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# Inhibition of vascular smooth muscle cell proliferation in vitro by genetically engineered marrow stromal cells secreting calcitonin gene-related peptide

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**Abstract**

Calcitonin gene-related peptide (CGRP) has a beneficial effect in pulmonary hypertension and is a target for cardiovascular gene therapy. Marrow stromal cells (MSCs), also known as mesenchymal stem cells, hold promise for use in adult stem cell-based ex vivo gene therapy. To test the hypothesis that genetically engineered MSCs secreting CGRP can inhibit vascular smooth muscle cell proliferation, rat MSCs were isolated, ex vivo expanded, and transduced with adenovirus containing CGRP. Immunocytochemical analysis demonstrated that wild type rat MSCs express markers specific for stem cells, endothelial cells, and smooth muscle cells including Thy-1, c-Kit, von Willebrand Factor and α-smooth muscle actin. Immunocytochemistry confirmed the expression of CGRP by the transduced rat MSCs. The transduced rat MSCs released 10.3 ± 1.3 pmol CGRP/$1 \times 10^6$ cells/48 h (mean ± S.E.M., $n = 3$) into culture medium at MOI 300 and the CGRP-containing culture supernatant from the transduced cells inhibited the proliferation of rat pulmonary artery smooth muscle cells (PASMCs) and rat aortic smooth muscle cells (ASMCs) in culture. Co-culture of the transduced rat MSCs with rat PASMCs or rat ASMCs also inhibited smooth muscle cell proliferation. These findings suggest that this novel adult stem cell-based CGRP gene therapy has potential for the treatment of cardiovascular diseases including pulmonary hypertension.


*Keywords:* Marrow stromal cells; Mesenchymal stem cells; Calcitonin gene-related peptide; Adenovirus; Vascular smooth muscle cells; Cell proliferation; Gene therapy; Cardiovascular diseases; Pulmonary hypertension

## Introduction

Vascular smooth muscle cell proliferation in response to vessel injury has been shown to play a role in the pathogenesis of cardiovascular diseases such as pulmonary hypertension and coronary artery restenosis following balloon angioplasty (Heckenkamp et al., 2002; Mandegar et al., 2004). Therefore, anti-proliferative strategies will be important in the treatment of vascular proliferative disorders (Kopp and de Martin, 2004; Nagaya, 2004). Calcitonin gene-related peptide (CGRP) is a 37-amino acid neuropeptide with many beneficial cardiovascular actions including potent vasodilator and antiproliferative effects on vascular smooth muscle cells (Amara et al., 1982; Li et al., 1997; Wang et al., 1999; Brain and Grant, 2004; Chattergoon et al., 2005). Therefore, the enhancement of local CGRP delivery should be a useful approach for the treatment of cardiovascular diseases (Li et al., 1997; Wang et al., 1999; Champion et al., 2000; Toyoda et al., 2000a,b; Bivalacqua et al., 2001, 2002; Gruchala et al., 2004; Chattergoon et al., 2005). However, the half-life of CGRP in human plasma is short (Struthers et al., 1986), and chronic infusion of CGRP in patients is difficult. Therefore, a gene therapy approach for the delivery of CGRP would be desirable.

Marrow stromal cells (MSCs), also known as mesenchymal stem cells, are multipotent adult stem cells from bone marrow that are easy to isolate, ex vivo expand, and gene engineer (Prockop, 1997; Pittenger et al., 1999; Bianco et al., 2001; Deng et al., 2001; Grove et al., 2004). Therefore, these cells

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can be used in adult stem cell-based ex vivo gene therapy for local delivery of a therapeutic gene product that promotes tissue repair and regeneration. To test the hypothesis that CGRP-secreting MSCs can be used to treat cardiovascular diseases, rat MSCs were transduced with an adenoviral vector containing CGRP and were incubated with vascular smooth muscle cells to determine if smooth muscle cell proliferation could be inhibited. The results of the present study demonstrate that MSCs genetically engineered to secrete CGRP inhibit the proliferation of vascular smooth muscle cells in culture, underscoring the potential of this novel approach for the treatment of vascular proliferative disorders.

## Materials and methods

### *Isolation, ex vivo expansion, and adenoviral transduction of MSCs*

MSCs were isolated from 6-week-old male brown Norway or Sprague–Dawley rats (Harlan, San Diego, CA) as described previously (Deng et al., 2003, 2004). MSCs were then ex vivo expanded in culture medium for MSCs (α-MEM, 20% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 25 ng/ml amphotericin B, and 2 mM L-glutamine, GIBCO Invitrogen, Grand Island, NY) and cells at passage 2 were used in the study. Adprepro-CGRP (an adenoviral vector carrying human prepro-CGRP gene driven by RSV promoter) and AdntlacZ (an adenoviral vector carrying nuclear-targeted β-galactosidase reporter gene ntlacZ driven by RSV promoter) from University of Iowa Gene Transfer Vector Core (Iowa City, IA) (Bivalacqua et al., 2002; Deng et al., 2003, 2004) were used to transduce MSCs as described previously (Deng et al., 2003, 2004). In brief, MSCs were plated in 6-well plates or T75 flasks at a density of $1\times10^4$ cells/cm$^2$ and cultured overnight. The cells were then exposed to fresh culture medium containing Adprepro-CGRP at 300 multiplicities of infection (MOI, defined as plaque-forming unit/cell) for 48 h. The virus-containing culture medium was removed and the cells were washed with PBS 3 times. These Adprepro-CGRP-transduced MSCs were either further incubated in fresh culture medium for 48 h for the collection of CGRP-containing culture supernatant or directly used for co-culture experiments after trypsinization. Wild type MSCs and AdntlacZ-transduced MSCs (MOI 300) were used as controls.

### *Immunocytochemical analysis for markers expressed by MSCs*

MSCs were plated at a density of 10,000 cells/cm$^2$ in Falcon 1-chamber culture slides (BD Bioscience, Bedford, MA) 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-Thy-1 (CD90) monoclonal antibody (BD PharMingen, San Diego, CA), rabbit anti-c-Kit (CD117, stem cell factor receptor) polyclonal antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA), mouse anti-CD45 (leukocyte common antigen) monoclonal antibody (BD PharMingen), rabbit anti-von Willebrand Factor (vWF) polyclonal antibody (DAKO Corporation, Carpinteria, CA), mouse anti-PECAM-1 (CD31) monoclonal antibody (BD PharMingen), mouse anti-endothelial nitric oxide synthase (eNOS) monoclonal antibody (BD Transduction Laboratories, San Diego, CA), mouse anti-α-smooth muscle actin (SMA) monoclonal antibody (Sigma, St. Louis, MO), and mouse anti-smooth muscle myosin heavy chain (SM-MHC) monoclonal antibody (Santa Cruz Biotechnology Inc.) at a dilution of 1:200 each. The secondary antibody was FITC-conjugated horse anti-mouse IgG (Vector Laboratories, Burlingame, CA) or Rhodamine-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) at a dilution of 1:200 each. The culture slides were then mounted with VECTASHIELD mounting medium for fluorescence with DAPI (Vector Laboratories) and checked under a Nikon Eclipse E800 fluorescent microscope.

### *Immunocytochemistry for CGRP expression in MSCs*

The expression of CGRP in Adprepro-CGRP-transduced MSCs was assessed by immunofluorescence staining. MSCs were plated at a density of $1\times10^4$ cells/cm$^2$ in Falcon 1-chamber culture slides (BD Bioscience) and cultured overnight. The cells were exposed to fresh culture medium containing Adprepro-CGRP or AdntlacZ at MOI 300 for 48 h and immunostained with rabbit anti-CGRP polyclonal antibody (Peninsula Laboratories Inc., San Carlos, CA) followed by Rhodamine-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories Inc., West Grove, PA). The culture slides were then mounted with VECTASHIELD mounting medium for fluorescence with DAPI (Vector Laboratories), and checked under a fluorescent microscope for CGRP-positive red cells.

### *Enzyme immunoassay for CGRP secretion*

For measurement of CGRP concentration in the culture supernatant of MSCs, the enzyme immunoassay (EIA) was used. Briefly, MSCs were transduced with Adprepro-CGRP at MOI 300 for 2 days. The virus-containing culture medium was removed, the cells were washed with PBS 3 times, and fresh culture medium was added. The cells were counted, cultured for 48 h, and the culture supernatant was collected. The supernatant was then assayed for the secretion of CGRP by the transduced MSCs using a competitive EIA kit for CGRP peptide (Peninsula Laboratories Inc., San Carlos, CA). The EIA data were expressed as the mean picomole of peptide/$1\times10^6$ cells/48 h ± S.E.M., with $n=3$ per group. Wild type and AdntalcZ-transduced MSCs at MOI 300 were used as controls.

### *Isolation and ex vivo expansion of pulmonary artery smooth muscle cells (PASMCs) and aortic smooth muscle cells (ASMCs)*

PASMCs and ASMCs were isolated from 6-week-old male Sprague–Dawley rats as described previously (D'Souza et al., 2003; Chattergoon et al., 2005). The cells were then ex vivo