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TITLE: Mesenchymal Stem Cells for Vascular Target Discovery in Breast Cancer-Associated Angiogenesis

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Cancer growth and spread is dependent on new blood vessel formation, i.e. angiogenesis. A tumor mass cannot develop into a life-threatening condition without angiogenesis. Obstructing the recruitment of new blood vessels to the tumor through administration of antiangiogenic agents will hinder cancer progression. We propose the use of marrow stromal cells (MSCs) for an investigative gene discovery program to identify new genes involved in blood vessel formation. MSCs, a normal cell type from the bone marrow, can spontaneously turn into blood vessels (MSC-mediated vasculogenesis) in experimental animals. Therefore, we propose that MSCs recapitulate the ontogeny of blood vessel formation and serve to identify novel angiogenesis promoters and potential new pharmacological targets. To test this hypothesis, we will utilize a cell biology and molecular genetic experimental approach. Products thus identified as involved in MSC-mediated vasculogenesis may become new cancer “antiangiogenesis” targets for either a classic pharmacological approach or for cell and gene therapy therapeutic strategies. The utilization of antiangiogenic agents for cancer treatment holds certain advantages over chemotherapeutic drugs, such as the destruction uniquely of tumor-associated normal blood vessels and not of other normal tissue such as bone marrow. Also, unlike chemotherapy, drug resistance is not an issue with antiangiogenic compounds.
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INTRODUCTION

Progression and metastasis of breast cancer is dependent on neoangiogenesis (Folkman J. *Journal of the National Cancer Institute* 82:4, 1990), and host-derived mesenchymal progenitor cells contribute to this phenomenon (Hombauer H and Minguell JJ. *British Journal of Cancer* 82:1290, 2000). Marrow-derived stromal cells (MSCs) display mesenchymal plasticity, are amenable to *ex vivo* tissue culture and can serve as a robust model of mesenchymal morphogenesis. We recently demonstrated that culture-expanded MSCs can transdifferentiate into blood vessels expressing the endothelial marker CD31 *in vivo* and that they also recruit a robust, vascular endothelial growth factor (VEGF)-dependent, angiogenic response from host-derived structures (Al-Khaldi A. et al. *Gene Therapy* 10(8): 621-629, 2003). This discovery may allow us unprecedented insights in the biology of postnatal vasculogenesis and angiogenesis, especially in the setting of cancer.

RATIONALE AND OBJECTIVES

We propose that MSCs recapitulate the ontogeny of vasculogenesis and that the analysis of this process will give novel insights in the molecular genetic events associated with cancer-driven angiogenesis. Gene products identified as involved in MSC-mediated vasculogenic response to cancer may then become new breast cancer “anti-angiogenesis” targets.

Specific aims are to perform gene array analysis of vascular differentiated murine MSCs in the setting of breast cancer. Both *in vitro* and *in vivo* MSC differentiation – and gene expression profiling - will be analyzed and validated.

PROPOSED METHODS

We have already shown that a mixed population of MSCs harvested from C57Bl/6 mice can be retrovirally-labelled to express the green fluorescent protein (GFP) and clonal MSC populations can be isolated and phenotypically and genotypically analyzed by flow cytometry and gene chip analysis, respectively. We have also shown that MSCs can then be stimulated to differentiate *in vitro* into an endothelial phenotype in presence of recombinant mitogens such as, VEGF 185 and basic fibroblast growth factor (bFGF). The input and output MSCs under different “controlled” *in vitro* conditions can be compared and analyzed by gene expression profiling as detailed below. Furthermore, $10^6$ clonal “endothelial-null” MSC cells can be admixed in a 0.5 ml Matrigel plug and embedded in the subcutaneous space in recipient C57Bl/6 mice. As we have shown (Al-Khaldi A. et al. *Gene Therapy* 10(8): 621-629, 2003), a vasculogenic/angiogenic response will occur as early as 2 weeks post-implantation and persists for at least 4 weeks. The Matrigel implant can be easily removed 2-4 weeks post-implantation and up to $10^5$ live GFP-positive MSCs retrieved by collagenase digestion of the Matrigel plug and sorted by flow cytometry. Sorting of these retrieved cells based on co-expression of the GFP marker and cell-surface endothelial markers (such as CD31) will allow purification of input MSCs [and eliminate contaminating GFP-null host-derived cells] having undergone endothelial differentiation *in vivo.*
The number of MSCs thus extracted is sufficient to allow for linear RNA amplification to quantities amenable to analysis by Affymetrix-based gene chip technologies. We propose that comparing the gene expression profile of input "GFP+, endothelial-null" MSCs with output "GFP+, endothelial-positive" MSCs will allow us to define molecular genetic events associated with post-natal vasculogenesis. The experiments here proposed can be done with MSCs alone or with MSCs admixed with cancer cell lines in vitro and within a Matrigel plug in vivo. We will thus determine whether cancer-associated angiogenic/vasculogenic responses are qualitatively distinct from vascular differentiation in the absence of tumor cells. To test this hypothesis, we will use mouse models of breast cancer. 4T1 and DA3 are murine breast cancer cell lines that generate metastatic and local breast carcinoma respectively in Balb/c mice and both these cell lines will model for breast cancer-associated vasculogenesis. MSCs derived from Balb/c mice will be admixed with these breast tumor cell lines and will be analyzed in vitro and in vivo, as described above.

RESULTS and REPORTABLE OUTCOMES

Although we have not yet completed many of the objectives of the approved Statement of Work (indicated here below in italics) due to unexpected technical delays, we have conducted several related experiments beneficial to the advancement of this proposal and understanding of vasculogenesis.

**Task 1. In Vitro experiments, Months 1-3:**

*Perform gene array analysis of in vitro-induced vasculogenesis with murine marrow stromal cells (MSCs) in the presence of recombinant VEGF 185 and recombinant bFGF.*

We have isolated primary murine MSCs from C57Bl/6 mice and genetically engineered them in vitro with retroviral particles to express the green fluorescent protein (GFP) reporter. We selected monoclonal populations of these GFP+ murine MSCs and conducted flow cytometry analysis to determine their phenotype. Specifically, we determined if these cells express the following cell surface antigens: CD31, CD34, CD44, CD45, CD117, Flk1 and Tie2. Upon noticing that all clones were CD31- and that most clones were CD34- but one was CD34+, we investigated if CD34 expression had an effect on the potential endothelial plasticity of MSCs and consequently performed the in vivo experiments described under Task 2 here below. Moreover, total RNA was isolated from CD34+ and CD34- MSCs and gene expression analysis was conducted (please see manuscript attached). Briefly, gene expression analysis of these recovered MSCs indicated no significant differences in the expression of VEGFs A and B, but showed that CD34+ MSCs upregulated several supplementary angiogenesis-associated genes (please see manuscript attached).

**Task 2. In Vivo experiments without tumor cells, Months 4-6:**

*Perform gene array analysis of in vivo-induced vasculogenesis with Matrigel-embedded murine MSCs in the absence of tumor cells in Balb/c mice.*
Although not yet conducted with MSCs derived from Balb/c mice, we did however perform related in vivo experiments with MSCs derived from C57Bl/6 mice. More specifically, we selected from the above-mentioned GFP gene-modified MSC populations, one that was CD34+ and one CD34− and utilized these in our vasculogenic assay, whereby 4 million MSCs were admixed with a typeIV collagen-based matrix Matrigel™ in a final volume of 0.5ml. This Matrigel-MSC mixture was then injected subcutaneously in syngeneic C57Bl/6 mice where it formed a semi-solid implant which was surgically removed 15 days later and collagenase digested to recover the cells. Flow cytometry analysis was then conducted on the GFP+ MSCs and revealed that 2-5 % of these MSCs had transdifferentiated from CD31− to CD31+ cells and that the MSCs that were CD34+ appeared more prone to becoming CD31+ and to recruiting hematopoietic cells from the host animal. Gene expression analysis of these recovered MSCs has not yet been carried out.

Task 3. In Vivo experiments with breast tumor cells, Months 7-12:

- Perform gene array analysis of in vivo-induced vasculogenesis with Matrigel-embedded murine MSCs in the presence of DA3 breast tumor cells (Balb/c mice).

- Perform gene array analysis of in vivo-induced vasculogenesis with Matrigel-embedded murine MSCs in the presence of 4T1 breast tumor cells (Balb/c mice).

We have prepared a manuscript on the findings summarized above related to the effect of CD34 cell surface antigen expression on the in vivo ability of MSCs to differentiate into endothelial cells. The manuscript entitled “CD34 expression by murine marrow stromal cells and neovascularization” was submitted to Gene Therapy in February 2005. Please see manuscript attached

CONCLUSION

As indicated by our submitted manuscript, even though we have not yet completed many of the objectives of the approved Statement of Work due to unforeseen technical difficulties, we have performed related experiments for the advancement of this proposal and understanding of vasculogenesis.

We are therefore grateful to have been granted a one-year no-cost extension on this Concept Award as seen below by the document send to us by Sherry M. Apperson, Procurement Technician. This extension will allow us to meet our objectives and accomplish the required tissue culture, in vivo studies, and in vitro analysis including gene expression profiling. We have however generated reagents, become more familiar with technical procedures and performed preliminary studies as indicated above that are related to this Concept Award proposal and that will facilitate the execution of the proposed tasks and improve the understanding of vasculogenesis.
4th Feb 2005

Dear Dr Galipeau:
Title: CD34 Expression by Murine Marrow Stromal Cells and Neovascularization
Corresponding Author: Dr. Galipeau

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CD34 Expression by Murine Marrow Stromal Cells and Neovascularization

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Summary

We have previously shown that anchorage-dependent Marrow Stromal Cells (MSCs) induce neovascularization in vivo. It has been observed that CD34 expression in a mixed population of mouse MSCs is variegated. Since CD34 identifies hemangiopoietic precursors, we tested whether its expression could serve as a surrogate marker of MSCs possessing neovascularization potential.

A mixed population of MSCs derived from C57Bl/6 mice (phenotype: CD44+/CD13-/CD31-/CD45-/CD117-/Flk1-/Flt4-/Tie2- and 10-15% of these expressed CD34) were transduced to express the green fluorescent protein (GFP). Pure MSC/CD34+ and MSC/CD34null clones were selected, admixed with Matrigel™, and injected subcutaneously into isogenic mice. Implants were excised at 15 days, dissociated, and the single cell preparation submitted to flow cytometric analysis. GFP-expressing MSCs were analyzed for the acquired expression of the CD31/PECAM-1 endothelial marker (10.7 ± 8.4% of MSC/CD34+ expressed CD31 de novo vs. 3.1 ± 0.6% by MSC/CD34null; p<0.05). A significantly greater proportion of host-derived endothelial cells (ECs) (CD31+/CD45-) were recruited in the MSC/CD34+ implants. This advantage was maintained with regards to blood vessel density (BVD). Though not as robust as MSC/CD34+, the MSC/CD34null group recruited a greater proportion of ECs and had a greater BVD compared to controls (p<0.05). Gene expression profiling revealed no significant differences in the expression of VEGFs A and B. However, CD34+ MSCs upregulated a number of supplementary angiogenesis associated genes.
CD34+ and CD34null MSCs have the ability to initiate post-natal vasculogenesis and induce a mostly host-derived neovascularization. CD34+ cells are more efficient at recruiting host-derived ECs and differentiating into CD31+ cells in vivo. This suggests that CD34 expression is not a mandatory surrogate marker of angiogenic plasticity of MSCs, but is associated with a more robust host-derived vascular response.

*Key Words:* Angiogenesis, vasculogenesis, bone marrow stromal cells, CD34
Introduction

It has been established that blood, marrow and cord blood derived endothelial progenitor cells (EPCs) may participate in post-natal vasculogenesis. These circulating cells of vasculogenic potential have been defined(1;2) as expressing vascular endothelial growth factor receptor 2 (VEGF-R2/Flk1) and the cell-surface sialomucin CD34, a widely utilized surrogate marker of hematopoietic stem cells(3).

We have also described that marrow-derived, anchorage-dependent, murine marrow-derived stromal cells (MSCs) display vascular plasticity in an in vivo Matrigel plug angiogenesis assay and have the ability to recruit a host-derived angiogenic response.(4) However, in distinction to EPCs and mesenchymal adult progenitor cells (MAPCs), there is marked heterogeneity in the expression of CD34 by cultured murine MSCs in vitro(5). Since CD34 is widely utilized as a surrogate marker of progenitor and stem cells with potential vascular plasticity(6-9), we investigated whether MSC CD34 expression also predicted for prospective vasculogenic properties.
Results

Phenotype of cultured Marrow Stromal Cells and clonal subsets

MSCs derived from C57Bl/6 mice were retrovirally labeled to express GFP. These GFP+ MSCs were subsequently analyzed for expression of commonly reported markers of endothelial progenitors and stem cells. As demonstrated by figure 1a, the phenotype of the polyclonal MSC population was: 15% CD34+, >96% CD44+, and did not express: CD13, CD31, CD45, CD117, Flk1, Flt3 and Tie2. We isolated twenty homogeneous clonal MSC subsets and analyzed expression of the CD34 antigen. We were able to detect CD34 expression in 3 of 20 clones analyzed and the level of CD34 expression amongst these clones was variable (figure 1b). The expression of all other markers was the same as in the parental mixed population from which they were derived (data not shown). The MSC clonal population with the highest CD34 expression level (hereafter MSC/CD34+) and a randomly chosen MSC/CD34null clonal subset were used for all subsequent experiments.

CD34 expression and MSC vascular plasticity in vivo

We have previously reported that Matrigel-embedded MSCs elicit a robust angiogenic response in normal mice.(4) We here use this same assay to determine whether CD34 expression by MSC clonal subsets correlates with vascular plasticity and host-derived angiogenic response. In the following series of experiments, two GFP-labeled MSC clonal subsets – MSC/CD34+ and MSC/CD34null - were compared to mouse embryonal fibroblasts for their in vivo
effects. As detailed in materials and methods, four million MSCs were admixed in 0.5 ml of Matrigel and the plug injected subcutaneously in normal C57Bl/6 mice (n=4 per group). At 15 days post-implantation, mice were sacrificed and matrigel plugs examined in situ. In Figure 2, representative examples of matrigel plugs in mice are shown. We observed that macroscopic neovascularization of the plugs occurred only with MSC implants, whereas matrigel only or fibroblast-containing matrigel plugs were consistently avascular.

Matrigel implants were surgically resected and collagenase-dissociated to allow for quantification and analysis of cellular content. The total number of nucleated cells isolated from collagenase dissociated Matrigel implants varied amongst the groups (figure 3a). A significantly greater number of cells were retrieved from the MSC/CD34+, MSC/CD34null, and embryonic fibroblast implants when compared to the Matrigel-alone implant (4.2±0.7 vs 1.9±0.5 vs 2.4±0.2 vs 0.2±0.1 x 10^6 cells respectively; p<0.05). However, the largest amount of cells was recovered from the MSC/CD34+ implant when compared to all other groups (p<0.05). Cells retrieved from the dissociated Matrigel plugs were subjected to flow cytometric analysis. The expression of the GFP reporter allowed us to identify input MSCs and their differentiated progeny, whereas all GFPnull events were deemed to be host-derived. As demonstrated by figure 3b, the percentage of GFP-expressing cells of total was 1.1% for MSC/CD34+ and 2.5% for MSC/CD34null implants, respectively. The great majority of cells populating the Matrigel are host-derived (>97%). Analysis of the GFP-expressing cells for CD31 (PECAM) marker expression was performed to assess vasculogenic plasticity of
MSCs (Figure 3c). We found that a subset of both MSC/CD34⁺ and MSC/CD34null cells expressed CD31 de novo. As shown in Figure 3d, MSC/CD34⁺ generated significantly more CD31-coexpressing progeny than MSC/CD34null (10.7±8.4% vs 3.1±0.6% respectively; p<0.05). As can be extrapolated from the above data, we found that less than 2% of input GFP-expressing MSCs remained detectable by day 15, a feature shared by both MSC/CD34⁺ and MSC/CD34null cells. To test whether cell death was involved in the observed loss of MSCs, we enumerated the number of live MSCs maintained in a Matrigel plug kept in a humidified incubator. Analysis of dissociated Matrigel revealed that up to 90% of MSCs are lost within 72 hours (data not shown).

**Hematopoietic and endothelial host-derived cellular response to MSCs**

When the total cellular content from the dissociated Matrigel implants were subjected to flow cytometric analysis, FSC/SSC scattergrams revealed that multiple distinct cellular subsets populate the implant. Amongst these GFPnegative cells the vast majority were CD45⁺ hematopoietic cells, followed by CD45⁻/CD31⁻ and lastly CD45⁻/CD31⁺ cells likely endothelial in origin (Figure 4a). Host-derived endothelial cells were here defined as GFPnegative/CD31⁺/CD45⁻ and we used this as a surrogate measure of host-derived angiogenic response to implanted MSCs. Figure 4b reveals that the mean proportion of host-derived endothelial cells recruited by either MSC/CD34⁺ or MSC/CD34null implants was found to be significantly (p<0.05) greater than that associated with embryonal fibroblasts or
Matrigel only. MSC/CD34^+ implants led to the highest host endothelial response when compared with MSC/CD34^null (4.2±0.5% vs 2.8±0.6%; p<0.05).

**MSC subsets and Matrigel plug vascularity**

Histochemical staining with isolectin B4 of fixed and sectioned Matrigel plugs was performed to identify capillaries within the implants at 15 days (figure 5a). The mean capillary density for each test group was assessed. Figure 5b shows that capillary density in MSC/CD34^+ or MSC/CD34^null implants was found to be significantly (p<0.05) greater than that associated with embryonal fibroblasts or Matrigel. MSC/CD34^+ implants led to the highest capillary density when compared with MSC/CD34^null (132 ± 22 vs. 85 ± 17 BVHPF p<0.05).

**MSC CD34 Expression and Differential Gene Expression.**

Total RNA was extracted from three distinct sets of culture expanded MSCs were processed as described in Methods and analyzed with Affymetrix Murine Genome U74v2 chips (Affymetrix, Santa Clara, California), which contains ~30,000 mouse genes (n = 3 for each group). A total of 1244 genes showed at least a 1.5 fold difference between the CD34+ and CD34- MSCs, 729 genes were higher in CD34+ cells, while 515 genes were higher in CD34- cells (Figure 6). For a complete list of differential gene expression between CD34+ and CD34- MSC see data supplement 1. Established pro-angiogenic genes such as the VEGFs, angiopoietins, and FGF2 were not significantly altered between CD34+ and CD34- cells (Table 1), however several well established
angiogenesis-related genes including FGF7, and hepatoma-derived growth factor, were significantly elevated in CD34+ cells (Table 1). In addition to these well-established angiogenesis-related genes, several angiogenesis-associated genes were also increased in the CD34+ cells (Table 1).
Discussion

In a previous study(4), we have demonstrated that MSCs from C57Bl/6 mice can undergo vasculogenesis *in vitro*, and induce a mostly host-derived, VEGF-dependent, neovascularization *in vivo*. MSCs differ from other reported vasculogenic EPCs in several aspects. Unlike EPCs, we and others(5) have shown that endothelial markers such as CD31/PECAM-1 are absent in MSCs and that in distinction to related MAPCs(10;11), CD34 is expressed by a subset of cultured C57Bl/6 MSCs. Also, there is no expression of the VEGF receptor Flk1, such as observed in MAPCs and EPCs. Therefore, MSCs represent a distinct stem cell subset to EPCs, yet share vasculogenic properties which may be exploited clinically. The expression of CD34 on culture expanded MSCs is a well described property in inbred mouse strains(5), in particular C57Bl/6 - a frequently used animal model of regenerative medicine. CD34 expression is widely utilized as a surrogate marker of stem cells in an array of clinical and pre-clinical regenerative medicine applications. In the field of cardiovascular regeneration in particular, the use of CD34 marker expression often defines and limits the cellular materials - either marrow or blood-derived EPCs - under study(6-9). The underlying assumption being that CD34 defines a wide subset of cells within which cardiovascular plasticity potential exists. However, marrow-derived, anchorage-dependent, CD34null mesenchymal adult progenitor cells (MAPCs) can also acquire endothelial markers *in vitro* with growth factor stimulation and will participate in cancer-associated angiogenesis *in vivo.*(10;11) Therefore, the utility of CD34 as a surrogate marker of vasculogenic plasticity
may vary with the type of blood/marrow stem cell under study, and this needs to be defined in MSCs.

To answer this question, we isolated homogeneous clonal populations of CD34\(^+\) and CD34\(^{null}\) MSCs and analyzed their post-natal vasculogenic and neo-angiogenic potential. We characterized 20 clonal MSC sets and found 3 which expressed CD34, all the others being CD34\(^{null}\). The average level of expression of CD34 varied widely between the three CD34\(^+\) clonal populations, but was homogeneous within each clonal population. All MSC clones studied did not express the CD45 hematopoietic marker or CD31/PECAM-1, a mature endothelial marker. We chose to further characterize *in vivo* the MSC subset which expressed the highest level of CD34 and compared its vasculogenic and angiogenic behavior to a randomly chosen CD34\(^{null}\) MSC clonal subset. Our results clearly reveal that MSCs will undergo vasculogenic differentiation and acquire CD31/PECAM-1 *in vivo* and initiate a robust host-derived angiogenic response independently of their CD34 status prior to implantation. However, MSC/CD34\(^+\) were significantly more efficient than MSC/CD34\(^{null}\) at this process. We noted that the majority of input MSCs is lost from the implant after 14 days. *In vitro* experiments suggest that most of this loss may be due to demise of the MSCs following their harvest and admixture in Matrigel within the first 72 hours. We may speculate that cell survival signaling may be disrupted when anchorage-dependent MSCs are suspended in matrix lacking appropriate microenvironmental cues.
We were also able to characterize host-derived cells that were recruited by MSCs to the implants. The vast majority of these cells were CD45+ hematopoietic cells (approximately 65% of total) and a small percentage of infiltrating cells were host-derived endothelial cells. We show that MSC clonal populations exhibited a significant advantage in recruiting host endothelial cells over controls such as fibroblasts. It remains possible that the origin of these host endothelial cells may actually be from circulating EPCs that underwent post-natal vasculogenesis in situ. It was interesting to find that embryonic fibroblasts also recruited host endothelial cells though significantly less than that recruited by MSCs and may represent a non-specific host response to syngenic cellular implants. Previous studies have demonstrated that fibroblasts are known to elaborate growth factors under hypoxic conditions which are likely to exist in the Matrigel implant assay, and these mechanisms may be at work in our system.(12;13)

Our data strengthens our prior observation that MSCs will differentiate in to endothelial cells in vivo, and we now demonstrate that this potential exists irrespective of their CD34 status, at least in C57Bl/6 mice. We have focused on CD34 expression as a putative marker for vascular plasticity since it is the only hemangiopoietic marker -amongst those we tested as part of this report - whose expression levels differs between murine MSC clonal subsets. It is conceivable that in addition to CD34 other MSC cell surface marker such as CD117(14) or CD140a(15) may predict for enhanced vascular plasticity in vivo. However, the MSCs we studied did not express CD117 and the significance of this marker in
our and other vasculogenic systems remains to be defined (16). We have also observed that the bulk of the in vivo angiogenic response to MSCs, independently of their CD34 status, is host-derived. This strongly supports the notion that MSCs mediate their in vivo angiogenic effect via a paracrine mechanism (4;17). In contrast to the use of enriched "CD34+ peripheral cells" as has been championed in pre-clinical and clinical cardiovascular cellular regenerative medicine studies (8;18), lack of CD34 expression does not adversely affect the vasculogenic capabilities of MSCs in mice. This observation highlights the inadequacy of CD34 expression as a universal surrogate marker for stem cells capable of vascular regeneration and caution must be exercised in cell regeneration research by omitting important yet unrecognized CD34null stem cell subsets as here described.

Despite the fact that CD34 expression does not preclude vasculogenic potential of MSCs, CD34+ MSCs did create a denser and thus more complex vascular network. This may be due to functional differences in the two clonal populations particularly in their ability to stimulate the host-derived angiogenic response. To gain insight into potential mechanisms underpinning these differences, we compared the gene mRNA profiles of CD34+ and CD34null cells in vitro. Our analyses clearly distinguished the two populations as evidenced by the 6 fold difference in CD34 expression we found between the cell types (See supplemental data set). In total we found 1244 genes to be differential regulated between the groups, but focused our attention on those genes which were increased in the CD34+ cells and were secreted proteins. From this list,
candidate genes were further selected according to literature evidence of their ability to act as angiogenic factors. From this we identified four growth factors (FGF7, HDGF, NGFb, and BDNF) which can influence angiogenesis.(19-27) In particular, FGF7 and HDGF are interesting targets as protein expression for both these mediators have been identified in the media of marrow-stem cells.(17;28) We also identified and increase in expression of the extracellular matrix protein Tenascin C, which influences angiogenesis through VEGF(29;30) and Glypican can also influence the actions of VEGF(31), while Apelin is a recently established VEGF-independent angiogenic factor.(32) Together these results suggest that modulation of the extracellular environment by CD34+ may enhances the host derive vascular response. It is of particular interest that there was no significant difference in the expression of VEGF A and B irrespective of their CD34 status. The shared VEGF gene expression profile may explain the ability of CD34null MSCs to initiate angiogenesis and conciliates with our prior observation that VEGF is an essential component in this phenomena in vivo.(4)

Indeed, there are other reports describing the contribution of CD34-negative peripheral blood stem cells to vascular regeneration.(33;34) Based on our results, we surmise that CD34 in itself play little to no direct role in neovascularization, especially since the CD34 knock-out mice had no defined vasculopathic phenotype.(35) In humans, the expression of CD34 on human fetal stromal progenitors has been reported, though the frequency of CD34-expressing stromal progenitors decreases as gestational age progresses(36). Postnatal human marrow stromal precursors can also be recovered within the
CD34 fraction of marrow nucleated cells(37). However, there is broad consensus that culture-expanded human MSCs do not express the CD34 antigen(38;39). We must conclude that CD34null marrow-derived stem cells, including MSCs, will likely lead to a clinically robust, host-derived, angiogenic response in those anatomical compartments - such as heart and limb - where therapeutic neovascularization would be clinically desirable.
Materials and Methods

1. Harvest and culture of murine MSCs

Female C57Bl/6 mice were purchased from Charles River Laboratory (Laprairie Company, Quebec) from which bone marrow was harvested as previously described by us.(4) In brief, mice were sacrificed by CO₂ inhalation. Femoral and tibial bone marrow plugs were hydrostatically expelled and cells were plated on tissue culture dishes in growth media [Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% decomplemented fetal bovine serum and antibiotics (50 U/ml Penicillin G and 50 μg/ml Streptomycin from Wisent Inc.)] and incubated at 37°C in room air with 5% CO₂. To eliminate the hematopoietic portion of cells, the non-adherent portion was discarded five days later and media was replaced once per week. Anchorage-dependent MSCs were passaged 1:2 when the cell confluency became 80% to 90%. MSCs were cultured for ~15 passages. Animals were handled under the guidelines promulgated by the Canadian Council on Animal Care and with the Animal Welfare Act Regulations and other Federal statutes relating to animals and experiments involving animals, and adhere to the principles set forth in the Guide for Care and Use of Laboratory Animals, U.S. National Research Council, 1996.

2. Marrow Stromal Cell retroviral labeling and clonal selection

Culture expanded MSCs were retrovirally labeled to express the green fluorescent protein (GFP) reporter as previously described by us.(40) In brief, MSCs were transduced with retroparticles from ecotropic GP+E86 producers
encoding the AP2 retrovector construct(41) in the presence of 5μg/ml lipofectamine reagent (Invitrogen/Life Technologies). Following transduction, 100% of MSCs expressed the GFP reporter stably over time (data not shown). It has been previously shown that MSC clonal subsets with varying phenotypes will form when MSCs are plated at a low density.(42) We isolated MSC clonal populations by a similar limiting dilution technique. Following retroviral GFP labeling, polyclonal MSC's were plated at a low density (approximately 5-10 cells per cm²). As visible colonies formed, they were collected with the use of filter disks immersed in trypsin 0.05%. After several minutes of incubation, the disks were removed and placed on individual culture plates with media. Twenty clonal MSC subsets were subsequently culture-expanded in the same manner as the mixed populations. Two clones: MSC/CD34+ and MSC/CD34- were subsequently characterized in vivo.

3. Characterization of MSC phenotype – Flow cytometric analysis

Flow cytometric analysis was used to characterize the phenotype of the MSCs (both mixed populations and clonal subsets). Immunostainings were performed by incubating 1 x 10⁶ cells for 30 min at +4°C with the following monoclonal antibodies: biotinylated anti-mouse Flt4 (VEGFR-3, clone AFL4), Tie-2 (clone TEK4) from ebioscience (San Diego, CA), CD31(clone 290), CD34 (clone RAM34) revealed with streptavidin-CyCr and PE labeled anti-mouse CD13 (clone R3-242), CD44 (clone IM7), CD45 (clone 30-F11), CD117 (clone 2B8) and Flk1(VEGFR-2, clone Avas12) from BD biosciences (Mississauga, ON,
Cells were acquired and analyzed with CellQuest Pro software on a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA).

4. *in vivo* Matrigel angiogenesis assay

We have previously described the subcutaneous implantation of matrix-embedded MSCs admixed in Matrigel™ (Becton Dickinson, Mississauga, Ontario, Canada) to ascertain their vasculogenic and angiogenic properties *in vivo*.(4) In brief, MSCs are admixed in chilled liquid Matrigel, syringe loaded and injected subcutaneously where it forms a semi-solid plug. The plug can be surgically retrieved at latter time points and subjected to histological analysis as well as protease dissociation to retrieve populating cells for FACS analysis.

We used 5 animals to test each described clonal MSC population (MSC/CD34* and MSC/CD34*). We also utilized embryonic fibroblast derived from C57Bl/6 (n=5) and cell-free Matrigel (n=5) as controls. At the time of implantation, 8x10⁶ cells were resuspended in 1 ml of Matrigel™, in liquid form at 4°C. A volume of 0.5 ml of this cell/Matrigel mixture (4 million cells) was injected subcutaneously in the right flank in each test C57Bl/6 mouse. Fifteen days following implantation, mice were sacrificed and Matrigel plugs were excised for analysis. With gentle dissection, the Matrigel™ plug was removed, taking care to avoid puncturing or dividing the Matrigel™. Four implants from each group were reserved for FACS analysis of phenotype while 1 implant from each group was reserved for immunohistochemistry.
Live cells were retrieved from Matrigel plugs utilizing a protocol adapted from Salomon and al.(43) Briefly, retrieved implants were cut into small fragments in a solution of PBS 1X supplemented with 1.6 mg/ml type IV collagenase and 200 μg/ml DNase I (Sigma-Aldrich, Oakville, Canada,) at 37°C for 30 min. Cells were dissociated by repeated pipetting, reincubated at 37°C for 20 min, and washed. The dissociated implants were then resuspended in staining buffer (3% FBS, PBS), filtrated through a cell strainer 70μm nylon mesh membrane (BD discovery labware). The expression of the GFP reporter allows for the FACS analysis of progeny cells derived from input GFP-expressing MSCs from a GFP-negative host-derived cellular response.

5. Immunohistochemistry and quantification of Blood Vessel Density

At the time of implant harvest (15 days post-implantation), one mouse from each group had 1% paraformaldehyde injected into the left ventricle prior to sacrifice. The implants were removed and placed in 1% paraformaldehyde for 24 hours. Subsequently, they were fixed in 10% buffered formalin, and embedded in paraffin to be sectioned (5μm). The sections were stained with isolectin B4 using the Vectastatin® Elite ABC system (Vector labs). Vascular density was assessed histologically with the aid of an Olympus BX60 microscope. The number of vessels (defined as tubular structures within the Matrigel with a patent lumen and lined with endothelium) was tallied and the vascular density was expressed as blood vessels/high power field (BVHPF). The mean number of
blood vessels per 5 (×100 magnification) fields of view was quantified by an independent observer in a blinded procedure.

6. Microarray Analysis

Total cellular RNA was extracted was from cultured MSCs by placing cell pellets in lysis buffer, homogenized and applied to RNA purification columns according to manufactures instructions (RNeasy®, Qiagen, Missassauga, ON, Canada). After washing the columns, the bound RNA was treated with DNAse I, washed and eluted. Reverse transcription, labeling and array hybridizations were performed by the microarray facility at the Ontario Genomics Innovation Centre (OGIC) within the Ottawa Health Research Institute (OHRI). Biotin-labeled complementary RNA was purified, fragmented, and hybridized to Affymetrix Murine Genome U74v2 chips (Affymetrix, Santa Clara, California), which contains ~30,000 mouse genes (n = 3 for each group).

Scanned raw data were processed with Affymetrix GeneChip version 5.0 software. The average intensity value for each probe set, which directly correlates to messenger RNA abundance, was calculated as an average of fluorescence differences for each perfectly matched probe versus single-nucleotide-mismatched probe. This software also gives each gene a qualitative assessment of "absent" or "present" calls. Data sets on each GeneChip were then imported into BRB ArrayTools version 3.2.2, developed by Dr. Richard Simon and Amy Peng Lam, (http://linus.nci.nih.gov/BRB-ArrayTools.html) for further analysis. When being imported, an intensity filter removed all genes with
a intensity value below 500, followed by a detection call filter that removed 
probesets with detection calls that have a value of "absent" in 50% or more of the 
samples. After these filters were applied the data was log transformed and 
subjected to median normalization. The gene expression database was then 
subjected to a variety of analysis tools (i.e. scatterplot, clustering gene ontology 
comparison), a differential gene expression of 1.5-fold or more between CD34+
and CD34- MSC was considered significant.

7. Statistics

All results are expressed as means ± standard error of the mean (s.e.m). 
Proportions were compared using Chi-square test and student's t-test was used 
for continuous variables. All statistical analysis was performed using SPSS data 
analysis software.
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Title and Legends to Figures

Figure 1
MSC phenotypic analysis. Figure 1(a) reveals the flow cytometric analysis our mixed population of MSCs from which clonal subsets was isolated. Expression profiles for CD13, CD31, CD34, CD44, CD45, flk1, flt4 and Tie2 were performed as detailed in materials and methods. The fraction of positive events is indicated in those analyses that had detectable marker expression. Figure 1(b) demonstrates the variable expression of the CD34 antigen amongst MSC clones isolated via limiting dilution. The left panel depicts the analysis of a representative CD34-negative clone, the three panels to its right reveal the level of CD34 expression in the three clones where its expression was detectable. In vivo studies were performed with the clone with highest average expression level of CD34 (last panel on right). Specific antibody profile (solid line) versus isotype-matched antibody control (hatched line) is depicted.

Figure 2
Matrigel plug assay in vivo – gross macroscopic appearance. At 15 days post matrigel plug implantation, experimental mice were sacrificed and subcutaneous plugs exposed. Representative examples are shown of mice implanted with MSC/CD34⁺ (top left panel) and MSC/CD34null (lower left panel). There was clear gross evidence that neovascularization had taken place within and about
the MSC matrigel implants. In contrast, the fibroblast (top right panel) and matrigel-only (lower right panel) implants appeared relatively avascular.

Figure 3
Analysis of GFP-labeled MSCs and their progeny in dissociated matrigel pugs. Matrigel plugs from individual mice were harvested at 15 days post-implantation and dissociated into single-cell preparations. The total mean number of cells retrieved from each implant group (n=5 per group) is represented in fig 3(a). Fig 3 (b) depicts the proportion of GFP positive cells relative to all live cells retrieved from dissociated MSC matrigel plugs. Total cells retrieved from each MSC-containing matrigel plug was labeled for CD31 and analyzed for co-expression of GFP and a representative example of analysis is shown in Fig 3 (c), whilst the aggregate data for each test group (n=5, average± sem) is depicted in Fig 3 (d).

Figure 4
Analysis of CD31-expressing cells in dissociated matrigel plugs. Using CD45 and CD31 co-labeling and gating on dominant populations defined by FSC/SSC profile, we queried the content in hematopoietic cells (CD45+/CD31- & CD45+/CD31+) and endothelial cells (CD45+/CD31+). A representative analysis is depicted in fig 4(a) and reveals the multiple host-derived cell types are present within the implants at 15 days post-implantation. The proportion of CD45+/CD31+ endothelial cells for each test group (n=5, average± sem) is represented in fig 4(b).
Figure 5
Histologic analysis of angiogenesis in excised matrigel plugs. Vascular density within each implant group was performed using isolectin B4 immunostaining as described in materials and methods. Blood vessels were defined as tubular structures within the matrigel with a patent lumen and lined with endothelium. The vascular density was expressed as blood vessels/high power field (BVHPF). Representative histological sections (magnification x100) are shown for each test group (panel 5a) and average blood vessel density for each test group (n=5, average± sem) is represented (panel 5b).

Figure 6
Scatterplot of CD34 phenotype class based on differential gene expression. By plotting the average log-ratio of each gene that passed our filtering criteria for CD34- MSCs on the x-axis versus the average log-ratio of each gene that passed our filtering criteria for CD34+ on the y-axis we compared the differential gene expression patterns in CD34+ vs. CD34- MSCs. The majority of genes were plotted as points that line up along a 45-degree diagonal line (black points), while genes that are differentially expressed between the two CD34+ and CD34- phenotypes fall outside the outlier lines (red points). These outlier lines indicate genes for that the fold-difference between the geometric mean of the expression ratios within each of the two classes is greater 1.5.
Acknowledgements

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Reference List


(17) Kinnaird T, Stabile E, Burnett MS, Lee CW, et al. Marrow-derived stromal cells express genes encoding a broad spectrum of arteriogenic cytokines and promote in vitro and in vivo arteriogenesis through paracrine mechanisms. [see comment]. *Circulation Research* 2004; **94**(5): 678-85.


Phenotypic Characterization of MSCs

CD31  CD34  Flt4

15%

CD44  CD45  Flk1

>96%

Tie2  CD13  CD117

Variable Expression of CD34 Antigen in Clonal Populations

Figure 1a

Figure 1b
Characterization of Cellular Infiltrate within Matrigel Implants

Figure 4a

Host-derived Endothelial Cells

Figure 4b

* p<0.05 compared with control
** p<0.05 compared with control, and fibroblasts
*** p<0.05 compared with control, fibroblasts, and CD34-

Implant Groups
Immunohistological Staining of Blood Vessels

Figure 5a

Blood Vessel Density of Implants

* p<0.05 compared with control
** p<0.05 compared with control, and fibroblasts
*** p<0.05 compared with control, fibroblasts, and CD34-

Figure 5b
Figure 6