

## Directed therapeutic angiogenesis by mesenchymal stem cells from umbilical cord matrix in preclinical model of ischemic limb disease

Prathibha Shetty,<sup>1</sup> Anirban M. Thakur,<sup>2</sup> Geeta Ravindran,<sup>1</sup> Chandra Viswanathan<sup>1</sup>

<sup>1</sup>Regenerative Medicine; <sup>2</sup>Laboratory Animal Research Services, Reliance Life Sciences Pvt Ltd Dhirubhai Ambani Life Sciences Centre, Navi Mumbai, India

### Abstract

Ischemic limb disease (ILD) is the end stage of peripheral vascular disease, progresses to leg amputation with associated morbidity and mortality. Therapeutic application of stem cell based angiogenesis may constitute a potential alternative treatment strategy for such patients. Mesenchymal stem cells (MSCs) obtained from various tissues have shown to elicit angiogenic activity that could potentially benefit patients with ILD and related indications. Recently, MSC derived from umbilical cord matrix (UCMSCs) has been proven to be a valuable alternate source of MSCs with good therapeutic potential. In this study, besides comparing UCMSCs and bone marrow derived MSCs (BMMSCs) for their morphology, surface marker expression, proliferative and differentiation potentials, we looked for its ability for angiogenesis. These cells secreted high amounts of HGF and upon induction with TNF $\alpha$  also secreted higher levels of VEGF as compared to the BMMSCs, which are the key factors in angiogenesis. Transplantation of these VEGF secreting UCMSCs in a preclinical model of ILD showed significant improvement as compared to the MSCs from other sources. In this study, we observed that the UCMSCs could be a new therapeutic cell type for clinical applications in the area of peripheral vascular diseases. Moving forward, it is also worth exploring the application of these MSCs in a clinical setting in the autologous and allogeneic settings.

### Introduction

Multipotent mesenchymal stem cells (MSCs) have been the subject of intensive research over the past several years because of their potential use in regenerative medicine and tissue engineering. The bone marrow derived MSCs (BMMSCs) have been the best

studied cell type so far. Although bone marrow has been the main source for isolation of multipotent MSCs, the BM aspiration is a highly invasive procedure. Moreover, the number of MSCs from the bone marrow and their ability to expand *ex vivo* and differentiate to cells of various lineage declines with age and under certain disease conditions.<sup>1</sup> As a result, alternative sources of MSC isolation have become a subject of intense investigation in the recent past. A recently reported alternative tissue as a source for MSCs is the connective tissue of human umbilical cord matrix.<sup>2,3</sup> Umbilical cord matrix (UCMSCs) represent a non-controversial source of primitive mesenchymal progenitor cells that can be harvested after birth, cryogenically stored, thawed, and expanded to large numbers for therapeutic use. These cells are of non haematopoietic origin and are phenotypically positive for CD73, CD105, CD44, CD106, CD90 and HLA class I expression and negative for haematopoietic and endothelial cell antigen expression. These cells also do not express HLA molecules (DP, DQ, DR) or co-stimulatory molecules CD80, CD86, CD40 that are usually present on the antigen presenting cells. In addition, UCMSCs have been shown to be immunologically privileged and possess the ability to modulate the immune response in various ways as reported earlier by us.<sup>4</sup>

The ability of human MSCs to mobilize into various organs after systemic administration and their ability to differentiate into their natural derivatives has led to the importance of these cells in repair of damaged organs. In addition, the MSCs have the potential to differentiate into other types of tissue forming cells such as hepatic, renal, cardiac and neural cells.<sup>5-8</sup>

Transplantation studies in rats showed clinical improvement after undifferentiated MSCs were transplanted into the Parkinsonian brain and spinal cord injury sites.<sup>9,10</sup> More recently, mesenchymal progenitor cells as well as MSCs were shown to differentiate to an endothelial phenotype and enhance vascularization. Enhanced neovascularization has been associated with regeneration of infarcted myocardium by MSCs.<sup>11</sup> BMMSCs have also shown to improve limb function, reduced the incidence of auto-amputation in an ischemic limb disease (ILD) animal model.<sup>12,13</sup>

The present study brings out our findings on the comparative functional efficacy of BMMSCs and UCMSCs for their angiogenic potentials both *in vitro* and *in vivo*. In order to correlate their phenotypic profile with *in vivo* functional activity of the MSCs, we used an established preclinical disease model for vascular diseases, namely the ILD model. Our results show that transplantation of UCMSCs was more efficacious than the BMMSCs in ILD mouse models.

Correspondence: Prathibha Shetty, Reliance Life Sciences Pvt Ltd, Dhirubhai Ambani Life Sciences Centre, R-282, TTC Area of MIDC, Thane Belapur Road, Rabale, Navi Mumbai-400701, India.  
Tel. +91.226.767.8436 - Fax: +91.226.767.8099  
E-mail: prathibha\_shetty@relbio.com

Key words: umbilical cord, mesenchymal stem cells, ischemic limb disease.

Received for publication: 3 September 2011.  
Accepted for publication: 3 October 2011.

This work is licensed under a Creative Commons Attribution NonCommercial 3.0 License (CC BY-NC 3.0).

©Copyright P. Shetty et al., 2011  
Licensee PAGEPress, Italy  
Stem Cell Studies 2011; 1:e16  
doi:10.4081/scs.2011.e16

## Materials and Methods

### Processing and culturing of mesenchymal stem cells from umbilical cord

Umbilical cords were obtained from mothers post normal or cesarean deliveries after a due consenting process. These umbilical cords were washed with PBS containing antibiotics, cut open longitudinally and blood vessels were removed. The cord matrix was then serially cut in a cross sectional manner and 4 to 5 explants of the matrix ranging from 1-2 cm in size were placed in 100 mm tissue culture dishes with 2-3 ml culture medium. The dishes were left undisturbed for 3-4 days after which the fresh cell culture media (MSC proliferation media) was added to the dishes. Adherent cells were allowed to expand with regular media changes. The cells were harvested at 80-90% confluence using trypsin EDTA, characterized and used for the further study.<sup>14</sup>

UCMSCs derived from 3 cords were extensively studied for the various *in vitro* characterization and differentiation studies.

### Isolation and expansion of mesenchymal stem cells from human bone marrow

Five to Ten mL of bone marrow was collected from the posterior superior iliac spines into heparinised vacutainers after a due consenting process. This was approved by the local hospital ethics committee. We included only those volunteers between the ages of 30 to 50 years. Marrow samples were transported to our laboratory at temperatures of 4-8°C by maintaining a validated cold chain. All the

processes on the sample began within 24 h of sample collection. The marrow was diluted with phosphate buffered saline and directly loaded onto a percoll (Sigma, St. Louis, MO, USA) density gradient (density 1073 g/mL). The cells were centrifuged at 400 g for 20 min and the mononuclear cells from the interface were harvested, washed and plated at a density of  $1 \times 10^6$  to  $10 \times 10^6$  cells/ml for isolation of mesenchymal stem cells.<sup>14</sup> BMSCs derived from two marrow aspirates were expanded, characterized and used for the various *in vitro* experiments as per our published reports.

### Characterization of the mesenchymal stem cells derived from umbilical cord and bone marrow after expansion

Immunophenotypic characterization of MSCs was done using flow cytometry (BD FACS Calibur) as per the protocol published.<sup>15</sup> Cells were incubated with CD45 PerCP (BD Biosciences Pharmingen, San José, CA, USA), CD73 PE (BD Biosciences Pharmingen), CD105 PE (Caltag Laboratories, Burlingame, CA, USA), SSEA4 PE (R&D Systems, Minneapolis, MN, USA), HLADR PE (BD Biosciences Pharmingen), HLAABC PE (BD Biosciences Pharmingen), CD14 PE (BD Biosciences Pharmingen), CD31 PE (BD Biosciences Pharmingen), CD29 PE (BD Biosciences Pharmingen), CD44 PE (BD Biosciences Pharmingen), purified vWF (BD Biosciences Pharmingen), using standard techniques. Appropriate isotype controls from BD Biosciences Pharmingen were used. Goat anti mouse FITC (BD Biosciences Pharmingen) was used as secondary antibody for vWF antibody. For viability determination, cells were stained with 7-Amino Actinomycin D (BD Biosciences Pharmingen) and acquired on the flow cytometer. Dead cells take up the fluorescent stain while live cells exclude this stain. Viability determination and surface antigen expression profiling was done at every passage.

### *In vitro* differentiation potential of mesenchymal stem cells to vascular lineage

MSCs derived from bone marrow and umbilical cords were expanded in MSC proliferation medium till 80% confluency was achieved. These expanded cells were treated with 50 ng/mL of TNF $\alpha$  (Sigma)<sup>16</sup> made in basal medium containing DMEM: F12 + 10%FBS for a period of 7 days to induce them to vascular lineage. The cells were fed every 2-3 days post TNF $\alpha$  treatment. Post induction, the culture supernatant was collected and checked for VEGF and HGF by ELISA (R&D Systems).

### Gene expression profiling and analysis of the vascular factors secreted by the induced and the uninduced umbilical cord and bone marrow derived mesenchymal stem cells

The cell pellets of induced and uninduced MSCs of both cell types UCMSCs and BMSCs were used for total RNA extraction. Total RNA was isolated from  $1 \times 10^6$  cells by using a RNeasy kit. (Qiagen, Singapore). Five  $\mu$ g of RNA was used for cDNA synthesis. The cDNA was synthesized using Superscript reverse-transcriptase II (Invitrogen, Chromos Singapore). Two  $\mu$ L of cDNA was amplified by using PCR master mix (ABgene, Surrey, UK) with appropriate primers. The list of primers is as given in Table 1. Cycling parameters are as follows: Initial denaturation at 94°C for 2 min, denaturation at 94°C for 30 s, annealing at 55-65°C for 30 s depending on the primer and elongation for 1 min and the number of cycles varied between 25 and 40. Final elongation was carried out at 72°C for 7 min. The culture supernatant of the undifferentiated and differentiated MSCs from umbilical cord and bone marrow were centrifuged to remove the particulate matter and stored at -20°C till assayed. The concentration of the secreted factors such as VEGF, HGF and bFGF in the supernatants was determined using Quantikine ELISA kit (R&D Systems) as per manufacturer's instructions.

### Creation of an ischemic limb disease preclinical model

Animals were handled in accordance with the Institutional Animal Ethical Committee, Reliance Life Sciences. We used 11- to 13-week old nude mice (immunodeficient mice) from our breeding facility. The nude mouse is valuable to research because it can receive many different types of tissue and tumor grafts, as it mounts no rejection response. These mice have been used for biomedical research involving tumour angiogenesis studies.<sup>17</sup> Surgeries were performed under general anesthesia with intraperitoneal xylazine (25 mg/kg) and ketamine (150 mg/kg). Surgical intervention was performed to create unilateral hind limb ischemia in the mice.<sup>18</sup> A skin incision on the medial aspect of the thigh was performed. The right femoral artery was exposed and ligated proximal to the caudal femoral artery, using non resorbable material (silk, 6-0). The skin was closed surgically with intradermal sutures to minimize the risk of skin irritation and prevent infections. All surgical procedures were performed inside a biosafety cabinet.

### Transplantation of mesenchymal stem cells in mice with ischemic limb disease

DiI labeled MSCs from human umbilical

cord and bone marrow ( $1 \times 10^6$  cells/animal in 200  $\mu$ L) were injected intramuscularly immediately after femoral artery ligation. The labeling of the MSCs was performed as per the protocol published.<sup>10</sup> These cells were injected into multiple sites in the right adductor muscle adjacent to and within 1 mm proximal or distal to the ligation site using a tuberculin syringe with 261/2 G needle (0.45 mm x 13 mm). The DiI labeled UCMSCs (n=9) and BMSCs (n=9), were injected into the muscle in a circular mode with multiple injection and each injection comprising of 25  $\mu$ L per site. Control groups were identically injected with medium (n=4, as control for medium effect) and human foreskin fibroblast (HFF) (n=5; as a negative control).

### Assessment of the efficacy posttransplantation

#### Functional measurement

The ILD preclinical models were assessed for their movements and the extent of necrosis pre and post ligation and post stem cell injection. Before and after surgery for serial measurements, spontaneous movement of the right hindlimbs was scored as either 0 (dragging of foot), 1 (no dragging but no active plantar flexion), 2 (moderately to severely reduced plantar flexion), or 3 (normal or only mildly abnormal use). A semi-quantitative functional assessment of the ischemic limb was performed by a blinded observer using a modification of a clinical score (0- toe flexion, 1-foot flexion, 2-no dragging but no plantar flexion, 3-foot dragging). Ischemic damage was also scored (0-no change, 1-mild necrosis, 2-moderate/severe necrosis, 3-necrosis, 4-amputation) before and after surgery and post cell injection.<sup>19</sup>

#### Radiography

Mice were anesthetized with ketamine as described previously. The left ventricle was fixed proximally and cannulated distally with a 26G polyethylene catheter. Warm heparinized saline (10 U/mL, 0.4 mL total volume) was injected into the aortic catheter. Iodine was then injected into the aortic catheter. The skin was removed from the mouse hind limbs to avoid imaging the dermal vasculature. Images were acquired by using Kodak *In Vivo* Fx at 35 kVp, and 0.5 s exposure. The aim of the study was to visualize femoral artery in general.

#### Histological analysis

At 12 weeks after the injection, tissue from the lower calf muscles of ischemic and healthy limbs was harvested and then fixed with 10% neutrally buffered formalin, and embedded in paraffin. Serial sections were cut at 4  $\mu$ m, placed on Superfrost plus slides (Fisher Scientific) and stored at room temperature. One section was processed for immunohistochemistry and the other for H&E staining. For

**Table 1. Primer sequences used for polymerase chain reactions.**

Gene	Primer sequence	Annealing temp	Size (bp)
GAPDH	5' TGAAGGTCGGAGTCAACGGATTGG 3' 3' CATGTGGCCATGAGGTCACCAC 5'	66°C	890 bp
Vascular endothelial growth factor (VEGF)	5' CTACCTCCACCATGCCAAG 3' 3' CACATCTGCAAGTACCTTCG 5'	55°C	513 bp
Hepatocyte growth factor (HGF)	5' TGGGAACCAGATGCAAGTAA 3' 3' GTGCATCCATAATTAGGTAA 5'	56°C	550 bp
Fibroblast growth factor (FGF)	5' TTCTTCTGCGCATCCAC 3' 3' CAGCTCTTAGCAGACATTGG 5'	55°C	354 bp
Transforming growth factor $\beta$ 1 (TGF $\beta$ 1)	5' ATCCACCTGCAAGACTATCGAC 3' 3' TGCTGTGTACTCTGCTTGAAC 5'	55°C	302 bp

immunohistochemical analysis, the sections were deparaffinized followed by antigen retrieval. The tissue sections were stained with human specific antibodies to check for the presence of transplanted MSCs expressing VEGF as per the published protocol.<sup>10</sup>

### Tumourgenecity assessment of the mesenchymal stem cells

SCID mice (n=15) were used for testing the safety of the MSCs. BMMSCs (n=5) and UCMSCs (n=5) were injected intramuscularly into the hindlimbs of the SCID mice using a tuberculin syringe with 261/2 G needle. As a positive control, human carcinoma cell line NTERA 2D cells were also injected into the SCID mice (n=5). All mice were observed for 12 weeks for the development of tumor if any.

## Results

### Expansion and characterization of mesenchymal stem cells

UCMSCs were isolated and expanded from the cord explants. From the explant stage (P0) to passage P10, the UCMSCs showed an 11 fold expansion.<sup>14</sup> Over 90% of these cells exhibited an MSC phenotype of CD73+/CD105+/SSEA4+/CD45-. The mesodermal lineage differentiation of these cells was confirmed by their ability to differentiate into osteocytes and chondrocytes. The osteogenic differentiation capability of the UCMSCs was also confirmed by immunofluorescence staining for Osteopontin, Osteonectin and Osteocalcin. We proved chondrogenic differentiation by alcian blue staining of proteoglycans. The ectodermal lineage differentiation of UCMSCs induced to differentiate along the neuronal lineage was confirmed by immunofluorescence analysis (14). Over 90% of the BMMSCs expressed the phenotype CD73+/CD105+/CD29+/CD44+/SSEA4+/HLA-ABC+/HLA-DR-/CD45-/CD14-/CD31-/WF-. The phenotypic characteristics exhibited by all the 3 cell lines of UCMSCs studied were very similar to those shown by the BMMSCs, excepting that we observed a

**Table 2. Culture supernatant of the HFF cells did not show presence of any secreted growth factors.**

Cell type	VEGF (pg/mL)	HGF (pg/mL)
UCMSCs (UN)	0.67	8838
UCMSCs (IN)	2061	12771
BMMSCs (UN)	1647	1342
BMMSCs (IN)	2291	624
HFF	ND	ND

**Table 3. Showing the details of stem cell injection and the status of the ischemic limb disease mice, 3 months post transplantation.**

Cell type injected	Total cell dose injected	No of ILD mice	Limb losses	Adverse events
UCMSCs	1.5 million	9 nos	None	2 deaths
BMMSCs	1.5 million	9 nos	None	None
HFF2 cell line [Control group]	1.5 million	9 nos	9 nos	None

ILD, ischemic limb disease; UCMSCs, umbilical cord derived mesenchymal stem cells; BMMSCs, bone marrow derived mesenchymal stem cells.

98% expression of SSEA4 by UCMSCs as compared to a low 27% expression of this marker by the BMMSCs (Figure 1). This observation was true upto passages 4 with UCMSCs while BMMSCs were studied only up to passage 3.

### Gene profiling and soluble factor estimation of the mesenchymal stem cells involved in angiogenesis and wound healing

The uninduced UCMSCs cell lines and BMMSCs showed a positive expression of VEGF, HGF, bFGF and TGF $\beta$ , the main angiogenic genes (Table 1). Dermal fibroblasts which were used as a positive control also expressed all these genes as expected (Figure 2A). ELISA analysis of the supernatant of the UCMSCs showed that these cells secreted very high levels of HGF and bFGF in their uninduced stage. It was observed that although the genes were present, surprisingly, expanded UCMSCs did not show any secretion of VEGF in the undifferentiated stage, until it was stimulated. In response to activation by TNF $\alpha$ , UCMSCs released VEGF which was significantly higher as compared to the pre activated

stage (Figure 2B). A point worth noting was that in case of BMMSCs, the VEGF was only marginally increased, and in sharp contrast, HGF secretion in fact reduced after the TNF $\alpha$  stimulation, in comparison to the unstimulated stage (Figure 2C) (Table 2).

### In vivo limb perfusion, functional improvement, and assessment of ischemic damage reduction, after cell transplantation

Table 3 brings out the fact that animals receiving MSCs from both the sources benefited as evident by prevention of limb loss from ischemic damage. However, the death of two mice in the UCMSCs group was perhaps due to the age of the mice. The animals died only at the end of the three months of the study, with the salvaged limb that was intact.

Functional assessment of the ischemic limb showed that the majority of the UCMSC transplanted animals had a score of 3. Ischemic damage assessment also showed a score of 0 to 1 in the UCMSC transplanted ILD mice (Figure 3).

## F - Limb function assessment score

A semi-quantitative functional assessment of the ischemic limb was performed by a blinded observer using a modification of a clinical score such as; 4- toe flexion, 3 -foot flexion, 2- no dragging but no plantar flexion, 1-foot dragging, 0 no movement.

## I - Ischemic damage score

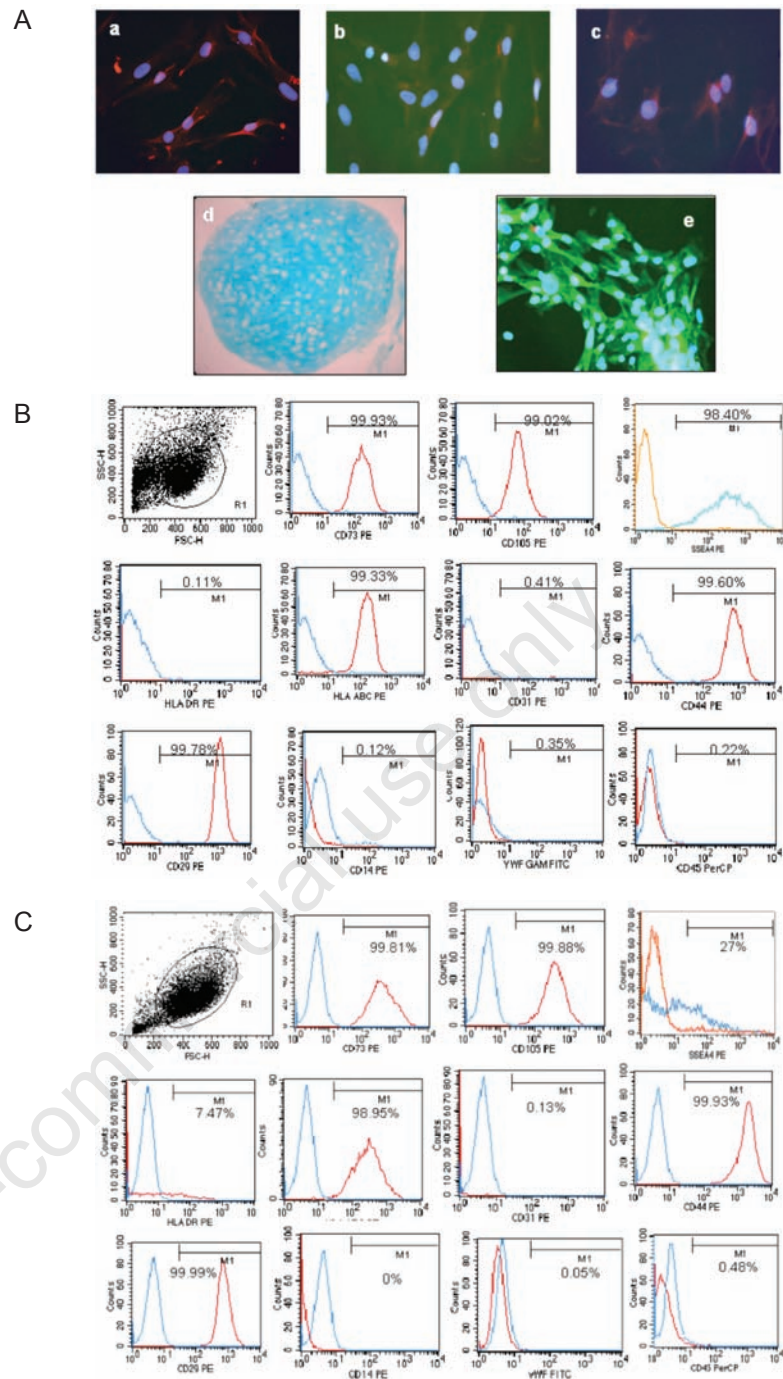
I - Ischemic damage score is 0- as compared to the normal, 1-mild diffuse or generalized necrosis, 2-moderate/severe necrosis, 3-necrosis, 4-amputation. The transition from 1 to 0 indicates that the degeneration has been arrested. The regeneration process has started earlier in UCMSC injected animals as compared to BMSCs. The functional assessment showed highest function at the control stage (normal mice) which later reduced to a score of 0 when the model (ILD mice) was dragging his feet and was total functionless (Table 4).

## Histological analysis of the umbilical cord derived mesenchymal stem cells transplanted ischemic limb disease mice

Sections from various parts of the muscles from both treated and untreated mice were checked for muscle architecture and histological changes. Sections from the MSC transplanted animals showed retention of good muscular architecture. Gross examination of the dead muscles in untreated mice were brown, necrotic and shrunken, under microscopic examination, confirmed a total loss of the muscle fibers (Figure 4). H and E sections showed uneven extensive areas of tissue loss and hyaline changes. The nuclei of the fascicles showed degeneration to varying degrees. The entire muscle section visualized was necrotic, its smooth muscle fibers having lost their close approximation and appearing as individual units whereas, the calf muscle sections of the UCMSC treated mice, showed regenerative fascicular clusters with new blood vessel sprouts in the form of small endothelial channels, with lesser fibrosis as compared to the controls. Similarly, BMSCs also showed similar findings, albeit improvement not as much as in the UCMSC groups (Figure 4D and 4F). Immunohistochemical staining of the sections showed the Dil labeled MSCs dispersed at different locations of the muscle bundles, traceable from the point of injection. There was evidence of migration all through out the muscle sections, but there was no remarkable pattern of movement and homing in. Human specific VEGF was also detected uniformly over the muscle section (Figure 4E and 4G).

## Umbilical cord derived mesenchymal stem cells was non tumorigenic in SCID mice

*In vivo* tumorigenic potential was deter-



**Figure 1. Differentiation potential of the umbilical cord derived mesenchymal stem cells (UCMSCs) to various lineages:** A) UCMSCs were differentiated to the mesodermal lineage such as the osteocytes and chondrocytes and the differentiated cells were characterized by immunofluorescence and alcian blue staining respectively. The osteocytes derived from the UCMSCs were positive for the markers such as the Osteopontin, Osteonectin and Osteocalcin as shown by immunofluorescence (a, b, c). The chondrocytes derived from the UCMSCs stained blue due to the presence of proteoglycans by alcian blue staining (d). The ectodermal lineage differentiation was demonstrated by immunofluorescence by using the antibody specific for Neurofilament (e). Comparative phenotypic characterization of mesenchymal stem cells (MSCs) by flow cytometry: Immunophenotype of UCMSCs have been studied and over 90% of these cells exhibited a MSC phenotype of CD73+/CD105+/CD29+/CD44+/SSEA4+/HLA-ABC+/HLA-DR-/CD45-/CD14-/CD31-/vWF-. The figure shows the representative mesenchymal and stem cell markers studied for the MSCs derived from human umbilical cord B) and bone marrow C) by flow cytometry. The expanded UCMSCs were CD45-/CD73+/CD105+/SSEA4+. Phenotypically UCMSCs were similar to the bone marrow derived MSCs (BMSCs). SSEA4 which is a pluripotent stem cell marker was found to be highly expressed by the UCMSCs as compared to the BMSCs.

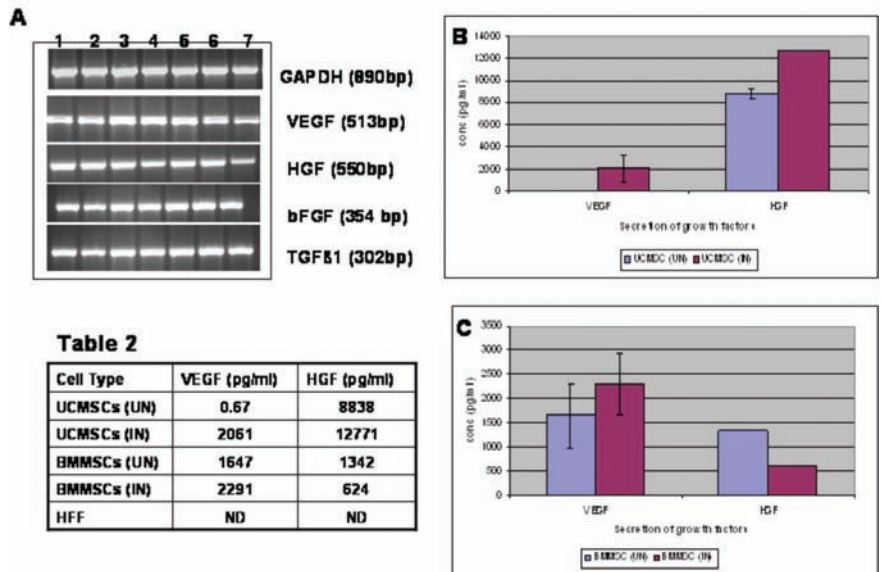
mined by injecting UCMSCs into SCID mice, and no tumors were detected in any of these animals even at 3 months. Similarly, BMMSCs also did not produce tumors in these immunocompromised animals. However, tumor formation was observed when Ntera-2D tumor cells were injected into SCID mice (*data not shown*).

## Discussion

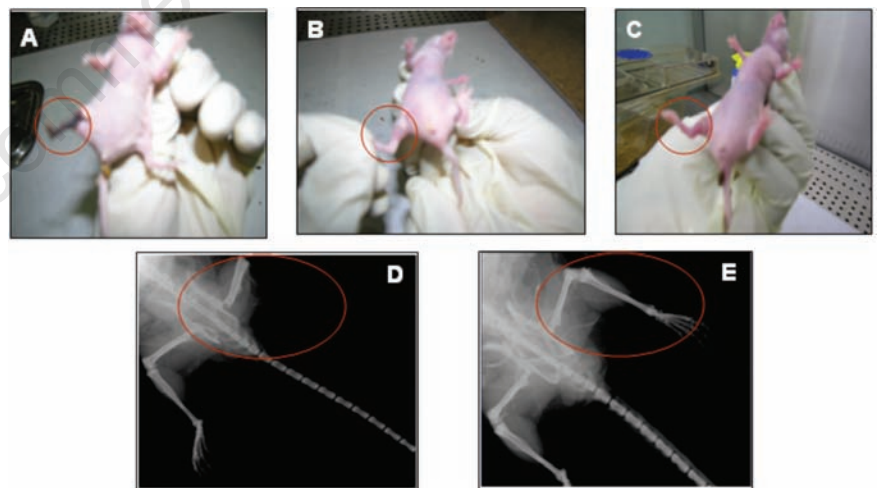
ILD is an intractable condition associated with high levels of amputation, leading to low quality of life and increased morbidity and mortality. It is often not treatable by standard therapeutic modalities. Neovascularization has been proposed as an alternate therapeutic strategy. VEGF and cytokine FGF-1 has been shown to elicit neovascularization. Early evidence has shown good therapeutic benefit with the administration of recombinant basic FGF. A variety of studies have confirmed the pro-angiogenic as well as independent muscle reparative properties of VEGF in ILD.<sup>12</sup> Cao *et al.*<sup>20</sup> demonstrated synergy between administration of PDGF-BB and FGF-2 in terms of increasing blood vessel formation and functional improvement in the femoral artery ligation model in rats and rabbits. Angiogenesis thus achieved has been described as a biological bypass, the idea being that through administration of agents capable of inducing collateralization, a more natural type of *bypass* can be achieved.

Cellular therapy for ILD is based on the rationale that delivery of endothelial progenitor cells into the areas of ischemia may result not only in differentiation of the cells into endothelium and thus contribute to angiogenesis through paracrine interactions with adjacent cells. Studies have shown MSC transplantation enhances neovascularization by supplying EPCs and angiogenic factors. To examine the potential role of MSCs in paracrine mechanisms in augmenting collateral remodeling we demonstrated the secretion of large amounts of angiogenic factors by the MSCs *in vitro*.

Asahara *et al.*<sup>21</sup> and Prockop *et al.*<sup>22</sup> showed that MSCs secrete many angiogenic cytokines, while Iba *et al.*<sup>23</sup> reported significant collateral vessel formation in the animal models with limb ischemia following injection of peripheral blood mononuclear cells. The favourable effect of autologous marrow stromal cells on neovascularization and blood flow in rat models was reported by Al-Khaldi *et al.*<sup>24</sup> A clinical trial report in four patients with limb ischemia using endothelial progenitor cells was reported by Yamamoto *et al.*<sup>25</sup> Tateishi *et al.*<sup>26</sup> compared the efficacy of bone marrow MNCs with the peripheral MNCs and demonstrated that stem cell injection for limb ischemia has shown sig-



**Figure 2.** *In vitro* angiogenic characteristics of mesenchymal stem cells (MSCs): RT-PCR analysis of the genes involved in angiogenesis and wound healing. (A) Three different lines of umbilical cord derived mesenchymal stem cells (UCMSCs) (Lanes 1,2,3) and two bone marrow derived MSCs (BMMSCs) (Lanes 4,5) derived from different donors along with the dermal fibroblasts (Lane 6) and HFF (Lane 7) were analyzed for genes such as VEGF, HGF, TGF 1 and bFGF. UCMSCs showed a good expression of all the genes which were analyzed. BMMSCs and dermal fibroblasts also were found to be positive for all the above genes. Analysis of VEGF and HGF secretion by the MSCs post induction with TNF $\alpha$ : UCMSCs and BMMSCs were induced with TNF and secretion of VEGF and HGF were checked by ELISA. The growth factors secreted by the uninduced (UN) and induced (IN) MSCs were compared. (B) The UN UCMSCs did not show any secretion of VEGF but upon induction a significant amount of VEGF was secreted which was almost equivalent to the BMMSCs. HGF secretion in the UCMSCs was also elevated post induction. (C) The IN BMMSCs did not show any significant difference in the secretion of both these factors as compared to the UN cells.



**Figure 3.** Transplantation of the mesenchymal stem cells into the hind limbs of the ischemic limb disease (ILD) mice model and contrast imaging of the transplanted limb. (A) HFF injected ILD mice showing necrosis of the ligated limb. The degenerated limb was then shed off within a week of transplantation of the HFF cells. (B) Umbilical cord derived mesenchymal stem cells (UCMSCs) were injected into the ligated limb of the ILD mice showed improvement of the limb within 48 hrs. The necrosis seen due to the ligation of the femoral artery was improved by the UCMSC injection. (C) Bone marrow derived MSCs (BMMSCs) treated ILD mice also showed improvement of the ligated limb post transplantation of the cells within a week. Skiagram of the hind limbs of the Nude Mice after the injection of Iodine Contrast Agent (Urographin<sup>TM</sup>) viewed in Kodak In-Vivo Image Analyzer (35kVp). ILD Model- Control (D) Control animal is showing degeneration of the femoral artery. The leg from the stifle joint has sloughed off due to stoppage of blood circulation (E) UCMSC injected into the ILD animal model is showing regeneration of the collateral blood circulation 12 weeks after MSC injection in which the femoral artery was permanently ligated.

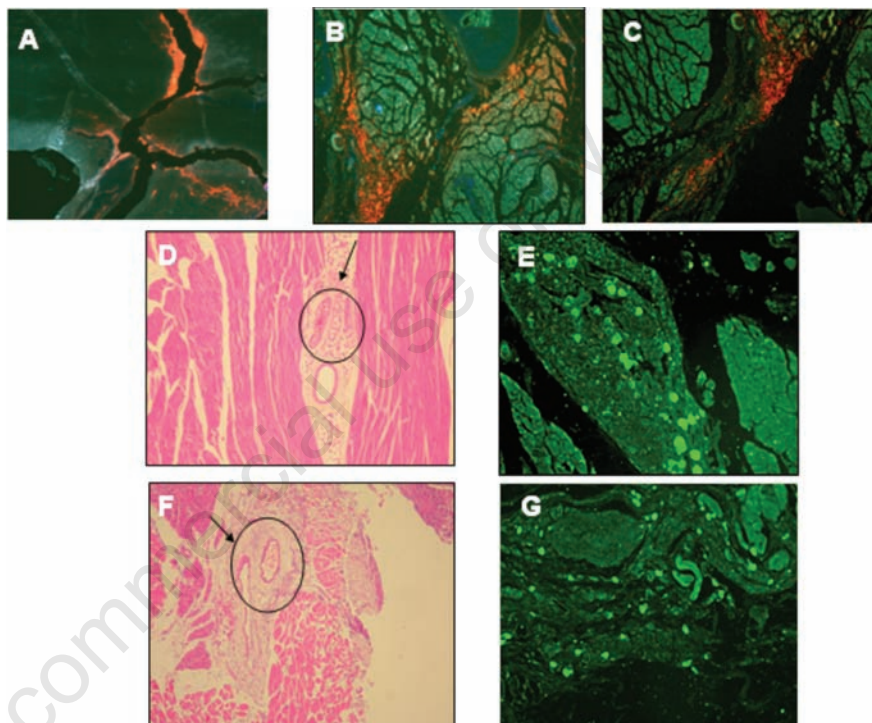
nificant improvement in patients treated with bone marrow MNC.

The use of autologous stem cells for treatment of patients has several advantages; however, it involves issues such as collection, patients age, isolation, preparation, and the consequent variations in quality and quantity. To overcome these inconsistencies, several groups including ours have investigated the use of an alternative source for deriving MSCs that overcomes all the above shortcomings and offer superior qualities to qualify its use in the allogeneic mode.<sup>4</sup> Mesenchymal stem cells derived from the umbilical cord has distinct advantages because of its good growth kinetics, differentiation potentials, banking ability, naivety and immunomodulatory functions, as compared to most other adult stem cell types. UCMSC being a unique cell type with its advantages, its transplantation may thus be a new therapeutic strategy for the treatment of peripheral vascular diseases.

In the present study, we demonstrate that MSCs secrete several important arteriogenic cytokines where they orchestrate processes that ultimately lead to collateral vessel growth. This is a comparative study between MSC derived from umbilical cord and bone marrow for their role in the treatment of peripheral vascular diseases. A higher expression of mesenchymal as well as pluripotent stem cell marker such as SSEA4 was seen in the UCMSCs demonstrating its naivety and primitiveness in comparison to the BMMSCs. In addition, these cells also expressed mRNAs that encode for the genes related to endothelial cell lineages. *In vitro* functional studies also showed UCMSCs secreting elevated levels of angiogenic factors such as vascular endothelial growth factor (VEGF) and hepatocyte growth factor (HGF). VEGF is a positive regulator and plays a crucial role in angiogenesis.<sup>27</sup> HGF secreted by the mesenchymal cells acts as a multi-functional cytokine and plays a central role in angiogenesis and tissue regeneration.<sup>28</sup> A marked difference was observed in the cytokine expression pattern between the two types of MSCs. In the uninduced state, the UCMSCs did not express any VEGF but showed higher levels of HGF while BMMSCs secreted both VEGF and HGF in the uninduced state. UCMSCs upon stimulation with  $TNF\alpha$ , the expression of VEGF increased significantly along with the HGF levels whereas reduced level of the cytokines was observed in the BMMSCs post stimulation. Many cytokines are elevated during conditions of ischemia or endotoxemia and stimulating stem cells with TNF or LPS creates a harsh condition in the *in vitro* conditions.<sup>29</sup> This suggests that the UCMSCs being from a neonatal source plays a significant role in mobilizing cytokines and stimulating angiogenesis during ischemia

Efficacy studies with both the types of MSCs were performed by injecting equal numbers into the abductor muscles of the ischemic hind limb. MSCs significantly enhanced perfusion of ischemic tissue and collateral remodeling, lessened tissue damage and improved limb function. Our results with the present study using multiple injections of MSCs around the ligated hind legs of nude mice is the first comprehensive comparative study ever done to prove the degree of efficacy of the two MSC types under consideration. The UCMSC transplantation induced angiogenesis and limb sal-

vage in a mouse model with hind limb ischemia. The extent of improvement in the ischemic limb and the prevention of limb loss were significantly greater than the BMMSCs. Even though equal number of BMMSCs and UCMSCs were injected into the ischemic hind limb, it was observed that the therapeutic angiogenesis was significant in the UCMSCs injected animals. The functional grading of the ischemic limb showed a higher functional score than that of the animals treated with UCMSCs. Animals in the BMMSCs treated group showed a slight necrosis at the tip of lig-



**Figure 4.** Histological analysis of the muscle section of the ischemic limb disease (ILD) mice. A) DiI labeled HFF injected into the limb of ILD mice. The mice injected with the cells lost the limb in a week. The muscles show total disintegration of the muscle bundles. B) DiI labeled umbilical cord derived mesenchymal stem cells (UCMSCs) injected into the ILD mice restored the limb of the ILD mice which was ligated. Muscle section of the UCMSCs injected limb shows good muscle integration which was similar to the normal muscle. C) DiI labeled bone marrow derived MSCs (BMMSCs) injected into the ILD mice also restored the limb of the ILD mice. The muscle section of the BMMSCs injected limb shows integrity of the muscle bundles. D) H&E section of the muscle injected with UCMSCs shows moderate atrophy of the fascicles, mild Zenker's degeneration with mild new blood vessel sprouts. Moderate to mild regenerative fascicular clusters was also observed in the UCMSCs. E) Muscle section injected with UCMSCs showing the transplanted cells expressing VEGF. F) H&E section of the muscle injected with BMMSCs shows regenerative fascicular clusters fascicular necrosis and mild new vessel sprouts. G) Muscle section injected with BMMSCs showing the transplanted cells expressing VEGF.

**Table 4.** Functional and ischemic assessment of transplanted limb.

Groups	1 week	2 weeks	3 weeks	4 weeks
Control	F0/14	F0/14	F0/14	F0/14
BMMSCs	F2/12	F2/11	F2/11	F2/11
UCMSCs	F2/11	F2/10	F2/10	F2/10

BMMSCs, bone marrow derived mesenchymal stem cells; UCMSCs, umbilical cord derived mesenchymal stem cells.

ated limb, and a bit higher ischemic score and a lower functional score. The improvement in the functional score was further supported by the contrast radiography which showed blood vessels of the ligated limb similar to the normal limb. Three months follow up showed no reversal of symptoms or any evidence of new fresh ischemia, and necrosis.

Histopathological analysis of the muscle sections was very encouraging showing well maintained muscle architecture, new blood vessel sprouts and regenerative fascicular clusters observed in both the groups of animals. The fascicular necrosis in the UCMSCs category was mild to moderate as compared to the BMMSCs injected animals. The detection of the DiI labeled cells expressing VEGF at the injected site post 3 months indicated the homing in and functionality of the MSCs which was similar to the observations made by Kim *et al.*<sup>13</sup> using umbilical cord blood derived MSCs. Wu *et al.*<sup>30</sup> based on its angiogenic capabilities and its ability to prevent limb in ischemic mice; however our study emphasises on both the *in vitro* and *in vivo* angiogenic potential using UCMSCs in comparison to the BMMSCs.

Based on our results, We can thus conclude i) UCMSCs produce a wide array of arteriogenic cytokines, ii) direct injection of UCMSCs into ischemic area promotes blood vessel formation, collaterals improves perfusion and remodeling, lessens tissue damage, and enhance limb function as observed in the mouse model of hind limb ischemia. The extent of improvement in the ischemic limb and the therapeutic angiogenesis was significant in the UCMSCs injected animals. This can be attributed to the increased secretion of VEGF and HGF post induction by the UCMSC as compared to the BMMSCs.

To the best of our knowledge, this is the first time, a comprehensive comparative study between MSC derived from umbilical cord and bone marrow in preclinical models of ILD is being reported. We chose to adhere to one dose and one volume in the animals, there could be scope for trying intra arterial route along with this, or extend up this to accelerate revascularization which will need, further set of experiments.

Based on our results and published data on use of other cell types, UCMSCs seems to be a more attractive source to plan clinical experiments. These could be extended to use as a vehicle for gene therapy, and for other unmet medical needs for such degenerative and life style disorders. However, we need to continue to conduct preclinical experiments with long term follow up. Thus, considering the immunological implications in terms of long term efficacy studies we need to arrive at the most optimum clinical protocol to initiate clinical trials.

## References

- Baksh D, Song L, Tuan RS, et al. Adult mesenchymal stem cells: characterization, differentiation and, application in cell and gene therapy. *J Cell Mol Med* 2004;8:301-16.
- Sarugaser R, Lickorish D, Baksh D, et al. Human umbilical perivascular cells (HUCPV) cells: A source of mesenchymal progenitors. *Stem Cells* 2005;23:220-9.
- Wang HS, Hung SC, Peng ST, et al. Mesenchymal stem cells in the Wharton's jelly of human umbilical cord. *Stem Cells* 2004;22:1330-7.
- Tipnis S, Viswanathan C, SenMajumdar A. Immunosuppressive properties of human umbilical cord derived mesenchymal stem cells Role of B7-H1 and IDO. *Immunol Cell Biol* 2010;88:795-806.
- Petersen BE, Goff JP, Greenberger JS, et al. Hepatic oval cells express the hematopoietic stem cell marker Thy-1 in the rat. *Hepatology* 1998;27:433-45.
- Poulsom R, Alison MR, Cook T, et al. Bone marrow stem cells contribute to healing of the kidney. *J Am Soc Nephrol* 2003;14:48-54.
- Orlic D, Kajstura J, Chimenti S, et al. Mobilized bone marrow cells repair the infarcted heart, improving function and survival. *Proc Natl Acad Sci U S A* 2001;98:10344-9.
- Mezey E, Chandross KJ, Harta G, et al. Turning blood into brain: cells bearing neuronal antigens generated *in vivo* from bone marrow. *Science* 2000;290:1779-82.
- Weiss ML, Medicetty S, Bledsoe AR, et al. Human umbilical cord matrix stem cells: Preliminary characterization and effect of transplantation in a rodent model of Parkinson's disease. *Stem Cells* 2006;24:781-92.
- Shetty P, Ravindran G, Sarang S, et al. Clinical grade mesenchymal stem cells transdifferentiated under xenofree conditions alleviates motor deficiency in rat model of Parkinson's disease. *Cell Biol Int* 2009;33:830-8.
- Davani S, Marandin A, Mersin N, et al. Mesenchymal stem cell progenitor cells differentiate into endothelial phenotype, enhance vascular density and improve heart function in rat cellular cardiomyoplasty model. *Circulation* 2003;108:253-8.
- Kinnaird T, Stabile E, Burnett MS, et al. Local delivery of the marrow derived stromal cells augments collateral perfusion through paracrine mechanisms. *Circulation* 2004;109:1543-9.
- Kim SW, Hoon H, Chae GT, et al. Successful stem cell therapy using umbilical cord blood derived Multipotent stem cells for beurger's disease and ischemic limb disease animal model. *Stem Cells* 2006;24:1620-6.
- Shetty P, Cooper K, Viswanathan C. Comparison of proliferative and multilineage differentiation potentials of cord matrix cord blood and bone marrow derived mesenchymal stem cells. *Asian J Transf Sci* 2010;4:14-24.
- Shetty P, Bharucha K and Tanavde V. Human umbilical cord blood serum can replace fetal bovine serum in the culture of mesenchymal stem cells. *Cell Biol Int* 2007;31:293-8.
- Wang M, Zhang W, Crisostomo P, et al. STAT3 mediates bone marrow mesenchymal stem cell VEGF production. *J mol Cell Cardiol* 2007;42:1009-15.
- Shao R, Xia W, Hung M. Inhibition of angiogenesis and induction of apoptosis are involved in E1A bystander effect and tumor suppression. *Cancer Res* 2000;60:3123-26.
- Iwase T, Nagaya N, Fujii T, et al. Comparison of angiogenic potency between mesenchymal stem cells and mononuclear cells in a rat model of hind limb ischemia. *Cardiovasc Res* 2005;66:543-51.
- Norgren L, Hiatt WR, Dormandy JA, et al. Inter-Society Consensus for the management of peripheral vascular disease (TASC II). *Eur J Vasc Endovasc Surg* 2007;33:S1-S75.
- Cao R, Brakenhielm E, Pawliuk R, et al. Angiogenic synergism, vascular stability and improvement of hind-limb ischemia by a combination of PDGF-BB and FGF-2. *Nat Med* 2003;9:604-13.
- Asahara T, Masuda H, Takahashi T, et al. Bone marrow origin of endothelial progenitor cells responsible for postnatal vasculogenesis in physiological and pathological neovascularization. *Circ Res* 1999;85:221-28.
- Prockop DJ. Marrow stromal cells as stem cells for non haematopoietic tissues. *Science* 1997;276:71-4.
- Iba O, Matsubara H, Nozawa Y. Angiogenesis by implantation of peripheral mononuclear cells and platelets into ischemic limbs. *Circulation* 2002;106:2019-25.
- Al-Khaldi A, Al-Sabti H, Galipeau J, et al. Therapeutic angiogenesis using autologous bone marrow stromal cells: Improved blood flow in Chronic limb ischemia model. *Ann Thorac Surg* 2003;75:204-9.
- Yamamoto K, Kondo T, Suzuki S, et al. Molecular evaluation of endothelial progenitor cells in patients with ischemic limbs – therapeutic effects by stem cell transplantation. *Arterioscler Thromb Vasc Biol* 2004;24:192-6.
- Tateishi-Yuyama E, Matsubara H, Muro-

- hara T, et al. Therapeutic angiogenesis for patients with limb ischemia by autologous transplantation of bone marrow cells: a pilot study and a randomised controlled trial. *Lancet* 2002;360:427-35.
27. Kagiwada H, Yashiki T, Ohshima A, et al. Human mesenchymal stem cells as a stable source of VEGF producing cells. *J Tissue Eng Regen Med* 2008;4:184-90.
28. Werner S, Grose R. Regulation of wound healing by growth factors and cytokines. *Physiol Rev* 2003;83:835-70.
29. Markel TA, Wang M, Crisostomo PR, et al. Neonatal stem cells exhibit specific characteristics in function, proliferation and cellular signaling that distinguishes them from their adult counterparts. *Am J Physiol Regul Intergr Comp Physiol* 2008;294: 1491-7.
30. Wu KH, Zhou B, Mo XM, et al. Therapeutic potential of umbilical cord derived stem cells in Ischemic diseases. *Transplant Proc* 2007;39:1620-2.

Non-commercial use only