STEM CELLS®

TRANSLATIONAL AND CLINICAL RESEARCH

Improved Autograft Survival of Mesenchymal Stromal Cells by Plasminogen Activator Inhibitor 1 Inhibition

IAN B. COPLAND,[a] SIMON LORD-DUFOUR,[b] JESSICA CUERQUIS,[a] DANIEL L. COUTU,[a] BORHANE ANNABI,[b] EUGENEA WANG,[c] JACQUES GALIPEAU[a]

[a]Sir Mortimer B. Davis Jewish General Hospital, McGill University, Montreal, Quebec, Canada; [b]Laboratoire d'Oncologie Moléculaire, Département de Chimie, Centre BIOMED, Université du Québec à Montréal, Montreal, Quebec, Canada; [c]Gheens Center on Aging and Department of Biochemistry and Molecular Biology, University of Louisville School of Medicine, Louisville, Kentucky, USA

Key Words. Plasminogen activator inhibitor 1 • Bone marrow stromal cells • Hypoxia • Ischemia • Cell transplantation

ABSTRACT

Mesenchymal stromal cells (MSCs) display robust reparative properties through their ability to limit apoptosis, enhance angiogenesis, and direct positive tissue remodeling. However, low in vivo survival of transplanted cells limits their overall effectiveness and significantly affects their clinical usage. Consequently, identifying strategies to improve cell survival in vivo are a priority. One explanation for their low survival is that MSCs are often transplanted into ischemic tissue, such as infarcted myocardium, where there is poor blood supply and low oxygen tension. Therefore, we examined how MSCs respond to a hypoxic, nutrient-poor stress environment to identify trophic factors that could be manipulated in advance of MSC transplantation. Combining microarray and proteomic screens we identified plasminogen activator inhibitor 1 (PAI-1) as one factor consistently upregulated in our in vitro ischemia-mimicking conditions. Subsequent genetic and chemical manipulation studies define PAI-1 as a negative regulator of MSC survival in vivo. Mechanistically, MSC-derived PAI-1 does not alter MSC survival through a plasmin-dependent mechanism but rather directly impacts on the adhesiveness of MSCs to their surrounding matrices. Thus we can conclude that post-transplantation, PAI-1 negatively impacts MSC survival by promoting anoikis via matrix detachment. STEM CELLS *2009;27:467–477*

Disclosure of potential conflicts of interest is found at the end of this article.

INTRODUCTION

Conceptually, cellular therapy has evolved from the initial premise that exogenous progenitor or stem cells regenerate injured tissue to a broader hypothesis that cell therapy facilitates complementary aspects of tissue repair [1]. Such complementary aspects might include augmentation of cell survival by paracrine release of antiapoptotic factors, improved tissue oxygenation by neoangiogenesis, and enhanced recovery of cellular and tissue function by positive remodeling. In numerous animal models, bone marrow-derived mesenchymal stromal cells (MSCs) have shown promise in treating ischemic disorders [2–5]. Indeed, MSCs have intrinsic features that identify them as an ideal cell type for cellular therapy. MSCs possess robust angiogenic and immunomodulatory properties, are a natural constituent of the host-derived tissue ischemia response, can be obtained in relatively large numbers through standard clinical procedures, and are easily expandable in culture [6]. At present, cell therapies are routinely administered via injections, either systemically or directly into tissues of interest [7, 8], and are generally considered safe. However, despite many cell transplantation studies showing beneficial effects, most studies report low levels of cell persistence. In particular, for those therapies where cells are injected directly into the tissue of interest, massive death of transplanted cells has been observed [7, 8]. This limits their overall effectiveness and significantly impacts their clinical use [9].

One explanation for the low survival rates of transplanted cells is that cells are often injected into a pre-existing inhospitable ischemic environment. Once transplanted, cells are exposed to a hypoxic, nutrient-poor environment and are often bombarded by a cytokine storm from existing or evolving inflammatory reactions. Two complementary approaches that could dramatically influence the survival of cells post-transplantation are manipulation of the cells prior to transplantation and/or manipulation of the transplantation site prior to cell delivery. In vivo manipulation of the transplantation site prior to delivery offers a complex series of hurdles and may not provide any clear benefit. As a result, strategies aiming to improve cell survival in vivo generally rely on ex vivo chemical, physical, or genetic manipulation of the cells prior to delivery [7]. Hypoxic preconditioning of MSCs increases their survival and angiogenic potential in vivo [10–12]. However, adding a nutrient-

Author contributions: I.B.C.: conception and design, collection and assembly of data, data analysis and interpretation, manuscript writing; S.L.-D., J.C., and D.L.-C.: collection and assembly of data; B.A. and E.W.: data analysis and interpretation, manuscript writing; J.G.: conception and design, manuscript writing, final approval of manuscript.

Correspondence: Jacques Galipeau, M.D., Sir Mortimer B. Davis Jewish General Hospital (McGill University), 3755 Cote Ste-Catherine Road, Montreal, Quebec H3T 1E2, Canada. Telephone: 514-340-8214; Fax: 514-340-8281; e-mail: jacques.galipeau@mcgill.ca Received May 27, 2008; accepted for publication November 10, 2008; first published online in STEM CELLS *EXPRESS* November 20, 2008. 1066-5099/2009/$30.00/0 doi: 10.1634/stemcells.2008-0520

poor component on top of this environment results in massive cell death [13]. As such, a preconditioning regime involving a hypoxia nutrient-poor environment is not feasible for MSC therapies. Therefore, we examined how MSCs responded to a hypoxic, nutrient-poor stress environment to identify mediators that could be manipulated in advance of MSC transplantation. In particular, since MSCs can regulate tissue regeneration via the secretion of trophic factors [14], we focused on how the secretome of MSCs was altered. Surprisingly, plasminogen activator inhibitor 1 (PAI-1) identified itself as a key regulator of MSC autograft survival in vivo, and this effect was mediated through PAI-1's ability to regulated MSC adherence.

MATERIALS AND METHODS

Condensed Materials and Methods are contained herein. Additional information regarding materials and methods can be found in the supporting information.

All animal experiments conducted at the Lady Davis Institute for Medical Research (Jewish General Hospital) were conducted with approval of the Lady Davis Institute for Medical Research Standing Committee on Animals. All animal protocols conformed to the Guide for the Care and Use of Laboratory Animals published by the NIH (NIH Publication No. 85-23, revised 1996).

In Vitro Characterization of Wild-Type and PAI-Null MSCs

Wild-type (WT) and PAI-null MSC isolation and culture was performed as previously described [15]. Briefly, female C57Bl/6 (Charles River Laboratories, St. Constant, QC, Canada, http://www.criver.com) or PAI-null mice (Jackson Immunoresearch Laboratories, West Grove, PA, http://www.jacksonimmuno.com) weighing approximately 15–20 g were euthanized by CO_2 asphyxiation. Whole bone marrow was harvested in cell culture dishes by flushing resected femurs and tibias with complete culture media (Dulbecco's modified Eagle's medium [DMEM] supplemented with 10% fetal bovine serum (FBS) (unselected lots) and 48 μg/ml Gentamicin [Sandoz Canada Inc., Boucherville, QC, Canada, http://www.sandoz.ca]) and placed at 37°C with 5% CO_2. Nonadherent hematopoietic cells were discarded 5 days later, and adherent stromal cells maintained in culture for seven passages to generate a polyclonal MSC population. MSCs were immunophenotyped by flow cytometric analysis at passage 8 and were differentiated, for 3 weeks, into adipocytes (complete culture media supplemented with 0.5 mM dexamethasone, 0.5 μM isobutylmethylxanthine, and 50 μM indomethacin), osteoblasts (complete culture media supplemented with 1 nM dexamethasone, 20 mM β-glycerol phosphate, and 50 μM L-ascorbic acid-2-phosphate) and chondrocytes (inverted-drop culture with serum-free culture media supplemented with 10 nM dexamethasone, 50 μM L-ascorbic acid-2-phosphate, and 5 ng/ml TGF-β1) [16, 17] (details in supporting information). MSCs were maintained and expanded in culture for no longer than 2 months.

In Vitro Characterization of Human MSCs

Adult human MSCs were collected from volunteer donors and isolated by Ficoll separation. Mononuclear cells were plated in cell culture dishes with complete culture medium (α-minimal essential medium supplemented with 16.5% unselected FBS, 2 mM L-glutamine, and 48 μg/ml Gentamicin [Sandoz Canada Inc.]) and placed at 37°C with 5% CO_2. Nonadherent hematopoietic cells were discarded 5 days later, and adherent stromal cells maintained in culture for up to 10 passages. Human MSCs were immunophenotyped by flow cytometric analysis at passage 4 and were differentiated, for 3 weeks, into adipocytes (complete culture medium supplemented with 0.5 mM dexamethasone, 0.5 μM isobutylmethylxanthine, and 50 μM indomethacin), osteoblasts (complete culture medium supplemented with 1 nM dexamethasone, 20 mM β-glycerol phosphate, and 50 μM L-ascorbic acid-2-phosphate), and chondrocytes (inverted-drop culture with serum-free culture media supplemented with 10 nM dexamethasone, 50 μM L-ascorbic acid-2-phosphate, and 5 ng/ml TGF-β1) [16, 17] (details in supporting information). Human MSCs were maintained and expanded in culture for no longer than 2 months.

Microarrays

Total cellular RNA isolation, reverse transcription, labeling and hybridization, and analysis were performed as previously described [18] (details in supporting information). Three separate array experiments were performed for each experimental condition (i.e., hypoxia vs. normoxia). The gene expression database was subjected to a variety of analysis tools, including scatterplot clustering, Significance Analysis of Microarrays (SAM), and gene ontology comparison (GoStat, http://gostat.wehi.edu.au/). On the basis of SAM analysis [19], a twofold change in gene expression between the normoxia and hypoxia experimental conditions was considered significant only when the false-positive discovery rate was set for $<1\%$.

Proteomics

Conditioned media from normoxia and hypoxia serum-deprived MSCs were collected and concentrated on 5-kDa exclusion spin columns (Millipore, Billerica, MA, http://www.millipore.com). iTRAQ labeling, tryptic digestion, and peptide chromatography were performed as previously described [20]. Isolated fractions were processed for identification and quantification by the QSTAR1 XL hybrid liquid chromatography (LC) tandem mass spectrometry (MS/MS) system (Applied Biosystems, Foster City, CA, http://www.appliedbiosystems.com) interfaced with an 1100 Series Capillary LC system (Agilent Technologies, Palo Alto, CA, http://www.agilent.com). Mass spectrometry (MS) time of flight scans were acquired from m/z 350 to 1,600, with up to two precursors selected for MS/MS from m/z 60 to 2,000 using information-dependent acquisition; rolling collision energy was used to promote fragmentation. Nanospray MS and MS/MS data from the QSTAR System were analyzed using ProQUANT 1.0 software (Applied Biosystems Inc.) for both identification and quantification. N terminus, lysine, tyrosine, and cysteine modifications were selected as fixed, methionine oxidation was selected as variable, one missed cleavage was allowed, precursor error tolerance was at <0.15 Da, and product ion error tolerance was at <0.1 Da.

Western Blotting

Proteins were then subjected to SDS-polyacrylamide gel electrophoresis (PAGE) using Tris-glycine gradient (4%–20%) gels (Invitrogen, Burlington, ON, Canada, http://www.invitrogen.com) and electroblotted to polyvinylidene difluoride membranes (Millipore). All incubations and washes were done at room temperature with constant agitation. Membranes were blocked in 5% (wt/vol) nonfat milk in Tris-buffered saline containing 0.1% (vol/vol) Tween 20 (TBST). Rabbit anti-mouse PAI-1, uPA, tPA, and uPAR polyclonal affinity purified antibodies (Santa Cruz Biotechnology Inc., Santa Cruz, CA, http://www.scbt.com) diluted in blocking solution 1:500 were added to the membranes for 2 hours. The membranes were washed in TBST and incubated for 1 hour with horseradish peroxidase-conjugated goat anti-rabbit IgG (R&D Systems Inc., Minneapolis, http://www.rndsystems.com) diluted 1:10,000 in blocking solution. Proteins were detected using an enhanced chemiluminescence kit (Amersham Biosciences, Buckinghamshire, United Kingdom, http://www.amersham.com).

Plasmin-Activity Assay

Plasmin activity in culture medium from MSC cultures was detected using an ANSN-based fluorogenic substrate (SN-5; Hematologic Technologies Inc., Essex Junction, VT, http://www.haemtech.com) [21]. In brief, 100 μl of culture medium was mixed with 10 μM SN-5 in a 96-well plate for 1 hour at 37°C. The relative change in fluorescence was assessed at 470 nm emission wavelength with a 352 nm excitation using a fluorescent plate reader. All experiments were performed in triplicate with cells that had been serum-deprived

for at least 24 hours. In all experiments exogenous plasmin was used as a positive control.

Zymography

Gelatinolytic activity in culture medium from MSC cultures was detected by zymography as described previously [22]. Briefly, an aliquot (20 μl) of the culture medium was subjected to SDS-PAGE with a 7.5% (wt/vol) polyacrylamide gel containing 0.1 mg/ml gelatin. Gels were then washed twice for 30 minutes in 2.5% (vol/vol) Triton X-100 to remove SDS and then rinsed in distilled water. Gels were then incubated at 37°C for 20 hours in a 20 mM $NaCl_2$, 5 mM $CaCl_2$, 0.02% (vol/vol) Brij-35, 50 mM Tris/HCl buffer (pH 7.6). The gels were then stained with 0.1% Coomassie Brilliant Blue R-250, followed by destaining in 10% (vol/vol) acetic acid, 30% (vol/vol) methanol in water. Gelatinolytic activity was detected as unstained bands on a blue background. All experiments were performed with cells that had been serum-deprived for at least 24 hours.

Reverse Transcription-Polymerase Chain Reaction

Two micrograms of total RNA was reverse-transcribed in a total volume of 50 μl containing 5 μl of reverse transcription buffer, 11 μl of $MgCl_2$ (25 mM), 5 μl of dNTP (10 μM each), 2.5 μl of random hexamers (50 μM), 1.5 μl of MULV reverse transcriptase (1 IU/μl), and 0.5 μl (5 IU/ml) of RNase inhibitor. Samples were first incubated at 25°C for 10 minutes and then at 37°C for 70 minutes. Reactions were stopped by heating to 70°C for 5 minutes. Polymerase chain reaction (PCR) was performed on 50 ng of cDNA to determine PAI-1 expression, whereas 5 ng of cDNA was amplified for 18S. The thermocycler parameters were 95°C for 3 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 30 seconds, followed by an extension at 72°C for 30 seconds.

Matrigel Implantation of WT and PAI-Null MSCs

We have previously described the subcutaneous implantation of MSCs admixed in Matrigel (Becton, Dickinson and Company, Mississauga, Ontario, Canada, http://www.bd.com) to ascertain neovascularization in vivo [23]. Briefly, MSCs were mixed with chilled liquid Matrigel, syringe-loaded, and injected subcutaneously to syngeneic C57Bl/6 mice where they formed a semisolid plug. The plugs can be surgically retrieved at latter time points and subjected to histological analyses for survival based upon β-galactosidase and PKH26 staining. Vascularity was assed through immunostaining for von Willebrand factor (vWF; NeoMarkers, Fremont, CA, http://www.labvision.com). For each experimental condition, six Matrigel implants were analyzed.

Vessel Density Determination

Capillary endothelial cells were identified by immunohistochemical staining for vWF (NeoMarkers). Following blocking, primary (vWF; NeoMarkers) and secondary antibody incubations (Alexa 488 goat anti-rabbit IgG; Invitrogen), samples were viewed using a Leica DM6000B (Leica, Wetzlar, Germany, http://www.leica.com) microscope. No signal was observed when primary antibodies were omitted or when samples were incubated with isotype control antibodies. For each Matrigel plug, quantitative assessment of vascular density with fluorescent microscopy was performed using 10 randomly selected high-power fields (magnification, ×200). The number of vessels from each section was averaged and expressed as the number of vessels per high-power field (HPF).

LacZ Transduction of WT and PAI-Null MSCs

In gene marking studies, WT and PAI-null MSCs were stably transduced with a LacZ integrating retroviral vector as previously described [24]. Following LacZ transduction, >90% of WT and PAI-null MSCs expressed detectable β-galactosidase activity as assessed by 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) staining. LacZ+MSCs were expanded in vitro for implantation. Prior to implantation, >90% LacZ positivity was confirmed for both the WT and PAI-null MSC populations. Two weeks postimplantation, surviving LacZ+MSCs and their progeny could be identified on histologic sections by a characteristic indigo-blue color when incubated with X-Gal staining solution. For each Matrigel plug, quantitative assessment of X-Gal with light microscopy was performed using 10 randomly selected high-power fields (magnification, ×200). The number of X-Gal-positive cells from each section was averaged and expressed as the number of X-Gal-positive cells per HPF.

PKH26 Labeling of MSCs

Culture expanded MSCs were trypsinized, washed, and resuspended in Diluent C buffer for general membrane labeling (Sigma-Aldrich, Oakville, ON, Canada, http://www.sigmaaldrich.com). Cells were then mixed with a 2× stock of PKH26 labeling solution and incubated at room temperature for 5 minutes. The reaction was stopped and cells were washed prior to injection.

In Vitro Matrigel Survival Assay

Cells were dislodged after brief trypsinization and dispersed into homogeneous single-cell suspensions. Cells were washed extensively and resuspended in serum-free DMEM to a concentration of 1×10^6 cells per milliliter. MSCs (2×10^5) were then mixed with an equal volume of cold Matrigel, added to microtiter plate, and placed into a tissue culture incubator at 37°C with 5% CO_2. Apoptosis was assessed 2 and 6 days postplating. Live cells were retrieved from in vitro Matrigel implants by cutting the implants into small fragments and incubating these fragments in a solution of 1× phosphate-buffered saline (PBS) supplemented with 1.6 mg/ml type IV collagenase and 200 μg/ml DNase I (Sigma-Aldrich) for 30 minutes at 37°C. Cells were dissociated by repeated pipetting, incubated for an additional 20 minutes at 37°C, collected, and washed. Intact cells were then resuspended in staining buffer containing annexin-V antibody and propidium iodide (PI) for 15 minutes at room temperature in the dark. Cells were then washed and placed into flow cytometric buffer and analyzed. Controls included untreated cells stained with annexin-V and PI, as well as untreated cells stained solely with annexin-V or PI.

Adhesion Assay

Microtiter plates were coated with either Matrigel (1:5 dilution in PBS) or vitronectin (5 μg/ml) for 18 hours at 4°C. Plates were then washed with PBS to remove excess matrices and incubated for 1 hour at 37°C with PBS containing 1% bovine serum albumin to block nonspecific binding. MSCs (5×10^4) in serum-free DMEM were added to each well, spun at 400*g* for 2 minutes, and allowed to attach for 20 minutes at 37°C. After incubation, the microtiter plates were agitated twice for 30 seconds and gently washed with PBS. The remaining cells were fixed (4% PFA), stained (0.1% crystal violet), and washed in water. The stain was extracted from the cells with 2% SDS, and the amount of extracted stain was quantified by absorbance at 590 nm.

Detachment/Anoikis Assay

Twenty-four-well plates were coated with either Matrigel (1:5 dilution in PBS) or vitronectin (5 μg/ml) for 18 hours at 4°C. Plates were then washed with PBS to remove excess matrices. MSCs (1×10^5) in DMEM containing 0.5% albumin were added to each well, spun at 400*g* for 2 minutes, and allowed to attach for 20 minutes at 37°C. After incubation, the plates were gently washed to remove nonadherent cells, and fresh DMEM containing 0.5% albumin was added. Specific wells then received either no treatment or rPAI-1 (25 μg/ml). Cells were then returned to the incubator for 72 hours, after which the adherent and floating cells were counted separately to determine the percentage of detached cells.

Migration Assay

Cells were dislodged after brief trypsinization and dispersed into homogeneous single-cell suspensions. Cells were washed extensively and resuspended in serum-free DMEM to a concentration of 1×10^6 cells per milliliter. To assess invasion from established monolayers, MSCs (1×10^5) were dispersed onto Matrigel-coated chemotaxis filters (8-μm pore size; BD Biosciences, San Diego,

http://www.bdbiosciences.com) within Boyden chamber inserts and allowed to adhere for 1 hour at 37°C, after which they were challenged by the addition of 600 μl of chemoattractant solution to the lower compartments composed of either DMEM alone or DMEM containing 10% FBS. Invasion was allowed to proceed for 16 hours at 37°C in 5% CO_2. Cells remaining attached to the upper surfaces of the filters were carefully removed with cotton swabs. Cells that had invaded to the lower surfaces of the filters were fixed with 3.7% formaldehyde, stained with 0.1% crystal violet/20% MeOH, and counted by microscopic examination. The average number of migrating cells per field was assessed by counting at least six random fields per filter.

Statistical Analysis

Statistical analyses were performed using either the two-tailed Student *t* test or nonparametric analysis of variance, as appropriate. A *p* value less than .05 was considered statistically significant. All statistical analysis was performed using SPSS data analysis software (SPSS, Chicago, http://www.spss.com).

RESULTS

Genomic and Proteomic Screens Identify PAI-1 as an Ischemia-Regulated Factor in MSCs

In our effort to identify factor(s) that were altered by MSCs under stress-like conditions, we cultured murine MSCs (passage 8; immunophenotype and multipotentiality are described in supporting information Fig. 1) to approximately 80% confluence, washed them several times with serum-free DMEM to remove exogenous growth factors, and then placed the MSCs into either normoxic (O_2 approximately 21%) or hypoxic ($O_2 < 3\%$) incubators for 24 hours. Consistent with Mylotte et al. [25], serum deprivation of MSCs for 24 hours was not associated with overt cell death, and hypoxia did not increase cell death at the 24-hour time period (data not shown). From these cells we collected both RNA for microarray analysis and conditioned serum-free DMEM for proteomic analysis.

Three separate RNA extractions were performed on each MSC treatment group ($n = 3$ for normoxia; $n = 3$ for hypoxia) and processed as described in Materials and Methods. Each MSC RNA isolate was hybridized to an Affymetrix Murine Genome U74v2 chip (Affymetrix, Santa Clara, CA, http://www.affymetrix.com), which contains ~30,000 mouse genes. A total of six separate array experiments were performed. When analyzing the expression levels from these experiments, we set exclusion criteria such that only those genes that showed at least a twofold change in expression and had a false-positive rate of <1% according to SAM analysis [19] were considered significantly different between the normoxia and hypoxia groups. As expected, the majority of genes from the two groups had similar expression levels and when plotted graphically lined up along a 45-degree diagonal axis (Fig. 1A, black points). However, 24 hours of hypoxia influenced the expression of 445 unique genes (Fig. 1A, gray points). Of these 445 genes, 174 were increased, whereas 271 were decreased (complete list of genes is given in supporting information Table 1). Since our focus was on secreted factors, we restricted our analysis to these genes. We submitted our list of 445 genes to GoStat [26] to identify only those genes whose proteins are found in the extracellular compartment. GoStat analysis identified 21 secreted proteins whose mRNAs were altered (12 upregulated, nine downregulated) when hypoxic stress was combined with nutrient deprivation (Fig. 1B). Within the upregulated group there were notable increases in vascular endothelial growth factor and members of the IGF-binding proteins.

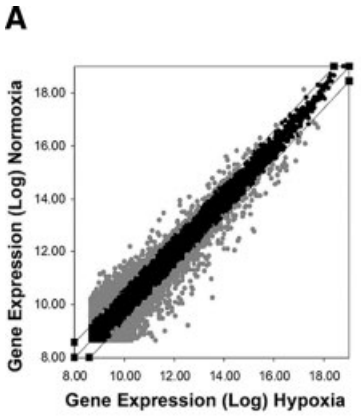
Since microarray data do not necessarily reflect protein production, we conducted several experiments using iTRAQ labeling followed by mass spectrometry to identify proteins secreted by MSCs in normoxic and hypoxic nutrient-poor conditions. Using this methodology, 24 hours of medium conditioning in the nutrient-poor environment was not conducive to identifying a large spectrum of secreted proteins. However, consistent with our microarray data, in each iTRAQ experiment the serine (or cysteine) proteinase inhibitor-clade E, member 1, also known as PAI-1, was increased due to hypoxia (Fig. 1C). Confirming this observation, Western blotting experiments showed that PAI-1 was increased not only in mouse MSCs under hypoxic conditions but in human MSCs (passage 5; immunophenotype and multipotentiality are described in supporting information Fig. 2) as well (Fig. 1D).

Plasminogen-Plasmin System in MSCs Under Ischemic-Like Conditions

PAI-1 is a primary regulator of the plasminogen activators (uPA and tPA), which regulates the conversion of plasminogen to plasmin [27]. Since ischemic-like conditions upregulated PAI-1 in our MSCs, we decided to further define the plasminogen-plasmin system in MSCs. From our mouse microarray data, we could infer that MSCs constitutively express the mRNA for PAI-1 and uPAR but do not normally produce high transcript levels for uPA or tPA (Fig. 2A). Western blotting, on 24-hour serum-free conditioned media from human mesenchymal stromal cells (hMSCs) (passage 6), did not reveal appreciable levels of either tPA or uPA (Fig. 2B, 2C); however, when these same hMSCs were stimulated with TGF-β1, small amounts of precursor uPA were detected (Fig. 2C). As expected, uPAR and PAI-1 from human (passage 6) and mouse (passage 8) MSCs were readily detected (Fig. 2D, 2E). These data suggested that MSCs do not actively convert plasminogen to plasmin. Consistent with this observation, when we assayed for plasmin activity in conditioned media from human and mouse MSCs (average relative fluorescence [RFI]: hMSC RFI, 166.58 ± 93.1; murine mesenchymal stromal cell [mMSC] RFI, 845 ± 119.5), regardless of whether MSCs were in a normoxic or hypoxic environment, or whether we exogenously primed the system with plasminogen and TGF-β1, substantial plasmin activity was not detected compared with our positive control (0.25 mIU plasmin per milliliter RFI = 28,969) (Fig. 2F). Since plasmin can directly alter matrix metalloproteinase (MMP) activity [28, 29], we used zymography as a second means of assessing whether MSCs could convert plasminogen to plasmin. Our experiments clearly demonstrated that exogenous addition of plasminogen and TGF-β1, onto human or mouse MSCs, did not alter MMP-2 activity (Fig. 2G, 2H). Notably, exogenous plasmin added, postcollection, to the MSC media effectively activated MSC-derived MMP-2 (Fig. 2G, 2H). In mouse MSCs (passage 8), we also noted that exogenous addition of recombinant PAI-1 or the inhibition of endogenous PAI-1, using a PAI-1-neutralizing antibody, did not affect MMP-2 activation (Fig. 2H).

Phenotypic and Functional Analysis of PAI-Null MSCs

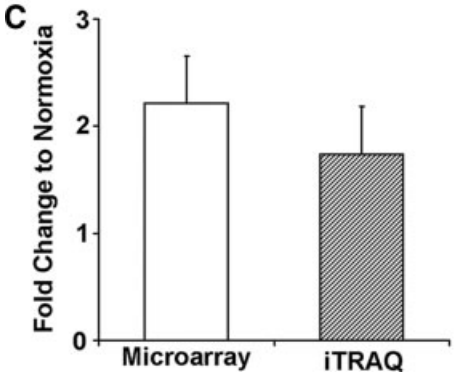
To better understanding the role that PAI-1 plays in MSC biology, we isolated MSCs from PAI-1 knockout mice (PAI-null) and compared them with MSCs from normal mice (WT-MSC). Upon plating, like WT bone marrow cells, PAI-null bone marrow cells formed discrete spindle colonies after 7 days (Fig. 3A) and could be propagated in vitro. PCR analysis showed that PAI-1 mRNA was readily detected in WT-MSCs but was absent in PAI-null MSCs (Fig. 3B). After eight passages, we immunophenotyped our PAI-null MSCs and exposed them to media



B

Up-Regulated	Symbol	Signal Hypoxia	Signal Normoxia	FoldDiff
Brevican	Bcan	12.33	10.05	4.88
IGF binding protein 4	Igfbp4	13.03	11.53	2.83
myeloperoxidase	Mpo	10.60	9.16	2.71
ceruloplasmin	Cp	10.79	9.38	2.66
IGF-2, binding protein 1	Igf2bp1	12.07	10.80	2.40
IGF binding protein 5	Igfbp5	12.46	11.21	2.38
biglycan	Bgn	11.00	9.85	2.22
serine proteinase inhibitor E1 (PAI-1)	Serpine1	12.89	11.74	2.21
vascular endothelial growth factor A	Vegfa	12.79	11.68	2.16
apelin	Apln	12.39	11.35	2.05
superoxide dismutase 3, extracellular	Sod3	9.97	8.94	2.05

Down-Regulated	Symbol	Signal Hypoxia	Signal Normoxia	FoldDiff
IGF-2, binding protein 3	Igf2bp3	9.87	11.43	2.95
serine hydroxymethyl transferase 1	Shmt1	9.43	10.88	2.73
Hepatoma-derived growth factor	Hdgf	10.92	12.32	2.65
chemokine (C-C motif) ligand 2	Ccl2	14.51	15.81	2.47
desmoplakin	Dsp	8.68	9.93	2.39
apolipoprotein D	Apod	8.93	10.13	2.30
Kit oncogene	Kit	8.94	10.13	2.28
secreted frizzled-related sequence protein 1	Sfrp1	9.14	10.33	2.28
interferon zeta	Ifnz	9.28	10.29	2.02



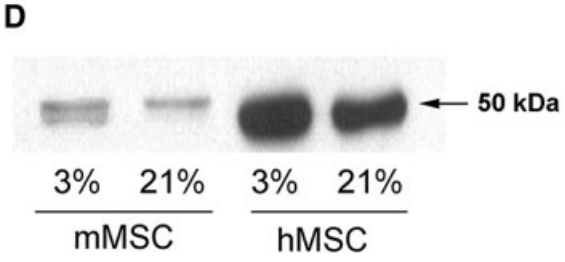


Figure 1. Impact of hypoxia on mesenchymal stromal cells. **(A):** Scatterplot of normoxia and hypoxia phenotype class based on differential gene expression in mMSCs. **(B):** Tabular representation of top 11 upregulated and top 9 downregulated genes for secreted proteins altered by hypoxia in mMSCs. **(C):** Comparison of PAI-1 expression alterations from microarray (mRNA) and iTRAQ (protein) experiments in mMSCs. **(D):** Representative Western blot of PAI-1 expression from media of mMSCs and hMSCs cultured in normoxia or hypoxia for 24 hours. (PAI-1 microarray, iTRAQ comparison; $n = 3$ separate experiments $\pm$ SD). Abbreviations: FoldDiff, fold difference; hMSC, human mesenchymal stromal cell; IGF, insulin-like growth factor; iTRAQ, isobaric tag for relative and absolute quantization; mMSC, mouse mesenchymal stromal cell; PAI, plasminogen activator inhibitor.

inductive for adipogenic, osteoblastic, and chondrocytic differentiation. PAI-null MSCs were similar to WT-MSCs regarding their cell surface expression for CD44, CD105, and MHC Class I and lack of expression for CD45, CD11b (Mac-1), MHC Class II, and CD31 (Fig. 3C). Like WT-MSCs (supporting information Fig. 1), PAI-null MSCs also readily differentiated into adipocytes, osteoblasts, and chondrocytes (Fig. 3D).

Using a Matrigel-embedded MSC subcutaneous assay, we have previously demonstrated that MSCs are capable of producing a robust host-derived angiogenic response in vivo [23]. Using this same assay system, we tested the host angiogenic response of PAI-null MSCs. Two weeks postimplantation, plugs embedded with either WT (passage 8) or PAI-null (passage 8) MSCs (2.0×10^6 cells per implant) were resected, fixed, and prepared for histologic analysis of blood vessel density. Immunostaining for vWF (green) identified blood vessels (Fig. 4A, 4B, arrows), and enumeration (Fig. 4C) demonstrated that PAI-null MSC implants had a significantly higher blood vessel density compared with WT-MSC implants. To determine whether the enhanced neovascularization of PAI-null MSC implants could be attributed to altered in vivo cell survival, we performed an additional set of subcutaneous Matrigel implants. For these experiments, we retrovirally transduced WT and PAI-null MSC populations with the LacZ gene and confirmed prior to implantation that >90% of cells were LacZ-positive, based on X-Gal staining (data not shown). At the time of implantation, 2×10^6 MSCs (WT and PAI-null MSCs, both at passage 12) were resuspended in Matrigel and injected subcutaneously in the right flank in each test mouse. Fourteen days postimplantation, mice were sacrificed and Matrigel implants were excised for analysis. Excised implants were briefly fixed, stained for X-Gal, and then prepared for histological analysis. Histologically, PAI-null MSC implants clearly had more X-Gal-positive cells compared with WT-MSC implants (Fig. 4D, 4E), and when enumerated they demonstrated a 3.7-fold increase in survival (Fig. 4F). Despite our WT and PAI-null MSC population having similar immunophenotypes, it was conceivable that our PAI-null MSC population possessed some unrealized difference that could account for its survival advantage. To preclude this possibility we performed additional subcutaneous Matrigel implants where WT-MSCs were first prelabeled with the membrane label PKH26. Prior to injection, one set of MSC implants was mixed with a PAI-1-neutralizing antibody (25 μg/ml; Molecular Innovation Inc., Novi, MI, http://www.mol-innov.com) and the other with an isotype control antibody (25 μg/ml IgG). These experiments confirmed our observations from the PAI-null MSC implants. At 2 weeks post-transplantation, there was clear evidence of more MSCs surviving in the implants mixed with the PAI-1-neutralizing antibody compared with those mixed with the IgG isotype control antibody (Fig. 4G, 4H).

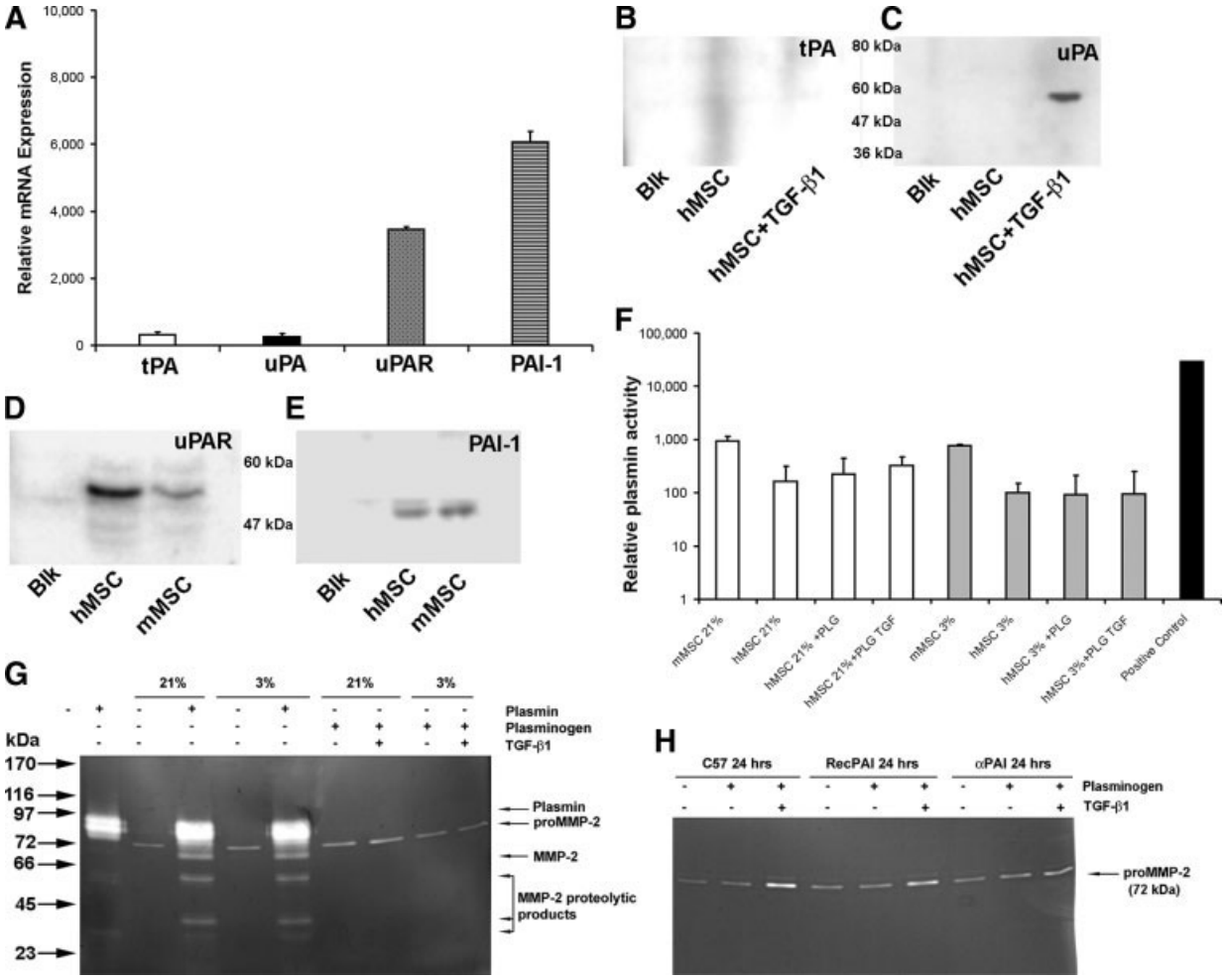


Figure 2. Plasminogen-plasmin system in mesenchymal stromal cells (MSCs). **(A):** Microarray data extrapolation of relative gene expressions for mMSCs. **(B–D):** Representative Western blots for in vitro-cultured MSC expression of tPA **(B)**, uPA **(C)**, uPAR **(D)**, and PAI-1 **(E)**. **(F):** Fluorometric in vitro plasmin activity assay on mMSC- (C57Bl/6) and hMSC-conditioned media. **(G):** Zymography of hMSC-conditioned media demonstrating that exogenously added plasmin can activate MMP-2. **(H):** Zymography of mMSC-conditioned media demonstrating that alterations in levels or activity of PAI-1 do not affect MMP-2 activation ($n = 4$ individual experiments $\pm$ SD) (plasminogen, 10 g/ml; plasmin, 0.25 mIU/ml; TGF, 1–10 ng/ml) (for **[B, C, E]**, Blk was unconditioned serum-free media; for **[D]**, Blk was loading dye only). Abbreviations: Blk, negative control blank; hMSC, human mesenchymal stromal cell; hrs, hours; MMP, matrix metalloproteinase; mMSC, murine mesenchymal stromal cell; PLG, plasminogen; PAI, plasminogen activator inhibitor; tPA, tissue plasminogen activator; TGF, transforming growth factor; uPA, urokinase plasminogen activator; uPAR, urokinase plasminogen activator receptor.

These data clearly indicate that extracellular PAI-1 negatively influences the survival of MSCs post-transplantation.

Local Extracellular PAI-1 Affects MSCs Adhesion

To determine the mechanism by which PAI-null MSCs have enhanced cell survival in vivo, we first assessed whether our PAI-null MSCs had altered MMP activity (supporting information Fig. 3a, 3b) or plasmin generation (supporting information Fig. 3c). Using the previously described assays, we could not detect a significant difference between our WT and PAI-null MSCs in regards to their MMP or plasmin activity. Thus the endogenous ability of PAI-null MSCs to convert plasminogen to plasmin does not explain the increased survival we observed in vivo. Supporting this observation, when we conducted an in vitro survival assay using MSCs embedded in Matrigel, where half the plugs were supplemented with mouse plasmin, those implants receiving exogenous plasmin showed reduced viability at 2 and 6 days postplating (Fig. 5A). These data suggest that a fine balance may exist between plasmin and PAI-1 activity in modulating cell survival post-transplantation.

Next we determined whether enhanced PAI-null MSC survival post-transplantation might be attributed to altered adhesiveness. Based upon previous work by Al-Fakhri et al. [30] and Czekay et al. [31], a series of short-term in vitro adhesion assays was conducted using tissue culture plates coated with either vitronectin (VN) or Matrigel (MTG). Vitronectin was chosen as a coating on the basis of evidence that PAI-1 can bind to VN and influence cell adhesion [32], whereas MTG-coated plates were used to allow direct comparisons of our in vitro and in vivo results. Compared with WT-MSCs, PAI-null MSCs had equal adhesion affinity to both VN and MTG, and blocking PAI-1 activity from MSCs did not overtly affect adhesion (Fig. 5B). Thus PAI-1 from MSCs does not alter initial adhesion of MSCs onto basement membranes regardless of whether they are adhering to a simple (i.e., vitronectin alone) or more complex (i.e., Matrigel) substrate. However, when recombinant PAI-1 (20 μg/ml) was added, to the VN or MTG matrix during the nonspecific blocking step, a significant decrease in MSC adhesion occurred on both substrates (Fig. 5B). This suggests that if PAI-1 levels are elevated at the site of MSC injection, survival

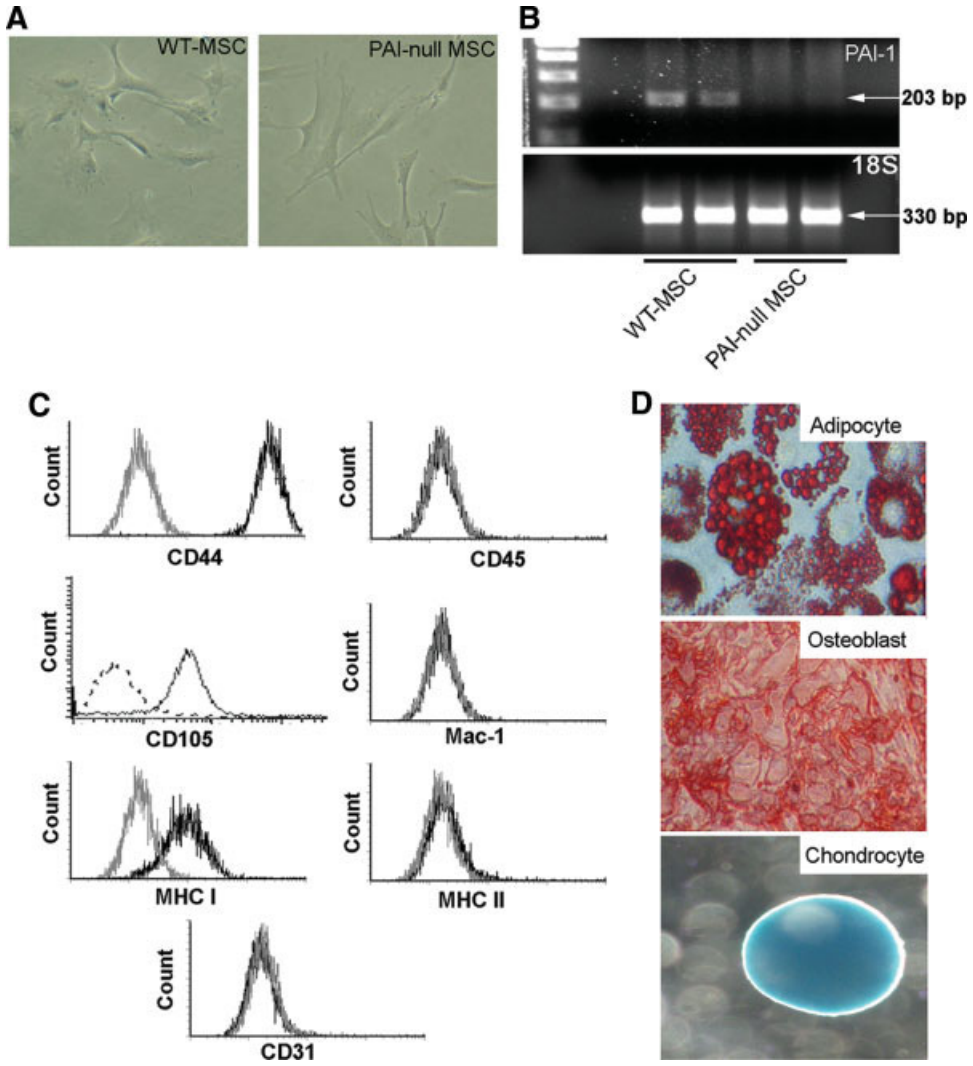


Figure 3. Isolation of mouse PAI-1-null MSCs. **(A):** Typical appearance of fibroblast-like colonies 7 days after plating of WT and PAI-1-null bone marrow cells. **(B):** Upper panel: Reverse transcription-polymerase chain reaction demonstrating presence of PAI-1 mRNA in WT-MSC and the lack thereof in PAI-null MSCs. Lower panel: 18S rRNA control. **(C):** Flow cytometry analysis on in vitro-cultured PAI-null MSCs determined cell surface antigen expression of CD44, CD105, MHC class I, CD45, Mac-1, MHC class II, and CD31, as described in Materials and Methods. **(D):** Undifferentiated PAI-null MSCs were cultured in conditions inductive of adipogenic (upper panel, stained with oil red O) osteogenic (middle panel, stained with alizarin Red S) and chondrocytic (lower panel, stained with Alcian Blue) differentiation. Abbreviations: bp, base pairs; MSC, mesenchymal stromal cell; PAI, plasminogen activator inhibitor; WT, wild-type.

of these cells will be compromised due to reduced adherence. Subsequently, we determined whether the accumulation of PAI-1 once MSCs had adhered to their matrices would promote cell detachment. In these experiments, we plated MSCs, onto both VN- and MTG-coated plates, and following their initial attachment period we added recombinant PAI-1 (20 μg/ml) at 0 and 12 hours. At all time points tested, the addition of recombinant PAI-1 increased MSC detachment (data not shown), and by 72 hours the addition of recombinant PAI-1 increased MSCs detachment on both VN- and MTG-coated plates by 74% and 86%, respectively (Fig. 5C). Thus, over time the accumulation of PAI-1 at the site of MSCs injection will promote cell detachment.

In the final set of experiments, we conducted in vitro invasion assays to determine whether abrogating the actions of PAI-1 influenced the migratory capacity of MSCs. The rationale for these experiments is rooted in the belief that MSCs are motile and will migrate toward environments more conducive to their survival. Thus, MSCs that are injected into an ischemic environment can potentially migrate away from their intended site of action. Using an MTG-coated transwell invasion assay system, we found that when MSCs were plated in the upper chamber and no chemoattractant applied to the lower chamber, few MSCs migrated over the 16-hour invasion period (Fig. 5D, negative control), whereas 10% FBS in lower chamber acted as a powerful chemoattractant for WT-MSCs (Fig. 5D). Comparatively, the migratory capacity of PAI-null MSCs was significantly lower than WT-MSCs, toward 10% FBS chemoattractant, and incubating WT-MSCs with a PAI-1-neutralizing antibody replicated the reduced migratory capacity of PAI-null MSCs (Fig. 5F). These data suggest that PAI-1 has a negative impact on MSC persistence at their injection site.

DISCUSSION

With the exception of a study by Mylotte et al. [25], very little is known regarding the ischemic response of MSCs. In partic-

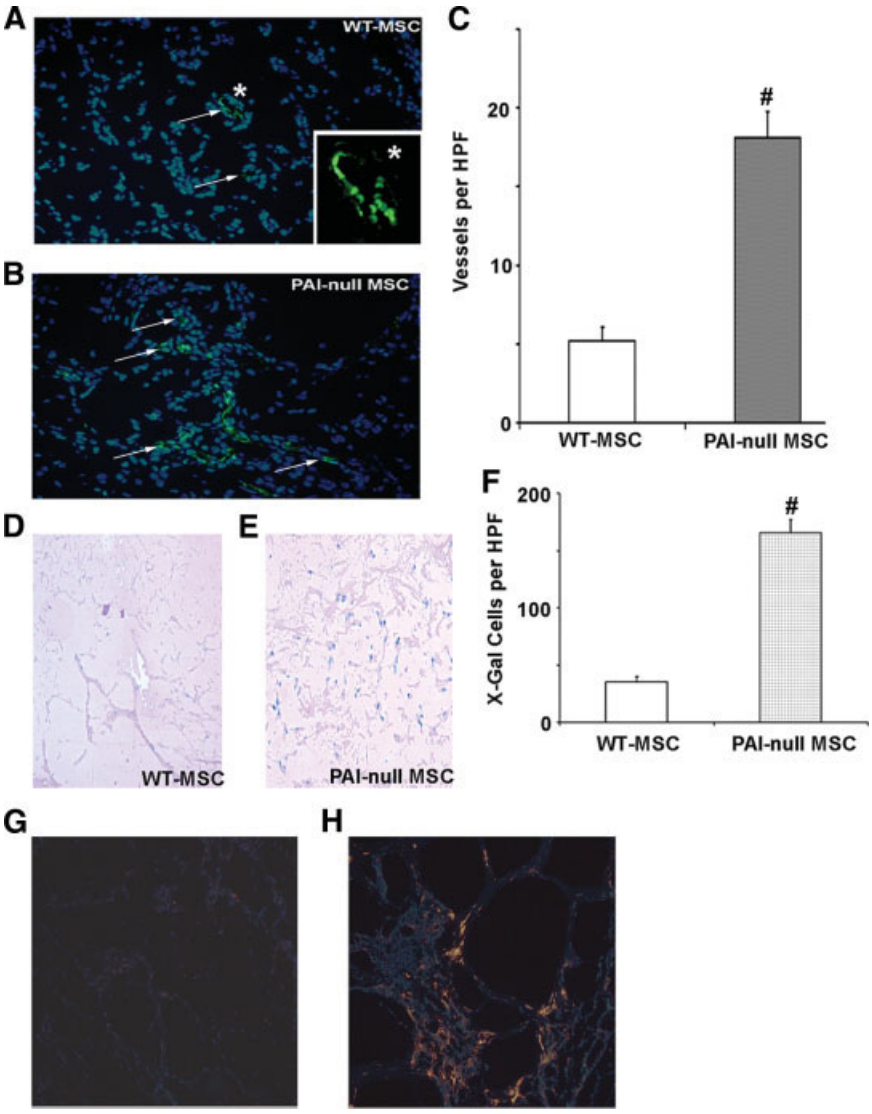


Figure 4. Regenerative potential of PAI-null MSCs. Morphometry (4,6-diamidino-2-phenylindole [Dapi], blue) and immunostaining for blood vessels (von Willebrand factor, green, arrows) of Matrigel implants from WT **(A)** and PAI-null MSCs **(B)**, 2 weeks postimplantation. **(C):** Quantification of blood vessel density for WT and PAI-null MSC implants. **(D, E):** Morphometry (red) and MSC survival (blue) of LacZ-transduced WT **(D)** and PAI-null **(E)** MSC implants, 2 weeks postimplantation. **(F):** Quantification of X-Gal-positive surviving WT and PAI-null MSCs. **(G, H):** Morphometry (Dapi, blue) and MSC survival (red) of PKH26-labeled WT-MSCs with isotype control antibody **(G)** or **(H)** PAI-1-neutralizing antibody (25 μg/ml), 2 weeks postimplantation. All graphs are presented as mean ± SD (n = 6 individual experiments) (#, $p < .01$ vs. WT-MSCs) (pictures are representative of six separate experiments; nuclei stained with Dapi, blue) (∗, enlargement of vessel in **[A]** without Dapi overlay). Abbreviations: HPF, high-power field; MSC, mesenchymal stromal cell; PAI, plasminogen activator inhibitor; WT, wild-type; X-Gal, 5-bromo-4-chloro-3-indolyl-β-D-galactoside.

ular, it is unclear how ischemia alters the secretome of MSCs. To better understand their true regenerative potential, investigators are trying to define the trophic factors that MSCs produce [33, 34]. However, as the list of factors MSCs secrete grows, the likelihood that MSCs secrete factors that are detrimental to their survival post-transplantation also grows. In an effort to identify secreted factors that could influence MSC survival, upon transplantation into ischemic tissues, we exposed murine MSCs to both a hypoxic and metabolic stress for 24 hours and profiled their secretomes using both genomic and proteomic approaches. Our gene array analyses identified a distinct set of genes whose expressions were altered when hypoxia was combined to nutrient deprivation. However, our mass spectrometry analyses showed that these changes were not readily detected in the media. Despite this, we found a correlation with PAI-1 and identified an unexpected causal role for extracellular PAI-1 levels influencing the survival of MSCs post-transplantation. Combined with our mechanistic data, we propose that modulating the activity of PAI-1, at the site of MSC transplantation, could enhance the efficacy of cellular therapy through enhanced persistence.

PAI-1 is a pleiotropic molecule, having both beneficial and detrimental effects. As a detrimental factor, PAI-1 influences the progression of metabolic disorders such as obesity and insulin resistance [35] and can also negatively influence tissue repair in the dermis [36], vasculature [37], and skeletal musculature [38]. PAI's best recognized function is preventing the conversion of plasminogen to plasmin by blocking the actions of tPA and uPA [27]. In the interstitium, where MSCs would be transplanted, plasmin can promote the release of latent growth factors and zymogens, which can promote local cell survival, proliferation, and migration [39]. Therefore, our initial hypothesis was that hypoxia-mediated MSC production of PAI-1 would negatively affect cell survival by preventing plasmin activation. Our data clearly suggest that this is not the case. The data revealed that MSCs do not normally produce tPA or uPA, and even when stimulated with TGF-β1, MSCs produce only the latent form of uPA. Furthermore, even when MSCs are stimulated with TGF-β1 and have plasminogen substrate available, MSCs cannot produce plasmin. Recently, Chiellini et al. also demonstrated that adipose-derived human MSCs actively produce PAI-1 but do not produce uPA or tPA [40]. Together these bodies of work imply that transplanted MSCs will not actively promote the conversion of plasminogen to plasmin. Although these data do not definitively disprove our original hypothesis, the fact that exogenous plasmin negatively affected MSC survival in our in vitro assays suggests that an alternative mechanism was at play. Furthermore, the negative effect plas-

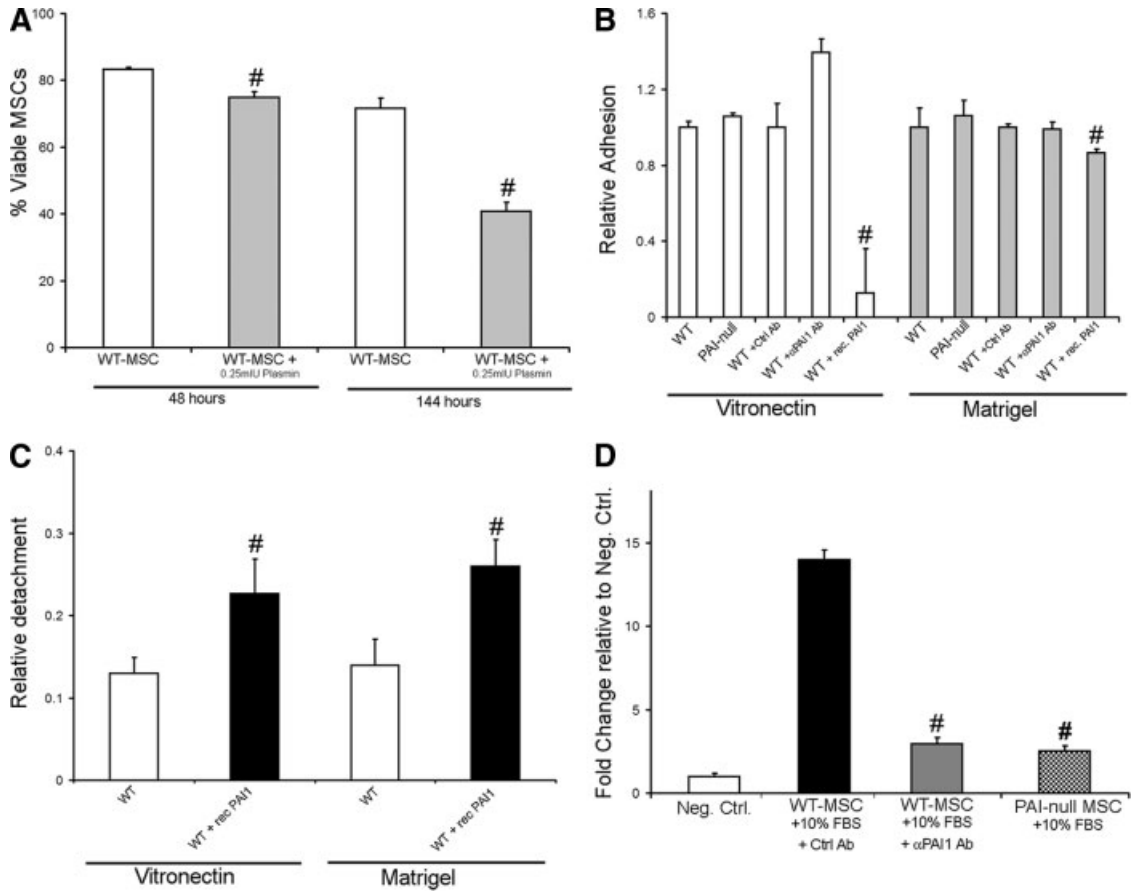


Figure 5. Neg actions of PAI-1 on MSC survival. **(A):** Annexin-V-propidium iodide staining of in vitro Matrigel plug survival assay demonstrating that exogenous plasmin is detrimental to MSC survival over time. **(B):** In vitro MSC adhesion assay on vitronectin- and Matrigel-coated plates. **(C):** In vitro MSC detachment assay on vitronectin and Matrigel-coated plates. **(D):** MSC invasion conducted on in vitro-cultured WT and PAI-null MSCs for 16 hours, exposed to serum-free media, media containing 10% FBS, and media containing PAI-1-neutralizing or isotype Ctrl ab (25 g/ml). All graphs are presented as mean ± SD ($n = 4$ individual experiments) (#, $p < .01$ vs. WT-MSC). Abbreviations: Ab, antibody; Ctrl, control; FBS, fetal bovine serum; MSC, mesenchymal stromal cell; Neg, negative; PAI, plasminogen activator inhibitor; rec., recombinant; WT, wild-type.

min had on MSC survival suggests that a balance must be achieved between plasmin and PAI-1 levels to maximize survival of transplanted MSCs. Indeed, plasmin has been shown to promote cell death (anoikis) in vascular smooth muscle cells [41, 42] via pericellular proteolysis, and although we did not investigate the mechanism of plasmin-induced MSCs death, we suspect that a similar mechanism was involved. Determining the balance between PAI-1 and plasmin levels to achieve optimal cell survival post-transplantation warrants further investigation.

Since MSCs do not actively convert plasminogen to plasmin and exogenous plasmin is actually detrimental to MSC survival, we sought an alternative explanation for why a lack of extracellular PAI-1 enhances MSC survival. From our in vitro mechanistic experiments, PAI-1's ability to influence adhesion and invasiveness clarifies why PAI-null MSCs and those WT-MSCs injected with a PAI-1-neutralizing antibody had a survival advantage in vivo. Studies have shown that uPAR and PAI-1 (both expressed by MSCs) can bind to the extracellular matrix protein vitronectin. However, since PAI-1 has a higher affinity to vitronectin, PAI-1 can out-compete uPAR for binding to vitronectin [43]. Thus, a high level of PAI-1 not only can reduce initial adhesion but also can actively promote cell detachment. When recombinant PAI-1 was added during the initial blocking step of our adhesion assays, MSC attachment was significantly reduced not only on vitronectin but also on the more complex matrix, Matrigel. Furthermore, when exogenous PAI-1 was added to WT-MSCs following their attachment, PAI-1 caused detachment of these cells. Finally, we found that when MSCs were exposed to a permissive chemotactic gradient, a lack of PAI-1 prevented the detachment of MSCs. Together these data suggest that PAI-1 acts as an antiadhesive molecule for MSCs and likely has a negative effect in vivo when MSCs are directly implanted.

As demonstrated in several cell types, controlling adhesive cues can dramatically increase cell survival post-transplantation by preventing anoikis [44–46]. Anoikis is defined as a process of programmed cell death induced by the loss of cell-matrix interactions [47, 48]. Our data support the theory that PAI-1 is an anoikis-inducing factor when MSCs are transplanted into ischemic tissue. Consistent with this view, Al-Fakhri et al. found the antiadhesive properties of PAI-1 proanoikic in vascular cells [30], whereas Czekay et al. found that PAI-1 not only caused cell detachment but also prevent detached cells from reattaching [31]. Thus one can hypothesize that transplanting MSCs into an ischemic environment, where PAI-1 may already be elevated, would reduce initial attachment, and the subsequent release of PAI-1 from surviving MSCs would prevent the long-term persistence of these cells.

Within this body of work, we acknowledge certain limitations. Foremost is the fact that our work was primarily conducted in the murine system. It is well known that differences exist between murine and human MSCs; however, by demonstrating that human MSCs behave similarly to murine MSCs in regards to their ability to elevate PAI-1 levels due to hypoxia and their inability to actively convert plasminogen to plasmin,

we believe our findings are transferrable. Supporting our assertion, recent work by Chiellini et al. showed that human adipose-derived MSCs produce PAI-1 and express uPAR but do not naturally produce tPA or uPA [40]. Thus, our observations can be applied to MSCs not only from different species but also from different tissue sources. A second limitation in our study is that our MSCs were exposed to a relatively mild and short duration of hypoxia. Conceivably, a longer duration and more severe hypoxia might have identified additional targets, but that does not preclude the importance of our findings. PAI-1 is a pleiotropic factor well known to be regulated not only by hypoxia, but also by a diverse range stimuli, including lipopolysaccharide, tumor necrosis factor-α, and interleukins [32]. Consequently, abrogating PAI-1 activity may enhance the survival and efficacy of MSC cellular therapy in a variety of settings. Furthermore, since PAI-1 is expressed by numerous cell types, abrogating PAI-1 activity may, also enhance the efficacy of other cell therapies, such as those involving myogenic stem cells [49, 50] or endothelial progenitor cells [51].

CONCLUSION

This study defines PAI-1 as an important mediator of MSC survival post-transplantation and supports ex vivo manipulation of PAI-1 to enhance the efficacy of MSC-based therapies for ischemic disorders. In the simplest approach, PAI-1-neutralizing antibodies or small molecule inhibitors could be physically encapsulated, covalently coupled, or associated via secondary bonding with the MSC transplantation vehicle. As a result, PAI-1-neutralizing factors would be present in vivo at high local concentration but have low systemic exposure [52], minimizing potential side effects. Essentially, local blockage of PAI-1 around MSCs would be advantageous for initial MSCs engraftment and adaptation to the ischemic environment but would not disrupt the long-term importance of PAI-1 in tissue repair and remodeling [53].

ACKNOWLEDGMENTS

This work was supported in part by the Canadian Institute of Health Research/Canadian Stem Cell Network/Heart and Stroke CARENET grant (to J.G.). I.B.C. is the recipient of a Canadian Institute of Health Research postdoctoral fellowship. J.G. is a Fond de la recherche en santé Quebéc chercheur boursier senior. The proteomic profiling was funded by Army Research Office of U.S. Department of Defense (to E.W.).

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicate no potential conflicts of interest.

REFERENCES

1 Caplice NM, Gersh BJ, Alegria JR. Cell therapy for cardiovascular disease: What cells, what diseases and for whom? Nat Clin Pract Cardiovasc Med 2005;2:37–43.
2 Wang Y, Haider HK, Ahmad N et al. Combining pharmacological mobilization with intramyocardial delivery of bone marrow cells overexpressing VEGF is more effective for cardiac repair. J Mol Cell Cardiol 2006;40:736–745.
3 Mangi AA, Noiseux N, Kong D et al. Mesenchymal stem cells modified with Akt prevent remodeling and restore performance of infarcted hearts. Nat Med 2003;9:1195–1201.
4 Shake JG, Gruber PJ, Baumgartner WA et al. Mesenchymal stem cell implantation in a swine myocardial infarct model: Engraftment and functional effects. Ann Thorac Surg 2002;73:1919–1925, discussion 1926.
5 Chen SL, Fang WW, Ye F et al. Effect on left ventricular function of intracoronary transplantation of autologous bone marrow mesenchymal stem cell in patients with acute myocardial infarction. Am J Cardiol 2004;94:92–95.
6 Stagg J, Galipeau J. Immune plasticity of bone marrow-derived mesenchymal stromal cells. Handb Exp Pharmacol 2007;45–66.
7 Mooney DJ, Vandenburgh H. Cell delivery mechanisms for tissue repair. Cell Stem Cell 2008;2:205–213.
8 Daley GQ, Scadden DT. Prospects for stem cell-based therapy. Cell 2008;132:544–548.
9 Muller-Ehmsen J, Krausgrill B, Burst V et al. Effective engraftment but poor mid-term persistence of mononuclear and mesenchymal bone marrow cells in acute and chronic rat myocardial infarction. J Mol Cell Cardiol 2006;41:876–884.
10 Li JH, Zhang N, Wang JA. Improved anti-apoptotic and anti-remodeling potency of bone marrow mesenchymal stem cells by anoxic pre-conditioning in diabetic cardiomyopathy. J Endocrinol Invest 2008;31:103–110.
11 Wang JA, Chen TL, Jiang J et al. Hypoxic preconditioning attenuates hypoxia/reoxygenation-induced apoptosis in mesenchymal stem cells. Acta Pharmacol Sin 2008;29:74–82.
12 Hung SC, Pochampally RR, Hsu SC et al. Short-term exposure of multipotent stromal cells to low oxygen increases their expression of CX3CR1 and CXCR4 and their engraftment in vivo. PLoS ONE 2007;2:e416.
13 Potier E, Ferreira E, Meunier A et al. Prolonged hypoxia concomitant with serum deprivation induces massive human mesenchymal stem cell death. Tissue Eng 2007;13:1325–1331.
14 Caplan AI, Dennis JE. Mesenchymal stem cells as trophic mediators. J Cell Biochem 2006;98:1076–1084.
15 Eliopoulos N, Al-Khaldi A, Crosato M et al. A neovascularized organoid derived from retrovirally engineered bone marrow stroma leads to prolonged in vivo systemic delivery of erythropoietin in nonmyeloablated, immunocompetent mice. Gene Ther 2003;10:478–489.
16 Pittenger MF, Mackay AM, Beck SC et al. Multilineage potential of adult human mesenchymal stem cells. Science 1999;284:143–147.
17 Phinney DG. Isolation of mesenchymal stem cells from murine bone marrow by immunodepletion. Methods Mol Biol 2008;449:171–186.
18 Copland I, Sharma K, Lejeune L et al. CD34 expression on murine marrow-derived mesenchymal stromal cells: Impact on neovascularization. Exp Hematol 2008;36:93–103.
19 Tusher VG, Tibshirani R, Chu G. Significance analysis of microarrays applied to the ionizing radiation response. Proc Natl Acad Sci U S A 2001;98:5116–5121.
20 Cong YS, Fan E, Wang E. Simultaneous proteomic profiling of four different growth states of human fibroblasts, using amine-reactive isobaric tagging reagents and tandem mass spectrometry. Mech Ageing Dev 2006;127:332–343.
21 Hockin MF, Kalafatis M, Shatos M et al. Protein C activation and factor Va inactivation on human umbilical vein endothelial cells. Arterioscler Thromb Vasc Biol 1997;17:2765–2775.
22 Annabi B, Lachambre M, Bousquet-Gagnon N et al. Localization of membrane-type 1 matrix metalloproteinase in caveolae membrane domains. Biochem J 2001;353:547–553.
23 Al-Khaldi A, Eliopoulos N, Martineau D et al. Postnatal bone marrow stromal cells elicit a potent VEGF-dependent neoangiogenic response in vivo. Gene Ther 2003;10:621–629.
24 Ory DS, Neugeboren BA, Mulligan RC. A stable human-derived packaging cell line for production of high titer retrovirus/vesicular stomatitis virus G pseudotypes. Proc Natl Acad Sci U S A 1996;93:11400–11406.
25 Mylotte LA, Duffy AM, Murphy M et al. Metabolic flexibility permits mesenchymal stem cell survival in an ischemic environment. STEM CELLS 2008;26:1325–1336.
26 Beissbarth T, Speed TP. GOstat: Find statistically overrepresented Gene Ontologies within a group of genes. Bioinformatics 2004;20:1464–1465.
27 Cesarman-Maus G, Hajjar KA. Molecular mechanisms of fibrinolysis. Br J Haematol 2005;129:307–321.
28 Monea S, Lehti K, Keski-Oja J et al. Plasmin activates pro-matrix metalloproteinase-2 with a membrane-type 1 matrix metalloproteinase-dependent mechanism. J Cell Physiol 2002;192:160–170.
29 Davis GE, Pintar Allen KA, Salazar R et al. Matrix metalloproteinase-1 and -9 activation by plasmin regulates a novel endothelial cell-mediated mechanism of collagen gel contraction and capillary tube regression in three-dimensional collagen matrices. J Cell Sci 2001;114:917–930.

30 Al-Fakhri N, Chavakis T, Schmidt-Woll T et al. Induction of apoptosis in vascular cells by plasminogen activator inhibitor-1 and high molecular weight kininogen correlates with their anti-adhesive properties. Biol Chem 2003;384:423–435.
31 Czekay RP, Aertgeerts K, Curriden SA et al. Plasminogen activator inhibitor-1 detaches cells from extracellular matrices by inactivating integrins. J Cell Biol 2003;160:781–791.
32 Balsara RD, Xu Z, Ploplis VA. Targeting plasminogen activator inhibitor-1: Role in cell signaling and the biology of domain-specific knock-in mice. Curr Drug Targets 2007;8:982–995.
33 Park HW, Shin JS, Kim CW. Proteome of mesenchymal stem cells. Proteomics 2007;7:2881–2894.
34 Schinkothe T, Bloch W, Schmidt A. In vitro secreting profile of human mesenchymal stem cells. Stem Cells Dev 2008;17:199–206.
35 Alessi MC, Juhan-Vague I. PAI-1 and the metabolic syndrome: Links, causes, and consequences. Arterioscler Thromb Vasc Biol 2006;26:2200–2207.
36 Chan JC, Duszczyszyn DA, Castellino FJ et al. Accelerated skin wound healing in plasminogen activator inhibitor-1-deficient mice. Am J Pathol 2001;159:1681–1688.
37 Carmeliet P, Moons L, Lijnen R et al. Inhibitory role of plasminogen activator inhibitor-1 in arterial wound healing and neointima formation: A gene targeting and gene transfer study in mice. Circulation 1997;96:3180–3191.
38 Koh TJ, Bryer SC, Pucci AM et al. Mice deficient in plasminogen activator inhibitor-1 have improved skeletal muscle regeneration. Am J Physiol Cell Physiol 2005;289:C217–223.
39 Alfano D, Franco P, Vocca I et al. The urokinase plasminogen activator and its receptor: Role in cell growth and apoptosis. Thromb Haemost 2005;93:205–211.
40 Chiellini C, Cochet O, Negroni L et al. Characterization of human mesenchymal stem cell secretome at early steps of adipocyte and osteoblast differentiation. BMC Mol Biol 2008;9:26.
41 Meilhac O, Ho-Tin-Noe B, Houard X et al. Pericellular plasmin induces smooth muscle cell anoikis. FASEB J 2003;17:1301–1303.
42 Michel JB. Anoikis in the cardiovascular system: Known and unknown extracellular mediators. Arterioscler Thromb Vasc Biol 2003;23:2146–2154.
43 Myohanen H, Vaheri A. Regulation and interactions in the activation of cell-associated plasminogen. Cell Mol Life Sci 2004;61:2840–2858.
44 Davis ME, Motion JP, Narmoneva DA et al. Injectable self-assembling peptide nanofibers create intramyocardial microenvironments for endothelial cells. Circulation 2005;111:442–450.
45 Hill E, Boontheekul T, Mooney DJ. Regulating activation of transplanted cells controls tissue regeneration. Proc Natl Acad Sci U S A 2006;103:2494–2499.
46 Delaney C, Varnum-Finney B, Aoyama K et al. Dose-dependent effects of the Notch ligand Delta1 on ex vivo differentiation and in vivo marrow repopulating ability of cord blood cells. Blood 2005;106:2693–2699.
47 Frisch SM, Francis H. Disruption of epithelial cell-matrix interactions induces apoptosis. J Cell Biol 1994;124:619–626.
48 Meredith JE Jr, Fazeli B, Schwartz MA. The extracellular matrix as a cell survival factor. Mol Biol Cell 1993;4:953–961.
49 Fibbi G, D'Alessio S, Pucci M et al. Growth factor-dependent proliferation and invasion of muscle satellite cells require the cell-associated fibrinolytic system. Biol Chem 2002;383:127–136.
50 Quax PH, Frisdal E, Pedersen N et al. Modulation of activities and RNA level of the components of the plasminogen activation system during fusion of human myogenic satellite cells in vitro. Dev Biol 1992;151:166–175.
51 Smadja DM, Basire A, Amelot A et al. Thrombin bound to a fibrin clot confers angiogenic and haemostatic properties on endothelial progenitor cells. J Cell Mol Med 2008;12:975–986.
52 Tessmar JK, Gopferich AM. Matrices and scaffolds for protein delivery in tissue engineering. Adv Drug Deliv Rev 2007;59:274–291.
53 Heymans S, Luttun A, Nuyens D et al. Inhibition of plasminogen activators or matrix metalloproteinases prevents cardiac rupture but impairs therapeutic angiogenesis and causes cardiac failure. Nat Med 1999;5:1135–1142.

See www.StemCells.com for supporting information available online.

Improved Autograft Survival of Mesenchymal Stromal Cells by Plasminogen Activator Inhibitor 1 Inhibition

Ian B. Copland, Simon Lord-Dufour, Jessica Cuerquis, Daniel L. Coutu, Borhane Annabi, Eugenea Wang and Jacques Galipeau

Stem Cells 2009;27;467-477
DOI: 10.1634/stemcells.2008-0520