Mimicking the functional hematopoietic stem cell niche *in vitro*: recapitulation of marrow physiology by hydrogel-based three-dimensional cultures of mesenchymal stromal cells

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The online version of this article has a Supplementary Appendix.

ABSTRACT

Background

A culture system that closely recapitulates marrow physiology is essential to study the niche-mediated regulation of hematopoietic stem cell fate at a molecular level. We investigated the key features that play a crucial role in the formation of a functional niche *in vitro*.

Design and Methods

Hydrogel-based cultures of human placenta-derived mesenchymal stromal cells were established to recapitulate the fibrous three-dimensional architecture of the marrow. Plastic-adherent mesenchymal stromal cells were used as controls. Human bone marrow-derived $CD34^+$ cells were co-cultured with them. The output hematopoietic cells were characterized by various stem cell-specific phenotypic and functional parameters.

Results

The hydrogel-cultures harbored a large pool of primitive hematopoietic stem cells with superior phenotypic and functional attributes. Most importantly, like the situation *in vivo*, a significant fraction of these cells remained quiescent in the face of a robust multi-lineage hematopoiesis. The retention of a high percentage of primitive stem cells by the hydrogel-cultures was attributed to the presence of CXCR4-SDF1α axis and integrin beta1-mediated adhesive interactions. The hydrogel-grown mesenchymal stromal cells expressed high levels of several molecules that are known to support the maintenance of hematopoietic stem cells. Yet another physiologically relevant property exhibited by the hydrogel cultures was the formation of hypoxia-gradient. Destruction of hypoxia-gradient by incubating these cultures in a hypoxia chamber destroyed their specialized niche properties.

Conclusions

Our data show that hydrogel-based cultures of mesenchymal stromal cells form a functional *in vitro* niche by mimicking key features of marrow physiology.

Key words: functional niche, 3D-cultures, hydrogel, mesenchymal stromal cells, bone marrow, hematopoietic stem cells, hypoxia-gradient.

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Introduction

A stem cell niche has many functions some of which require the activation of conflicting mechanisms. While the niche is expected to preserve the stem cell pool, it also has to promote a continuous formation of differentiated progenitors to achieve a steady-state hematopoiesis. How these apparently contradictory and amazingly dynamic processes are driven by the niche continues to be an intriguing issue in stem cell research.

The importance of the niche-mediated regulation of hematopoietic stem cells (HSCs) became evident with the development of the Dexter-type long-term cultures,[1] a landmark technological innovation in the field. These cultures provided the first cellular platform to study the role of the microenvironment in controlling the fate of HSCs. Variants of these cultures, made up of irradiated/inactivated stromal cell lines (with or without genetic modifications) seeded with marrow cells, either un-fractionated mononuclear cells (MNCs) or purified HSCs, became popular tools to study stromal function. Though extremely useful, these cultures lacked the three-dimensional (3D) architecture of the marrow and, therefore, failed to mimic the *in vivo* niche. It became apparent that acquiring an in-depth understanding of the complex niche-functions, under both steady state and diseased conditions, requires the creation of an experimental system recapitulating the specialized properties of the marrow microenvironment.[2] Recent studies have clarified the role of the marrow microenvironment in the pathogenesis of hematologic tumors, underscoring the need for therapeutic targeting of the niche to achieve a complete, or at least a long-term, remission.[3,4] Therefore, the identification of target molecules that can be exploited to eradicate the leukemic stem cells from the niche has become the focus of intensive research. The availability of a culture system that closely mimics marrow physiology may speed up the development of new strategies to specifically target leukemic stem cells without adversely affecting normal stem cell self-renewal.

Accordingly, 3D-cultures using specialized scaffolds[5] or extra-cellular-matrix (ECM) molecules, like collagen and/or fibronectin and spheroid cultures of mesenchymal stromal cells (MSCs), were developed. MSCs form an important constituent of the marrow niche. Sacchetti *et al.* have shown that human $CD45^{-}146^{+}$ osteoprogenitor cells are able to transfer hematopoietic activity to an ectopic site.[6] An essential function of $Nestin^{+}$ MSC in the HSC niche has been documented in a mouse system.[7] These authors demonstrated that purified HSCs specifically home to $Nestin^{+}$ MSCs in the bone marrow of irradiated mice and $Nestin^{+}$ cell depletion results in a significantly compromised homing process. These reports, together with published data showing that the MSCs support the maintenance of HSCs *in vitro*,[8] suggest that MSCs are a suitable candidate to study niche function. The 3D-cultures of MSCs were shown to be superior to the traditional two-dimensional (2D) feeder layer cultures.

In the last decade, creating 3D-cellular micro-environments with hydrogels (a network of interacting polymer chains that are highly hydrated, with an elasticity similar to natural tissues) has progressed remarkably.[9] These microenvironments can be suitably tailored to achieve tissue-like structures *in vitro*. In the present study, we used the hydrogel-based 3D-culture system of MSCs (3D-MSCs) to investigate the features that are required to create the HSC niche equivalent *in vitro*.

Design and Methods

The mice, murine cells and humans cells used in this study are described in the *Online Supplementary Appendix*, as are details of CFU, LTC-IC and migration assays, flow cytometry, cell cycle analysis, division tracking studies, side population analysis, immuno-fluorescence studies, gene expression studies and statistical analysis.

Establishment of 2D and 3D cultures

The MSCs were seeded on a pre-set, medium-equilibrated 0.5% Puramatrix gel (BD; 3D-MSCs). The MSCs grown on plastic surfaces (2D-MSCs) were used as controls. Both 2D- and 3D-MSCs were characterized for the expression of MSC-specific markers. The adipogenic, osteogenic, as well as chondrogenic differentiation, of 2D- and 3D-MSCs was revealed by oil red O, alizarin red and alcian blue, respectively, after culturing them in differentiation media (InVitrogen; *Online Supplementary Appendix*).

Freshly isolated human $CD34^{+}$ cells (or Lin^{-} murine cells) were seeded in 7-day old 2D- or 3D-cultures. The cultures were fed every third day with growth medium supplemented with human (or murine) specific growth factors (Stem Cell Factor-(SCF), 50ng/mL; Interleukin (IL)-6, 50ng/mL and IL-3, 20ng/mL; Peprotech, Rocky Hill, NJ, USA). The cultures were harvested after seven days of co-culture and the output hematopoietic cells were subjected to various phenotypic and functional assays (*Online Supplementary Figure S2*).

In one set of experiments, MSCs were treated with a synthetic oxygen-carrier, perfluorotributylamine (5% w/v), (PFTBA, Sigma),[10] before seeding the $CD34^{+}$ cells in them. In another set, pre-set co-cultures were incubated with 50 μM of AMD3100, (Sigma) for 24 h before harvesting the hematopoietic cells. Supernatant and matrix-adherent fractions were collected and analyzed separately.

A schematic representation of the experimental design is shown in *Online Supplementary Figure S2*.

Results

3D-MSCs form an ECM- and integrin-rich microenvironment

Since the marrow microenvironment is enriched with ECM molecules, such as fibronectin, collagen IV, vitronectin and laminin, the 7-day old 3D-MSCs were subjected to immunofluorescence studies to examine the presence of these molecules. It was observed that the 3D-MSCs highly expressed various ECM molecules (Figure 1A).

The integrins, transducers of the 'outside in' signaling, form alpha-beta dimers when engaged by specific ECM molecules that act as their ligands and induce cell surface clustering. Immunostaining of the cells with antibodies that specifically detect dimeric forms of $\alpha5\beta1$, $\alpha4\beta1$, $\alpha2\beta1$ and $\alpha v\beta3$ integrins showed that, consistent with the high expression of various ECM molecules, the 3D-MSCs expressed high levels of active integrins on their surface (Figure 1B; *Online Supplementary Figure S1B*).

Quantitative PCR experiments (*Online Supplementary*

Table S3, Applied Biosystems, Foster City, CA, USA) performed to examine the mRNA levels of αv, α2, α5, α4 as well as β1 subunits showed that these subunits were upregulated under 3D conditions (*Online Supplementary Figure S1D*), indicating that the formation of dimeric active integrins on the surface of the 3D-MSCs by ligand-mediated clustering on the cell surface was also accompanied by transcriptional upregulation of integrin subunits.

Abundant growth of CD34⁺ cells in 3D-MSCs

At a concentration of 0.5%, Puramatrix (BD) allowed an efficient cell migration into the matrix, avoiding the stressful encapsulation step. The MSCs grew along with the matrix fibers assuming the matrix architecture (*Online Supplementary Figure S1C*) and formed a stable fibrous 3D structure (3D-MSCs) in seven days. The MSCs were analyzed for their capacity to undergo tri-lineage differentiation potential under 2D as well as 3D conditions to establish their multi-potency. The results showed that the tri-lineage differentiation capacity of the MSCs was not affected by the growth in hydrogel (*Online Supplementary Figure S1H*).

CD34⁺ cells were seeded on 7-day old 2D- or 3D-MSCs. Within a week, the hematopoietic cells were seen growing as large compact clusters in the 3D-cultures, whereas the 2D ones had scattered growth (*Online Supplementary Figure S1E*). The compact clusters were found to be populated by CD34⁺ cells (*Online Supplementary Figure S1F*). An abundant growth of CD34⁺ cells in 3D-cultures was observed compared to the sparse growth in 2D-cultures (*Online Supplementary Figure S1G*). The retention of a large number of CD34⁺ cells even after the extensive washing involved in the immunostaining procedure indicated that these cells were perhaps held in the matrix by stronger mechanisms than mere physical entrapment.

The hydrogel may provide an increased surface area, leading to a significantly increased number of MSCs present in the 3D-cultures as compared to the conventional plastic-adherent MSCs. It was possible that a larger number of MSCs in the 3D-cultures as a result of an increased surface area itself could explain the increase in numbers of hematopoietic cells observed under these conditions. In order to rule this out, we estimated the number of MSCs recovered from both types of cultures. The number of MSCs harvested from the hydrogel cultures was comparable with the number of MSCs present in the conventional culture (2D $5.04\times10^4\pm0.111$ *vs.* 3D $4.686\times10^4\pm0.22$; $P=0.21$, NS; n=7), ruling out the possibility that the 3D-MSCs provide a better hematopoietic support simply by being more numerous.

The harvested cells were subjected to flow cytometric analysis to count the number of CD34⁺ cells in them. The total yield of hematopoietic cells (*Online Supplementary Figure S1I*) and CD34⁺ cells (Figure 1C) from the 3D cultures was significantly higher than that from the 2D cultures, indicating that the hydrogel-based cultures of MSCs provide a highly supportive microenvironment for the growth of CD34⁺ cells (Figure 1C).

3D-MSCs foster a robust multi-lineage hematopoiesis

To examine whether the output CD34⁺ progenitors are functional, we subjected these cells to *in vitro* functional assays (CFU and LTC-IC). This showed that the 3D-HSCs indeed contained a significantly high number of CFU and LTC-IC units in them (*Online Supplementary Figure 1J and K*) confirming that the 3D-cultures contain a significantly high number of functional primitive progenitors.

Since these functional assays detect only myeloid progenitors, we used flow cytometry to enumerate lymphoid progenitors. The 3D-CD34⁺ population had a significantly higher percentage of progenitors committed to both lymphoid as well as myeloid lineages as compared to the 2D ones (*Online Supplementary Figure S1L*). This indicated that the 3D-MSCs indeed provide a superior support for an active multi-lineage hematopoiesis to take place *in vitro*.

3D-MSCs harbor primitive HSCs

The next obvious experiment to carry out was to examine whether the 3D-cultures could maintain the primitive stem cells, or whether the observed robust multi-lineage commitment was taking place at their expense. For this, we analyzed the percentage of $CD45^+34^+38^-Lin^-$ cells in the output population. We observed that the 3D-cultures were enriched for $CD45^+34^+38^-$ Lin^- cells as compared to the output of the 2D ones (Figure 1D and E). The net yield of $CD45^+34^+38^-Lin^-$ cells under 3D conditions was significantly higher than that under 2D conditions (Figure 1F, *Online Supplementary Figure S3D*).

The MSCs obtained from different sources can have different properties. We compared the hematopoietic support given by placenta- or marrow-derived MSCs to determine whether the source of the MSCs affected the outcome. It was observed that MSCs obtained from both sources show the same effect on hematopoiesis under 2D as well as 3D conditions (*Online Supplementary Figure S3A and B*)

We then analyzed the output cells for the expression of two important markers of primitive HSCs: CD133 and CXCR4. We found that the CD34⁺ cells from 3D-cultures contained a significantly higher percentage of cells expressing these markers (Figure 1G, *Online Supplementary Figure S3C*), indicating that the 3D-cultures retain a significantly higher number of primitive HSCs and that the formation of a large number of committed progenitors was not leading to an exhaustion of the stem cell pool. Consistent with their high expression of CXCR4, the 3D-CD34⁺ cells migrated towards SDF1α in significantly higher numbers compared to the 2D-CD34⁺ cells (*Online Supplementary Figure S3E*).

Although its role in HSC maintenance and function *in vivo* is still a subject of debate,[11] it is likely that N-Cadherin is an important component for anchoring HSCs in their niche.[12,13] In a co-culture model, N-Cadherin was found to be necessary for the interaction of the human CD34⁺ cells with the MSCs.[14] We, therefore, examined the expression of N-Cadherin in the CD34⁺ cells grown in 2D- or 3D-MSCs by performing immunofluorescence experiments. We found that most 3D-CD34⁺ cells expressed N-Cadherin, albeit at varying levels, whereas such cells were nearly absent in the 2D-cultures (*Online Supplementary Figure S3F and G*).

Side population (SP) phenotype is yet another marker of primitive stem cells with the ability to repopulate.[15] To ascertain whether the 3D-cultures harbor this population, we analyzed the output cells for the presence of SP cells. As seen in Figure 1H, the 3D-cultures harbored an approximately 2.5-fold higher percentage of SP cells compared to that in the 2D cultures (*Online Supplementary Figure S3H*).

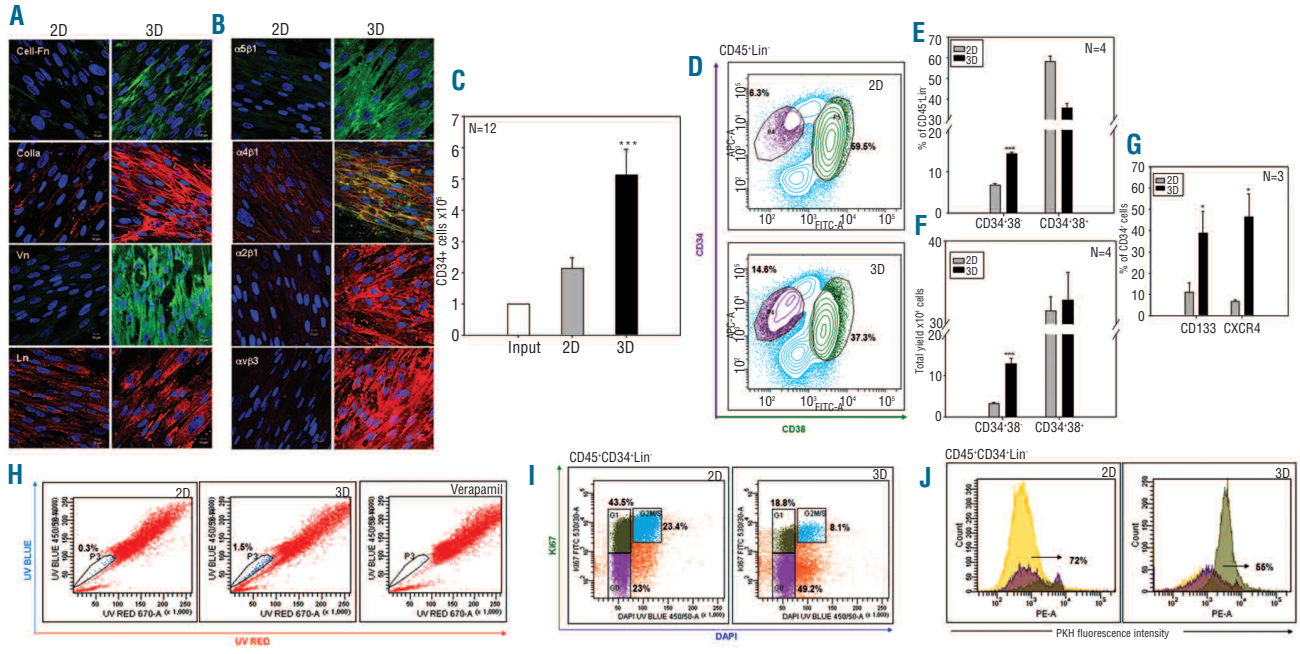

Figure 1. 3D-MSCs mimic marrow microenvironment and foster primitive stem cell pool. (A) Confocal microscopy analysis showed that the 3D-MSCs express high levels of ECM molecules compared to the 2D-MSCs. (B) Use of antibodies specifically detecting dimeric forms of α5β1 (FITC), α2β1 (Cy3) and αvβ3 (Cy3) integrins in the confocal microscopy analysis revealed that the 3D-MSCs express active integrins on their surface. Formation of α4β1 dimer (yellow) has been documented by double immuno-fluorescence staining using antibodies to α 4 (Cy3) and β1 (FITC). (C) 3D-MSCs support robust hematopoiesis and there is extensive proliferation of $CD34^+$ cells. Quantification of the total number of $CD34^+$ cells revealed that the 3D-MSCs yield a significantly higher number of total hematopoietic cells compared to their 2D counterparts. (D) Flow cytometric analyses of the output populations show that the 3D-MSCs yield a higher % of $CD45^+34^+38^-Lin^-$ HSCs compared to the 2D-MSCs. Analysis of 4 independent experiments (mean ± S.E.M.) shows that the percentage (E) and the total yield (F) of the $CD34^+38^-Lin^-$ HSCs is significantly higher in the 3D-MSCs compared to the 2D- MSCs (n=4; ***$P<0.001$). (G) 3D-HSCs are primitive. The data obtained in 3 independent experiments (mean ± S.E.M.) show that the % $CD45^+34^+133^+$ and $CD45^+34^+CXCR4^+$ cells in the 3D-MSCs are significantly higher compared to the 2D-MSCs (n=3; *$P<0.05$). (H) The flow panel shows that the 3D-MSCs harbor a higher percentage of SP cells (middle panel) compared to the 2D (left-hand panel). The verapamil-sensitivity of the gated population is shown in the far right panel (n=4; $P<0.05$). (I) HSC-quiescence is maintained in the 3D-MSCs. Cell cycle analysis of the gated $CD45^+34^+Lin^-$ population shows that a higher % of these cells was present in G0 state (violet) compared to the 2D (n= 3; $P<0.01$). (J) Division history tracking of HSCs. Flow cytometric analyses of the PKH26-labeled $CD34^+$ cells after seven days of co-culture showed that a majority of the $CD45^+34^+Lin^-$ population from 3D-MSCs (green histogram) did not divide during the culture period, whereas more than 70% of their 2D counterparts (yellow histogram) had undergone proliferation (n=7; $P\leq0.001$).

3D-MSCs form a functional niche

Maintaining a large pool of quiescent stem cells is a critical niche characteristic. To examine whether the 3D-cultures mimic the *in vivo* niche in this respect, we analyzed the cell cycle status of the output $CD45^+34^+Lin^-$ cells from both 2D- and 3D-cultures. A much larger percentage of the 3D-HSCs was maintained in the GO stage of the cell cycle compared to that in the 2D-HSCs (Figure 1I). The data obtained in 3 independent experiments showed that the result was reproducible and statistically significant (*Online Supplementary Figure S3I*).

Though the 3D-cultures possessed a large proportion of HSCs in quiescent state, it was not apparent whether they had acquired a post-cycling quiescence or whether they had not entered the cell cycle at all. In order to address this, we seeded PKH26-labeled $CD34^+$ cells in the cultures; after seven days, we tracked the division history of $CD45^+34^+$ Lin- HSCs by flow cytometry. Our results show that approximately 50% of the gated 3D-HSCs had retained a high PKH-fluorescence (green), indicating that the majority of them had not divided during the culture period, while over 70% of their 2D counterparts had undergone more rounds of proliferation (yellow) indicated by their low PKH26-fluorescence (Figure 1J). Our analysis of 7 independent experiments showed that the result was consistent and statistically highly significant (*Online Supplementary Figure S3J*). This shows that the 3D-MSCs form a functional niche and foster a significantly large pool of quiescent HSCs by actively preventing their cycling.

3D-MSCs foster stem cells having superior in vivo engraftment potential

Engraftment in NOD/SCID mice, especially in the secondary recipients, reflects the functionality of the human LT-HSCs. We injected sublethally irradiated NOD/SCID mice with 5×10^5 cells harvested from 2D-/3D-cultures and monitored their engraftment after 12 weeks. The 3D-HSCs engrafted more efficiently compared to the 2D-HSCs (Figure 2A and B).

To further monitor the ability of primary engrafted HSCs to maintain long-term repopulation, we performed secondary transplant assays; 3D-HSCs gave a 1.8-fold greater engraftment in secondary recipients as compared to that given by the 2D-HSCs (*Online Supplementary Figure S4A and B*). This shows that the 3D-cultures harbored more LT-HSCs than their 2D counterparts.

3D-HSCs exhibit a strong competitive engraftment potential

Since we could not assess the competitive repopulation ability of the HSCs in a NOD/SCID model, these studies were performed using the CD45.1/45.2 chimera model. The model was first validated by creating 3D-cultures with murine MSCs and seeding murine Lin$^-$ cells in them. The output cells were analyzed for primitive stem cells by multi-color phenotypic analyses; 3D-cultures of murine MSCs yielded a much higher proportion of LT-HSCs (LSK-CD34^{low})[16] compared to those harvested from 2D-cultures (*Online Supplementary Figure S4C*).

CD45.1$^+$ cells grown in 2D-cultures were mixed with an equal number of CD45.2$^+$ cells grown in 3D-cultures along with unmanipulated marrow cells from the F1 mice and were injected intravenously into lethally irradiated (900 rads) F1 recipients (CD45.1 x CD45.2) to assess their head-to-head competitive engraftment potential;[17] 3D-HSCs

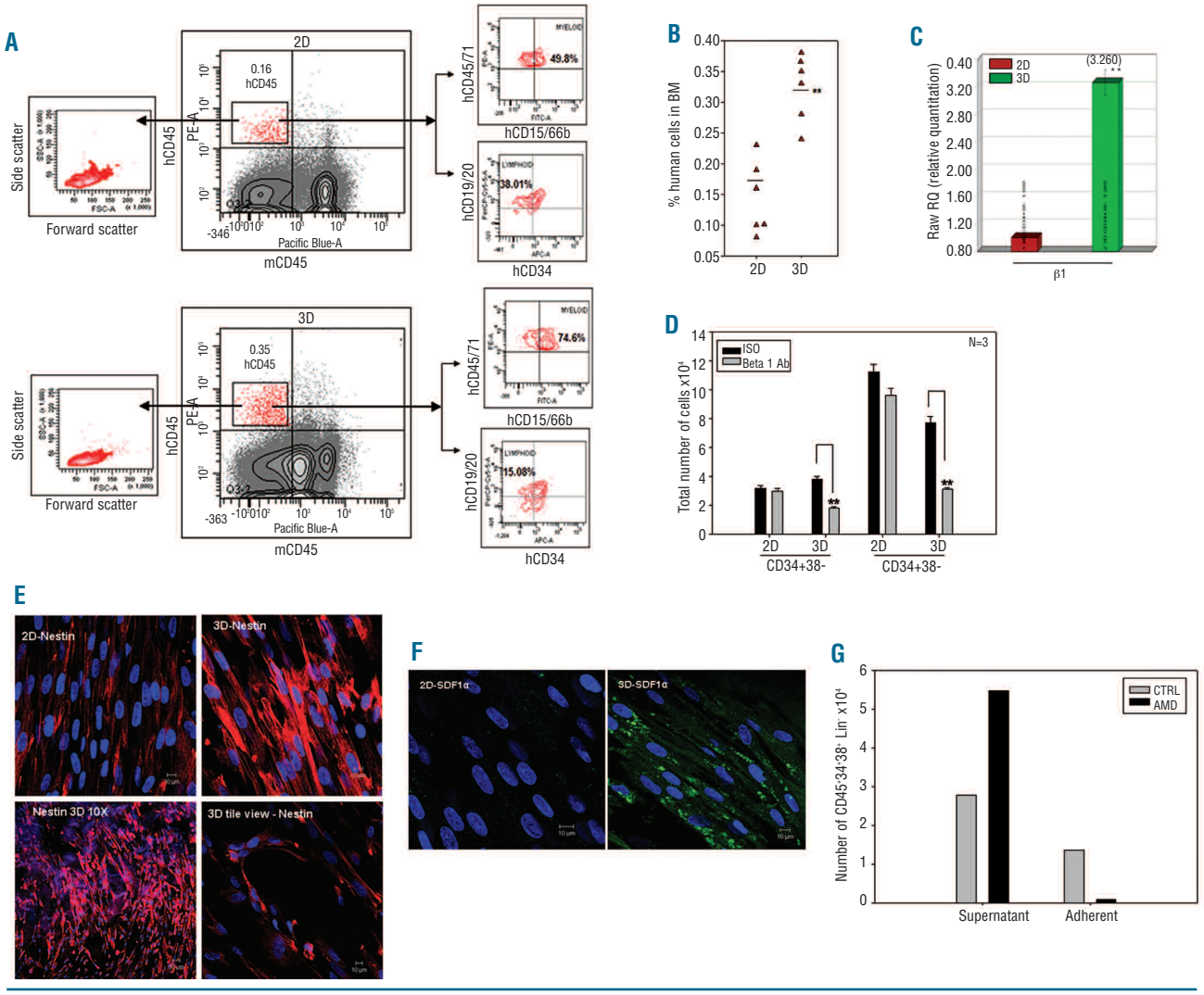

Figure 2. 3D-HSCs exhibit superior *in vivo* attributes and 3D-MSCs express HSC-supportive transcriptome and proteome. (A) 3D-HSCs contain a higher number of SCID-mouse repopulating (SRC) units. Output hematopoietic cells were injected into sublethally irradiated NOD/SCID mice. Twelve weeks after the transplantation, the percentage of human CD45$^+$ cells was analyzed using a flow cytometer. The hCD45$^+$ gated population was further analyzed for multi-lineage hematopoiesis using myeloid- and lymphoid-specific markers. Both populations gave rise to myeloid and lymphoid cells, with the 3D-HSCs showing a slight bias towards the myeloid side. Representative flow cytometric analysis of the recipient bone marrow cells is shown. (B) The scatter plot shows that the engraftment by the 3D-cells was significantly higher compared to the 2D-cells (n=6; ***P*<0.01). (C) CD45$^+$34$^+$38$^-$Lin$^-$ cells were sorted from 2D, 3D cells cultures and checked for the expression of β1 integrin by real time PCR. 3D-HSCs express higher levels of β1 integrin mRNA compared to the 2D-ones (n=3; ***P*<0.01). (D) Integrin β1-mediated adhesive interactions are involved in the 3D-cultures. Antibody-mediated neutralization of β1 integrin function significantly reduced the output of primitive cells from the 3D-MSCs (n=3; ***P*<0.01). The output of HSCs from the 2D-cultures remained unaffected. (E) 3D-MSCs are Nestin-positive. Confocal microcopy analysis revealed that the 3D-MSCs strongly express Nestin compared to the 2D-MSCs. Both low power and tile view (pixel size 1024X1024) images of Nestin-positive 3D-MSC are shown in the lower panel. DAPI (blue) demarcates nuclei. (F) The confocal laser micrograph shows a strong intracellular fluorescence (FITC) for SDF1α in the 3D-MSCs compared to the 2D, indicating their potential to exert a chemotactic attraction on the HSCs. (G) 3D-MSCs mimic marrow physiology. Flow cytometric analysis of the cells collected after 24 h of AMD3100 treatment from the supernatant *vs.* the matrix-adherent fractions showed that the addition of AMD3100 in the 3D-cultures resulted in mobilization of almost 2-fold higher number of CD45$^+$34$^+$38$^-$Lin$^-$ HSCs from the matrix to the supernatant fraction, indicating the presence of SDF1α-CXCR4 axis in these cultures (n=2).

showed greater engraftment potential than the 2D-HSCs in F1 recipients (*Online Supplementary Figure S4D-F*) and gave rise to both myeloid and lymphoid progeny (*Online Supplementary Figure S4D, right hand panel*), indicating their biological superiority.

3D-MSCs express HSC-supportive transcriptome and proteome

The phenotypic profile of the MSCs grown under 2D *vs.* 3D conditions was assessed using MSC-specific markers to examine whether a growth in hydrogel leads to any

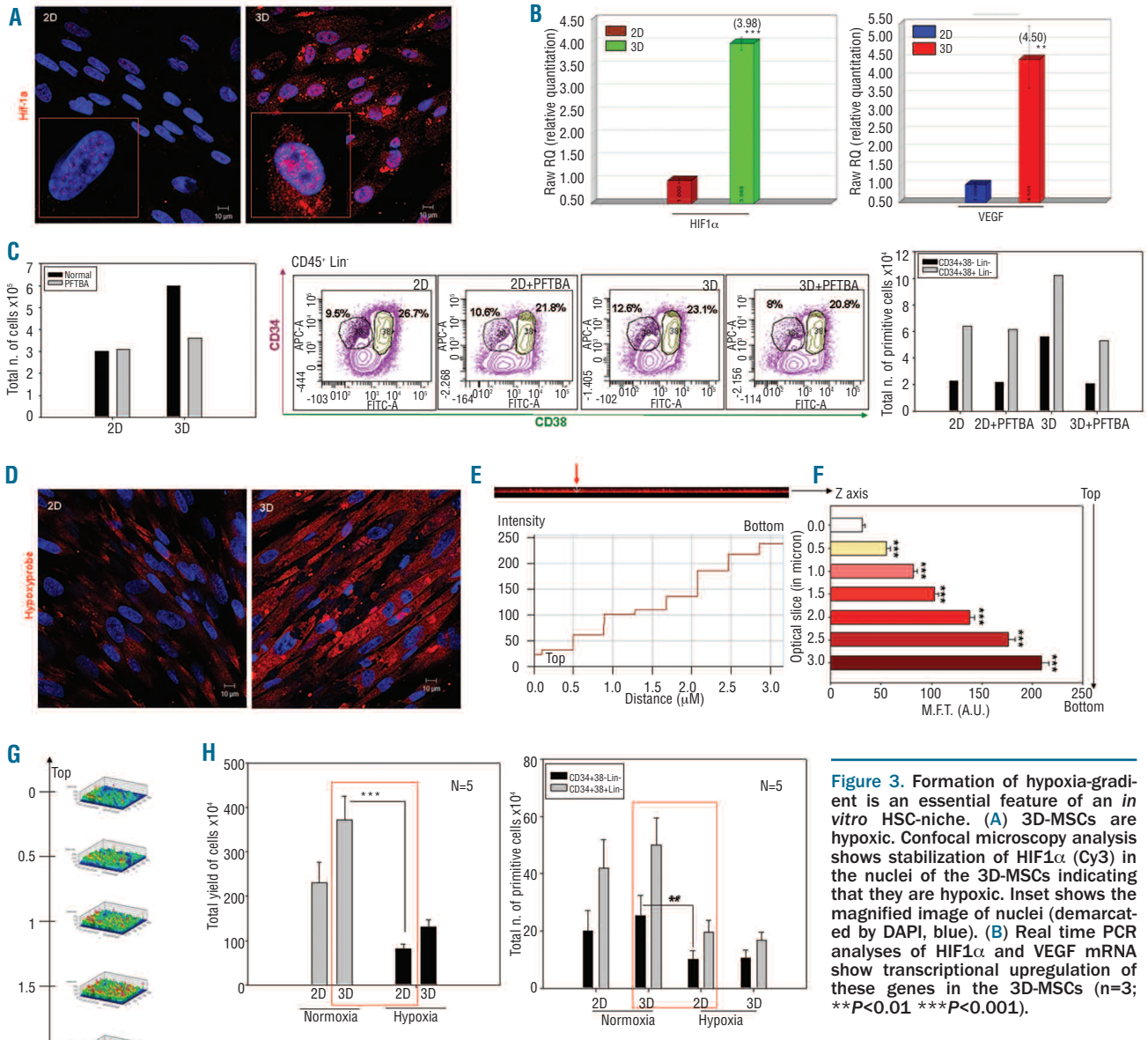

Figure 3. Formation of hypoxia-gradient is an essential feature of an *in vitro* HSC-niche. (A) 3D-MSCs are hypoxic. Confocal microscopy analysis shows stabilization of HIF1α (Cy3) in the nuclei of the 3D-MSCs indicating that they are hypoxic. Inset shows the magnified image of nuclei (demarcated by DAPI, blue). (B) Real time PCR analyses of HIF1α and VEGF mRNA show transcriptional upregulation of these genes in the 3D-MSCs (n=3; $^{**}P<0.01$ $^{***}P<0.001$). (C) Oxygenation of the 3D-cultures with a synthetic oxygen carrier, PFTBA, offsets their HSC support. PFTBA (5% w/v) was added in the MSCs before seeding the $CD34^+$ cells in them. The output cells were analyzed for the % $CD45^+34^+38^-Lin^-$ HSCs. The left hand panel shows the total cell yield and the middle panel shows representative flow data. The right hand panel shows that PFTBA affected the output of HSCs from the 3D-MSCs, but had only a marginal effect on the 2D output (n=2). (D) 3D-MSCs possess hypoxia-gradient. Confocal microscopy analysis of the Hypoxyprobe-stained (Cy3) cultures revealed that the 3D-MSCs are hypoxic. (E) A series of optical sections were captured to quantify the fluorescence intensity as a function of distance from the top of the 3D-cultures. A z axis view of a representative stack is depicted in the panel and the point of analysis is marked by a red arrow. (F) The software-generated analysis of mean fluorescence intensity (M.F.I.; measured as arbitrary units - a.u.) as a function of distance at the selected point on the stack is shown (n=14; $^{***}P<0.001$). The z axis analyses and the corresponding 2.5D analyses of the optical sections (G) using LSM5 image examiner revealed that the 3D-MSCs possess a "hypoxia-gradient". (H) Presence of hypoxia-gradient, rather than total hypoxia is necessary to create an HSC-niche *in vitro*. Both 2D- and 3D-MSCs were incubated under normoxia (20% O_2) or hypoxia (1% O_2). The yield of hematopoietic cells from both 2D- and 3D-cultures was drastically affected under the hypoxic conditions (left hand graph). The $CD45^+34^+38^-Lin^-$ and $CD45^+34^+38^+Lin^-$ HSCs were quantified in the output population (right hand graph). The near identical yield of primitive population from 2D- and 3D-cultures incubated under hypoxia (last two paired bars, right hand panel) indicates that a complete hypoxia offsets the HSC-supportive ability of the 3D-MSCs. Data marked with red boxes highlight results obtained with 3D-cultures incubated under normoxia (our system) *vs.* 2D-cultures incubated in hypoxia chamber (standard approach) (n=5, $^{**}P\leq0.01$, $^{***}P\leq0.001$).

change in the profile. It was observed that the phenotypic profile of the 3D-MSCs was comparable to that of the 2D-MSCs. A difference in the expression level of CD146 was, however, noted. While a large percentage of the 2D-MSCs was CD146$^+$, the vast majority of the 3D-MSCs did not express CD146 (*Online Supplementary Figure S5A*).

The BM niche is known to express some important key molecules playing a critical role in the development of effective hematopoiesis: osteopontin,[18] runx-2[19] and angiopoietin-1.[20] It was, therefore, essential to quantify the expression of these genes in the 3D-MSCs at mRNA levels. The transcripts of all these molecules were higher in the 3D-MSCs compared to those in the 2D-MSCs (*Online Supplementary Figure S5B*). Osteopontin-β1[18-21] and angiopoietin-Tie-2[20] axes are known to anchor the HSCs to the niche and also to maintain their quiescence. Correspondingly, the 3D-HSCs expressed high levels of Tie-2 (*Online Supplementary Figure S5D*) and β1 integrin (Figure 2C) mRNA suggesting that perhaps both axes are operative in the 3D-cultures.

Expression of β1 integrin on stem cells has been shown to play a major role in their anchoring to the niche.[8,21] In order to examine the role of β1 integrin in the retention of stem cells in the 3D-cultures, 10 μg/mL anti-β1 neutralizing antibody (or its isotype; R&D Systems Inc. Minneapolis, USA.) was added in both 2D and 3D-MSCs. They were seeded with CD34$^+$ cells pre-treated with anti-β1 antibody (or its isotype). The incorporation of the neutralizing antibody to β1 integrin resulted in a significant reduction in the yield of both CD45$^+$CD34$^+$38$^-$Lin$^-$ and CD45$^+$CD34$^+$38$^+$Lin$^-$ cells from the 3D-MSCs, but not from the 2D-MSCs, suggesting that β1 integrin-mediated interactions contribute towards the retention of HSCs in 3D-MSCs (Figure 2D).

Since the β1 integrin subunit partners with several alpha subunits, we also examined their expression; 1.5-fold upregulation of α4 was seen in 3D-HSCs compared to 2D-HSC. Integrins α2, α5 and α2b were found to be down-regulated in 3D-HSCs (*Online Supplementary Figure S5C*). The significantly high expression of α4 and β1 in 3D-HSCs show that they are more primitive in nature and more competent to interact with the niche.[21,22]

Nestin-positive MSCs have been recently shown to form an important HSC-niche component.[7] We, therefore, examined the expression of nestin in our cultures. The 3D-MSCs were more strongly positive for nestin compared to the 2D-MSCs at both translational (Figure 2E) and transcriptional (*Online Supplementary Figure S5E*) levels.

The data indicate that the expression of HSC supportive transcriptome by the 3D-MSCs contributes substantially to the maintenance of a quiescent HSC pool in these cultures.

3D-MSCs retain the HSCs via the SDF1α/CXCR4 axis

The HSCs are retained in the marrow via the SDF1α/CXCR4 axis. A disruption of this axis leads to an egress of the HSCs from marrow to peripheral blood circulation.[23] The high yield of functionally and phenotypically superior HSCs from the 3D-cultures suggested that these cultures not only supported the growth of HSCs, but also efficiently retained them. Earlier experiments have already shown that a high percentage of CD34$^+$ cells expressed CXCR4 and exhibited an increased migration towards the SDF1α gradient. In these experiments, we assessed the SDF1α expression in the MSCs by immunofluorescence and real time PCR and found that the 3D-MSCs expressed a high level of this chemokine as compared to the 2D-MSCs (Figure 2F, *Online Supplementary Figure S5F*). These data suggested that the presence of such active chemokine axis may be responsible for a high content of HSCs in these cultures. To validate this, we added AMD-3100, a CXCR-4 antagonist, in the pre-set 3D-co-cultures and assessed the percentage of CD45$^+$34$^+$38$^-$Lin$^-$ HSCs present in the supernatant *vs*. the matrix-adherent fraction. We observed that the addition of AMD-3100 resulted in a mobilization of HSCs from the matrix into the supernatant (Figure 2G), supporting our belief that the 3D-MSCs retain the HSC pool via an active SDF1α/CXCR4 axis, and mimic the *in vivo* marrow physiology.

3D-MSCs are hypoxic

The presence of hypoxia is a striking feature of the BM niche. Several reports have underscored its importance in HSC biology.[24-26] We, therefore, conjectured that the superior HSC-supportive ability of the 3D-cultures may be related to hypoxia. Nuclear localization and transcriptional upregulation of HIF1α in the 3D-MSCs (Figure 3A and B) clearly supported our interpretation. Consistent with these data, the 3D-MSCs expressed a 4.5-fold higher expression of VEGF at mRNA level (Figure 3B), a downstream target of HIF1α and a cytokine having an important role in HSC maintenance.[27] Re-oxygenation of cultures using PFTBA[10] abolished the advantage offered by the 3D-MSCs (Figure 3C, left-hand panel). The percentage and total yield of CD45$^+$34$^+$38$^-$Lin$^-$ primitive HSCs in the 3D-MSCs were specifically affected (Figure 3C, middle and right hand panels), indicating that the hypoxia prevailing in the 3D-MSC cultures was perhaps responsible for their superior HSC support.

CD146 has been shown to be down-regulated under hypoxic conditions.[28] The phenotypic characterization of MSCs grown under 2D- and 3D-conditions had shown that most 2D-MSCs were CD146$^+$, while most 3D-MSCs did not express CD 146 (*Online Supplementary Figure S5A*), further supporting our contention that the 3D-MSCs are hypoxic.

Hypoxia-gradient is crucial to mimic the niche function in vitro

The presence of 'hypoxia-gradient' in the marrow microenvironment plays a critical role in the preservation of the stem cell pool while millions of committed progenitors and differentiated cells are being formed continuously.[29] Hypoxyprobe (pimonidazole hydrochloride, Chemicon International, Temecula, CA, USA) has been used to quantitatively detect hypoxia in cells and tissues.[30] Immunostaining of 2D- and 3D-MSCs with hypoxyprobe not only confirmed that the 3D-MSCs are hypoxic (Figure 3D), but the image analysis of optical stacks through the z axis and 2.5D analysis of serial optical sections (Figure 3E-G) clearly showed that, like the *in vivo* marrow microenvironment, a steep hypoxia-gradient was present in them.[29] The mean fluorescence intensity of the hypoxyprobe was seen to increase from the surface of the culture to the bottom of the culture and the intensity difference at each optical slice captured at 0.5 micron was statistically highly significant (Figure 3E and F). The destruction of the hypoxia gradient by the incubation of cultures in hypoxia chambers (1% oxygen) resulted in a drastic decrease in the total hematopoietic cell output and the CD45$^+$34$^+$38$^-$Lin$^-$ /

$CD45^+34^+38^+Lin^-$ cell output from both 2D- and 3D-cultures (Figure 3H).

The data show that an ECM- and integrin-rich environment, HSC-supportive transcriptome, SDF1α-CXCR4 chemokine axis, beta1-mediated adhesive interactions and hypoxia-gradient are the crucial parameters required to mimic the HSC niche *in vitro*.

Discussion

Though the importance of the microenvironment in HSC biology had been shown through *in vitro* experimental systems, the specialized niche properties have been difficult to recapitulate in culture. Therefore, the search for an experimental system that closely resembles its *in vivo* counterpart still continues.[5,9] The development of 3D-culture systems gained importance with the realization that the behavior of normal and neoplastic cells growing under 3D conditions vastly differs from the behavior of cells growing on flat surfaces.[31] The 3D-cultures allow a reconstruction of the complex tissue architecture, thus providing a better platform to study cellular biology.

A culture of multiple cell types on hydrogels improves their function relative to the conventional cultures.[9] In the present study, we used hydrogel-based cultures to investigate all the important features that are necessary to create an *in vitro*-equivalent of the HSC-niche. Instead of tailoring the composition of this matrix with purified ECM molecules, we formulated these cultures with MSCs to facilitate *in situ* analyses of the HSCs within this *in vitro* niche. The hydrogel-grown MSCs were found to secrete high levels of ECM molecules showing that they themselves were capable of adding a physiologically relevant dimension to the culture system.

We observed that the MSCs grew with the matrix fibers and formed a meshwork-like structure similar to the *in vivo* marrow-microenvironment[32] while the HSCs grew in the intercellular spaces and formed large, compact clusters. This system offers a distinct advantage in that it can be used to generate specialized micro-environments made up of various types of niche-cells,[33] having a specific stage of differentiation or possessing a specialized signaling status,[34] to investigate how the differentiation stage or the biochemical make-up of the niche cells affects the HSC fate. Yet another advantage of this system is that the MSCs can be sourced from marrows harvested from patients suffering from aplastic anemia, leukemia, myelodysplastic syndrome, etc., to identify the deregulated niche functions at the molecular level and to develop strategies to target them.

Though the MSCs are known to support HSC growth *in vitro*, their participation in the *in vivo* HSC niche remained uncertain till it was shown that nestin-positive MSCs form a specialized HSC niche.[7] The 3D-MSCs showed a very high expression of this niche-molecule at both gene and protein levels, supporting our claim that the 3D-MSCs form an equivalent of the HSC-niche. $Nestin^+$ MSCs were shown to play an important role in maintaining the HSCs in the marrow compartment and, therefore, it may be logical to conclude that the nestin-mediated interactions contributed to the high level of retention of HSCs in the 3D-cultures.

We carried out extensive analyses of the cells growing under 3D conditions using HSC-specific phenotypic and functional assays to establish that these 3D-cultures recapitulate the niche physiology *in vitro*. The most important finding from these analyses was that the 3D-MSCs fostered a large pool of quiescent HSCs. The development of a robust multi-lineage hematopoiesis and the simultaneous maintenance of such a quiescent stem cell pool in the 3D systems suggest that, similar to the *in vivo* niche, the 3D-cultures activate only a few stem cell clones to produce committed progenitors[35] to prevent stem cell exhaustion. Alternatively, the 3D-MSCs may harbor separate pools of quiescent *versus* proliferating stem cells having distinct functions.[36] It would be interesting to examine these issues more specifically.

The role of N-Cadherin in HSC function has triggered much intense debate.[11] Using a germ-line knockout mouse model, Kiel *et al.* have conclusively shown that N-Cadherin is not required for HSC function.[37] On the other hand, Hosokawa *et al.* showed that shRNA-mediated knockdown of N-Cadherin suppressed the long-term engraftment ability of the stem cells.[38] In a murine system, angiopoietin1-Tie2 axis was shown not only to induce quiescence, but also to increase N-Cadherin expression in the HSCs.[20] Human $CD34^+$ cells were found to express moderate levels of N-Cadherin when co-cultured with the human bone marrow-derived MSCs and a genetic knockout or functional blocking of N-Cadherin resulted in the loss of primitive stem cell population,[14] suggesting that, at least *in vitro*, N-Cadherin is an important niche-interacting molecule that is needed for HSC maintenance. In our 3D-cultures, a large proportion of $CD34^+$ cells were seen to be positive for N-Cadherin, albeit at variable levels. Surprisingly, these cells were not found in 2D-cultures. In a murine system, $N\text{-}Cadherin^{int/low}$ cells, but not $N\text{-}Cadherin^{high}$, represented LT-HSCs.[39] Such studies have not been reported for human cells. It would be interesting to see whether the results obtained in the mouse system hold true for human HSCs.

It is well known that HSCs are retained in the marrow environment via the SDF1α/CXCR4 axis[40] and the use of pharmacological agents to disrupt this interaction leads to their egress from the marrow.[23] The high expression of SDF1α by the 3D-MSCs at both the RNA and protein levels indicated that perhaps they retain a high number of HSCs via forming a chemokine-rich environment. Indeed, the addition of AMD3100 in 3D-cultures resulted in the mobilization of HSCs from the matrix into the supernatant, confirming that the 3D-cultures retain the HSC pool via active mechanisms that are analogous to the *in vivo* situation and, therefore, this system can be used as a primary screen in drug-discovery programs for HSC mobilization agents. In addition to acting as a chemo-attractant for the HSCs and facilitating their homing process, SDF1α has also been shown to play an important role in the maintenance of the HSC quiescence.[40] Therefore, the elevated levels of SDF1α present in the hydrogel-cultures may also have contributed towards fostering the quiescent HSCs.

A unique characteristic of the marrow-environment is the presence of hypoxia-gradient, wherein the stem cells reside in the most hypoxic regions while the progenitors occupy the regions with higher oxygen contents and proliferate.[24-25, 29, 41] Hypoxia preserves the stem cells by inducing quiescence in them and also protects them from oxidative stress.[26] In order to mimic this situation *in vitro*, specialized incubators and hypoxia chambers were designed

to maximize the yield of HSCs. We found that the 3D-MSCs fostered a large stem cell pool and also supported a robust multi-lineage hematopoiesis, indicating that these cultures must have an oxygen-gradient analogous to the *in vivo* micro-environment; a conjecture supported by the image analysis of the hypoxyprobe-stained 3D-cultures. Hypoxyprobe (pimonidazole) specifically binds to proteins in hypoxic cells at an oxygen pressure that is equal to or lower than 10 mmHg. The protein adducts thus formed are detected by staining with specific monoclonal antibodies. The amount of adducts formed is proportional to the level of hypoxia and thus the intensity of the signal can be taken as an indirect correlate of the level of hypoxia.[42] In the present study, no comparison was made of the intensity of hypoxyprobe with the actual percentage of O_2 present *in situ*. Such analyses may help to interpret the results more conclusively. Nonetheless, the destruction of this gradient by the incubation of the 3D-MSCs in hypoxia chambers abolished their niche-like behavior, emphasizing that the 3D-MSCs are more representative of the HSC niche than the hypoxia-based cultures.

Tormin *et al.*[28] have reported that hypoxia leads to a downregulation of CD146 in MSCs. Lack of CD146 expression by most 3D-MSCs further supported our hypothesis that the 3D-MSCs are hypoxic. Sachetti *et al.*[6] have shown that human $CD45^-CD146^+$ cells contained all bone marrow CFU-F and these cells were able to transfer the hematopoietic activity to an ectopic site. Tormin *et al.*,[28] however, found a similar recovery of the CFU-F from the $CD45^-CD146^-$ cells residing in the endosteal region, where the HSCs are preferentially located. Our data clearly show that the HSCs are better supported by the $CD146^-$ 3D-MSCs, underscoring that they are indeed true representatives of the HSC niche.

Osteoblasts express HSC-supportive molecules like osteopontin and angiopoietin-1, which are known to promote HSC quiescence.[18, 20] Cultivation of MSCs in low oxygen is known to increase osteogenesis.[43] However, in spite of an upregulation of Runx-2, a transcription factor required for osteoblastic differentiation, and the prevailing hypoxic conditions in the 3D-MSCs, no spontaneous osteoblastic differentiation was observed in the 3D-MSCs (*data not shown*), indicating that the HSC-supportive properties of the 3D-MSCs were independent of the cell commitment towards the osteoblastic lineage.

Our research is now focused on acquiring a more detailed understanding of the specific pathways that explain the benefits of hydrogel over traditional MSC culture for HSC support. Such studies may help to create specialized *in vitro* niches (IVNs) with the desired signaling gamut using pharmacological or genetic approaches for an *in vitro* modulation of stem cell functions.

Authorship and Disclosures

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