Online supplement

Transplantation of mesenchymal stem cells into the renal medulla attenuated salt-sensitive hypertension in Dahl S rat

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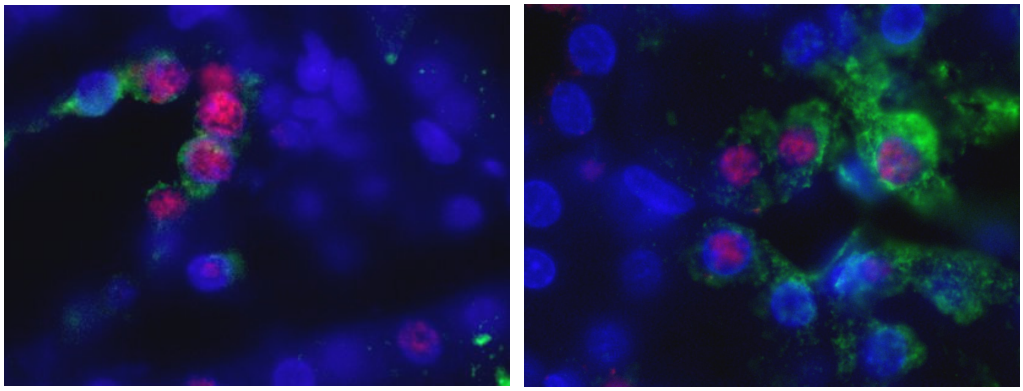
Supplement Figure 1

Fluorescent images showing double staining of Oct-4 (**red**) and CD133 (**green**) in the renal medulla. Nuclei are stained with DAPI (**Blue**). (Representative photomicrographs from 2 rats, 400x)



Detailed Materials and Methods

Animals. Experiments used male Sprague-Dawley rats, Dahl S rats (Harlan, Madison, WI) and SS-13BN rats (Charles River, Wilmington, MA), weighing 250 to 350 g. Animals were kept on a low-salt diet (0.4%NaCl) and some of them were fed with a high-salt diet (8% NaCl) (Dyets, Inc) during experiments as indicated in the results section. All the animal procedures were approved by the Institutional Animal Care and Use Committee of the Virginia Commonwealth University.

Immunohistochemistry analysis of CD133+, CD90+ and CD43+ cells. CD133+ cells were examined in the kidneys from Sprague-Dawley, SS-13BN rats and Dahl S rats that were fed with a low salt diet. CD90+ and CD43+ cells were detected in the kidneys from Dahl S rats that were treated with control or MSC cells and fed with a low or high salt diet as indicated in result description. The kidneys were fixed in 10% formalin, paraffin embedded and cut into 4μm sections. Immunostaining was performed as we described previously [1-2], using primary antibodies against rat CD133 (rabbit polyclonal, Abcam, 1:50), CD90 (rabbit monoclonal, Novus, 1:50) and CD43 (goat polyclonal, Santa Cruz, 1:50), and then secondary antibodies (1:200). At least 10 microscope fields (400×) were examined by two independent examiners who were blinded as to animal groups and the number of CD133 and CD90 positive cells was counted and calculated per mm^2. A large amount of CD43+ cells were detected and it was difficult to count the number of CD43+ cells. Therefore, the quantitation of CD43+ cell was analyzed using the percentage of positive staining area, which was calculated using a computer program (Image-Pro Plus) as described previously [3].

Indirect immunofluorescent staining and confocal microscopy for colocalization of CD133 and Oct-4 in the renal medulla. Rat kidney tissue slides were first incubated with antibodies against CD133 (rabbit) and Oct-4 (goat) overnight at 4°C and then HRP-conjugated secondary antibodies for 1 h at room temperature. A Tyramide Signal Amplification (TSA™) kit containing fluorescent substrate was used for visualization (PerkinElmer, Santa Clara, CA). After staining, the slides were examined using a fluorescent microscope and analyzed with the PDManager program (Olympus, Japan).

Preparation of single cell suspension from the renal medulla and flow cytometric analysis of CD133+ cells. Single cell suspension was prepared by collagenase digestion and cell strainer sieving as described before [4]. In brief, the renal issue was minced and then incubated in solution containing collagenase II (1mg/mL) and trypsin inhibitor (0.5mg/mL), which was pre-bubbled by 95%O_2/5%CO_2 gas and pre-warmed for 20 min at 37^{o}C. Digested tissue was passed through a 35μm cell-strainer (BD, Franklin Lakes, NJ), centrifuged, washed with PBS and transferred into a 1.5ml Eppendorf tube. The cells were then stained with anti-CD133 primary antibody and subsequently FITC-conjugated goat-anti-rabbit secondary antibody. After fixed in 2% paraformaldehyde, the cells were subjected to flow cytometric analysis on a Becton Dickinson FACScan Flow Cytometer. The data were analyzed using CELLQuest software. A minimum of 10,000 events were acquired. The appropriate isotype controls were included to assist in gating in the analyses, and the forward scatter threshold was set to eliminate cell debris.

Ex-vivo expansion of rat mesenchymal stem cells and renal medullary interstitial cells. Rat mesenchymal stem cells (MSCs) were obtained from Institute for Regenerative Medicine at

Texas A&M Health Science Center College of Medicine. Passage 6 MSCs were cultured according to the instruction. In brief, cells were cultured with Eagle's alpha minimum essential medium (α-MEM, Sigma) supplemented with 20% FBS (Invitrogen), 2mM L-glutamine (Invitrogen-Gibco), 100U/ml penicillin and 100ug/ml streptomycin (Invitrogen-Gibco) at 37°C in an incubator with 5% CO_2 and 95% air. The ability of these MSCs to differentiate into adipocytes or osteoblasts was confirmed. Renal medullary interstitial cells (RMIC) were isolated from Sprague Dawley rats as described previously [5-6] and cultured the same as MSCs. When reaching sub-confluence, the cells were trypsinized in 0.25% Trypsin-EDTA, washed and then suspended (5×10^6 cells in 600 ml 0.9% saline) [7-10] for transplantation into the renal medulla as described below.

Transplantation of MSCs or RMICs into renal medulla in Dahl S rats. Dahl S rats were uninephrectomized 1 week before. Cell suspensions prepared above were infused into the renal medulla of the remaining left kidney similar to what we described before for renal medullary infusion or DNA delivery [2, 11-14]. In brief, after rats were anesthetized with 2.5% isoflurane, an interstitial catheter was placed into the renal medulla (5 mm in depth) and the cell suspension was infused into renal medulla at a speed of 20μl/min using a syringe pump. During the infusion, the syringe was kept in an upright position with tip down to ensure the cells were infused into the kidneys. After infusion, the catheter site was blocked by a piece of fat tissue with Vetbond Tissue Adhesive. A second cell infusion was performed 2 weeks later. RMICs were used in control animals. Animal groups included rats treated with RMICs + low salt diet (Ctrl + LS), RMICs + high salt diet (Ctrl + HS), 0.9% saline + high salt diet (Saline + HS) and MSCs + high salt diet (MSC + HS).

Measurement of sodium balance. Additional groups of Dahl S rats were prepared and treated with MSCs or RMICs as described above, and then housed in metabolic cages 5 days after cell transplantation. Daily indexes of sodium balance were computed by subtracting urinary sodium excretion from total sodium intake and cumulative sodium balance was calculated. After a control day measurement, the animals were switched from tap water to 2% NaCl water and sodium balance measurements were continued for additional 3 days [2, 13, 15-16].

Chronic monitoring of arterial blood pressure in conscious rats. Mean arterial pressure (MAP) were monitored with a telemetry blood pressure measuring system (Data Sciences International, DSI) as we described before [11, 17]. MAP was recorded for 3 hrs each day at the same time period (12-3 pm). After baseline MAP was recorded on 3 consecutive control days while the rats remained on a low salt diet, animals were then either remained on a low salt diet or switched to a high salt diet and MAP record continued for 20 more days. A second cell infusion was performed 2 weeks later. At the end of experiment, the kidneys were removed and cut longitudinally; half of the kidney was fixed in 10% neutral buffered formalin for immunostaining of CD90+ and CD43+ cells as described above. The other half of kidney was dissected into cortex and medulla, frozen in liquid N_2 and stored at-80°C for protein and RNA isolation later.

Preparation of tissue homogenate and Western blot analyses for protein level of monocyte chemoattractant protein-1 (MCP-1). Renal tissue homogenates and Western blot analyses were performed as described previously [14]. Primary antibody used was anti-rat MCP-1 (rabbit

polyclonal, Abcam, 1:1000). The intensities of the blots were determined using an imaging analysis program (ImageJ, free download from http://rsbweb.nih.gov/ij/). The levels of β-actin were used as internal control.

Enzyme-linked immunosorbent assay (ELISA) analysis of interleukin (IL)-1β level. The dissected medullary tissues were homogenized with a glass homogenator in ice-cold sucrose buffer (pH7.2) containing (in mmol/L) Tris-HCl, 20; sucrose, 250; and protease inhibitor cocktail, 2μg/ml. After centrifugation of the homogenate at 10,000 x g for 10 minutes at 4°C, the supernatant containing 50 μg protein was subjected for IL-1β assay using an ELISA kit (R&D system, Minneapolis, MN).

RNA extraction and quantitative RT-PCR analysis of CD90 mRNA. Total RNA from the renal medulla was extracted using TRIzol solution (Life Technologies, Inc. Rockville MD) and then reverse transcribed (RT) (cDNA Synthesis Kit, Bio-Rad, Hercules, CA). The RT products were amplified using TaqMan Gene Expression Assays kits (Applied Biosystems). The level of 18S ribosomal RNA was used as an endogenous control. The relative gene expressions were calculated in accordance with the ΔΔCt method. Relative mRNA levels were expressed by the values of $2^{-\Delta\Delta Ct}$.

Statistics. Data are presented as means ± SE. The significance of differences in mean values within and between multiple groups was evaluated using an ANOVA followed by a Duncan's multiple range test. Student's t-test was used to evaluate statistical significance of differences between two groups. $P<0.05$ was considered statistically significant.

Reference

1. Zhu Q, Wang Z, Xia M, Li PL, Van Tassell BW, Abbate A, Dhaduk R, Li N (2011) Silencing of Hypoxia-Inducible Factor-1{alpha} Gene Attenuated Angiotensin II-Induced Renal Injury in Sprague-Dawley Rats. Hypertension 58: 657-664. DOI HYPERTENSIONAHA.111.177626 [pii]
10.1161/HYPERTENSIONAHA.111.177626

2. Wang Z, Zhu Q, Xia M, Li PL, Hinton SJ, Li N (2010) Hypoxia-inducible factor prolyl-hydroxylase 2 senses high-salt intake to increase hypoxia inducible factor 1alpha levels in the renal medulla. Hypertension 55: 1129-1136. DOI HYPERTENSIONAHA.109.145896 [pii]
10.1161/HYPERTENSIONAHA.109.145896

3. Turnberg D, Lewis M, Moss J, Xu Y, Botto M, Cook HT (2006) Complement Activation Contributes to Both Glomerular and Tubulointerstitial Damage in Adriamycin Nephropathy in Mice. J Immunol 177: 4094-4102

4. Oliver JA, Maarouf O, Cheema FH, Martens TP, Al-Awqati Q (2004) The renal papilla is a niche for adult kidney stem cells. J Clin Invest 114: 795-804

5. Li N, Yi F, Sundy CM, Chen L, Hilliker ML, Donley DK, Muldoon DB, Li PL (2007) Expression and actions of HIF prolyl-4-hydroxylase in the rat kidneys. Am J Physiol Renal Physiol 292: F207-216. DOI 00457.2005 [pii]
10.1152/ajprenal.00457.2005

6. Wang Z, Tang L, Zhu Q, Yi F, Zhang F, Li PL, Li N (2011) Hypoxia-inducible factor-1alpha contributes to the profibrotic action of angiotensin II in renal medullary interstitial cells. Kidney Int 79: 300-310. DOI ki2010326 [pii]

10.1038/ki.2010.326

7. Nishimatsu H, Suzuki E, Kumano S, Nomiya A, Liu M, Kume H, Homma Y (2012) Adrenomedullin mediates adipose tissue-derived stem cell-induced restoration of erectile function in diabetic rats. J Sex Med 9: 482-493. DOI 10.1111/j.1743-6109.2011.02469.x

8. Phulpin B, Dolivet G, Marie PY, Poussier S, Huger S, Bravetti P, Graff P, Merlin JL, Tran N (2011) Feasibility of treating irradiated bone with intramedullary delivered autologous mesenchymal stem cells. J Biomed Biotechnol 2011: 560257. DOI 10.1155/2011/560257

9. Ninichuk V, Gross O, Segerer S, Hoffmann R, Radomska E, Buchstaller A, Huss R, Akis N, Schlondorff D, Anders HJ (2006) Multipotent mesenchymal stem cells reduce interstitial fibrosis but do not delay progression of chronic kidney disease in collagen4A3-deficient mice. Kidney Int 70: 121-129. DOI 5001521 [pii]

10.1038/sj.ki.5001521

10. Morigi M, Imberti B, Zoja C, Corna D, Tomasoni S, Abbate M, Rottoli D, Angioletti S, Benigni A, Perico N, Alison M, Remuzzi G (2004) Mesenchymal stem cells are renotropic, helping to repair the kidney and improve function in acute renal failure. J Am Soc Nephrol 15: 1794-1804

11. Zhu Q, Wang Z, Xia M, Li P-L, Zhang F, Li N (2012) Overexpression of HIF-1α transgene in the renal medulla attenuated salt sensitive hypertension in Dahl S rats. Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease 1822: 936-941

12. Zhu Q, Liu M, Han W, Li P-L, Wang Z, Li N (*2012*) **Overexpression of HIF Prolyl-Hydoxylase-2 transgene in the renal medulla induced a salt-sensitive hypertension**. J Cell Mol Med (in press)

13. Li N, Chen L, Yi F, Xia M, Li P-L (2008) Salt-Sensitive Hypertension Induced by Decoy of Transcription Factor Hypoxia-Inducible Factor-1{alpha} in the Renal Medulla. Circ Res 102: 1101-1108

14. Li N, Yi F, dos Santos EA, Donley DK, Li P-L (2007) Role of Renal Medullary Heme Oxygenase in the Regulation of Pressure Natriuresis and Arterial Blood Pressure. Hypertension 49: 148-154

15. Dibona GF, Jones SY, Sawin LL (1998) Angiotensin receptor antagonist improves cardiac reflex control of renal sodium handling in heart failure. Am J Physiol Heart Circ Physiol 274: H636-641

16. Li P, Morris M, Ferrario CM, Barrett C, Ganten D, Callahan MF (1998) Cardiovascular, endocrine, and body fluid-electrolyte responses to salt loading in mRen-2 transgenic rats. Am J Physiol Heart Circ Physiol 275: H1130-1137

17. Zhu Q, Liu M, Han WQ, Li PL, Wang Z, Li N (2012) Overexpression of HIF prolyl-hydoxylase-2 transgene in the renal medulla induced a salt sensitive hypertension. J Cell Mol Med 16: 2701-2707. DOI 10.1111/j.1582-4934.2012.01590.x

18. Simone S, Cosola C, Loverre A, Cariello M, Sallustio F, Rascio F, Gesualdo L, Schena FP, Grandaliano G, Pertosa G (2012) BMP-2 induces a profibrotic phenotype in adult renal progenitor cells through Nox4 activation. Am J Physiol Renal Physiol 303: F23-34. DOI ajprenal.00328.2011 [pii]
10.1152/ajprenal.00328.2011

19. Kanno H, Sato H, Yokoyama TA, Yoshizumi T, Yamada S (2013) The VHL tumor suppressor protein regulates tumorigenicity of U87-derived glioma stem-like cells by inhibiting the JAK/STAT signaling pathway. Int J Oncol 42: 881-886. DOI 10.3892/ijo.2013.1773