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Expansion and Angiogenic Potential of Mesenchymal Stem Cells from Patients with Critical Limb Ischemia

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Abstract

Introduction—Critical limb ischemia (CLI) is a life and limb-threatening condition affecting 1–10% of the peripheral arterial disease (PAD) population. Traditional revascularization options are not possible for up to 50% of CLI patients, in which case, the use of cellular therapies, such as bone marrow derived mesenchymal stem cells (MSCs), hold great promise as an alternative revascularization therapy. However no randomized controlled phase 3 trials to date have demonstrated an improvement in limb salvage with cellular therapies. This may be due to either: poor cell quality (i.e. inability to generate a sufficient number of angiogenic MSCs) and/or the inadequate retention and viability of MSCs after delivery. Because concerns remain about the expansion and angiogenic potential of autologous MSCs in the CLI population, the objective of this study was to examine: the impact of our novel culture media supplement, pooled human platelet lysate (PL), in lieu of the standard fetal bovine serum (FBS), to improve the expansion potential of MSCs from CLI patients. We also characterized the *in vitro* angiogenic activity of MSCs from the tibia of amputated CLI limbs compared to MSCs from healthy donors.

Methods—MSCs from CLI patients were obtained from the tibia of patients undergoing major amputation; n=4 ischemic (ISC); n=4 ischemic and diabetic (ISC + DM). Healthy MSCs (n=4) were aspirated from the iliac crest of young and healthy donors. MSCs were successfully isolated and expanded in culture with PL or FBS. MSCs from passage 3–6 were used for phenotypic

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marker expression, adipogenic and osteogenic differentiation, and tested for their *in vitro* angiogenic activity on human microdermal endothelial cells (EC). In parallel MSCs were cultured to passage 11 for population doubling calculations.

Results—MSCs from both ischemic and healthy patients exhibited appropriate expression of cell surface markers and differentiation capacity. Population doublings were significantly greater for PL-stimulated compared to FBS-stimulated MSCs in all groups. The secretome of all MSCs had biologically active amounts of angiogens identified without consistent trends between groups. PL expansion did not adversely affect MSCs' angiogenic activity compared to FBS, and both ISC and ISC + DM MSCs demonstrated similar angiogenic effects on ECs by healthy and ischemic MSCs.

Conclusion—PL promotes the rapid expansion of MSCs from CLI and healthy persons. Importantly, MSCs expanded from CLI patients demonstrate the desired angiogenic activity as compared to their healthy counterparts. We conclude that autologous MSCs from CLI patients can be sufficiently expanded with PL and be expected to deliver requisite angiogenic effects *in vivo*. We expect the improved expansion of both ISC and ISC + DM with PL to be helpful in improving the successful delivery of autologous MSCs to patients with CLI.

Introduction

Critical limb ischemia (CLI) is the most severe form of peripheral arterial disease (PAD). CLI patients have inadequate perfusion, leading to rest pain, tissue loss, and ~30% one year amputation rates[1]. Given the increasing incidence of PAD worldwide[2], proportion of the population over 70 years of age, and longevity of cardiovascular patients, CLI is likely to increase substantially in the future[3]. Since many patients with CLI do not have appropriate anatomy or conduits for traditional revascularization[4, 5], major amputation with its significant morbidity and 30 day mortality rate[6] can be the only definitive treatment option. Thus novel treatments for CLI, such as cellular therapy, are critically needed[7].

Cellular therapies are a promising technology that may be capable of improving limb salvage by preventing major amputation in CLI patients[8]. Mesenchymal stem cells (MSCs) are particularly well suited for CLI as they promote angiogenesis and arteriogenesis through stromal and paracrine activity[9, 10]. To date, several early phase clinical trials of MSC therapies have shown benefit[11, 12], but there have been no phase III studies demonstrating improved limb salvage (i.e. decreased amputation rate) in CLI patients[11]. This has led to a call for deliberate testing and optimization of protocols, including MSC sourcing and expansion protocols[13].

Despite the robust capacity of *in vitro* testing of MSC quality and expansion, there is an imbalance in the number of bench studies of human MSCs in CLI compared to that of small clinical series[14]. Spurred by the inability to limit amputation rates in recent phase III clinical trials (Juventas and Restore-CLI[12, 15]), we believe further investigation into MSC quality and expansion protocols using clinically relevant MSCs is warranted (i.e. moving from the bedside to the bench). Here we utilize our access to MSCs from amputated limbs of CLI patients, as a readily available and clinically relevant human source of MSCs, to test their expansion and angiogenic capacity.

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In order to improve the expansion capacity of MSCs in CLI patients, we have generated and validated a MSC manufacturing process that uses pooled human platelet lysate (PL) as a human serum supplement to stimulate rapid MSC expansion. PL avoids the xenogeneic impact of fetal bovine serum (FBS) on MSCs, which can even result in the host rejecting autologous cells[16]. PL is generated following rigorous testing for contamination by the American Red Cross. Most importantly we already have approval to use PL in a current MSC human trial (IND14825; NCT 01659762). Finally since PL has been used to salvage MSCs from less robust cell sources[17], it may provide an advantage to the expansion of MSCs in CLI patients.

Similarly, autologous sourcing of MSCs expanded in human PL may be favored over allogeneic sourcing due to decreased concern for immunogenic rejection in CLI[18] and with repeat dosing. However there has been a persistent concern in the field of cellular therapy that autologous sourcing of cells in patients with cardiovascular disease may not provide the desired angiogenic effects. Heeschen et al. first demonstrated this discrepancy using bone marrow-derived mononuclear cells from patients with chronic ischemic cardiomyopathy[19]. This work led investigators to focus on the MSC component of the bone marrow mononuclear cells,[20] which are more angiogenic[21], but Neef et al. have recently demonstrated impaired quality of MSCs harvested from the sternum of coronary artery bypass grafting patients[22]. Interestingly other investigators have not found these deficits in dialysis patients[23] and other cardiovascular patients[24]. Thus this is a topic of intense debate and experimental equipoise requiring greater investigation.

In this study, we used human MSCs from amputated CLI limbs to test the hypotheses that PL promotes a more rapid expansion of MSCs than FBS and that autologous MSCs cultured from patients with CLI stimulate angiogenesis *in vitro* in a comparable fashion to that of MSCs from healthy donors. Our long-term aim is to develop improved pathways for cell expansion that enables expedited use of autologous MSCs from patients with CLI by utilizing PL as a novel, human-based, blood product source.

Methods

Cell harvest and culture

Healthy MSCs were retrieved from the iliac crest of young healthy donors (3 male; 1 female). Following IRB approval and informed consent, MSC were isolated from 10–20 mL of bone marrow aspirated from the iliac crest of these volunteer donors. Bone marrow aspirates were diluted 1:2 with phosphate-buffered saline and layered by a Ficoll density gradient. The cells were centrifuged at 400 g for 20 minutes and thereafter the mononuclear cells were plated in complete human MSC medium (α-MEM, 10% human platelet lysate or 10% FBS, 100 U/ml penicillin/streptomycin) at 100,000–300,000 cell/cm^2. Non-adherent hematopoietic cells were removed by changing the medium after 3 days of culture and MSCs were allowed to expand for 7–12 days. Thereafter, the cells were passaged and reseeded in fresh MSC medium at 1000 cells/cm^2.

Ischemic MSCs were harvested from the marrow of transected tibia after patient consent under an IRB approved protocol at Emory University Hospital. The transected bones were

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brought to the laboratory and cultured under the above conditions. PL was provided by the Emory Personalized Immunotherapy Center. Raw marrow was collected from the tibias and cells were disaggregated and strained through a 30 micron nylon mesh with α-MEM (Corning). Marrow volumes were calculated by volumetric measurement of water in the bone sections utilized and ranged from 2–6mL between patients. Cells were centrifuged, pelleted, and resuspended in culture. After 48 hours, non-adherent cells were discarded, and fresh media was added. Cells were passaged and reseeded as above.

Population Doubling and Differentiation assays

Healthy, ischemic (ISC), or ischemic and diabetic (ISC + DM) MSCs were plated at 1000 MSCs/cm^2 and serially passaged with either 5% PL or 5% FBS. Total cell count was recorded every 7 days, and population doubling times were calculated by dividing the number of days over which the cells were allowed cells to grow (7) by the doubling time calculated as published[17]. Cumulative population doublings were determined by the sum of populations up to and including each week, which was synonymous with passage number (week 4=passage 4). Colony forming units (CFU) were performed as described[25]. Briefly, MSCs were serially diluted in cell culture medium to obtain 100 cells/10 mL of media and plated in triplicate on a 100 mm tissue culture dish (Falcon). After 10 days, the colonies were fixed with methanol and stained with 0.5% crystal violet solution (Sigma). The dishes were placed on a grid and visible colonies were counted by a blinded observer.

adipogenic differentiation

MSCs were cultured in αMEM media was supplemented with 20% FBS, 100 unit/ml penicillin/100 μg/ml streptomycin, and 2 mM L-glutamine, 5 μg/ml insulin, 50μM indomethacin, $1{\times}10^{-6}$ M dexamethasone, and 0.5 μM IBMX. After 3 weeks, MSCs were fixed and stained with 1.5% w/v Oil Red O for quantification.

osteogenic differentiation

MSCs were cultured in αMEM media was supplemented with 20% FBS, 100 unit/ml penicillin/ 100 μg/ml streptomycin, and 2 mM L-glutamine, 6 mM β-glycerol phosphate, 50 ng/ml thyroxine, 1 nM dexamethasone, and 0.5 μM ascorbate 2-phosphate. After 3 weeks, MSCs were fixed and stained with 0.4mM Alizarin red for quantification.

FACS Profiles of MSCs

Passage 3 MSCs were cultured for 7 days in 5% FBS media or 5% PL in standard media, harvested and resuspended at a concentration of 1×10^6 cells/ml and analyzed by flow cytometry for the expression of CD45, CD34, CD44, CD73, CD90, CD105 and HLA-I (BD BioSciences). All samples were run on a Canto II flow cytometry with the appropriate isotype controls. Data is presented as fold change (total mean fluorescent intensity (MFI) of marker/ MFI) of the marker and isotype. Similarly all MSCs from each group were grown out to passage 8 in FBS and FACS analysis performed again to demonstrate retention of MSC markers.

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Secretome

MSC secretion profiles were determined by plating MSCs (passage 4–5) on to 6-well plates, at a density of 200,000 cells per well, for each donor and media condition (PL or FBS) (n=4). To maintain concurrent sampling of the secretome and account for the more rapid proliferation of PL treated cells, MSCs were cultured in either 2% PL or 10% FBS. After 24 h of seeding, culture media was removed from each well and wells were washed with 2 ml phosphate buffered solution (PBS) and replaced with 2 ml of serum free media with 0.2 % human serum albumin (HSA). After 1h, media was removed and a fresh 2 ml of serum free media with 0.2% HSA was placed on each well for 24 hours. Secretion of angiogenic factors in MSC conditioned media was determined by ELISA according to the manufacturer's instructions for bFGF, HGF, MCP-1 and VEGF (R&D Systems, USA). All statistical analyses were performed using Prism software (GraphPad, San Diego, CA, USA).

MSC Stimulation of EC invasion, proliferation, and migration

Angiogenic effect of MSCs on EC in 3-D assay—We adapted our 3-D *in vitro* cell invasion assay[26] by creating single multicellular cell aggregates of healthy, ISC, and ISC + DM MSCs were used with microdermal endothelial cells (ECs) or ECs alone to form the aggregate pellet. MSCs were labeled red with PKH-26 (Sigma) and ECs were labeled green with PKH-67 (Sigma) prior to pelleting in cellulose overnight to form a single cell pellet. The invading cells were imaged by light (bright field) and fluorescence microscopy (for PKH labeled cells). The invasion area semi-automated segmented and was defined as the difference between the total invasion area and normalized to the pellet area.

To compare the effect of FBS and PL on MSC invasion and MSC angiogenic effect on ECs, ECs were labeled with PKH-26 (Sigma) prior to co-culture aggregation. Here we quantified invasion by sprout length using a semi-automated program (MATLAB), and variability in pellet size was accounted for by normalizing sprout length to pellet diameter. Each experiment was performed with 6 replicates, and the experiments were performed in triplicate. Comparisons were made between healthy, ischemic, and ischemic diabetic MSCs in a blinded fashion over several passages (P3-5).

Endothelial cell proliferation

ECs were cultured under standard conditions to 80% confluence, serum starved for 24 hours, and then stimulated with MSC conditioned media (CM) from the various MSCs (healthy, ischemic, ischemic diabetic). Positive control cells were stimulated with complete media and PBS was added for a negative control. Cell growth was quantified (4 wells/time/analysis) after 24–48 h by Celltiter 96 Aqueous reagent and quantified by absorbance in microplate reader. Results were performed in triplicate and analyzed in a blinded fashion.

Endothelial cell chemotaxis

A Boyden chamber was modified by coating the upper surface of the membrane with fibronectin before seeding of ECs. The lower chamber was replaced with the above CM. Relative EC chemotaxis was quantified as described[27]. The positive control was complete

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media and the negative control was PBS. Results were obtained in triplicate and analyzed in a blinded fashion.

Statistical Analysis

Planned comparisons were analyzed with students paired t tests. Welch's correction were used when variances between groups were unequal. Comparisons between multiple groups were normalized and then analyzed with ANOVA. An alpha level of .05 was used to determine significance between groups. Donor and media type were analyzed using Tukey post hoc on a repeated measures two-way analysis of variance (ANOVA) at a 95% confidence interval.

Results

Expansion of MSCs in PL and FBS

MSCs were cultured from the iliac crest of healthy donors or from the tibia of amputated limbs of ISC and ISC-DM patients. (Table I)

The efficiency of MSC isolation from healthy volunteers was calculated by comparing the initial seeding of MNC to the number of MSCs recovered at passage 1. Based upon 20 isolations of BM-MSCs from healthy donors, a recovery of 1 MSC requires plating 16.68 +/−2.92 MNCs over 9.5 +/−1.98 days. Thus, from bone marrow volumes of 10–20 mLs, we acquire >20 million MSCs at the time of first passage.

CLI MSCs were cultured from marrow volumes ranging from 2–6 mL (ISC averaged 4.75 mL; Isc + DM averaging 3.5 mL). Calculating the efficiency of MSC isolation was not possible as MSCs were sorted by adhesion in tissue culture and not pre-selected by a Ficoll gradient. However colony forming units between all MSCs were similar between healthy and ischemic MSC groups. (Supplemental Figure 1)

MSC proliferation was robust in all groups at early passages but declined in the ischemic groups in later passages. (Figure 1a) PL significantly increased population doublings at multiple time points over that of FBS. (Figure 1b) PL demonstrated significant benefit over FBS when grouping all MSCs (healthy, ISC, and ISC+DM) and comparing mean population doublings. (Figure 1c) With serial passaging, there was a gradual stagnation of cumulative population doublings in both the ISC and ISC +DM groups as segregated for FBS (Figure 2a) and PL treated MSCs (Figure 2b). Importantly, PL culture significantly increased the cumulative population doublings over FBS treatment in all groups from week 8 onwards. (Figure 2c–e) ISC and ISC + DM MSCs have reduced expansion potential compared to healthy MSCs, but they can still yield clinically relevant cell numbers that are superior with PL compared to FBS. Table II compares the impact of PL or FBS on the relative fold expansion of MSCs from each group.

MSC Differentiation and Cell Markers

All MSCs retained their ability to differentiate when cultured in adipogenic and osteogenic media, (Figure 3) and MSCs at passage 3 retained typical MSC markers by flow cytometery analysis without differences between MSCs cultured in FBS and PL. (Figure 4) Similarly all

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MSCs from each group were expanded out to passage 8 in FBS and FACS analysis demonstrated retention of MSC surface markers.

MSC secretome analyses

To determine the angiogenic factor secretion profiles of MSCs, conditioned media from MSCs was collected after 24 h under serum free conditions and quantified via ELISA. All MSCs secreted detectable levels of the angiogens and chemokines examined (bFGF, HGF, MCP-1 and VEGF) from both PL and FBS media conditions. No significance differences were detected between either donor group or serum supplementation (FBS vs. PL) in the secretion of HGF or VEGF, but there was wide variability in the MSC secretion of these angiogens between donors. Significance differences were detected in MCP-1 were identified between FBS and PL in the healthy donors ($p<0.01$), and ISC + DM MSCs secreted significantly more bFGF than healthy donors under PL serum supplementation. (Figure 5)

MSC invasion and stromal support for EC ingrowth

MSCs significantly increased EC invasion area over EC alone pellets at days 2 and 3. Differentially stained MSCs (red) and ECs (green) were used to define the cellular interaction and quantify 3-D invasion of respective cells in this assay. (Supplementary Figure 2 a–c). We then tested for differences in MSC invasion by bright field (BF) and EC invasion by fluorescent imaging (red PKH) between PL and FBS with the same 3-D assay. (Figure 6a–b) Here PL culture of MSCs did not have an overally advantage over FBS. However ISC MSCs cultured in FBS had decreased invasion compared to both FBS and PL-stimulated healthy donor and ISC + DM MSCs. (Figure 6c) For MSC stimulation of EC invasion, there was increased EC ingrowth when ECs were co-cultured with ISC MSCs cultured in PL compared to healthy MSCs cultured in FBS and compared to ISC + DM MSCs cultured in either FBS or PL. (Figure 6d) CM from all MSC groups was able to stimulate EC proliferation over that of negative control (PBS); this was not influenced by PL vs. FBS. (Figure 7a) Similarly the CM from all MSC groups, except healthy MSCs in PL, stimulated increased EC migration compared to the negative control (PBS). (Figure 7b) Of note, the CM was far inferior to that of complete EC media in stimulating both EC proliferation and migration.

Discussion

This is the first report of *in vitro* testing of the expansion potential and angiogenic potency of bone marrow MSCs from amputated CLI limbs. We have identified that: 1) PL increased the expansion potential of all MSCs compared to standard FBS; 2) the expansion of MSCs from CLI patients, even at modest yields ~5 per million cells reported in cardiovascular patients[22], would expanded in PL 500,000 fold for ISC + DM and 2 million fold for ISC, providing sufficient numbers for clinical use; 3) we did not identify any obvious limitations to autologous MSC sourcing from CLI patients in this study as MSCs from both CLI patient groups had a similar secretome and angiogenic activity compared to that of healthy MSCs.

While the tibia will not replace the iliac crest as a marrow harvest site, it could be a source of MSCs for assisting in secondary healing of amputation wounds or for storage for future

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use in other tissue beds. Here we expanded the MSCs for this work using relatively small volumes of tibia marrow (2–6 mL). In the iliac crest, where larger volumes (10×) are harvested, an even greater MSC yield would be expected. Finally the similarities between healthy iliac crest MSCs and CLI tibia MSCs in this study provides proof of concept that amputated limbs are a suitable source of human MSCs for translational experimentation. This is important as human MSCs have many differences compared to murine and rodent MSCs, both in cell markers and function that can complicate translation to the bedside of murine findings[28, 29]. Further we recognize that the environment and MSC quality could be different between the tibia and iliac crest, but to date, the lone report was performed in patients with rheumatoid arthritis, where the authors linked a relative decrease in CFUs in the tibia to a possible deficit in osteogenic differentiation[30]. While such an investigation in CLI would have merit, our approach is desirable as it allows patients to donate tissue for research that would otherwise be discarded without adding a subsequent and painful procedure or additional surgeon time.

The promising results of PL and the desired angiogenic activity of MSCs from the tibia of patients with CLI provides motivation to address remaining concerns before testing this approach clinically. First, the impact of CLI on the quality of the MSC at harvest and the impact of hypoxia on MSC expansion was not evaluated. While it is well known that MSCs in bone marrow live normally under a low oxygen tension (~0–6%)[31, 32] and that MSC expansion is augmented by relative hypoxia[33, 34], a recent publication by Gremmels et al.[35] suggests that hypoxia did not play a significant or discriminating role in the conditioned media of healthy and ISC MSCs. Secondly we segregated MSCs from CLI patients into ISC and ISC + DM; this even split of CLI patients with ISC and ISC + DM is representative of the CLI population undergoing amputation at our institution and our future target patients. Next our sample size (n=4 per group), while consistent with other studies[36], is small, and may not have been large enough to identify subtle differences between groups. However, given the significant patient-to-patient variability and reproducibility of our results across groups, we are confident that autologous MSCs from ISC and ISC + DM patients can be expanded with PL to sufficient number and expected to provide the desired angiogenic activity. Excitingly, while we kept this focus in this initial work, two recent reports on MSCs from CLI patients (albeit with sample sizes of 11 and 12 patients in the ischemic and healthy groups) have demonstrated *in vivo* angiogenesis in mouse models that was equivalent to that of healthy donors[35, 37]. The similarities between these publications and our work certainly lessen the potential concern about the transferability of our results using MSCs from the tibia to that expected clinically from the iliac crest.

There are also changes that may have occurred in the MSCs during transection of the bone during amputation that were not controlled for in this study; here it would be expected that any disturbance of MSCs at harvest would lessen with serial passaging. Also while our study did attempt to represent both genders in all groups (ISC: 2 male/2 female patients; ISC + DM: 2 male/2 female; Healthy: 3 male/1 female), this work was not designed to add to the building body of literature on the role of gender on MSC function[38, 39].

In conclusion, we demonstrate that PL expansion of MSCs from the tibia of CLI patients is superior to that of FBS, and that ischemic MSCs have suitable expansion profiles and similar

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in vitro angiogenic capacity on human microdermal endothelial cells compared to that of healthy MSCs. Excitingly the correlation between these findings and that of the existing literature, supports the use of marrow from amputated limbs as a clinically relevant source of MSCs for translational investigation. This intentional step from bedside to bench may be useful in improving the likelihood of clinical translation of cellular therapies in patients with CLI through novel investigative new drug (IND) applications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Clinical relevance

Despite widely held belief that MSCs from patients with multiple vascular co-morbidities (eg. diabetes; aged patient) are inferior in quality compared to healthy patients, we demonstrate that MSCs from CLI patients have similar angiogenic function *in vitro* to that of healthy patients and that PL supplementation of culture media improves expansion potential of these MSCs. These findings support the use of autologous MSCs to promote angiogenesis and our novel MSC expansion protocol to increasing the number of suitable MSCs from patients with CLI for cellular therapy.

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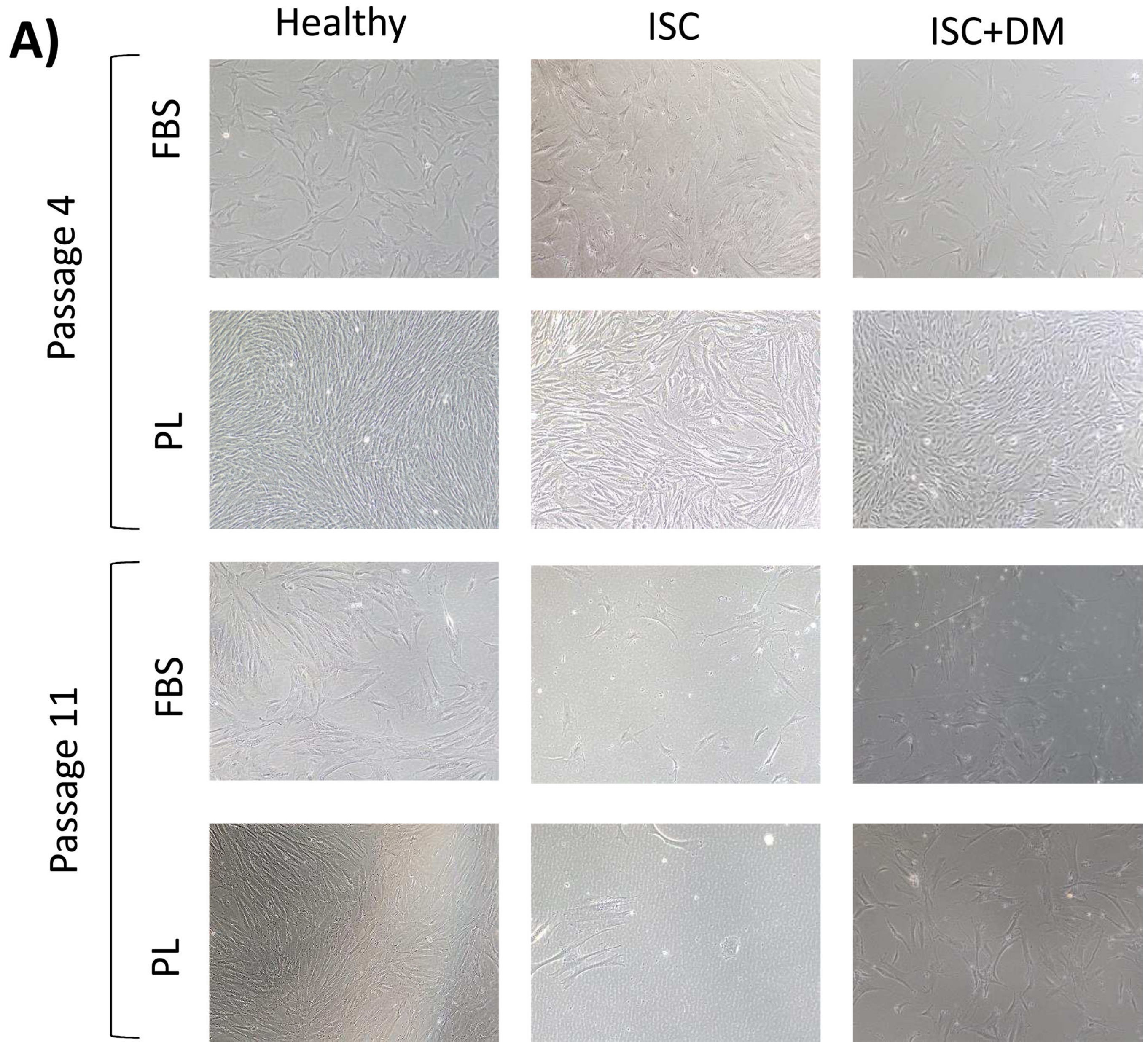

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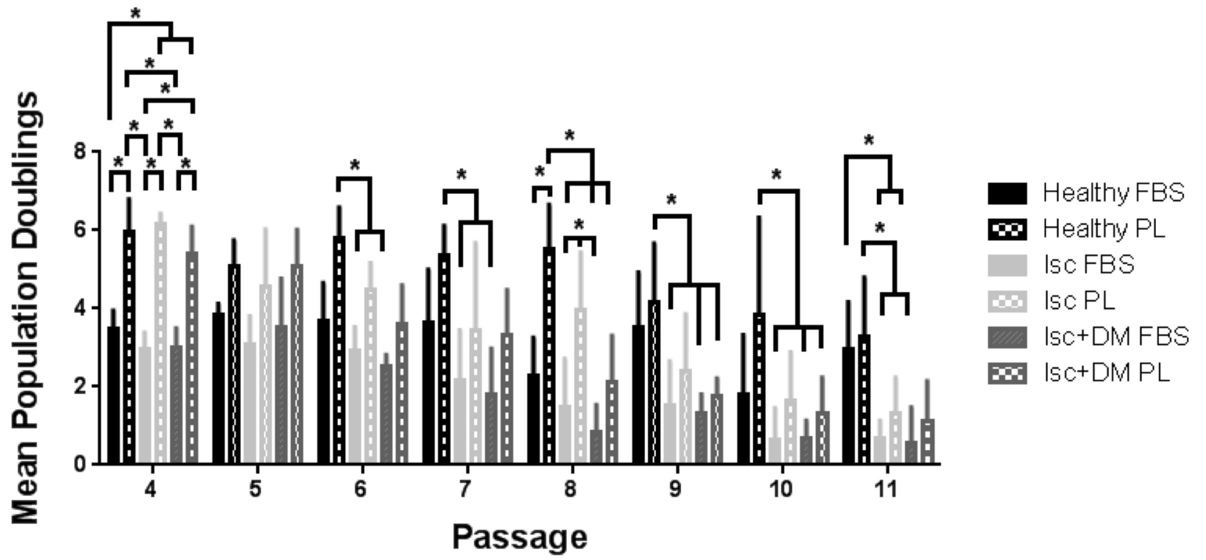

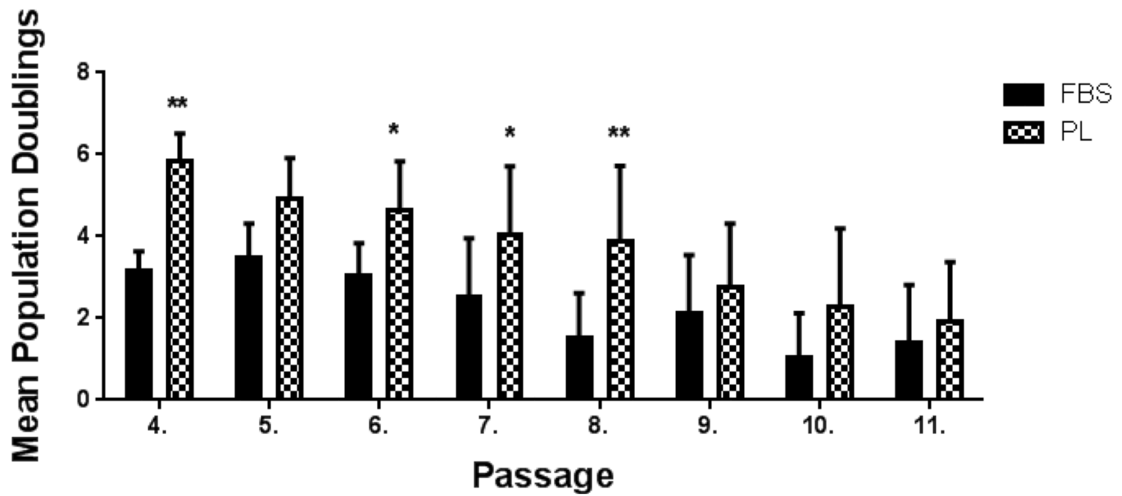

Figure 1. Impact of PL on MSC Expansion and Population Doubling

MSCs were cultured in α-MEM with supplementation of either fetal bovine serum (FBS) or platelet lysate (PL). Representative images of MSCs from all groups are shown. Here we show robust proliferation at passage 4, but the ISC and ISC + DM demonstrate decreased confluence at passage 11. **(1a)** Mean population doublings within each passage were then compared between groups (Healthy, ISC, ISC +DM) and media supplement (FBS and PL). Here there are a number of differences that support the benefit of PL over FBS within and between groups at most passages, and the superiority of healthy over ISC and ISC + DM at

later passages, regardless of media supplement. **(1b)** When grouping all MSCs by media supplement (FBS vs. PL), the benefit of PL over FBS within passages 4, 6–8 is demonstrated. **(1c)**

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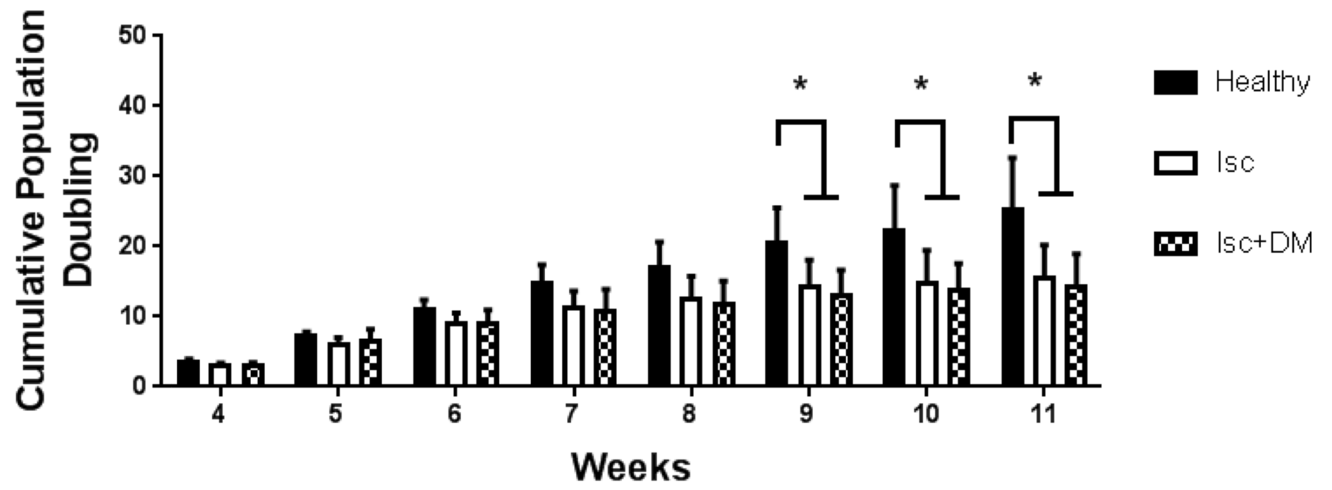

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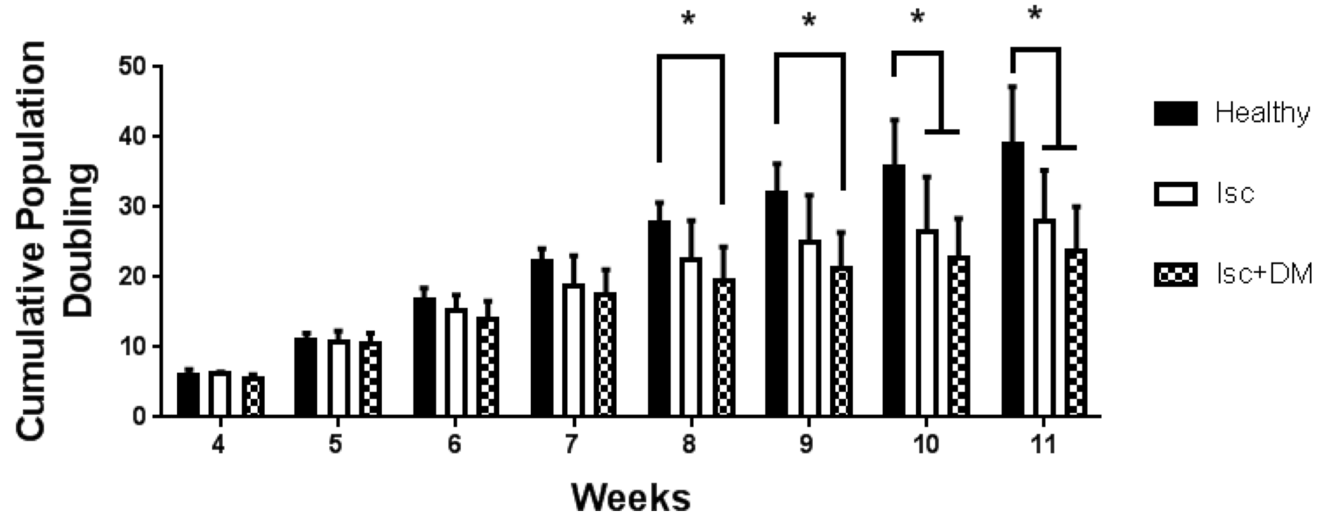

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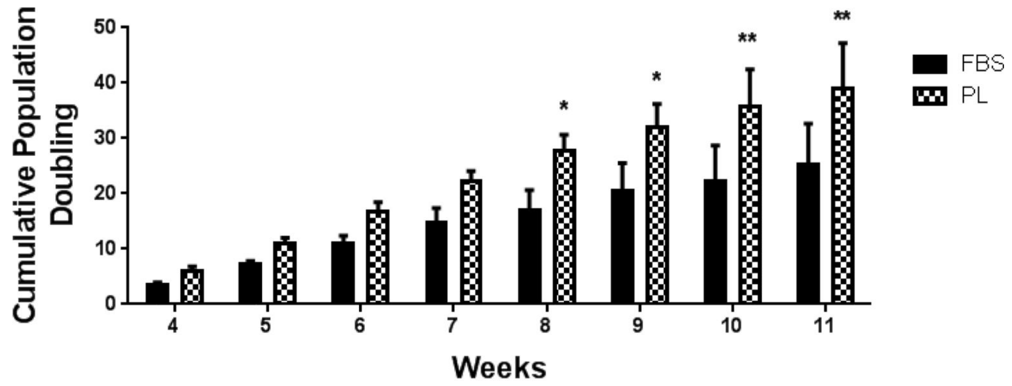
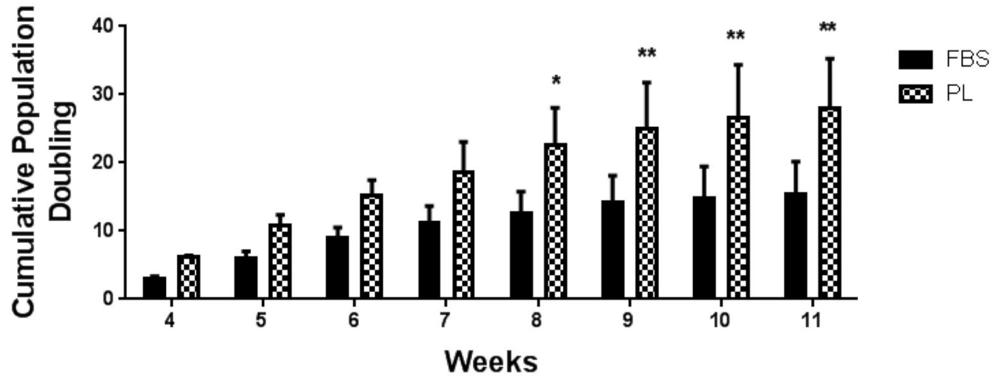
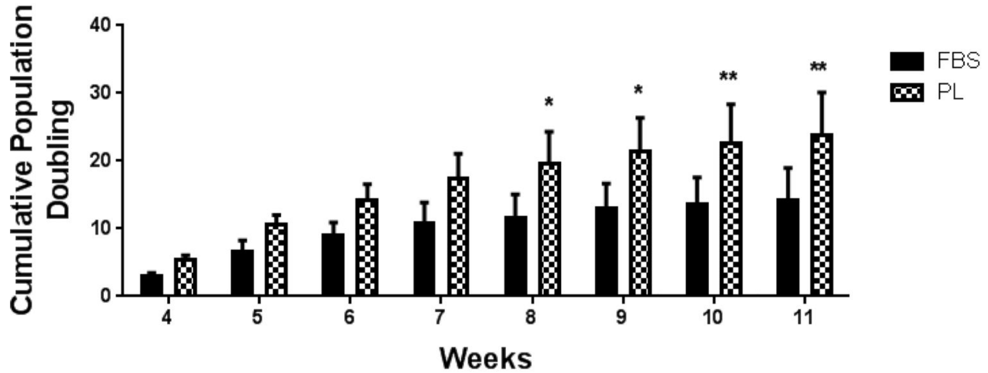


c. Healthy



d. ISC



e. ISC + DM

Figure 2. Differences in Cumulative MSC Population Doubling Are Dependent on Both the Donor and Media Supplement

There is a significant increase in the cumulative MSC population doubling for Healthy versus ISC and ISC + DM groups beginning at week (and passage) 9 in the FBS media. **(2a)** Similarly cumulative population doubling in healthy donor MSCs were significantly increased over ISC + DM MSCs in PL media beginning at week (and passage) 8. By week (and passage) 10, healthy MSCs' cumulative population doubling was also significantly increased over the ISC MSCs. **(2b)** When comparing media supplement (FBS vs. PL) within

each group, all groups demonstrate a significant increase with PL in cumulative population doubling by week (and passage) 8. **(2c–e)** Of note the duration (weeks) were chosen by design and passaging for expansion in culture can be sped up to obtain multiple passages in a single week.

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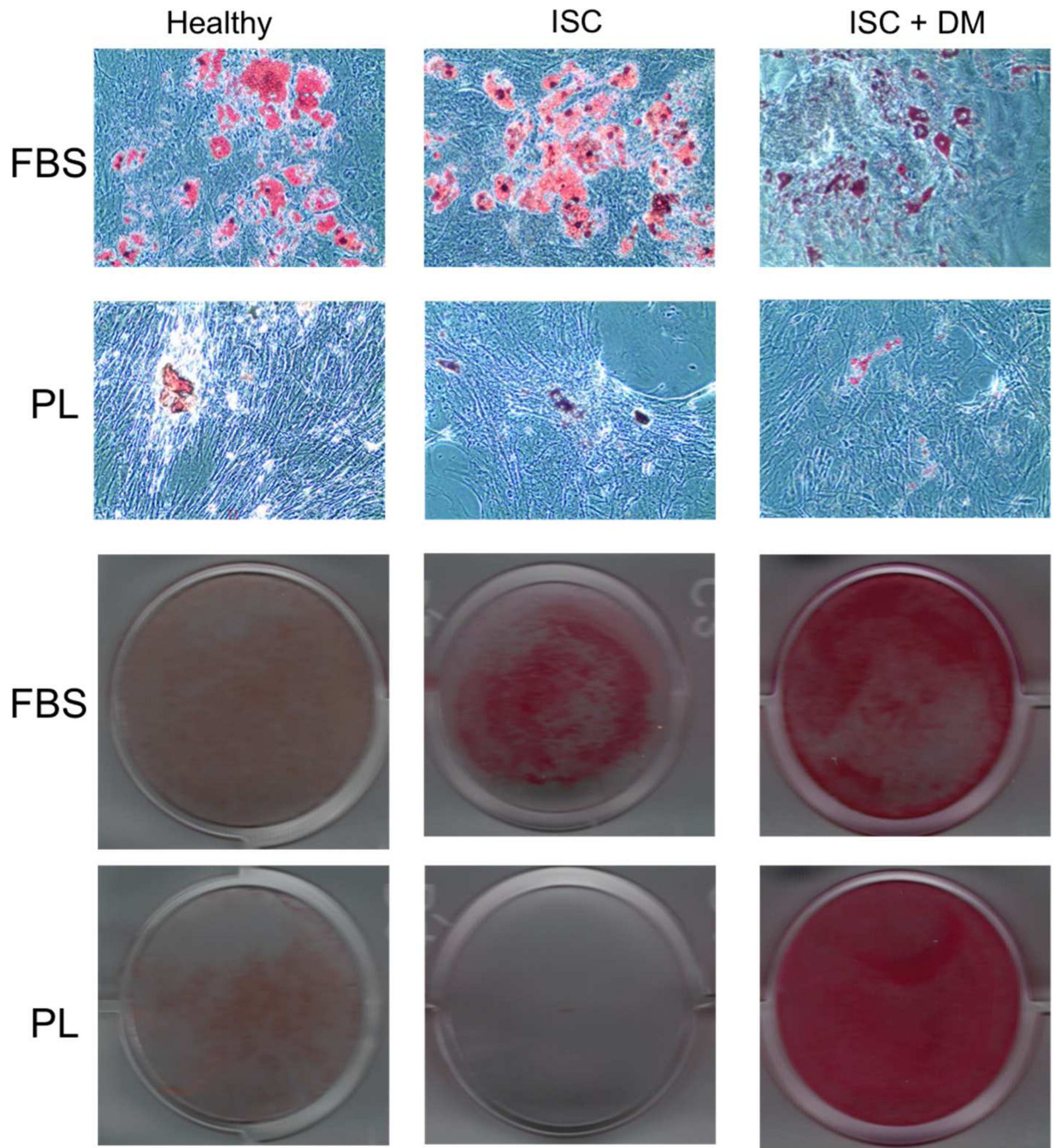

Figure 3. Healthy, ISC, and ISC + DM MSCs Demonstrate Adipogenic and Osteogenic Differentiation

Representative images of FBS and PL expanded healthy donor (healthy), ischemic (ISC) and ischemic and diabetic (ISC + DM) MSCs after culture in adipogenic and osteogenic conditions. All MSCs retained both adipogenic (top 2 panels) and osteogenic differentiation (bottom 2 panels) as determined by Oil Red O and Alizarin red staining, respectively. While our representative images show differences within the same patients MSCs with FBS and PL media, there were not consistent differences in differentiation identified between FBS and PL media or between patient groups.

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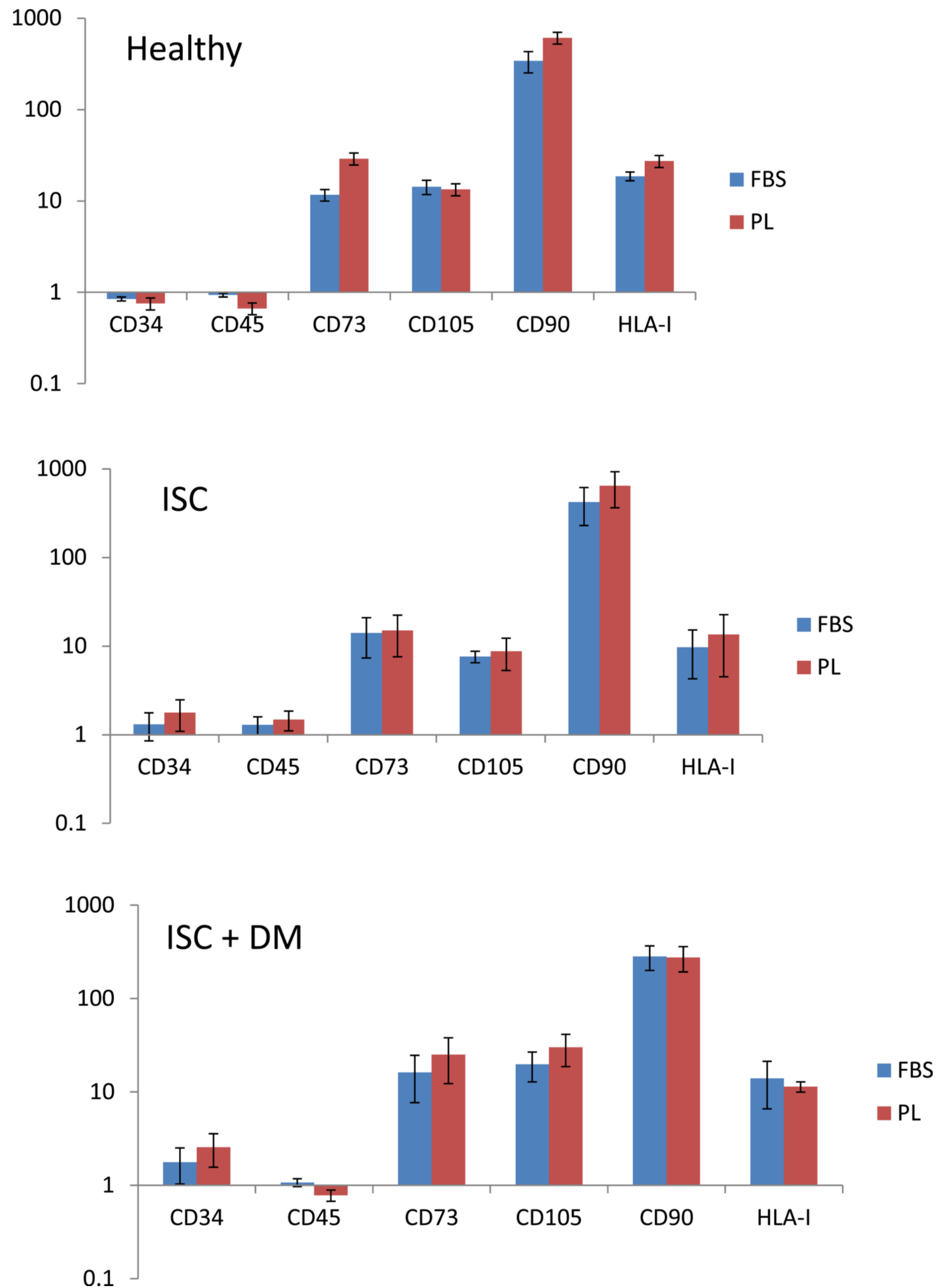

Figure 4. All MSCs in Both FBS and PL Media Maintain Appropriate FACS Markers

The mean fluorescent intensity (MFI) of selected FACS markers are displayed for each group of patients with FBS expanded MSCs in blue and PL expanded MSCs in red. All MSCs demonstrated the appropriate cell markers, and no differences were identified according to type of media supplement (FBS vs. PL).

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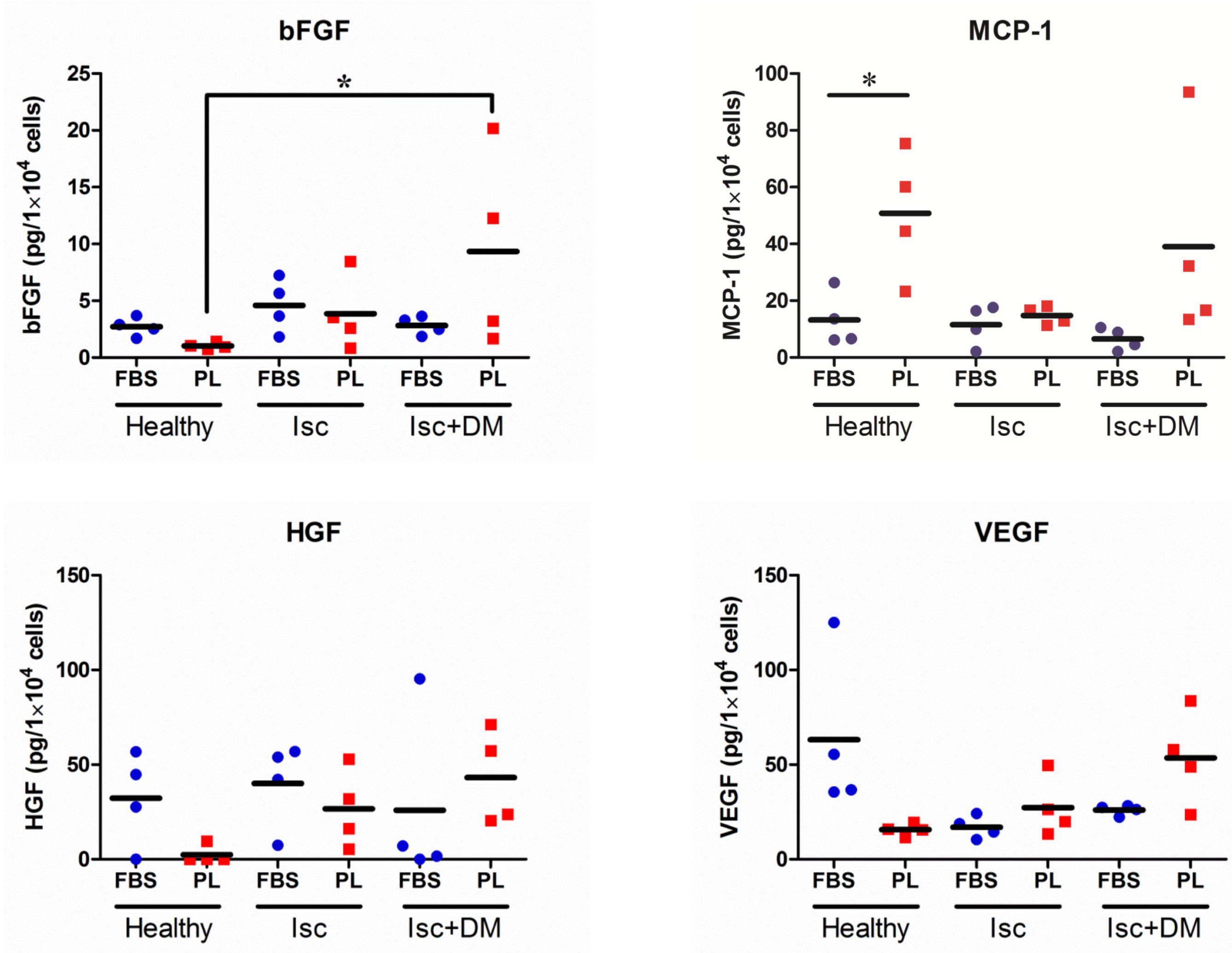

Figure 5. Robust Angiogenic Secretome Profiles for All MSC Groups

Basic fibroblast growth factor (bFGF), monocyte chemoattractant protein-1 (MCP-1), hepatic growth factor (HGF), and vascular endothelial growth factor (VEGF) were quantified as picograms per 10,000 MSCs (pg/10^4 cells). MSC secretion profiles from bone marrow of healthy (Healthy), ischemic (ISC) ischemic and diabetic mellitus (ISC-DM); n=4 in all groups. Results are shown from each patient from samples collected from passages 4 and 5 of MSCs that were isolated and expanded in FBS or PL media supplement. All MSCs had media removed and were maintained in 0.5% human serum albumin for 24 hours prior to media collection for secretome analysis. Mean of donors is shown by black bar and * $p<0.05$. While significant increases were identified in bFGF concentrations between healthy and ISC + DM MSCs cultured in PL and in MCP-1 in healthy MSCs cultured in PL, no clear trends were identified demonstrating a superiority or inferiority between cell groups or serum supplement as it relates to the secretome.

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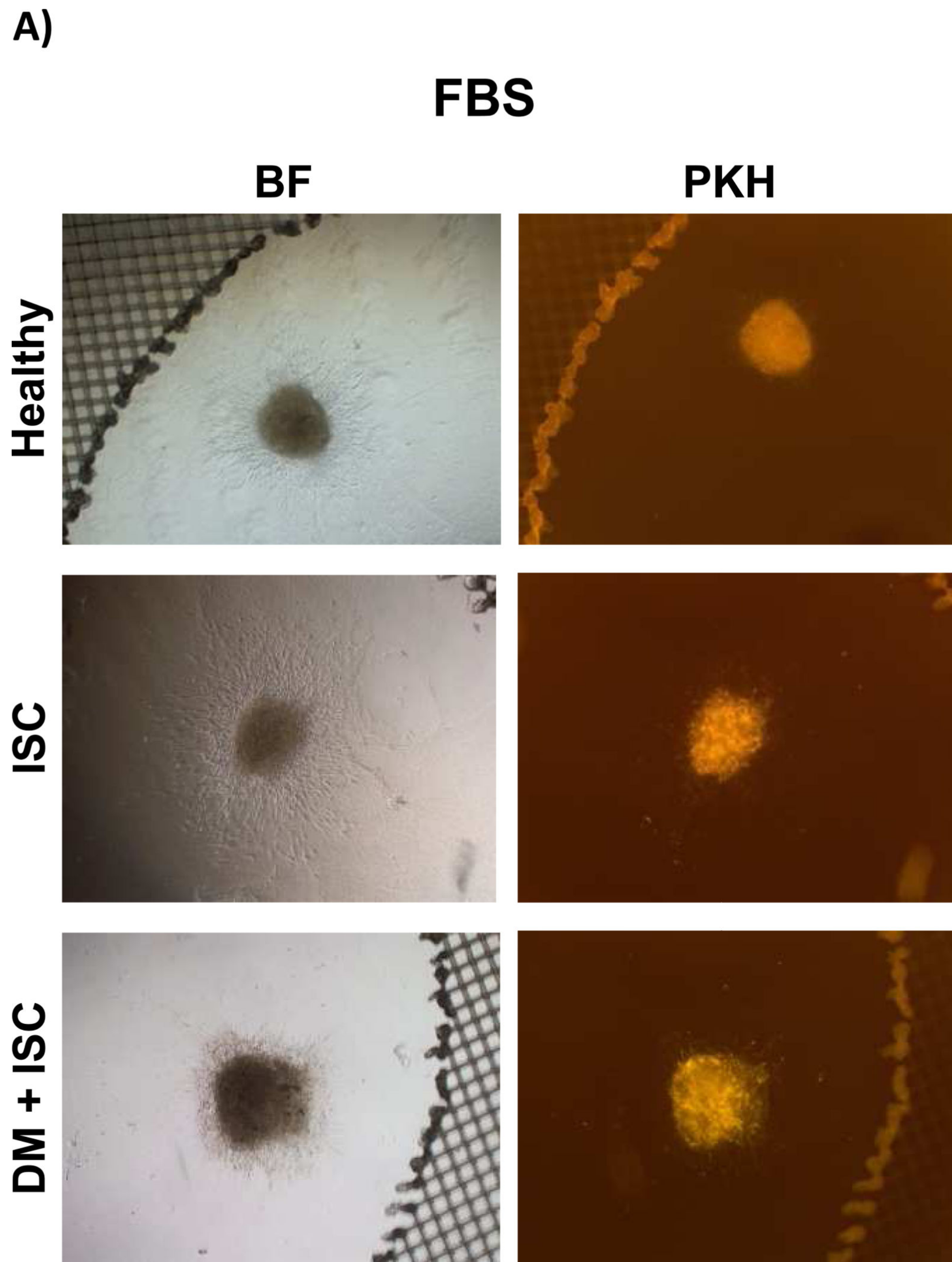

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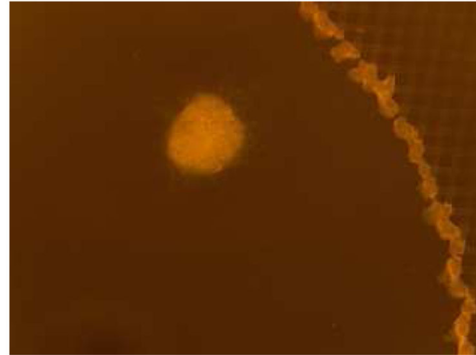
B)

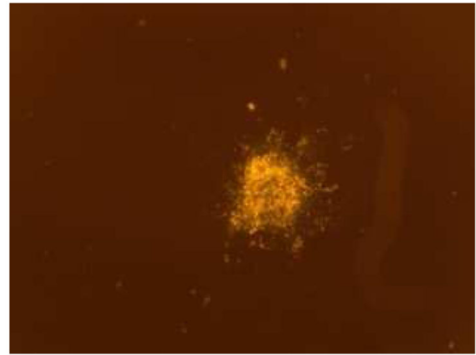
PL

BF PKH

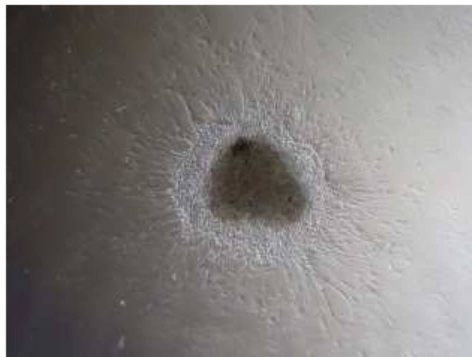
Healthy

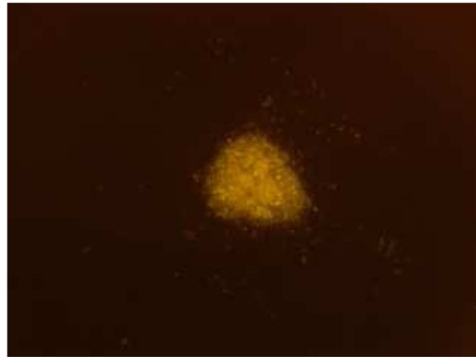


ISC



DM + ISC





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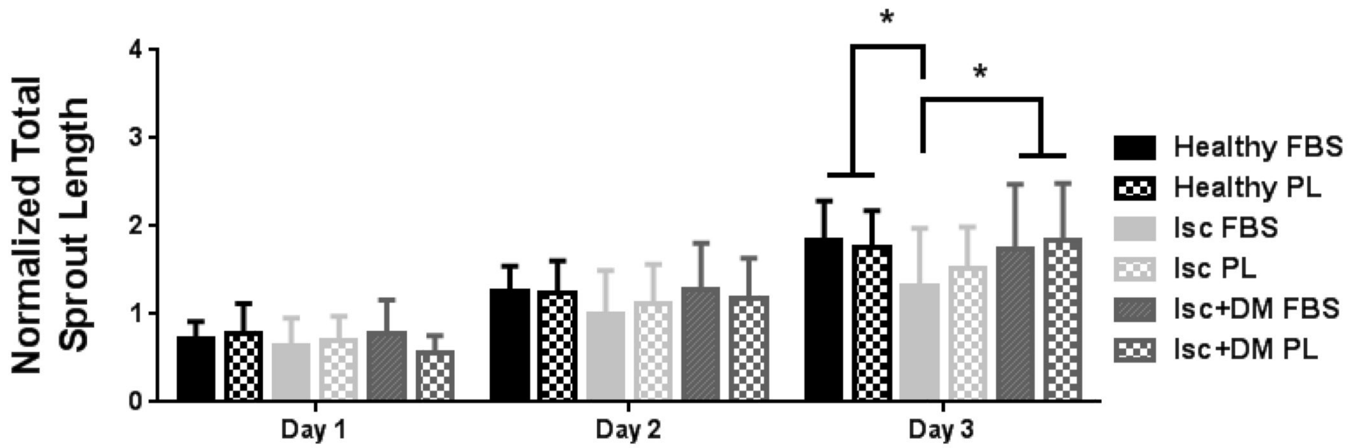

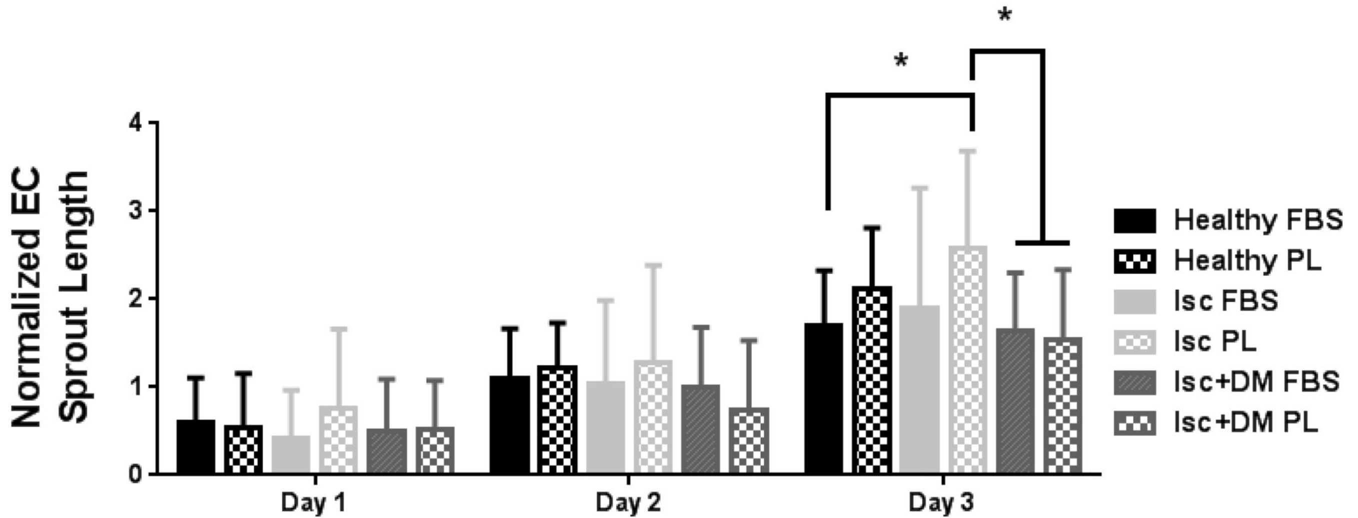

Figure 6. MSCs From All Groups Support EC Invasion in 3-D Assay

Co-culture assay of respective MSCs from each group with PKH-26 stained human microdermal endothelial cells (EC) demonstrate robust invasion of MSCs (unstained) as visualized by bright field imaging (BF) and ECs (PKH-stained red and imaged under fluorescence). **Figure 6a** demonstrates representative BF and fluorescent (PKH) imaging of MSCs expanded in FBS. **Figure 6b** demonstrates the same comparison for MSCs expanded in PL. Quantification of MSC invasion as captured under BF demonstrates at day 3 decreased invasion in ISC MSCs cultured in FBS compared to both FBS and PL stimulated

healthy donor and ISC + DM MSCs. **Figure 6c** EC invasion as captured by PKH-staining under fluorescence microscopy (PKH) at day 3 demonstrates increased MSC support of EC ingrowth in ISC MSCs cultured in PL compared to healthy MSCs cultured in FBS and ISC + DM MSCs cultured in either FBS or PL. **Figure 6d**

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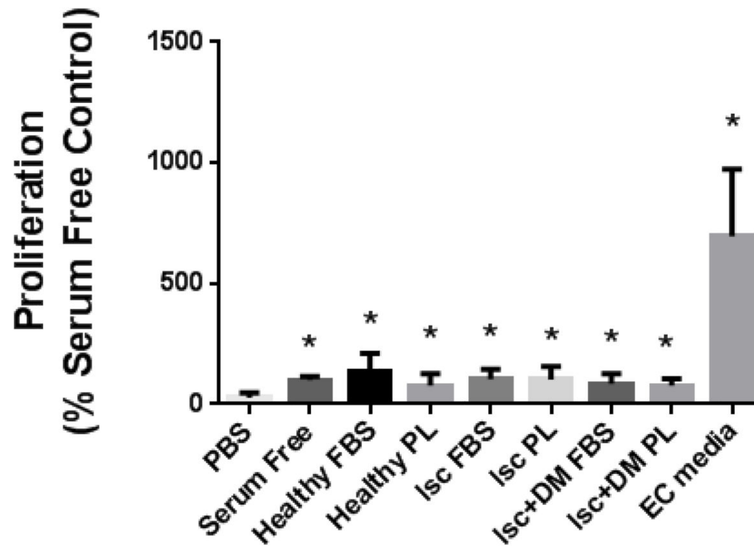

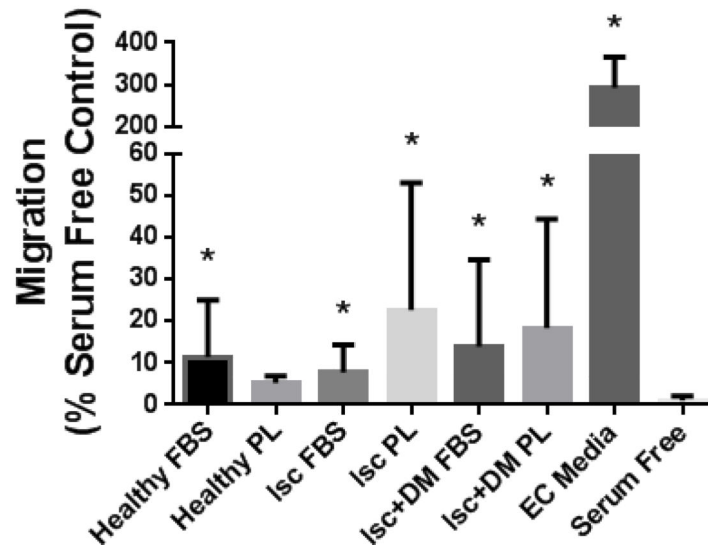

Figure 7. MSC Conditioned Media from All Groups Stimulates EC Proliferation and Migration over Negative Control

Cultured media was collected from MSCs from each group cultured with FBS or PL and frozen in −80 Celsius freezer until being thawed on day of use. Human microdermal endothelial cells (EC) were tested for proliferation (Celltiter 96 Aqueous reagent) and migration (modified boyden chamber). PBS was used as negative control and EC complete media as positive control. Conditioned media from all groups and both FBS and PL supplementation was able to stimulate EC proliferation over that of negative control (PBS).

(Figure 7a) Similarly the conditioned media of all groups, except healthy MSCs in PL, stimulated increased EC migration compared to negative control (PBS). **(Figure 7b)**

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Table I

BM MSC Donor Characteristics.

Patient Groups	1	2	3	4
Healthy	22 year old healthy female Non-smoker	26 year old healthy male Non-smoker	34 year old healthy male Non-smoker	28 year old healthy male Non-smoker
ISC	76 year old male with CLI. Comorbidities: hypertension, atrial fibrillation, dyslipidemia. Smoker	46 y/o male with CLI. Comorbidities: Hypertension, Chronic Obstructive Pulmonary Disease, Obesity. Smoker	81 year old female with CLI. Comorbidities: Hypertension, Prior peripheral revascularizations, Prior smoker>1 year ago	85 year old female with CLI. Comorbidities: Hypertension, Congestive Heart Failure, Coronary Artery Disease. Non-smoker
ISC + DM	85 year old diabetic female with CLI. Comorbidities: Hypertension, Prior stroke, Prior distal leg bypass. Non-smoker	67 year old diabetic female with CLI. Comorbidities: Coronary artery disease, Chronic obstructive Pulmonary Disease, Congestive heart failure, dyslipidemia Non-smoker	69 year old diabetic male with CLI Comorbidities: Dyslipidemia, Coronary artery disease. Prior smoker> 1 year ago	81 year old diabetic male with CLI. Comorbidities: Hypertension, Congestive heart failure, Dyslipidemia. Prior smoker> 1 year ago

4 persons were included in each group. Groups were labeled as: Healthy, ischemic (ISC), or ischemic and diabetic (ISC + DM). The healthy donors had bone marrow harvested from their iliac crest, and the ISC and ISC + DM had bone marrow harvested from their tibia after major amputation. Ages, gender, comorbidities, and smoking status are all included in table. Of note, only 2/12 patients were active smokers at time of harvest; both of these persons were in the ISC group.

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Table II

Estimated Fold Expansion Benefits of PL over FBS.

PL vs. FBS	Healthy	ISC	ISC + DM
PL	134 million fold expansion	4 million fold expansion	500,000 fold expansion
FBS	65,000 fold expansion	2000 fold expansion	2000 fold expansion

Based upon the calculated cumulative population doubling times for PL and FBS, we could estimate that for a similar culture duration time, culturing healthy MSCs in PL would allow for a 134-million fold expansion whereas for FBS the fold expansion would only be 65,000. ISC and ISC + DM MSCs have reduced expansion potential compared to healthy MSCs, but they can still yield clinically relevant cell numbers that are superior with PL compared to FBS. Specifically for ISC MSCs, PL allowed for a 4 million fold expansion, while FBS facilitated 2000 fold expansion. For ISC + DM, PL was capable of a 500,000 fold expansion of MSCs while FBS remained at 2000 fold expansion.