

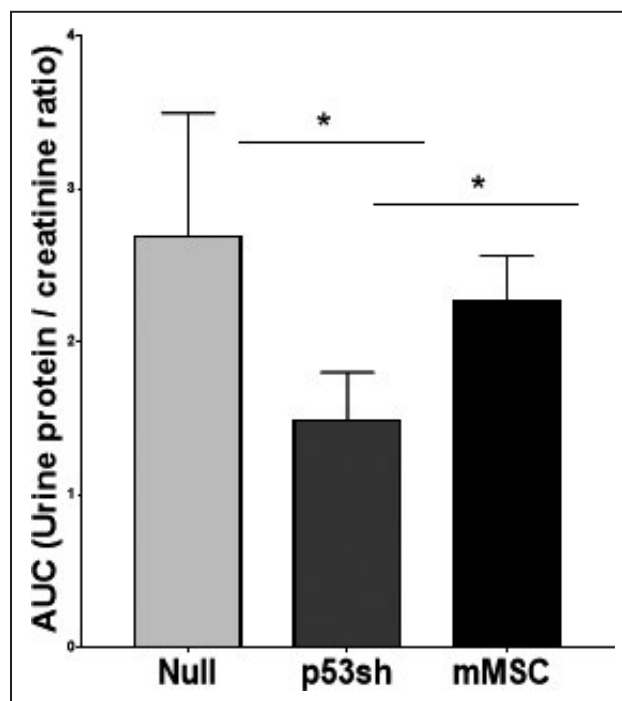


Figure 1. Increased renal function post p53sh-EPC transplantation: reduction of area under curve of urine protein/creatinine ratio posttransplantation of p53sh-EPCs compared with null-EPCs indicates improved renal function compared with control.

A comparison of urine protein/creatinine ratio between p53sh-EPC transplantation and mMSC transplantation showed increased renal function in the p53sh-EPC group. Statistical analysis was done based on 4 biological repetitions. $*P < 0.05$. AUC indicates area under curve; EPCs, endothelial progenitor cells; and mMSC, mouse mesenchymal stromal cell.

in kidney tissue. The mRNA gene expression analysis showed regulation of the angiogenesis markers. Vascular endothelial growth factor-A expression was reduced (7-fold, $P = 0.0004$) significantly on mMSC therapy compared with p53sh EPC transplantation (Figure 2A). Endothelial nitric oxide synthase gene expression showed improved expression in the p53shEPC group compared with the null-EPC group (1.7-fold). Kinase insert domain receptor, also known as vascular endothelial growth factor receptor-2, showed statistically significant improved expression in the p53shEPC group compared with the null-EPC group (1.2-fold, $P = 0.04$), $n = 4$ in each group.

Renal Perfusion by Renal Ultrasound Analysis

Renal artery blood flow measured by ultrasound analysis showed a rise in mean and peak intrarenal blood velocity (1.3-fold, $P = 0.01$, 1.4-fold, $P = 0.001$, respectively) in the p53sh EPC transplant group compared with the null group (Figure 2B). Mean and peak intrarenal blood

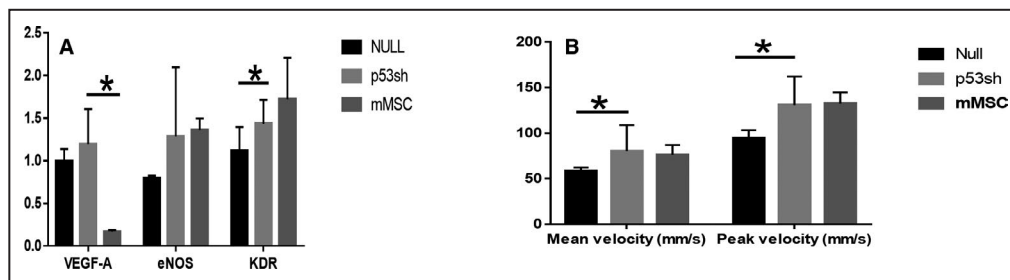


Figure 2. Increased perfusion post p53sh-EPC transplantation: (A) gene expression analysis showed upregulation of angiogenesis marker KDR in p53sh-EPC transplanted group compared with null-EPCs, whereas reduced expression of VEGF-A was observed in the mMSC-transplanted group compared with both EPC transplanted groups.

B, Increased blood flow of the renal artery was observed in the p53sh-EPC-transplanted group compared with null-EPCs. Statistical analysis was done based on 4 biological repetitions. * $P < 0.05$. eNOS indicates endothelial nitric oxide synthase; EPC, endothelial progenitor cell; KDR, kinase insert domain receptor; mMSC, mouse mesenchymal stromal cell; and VEGF-A, vascular endothelial growth factor-A.

velocity also increased with mMSC treatment, similar to that found with p53sh EPCs, $n=4$ in each group.

Systolic and Diastolic Blood Pressures

There were no differences in both systolic and diastolic pressure among the groups at baseline and week 4 posttransplantation. The heart rates were also not different among the groups at baseline, but the heart rate of the mMSC-transplanted group was significantly higher (1.2-fold, $P=0.03$) than that of the p53sh-EPC transplanted group at week 4, posttransplantation, $n=4$ in each group.

Histology

H&E staining (Figure 3) of the kidney sections followed by isolectin $\beta 4$ showed improvement of the sclerosed glomerular vascular tufts with p53sh EPC delivery, compared with null EPCs and mMSCs. Isolectin $\beta 4$ staining is used to detect endothelial lineage cells,²⁹ $n=4$ in each group.

Scratch Test or Wound Healing Test (Test for Endothelial Proliferation)

To understand the paracrine effect of p53sh EPCs, we compared CM obtained from null-EPC and p53sh-EPC on mouse endothelial cell wound healing capacity. Wound healing property or endothelial proliferation capacity of the endothelial cells was greater with p53sh-EPC CM compared with null-EPC CM and saline control (Figure 4). These set of experiments were repeated twice.

DISCUSSION

One of the major vascular complications of diabetes mellitus is diabetic nephropathy and is associated

with impaired renal perfusion. In our previous study,²⁷ we reported that transplantation of apoptosis-resistant EPCs improved the ischemic hindlimb by increasing blood flow and angiogenesis in a peripheral vascular disease mouse model. Moreover, transplanting p53-silenced EPCs also efficiently overcame the high mortality rate of EPCs in a hyperglycemic environment.²⁷

Previous studies showed that a therapy with MSC transplantation improves renal function.²⁰⁻²² Ni et al showed that MSC transplantation ameliorates diabetic nephropathy by activating klotho and inhibiting the Wnt/ β -catenin pathway.²¹ Ezquer et al reviewed and discussed the direct and indirect impact of transplanting MSC to reverse the renal damage caused by diabetes mellitus.²² Griffin et al also reported on the promise of MSC therapy for DKD.²⁰ However, all these studies also discussed the limitation and the knowledge gap of these studies. In addition, studies also showed adverse effect of endothelial-to-mesenchymal transition.²³ Thus, EPCs could be a great tool for cell therapy.

Individuals with end-stage renal disease secondary to DKD developed renal macro- and microvascular complications, depending on level of albumin secretion.³⁰⁻³² A key determinant of diabetic nephropathy is poor glomerular filtration rate. In this study, we found that transplantation of p53sh-EPC reduced the protein/creatinine ratio compared with mMSC treatment. This result indicates improved renal function when apoptosis-resistant EPCs are used as a therapy.

Diabetes mellitus is known to adversely affect glomerular structure and function. DKD is associated with primary and secondary pathological changes in the vascular and tubulo-interstitial compartments. The most consistent pathological features of DKD are glomerular basement membrane thickening

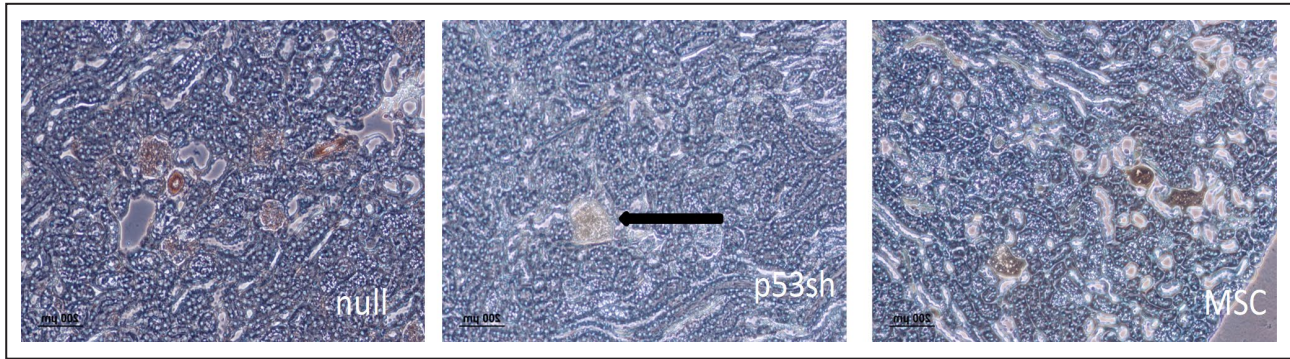


Figure 3. Improved renal structure post p53sh-EPC transplantation by H&E staining.

Hematoxylin staining of right and left kidney showed improved renal structure in the p53sh-EPC-transplanted group compared with null-EPCs. Following H&E staining, the sections were stained with isolectin-β4 to demonstrate renovascular structures. The p53sh-EPC-transplanted group showed better histological architecture compared with null-EPCs and m-MSC. These are representative images. arrow. Arrow indicates intact glomerulus stained by isolectin β4; EPC, endothelial progenitor cell; H&E, hematoxylin and eosin; mMSC, mouse mesenchymal stromal cell; and MSC, mesenchymal stromal cell.

and glomerulosclerosis.³³ Renal fibrosis is one of the key changes in DKD. On the other hand, mediators of renal fibrosis are ischemia and inflammation.³⁴ Hyperglycemia augments glomerular sclerosis and impairs cell growth by reducing growth factors. Moreover, ischemia is directly related to free radical production. Hence, the presence of high glucose generates reactive oxygen species and causes poor mitochondrial function and inflammation.³⁵⁻³⁷ In addition, prolonged hyperglycemia causes advanced glycosylated end products production and eventually, thickening of the glomerular basement membrane, and increased extracellular matrix. Studies showed that renal microvascular abnormalities often started before microalbuminuria.¹⁹ Therefore, improvement of renal vascular health could be a better approach. In this study, we noticed upregulation of angiogenesis markers, endothelial nitric oxide synthase and kinase insert domain receptor, which indicate improvement of the ischemic condition. To

support this outcome, when we analyzed ultrasound data of renal artery flow, we noticed improvement when transplanted with p5sh-EPCs, relative to null EPCs. However, we did not find any difference of renal artery flow between mMSC and p53sh-EPC-transplanted groups. Interestingly, we did not notice any differences in systolic and diastolic blood pressures among the groups. These data suggest renal blood flow can be increased without any systemic blood pressure changes. When we stained a kidney section with angiogenesis marker isolectin beta4, we have observed that renal architecture was preserved better in the p53sh-EP-transplanted group than the null and mMSC groups. In combination, these data suggest that increased renal blood flow and angiogenesis occurred with transplanted p53sh-EPCs.

Adult stem cells release a variety of growth factors, cytokines, and chemokines. Urbich et al reported that conditioned medium of EPCs induced a strong migratory response by activating angiogenic

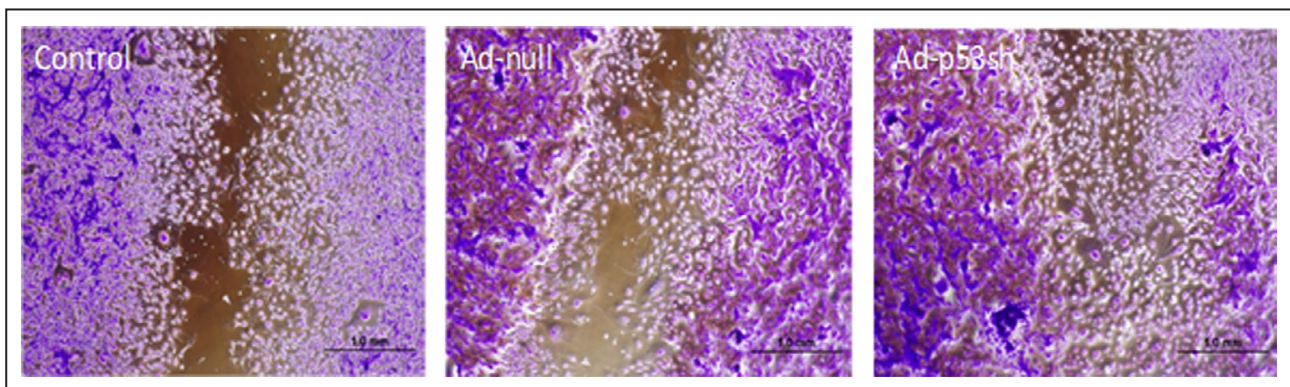


Figure 4. Increased wound healing or endothelial cell regeneration with addition of p53sh-EPC conditioned media: mouse endothelial cells were cultured in a wound healing plate, and condition media from null and p53sh transduced EPC were added in 1:20 ratio to the exosome-free culture media.

Increased wound healing was noticed, when condition media from p53sh-EPC was added to the culture media compared with null and control (n=2). These are representative images. EPC indicates endothelial progenitor cell.

factors.²⁴ When we compared wound-healing capacity of null-EPC and p53sh-EPC-CM, we noticed p53sh silencing favored wound healing. This outcome indicates that paracrine factors,³⁸ secreted from p53sh-EPC, have better regenerative capability compared to null EPCs.

This study is focused on renal complications in the type 1 disease model, although, in our previous study, we were unable to identify clear improvement in the type 2 peripheral vascular disease mouse model.²⁷ Irrespective of the previous result, information on the effect of this therapy on the type 2 CKD model is essential and could be a future approach. In this pilot study, we showed that transplantation of p53-silenced apoptosis-resistant EPCs improves renal perfusion and renal function in a diabetic milieu and transplantation of p53shEPCs is more beneficial than transplantation of mMSC, in terms of proteinuria and renal structure. Moreover, p53sh-EPCs help to rescue damage caused by hyperglycemia through their paracrine effect. Therefore, in DKD, p53sh-EPC transplantation could be a promising therapy.

ARTICLE INFORMATION

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Disclosures

None.

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