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Hypoxic Niche-Mediated Regeneration of Hematopoiesis in the Engraftment Window Is Dominantly Affected by Oxygen Tension in the Milieu

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The bone marrow (BM) microenvironment or the hematopoietic stem cell (HSC) niche is normally hypoxic, which maintains HSC quiescence. Paradoxically, transplanted HSCs rapidly proliferate in this niche. Pre-transplant myelosuppression results in a substantial rise in oxygen levels in the marrow microenvironment due to reduced cellularity and consequent low oxygen consumption. Therefore, it may be construed that the rapid proliferation of the engrafted HSCs in the BM niche is facilitated by the transiently elevated oxygen tension in this milieu during the ''engraftment window.'' To determine whether oxygen tension dominantly affects the regeneration of hematopoiesis in the BM niche, we created an ''oxygen-independent hypoxic niche'' by treating BM-derived mesenchymal stromal cells (BMSCs) with a hypoxia-mimetic compound, cobalt chloride ($CoCl_2$) and cocultured them with BM-derived HSC-enriched cells under normoxic conditions (HSCs; $CoCl_2$-cocultures). Cocultures with untreated BMSCs incubated under normoxia (control- cocultures) or hypoxia (1% O_2; hypoxic-cocultures) were used as comparators. Biochemical analyses showed that though, both $CoCl_2$ and hypoxia evoked comparable signals in the BMSCs, the regeneration of hematopoiesis in their respective cocultures was radically different. The $CoCl_2$-BMSCs supported robust hematopoiesis, while the hypoxic-BMSCs exerted strong inhibition. The hematopoiesis-supportive ability of $CoCl_2$-BMSCs was abrogated if the $CoCl_2$-cocultures were incubated under hypoxia, demonstrating that the prevalent oxygen tension in the milieu dominantly affects the outcome of the HSC-BM niche interactions. Our data suggest that pharmacologically delaying the re-establishment of hypoxia in the BM may boost post-transplant regeneration of hematopoiesis.

Introduction

The bone marrow (BM) microenvironment is hypoxic under steady-state conditions, with oxygen gradients ranging from ~1% to 6% [1,2]. Hypoxia plays an essential role in the regulation of hematopoiesis, primarily by protecting the hematopoietic stem cells (HSCs) from oxidative stress, which is believed to be an important mediator of HSC aging, dysfunction, and senescence [3,4]. In the hypoxic niche, the HSCs rely on glycolysis, have a lower rate of oxygen consumption and possess a low metabolic profile [3,5]. These attributes help them to remain in a quiescent state. Hypoxia-induced autocrine secretion of VEGF-A is needed to regulate HSC function [6]. HIF-1, a major transcriptional regulator of hypoxic response, plays an important role in HSC biology. The loss of HIF-1α results in HSC dysfunction, while its over-stabilization drives the HSCs into deep quiescence [7] and also affects their reconstitution ability [8], showing that the precise regulation of HIF-1α levels is required for optimal HSC function [9]. It also regulates the Cripto-GRP78 axis, which is required for glycolytic metabolism-related proteins, and lowers mitochondrial potential in the HSCs [10]. A pharmacological increase in HIF-1α in the HSCs has been shown to enhance their homing and engraftment [11], and also protect them from irradiation-induced toxicity [12]. In situ tissue analysis has revealed that HSCs exhibit a hypoxic profile regardless of localization anywhere in the BM suggesting that the characteristic hypoxic state of HSCs may be partially regulated by cell-specific mechanisms [13].

In addition to these cell-autonomous effects of hypoxia, the non-cell-autonomous effects of HIF-1α-mediated signaling via the niche cells have also been reported. Stabilization of HIF-1α in the stromal cells leads to secretion of hematopoiesis-supportive cytokines and chemokines [14,15]. Overexpression of HIF-1α in human mesenchymal stem cells (MSCs) has been shown to enhance their hematopoiesis-supportive functions in vitro [16] and promote proangiogenic properties in them [17]. BM endosteal mesenchymal

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progenitors also depend on HIF-1α and HIF-2α to regulate and maintain hematopoiesis [18].

BM transplantation (BMT) presents some unique features as compared to steady-state conditions. While the HSC numbers remain steady under the latter conditions, their numbers substantially increase after BMT [19]. The pretransplant myeloablation results in a significant elevation of oxygen tension in the marrow compartment due to reduced cellularity and consequent low oxygen consumption [2]. These observations suggest that under transplantation settings, as opposed to the steady-state conditions, the exposure of the infused HSCs to the relatively higher oxygen tension in the resident niche probably results in their rapid proliferation.

To test this hypothesis, we studied the outcome of interactions of HSCs with BM-derived MSCs (BMSCs) under normoxia vis-à-vis hypoxia. Using an ''oxygen-independent hypoxic niche'' model, we show here that while the hypoxic niche is by default equipped with a hematopoiesis-supportive signaling gamut, it is the oxygen tension in the milieu that predominantly determines the extent of regeneration. Based on our data, we speculate that pharmacologically delaying the reestablishment of hypoxia in the BM may boost post-transplant regeneration of hematopoiesis.

Materials and Methods

Mice

Six to 8-weeks-old C57BL/6 mice (CD45.2) and their congenic variant, B6.SJL-ptprc Pep3/Boy J (CD45.1) were supplied by the Experimental Animal Facility, NCCS. All protocols were approved by the Institutional Animal Ethics Committee (IAEC).

Generation of BMSCs

BM cells were isolated from the hind limbs (tibia and femur) of C57BL6 mice (CD45.2) by flushing the marrow in Iscove's modified Dulbecco's medium (IMDM) (Hi Media, Mumbai, India) supplemented with 20% MSC-qualified fetal bovine serum (Mesen-FBS; Stem Cell Technology, Vancouver, Canada) using 25G-needles. The cells were cultured in the same medium at 37°C in 5% CO_2 for 1 week with removal of nonadherent floating cells at intervals and allowing the stromal cells (BMSCs) to adhere. The cells were allowed to reach near-confluence before subculturing.

Isolation of lineage-negative cells

Mononuclear cells (MNCs) were isolated from the BM cells (CD45.1) by ficoll hypaque density gradient centrifugation (HiSep™ from Hi Media). These MNCs were depleted of lineage-positive cells by immunomagnetic separation using a biotin-labeled anti-mouse lineage antibody cocktail (BD Biosciences, San Jose, CA) and Dynabeads® biotin binder (Invitrogen, Carlsbad, CA).

Cell treatments

1×10^5 BMSCs or 7×10^5 M210B4 cells/well were cultured overnight in 12-well plates (BD Falcon, Bedford, MA) in their respective media. The cells were treated with fresh medium supplemented with 100 μM of cobalt chloride ($CoCl_2$) (Sigma-Aldrich, St. Louis, MO) for 48 h, following which the medium was replaced with fresh medium without $CoCl_2$ ($CoCl_2$-BMSCs) and the cultures were incubated under normoxia (21% oxygen, which is the standard cell culture condition). In hypoxia treatments, the cells were either continuously cultured under 1% oxygen (hypoxic-BMSCs) or under normoxia after preincubation for 48 h under hypoxia (BMSCs-48 h-hypoxia). For the treatment of focal adhesion kinase (FAK) inhibitor PF573228 (PF228; Sigma-Aldrich), overnight-cultured BMSCs (1×10^5/well of 12-well plate) were pretreated with 300 nM PF228 for 2 h, followed by the addition of 100 μM of $CoCl_2$ for 48 h. Both compounds were removed before seeding the HSCs.

The cell viability in all cases was >98% as determined by Trypan Blue dye exclusion method.

Generation of stable clones expressing shRNA specific to Hif-1α and N-cadherin

M210B4, a mouse BM stromal cell line (ATCC, Manassas, VA) was maintained in RPMI1640 (Hi Media) containing 10% FBS (Invitrogen).

The details of plasmid constructs, transfection, selection, and screening of the clones are described in the Supplementary Data (Supplementary Data are available online at www.liebertpub.com/scd).

Coculture studies

2×10^5 lineage-negative cells (HSCs) were seeded over the BMSCs/M210B4 in IMDM supplemented with 20% Mesen-FBS (Stem Cell Technology), 25 ng/mL murine IL-6, 25 ng/mL murine stem cell factor and 10 ng/mL murine IL-3 (Peprotech, Rocky Hill, NJ), and the cocultures were maintained 7 days at 37°C under either normoxia (control-cocultures) or hypoxia (hypoxic-cocultures). Cocultures with $CoCl_2$-BMSCs were incubated under normoxia ($CoCl_2$-cocultures), unless stated otherwise.

After 7 days, the cocultures were harvested and viable cell counts were taken using the Trypan Blue dye exclusion method.

Phenotypic analysis

The antibodies used for various experiments are listed in Supplementary Table S1. 1×10^6 cells were suspended in phosphate-buffered saline containing 0.1% bovine serum albumin and 0.01% sodium azide, and incubated with fluorescence-conjugated antibodies for 1 h at 4°C. After washing, the cells were acquired on FACS canto II (Becton and Dickinson, Franklin Lakes, NJ) and the data were analyzed using the BD FACS-DIVA software version 5.0.

BM transplantation

For primary transplantation, 1×10^6 cocultured donor cells (CD45.1) admixed with 2×10^5 fresh competitor BM cells (CD45.2) were intravenously infused through the tail vein into lethally irradiated (9.5 Gy, γ irradiation Co^{60}; split doses given 4 h apart) C57BL6 (CD45.2) recipient mice. This dose of competitor cells ensured the survival of mice. After 4 and 16 weeks, the peripheral blood from the recipient mice was collected by orbital plexus bleeding and immunostained with various antibodies (Supplementary Table

S1). The cells were analyzed using FACS canto-II (Becton and Dickinson) to assess donor cell engraftment. The data were analyzed using the BD FACS-DIVA software version 5.0.

For secondary transplantation, MNCs were isolated from the tibia and femur bone of the primary recipient mice (CD45.2) and the donor cells (CD45.1) were sorted using FACS Aria II. 2×10^5 sorted donor cells admixed with 2×10^5 fresh competitor BM cells (CD45.2) were infused into individual irradiated secondary recipients (CD45.2).

Statistical analysis

Results are expressed as mean ± standard error of the mean. Data were analyzed with one-way repeated measure analysis of variance using Sigma Stat software (Jandel Scientific, San Rafael, CA). A $P \le 0.05$ was considered significant. $^*P \le 0.05$, $^{**}P \le 0.01$, $^{***}P \le 0.001$.

Results

$CoCl_2$-treated BMSCs form an "oxygen-independent hypoxic niche" in vitro

To study the interaction of HSCs with the BM niche having an active HIF-1α signaling under normoxia, it was necessary to create an ''oxygen-independent hypoxic niche.'' For this purpose, we treated the BMSCs with a hypoxia-mimetic compound, $CoCl_2$ ($CoCl_2$-BMSCs). BMSCs incubated under hypoxia (1% O_2; ''hypoxic-BMSCs'') [20–22] or normoxia (21% O_2; control-BMSCs) were used as comparators. Image analysis of cells immunostained with an anti-HIF-1α antibody confirmed that both, $CoCl_2$-BMSCs and hypoxic-BMSCs, showed a significantly high level of HIF-1α in their nuclei (Fig. 1A). Western blot analyses revealed strong expression of HIF-1α in both, $CoCl_2$-treated and hypoxic-BMSCs (Fig. 1B). Importantly, the $CoCl_2$-BMSCs showed a persistent upregulation of HIF-1α even after removal of $CoCl_2$ from the medium post-48 h exposure (Fig. 1C; $CoCl_2$-48 h), but the hypoxic BMSCs lost the signal when they were further incubated under normoxia (Fig. 1C; hypoxia-48 h). Therefore, further experiments were done using BMSCs cultured under normoxia with a 48 h pretreatment with $CoCl_2$ ($CoCl_2$-BMSCs), BMSCs continuously cultured under hypoxia (1% O_2; hypoxic-BMSCs), and untreated BMSCs cultured under normoxia (control-BMSCs).

These data confirmed that the $CoCl_2$-BMSCs could form a suitable in vitro model to examine the effect of a hypoxic niche on the HSC fate under varying O_2 tensions.

$CoCl_2$-treated BMSCs boost HSC numbers and support robust hematopoiesis in vitro

To examine the effect of these variously treated BMSCs on the regeneration of hematopoiesis, we cocultured them with the HSC-enriched lineage-negative (Lin^-) fraction of the BM cells (hereafter referred to as HSCs; Supplementary Fig. S1A) as described previously. We found that the $CoCl_2$-cocultures gave the highest output of total hematopoietic cells (Fig. 2A, B). HSCs are phenotypically defined as $Lin^-Sca1^+c\text{-}Kit^+$ (LSK) cells. Within this population, the HSCs having an ability to give rise to long-term

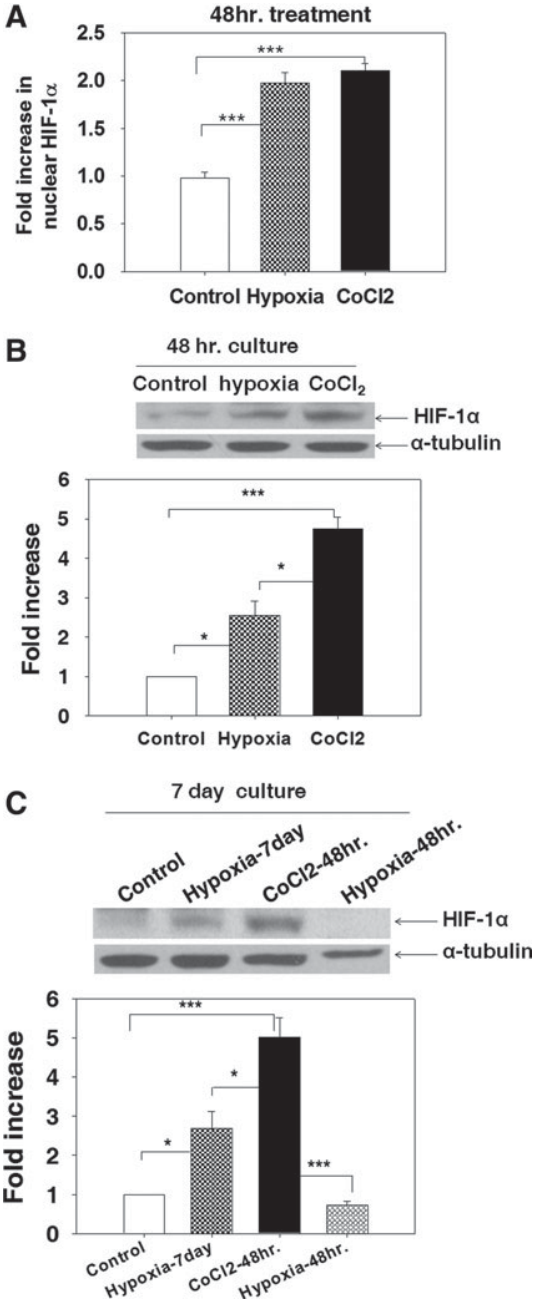

FIG. 1. $CoCl_2$ induces a persistent stabilization of HIF-1α. Murine BMSCs were cultured under hypoxia (1% O_2) or treated with 100 μM of $CoCl_2$ for 48 h. **(A)** Image analysis data collected on cells immunostained with an anti-HIF-1α antibody is graphically illustrated. MFI in 20 nuclei in nonoverlapping fields was measured. **(B, C)** *Upper panels* show representative western blots of HIF-1α and α-tubulin in the cells treated for the indicated time periods. Graphs in the *lower panels* represent HIF-1α levels normalized to α-tubulin. Data show that $CoCl_2$ induces a persistent upregulation of HIF-1α till 7 days even after its removal, while removal of hypoxia leads to waning of the signal (hypoxia-48 h, *last lane* in **C**). Results are expressed as mean ± SEM of three independent experiments. $^*P \le 0.05$ and $^{***}P \le 0.001$. BMSCs, bone marrow-derived mesenchymal stromal cells; $CoCl_2$, cobalt chloride; MFI, mean fluorescence intensity; SEM, standard error of the mean.

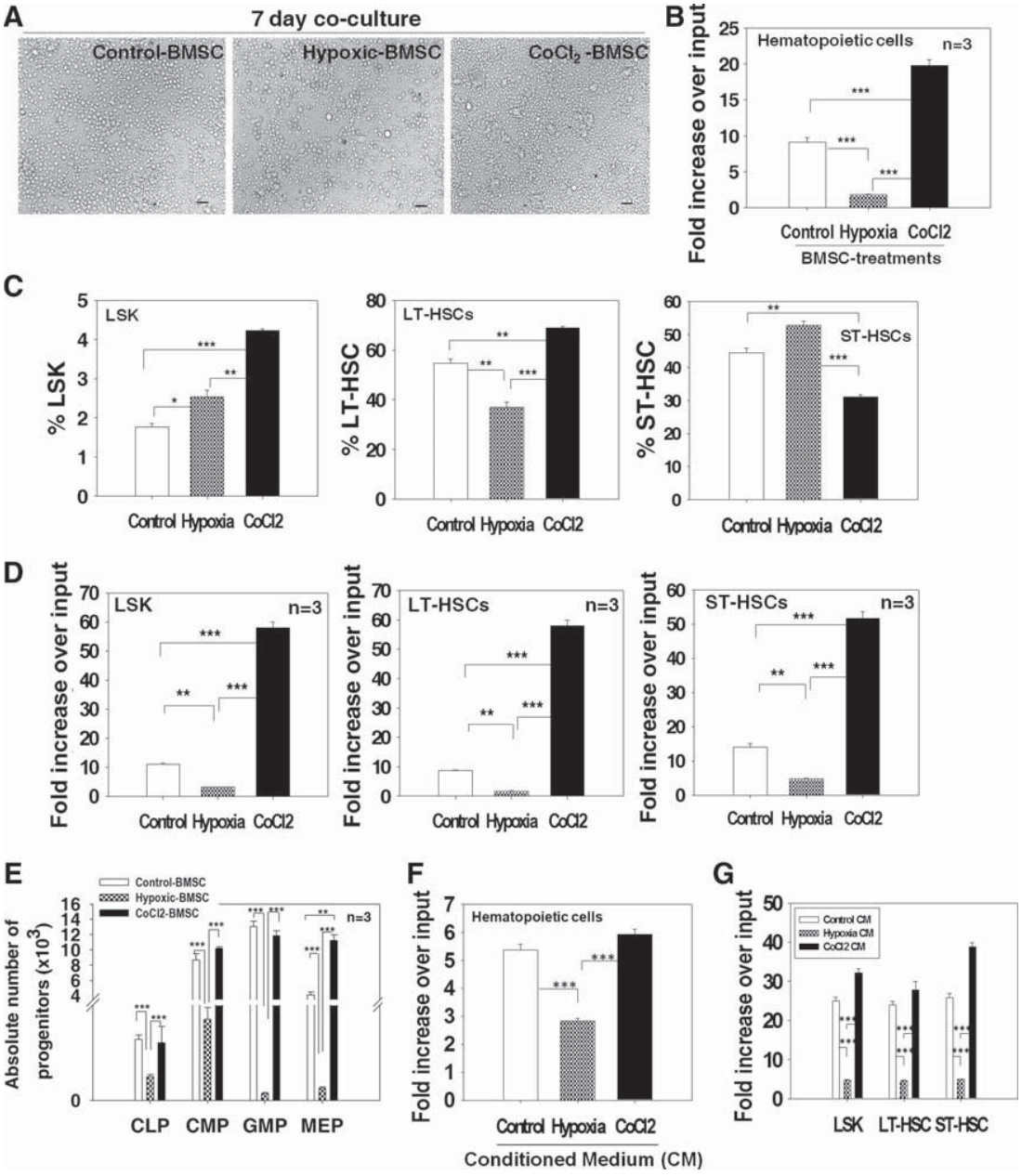

FIG. 2. $CoCl_2$-treated BMSCs support robust regeneration of hematopoiesis in vitro. BM-derived lineage$^-$ cells (Lin^-) were cocultured with BMSCs pretreated with $CoCl_2$ ($CoCl_2$-BMSCs) for 48 h or not (Control-BMSCs) and incubated under normoxia or with hypoxia-treated BMSCs (hypoxic-BMSCs) and incubated under hypoxia. **(A)** Phase contrast images of cocultures are depicted. Bar represents 1 μm. **(B)** Graph represents fold increase over input for the total cells harvested after 7 days of coculture and manually counted using Trypan Blue dye exclusion method. **(C)** Frequency of various HSC subsets in the output populations is graphically represented. **(D)** Graphs represent the fold increase in the HSC populations over input. **(E)** Graph represents absolute numbers of various committed progenitors present in the output population. Absolute number of each of the respective cell population was calculated from the total hematopoietic output and the percentage obtained in flow cytometry analysis. **(F, G)** Lin^- cells were cultured with CM collected from control, $CoCl_2$- or hypoxia-treated BMSCs for 7 days. Graphs represent fold increase over input for the total cells harvested **(F)** and fold increase in the HSC populations over input **(G)**. Absolute number of each of the respective cell population was calculated from the total hematopoietic output and the percentage obtained in flow cytometry analysis. Data show that the HSC-supportive activity of $CoCl_2$-BMSCs is contact-dependent, while hypoxia exerts a strong suppressive effect irrespective of the presence of BMSCs. Data shown in graphs represent mean ± SEM. The experiments were repeated at least three times. $^*P \le 0.05$, $^{**}P \le 0.01$ and $^{***}P \le 0.001$. Also see Supplementary Fig. S1. CM, conditioned medium; HSC, hematopoietic stem cells.

hematopoietic reconstitution after transplantation are defined as LSK-CD34$^-$ (LT-) HSCs while the HSCs giving rise to short-term engraftment are defined as LSK-CD34$^+$ (ST) HSCs. Phenotypic analyses of the output cells by multi-color flow cytometry showed that the frequency of LSK and LT-HSCs was significantly high in $CoCl_2$-cocultures, whereas the frequency of ST-HSCs was the highest in hypoxic-cocultures (Fig. 2C and Supplementary Fig. S1B), indicating that the $CoCl_2$-BMSCs support expansion of HSCs having a long-term reconstitution ability. $CoCl_2$-cocultures yielded the highest output of various HSC subsets (Fig. 2D). In sharp contrast, the hypoxic-cocultures yielded the lowest output of all these HSC subsets.

The output cells were also analyzed for various hematopoietic progenitor cells (HPCs) such as common lymphoid progenitors [23], common myeloid progenitors, granulocyte/macrophage progenitors, and megakaryocyte/erythrocyte progenitors [24]. A multi-color flow cytometry analysis showed that both the normoxic cocultures, that is, control and $CoCl_2$, produced significantly higher numbers of the various HPCs, compared to hypoxic-cocultures, showing that normoxia promoted the formation of committed progenitors (Fig. 2E and Supplementary Fig. S1C).

Flow cytometry analyses done to enumerate terminally differentiated cells showed that $CoCl_2$-cocultures produced the highest numbers of T-cells and myeloid cells, but the B-cell output was comparable in the $CoCl_2$- and control-cocultures (Supplementary Fig. S1D). Collectively, the data demonstrate that $CoCl_2$-BMSCs support robust regeneration of hematopoiesis, while hypoxia exerts strong suppression.

We next examined whether cell–cell contact was necessary for the HSC-stimulatory effect of $CoCl_2$-BMSCs, by incubating the HSCs with conditioned media (CM) prepared from control, $CoCl_2$-treated and hypoxia-treated BMSCs and performing phenotypic analyses of the output population. We found that the CM of both control- and $CoCl_2$-treated BMSCs supported a five to sixfold increase in hematopoietic cell proliferation (Fig. 2F). However, cell–cell contact induces approximately threefold increase in hematopoietic cell proliferation over CM alone (Fig. 2B), suggesting that maximal hematopoietic cell proliferation requires both cell–cell contact and soluble factors. It was also interesting to find that the CM of the control cells increases LSK, LT-HSC, and ST-HSC proliferation (almost twofold increase; Fig. 2G) when compared with the control cocultures (Fig. 2D), which may suggest that cell–cell contact is required to more robustly limit HSC proliferation under normoxia. The CM of normoxic control BMSCs and $CoCl_2$-BMSCs have very similar effects on HSC proliferation (Fig. 2G), but when cell–cell contact was added in, the $CoCl_2$-BMSCs showed an advantage in boosting HSC proliferation (Fig. 2D).

The output of total hematopoietic cells and HSC subsets was the least in the HSCs incubated under hypoxia with CM collected from hypoxic-BMSCs, suggesting that hypoxia exerts an HSC-suppressive effect irrespective of the presence of BMSCs.

$CoCl_2$-BMSCs and hypoxic-BMSCs possess a comparable signaling gamut

Signaling pathways operative in the niche are known to regulate the stem cell fate [25]. To identify the signaling pathways activated in the BMSCs by hypoxia, we investigated and compared the signaling pathways activated in them by hypoxia (1% O_2 tension) and $CoCl_2$. Western blot analyses performed at both early (48 h—time of seeding of the HSCs) and late (7 days—time of harvesting) time points showed that hypoxia- and $CoCl_2$-treated BMSCs showed strong activation of FAK and its downstream target, Akt [26] (Fig. 3A, B). In $CoCl_2$-BMSCs, the level of native FAK protein was significantly higher compared with hypoxic-BMSCs at both time points, but the levels of phospho-FAK (Y397) were comparable (Fig. 3A). Importantly, in $CoCl_2$-BMSCs, the activation of both FAK and Akt persisted even after the removal of $CoCl_2$ after treatment for 48 h, but subsequent incubation of hypoxic-BMSCs under normoxia resulted in waning of both signals (Fig. 3A, B, right panels, hypoxia-48 h).

Upregulation of N-cadherin by $CoCl_2$ and hypoxia

Our earlier studies using three-dimensional (3D) cultures of placenta-derived MSCs (PMSCs) had shown that the 3D-PMSCs were hypoxic and showed a strong upregulation of N-cadherin [27]. Here, we examined whether hypoxia upregulates N-cadherin expression in BMSCs. Western blot analyses revealed that both $CoCl_2$ and hypoxia strongly upregulated N-cadherin expression in the BMSCs at both time points examined (Fig. 3C). Here, again, this effect persisted even after the removal of $CoCl_2$ post-48 h, but removal of hypoxia resulted in loss of the N-cadherin signal (Fig. 3C, right hand panel, hypoxia-48 h). Whether the upregulation of N-cadherin by $CoCl_2$ or hypoxia is dependent on HIF-1α-mediated signaling mechanisms needs to be examined, and we propose to address this in the near future.

Since membrane expression of N-cadherin is crucial for cell–cell interactions, we performed immunofluorescence staining of the treated cells and found that N-cadherin was localized in the membranes of both $CoCl_2$- and hypoxic-BMSCs (Fig. 3D). In control cells, a distinct nuclear signal for N-cadherin was observed, suggesting that perhaps this protein shuttles between the nucleus and the membrane depending on the external signal. This aspect needs to be studied in detail.

A 48-h interaction with the $CoCl_2$-BMSCs enhances the long-term functionality of HSCs

It was felt necessary to ensure that the interaction of the HSCs with $CoCl_2$-BMSCs under normoxia did not compromise their functionality. We determined the long-term engraftment and regenerative capacity of the output HSCs from 48 h cocultures, since the output cell numbers were comparable in all sets at this time point (data not shown), and the signaling mechanisms were fully activated in the BMSCs (Fig. 3). We transplanted an equal number of output cells (1×10^6 CD45.1 donor cells/mouse) collected from the cocultures into lethally irradiated C57Bl6 (CD45.2) recipients and monitored engraftment levels at 4 and 16 weeks post-transplant.

We found that the cells from $CoCl_2$-cocultures showed significantly higher levels of peripheral blood chimerism than those harvested from hypoxic- or control-cocultures, both at 4 weeks (Fig. 4A) and 16 weeks (Fig. 4B) post-

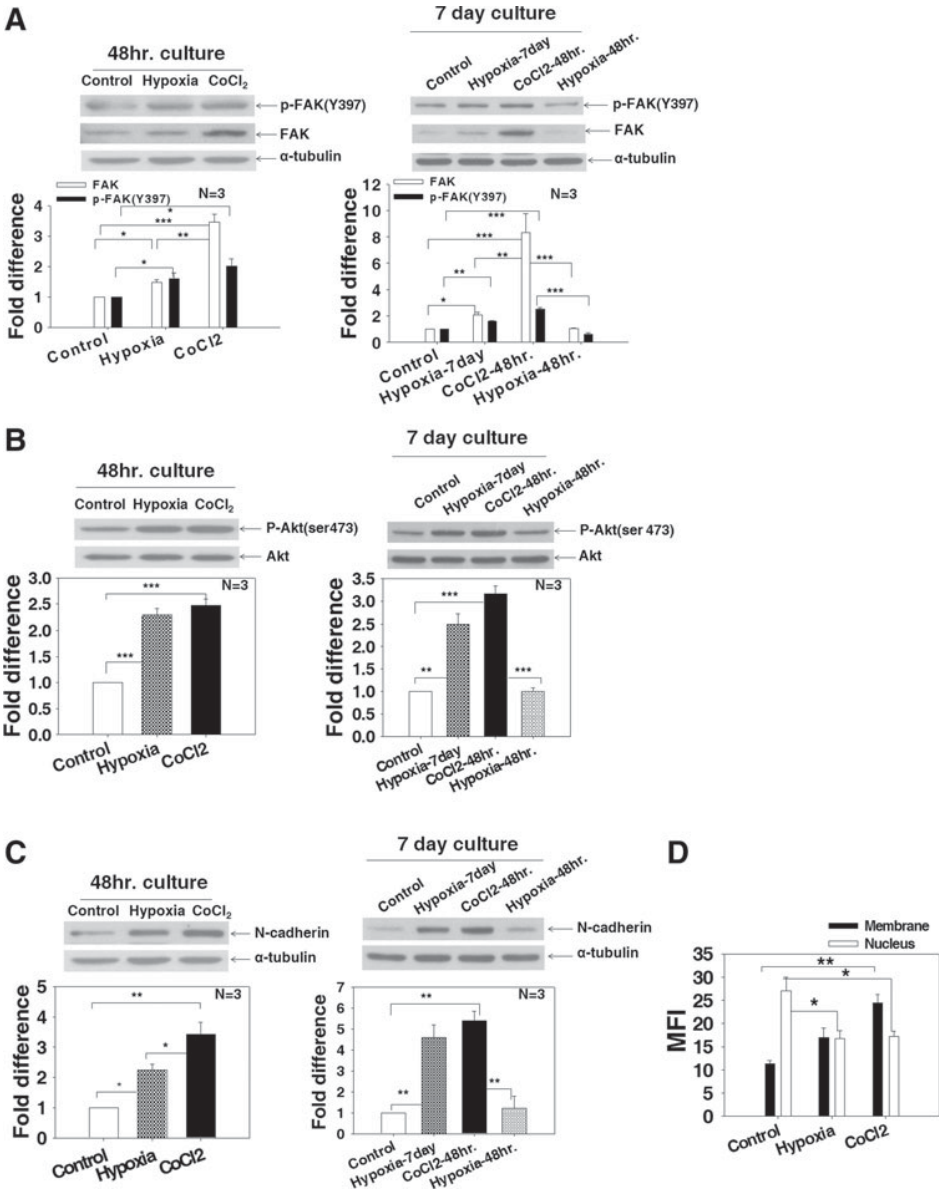

FIG. 3. Hypoxia and $CoCl_2$ evoke comparable signaling in BMSCs. BMSCs were treated with hypoxia or $CoCl_2$ (100 μM) and lysed at the indicated time points. The lysates were subjected to western blot analysis for p-FAK (Y397), FAK, and α-tubulin **(A)**, for p-Akt-ser473 and Akt **(B)**, and N-cadherin and α-tubulin **(C)**. *Upper panels* depict representative western blots and *lower panels* depict the fold difference over control. Except in case of p-Akt, which was normalized with respect to its native form, all other data were normalized with respect to α-tubulin. Data depicted graphically are represented as mean ± SEM of three independent experiments performed. **(D)** BMSCs treated with hypoxia or $CoCl_2$ (100 μM) were immunostained with anti-N-cadherin and Cy3-conjugated secondary antibodies. DAPI was used to demarcate the nuclei. The fluorescence intensity at nuclear and membrane region was measured in 50 cells selected from nonoverlapping fields using Image J software and the data are graphically illustrated. $*P \leq 0.05$, $**P \leq 0.01$, and $***P \leq 0.001$.

transplant. The level of engraftment in the BM after 16 weeks post-transplant was significantly higher in the $CoCl_2$-set (Fig. 4C), although modestly so. This modest increase in the HSC numbers in the BM suggests that these cells are responsive to the natural regulatory mechanisms that contain the stem cell pool, and do not undergo unwarranted proliferation upon transplantation. On the other hand, the stimulated progenitors produced a much higher level of chimerism in the peripheral blood (Fig. 4B). Analyses for lymphoid and myeloid cells showed that there was no lineage bias (Supplementary Fig. S2A, B). After 16 weeks, the donor cells sorted from the marrow of primary recipients were infused into irradiated secondary recipients. We found that the engrafted donor cells collected from the primary recipients infused with cells from the $CoCl_2$-cocultures engrafted the secondary recipients (Fig. 4D). The cells from control-cocultures showed a marginal level of engraftment in the secondary recipients whereas the cells from hypoxic-cocultures did not engraft in the secondary recipients.

CXCR4 expression reflects HSC functionality and plays a vital role in the engraftment of HSCs. Therefore, we enumerated $CXCR4^+$ cells in the output HSCs from the 48 h-cocultures. The number of $CXCR4^+$ HSCs was significantly high in the $CoCl_2$- and hypoxic-cocultures compared with the control-cocultures (Fig. 4E). Consistent with these data, the HSCs collected from both, $CoCl_2$- and hypoxic-cocultures migrated in a significantly higher numbers toward SDF-1α compared with their control counterparts (Fig. 4F). These data show that the engraftment defect exhibited by the output HSCs from hypoxic cocultures is perhaps related to their failure to proliferate in the recipients' marrow, and not as a result of a defect in CXCR4 expression or function.

Since LSK ($Lin^-Sca\text{-}1^+c\text{-}Kit^+$) HSCs with high reactive oxygen species (ROS) levels have limited serial

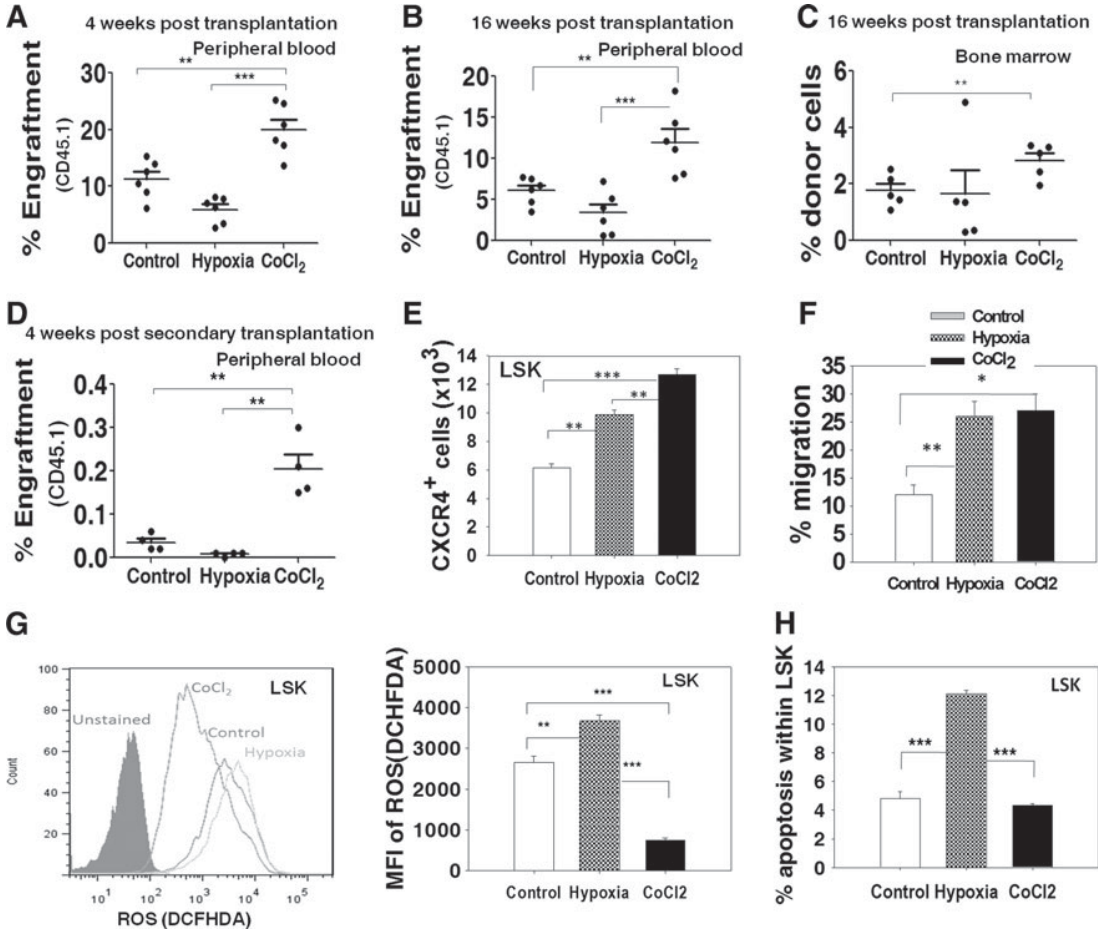

FIG. 4. $CoCl_2$-BMSCs boost engraftment potential of HSCs cocultured with them. HSC-enriched Lin^- fraction of BM cells (HSCs) was cocultured with control, $CoCl_2$-treated and hypoxia-treated BMSCs for 48 h and the output cells (CD45.1) were intravenously infused in irradiated mice (CD45.2). Engraftment levels were assessed by multicolor flow cytometry analyses of PB at 4 and 16 weeks post-transplant. Scatter plots show the level of engraftment by the output cells at 4 weeks **(A)** and at 16 weeks post-transplant **(B)** in the PB of recipients. $n = 6$. **(C)** Engraftment level in the BM of primary recipients at 16 weeks post-transplant is depicted. $n = 5$. **(D)** The engrafted donor cells from the primary recipients were sorted and infused into irradiated secondary recipients. Scatter plot shows the level of engraftment in the PB of secondary recipients ($n = 4$). **(E)** Graphs represent absolute numbers of output CXCR4-expressing HSCs from the various 48 h-cocultures. $n = 3$. **(F)** SDF-1α-mediated chemotaxis of the output cells. The output cells from $CoCl_2$- and hypoxic-cocultures migrate in significantly higher numbers than those from control cocultures. **(G)** Representative flow panel depicts the ROS levels in the LSK HSCs as determined by the DCHFDA fluorescence. Graph represents the comparative MFI of DCHFDA fluorescence (ROS levels) in the output LSK HSCs collected at 48 h from various cocultures. $n = 3$. **(H)** Graph represents the % apoptotic LSK cells (Annexin V^+) in the output cells from 48 h cocultures. $n = 3$. Data in all graphs are represented as mean ± SEM. $*P \leq 0.05$, $**P \leq 0.01$, and $***P \leq 0.001$. Also see Supplementary Fig. S2. DCHFDA, 5-(and-6)-carboxy-2′,7′-dichlorofluorescein diacetate; PB, peripheral blood; PI, propidium iodide; ROS, reactive oxygen species.

transplantation ability due to increased senescence and apoptosis [3], we determined the ROS levels and percentage of apoptotic cells in the output cells collected from 48 h cocultures. We found that both were significantly higher in the LSK HSCs collected from hypoxic-cocultures than those collected from the control- and $CoCl_2$-cocultures (Fig. 4G, H and Supplementary Fig. S2C). These data indicate that higher levels of ROS and apoptosis in hypoxic HSCs could contribute to their loss of engraftment potential. The lowest level of ROS was seen in the LSK HSCs from $CoCl_2$- cocultures (Fig. 4G).

These data strongly suggest that the $CoCl_2$-BMSCs boost the functionality of the HSCs within a very short period of interaction via reduction in ROS and apoptosis levels in them.

Contributions of FAK, HIF-1α and N-cadherin in regeneration of hematopoiesis by $CoCl_2$-BMSCs

Since biochemical analyses had shown upregulation of HIF-1α, FAK, and N-cadherin in $CoCl_2$-BMSCs, we sought to determine the individual contribution of these signaling pathways in the hematopoietic support provided by them.

To study the role of FAK activation, we employed a pharmacological FAK inhibitor, PF228 (Sigma-Aldrich), a compound that targets FAK catalytic activity by inhibition

of FAK phosphorylation at Y397 [28]. After confirming that the inhibitory effect of PF228 persists even after its removal from the medium (Supplementary Fig. S3A), we performed coculture experiments with the $CoCl_2$-BMSCs pretreated with PF228. We found that in the cocultures set up with the $CoCl_2$-BMSCs pretreated with PF228, the number of the total hematopoietic cells and HSCs subsets was drastically reduced compared to the cocultures set with $CoCl_2$-BMSCs that were not treated with the inhibitor (Fig. 5A). These data indicate that the $CoCl_2$-BMSCs boost hematopoiesis through activated FAK signaling.

To examine the role of HIF-1α in the regeneration of hematopoiesis, we used the M2-shHif-1α and M2-Scr clones of M210B4, a MSC line of murine origin (ATCC). The clones showing more that 80% reduction in HIF-1α expression (Supplementary Fig. S3B, left panel) were used in the experiments and their effect on the cocultured HSCs was examined by flow cytometry. We found that the treatment

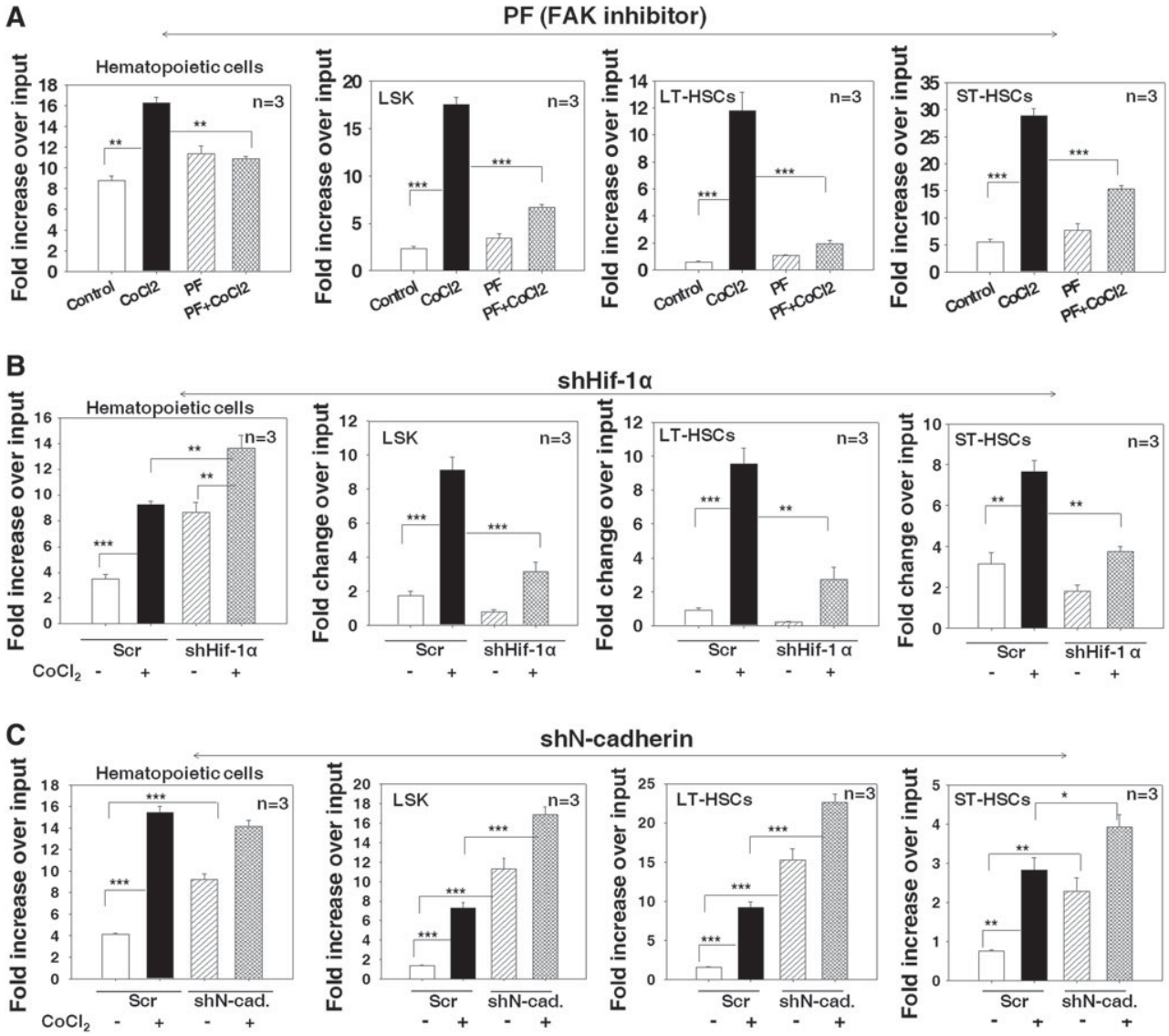

FIG. 5. HSC-supportive ability of $CoCl_2$-BMSCs involves FAK-, HIF-1α- and N-cadherin-mediated signaling mechanisms. BMSCs were treated with an inhibitor of FAK signaling PF228 (300 nM), both before and during $CoCl_2$-treatment, and the HSCs were cocultured with them after removal of both treatments. Output cells collected after 7 days of cocultures were analyzed by flow cytometry. **(A)** Graphs represent the fold increase in the absolute numbers of total cell yield, LSK, LT-HSCs and ST-HSCs over input. $n = 3$. **(B, C)** Stable clones of M210B4 cells expressing shRNA specific to *Hif-1α* (M2-shHif-1α) and *N-cadherin* (M2-shN-cadherin) were used. M210B4 cells stably transfected with plasmid constructs expressing respective scrambled sequences were used as controls (M2-Scr). The cells treated or not with $CoCl_2$ were cocultured with HSCs for 7 days. The output cells were analyzed by flow cytometry to enumerate HSC subsets. Graphs represent the fold change in the output of total hematopoietic cells and various HSC subsets over input after HIF-1α **(B)** or N-cadherin **(C)** was silenced in the stromal cells. $n = 3$. Data are represented as mean ± SEM. The experiments were repeated twice with similar results. $^*P \le 0.05$, $^{**}P \le 0.01$, and $^{***}P \le 0.001$. Also see the Supplementary Fig. S3.

with $CoCl_2$ upregulated HIF-1α expression in M2-Scr cells (Supplementary Fig. S3B, left panel, first and second bars) but not in the M2-shHif-1α clone, confirming the ability of the short hairpin RNA sequences to silence HIF-1α expression even after stimulation (Supplementary Fig. S3B, left panel, third and fourth bars). We found that silencing of HIF-1α resulted in a significant increase in the total hematopoietic cell output and this was further enhanced by $CoCl_2$-treatment of M2-shHif-1α cells (Fig. 5B, left panel). However, the HSC subsets were significantly reduced (Fig. 5B). These data demonstrate that activation of HIF-1α in the stromal cells actively participates in the maintenance of the stem cell pool by $CoCl_2$-BMSCs, and that its loss favors the production of higher number of hematopoietic cells in general at the expense of the stem cell pool.

To examine the role of N-cadherin, we generated stable clones of M210B4 expressing N-cadherin-specific shRNA (Supplementary Fig. S3B, right panel). Clones showing more than 80% reduction in the N-cadherin levels (M2-shN-cadherin) were selected for the coculture experiments. The cells transfected with vector containing scrambled sequences were used as controls (M2-Scr). To our surprise, we found that silencing of N-cadherin in M210B4 cells resulted in a significantly higher output of HSCs compared with the M2-Scr and this was further enhanced by the $CoCl_2$-treatment (Fig. 5C). The total hematopoietic cell yield was higher in the M2-shN-cadherin cocultures, but the treatment of M2-Scr and M2-shN-cadherin with $CoCl_2$ did not cause any further increase (Fig. 5C, left panel). These data suggest that under normoxic conditions N-cadherin acts as a negative regulator of HSC proliferation.

These unexpected findings prompted us to examine whether similar effects would also be observed in the hypoxic-cocultures. Under these conditions we found that the effect of N-cadherin silencing in the stromal cells was not apparent due to the overall suppression of hematopoiesis, though a slight decrease in the HSC subsets was seen (Supplementary Fig. S3C).

Collectively, these data reveal that $CoCl_2$-BMSCs support the regeneration of hematopoiesis via activation of FAK and upregulation of HIF-1α and N-cadherin.

Non-cell-autonomous N-cadherin-mediated regulation of CXCR4 expression by HSCs interacting with BMSCs with active HIF-1α signaling

Having determined that N-cadherin downregulates hematopoiesis, we further examined whether the loss of N-cadherin also affects the functionality of the HSCs. Since CXCR4 plays a vital role in the homing and the engraftment of HSCs, we enumerated $CXCR4^+$ cells in the output population collected from the normoxic cocultures with M2-Scr and with M2-shN-cadherin cells (Fig. 6A). Depletion of N-cadherin in the stromal cells (M2-shN-cadherin) resulted in a significant increase in the absolute numbers of CXCR4-expressing HSCs (Fig. 6B, first panel) compared with the M2-Scr cells. In contrast, $CoCl_2$-treatment of M2-shN-Cadherin cells resulted in a significant reduction in the $CXCR4^+$ HSCs in the output cells, compared to untreated M2-shN-cadherin cells (Fig. 6B, second panel). The difference between absolute numbers of $CXCR4^+$ HSCs in cocultures of $CoCl_2$-treated M2-Scr and M2-shN-cadherin was not significant (Fig. 6B, third panel). $CoCl_2$-treatment of M2-Scr under normoxia significantly increased the absolute numbers of $CXCR4^+$ LSK and ST-HSCs in the output cells (Fig. 6B, fourth panel).

Flow cytometry analysis of the output cells from hypoxic-cocultures revealed that silencing of N-cadherin in the stromal cells (M2-shN-cadherin) significantly affected the output of CXCR4-expressing HSCs (Fig. 6C). Consistent with these data, significantly fewer cells cocultured with M2-shN-cadherin clones migrated toward SDF-1α compared with those cocultured with M2-Scr cells under hypoxic conditions (Fig. 6D).

These data demonstrate that N-cadherin in the microenvironment plays a crucial role in the regulation of CXCR4 expression by the HSCs interacting in a niche having an active HIF-1α signaling.

Differential decay kinetics of HIF-1α and N-cadherin may facilitate HSC proliferation in the engraftment window

From the data obtained so far, it was clear that the BMSCs having an active HIF-1α-mediated signaling and reduced levels of N-cadherin would support HSC proliferation. But, we also found that hypoxia upregulates N-cadherin expression, whereas normoxia downregulates it along with HIF-1α. Therefore, we asked a question how the transiently elevated oxygen content in the BM that arises due to myelosuppression facilitates HSC proliferation in the BM niche, where the stromal cells are also experiencing normoxic conditions.

We speculated that the answer to this paradox may lie in the differential kinetics of oxygen-mediated decay of HIF-1α and N-cadherin in the stromal cells. To examine this possibility, we transferred the BMSCs that were pre-incubated under hypoxia to normoxia and lysed the cells after specific time intervals. The lysates were subjected to western blot analyses using antibodies to N-cadherin and HIF-1α. As seen in the Fig. 7A, the decay rate of HIF-1α was much slower than that of N-cadherin, giving a perfect window of >24 h, wherein stromal cells still had higher levels of HIF-1α, but lower levels of N-cadherin—a situation very much conducive to the HSC proliferation.

Based on these in vitro data, we speculate that such situation arising in vivo would allow a rapid proliferation of HSCs in the engraftment window before the hypoxia gets reestablished.

Oxygen tension in the milieu plays a dominant role in the regeneration of hematopoiesis

The results obtained so far showed that both $CoCl_2$- and hypoxic-BMSCs possessed an identical hematopoiesis-supportive signaling gamut, and yet, the regeneration of hematopoiesis in the output of their cocultures was radically different. Since the only major difference in these two types of cocultures was the oxygen tension in the milieu, we examined whether hypoxia dominantly affects/reverses the regenerative capacity of the $CoCl_2$-BMSCs. We incubated the $CoCl_2$-cocultures under hypoxia, and enumerated the output hematopoietic population. The total hematopoietic cell output in $CoCl_2$-cocultures incubated under hypoxia

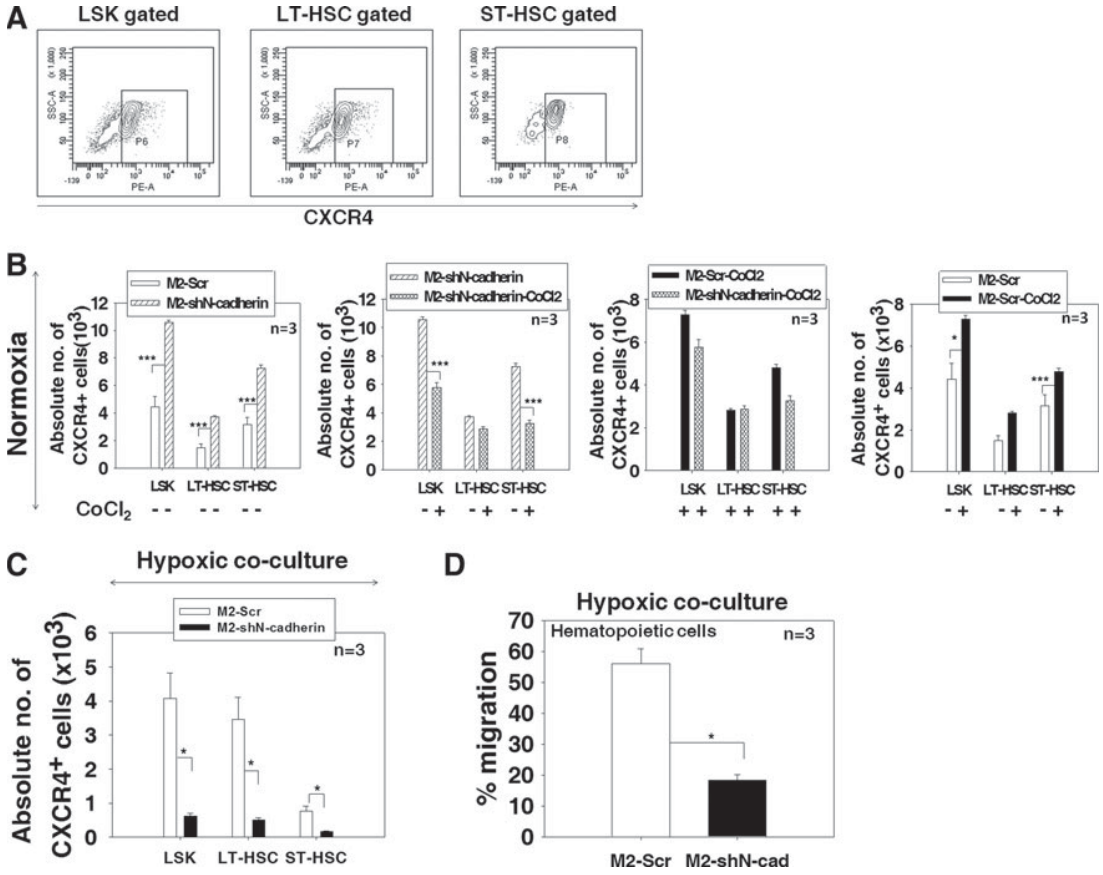

FIG. 6. Stromal N-cadherin regulates CXCR4 expression on cocultured HSCs. HSCs (Lin^- fraction) were cocultured with M2-Scr or M2-shN-cadherin cells, treated or not with $CoCl_2$, for 7 days under normoxia or hypoxia, and the output HSCs were analyzed for CXCR4 expression by flow cytometry. **(A)** Representative flow panel illustrates the gating strategy used to examine the CXCR4 expression in the output HSCs. **(B)** Silencing of N-cadherin increases the absolute numbers of $CXCR4^+$ HSCs under normoxia (*first panel*). $CoCl_2$-treatement of M2-shN-cadherin significantly reduces the absolute numbers of $CXCR4^+$ HSCs (*second panel*). *Third panel* compares the absolute numbers of $CXCR4^+$ HSCs in M2-Scr and M2-shN-cadhrin cells treated with $CoCl_2$. $CoCl_2$-treatment of M2-Scr increases the absolute numbers of $CXCR4^+$ HSCs (*fourth panel*). $n=3$. **(C)** Data show that loss of N-cadherin in the stromal cells significantly lowers the absolute numbers of $CXCR4^+$ HSCs in the output cells from hypoxic-cocultures. $n=3$. **(D)** SDF-1α-mediated chemotaxis of output cells from hypoxic-cocultures set with M2-Scr and M2-shN-cadherin. The output cells from M2-shN-cadherin cocultures migrate in significantly lower numbers than those from M2-Scr cocultures. $n=3$. Data are represented as mean ± SEM. The experiments were repeated twice with similar results. $*P \le 0.05$ and $***P \le 0.001$.

was drastically reduced (Fig. 7B). Phenotypic analyses of the output population showed that incubation of $CoCl_2$-cocultures under hypoxia abrogated their HSC-supportive ability as seen by the drastic decrease in the total output of HSC subsets (Fig. 7C). Similar results were also obtained with 5% oxygen (data not shown).

These data clearly reveal that the oxygen tension in the milieu plays a dominant role in the regeneration of hematopoiesis in spite of active hematopoiesis-supportive signals present in the hypoxic niche.

Discussion

The marrow is a hypoxic tissue with an oxygen gradient ranging from 1% to 6% [1,2]. Most quiescent HSCs are found at the lowest end of this gradient [29,30], suggesting that the primary function of marrow hypoxia is to maintain a pool of quiescent HSCs. Using 3D cultures of PMSCs we have earlier shown that a hypoxia-gradient, and not merely complete hypoxia, promotes robust regeneration of hematopoiesis without affecting the stem cell pool [27].

Most in vitro studies reported so far have examined the effect of hypoxia on hematopoiesis by using hypoxia chambers, where either the whole marrow cells or purified HSCs were incubated under hypoxic conditions and analyzed [21,22,31–36]. These experimental systems did not permit the delineation of the indirect effect of the hypoxic niche on the HSCs vis-à-vis the direct effect of hypoxia on the HSCs themselves. One of the objectives of this study was to identify the signaling mechanisms operative in the hypoxic niche and to understand the effect of this signaling on HSC fate under different oxygen tensions. To enable us to study this, we created an in vitro ''oxygen-independent hypoxic niche.''

The coculture studies revealed radically different regenerative capacities of $CoCl_2$-BMSCs and hypoxic-BMSCs.

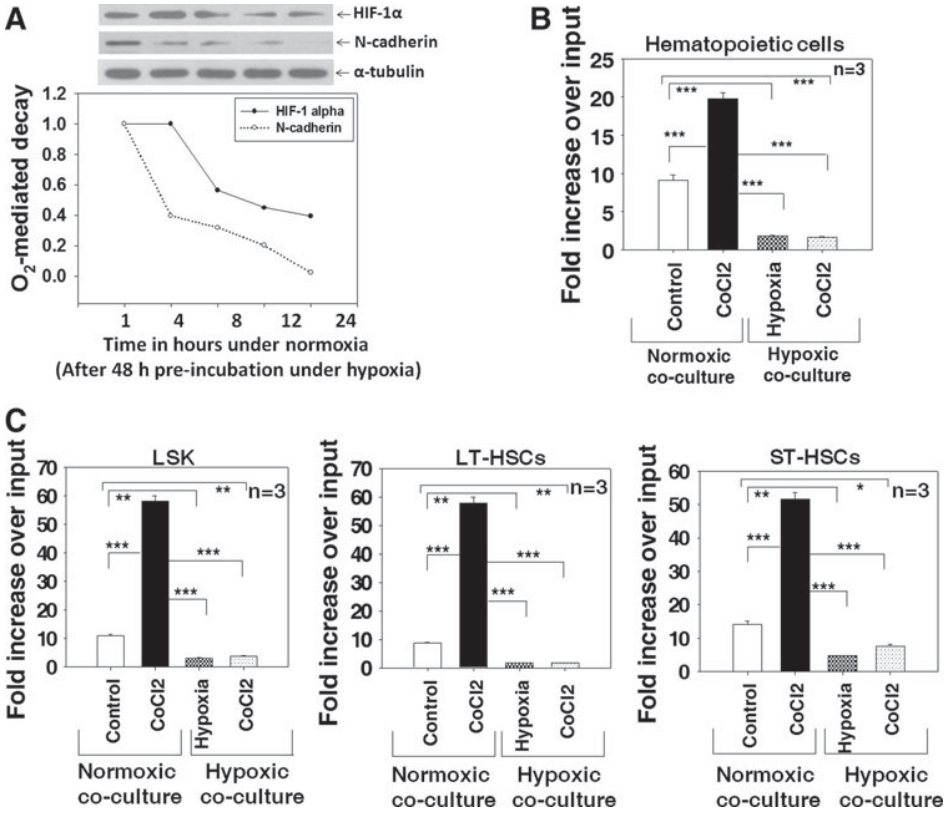

FIG. 7. Oxygen tension in the milieu plays a dominant role in the regeneration of hematopoiesis. **(A)** Differential rate of oxygen-mediated decay of HIF-1α and N-cadherin is graphically illustrated. Incubation of $CoCl_2$-cocultures under hypoxia significantly reduces the total hematopoietic cell output **(B)** and total numbers of various HSC subsets **(C)** compared to their counterparts incubated under normoxia. $n = 3$. The experiments were repeated twice with similar results. Data are represented as mean ± SEM. $*P \leq 0.05$, $**P \leq 0.01$, and $***P \leq 0.001$.

Since both BMSCs possessed a comparable signaling gamut, we inferred that the oxygen tension in the milieu plays a dominant role in the process. We did indeed find that when the $CoCl_2$-cocultures were incubated under hypoxia, their regenerative capacity was suppressed, clearly showing that the oxygen tension in the milieu plays a dominant role in the regenerative process, despite active hematopoietic-supportive signaling in the niche.

It is generally believed that the hypoxic niche protects the stem cells from oxidative stress, and thus, the HSCs present in such niches possess low ROS levels in vivo [3]. Contrary to this, we found that the HSCs from hypoxic-cocultures possessed very high levels of ROS and a high percentage of apoptotic cells. These data are consistent with their deficient engraftment. Though hypoxia-associated ROS appears paradoxical, it is known to occur in various systems, and a bimodal distribution of ROS formation as a function of pO_2 has been proposed, where both hypoxia and hyperoxia supported elevations in ROS formation [37]. Such a phenomenon may be operative in the hypoxic-cocultures used in our experiments. Actively proliferating HSCs are known to accumulate less ROS and possess a more efficient DNA damage repair mechanism than quiescent HSCs [38]. The high level of proliferation induced by $CoCl_2$-BMSCs may be the reason behind the low ROS and apoptosis levels seen in the HSCs from $CoCl_2$-cocultures.These findings collectively suggest that the use of a normoxic coculture with an ''oxygen-independent hypoxic niche'' may be a useful strategy for ex vivo expansion of HSCs.

Although the effect of hypoxia on HSCs has been studied extensively, very few reports are available that specifically deal with signaling mechanisms operative in the hypoxic niche. The role of FAK signaling in steady-state and stress hematopoiesis has been reported [39,40]. Here, we demonstrate that both, hypoxia- and $CoCl_2$-treatment activate FAK signaling in the BMSCs, and a pharmacological inhibition of this signaling significantly reduces the hematopoietic-supportive ability of $CoCl_2$-BMSCs, thus underscoring the role of FAK signaling in the regeneration of hematopoiesis.

The role of micro-environmental HIF subunits in stem cell biology has not been extensively studied. Several BM niche cells such as endothelial cells and osteoblasts have been shown to express HIF-2α, whereas HSCs express HIF-1α [41]. Exogenous-expression of HIF-1α in the BMSCs has been shown to promote hematopoiesis in vitro [16]. Similarly, overexpression of HIF-1α in human fetal liver stromal cells has been shown to maintain self-renewal and pluripotency of human embryonic stem cells [42]. We report here that both, hypoxia- and $CoCl_2$-treatment of BMSCs strongly increase HIF-1α levels in them. ShRNA-mediated silencing of HIF-1α in the stromal cells resulted in diminished ability of these BMSCs to expand HSCs after $CoCl_2$-treatment, showing that HIF-1α expression was crucial for the expansion of HSC pool by $CoCl_2$-treated BMSCs. Importantly, silencing of HIF-1α in the stromal cells led to production of higher number of hematopoietic cells in general at the expense of stem cell pool, underscoring the importance of stromal HIF-1α in the maintenance of the stem cell pool.

The role of N-cadherin in the regulation of hematopoiesis has been controversial [43–46]. We found that treatment of BMSCs with $CoCl_2$ or hypoxia strongly upregulated

N-cadherin in them. We examined the role of this upregulated N-cadherin on the HSCs cocultured with N-cadherin-silenced clones of M210B4 stromal cells. The data obtained with these clones revealed an important facet of stromal N-cadherin in the regulation of hematopoiesis. Silencing of N-cadherin in the stromal cells further stimulated the hematopoietic output and further increased the HSC pool in $CoCl_2$-cocultures under normoxic conditions. These data suggest that N-cadherin acts as a negative regulator of hematopoiesis under higher oxygen tensions. These data also suggest that the transient elevation of oxygen tension in the niche postmyelosupproession may downregulate microenvironmental N-cadherin, thereby temporarily relieving the effect of this negative regulator, thus facilitating the regeneration of hematopoiesis.

Most significantly, our data demonstrate the important role of stromal N-cadherin in the regulation of CXCR4 expression by the HSCs. We found a significant decrease in the frequency and absolute numbers of CXCR4-expressing HSCs when the cells were cocultured with M2-shN-cadherin clones, with HIF-1α activated either via hypoxia or by $CoCl_2$-treatment. Pharmacological or hypoxia-mediated stabilization of HIF-1α in the HSCs has been shown to induce high expression of CXCR4 in them [11,47]. However, our data provide the first evidence for the critical role of N-cadherin-mediated microenvironmental signaling on the CXCR4 expression in the cocultured HSCs, especially when they are interacting within a hypoxic niche. In other words, a hypoxic niche lacking N-cadherin expression appears to be detrimental to the HSC function and it may be important to see whether this is a case in any of the hematological disorders. The mechanism involved in the regulation of CXCR4 expression in the HSCs by the microenvironmental N-cadherin forms the basis of our future research.

Another objective of these studies was to establish an experimental system that would help us to understand the events taking place in the engraftment window. We were specifically interested in learning why the infused HSCs proliferate after homing to the endosteal niche that otherwise maintains HSCs in a quiescent stage. Pretransplant myeloablation leads to a transient increase in the marrow oxygen tension due to the lower consumption of oxygen resulting from cell death [2]. Therefore, under this situation, the infused HSCs would interact with the BM niche under a relatively higher oxygen tension. However, once the regeneration of hematopoiesis takes place, the oxygen consumption would increase, leading to reestablishment of hypoxia and the regenerative process would reach steady-state conditions. A recent report shows that hypoxia activates the TGF-β pathway in the HSCs and induces cell cycle arrest in them via the cyclin-dependent kinase inhibitor CDKN1C/p57kip2 [48]. These reports, coupled with our data, which show that higher levels of oxygen are needed for the regenerative process, suggest that the kinetics of reestablishment of hypoxia may be the limiting factor in the post-transplant regeneration of hematopoiesis.

A point of confusion that may arise in this proposed scenario would be that the normoxic cocultures, wherein both BMSCs and HSCs experience conditions that mimic the engraftment window (ie, higher oxygen content) do not support robust HSC proliferation and differentiation. Only when the BMSCs are ''frozen'' in the hypoxic state (ie, treated with $CoCl_2$) can they support HSC proliferation and differentiation when cocultured under normoxic conditions. Then, the question arises as to how the transiently elevated oxygen content in the BM that arises due to myelosuppression facilitates HSC proliferation in the BM niche, where the stromal cells are also experiencing normoxic conditions. We tried to find a solution to this paradox by studying the rate of oxygen-mediated decay of HIF-1α vis-à-vis N-cadherin in the BMSCs, and found that rate of degradation of HIF-1α is much slower than that of N-cadherin (Fig. 7A). Thus, the stromal cells would lose HIF-1α signaling at much slower rate compared with the loss of N-cadherin under the transiently elevated oxygen tension postmyelosuppression, giving a perfect window for the transplanted HSCs to proliferate (Supplementary Fig. S4). These data suggest that perhaps in the engraftment window, the stromal cells would have relatively higher levels of HIF-1α (so signaling is still active which will support HSC proliferation under higher oxygen tension), but lower levels of N-cadherin (which will result in temporarily removing its negative influence).

In conclusion, our data reveal that the hypoxic niche by default possesses a hematopoiesis-supportive signaling gamut, but the regenerative process is dominantly regulated by the oxygen tension in the milieu. An approach to modulate the kinetics of hypoxia reestablishment in the BM may lead to better regeneration of post-transplant hematopoiesis.

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Author Disclosure Statement

The authors declare that no competing financial interests exist.

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