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Mesenchymal stem cells alone or ex vivo gene modified with endothelial nitric oxide synthase reverse age-associated erectile dysfunction

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Bivalacqua TJ, Deng W, Kendirci M, Usta MF, Robinson C, Taylor BK, Murthy SN, Champion HC, Hellstrom WJ, Kadowitz PJ. Mesenchymal stem cells alone or ex vivo gene modified with endothelial nitric oxide synthase reverse age-associated erectile dysfunction. *Am J Physiol Heart Circ Physiol* 292: H1278–H1290, 2007. First published October 27, 2006; doi:10.1152/ajpheart.00685.2006.—Mesenchymal stem cells (MSCs) can be used in adult stem cell-based gene therapy for vascular diseases. To test the hypothesis that MSCs alone or endothelial nitric oxide synthase (eNOS)-modified MSCs can be used for treatment of erectile dysfunction (ED), syngeneic rat MSCs (rMSCs) were isolated, ex vivo expanded, transduced with adenovirus containing eNOS, and injected into the penis of aged rats. Histological analysis demonstrated that rMSCs survived for at least 21 days in corporal tissue after intracavernous injection, and an inflammatory response was not induced. Intracavernous administration of eNOS-modified rMSCs improved the erectile response in aged rats at 7 and 21 days after injection. The increase in erectile function was associated with increased eNOS protein, NOS activity, and cGMP levels. rMSCs alone increased erectile function of aged rats at *day 21*, but not at *day 7*, with the transplanted cells exhibiting positive immunostaining for several endothelial and smooth muscle cell markers. This change in rMSC phenotype was accompanied by upregulation of penile eNOS protein expression/activity and elevated cGMP levels. These findings demonstrate that an adenovirus can be used to transduce ex vivo expanded rMSCs to express eNOS and that eNOS-modified rMSCs improve erectile function in the aged rat. Intracavernous injection of unmodified wildtype rMSCs improved erectile function 21 days after injection through mechanisms involving improved endothelium-derived NO/cGMP signaling and rMSC differentiation into penile cells expressing endothelial and smooth muscle markers. These data highlight the potential clinical use of adult stem cell-based therapy for the treatment of ED.

endothelium; gene therapy; cGMP; phosphodiesterase type 5; neuronal nitric oxide synthase

PENILE ERECTION IS A COMPLEX neurovascular response that requires an increase in arterial inflow, relaxation of corporal smooth muscle, and restriction of venous outflow (29, 35). Relaxation of corporal smooth muscle is essential for normal erectile activity, and evidence has accumulated to implicate nitric oxide (NO) as a major mediator of corporal smooth muscle relaxation and penile erection (14, 44). The release of NO from the endothelium and nitrergic nerves innervating the penile vasculature serves to activate NO-sensitive guanylyl cyclase and increase penile tissue cGMP levels. cGMP activates a cGMP-dependent protein kinase (PKG), and the phosphorylation of downstream proteins results in decreased intracellular calcium concentration and vasodilation (4). cGMP is subsequently hydrolyzed by type 5 phosphodiesterase (PDE5), and PDE5 inhibitors have been shown to successfully treat male erectile dysfunction (ED) (46).

As men age, a significant decline in erectile function occurs (24, 41). Aging is recognized to alter endothelial cell function, and age-related impairments in erectile function have been attributed to multiple factors including increased penile vascular tone, endothelial dysfunction, and reduced NO bioavailability (4, 15, 27). The decreased NO bioavailability has been associated with the formation of reactive oxygen species (ROS), and, when formed in excess, superoxide anion interacts with NO to cause endothelial dysfunction (1, 5, 10, 31, 48). Recently, age-related ED has been shown to be associated with endothelial NO synthase (eNOS) inactivation through a decrease in phosphorylation of its positive regulatory site (Ser^{1177}) and an increase in phosphorylation of its negative regulatory site (Thr^{495}) in the penile vasculature (39). Altered expression and regulation of eNOS may be a major determinant of compromised vascular function in the aged penis and therefore serves as an ideal molecular target to improve penile vascular homeostasis.

Overexpression of eNOS via adenoviral gene therapy to the corpora cavernosa with co-administration of the PDE5 inhibitor sildenafil has been shown to have a beneficial effect on erectile function in the diabetic and aged rat by improving endothelium-derived NO formation (3, 6–8, 16). However, the use of adenoviral vectors raises the possibility of an inflammatory response and random expression of the transgene in almost all cell types (13). A new approach for the treatment of ED that could avoid random distribution of the transgene and reduce the possibility of an inflammatory response could involve the use of mesenchymal stem cells (MSCs), also known as marrow stromal cells, alone or ex vivo genetically modified with eNOS (8, 18–20). MSCs can easily be isolated, readily ex vivo expanded, and efficiently gene modified (18, 19, 21, 25, 26, 32, 43). MSCs do not cause an immune response and differentiate into multilineage cells that can survive for long periods after autologous transplantation (2, 42). Therefore, the present study was undertaken to investigate the effect of

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intracavernous administration of syngeneic MSCs alone or ex vivo gene modified with eNOS in aged Brown Norway rats to determine the physiological and biochemical effects of a novel cell-based therapy for ED.

MATERIALS AND METHODS

Isolation and ex vivo expansion of rat MSCs. Rat MSCs (rMSCs) were isolated as previously described (18, 19). Six-week-old male Brown Norway rats were euthanized with CO_2, and the femurs and tibias were removed. Both ends of the bones were cut, and bone marrow was flushed out using a 21-gauge needle with culture medium for rMSCs (α-MEM containing 20% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 25 ng/ml amphotericin B, and 2 mM L-glutamine). The bone marrow cells were filtered through a cell strainer with 70-μm nylon mesh and were plated in a T75 flask. The cells were incubated in the culture medium for rMSCs at 37°C with 5% humidified CO_2, and rMSCs were isolated by adherence to tissue culture plastic. Fresh culture medium was added and replaced every 2–3 days. The adherent rMSCs were grown to 90% confluency, harvested with a 1:3 dilution of 0.25% trypsin-1 mM EDTA, replated in tissue culture flasks, and again grown to 90% confluency for further ex vivo expansion. rMSCs at passages 0–3 were used for all the experiments.

Immunocytochemical analysis for rMSCs ex vivo. rMSCs were plated at a density of 10,000 cells/cm^2 in one-chamber culture slides and cultured overnight. Cells were rinsed with PBS and fixed with 4% paraformaldehyde in PBS for 5 min. Cells were then immunostained with mouse anti-smooth muscle actin (SMA; Sigma), rabbit anti-von Willebrand factor (vWF; DAKO, Carpinteria, CA), mouse anti-platelet endothelial cell adhesion molecule-1 (PECAM-1, CD31; BD Pharmingen), mouse anti-smooth muscle myosin heavy chain (SM-MHC, Santa Cruz Biotechnology), mouse anti-CD45 (BD Pharmingen), and mouse anti-eNOS (BD Transduction Laboratories) at a dilution of 1:200 each. Secondary antibody was FITC-conjugated horse anti-mouse IgG (Vector Laboratories) or rhodamine-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories) at a dilution of 1:200 each. The culture slides were mounted with VECTASHIELD mounting medium for fluorescence with 4′,6-diamidino-2-phenylindole (DAPI) and checked under a fluorescent microscope.

Western blot analysis for rMSCs ex vivo. rMSCs were plated at a density of 10,000 cells/cm^2 in six-well plates and cultured overnight. Culture medium was removed, and the cells were further incubated in fresh culture medium for 48 h. Cells were rinsed with PBS, lysed with a PBS solution containing 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 0.1 mg/ml leupeptin, and 574 μM phenylmethylsulfonyl fluoride, and prepared for Western blot analysis as previously described (18, 19). Immunodetection was performed using mouse anti-SMA (Sigma), rabbit anti-vWF (DAKO), mouse anti-PECAM-1 (BD Pharmingen), mouse anti-SM-MHC (Santa Cruz Biotechnology), mouse anti-CD45 (BD Pharmingen), and mouse anti-β-tubulin (Santa Cruz Biotechnology) at a dilution of 1:2,500 each. Secondary antibody was horseradish peroxidase conjugated to goat anti-mouse IgG (Santa Cruz Biotechnology, 1:4,000 dilution) or goat anti-rabbit IgG (Santa Cruz Biotechnology, 1:4,000 dilution). Five micrograms of whole cell lysate of rat pulmonary artery smooth muscle cells or rat pulmonary artery endothelial cells were used as positive control.

Adenoviral vectors. AdeNOS, an adenoviral vector containing bovine eNOS gene under the control of Rous sarcoma virus (RSV) promoter, and AdntlacZ, an adenoviral vector containing nuclear-targeted β-galactosidase gene ntlacZ under the control of RSV promoter, were obtained from University of Iowa Gene Transfer Vector Core (Iowa City, IA) and have been used in previous studies from our laboratory (3, 8, 16). The AdeNOS virus concentration was 1.2×10^{12} viral particles (vp)/ml and a titer of 3.0×10^{10} plaque-forming units (pfu)/ml. The AdntlacZ virus concentration was 1.4×10^{12} vp/ml and a titer of 1.0×10^{10} pfu/ml.

Transduction with adenoviral vectors. The transduction of rMSCs with adenovirus was carried out as previously described (18, 19). Briefly, rMSCs were plated at a density of 10,000 cells/cm^2 in six-well plates or T75 flasks and cultured overnight. The cells were exposed to fresh culture medium for rMSCs containing AdntlacZ or AdeNOS at 300 multiplicities of infection (MOI; defined as pfu/cell) for 48 h. Cells were then analyzed for transgene expression or used for ex vivo gene therapy. Cell viability was determined by the Trypan blue exclusion method.

X-gal staining for β-galactosidase expression in culture. X-gal cytochemistry for β-galactosidase activity in rMSCs was carried out as previously described (18, 19). The expression of the ntlacZ transgene in rMSCs was evaluated by light microscopy, and nuclear-targeted β-galactosidase-positive blue cells found in two to three microscopic fields were counted and expressed as a percentage of the total number of cells in the field.

Western blot analysis for eNOS and constitutive NOS activity ex vivo. Western blot analysis for eNOS expression and constitutive NOS activity in rMSCs was carried out as previously described (6, 18, 22). Immunodetection was performed using a mouse anti-eNOS monoclonal antibody (BD Transduction Laboratories, 1:2,500 dilution) and a rabbit anti-β-tubulin polyclonal antibody (Santa Cruz Biotechnology, 1:2,500 dilution). The secondary antibodies were horseradish peroxidase conjugated to goat anti-mouse IgG (Santa Cruz Biotechnology, 1:4,000 dilution) or goat anti-rabbit IgG (Santa Cruz Biotechnology, 1:4,000 dilution). For the determination of constitutive NOS activity (Calbiochem-Novabiochem, La Jolla, CA), radiolabeled L-arginine-to-L-citrulline conversion was assayed in rMSC extracts as previously described. Enzyme activity was expressed as L-citrulline production (in pmol·min^{-1}·mg protein^{-1}).

Intracavernous injection of rMSCs into aged rats and histological analysis. rMSCs were transduced with AdeNOS or AdntlacZ at MOI 300 for 48 h. The virus-containing culture medium was removed, and the cells were washed with PBS three times. AdeNOS-transduced rMSCs or AdntlacZ-transduced rMSCs were then harvested with 0.25% trypsin-1 mM EDTA and washed with PBS, and a cell suspension at a concentration of 12,500 cells/μl was prepared in PBS. Twenty-five-month-old male Brown Norway rats obtained from Harlan Sprague Dawley were anesthetized with pentobarbital sodium (30 mg/kg, ip) and placed in a supine position on a thermoregulated surgical table. The penis was exposed, and 40 μl of cell suspension or 40 μl of PBS were injected into the corpora cavernosum with a 25-gauge needle. A total of 500,000 cells or 40 μl of PBS was injected, and, immediately before injection, blood drainage via the dorsal vein was halted by circumferential compression of the penis at the base with an elastic band. The compression was released 1 min after the injection of rMSCs. This method for intracavernous stem cell therapy has been used previously (8, 16).

Seven and twenty-one days after intracavernous injection of AdeNOS-transduced rMSCs (MOI 300), AdntlacZ-transduced rMSCs (MOI 300), wildtype rMSCs, or PBS, the rats were deeply anesthetized with pentobarbital sodium (80 mg/kg, ip) and perfused through the heart with 200 ml of PBS. For X-gal histochemical analysis of AdntlacZ-transduced rMSCs in rat corpora cavernosum, the tissue was removed, cut into 5-mm sagittal fragments, and fixed in 4% paraformaldehyde in PBS for 10 min. The tissue was washed with PBS three times at 10-min intervals and incubated in X-gal staining solution at 37°C overnight in the dark. The tissue was washed with PBS for 5 min, postfixed in 4% paraformaldehyde in PBS at 4°C, and stored overnight and then transferred to 30% sucrose in PBS at 4°C and stored overnight. The tissue was embedded in OCT compound, snap-frozen in liquid nitrogen, and stored at −70°C. Fifteen-micrometer cross sections were prepared with a cryostat and mounted with VectaMount mounting medium, and the localization of the blue β-galactosidase-positive AdntlacZ-transduced rMSCs in rat corpora

cavernosum was analyzed by phase-contrast microscopy. Hematoxylin and eosin staining was used to determine the presence of inflammatory cells. The tissue was removed, cut into 5-mm sagittal sections, and fixed in 4% paraformaldehyde in PBS at 4°C and stored overnight. The tissue was transferred to 30% sucrose in PBS at 4°C and stored overnight. The tissue was embedded in OCT compound, snap-frozen in liquid nitrogen, and stored at −70°C. Fifteen-micrometer tissue cross sections were prepared with a cryostat and mounted on Superfrost Plus slides (Fisher Scientific, Pittsburgh, PA). Lymphocyte infiltration in rat corpus cavernosum was analyzed by phase-contrast microscopy. β-Galactosidase activity was performed as previously described in our laboratory (3, 6, 16).

Western blot analysis of eNOS and constitutive NOS activity in vivo. Seven and twenty-one days after intracavernous injection of AdeNOS-transduced rMSCs (MOI 300), AdntlacZ-transduced rMSCs (MOI 300), rMSCs, or PBS, rats were deeply anesthetized with pentobarbital sodium (80 mg/kg body wt ip) and perfused through the heart with 200 ml of PBS. To prepare corporal homogenate, the penis was excised, snap frozen in liquid nitrogen, and stored at −70°C. Corporal tissue alone was then homogenized in an ice-cold buffer, and both cytosolic and membrane fractions were isolated and prepared for Western blot analysis as previously described (6, 9). The detergent-extracted membrane fraction contained ~80% eNOS and was used for Western blot analysis in the present study (6, 9). For the determination of constitutive NOS activity (Calbiochem-Novabiochem, La Jolla, CA), radiolabeled L-arginine-to-L-citrulline conversion was assayed as previously described (6, 9). To determine the role of inducible NOS activity, radiolabeled L-arginine-to-L-citrulline conversion was assayed under calcium-free conditions.

Double immunostaining for AdntlacZ-transduced rMSCs after intracavernous injection. Twenty-one days after injection of 500,000 AdntlacZ-transduced rMSCs (MOI 300), the rat was deeply anesthetized with pentobarbital sodium (80 mg/kg body wt ip) and perfused through the heart with 200 ml of PBS. The penis was removed, and the tissue was cut into 5-mm sagittal fragments and fixed in 4% paraformaldehyde in PBS (USB, Cleveland, OH) at 4°C overnight. The tissue was transferred to 30% sucrose in PBS at 4°C overnight. The tissue was embedded in OCT compound (Triangle Biomedical Sciences, Durham, NC), snap frozen in liquid nitrogen, and stored at −70°C. The tissue was cut transversely at a thickness of 40 μm with a cryostat. Penile sections were then double immunostained with mouse anti-SMA/rabbit anti-β-galactosidase, mouse anti-β-galactosidase/rabbit anti-vWF, mouse anti-eNOS/rabbit anti-β-galactosidase, mouse anti-PECAM-1/rabbit anti-β-galactosidase, mouse anti-SM-MHC/rabbit anti-β-galactosidase, or mouse anti-CD45/rabbit anti-β-galactosidase at a dilution of 1:200 each. Secondary antibodies were FITC-conjugated horse anti-mouse IgG and rhodamine-conjugated donkey anti-rabbit IgG at a dilution of 1:200 each. Penile sections were then mounted with VectaShield mounting medium for fluorescence with DAPI and viewed under a deconvoluted fluorescent microscope (Nikon Eclipse E800).

Measurement of cGMP levels in penile tissue. Penile tissue was homogenized in 1.0 ml of ice-cold 6% trichloroacetic acid, pH 4.0, and each sample was centrifuged at 1,500 *g* for 10 min at 4°C. The supernatant was transferred to a 10-ml tube, and the trichloroacetic acid was extracted with H_2O-saturated diethyl ether. The samples were assayed for cGMP using an enzyme immunoassay kit (Cayman Chemicals, Ann Arbor, MI). Tissue cGMP levels were expressed as fmol cGMP/mg protein (3, 16).

Evaluation of erectile function in aged rats. Seven and twenty-one days after intracavernosal injection of AdeNOS-transduced rMSCs (MOI 300), AdntlacZ-transduced rMSCs, rMSCs, or PBS, the response to cavernous nerve stimulation was evaluated as previously described (6, 9). The rats were anesthetized with pentobarbital sodium (30 mg/kg, ip) and placed on a thermoregulated surgical table. Supplemental doses of pentobarbital were administered as needed to maintain a uniform level of anesthesia. The trachea was cannulated (PE-240 tubing) to maintain a patent airway, and the animals breathed room air enriched with 95% O_2-5% CO_2. A carotid artery was cannulated (PE-50 tubing) for measurement of mean systemic arterial pressure (MAP), which was measured with a Viggo-Spectramed transducer (Viggo Spectramed, Oxnard, CA), a data acquisition system, and a computer. The left jugular vein was cannulated (PE-50 tubing) for administration of fluids. The shaft of the penis was freed of skin and fascia, and, by removing part of the overlying ischiocavernous muscle, the right crus was exposed. A 25-gauge needle filled with 250 U/ml heparin and connected to PE-50 tubing was inserted into the right crura and connected to a pressure transducer for measurement of intracavernosal pressure (ICP). The bladder and prostate were exposed through a midline abdominal incision. The right major pelvic ganglion and cavernous nerve were identified posterolateral to the prostate on one side, and a stainless steel bipolar hook electrode was placed around the cavernosal nerve. MAP and ICP were measured with pressure transducers connected to a data acquisition system (Biopac MP 100 Systems, Santa Barbara, CA) and a computer. The nerve was stimulated with a square-wave stimulator (Grass Instruments, Quincy, MA) at a frequency of 15 Hz with a pulse width of 30 ms. The nerve was stimulated at 2.5, 5.0, and 7.5 V for 1 min with rest periods of 2–3 min between subsequent stimulations. Total erectile response or total ICP was determined by the area under the erectile curve (AUC; mmHg·s) from the start of nerve stimulation until the ICP pressure returned to baseline or prestimulation values. The ratio between the maximal ICP and MAP obtained at the peak of erectile response was calculated to control for changes in systemic arterial pressure. The Tulane University School of Medicine Animal Care and Use Committee approved all procedures used in the present study.

Statistics. All hemodynamic data are expressed as means + SE and were analyzed using one-way analysis of variance (ANOVA) with repeated measures and Newman-Keuls post hoc test for multiple group comparisons (Statview; Abacus Concepts, Berkeley, CA). P value < 0.05 was used as the criterion for statistical significance.

RESULTS

Expression of cell markers in rMSCs. MSCs were isolated from Brown Norway rats by cell adherence to tissue culture plastic. The adult stem cells were then readily ex vivo expanded, and the multipotentiality of rMSCs was confirmed by evaluating the in vitro differentiation of the cells into cells with osteoblast and adipocyte lineages, as previously demonstrated in MSCs from Sprague Dawley and Brown Norway rats (18, 19, 21). In the first series of experiments, rMSCs were immunostained with markers for endothelial, smooth muscle, and hematopoietic phenotypes. Immunofluorescence analysis showed that rMSCs stained positive for SMA and vWF, markers for smooth muscle and endothelial cell lineages, and was consistent with previous studies from our laboratory (21) (Fig. 1*A*). rMSCs did not stain positive for SM-MHC, PECAM-1, eNOS, or CD45 (a marker specific for hematopoietic cells; Fig. 1*A*). Further molecular analysis of these cells was conducted using Western blot analysis, and these data are shown in Fig. 1*B*. Western blot analysis confirmed the above findings with robust protein expression for SMA and vWF and no protein expression for SM-MHC, PECAM-1, eNOS, or CD45 (Fig. 1*B*), confirming the immunofluorescence analyses. Therefore, wildtype rMSCs express limited markers for smooth muscle and endothelial cells (SMA and vWF) but do not endogenously express a multiplicity of smooth muscle- and endothelium-specific markers in vitro.

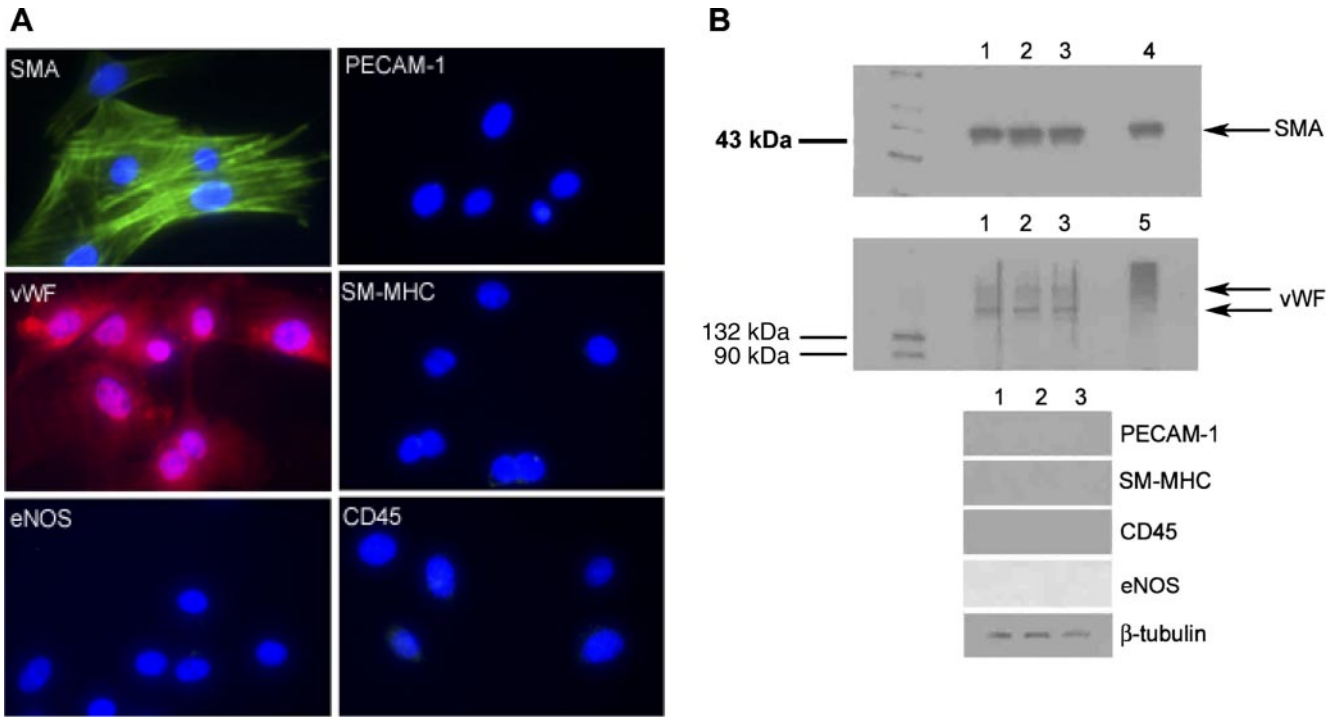

Fig. 1. *A*: photomicrographs showing mesenchymal stem cells (MSCs) express markers specific for endothelial and smooth muscle cells. Rat MSCs (rMSCs) were plated at a density of 10,000 cells/cm^2 in 1-chamber culture slides and cultured overnight. Cells were rinsed with PBS, fixed with 4% paraformaldehyde in PBS, and immunostained with antibodies for markers specific for hematopoietic stem cells (CD45), endothelial cells [von Willebrand factor (vWF), platelet endothelial cell adhesion molecule-1 (PECAM-1), and endothelial nitric oxide synthase (eNOS)], and smooth muscle cells [smooth muscle actin (SMA) and smooth muscle myosin heavy chain (SM-MHC)]. The culture slides were then mounted with mounting medium for fluorescence with DAPI and checked under a fluorescent microscope. Magnification, ×40. *B*: Western blot analysis for protein expression of CD45, vWF, PECAM-1, eNOS, SMA, SM-MHC, and β-tubulin in rMSCs. *Lanes 1–3*, rMSCs; *lane 4*, rat pulmonary artery smooth muscle cells; *lane 5*, rat lung endothelial cells. Analysis for β-tubulin was carried out to ensure that sample loading was similar in all lanes; $n = 3$.

Expression of β-galactosidase in AdntlacZ-transduced rMSCs. As shown in Fig. 2, *A–C*, at MOI 300, >90% of rMSCs were stained positive for β-galactosidase 48 h after transduction. Cell viability was determined to be >90%. The percent β-galactosidase-positive cells declined over a 21-day period in AdntlacZ-transduced rMSCs but was >50% on *day 21* (Fig. 2*C*).

eNOS expressed in transduced rMSCs is biologically active. AdeNOS, a replication-deficient adenovirus containing the bovine eNOS gene, was used to transfect rMSCs, and, as shown in Fig. 2, *D–F*, constitutive eNOS protein expression was not detected in wildtype rMSCs or AdntlacZ-transduced rMSCs. Expression of eNOS was detected in AdeNOS-transduced rMSCs 48 h after transduction (Fig. 2*F*). To determine whether eNOS expressed in AdeNOS-transduced rMSCs is biologically active, rMSCs were incubated with AdeNOS for 48 h, and the cell lysate was assayed for eNOS protein abundance by Western blot analysis and constitutive NOS activity by measuring the conversion of L-arginine to L-citrulline in the presence of calcium. eNOS protein and NOS activity were not detected in wildtype rMSCs or AdntlacZ-transduced rMSCs (Fig. 2, *G* and *H*). However, the presence of eNOS protein and upregulated calcium-dependent NOS activity as demonstrated by significant L-arginine-to-L-citrulline conversion was detected in AdeNOS-transduced rMSCs (Fig. 2, *G* and *H*).

rMSCs survive in rat corpora cavernosum, and inflammation was not induced. To ascertain whether rMSCs survive after intracavernosal injection, 5×10^5 AdntlacZ-transduced rMSCs in 40 μl of PBS were injected into the corpora cavernosum of aged rats. Seven and twenty-one days later, the rats were killed, and penile tissue was processed for X-gal staining to demonstrate blue β-galactosidase-positive activity in AdntlacZ-transduced rMSCs. As seen in Fig. 3, significant numbers of transplanted AdntlacZ-modified rMSCs were identified, and β-galactosidase activity was significantly higher in corporal tissue 7 days (Fig. 3, *A–C*) and 21 days (Fig. 3, *D–F*) after intracavernous injection, indicating that rMSCs survived in the corpora cavernosum for at least 21 days. As shown in Fig. 3, *B* and *C*, the rMSCs in corporal tissue appeared as small round-to-oval cells on *day 7*. However, by *day 21*, the phenotype had changed and the rMSCs appeared elongated and incorporated into the tissue (Fig. 3, *E* and *F*). In addition, penile tissue from aged rats killed on *days 7* and *21* was stained with hematoxylin and eosin to determine whether an inflammatory response had occurred. Lymphocytic infiltrates were not seen in the corpora cavernosum of rats injected with eNOS-transduced rMSCs, lacZ-transduced rMSCs, wildtype (unmodified) rMSCs, or 40 μl of PBS, the vehicle for the rMSCs in all experiments (Fig. 3, *G–M*). There was no evidence of fibrosis as measured by trichrome stain (data not shown) in sections of corpora cavernosum from the four groups of rats, indicating that a local inflammatory response or histological changes had not occurred in animals treated with unmodified or gene-modified rMSCs.

Fate of rMSCs in rat penis. Twenty-one days after injection of AdntlacZ-transduced rMSCs into the corpora cavernosum, the rats were killed and the penile tissue was removed. The fate

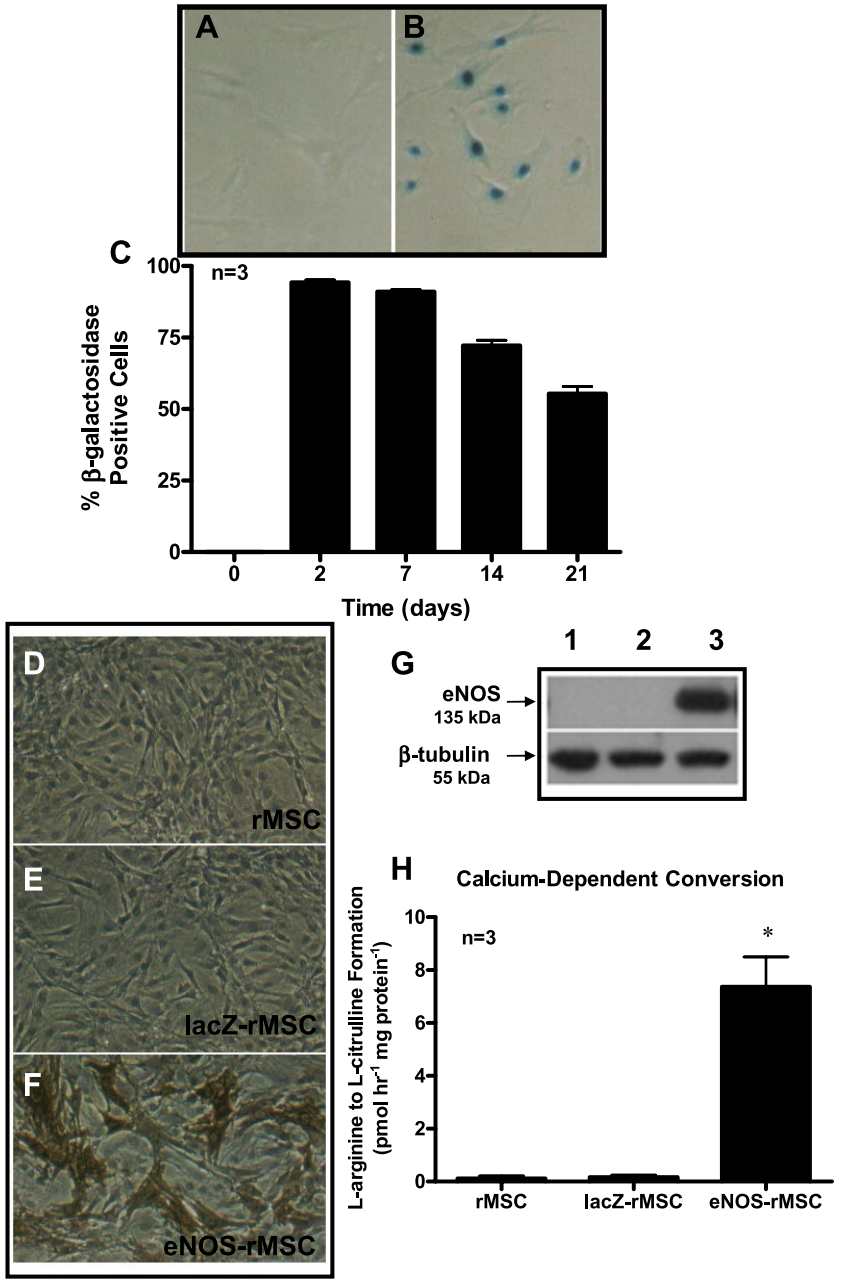

Fig. 2. Photomicrographs showing expression of ntlacZ or eNOS in adenoviral-transduced rMSCs. Photomicrograph showing β-galactosidase-positive blue AdntlacZ-transduced rMSCs. rMSCs were transduced with AdntlacZ at multiplicities of infection (MOI) 300 for 48 h. The cells were X-gal stained for nuclear-targeted β-galactosidase activity. *A*: wildtype rMSCs. *B*: rMSCs transduced with AdntlacZ. *C*: the percentage of nuclear-targeted β-galactosidase-positive cells declines over the 21-day period. Photomicrograph showing eNOS-positive brown AdeNOS-transduced rMSCs. rMSCs were transduced with AdeNOS at MOI 300 for 48 h. The cells were immunoperoxidase stained for eNOS expression. *D*: wildtype rMSCs immunoperoxidase stained for eNOS expression. *E*: rMSCs transduced with AdntlacZ (MOI 300) and immunoperoxidase stained for eNOS expression. *F*: rMSCs transduced with AdeNOS (MOI 300) and immunoperoxidase stained for eNOS expression. Magnification, × 25. eNOS protein and calcium-dependent NOS activity are upregulated in AdeNOS-transduced rMSCs. rMSCs were transduced with AdeNOS at MOI 300 for 48 h. The cell lysate was then assayed for eNOS protein abundance by Western blot analysis (*G*) and for calcium-dependent NOS activity (*H*) using the L-[^{3}H]arginine-to-L-[^{3}H]citrulline conversion. Wildtype rMSCs (*G*, *lane 1*), AdntlacZ-transduced rMSCs (MOI 300; *G*, *lane 2*), and AdeNOS-transduced rMSCs (MOI 300; *G*, *lane 3*). Analysis for β-tubulin was carried out to ensure that sample loading was similar in all lanes. Each value represents mean ± SE ($n = 3$). *$P < 0.01$ vs. wildtype rMSCs or AdntlacZ-transduced rMSCs (ANOVA).

of rMSCs in penile tissue of the aged rat was assayed by double immunostaining of penile tissue with antibodies for β-galactosidase and for a variety of smooth muscle and endothelial cell markers. As shown in Fig. 4, significant numbers of β-galactosidase-positive rMSCs were identified in the corpus cavernosum of the aged rat, suggesting the survival of rMSCs in the rat penis for 21 days after intracavernosal injection. Furthermore, β-galactosidase-positive rMSCs were still negative for CD45 and positive for SMA and vWF, which is similar to observations in wildtype rMSCs in vitro. However, immunofluorescence double-staining experiments demonstrated that β-galactosidase-positive rMSCs in the aged rat penis changed phenotype and converted from negative to positive for eNOS, PECAM-1, and SM-MHC markers specific for endothelial and smooth muscle lineages (Fig. 4). These data suggest that transplanted rMSCs differentiated into new cells that stained positive for both smooth muscle (SM-MHC) and endothelial cell (eNOS and PECAM-1) markers in the corpora cavernosa of the aged rat.

eNOS protein, NOS activity, and cGMP levels in corporal tissue are increased after intracavernous injection of rMSCs alone or AdeNOS transduced. To determine whether intracavernous injection of rMSCs alone or AdeNOS-transduced rMSCs increases eNOS protein abundance, calcium-dependent NOS activity, and cGMP levels in aged corporal tissue, 5×10^5 AdeNOS-transduced rMSCs (MOI 300) in 40 μl of PBS, 5×10^5 AdntlacZ-transduced rMSCs (MOI 300) in 40 μl of PBS, 5×10^5 rMSCs in 40 μl of PBS, or 40 μl of PBS were injected into the penis of aged rats. Seven and twenty-one days later, the rats were killed and corporal tissue was assayed for

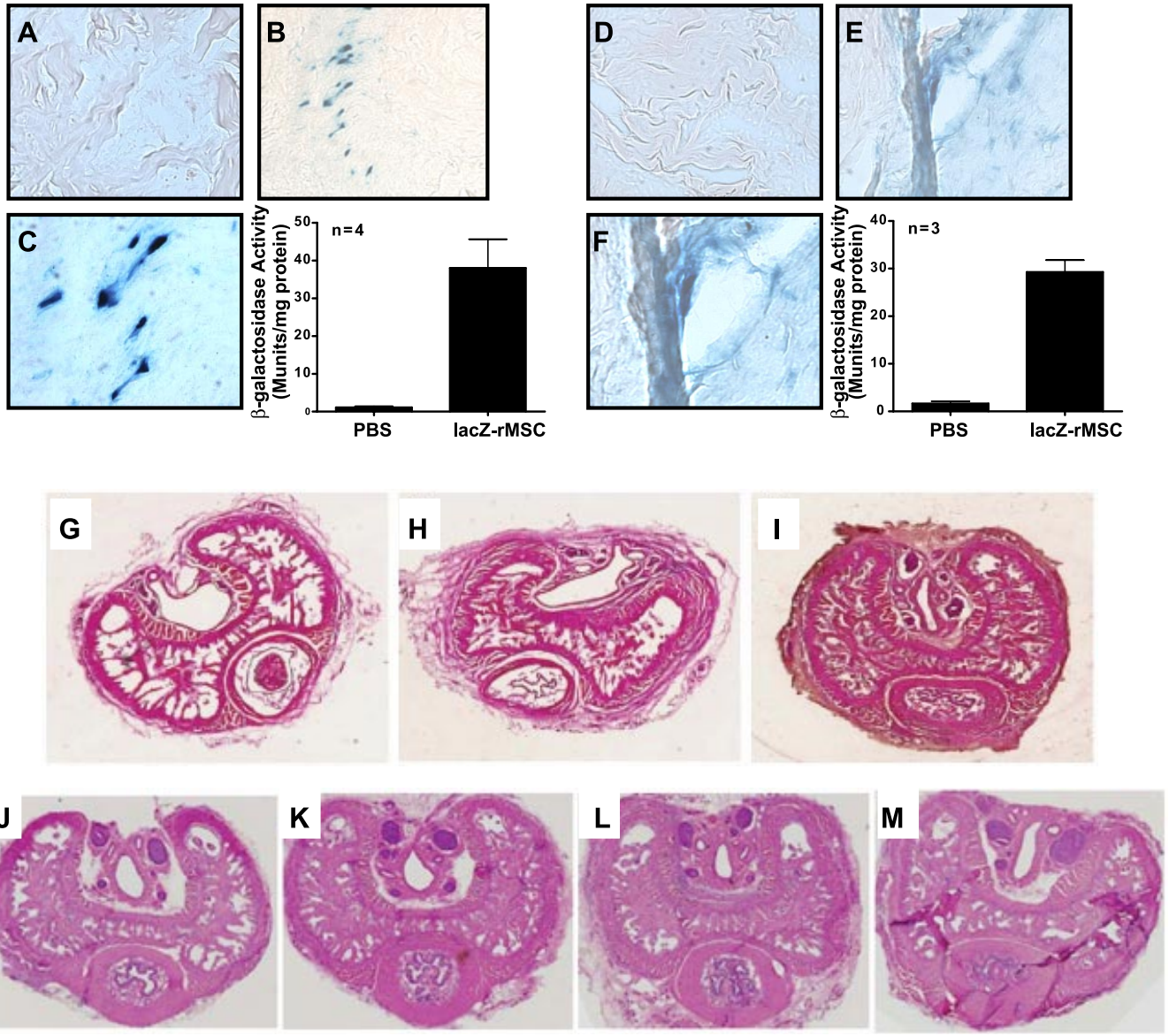

Fig. 3. *Left*: photomicrographs showing β-galactosidase-positive blue AdntlacZ-transduced rMSCs in rat corpus cavernosum at 7 days after intracavernous injection. Seven days after intracavernous injection of AdntlacZ-transduced rMSCs (MOI 300), rats were killed. Cavernosal tissues were X-gal stained for the identification of β-galactosidase-positive blue AdntlacZ-transduced rMSCs. *A*: cavernosal tissue of rats treated with PBS; magnification, ×20. *B*: cavernosal tissue of rats treated with AdntlacZ-transduced rMSCs; magnification, ×20. *C*: cavernosal tissue of rats treated with AdntlacZ-transduced rMSCs; magnification, ×40. *Right*: photomicrographs showing β-galactosidase-positive blue AdntlacZ-transduced rMSCs in rat corpus cavernosum at 21 days after intracavernous injection. Twenty-one days after intracavernous injection of AdntlacZ-transduced rMSCs (MOI 300), rats were killed. Cavernosal tissues were X-gal stained for the identification of β-galactosidase-positive blue AdntlacZ-transduced rMSCs. *D*: cavernosal tissue of rats treated with PBS; magnification, ×20. *E*: cavernosal tissue of rats treated with AdntlacZ-transduced rMSCs; magnification, ×20. *F*: cavernosal tissue of rats treated with AdntlacZ-transduced rMSCs; magnification, ×40. *Right* panels show that β-galactosidase activity is increased significantly at 7 and 21 days. *Bottom*: photomicrographs showing rat penile sections stained with hematoxylin and eosin. Seven days after intracavernous injection of PBS, AdntlacZ-transduced rMSCs (MOI 300), or AdeNOS-transduced rMSCs (MOI 300), the rats were killed and cross sections of the penis were stained with hematoxylin and eosin. *G*: penile section of rats treated with PBS. *H*: penile section of rats treated with AdntlacZ-transduced rMSCs. *I*: penile section of rats treated with AdeNOS-transduced rMSCs. Magnification, ×50. Twenty-one days after intracavernous injection of PBS, rMSCs, AdntlacZ-transduced rMSCs (MOI 300), or AdeNOS-transduced rMSCs (MOI 300), the rats were killed and tissue cross sections were stained with hematoxylin and eosin. *J*: tissue section from rats treated with PBS. *K*: tissue section from rats treated with rMSCs. *L*: tissue section of rats treated with AdntlacZ-transduced rMSCs. *M*: sections from rats treated with AdeNOS-transduced rMSCs. Magnification, ×50.

eNOS protein expression, constitutive and inducible NOS activity, and tissue cGMP levels. As seen in Figs. 5 and 6 and Table 1, eNOS protein abundance, calcium-dependent NOS activity, and cGMP levels were increased in corporal tissue 7 and 21 days after intracavernous injection of AdeNOS-transduced rMSCs. Twenty-one days after injection of wildtype (unmodified) rMSCs or AdntlacZ-transduced rMSCs, eNOS protein abundance, calcium-dependent NOS activity, and cGMP levels were also increased in the penis of aged rats (Fig. 6 and Table 1). There were significantly greater constitutive NOS activity and cGMP levels in penile tissue from rats treated with AdeNOS-transduced rMSCs compared with the PBS-treated rats, rats treated with rMSCs alone, or AdntlacZ-transduced rMSC-treated rats (Fig. 6*C* and Table 1). In addition, inducible (calcium inde-

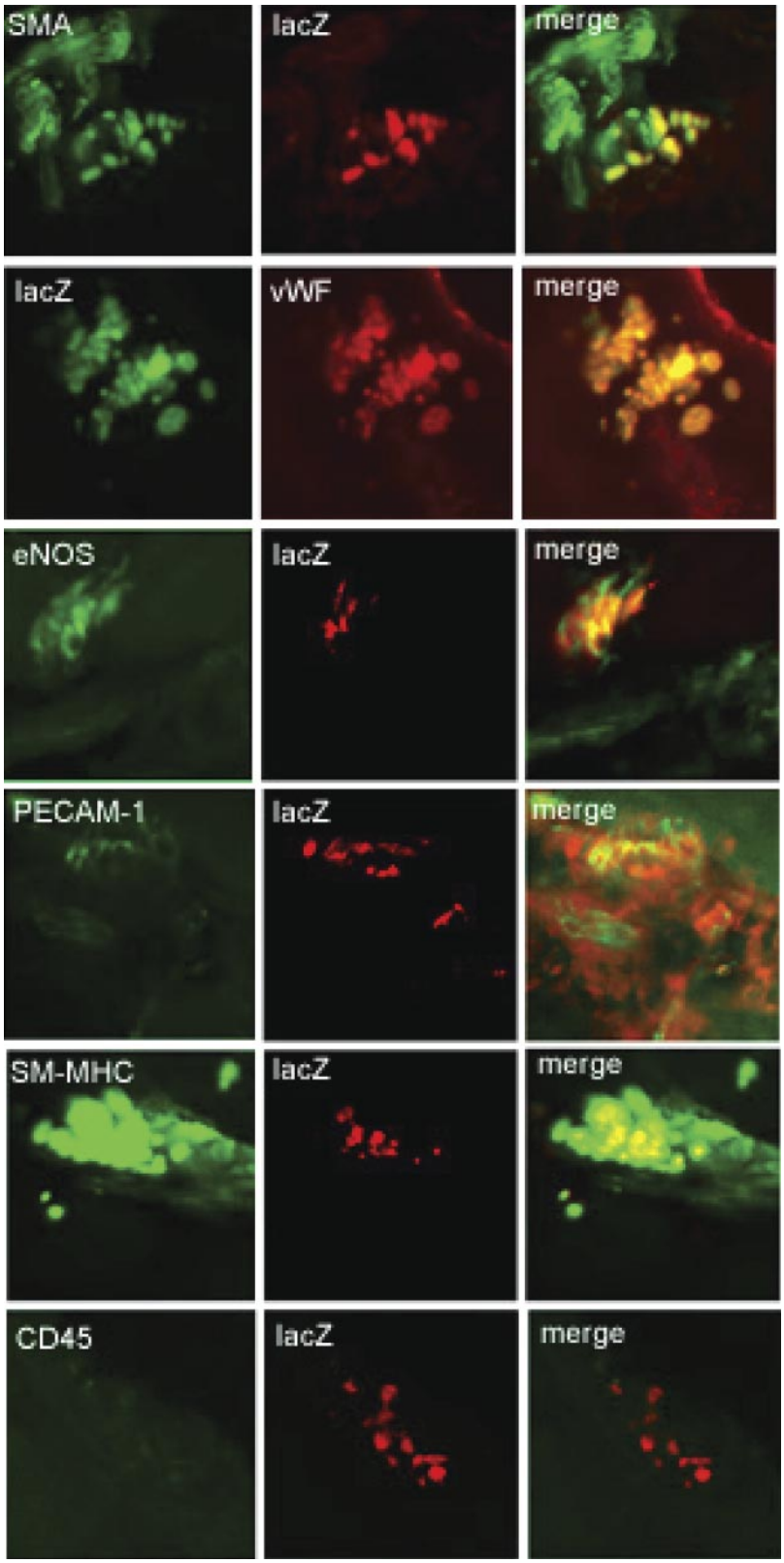

Fig. 4. Colocalization of β-galactosidase, smooth muscle, endothelial, and hematopoietic cell markers in the corpora cavernosum of aged rats 21 days after intracavernous injection of lacZ-transduced rMSCs. Sections of corpora cavernosum were immunolabeled with antibodies for SMA, vWF, eNOS, PECAM-1, SM-MHC, and CD45 and visualized with deconvoluted fluorescent microscope. *Right* column shows an overlay image of each cell marker with β-galactosidase (yellow stain). Magnification, ×400. Photomicrograph is representative of 3 experiments.

pendent) NOS activity was significantly lower in the corporal tissue from aged rats treated with AdeNOS-transduced rMSCs compared with rats treated with PBS, rMSCs alone, or AdntlacZ-transduced rMSCs when measured at 21 days (Fig. 6*D*). These data suggest that rMSCs alone or eNOS gene-modified rMSCs increase endothelial-derived NO biosynthesis with a subsequent increase in cGMP signaling in the aged penile vasculature.

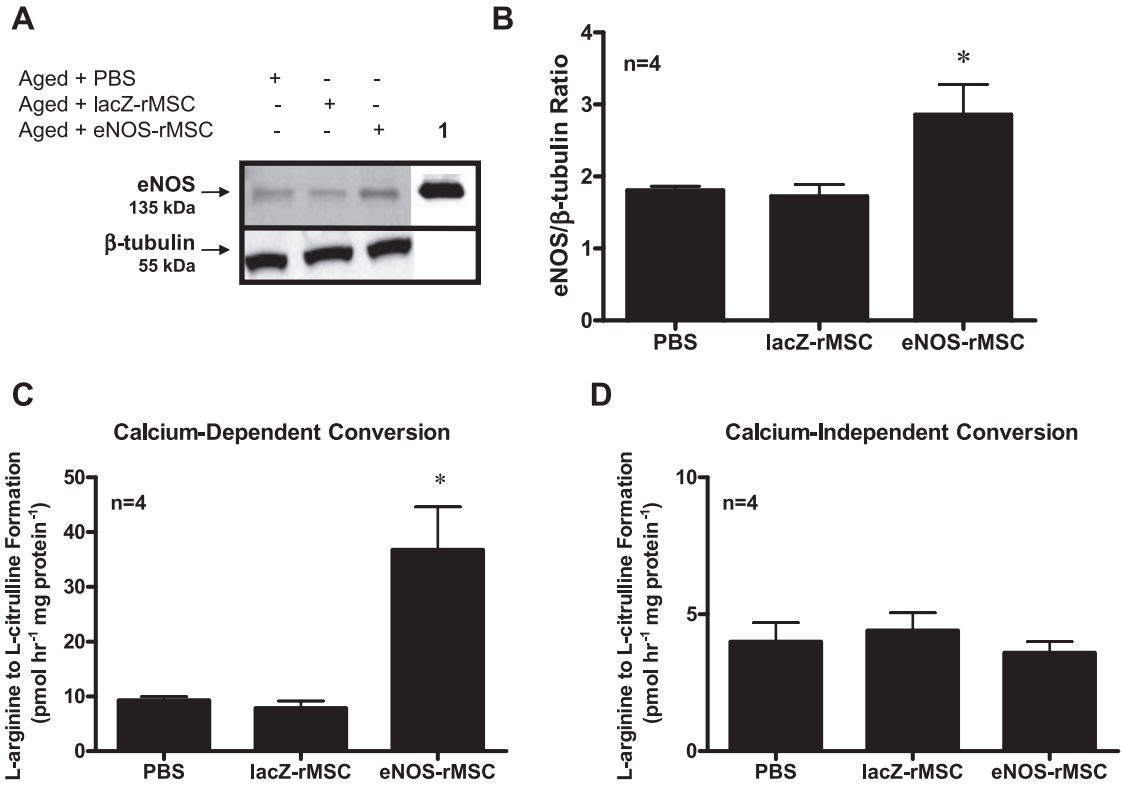

Fig. 5. Changes in eNOS protein abundance and calcium-dependent and -independent NOS activity in aged rat corporal tissue 7 days after intracavernous injection of PBS or tlacZ- or eNOS-transduced rMSCs. Western blot analysis showing changes in eNOS protein expression. β-Tubulin analysis was carried out to ensure similar sample loading in all lanes. *A*: *lane 1*, whole lung cell lysate representing positive control. *B–D*: bar graphs showing the ratio of eNOS to β-tubulin in aged rat corporal tissue (*B*) and calcium-dependent (*C*) and calcium-independent (*D*) L-[^{3}H]arginine-to-L-[^{3}H]citrulline conversion in corporal tissue from aged rats injected with PBS, lacZ-transduced rMSCs, and eNOS-transduced rMSCs; n = no. of experiments. *Significantly different from PBS.

Erectile responses in aged rats after intracavernous injection of AdeNOS-transduced rMSCs, AdntlacZ-transduced rMSCs, or wildtype rMSCs. The effect of intracavernous injection of rMSCs alone and eNOS-modified rMSCs on the increase in ICP, ICP/MAP, and ICP-AUC in response to electrical stimulation of the cavernous nerve in the aged rat was investigated, and these data are shown in Figs. 7 and 8. The increase in ICP, ICP/MAP, and ICP-AUC in response to cavernous nerve stimulation was significantly less in the aged rat compared with responses in the young rat (Figs. 7 and 8). The injection of lacZ-transduced rMSCs into the corpora cavernosum of the aged rat had no significant effect on the erectile parameters studied in response to cavernous nerve stimulation on *day 7* (Fig. 7). The injection of AdeNOS-transduced rMSCs into the corpora cavernosum of the aged rat significantly increased the rise in ICP, ICP/MAP, and ICP-AUC in response to cavernous nerve stimulation compared with responses in PBS-treated and AdntlacZ-transduced rMSC-treated aged rats on *day 7* (Fig. 7).

Twenty-one days after intracavernous injection of wildtype rMSCs alone, AdntlacZ-transduced rMSCs, and AdeNOS-transduced rMSCs, neurogenic-mediated erectile responses to cavernous nerve stimulation as measured by the rise in ICP, ICP/MAP, and ICP-AUC were investigated, and these data are summarized in Fig. 8. Neurogenic-mediated erectile responses were significantly improved in rats treated with wildtype rMSCs, AdntlacZ-transduced rMSCs, and AdeNOS-transduced rMSCs compared with responses in PBS-injected aged rats (Fig. 8). Rats injected with AdeNOS-transduced rMSCs had significantly greater increases in peak ICP in response to cavernous nerve stimulation at 5 and 7.5 V and a greater increase in ICP-AUC at 7.5 V compared with responses in rats treated with PBS, wildtype rMSCs, or AdntlacZ-transduced rMSCs on *day 21* after rMSC therapy (Fig. 8).

Effect of rMSC on resting systemic arterial pressure, heart rate, and ICP. There was no significant difference in baseline MAP and heart rate in the four experimental groups of rats on *day 7* or *21* (Table 2). There was a significant decrease in baseline ICP in aged rats treated with PBS, wildtype rMSCs, or AdntlacZ-transduced rMSCs on *day 7* or *21* compared with pressure in young rats (Table 2). However, treatment with AdeNOS-transduced rMSCs significantly improved resting ICP on *days 7* and *21*, suggesting that ex vivo gene-modified rMSCs increase ICP by increasing the release of endothelium-derived NO and thereby decreasing penile vascular tone.

DISCUSSION

The results of the present study show that intracavernous injection of wildtype rMSCs alone or eNOS gene-modified syngeneic rMSCs improves endothelium-derived NO biosynthesis and neurogenic-mediated erectile responses in the aged rat. The augmented erectile response to cavernous nerve stimulation 7 days after administration of eNOS-modified rMSCs is in agreement with our previous studies (18). New findings in this study are that the enhanced responses to cavernous nerve

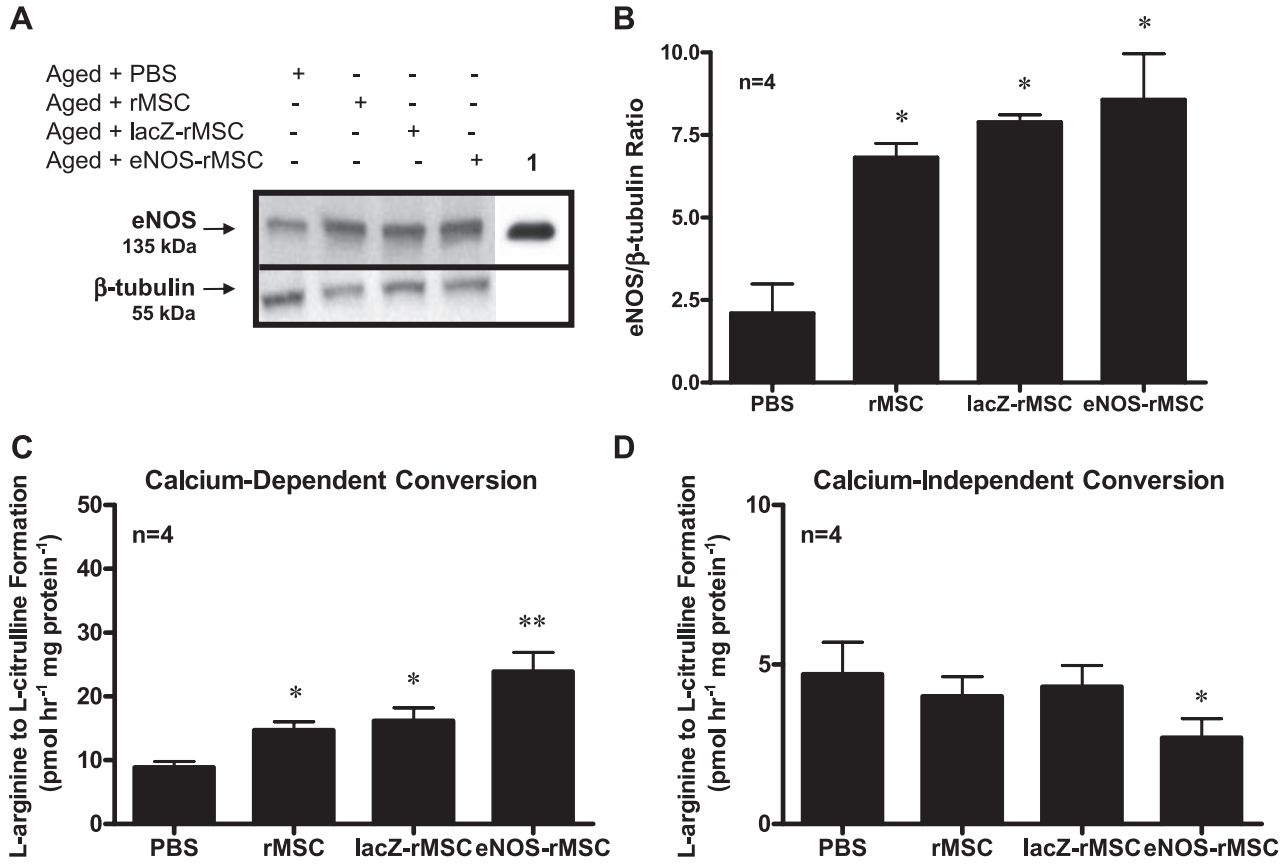

Fig. 6. Changes in eNOS protein abundance and calcium-dependent and -independent NOS activity in aged rat corporal tissue 21 days after intracavernous injection of PBS, rMSCs, lacZ-transduced rMSCs, or eNOS-transduced rMSCs. *A*: Western blot analysis showing changes in eNOS protein expression. Analysis for β-tubulin was carried out to ensure similar sample loading in each lane. *Lane 1*, whole lung cell lysate representing positive control. *B–D*: bar graphs showing the ratio of eNOS to β-tubulin in aged rat corporal tissue (*B*) and calcium-dependent (*C*) and -independent (*D*) L-[^{3}H]arginine-to-L-[^{3}H]citrulline conversion in corpora cavernosum from aged rats injected with PBS and rMSCs; n = no. of experiments. *Significantly different from PBS. **Significantly different from PBS, rMSCs, and lacZ-transduced rMSCs.

stimulation 7 and 21 days after administration of rMSCs alone or eNOS-transduced rMSCs were associated with increased eNOS protein expression, calcium-dependent NOS activity, and cGMP levels in aged corporal tissue. These molecular changes in the penis in response to rMSC therapy caused physiologically relevant changes in neurogenic-mediated erectile function. Importantly, wildtype rMSCs administered intracavernosally differentiated into new endothelial and smooth muscle cells that improved erectile physiology in the aged rat. These data are consistent with the hypothesis that administration of eNOS-transduced rMSCs improves the erectile response to cavernous nerve stimulation by enhancing the release of endothelium-derived NO.

Table 1. *cGMP levels in corporal tissue of aged rats treated with PBS and wildtype and modified rMSCs*

Group	Tissue, pmol/mg protein
7 days	
Aged + PBS	0.067±0.05
Aged + AdlacZ-rMSC	0.073±0.02
Aged + AdeNOS-rMSC	0.219±0.03*
21 days	
Aged + PBS	0.058±0.01
Aged + rMSC	0.152±0.06†
Aged + AdlacZ-rMSC	0.168±0.04†
Aged + AdeNOS-rMSC	0.237±0.05‡

Values are means ± SE; n = 4–6. rMSCs, rat mesenchymal stem cells; AdlacZ (AdntlacZ) and AdeNOS, adenoviral vectors (see MATERIALS AND METHODS for more detail). *$P < 0.05$, value significantly greater than aged rats treated with PBS or AdlacZ-rMSC; †$P < 0.05$, value significantly greater than aged rats treated with PBS; ‡$P < 0.05$, value significantly greater than aged rats treated with rMSC or AdlacZ-rMSC.

MSCs are found in the adult bone marrow together with hematopoietic stem cells. Recent evidence has shown the MSCs found in tissues other than bone marrow are highly plastic and may differentiate into muscle and cardiac myocytes, which alter cellular function (2, 25, 37, 47). In the present study, injection of lacZ-transduced rMSCs had no significant effect on the erectile function in vivo on *day 7*. However, on *day 21* after injection of lacZ-modified or wildtype rMSCs, the increase in ICP in response to cavernous nerve stimulation was augmented significantly in vivo. Additionally, resting ICP was significantly augmented in eNOS-transduced rMSC rats, suggesting increased corporeal perfusion as a result of improved NO biosynthesis with enhanced relaxation of corporal smooth muscle and increased resting ICP. The mechanism by which lacZ-modified and wildtype rMSCs improve erectile function was investigated, and 21 days after injection

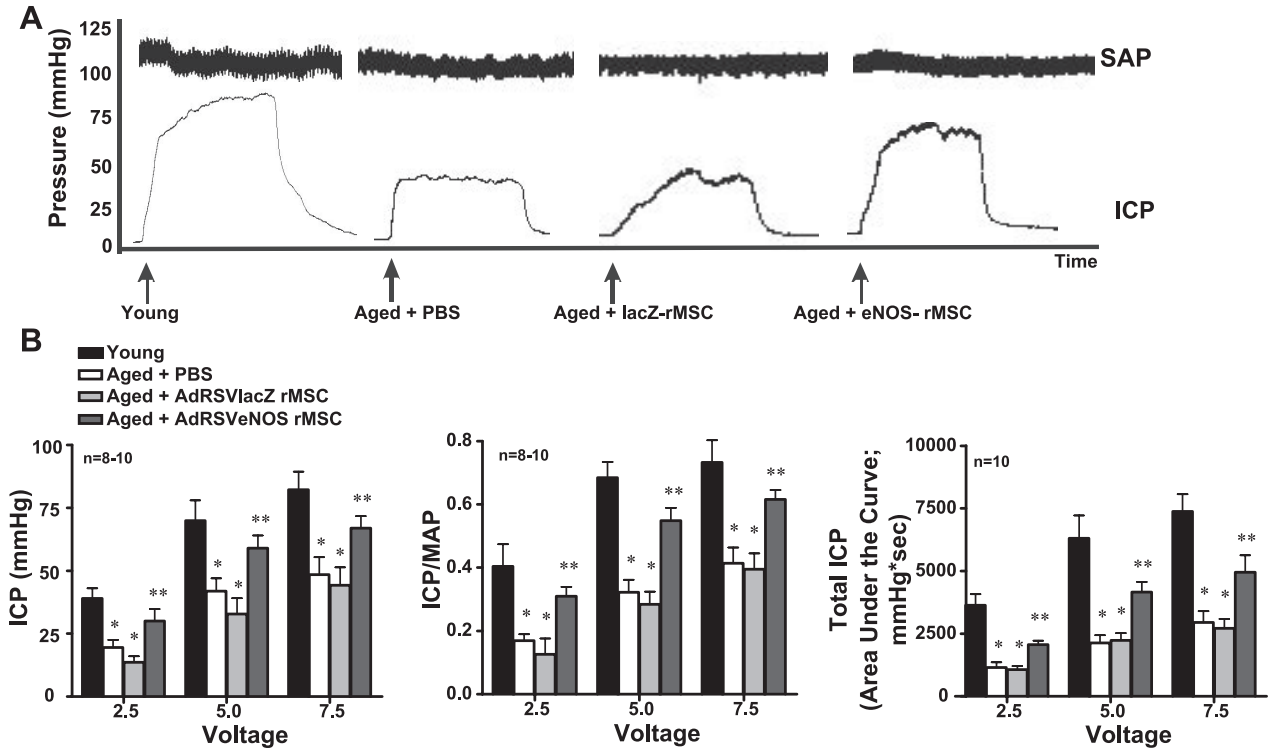

Fig. 7. Improvement in erectile response 7 days after intracavernous injection of eNOS-transduced rMSCs in the aged rat. *A*: records showing representative changes in intracavernous pressure in response to cavernous nerve stimulation (5 V). *Top*: systemic arterial pressure (SAP). *Bottom*: intracavernosal pressure (ICP). *B*: bar graph showing the increase in ICP, ICP/mean systemic arterial pressure (MAP), and ICP-area under the erectile curve (AUC) in response to cavernosal nerve stimulation in young rats and in aged rats treated with PBS, lacZ-transduced rMSCs, and eNOS-transduced rMSCs. The cavernous nerve was stimulated at 2.5, 5, and 7.5 V; n = no. of experiments. *Significantly different from value in young rats. **Significantly different from value in aged PBS-treated and aged lacZ-transduced rMSC-treated rats. Data from young (3 mo old) rats were used as a reference for normal erectile function.

of lacZ-modified cells, β-galactosidase-positive rMSCs were identified in sections from the corpora cavernosa, indicating that transplanted cells are capable of surviving for at least 21 days. Histological examination of tissue sections suggested that the morphology of the transplanted rMSCs had changed and the cells appeared to be elongated and incorporated into the tissue. Moreover, immunohistochemical studies suggested that a change in phenotype had occurred. In addition to exhibiting positive immunostaining for vWF and SMA, the transplanted rMSCs now exhibited positive immunostaining for eNOS, PECAM-1, and SM-MHC. Of note, these stem cells now exhibited endothelial- and smooth muscle cell-specific markers that were not present in vitro before injection into the aged rat penis. These data can be interpreted to suggest that the transplanted cells may have differentiated into cells that now express several markers for endothelial and smooth muscle cell lineages. The absence of positive staining for CD45 suggests that the transplanted cells do not have hematopoietic cell lineage.

Penile erection is a complex response requiring the functional integrity of nitrergic nerves, endothelium, and smooth muscle in the penis (29). Endothelial- and neuronal-derived NO plays a prominent role in the regulation of erectile physiology in healthy and diseased penile vasculature (4, 23, 35, 40). NO directly binds to the heme moiety of soluble guanylyl cyclase, thus increasing intracellular levels of cGMP, thereby activating PKG to reduce intracellular levels of Ca^{2+} and cause hyperpolarization of the corporal myocyte via an efflux of K^+. This series of cellular events results in corporal smooth muscle relaxation and penile erection. Most pharmacological and gene therapies have focused on improving NO biosynthesis in the penis and/or inhibiting the breakdown of cGMP. In the present study, erectile responses in the aged rat were improved by injection of eNOS-transduced and unmodified rMSCs without the appearance of an inflammatory response. The syngeneic MSCs were isolated from bone marrow by adherence to tissue culture plastic and were readily expanded by simple tissue culture techniques (43). This novel cell-based therapy using rMSCs alone or ex vivo gene modified with eNOS shows for the first time that MSCs can be successfully injected into the penis and differentiate into new cells that are biologically active and/or influence erectile mechanisms by improving NO/cGMP signaling in the penis. Importantly, the results of these studies show that treatment with rMSCs improves erectile function without inducing inflammation. Longer-term studies evaluating erectile function are warranted to determine the feasibility of this form of cell-based therapy.

Endothelium-derived NO is vital in initiating and maintaining normal erectile responses (30). Endothelial dysfunction as a result of diminished phosphorylation of eNOS and reductions in eNOS activity and endothelial NO bioavailability in the aging penile vascular bed have been reported as causes of age-associated ED (4, 40). It has been reported that eNOS gene therapy can improve neurogenic- and endothelial-dependent erectile responses in the age rat (3, 4, 8, 16). Although adeno- or adeno-associated gene transfer of eNOS, neuronal NO

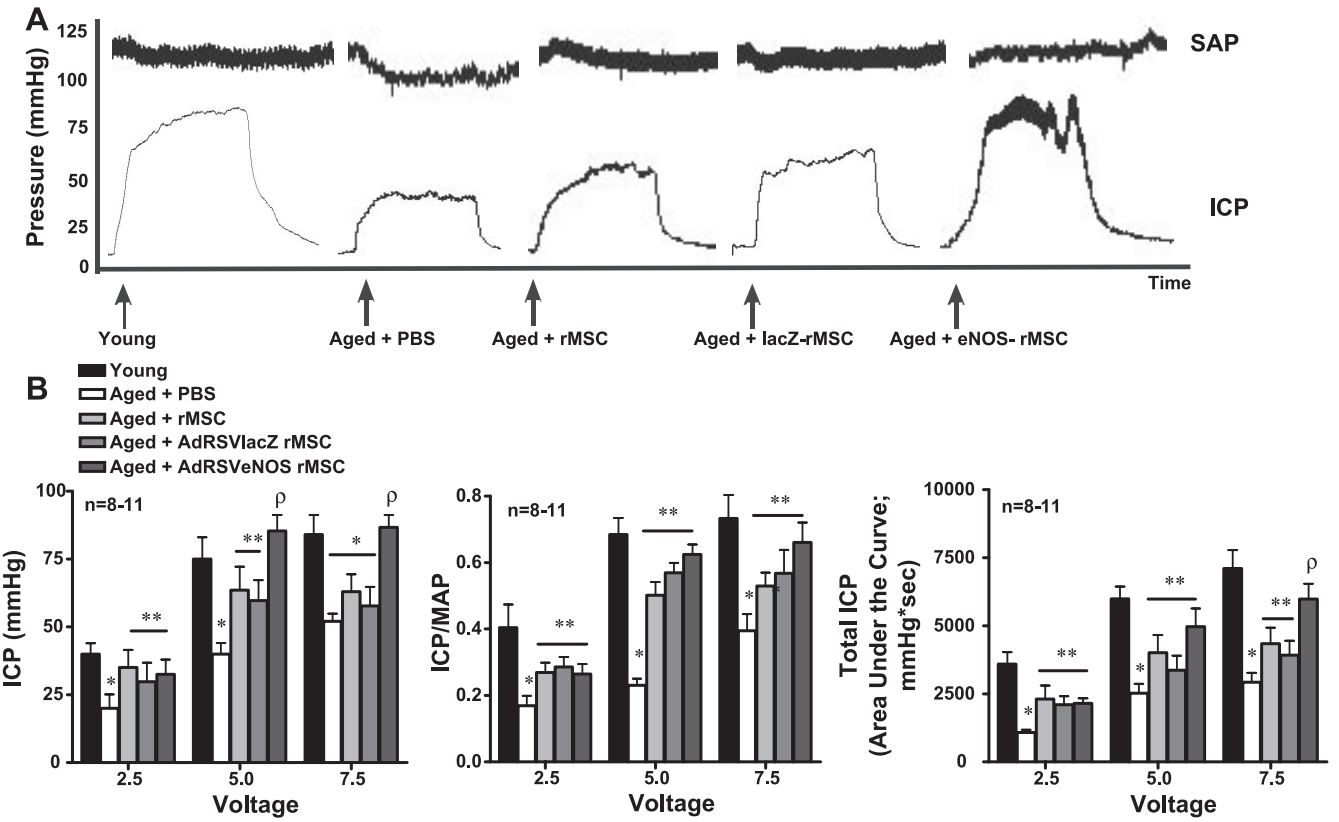

Fig. 8. Improvement of erectile response 21 days after intracavernous injection of rMSCs alone, lacZ-transduced rMSCs, and eNOS-transduced rMSCs in the aged rat. *A*: records showing representative changes in ICP in response to cavernous nerve stimulation (5 V). *Top*: SAP. *Bottom*: ICP. *B*: bar graph showing the increase in ICP, ICP/MAP, and ICP-AUC in response to cavernosal nerve stimulation in young rats and in aged rats treated with PBS, rMSCs, lacZ-transduced rMSCs, and eNOS-transduced rMSCs. The cavernous nerve was stimulated at 2.5, 5, and 7.5 V; n = no. of experiments. *Significantly different from value in young rats. **Significantly different from value in aged PBS-treated rats. ρSignificantly different from value in aged PBS-treated, rMSC-treated, and lacZ-transduced rMSC-treated rats. Data from young (3 mo old) rats were used as a reference for normal erectile function.

synthase, VEGF, brain-derived neurotrophic factor, SOD, or a dominant-negative RhoA mutant can augment erectile responses in aged or diabetic rat models, the possible occurrence of an inflammatory response and random expression of the transgene may limit the clinical utility of these interventions (3–10, 16, 28, 36, 38). In the present study, the administration of rMSCs alone or eNOS gene-modified rMSCs improved erectile physiology as measured by increases in ICP, ICP/MAP, and ICP-AUC in response to cavernous nerve stimulation in the aged rat without evidence of an inflammatory response. In these experiments, the presence of inflammatory cells and an increase in calcium-independent NOS activity were not detected in the penis, suggesting that stem cell-based therapy may have an advantage in the clinical treatment of ED. However, further preclinical studies are warranted before this cell-based therapy can be utilized in human clinical trials.

Table 2. *Baseline mean SAP, HR, and ICP in young rats and aged rats treated with PBS and stem cell therapy cohorts*

Group	SAP, mmHg	HR, beats/min	ICP, mmHg
	7 days		
Young	114.4±5.9	312.3±7.8	10.4±0.6
Aged + PBS	118.3±4.1	321.6±11.5	3.4±0.4*
Aged + AdlacZ-rMSC	112.5±3.9	312.7±6.8	4.1±0.7*
Aged + AdeNOS-rMSC	119.8±5.4	313.6±6.2	7.4±0.5†
	21 days		
Young	109.8±11.6	315.1±3.2	11.1±0.8
Aged + PBS	109.7±5.1	315±8.8	3.5±0.4*
Aged + rMSC	114.6±3.6	301±12.5	5.6±0.7*
Aged + AdlacZ-rMSC	115.5±7.9	307.7±5.6	5.8±0.6*
Aged + AdeNOS-rMSC	120±15.5	313.2±8.6	8.7±1.1†

Values are means ± SE; n = 8–11. SAP, systemic arterial pressure; HR, heart rate; ICP, intracavernosal pressure. *$P < 0.05$, value significantly lower than young; †$P < 0.05$, value significantly higher than aged rats treated with PBS, rMSC, or AdlacZ-rMSC.

The results of biochemical studies after intracavernous injection of rMSCs alone or eNOS gene-modified rMSCs demonstrated that eNOS protein expression, calcium-dependent NOS activity, and cGMP levels are upregulated in corporal tissue 21 days after injection of syngeneic rMSCs and suggest that eNOS is biologically active with improved endothelium-derived NO biosynthesis, since these molecular changes significantly improved erectile function. These data provide evidence in support of the hypothesis that the improved erectile response to cavernous nerve stimulation may involve upregulation of endothelium-derived NO release in the penis of the aged rat after rMSC therapy. The mechanism by which eNOS expression and NO release are upregulated in the corpora cavernosum of the aged rat is unknown, but it is possible that an

unidentified paracrine action (growth factor secretion to promote NO signaling) by rMSCs may occur when rMSCs are injected and transplanted into the microenvironment of the aged penile vascular bed (34, 45, 47, 49). Theoretically, improving penile vascular function could occur by production of new endothelial and smooth muscle cells in the penis with full genetic and cellular properties. The present study demonstrates stem cell differentiation into new penile cells exhibiting endothelial- and smooth muscle cell-specific markers. Future experiments defining the exact mechanism of restoration of penile vascular function by rMSCs in the aging penis are necessary.

In the present study, rMSCs from passages 0–3 were used for all in vitro and in vivo experiments, because in early passage, MSCs retain differentiation capability. This experimental design was conducted because stem cells in vitro become quiescent in later passages (12). The differentiation potential to adipocyte and osteocyte drops in late passages, supporting the concept that MSCs become dormant in late passages (17). The effects of later passage on stem cell differentiation in vivo and on erectile physiology are unknown at this time and warrant further investigation.

Stem cell-based techniques have been used to improve erectile function after cavernous nerve injury (11, 33). Embryonic stem cells have the capability to promote regeneration of damaged nerve cells and increase growth; therefore, an embryonic stem cell-based therapy has been used to restore erectile function after cavernous nerve injury (11). Additionally, skeletal muscle-derived cells have also been utilized to facilitate recovery of injured nerves after cavernous nerve injury (33). Both of these experimental cell-based therapies did show some efficacy in improving erectile function after cavernous nerve injury. However, the exact mechanism by which nerve injury was prevented or protected against by either embryonic stem cells or skeletal muscle-derived cell therapy was not fully identified. In the present study, we have demonstrated that rMSC therapy improved endothelium-derived NO biosynthesis in the penis with subsequent increases in penile tissue cGMP to restore normal erectile physiology in the aging penile vascular bed.

In summary, the present results show that eNOS-transduced rMSCs improve erectile function 7 and 21 days after injection into the corpora cavernosa of the aged rat. The augmented response was associated with upregulated eNOS protein expression, calcium-dependent NOS activity, and tissue cGMP levels. Moreover, when wildtype rMSCs or lacZ-transduced rMSCs were administered, the response to cavernous nerve stimulation was improved on *day 21*, and immunofluorescent studies suggested that the rMSCs had undergone a change in phenotype. The transplanted cells retained expression of vWF and SMA and also expressed eNOS, SM-MHC, and PECAM-1 immunoreactivity. The augmented erectile response after rMSC therapy alone was associated with increased eNOS expression, calcium-dependent NOS activity, and tissue cGMP levels. These data suggest that wildtype rMSCs alone or eNOS-transduced syngeneic MSCs can improve erectile function through mechanisms involving improved endothelial-derived NO biosynthesis in the aged penile vascular bed and point out the clinical potential of adult stem cell-based therapy for the treatment of ED.

GRANTS

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