Uremia induces functional incompetence of bone marrow-derived stromal cells

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Abstract

Background. Chronic kidney disease (CKD) is associated with increased risk for cardiovascular diseases (CVD). We hypothesized that inadequate angiogenic response in uremic patients could result from dysfunction of bone marrow-derived stromal cells [mesenchymal stem cells (MSCs)].

Methods. We investigated whether MSCs are functionally competent in uremia induced by partial kidney ablation in C57Bl/6J mice.

Results. Uremic MSCs showed decreased expression of vascular endothelial growth factor (VEGF), VEGF receptor (VEGFR)1 and stromal cell-derived factor (SDF)-1α, increased cellular senescence, decreased proliferation, defects in migration in response to VEGF and SDF-1α and in vitro tube formation. Interestingly, the expression of fibroblast-specific protein-1 was higher in uremic MSCs. Uremia decreased hypoxia-inducible factor-1α, VEGF and VEGFR1 expression under hypoxia and Akt phosphorylation in both basal and VEGF-stimulated states. A diminished mitogenic effect on endothelial proliferation was observed in conditioned media from uremic MSCs. In addition, intravital microscopic analysis showed decreased angiogenesis in uremic MSCs.

Conclusion. These results clearly demonstrate the functional incompetence in MSCs under uremic conditions and may significantly contribute to the disproportionately high risk for CVD in patients with CKD.

Keywords: angiogenesis; bone marrow-derived stromal cells; cardiovascular disease; chronic kidney disease

Introduction

Cardiovascular disease (CVD) is the most common cause of mortality in patients with chronic kidney disease (CKD) [1]. Mortality rates in CKD patients on dialysis remain high with more than half of the deaths being related to CVD [2, 3]. Recently, it has been reported that there is an independent and graded association between reduced renal function and the risk of death and cardiovascular events in a large community-based population [4]. Risk factors for vascular changes in CKD include diabetes [5], hypertension and dyslipidemia [6]. CKD is also associated with increased levels of inflammatory factors [7, 8], abnormal apolipoprotein levels [7], elevated plasma homocysteine [7, 8], enhanced coagulability [8], anaemia [9], left ventricular hypertrophy [10], increased arterial calcification [11], endothelial dysfunction [12] and arterial stiffness [13].

Decreased collateral vessel formation in the ischemic myocardium and peripheral limbs is an important cardiovascular pathology in the uremic state. Angiogenic responses to ischemia depend on complex interactions between multiple growth factors, vascular cells and possibly vasculotropic stem/progenitor cells, such as bone marrow (BM)-derived stromal cells (mesenchymal stem cells: MSCs), endothelial progenitor cells (EPC) or resident local stem cells [14, 15]. Previous studies have proposed that MSCs are vasculotropic stem cells which can differentiate into vascular...
cells, including endothelial cells [14, 15], smooth muscle cells [15] or pericytes [16]. These observations suggest that MSCs have the potential to improve blood flow and function although documentation of infused MSCs incorporating into the newly formed vessels has only rarely been observed. Therefore, it has been suggested that MSCs’ contribution to enhancing angiogenesis is mediated by stimulating expression and action of cytokines and growth factors in a paracrine manner [17–19]. It is not known, however, if MSCs are functionally competent in uremia. In this study, we hypothesized that inadequate angiogenic response in the uremic state could result from dysfunction of MSCs.

Materials and methods

**Animals**

All animal studies were conducted with the approval of the Institutional Care and Use Committee of Soon Chun Hyang University Hospital in Seoul, Korea, and our study complied with the National Institutes of Health guidelines for the care and use of experimental animals. Male C57Bl/6J mice were obtained from Charles River Laboratories Japan (Yokohama, Japan). The mice were housed in a pathogen-free facility set on a 12-h light–dark cycle and given free access to water and regular laboratory chow (Cargill Agri Purina, Seongnam, Korea).

**Creation of CKD**

At 7 weeks of age, C57Bl/6J mice were randomly assigned into uremic and nonuremic control groups. Uremia was produced as previously published [20, 21]. In brief, the animals were subjected to electrocoagulation of the left kidney surface leaving a small portion of the hilum intact, and 2 weeks later, the animals received surgical ablation of the contralateral right kidney. Control animals received a sham operation. Special care was taken to avoid damaging the adrenal glands. Six weeks after surgery, the mice were sacrificed by exsanguination under tiletamine (15 mg/kg; Virbac Japan) anesthesia. Blood samples were collected from the abdominal aorta and used for determination of hemocrit (Hct) via a standard micro-Hct method and of plasma urea nitrogen and creatinine concentration by colorimetric method using a Hitachi 7600-10 automated chemistry analyzer (Hitachi, Tokyo, Japan).

**MSCs isolation and culture**

Primary MSCs were generated from pooled BM from three mice. Six and five independently isolated primary MSCs were used for the control and uremic groups, respectively. BM was collected by flushing femurs and tibias with 2% FBS and plated in a 75-cm² flask with Dulbecco’s modified serum (FBS; Gibco, Grand Island, NY). The cells were washed with HBSS uremic groups, respectively. BM was collected by flushing femurs and five independently isolated primary MSCs were used for the control and nonuremic control groups. Uremia was produced as previously published.

**Real-time reverse transcription–polymerase chain reaction (RT–PCR)**

Total RNA was prepared from MSCs using TRIzol reagent (Sigma) according to standard protocol. For real-time reverse transcription–polymerase chain reaction (RT–PCR), the complementary DNA product was synthesized and amplified by polymerase chain reaction (PCR) using 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA). As an internal control, 18S ribosomal RNA expression was quantified in parallel with target genes. The PCR primers are listed in the Supplemental Table.

**Western blot analysis**

Cell lysates were centrifuged to remove cell debris, and supernatants were mixed with sodium dodecyl sulfate (SDS) loading buffer. Samples were then heated at 100°C for 5–10 min before loading, separated through SDS–polyacrylamide gels and subjected to western blot. Membranes were immunoblotted with vascular endothelial growth factor (VEGF) (Milipore, Temecula, CA), VEGF receptor (VEGFR1 [Flt-1]; Santa Cruz Biotechnology, Santa Cruz, CA), VEGF, and hypoxia-inducible factor (HIF)-1α (Novus Biologicals, Littleton, CO) or Akt (Cell Signaling) antibodies. Horseradish peroxidase-conjugated secondary antibody was used and the signal was visualized using enhanced chemiluminescence reagents (Amer sham Biosciences, Louisville, UK).

**Enzyme-linked immunosorbent assay (ELISA)**

VEGF and stromal cell-derived factor (SDF)-1α protein expression in MSCs culture media was assessed by commercially available ELISA kit (R&D Systems, Minneapolis, MN) according to the manufacturer’s recommendation.

**Proliferation assay, senescence-associated β-galactosidase (SA-β-gal) staining and migration assay**

MSCs were cultured in DMEM containing 0.1, 1 or 10% FBS and seeded in a 96-well plate at a density of 10 000 per well and left overnight to attach. Bromodeoxyuridine (BrDU) incorporation was measured using cell proliferation enzyme-linked immunosorbent assay (ELISA) for BrDU (Roche, Penzberg, Germany) according to the manufacturer’s instruction. To assess cell senescence, SA-β-gal activity was measured using standard senescence detection kit (Biovision, Mountain View, CA) according to manufacturer’s instruction. Cell migration was measured with QCM™ Chemotaxis 96-Well Cell Migration Assay (Millipore, Billerica, MA), consisting of two chambers separated by a ChemoTx filter (8 μm pore size). Cells were adjusted into the upper chamber (25 000 per well) and the lower chamber was filled with recombinant human VEGF (R&D Systems) or SDF-1α (R&D Systems). The chambers were incubated at 37°C for 4 h then the cells that had migrated to the lower surface of the filter were detached with cell detachment buffer, lysed and stained with CyQuant GR. The cells were fixed with 40% formalin for 20 min at room temperature and stained with oil red O (Sigma). For osteogenic differentiation, subconfluent MSCs were cultured in DMEM containing 10% FCS, 10% HS, 100 U/mL penicillin, 100 μg/mL streptomycin, 12 mM l-glutamine, 5 μg/mL insulin, 1 μM dexamethasone and 0.5 μM 3-isobutyl-1-methylxanthine (Sigma, St. Louis, MO) for 3 weeks. The cells were fixed with 10% formalin for 20 min at room temperature and stained with Alizarin Red (Sigma).

**MSCs treatment with indoxyl sulfate (IS) or p-cresol**

To determine whether uremic toxins are directly involved in functional abnormalities of MSCs isolated from uremic mice, we analyzed the effect of IS (Sigma) or p-cresol (Sigma) on the proliferation and the messenger RNA (mRNA) expression of angiogenic growth factors. IS and p-cresol were chosen because they are known to induce endothelial dysfunction [24]. For these experiments, the control MSCs were treated with various concentrations of IS or p-cresol for 48 h. The concentrations we tested were similar to the blood level in dialysis patients [25].

**Matrigel assay**

MSCs were seeded (8000 per well) on growth factor-reduced matrigel (BD Biosciences, San Jose, CA) and the plates placed in a humidified atmosphere of 5% CO₂ at 37°C. Identical fields in each well were photographed.
at 4 and 6 h after plating. The photographs were digitized and image analysis software (Image and Microscope Technology) was used to measure total complete tube length and number of closed networks per unit. All conditions were tested in triplicate wells.

*Human umbilical vein endothelial cell (HUVEC) proliferation with conditioned media*

Conditioned medium generated by identical number of control and uremic MSCs was collected after 48 h of culture, centrifuged at 700 g for 10 min, and passed through a 0.2-µm filter. For HUVEC proliferation, the media was replaced with varying dilutions of conditioned media from either control or uremic MSCs and DMEM with 10% FBS. Endothelial growth medium-2 (Lonza, Walkersville, MD) was used as positive control. After 72 h of culture, the cells were recovered and counted.

*Spheroid formation and animal model for intravital microscopy*

Spheroids were generated with $2.5 \times 10^4$ MSCs either from control or CKD mice dispersed (100 µL per well) into 96-well round bottom plates coated with 1.0% agarose for a liquid overlay. The spheroids were allowed to compact for 48–72 h followed by washing in serum-free media for implantation. The dorsal skin window chamber in the mouse was prepared as previously described [26]. Briefly, male athymic nude mice (25–35 g body weight) were anesthetized with tiletamine (15 mg/kg; Virbac Laboratories)/zolazepam (15 mg/kg; Virbac Laboratories)/xylazine (9 mg/kg; Bayer), intraperitoneally, and placed on a heating pad. Two symmetrical titanium frames were implanted into a dorsal skinfold, to sandwich the extended double layer of skin. A 15 mm full thickness layer was excised. The underlying cutaneous maximum muscle and subcutaneous tissues were covered with a glass cover slip incorporated in one of the frames. Small circular Band-Aids were applied on the backside of the chamber after surgery to prevent scratching.

**Statistical analyses**

The mean values obtained from each group were compared by analysis of variance with subsequent use of the Fisher’s least significant difference method. Unpaired two-tailed Student’s *t*-tests were also used where appropriate. Data are presented as mean ± SE. A P-value <0.05 was used as the criterion for a statistically significant difference.

![Fig. 1](image.png)

**Expression of angiogenic factors in MSCs**

Since there is a growing body of evidence that supports the hypothesis that paracrine mechanisms mediated by factors released by MSCs play an important role in the reparative process [17–19], we measured angiogenic growth factors in

**Results**

**Creation of CKD**

We created uremic mice as described previously [20, 21]. Six weeks after surgery, serum urea nitrogen (89.3 ± 3.5 versus 35.4 ± 1.2 mg/dL, *P* < 0.0001) and creatinine (0.5 ± 0.05 versus 0.3 ± 0.02 mg/dL, *P* < 0.0001) concentrations were significantly increased in uremic mice as compared with control animals. Hct levels were significantly lower in uremic mice (40.8 ± 2.4 versus 53.7 ± 1.3% *P* = 0.001). Body weight did not differ between the uremic (23.4 ± 0.3, 24.3 ± 0.2 and 24.3 ± 0.6 g at 0, 2 and 6 weeks after surgery, respectively) and control (22.7 ± 0.2, 24.7 ± 0.3 and 24.9 ± 0.5 g at 0, 2 and 6 weeks after surgery, respectively) mice throughout the study.

**Characterization of MSCs**

Both uremic and control MSCs were positive for the cell surface antigens Sca-1, CD44 and PDGFR-α and negative for CD45 and CD11b (Supplemental Figure 1A and B) as previously reported [27, 28]. MSC multipotency was confirmed by positive differentiation into adipocytes (Supplemental Figure 1C) and osteocytes (Supplemental Figure 1D).

![Fig. 1](image.png)
both uremic and control MSCs. Uremia was associated with decreased mRNA expression of VEGF and its receptor VEGFR1 (Figure 1A). As previously reported [22], murine MSCs did not express VEGFR2 (data not shown). Similarly, immunoblot analysis of VEGFR1 and ELISA for VEGF showed significant reductions as compared with control MSCs (Figure 1B). The expression of SDF-1α was significantly decreased in uremic MSCs at both mRNA and protein levels (Figure 1C). Additionally, mRNA expression of fibroblast-specific protein-1, which is a marker of myofibroblast differentiation, was significantly higher in uremic MSCs than nonuremic control MSCs (Figure 1D), suggesting that uremia induces a myofibroblast-like phenotype in MSCs. In order to determine whether uremic toxin is directly involved in the reduced expression of VEGF, VEGFR1 and SDF-1α in the uremic MSCs, we examined the effects of uremic toxins on the expression of those factors. Since IS has been reported to be taken up into cells by organic anion transporters (OAT) [29], we first confirmed that OAT-3 was expressed in MSCs using real-time RT–PCR (data not shown). Both IS and p-cresol significantly decreased mRNA expression of VEGF, VEGFR1 and SDF-1α (Supplemental Figure 2A and B).

**MSC proliferation and senescence**

The effect of uremia on the proliferation of MSCs was passage dependent. At Passage 6, MSC proliferation was not different between the uremic and control groups. However, at Passage 11, uremia significantly reduced cell proliferation (Figure 2A). Similarly, a concentration–response relationship between IS and MSC proliferation was observed between 0.25 and 5 mM IS (Supplemental Figure 2C). p-cresol at 0.1 mM also induced a decrease in MSC proliferation (Supplemental Figure 2D). We reasoned that the lack of proliferation in uremic MSCs was due to their high rate of intrinsic senescence. Using the senescence biomarker SA-β-gal, we measured the degree of senescence in uremic and control MSCs at matched passages. At Passages 6, 9 and 11, uremic MSCs contained 1.4, 2.6 and 17.9% SA-β-gal-positive cells, whereas control MSCs contained 0.4, 0.5 and 1.4% SA-β-gal-positive cells (P < 0.05, Figure 2B), suggesting premature senescence of MSCs in uremia.

**MSC migration and in vitro tube formation**

To further analyze the effect of uremia on the biological function of MSCs, its effect on MSC migration was studied. As shown in Figure 3A, migration toward VEGF or SDF-1α was significantly reduced in uremic MSCs as compared to control cells. We next tested whether uremia diminishes tube formation of MSCs using matrigel assays. A significant reduction in tube formation was observed in uremic MSCs when compared with control MSCs at both 4 and 6 h after incubation (Figure 3B).

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**Fig. 2.** Cell proliferation and senescence in uremic and nonuremic MSCs. (A) Cell proliferation was measured by BrdU incorporation. Data are mean ± SE; n = 3 experiments performed in triplicate using independently isolated MSC lines; *P < 0.05 versus sham. (B) The degree of senescence was measured using the senescence biomarker SA-β-gal in uremic and nonuremic MSCs at different passages. Representative phase-contrast images of control and uremic MSCs are shown on the left (×40), and the quantitative assessment is on the right. Data are mean ± SE; n = 6 for control and n = 5 for uremic MSC lines isolated independently. The experiments were performed in triplicate; *P < 0.05 versus sham.
HIF-1α, VEGF and VEGFRI expression under hypoxia and VEGF-induced Akt phosphorylation

Since HIF-1 is the central mediator of angiogenesis by controlling the expression of multiple angiogenic growth factors, we investigated the effect of uremia on HIF-1α expression in MSCs. Immunoblot assays detected a high level of HIF-1α protein expression in control MSCs under hypoxic conditions but not in uremic MSCs (Figure 4A). Enhancement of VEGF and VEGFRI expression under hypoxic condition, which is regulated by HIF-1α [22], was also significantly decreased by uremia (Figure 4B and C). Since Akt is not only a general mediator of survival gene [30] upregulating the expression of secreted factors [17] but also a downstream signaling molecule of VEGF/VEGFRI in MSCs [22], we assessed the phosphorylation of Akt in both basal and VEGF-stimulated states. As shown in Figure 4D, uremia significantly reduced Akt phosphorylation in the basal state without producing any enhancement in response to VEGF.

Paracrine effect on endothelial proliferation

Having demonstrated the effects of a uremic environment on MSCs, we next tested whether conditioned media from uremic MSCs would have reduced effect on endothelial cell proliferation. Conditioned media from both control and uremic MSCs significantly enhanced endothelial proliferation in a dose-dependent manner over DMEM with 10% FBS. However, the mitogenic effect of uremia-conditioned media was significantly lower than that of control-conditioned media (Figure 5).

In vivo angiogenesis

To assess the effect of uremia on in vivo angiogenesis, cell spheroids of control or uremic MSCs were implanted on dorsal skinfold chambers of athymic nude mice. From Day 0 of implantation, the growth of the vascular network was evaluated every 1–2 days using intravital microscopy. Implantation of control MSCs could lead to the formation of a dense microvascular network by Day 9 (Figure 6A). In contrast, when uremic MSCs were implanted, some vessels sprouted but did not form a dense vascular network (Figure 6B).

Discussion

Recent evidence has suggested that MSCs play a role in angiogenesis [14–16], therefore their dysfunction may be important in the development of CVD associated with CKD. In this study, we clearly demonstrated that MSCs from CKD mice were functionally incompetent. They showed decreased expression of VEGF, VEGFRI and SDF-1α, decreased proliferative capacity, increased...
without VEGF stimulation, using conditioned media from different MSC lines; *P < 0.05 versus control and uremic MSCs. Cells were cultured for 10 h under normoxic or hypoxic conditions. Data are mean ± SE; n = 5 MSC lines isolated independently; †P < 0.05 versus normoxia, ‡P < 0.05 versus control. Akt phosphorylation in MSCs. Cells were stimulated with VEGF (100 ng/mL) for 10 min after overnight starvation in 0.1% bovine serum albumin. Immunoblot analysis was performed using antibodies specific for Akt phosphorylated at serine 473 or total Akt. Data expressed as % of the cells cultured in EGM-2. Data are mean ± SE; n = 4 MSC lines isolated independently; *P < 0.05 versus nonuremic controls.

**Fig. 4.** (A–C) Immunoblot analysis of HIF-1α, VEGF and VEGFR1 under normoxia (N) and 1% hypoxia (H) in control and uremic MSCs. Cells were cultured for 10 h under normoxic or hypoxic conditions. Data are mean ± SE; n = 5 MSC lines isolated independently; †P < 0.05 versus normoxia, ‡P < 0.05 versus control. (D) Akt phosphorylation in MSCs. Cells were stimulated with VEGF (100 ng/mL) for 10 min after overnight starvation in 0.1% bovine serum albumin. Immunoblot analysis was performed using antibodies specific for Akt phosphorylated at serine 473 or total Akt. Data are mean ± SE; n = 4 MSC lines isolated independently; *P < 0.05 versus without VEGF stimulation, †P < 0.05 versus nonuremic controls.

**Fig. 5.** Effect of conditioned media from uremic and nonuremic MSCs on the proliferation of HUVEC. HUVEC were cultured under varying dilutions of conditioned media from either control or uremic MSCs and DMEM with 10% FBS for 72 h then recovered and counted. Values were expressed as % of the cells cultured in EGM-2. Data are mean ± SE; n = 4 using conditioned media from different MSC lines; †P < 0.05 versus DMEM with 10% FBS, ‡P < 0.05 versus nonuremic conditioned media. EGM-2, endothelial growth media-2.

cellular senescence, defects in migration in response to VEGF and SDF-1α, decreased in vitro tube formation as well as decreased angiogenesis in vivo. A diminished proliferative effect on the endothelial cells was also observed in conditioned media from uremic MSCs as compared to that of control MSCs.

VEGF is a major mediator of angiogenesis in both physiological and pathological conditions, and it plays critical developmental roles in blood vessel formation and regulation of ischemia-induced tissue angiogenesis [31–33]. Since it is well known that HIF-1α is a key regulator of angiogenesis and controls the expression of multiple angiogenic factors including VEGF/VEGFR [22, 34] and SDF-1α [35] in vascular or vasculotropic stem cells, we investigated the effect of uremia on HIF-1α expression in MSCs. Our data demonstrated that uremia significantly decreased hypoxia-induced HIF-1α expression as well as VEGF and VEGFR1 expression. Moreover, we found that both basal- and VEGF-induced Akt phosphorylation were significantly reduced in uremic MSCs. Previous studies reported that MSCs that overexpress Akt improve cardiac function after myocardial infarction by enhancing the survival of MSCs and the upregulation of angiogenic factors, including VEGF [17, 30]. Therefore, decreased Akt phosphorylation may underlie abnormal cell survival and angiogenic function of MSCs in uremia. The finding of decreased Akt phosphorylation induced by VEGF along with the decreased expression of VEGF and VEGFR1 suggest that there are critical defects of angiogenic functions in uremic MSCs.

SDF-1α is a CXC chemokine known to play a crucial role in the trafficking of hematopoietic and lymphopoietic cells and stem cell progenitors [36, 37]. Interestingly, recent studies have shown that the induction of myocardial SDF-1α with ischemic preconditioning confers myocardial protection by recruiting the antiapoptotic kinases extracellular signal-regulated kinases and Akt [38], and that the infusion of SDF-1α-overexpressing MSCs via tail veins 24 h following myocardial infarction significantly increases cardiomyocyte survival and vascular density within the infarct zone [39]. In this regard, our observation demonstrating diminished expression of SDF-1α in uremic MSCs suggests that the defect of vasculotropic stem cells might be involved in the pathogenesis of CVD in uremia. Since SDF-1α is cleaved and inactivated by CD26/dipeptidylpeptidase IV (DPP-IV), pharmacologic inhibition of DPP-IV would decrease DPP-IV activity, stabilize SDF-1α, increase homing of MSCs and eventually improve heart function, as recently shown by Zaruba et al. [40].

Decreased proliferation, premature senescence and compromised ability to migrate and form tube-like structures are other characteristics of stem cell incompetence. In the present study, we found that uremia induces all of these features. Furthermore, we clearly showed impaired angiogenesis induced by uremic MSCs using intravital microscopy. Although we did not investigate underlying mechanisms for the altered biology of uremic MSCs in this study, excessive oxidative stress, which is known to be related to increased cardiovascular risk in CKD, would be one of the possible mechanisms. Reactive oxygen species have been suggested to mediate stem cell aging through p38 mitogen-activated protein kinase (MAPK) pathway [41] and cyclooxygenase inhibitor p16(Ink4a) [42]. In diabetes, hyperactivation of the reactive oxygen species/p38 MAPK appears to be a major
contributor to the reduced number, premature senescence and dysfunction of EPC. Recently, it has been demonstrated that high glucose-induced p16^{INK4a} expression and senescence of EPC is attenuated by either antioxidants or p38 MAPK inhibitor [43].

A major unanswered question is whether the homing of MSCs is decreased in the uremic state. Since the current experimental design did not involve an ischemic injury such as coronary artery ligation, we could not directly address this issue. Further studies using ischemic injury model will be needed to determine whether the recruitment of MSCs is decreased by uremia.

Interestingly, dysfunction of MSCs was sustained even after we cultured the cells in nonuremic conditions and qualitatively similar to the acute effects of uremic toxins. We suspect that this is a phenomenon similar to that of metabolic or hyperglycemic memory, whereby chronic exposure to hyperglycemia has prolonged effects manifested as diabetic complications that persist during subsequent periods of improved glycemic control. Our results may support the concept of uremic memory suggested by Golestaneh et al. [44] which is based on clinical observations showing that an episode of dialysis-requiring acute kidney injury leaves a metabolic imprint regardless of complete recovery. Epigenetic change such as DNA methylation, histone acetylation/deacetylation, histone methylation and RNA interference may be one of the responsible factors for this phenomenon.

Taken together, the results from our present study may be of some relevance in understanding the molecular underpinnings for the increased risk of cardiovascular complications that are associated with CKD. These novel observations also call for further studies to identify the mechanisms leading to the changes in MSCs induced by uremia. Therapeutic approaches targeting dysfunctional MSCs may have benefits for countering CVD in CKD.

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Conflict of interest statement. None declared.

Supplementary data

Supplementary Figures 1 and 2 and Table are available online at http://ndt.oxfordjournals.org.

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