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Bone Marrow Endosteal Mesenchymal Progenitors Depend on HIF Factors for Maintenance and Regulation of Hematopoiesis

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SUMMARY

Maintenance and differentiation of hematopoietic stem cells (HSCs) is regulated through cell-autonomous and non-cell-autonomous mechanisms within specialized bone marrow microenvironments. Recent evidence demonstrates that signaling by HIF-1α contributes to cell-autonomous regulation of HSC maintenance. By investigating the role of HIF factors in bone marrow mesenchymal progenitors, we found that murine endosteal mesenchymal progenitors express high levels of HIF-1α and HIF-2α and proliferate preferentially in hypoxic conditions ex vivo. Inactivation of either HIF-1α or HIF-2α dramatically affects their phenotype, propagation, and differentiation. Also, downregulation of HIF factors provokes an increase in interferon-responsive genes and triggers expansion and differentiation of hematopoietic progenitors by a STAT1-mediated mechanism. Interestingly, in conditions of demand-driven hematopoiesis HIF factors are specifically downregulated in mesenchymal progenitors in vivo. In conclusion, our findings indicate that HIF factors also regulate hematopoiesis non-cell-autonomously by preventing activation of a latent program in mesenchymal progenitors that promotes hematopoiesis.

INTRODUCTION

Hematopoiesis is a tightly regulated process orchestrated by cell-autonomous and non-cell-autonomous signals emanating from a variety of cell types within specialized bone marrow (BM) microenvironments (Wang and Wagers, 2011; Frenette et al., 2013). Coordinated signals instruct hematopoietic stem cells (HSCs) to maintain their undifferentiated status or to commit and differentiate into mature hematopoietic cells (Kiel and Morrison, 2008; Wilson and Trumpp, 2006).

A number of recent reports suggest that signaling by hypoxia-inducible transcription factors (HIFs) regulate HSC maintenance in a cell-autonomous manner. HIF factors are heterodimeric transcription factors composed of α and β subunits: the β subunit (ARNT) is constitutively expressed, whereas the α subunit is degraded through an oxygen-dependent mechanism and is stabilized at low oxygen concentrations (Schofield and Ratcliffe, 2004). Three α subunits have been identified: HIF-1α, HIF-2α, and HIF-3α, with HIF-1α and HIF-2α being the most extensively characterized (Keith et al., 2012). Despite sharing a high degree of sequence identity, HIF-1α and HIF-2α are not redundant, because they are expressed at least partly in a tissue-specific manner and regulate a number of unique target genes (Ratcliffe, 2007; Keith et al., 2012). In hypoxic conditions, HIF transcription factors trigger a variety of adaptive responses that include induction of anaerobic metabolism, cell migration, and neo-angiogenesis (Semenza, 2003). More recently, HIF factors are being increasingly implicated in regulating stem cells homeostasis (Mohyeldin et al., 2010; Suda et al., 2011), particularly in the hematopoietic system where HIF-1α is expressed in HSC (Takubo et al., 2010) and promotes HSC maintenance by enforcing a glycolytic metabolic state (Takubo et al., 2013).

Quiescent, long-term repopulating HSC (LT-HSC) are believed to reside predominantly in periendosteal areas of the BM characterized by low oxygen levels (Mohyeldin et al., 2010; Suda et al., 2011; Eliasson and Jönsson, 2010). Moreover, it is recently being suggested that HSC and hematopoietic progenitors may exhibit a hypoxic state and express high levels of HIF-1α also through oxygen-independent mechanisms (Nombela-Arrieta et al., 2013). Different cell populations reside in close proximity to HSC in the BM and participate to the regulation of HSC maintenance and differentiation (Wang and Wagers, 2011). Within these cell types, a number of mesenchymal progenitors are being described as important non-cell-autonomous regulators of HSC maintenance (Wang and Wagers, 2011). Mesenchymal progenitors are functionally defined as clonogenic populations that can differentiate toward mesenchymal lineages adipocytes, osteoblasts, and chondrocytes ex vivo (Uccelli et al., 2008; Nombela-Arrieta

et al., 2011). Among these, BM stromal cells expressing SCA-1 and PDGFRα (from now on referred to as PαS$^+$ cells) localize to the perivascular spaces of endosteal BM (Morikawa et al., 2009; Nakamura et al., 2010) and are described as important regulators of HSC maintenance (Nakamura et al., 2010; Ding and Morrison, 2013; Greenbaum et al., 2013).

Here, we demonstrate that similarly to HSC, PαS$^+$ cells are characterized by a hypoxic gene expression profile, as measured by expression of HIF-1α, HIF-2α, and HIF-target genes and have increased capacity to proliferate and form colonies in hypoxic conditions ex vivo. We find that expression of HIF-1α and HIF-2α in PαS$^+$ progenitors is necessary to maintain their colony-forming capacity, differentiation competence and phenotype. Moreover, expression of HIF factors in PαS$^+$ progenitors is necessary to promote non-cell-autonomous regulation of hematopoiesis by a molecular mechanism involving repression of STAT1-induced soluble factors.

RESULTS

Endosteal Mesenchymal Progenitors Expressing SCA-1 and PDGFRα Exhibit a Hypoxic Profile

It was recently reported that HIF-1α is highly expressed in HSC (Takubo et al., 2010). Although oxygen levels are generally low in the BM, HIF-1α expression in HSC appears to be regulated not only by hypoxic protein stabilization but also by additional mechanisms, because *Hif-1α* is highly expressed at the mRNA level in HSC (Takubo et al., 2010), and HIF-1α expression is detected in HSC residing in BM compartments characterized by different oxygen levels (Nombela-Arrieta et al., 2013). Although the molecular mechanisms driving HIF-1α expression in HSC are not fully elucidated and may involve secreted factors within HSC niches, mesenchymal progenitors localizing in close proximity to HSC in the BM are exposed to the same environmental conditions. Therefore, we hypothesized that similarly to HSC, mesenchymal progenitors may also express HIF factors and depend on hypoxia signaling for their cell-autonomous and non-cell-autonomous functions.

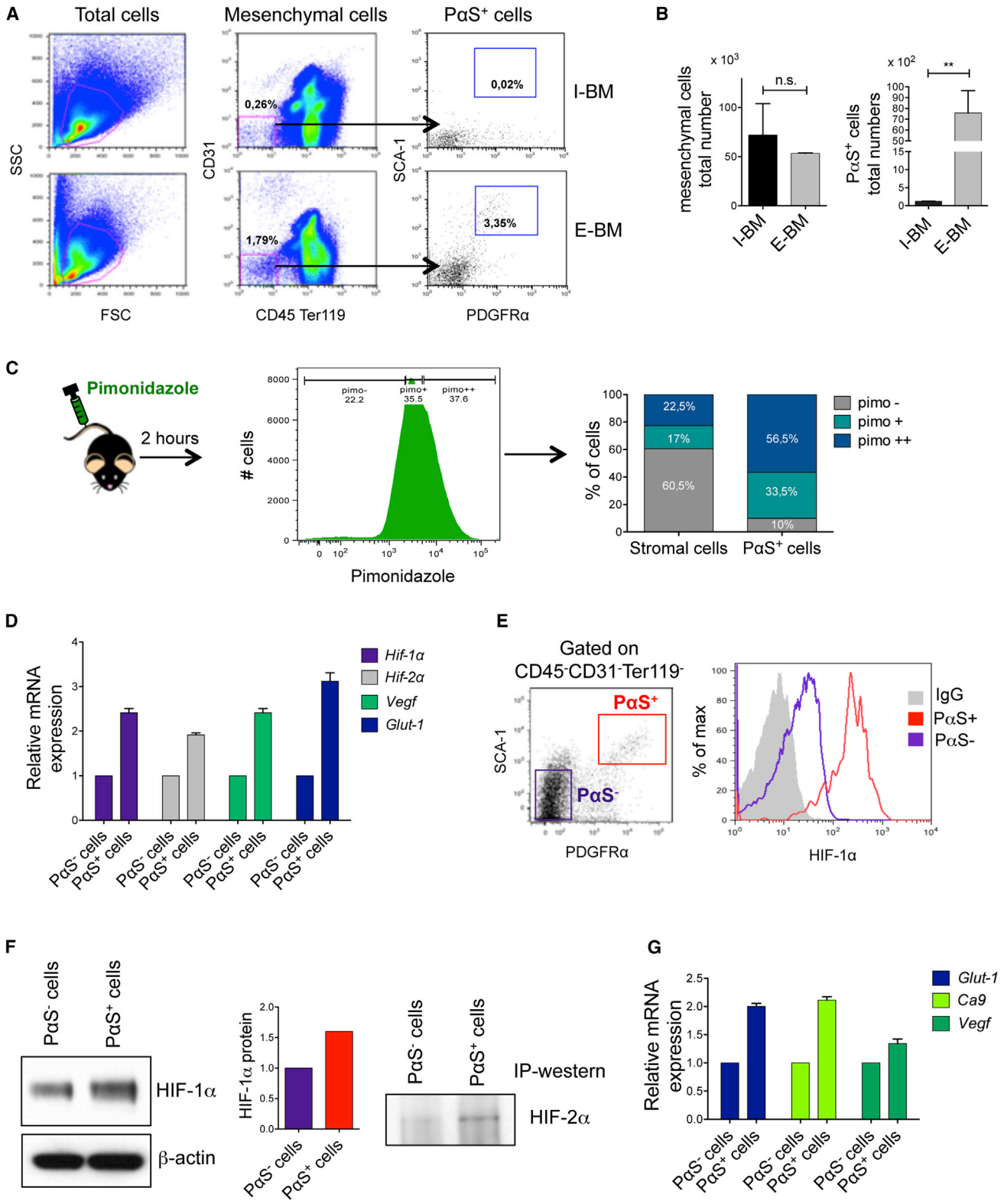
To test this hypothesis, we focused on stromal BM cells (CD45$^-$CD31$^-$Ter119$^-$) expressing SCA-1$^+$ and PDGFRα$^+$ (PαS$^+$ cells) (Morikawa et al., 2009; Houlihan et al., 2012). PαS$^+$ cells are enriched in mesenchymal progenitors because they give rise to fibroblasts colonies when plated as single cells in vitro (colony forming unit-fibroblast assay, or CFU-F) and differentiate into different mesenchymal cell types (Morikawa et al., 2009). In vivo PαS$^+$ cells localize to perivascular spaces at the endosteal surface of the BM (Morikawa et al., 2009; Houlihan et al., 2012; Nombela-Arrieta et al., 2013) and participate to non-cell-autonomous regulation of hematopoiesis by inducing HSC maintenance (Nakamura et al., 2010; Ding and Morrison, 2013; Greenbaum et al., 2013).

To assess if PαS$^+$ cells are characterized by a hypoxic profile, we measured pimonidazole incorporation and expression of HIF factors. The inner fraction of the BM (I-BM) was collected by flushing out the BM and treating crushed bone fragments with collagenase to isolate cells associated with the endosteal surface (E-BM) (Grassinger et al., 2010; Nakamura et al., 2010). The percentage of mesenchymal cells (CD45$^-$Ter119$^-$CD31$^-$) was higher in E-BM compared to I-BM (Figure 1A), although total mesenchymal cell numbers were similar in the two BM fractions (Figure 1B, left graph). Conversely, PαS$^+$ mesenchymal cells were found almost exclusively in E-BM (Figures 1A and 1B, right graph).

To assess the hypoxic profile of endosteal mesenchymal cells, E-BM cells were divided in pimo$^-$, pimo$^+$, and pimo^{++} based on incorporation of the hypoxic probe pimonidazole (pimo; Figure 1C), and stromal cells were analyzed within each fraction. As shown in Figure 1C (right graph), endosteal mesenchymal cells (CD45$^-$Ter119$^-$CD31$^-$) were found mainly in the pimo$^-$ fraction (60.5%), with smaller fractions residing in the pimo$^+$ (16.9%) or pimo^{++} regions (22.5%). On the contrary, PαS$^+$ cells were specifically enriched in the pimo^{++} (56.5%) and pimo$^+$ fraction (33.5%), with only a minority of PαS$^+$ cells being pimo$^-$ (10%). These data suggest that similarly to HSC (Nombela-Arrieta et al., 2013), the hypoxic phenotype of mesenchymal progenitors is a cell-specific phenomenon, rather than simply depending on endosteal localization.

We next analyzed expression of HIF-1α, HIF-2α, and bona fide HIF-target genes in PαS$^+$ cells compared to BM endosteal stromal cells negative for SCA-1 and PDGFRα (PαS$^-$). PαS$^+$ cells expressed higher mRNA levels of *Hif-1α*, *Hif-2α*, and HIF-target genes *Vegf* and *Glut-1*, and higher HIF-1α protein than PαS$^-$ cells (Figures 1D and 1E), thus indicating that in PαS$^+$ cells expression of HIF-1α is regulated at least in part at the transcriptional level, coherently with a similar level of regulation in human mesenchymal stem cells (Palomäki et al., 2013). Interestingly, higher expression of HIF-1α and HIF-2α protein (Figure 1F) and HIF-target genes (Figure 1G) in PαS$^+$ cells was also maintained after 1 week culture in hypoxic conditions, thus indicating that even when exogenously subjected to the same oxygen concentrations PαS$^+$ cells maintain a stronger hypoxia signature than PαS$^-$ cells.

Suda and coworkers have demonstrated that in the hematopoietic hierarchy HSC express highest HIF-1α levels compared to more committed cells, while expressing low levels of HIF-2α (Takubo et al., 2010). Interestingly, by comparing the expression of HIF-1α and HIF-2α in BM hematopoietic progenitors (HPC: CD45$^+$Lineage$^-$) and total



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endosteal stromal cells (BM-SC: CD45$^-$Ter119$^-$CD31$^-$) we found that BM-SC express higher levels of both *Hif* factors, with the biggest difference found in *Hif-2α* expression (Figure S1A available online), although *Hif-1α* is expressed at higher levels than *Hif-2α* in BM-SC (Figure S1B). Also, HIF-1α protein levels are higher in PαS$^+$ mesenchymal progenitors than in CD45$^+$C-KIT$^+$SCA-1$^+$ hematopoietic stem/progenitor cells (Figure S1C). Taken together, these data indicate that PαS$^+$ cells express higher levels of HIF factors than hematopoietic cells and other stromal cell types residing in the same BM location and maintain a stronger hypoxia signature also when subjected to similar oxygen concentrations.

Mesenchymal Progenitors Expressing SCA-1 and PDGFRα Expand Preferentially in Hypoxic Conditions

Because PαS$^+$ cells display a hypoxic phenotype, we analyzed their propagation and maintenance in vitro in hypoxic conditions. When plated at single-cell dilution (Morikawa et al., 2009; Houlihan et al., 2012) at 20% or 1% O_2 for 7 days, PαS$^+$ cells expressed higher levels of HIF-target genes (Figure 2A) and proliferated more efficiently at 1% O_2 (Figure 2B). Furthermore, the morphology of PαS$^+$-generated colonies and cells within these colonies differed significantly at different O_2 concentrations, with CFU-F colonies composed of tightly adherent cells at 1% O_2, whereas cells were sparse, elongated, and detached in 20% O_2 colonies (Figure 2C). In addition, hypoxic conditioning contributed to maintenance of a transcriptional profile typical of undifferentiated mesenchymal progenitors (Tsai et al., 2012a; Yoon et al., 2011), such as higher expression of the transcription factors *Nanog*, *Oct-4*, and *Sox2* (Figure 2D). These data indicate that PαS$^+$ cells expand preferentially in hypoxic conditions while also maintaining higher expression of genes characterizing stem/progenitor cells.

We next asked whether PαS$^-$ endosteal mesenchymal cells acquired properties of mesenchymal progenitors if cultured at low oxygen concentrations. Endosteal PαS$^+$ and PαS$^-$ stromal cells were plated at the same concentration and cultured at 1% O_2. Interestingly, we found that after 1 week in culture PαS$^-$ cells acquired surface expression of both SCA-1 and PDGFRα, although at lower levels than PαS$^+$ cells (Figure 2E). Surprisingly, this occurred independently of oxygen concentrations, because it also occurred at 20% O_2 (data not shown). Furthermore, although it was previously shown that PαS$^-$ cells have limited colony-forming capacity at 20% O_2 (Morikawa et al., 2009), they formed colonies at 1% O_2 (Figure 2E), although less than PαS$^+$ cells (Figure 2F) and with a different morphology, being smaller and composed of round cells (Figure 2E). Because both PαS$^+$ and PαS$^-$ cells form colonies and express SCA-1 and PDGFRα in vitro in hypoxia, we next analyzed the expression of genes associated with mesenchymal stem cell maintenance or differentiation. Specifically, *Oct-4* and *Sox2* are expressed in undifferentiated mesenchymal progenitors (Tsai et al., 2012a; Yoon et al., 2011), whereas PDGF specifies commitment to mesenchymal lineages (Ball et al., 2012). As shown in Figure 2G, PαS$^-$ cells express lower levels of *Oct-4* and *Sox2*, and higher levels of *Pdgf* than PαS$^+$ cells even upon culture in hypoxia.

Altogether, these data indicate that although hypoxic conditioning stimulates colony-forming capacity by endosteal mesenchymal cells, gene expression programs and growth properties remain different in distinct mesenchymal cell types, thus suggesting that hypoxic conditioning does not per se endorse all mesenchymal cells with a progenitor's profile, but possibly reinforces programs that are built in within specific cell types. Importantly, our data also indicate that expression of surface markers currently utilized to define mesenchymal

Figure 1. Mesenchymal Progenitors Expressing SCA-1 and PDGFRα Reside at the Endosteal Surface of the BM and Display a Hypoxic Profile

(A) Analysis of the percentage of mesenchymal cells (CD31$^-$CD45$^-$Ter119$^-$) and PαS$^+$ cells (CD31$^-$CD45$^-$Ter119$^-$SCA-1$^+$PDGFRα$^+$) at the endosteal BM surface (E-BM) and in the inner BM fraction (I-BM) (n = 6 mice).

(B) Total number of mesenchymal cells (left graph) or PαS$^+$ cells (right graph) at endosteal or inner BM (E-BM, I-BM) (n = 6 mice, mean ± SEM).

(C) Pimonidazole incorporation by endosteal BM cells. E-BM cells were divided in pimo^{++}, pimo$^+$, and pimo$^-$ and stromal cells (CD31$^-$CD45$^-$Ter119$^-$) or PαS$^+$ cells were analyzed in each fraction (one representative experiment out of two with similar results is shown).

(D) Ex vivo RT-PCR analysis of *Hif-1α*, *Hif-2α*, *Vegf*, and *Glut-1* mRNA levels in PαS$^-$ cells or in PαS$^+$ E-BM cells (data are pooled from two independent experiments, mean ± SEM).

(E) Ex vivo flow cytometry analysis of HIF-1α expression in PαS$^+$ cells or PαS$^-$ E-BM cells (n = 3, representative analysis of one mouse).

(F) Western blot analysis of HIF-1α (left blot) and IP-western blot analysis of HIF-2α (right blot) in PαS$^+$ or PαS$^-$ E-BM cells. Middle graph represents quantification of HIF-1α levels.

(G) RT-PCR analysis of *Glut-1*, *Ca9*, and *Vegf* mRNA levels in PαS$^-$ cells or in PαS$^+$ cells cultured for 7 days at 1% oxygen (data are pooled from two independent experiments, mean ± SEM).

See also Figure S1.

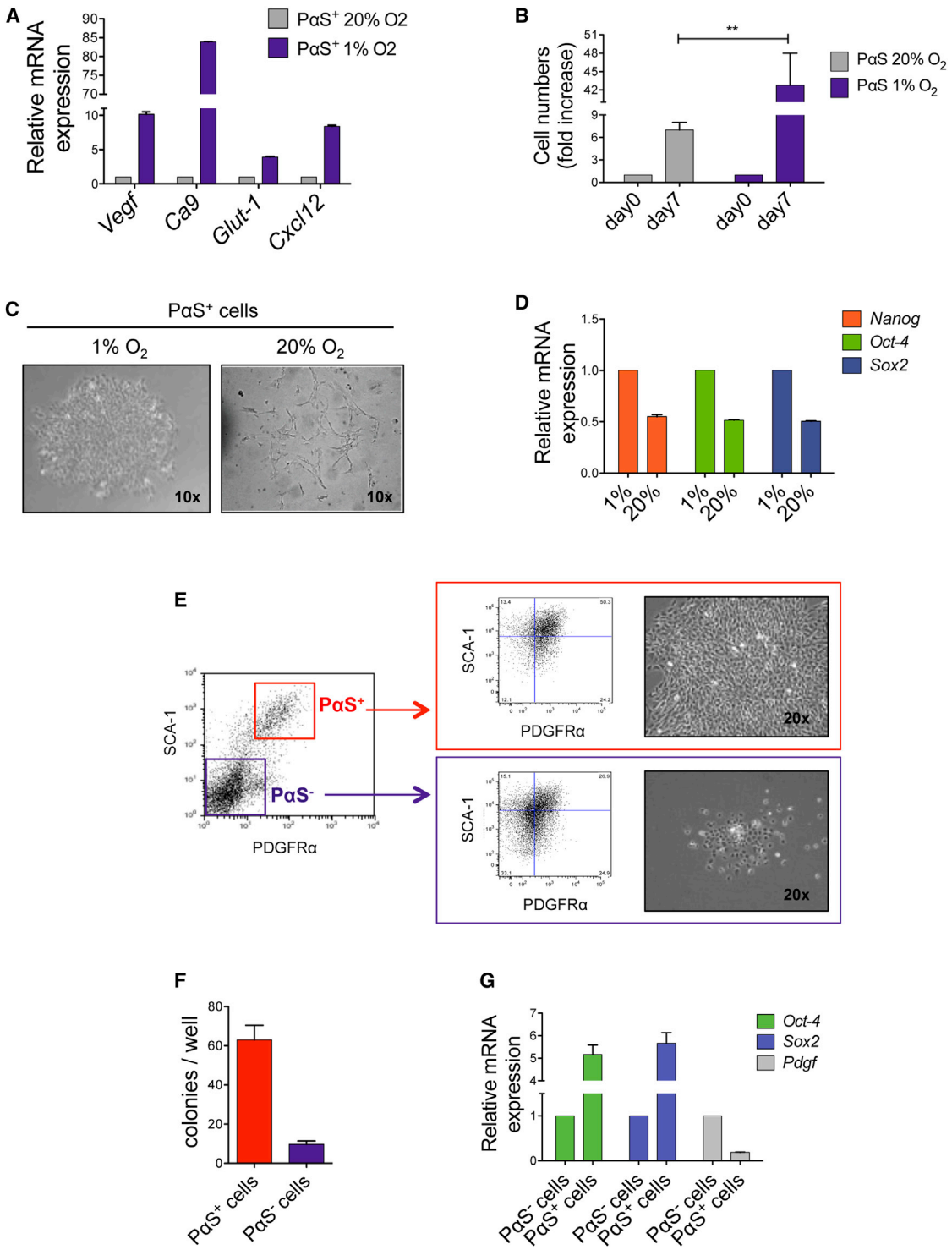

Figure 2. Mesenchymal Progenitors Expressing SCA-1 and PDGFRα Expand Preferentially in Hypoxic Conditions

(A) Relative mRNA expression of *Vegf*, *Ca9*, *Glut-1*, and *Cxcl12* in PαS$^+$ cells cultured for 7 days at 20% or 1% oxygen (data show one representative experiment out of two independent experiments performed in triplicate with similar results ±SD).

(B) Number of PαS$^+$ cells after a 7 day culture at 20% or 1% oxygen expressed as fold increase over the number of cells seeded at day 0 (data are pooled from three independent experiments, mean ± SEM).

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progenitors in vivo (e.g., SCA-1) can be acquired in culture, therefore suggesting that care should be taken when defining cultured cells by the expression of these markers.

HIF-1α and HIF-2α Regulate Expansion and Differentiation of Mesenchymal Progenitors

To understand if HIF factors regulate proliferation and maintenance of PαS$^+$ mesenchymal progenitors, HIF-1α and HIF-2α expression was downregulated ex vivo with specific small hairpin RNAs (shRNAs) (Figures S2A and S2B). PαS$^+$ cells transduced with either HIF-1α or HIF-2α shRNA showed lower CFU-F capacity and proliferation in hypoxic conditions (Figure 3A), without undergoing apoptosis (Figure S2C) or senescence (data not shown). HIF silencing also affected the morphology of colonies and colony-forming cells, with colonies formed by sparse and elongated cells, less adherent to one another (Figure 3B), and similar to colonies formed by PαS$^+$ cells in 20% O_2 (Figure 2C). As PαS$^+$ cells lacking HIF-1α or HIF-2α engaged in fewer cell-cell contacts, we investigated whether they had increased motility. Cell migration assays revealed that reduced expression of either HIF-1α or HIF-2α favored PαS$^+$ cell migration (Figure 3C).

Mesenchymal progenitors are defined as cells able to differentiate toward the mesodermal lineages adipocytes, osteoblasts, and chondrocytes (Uccelli et al., 2008; Nombela-Arrieta et al., 2011). As reported in Figures 3D–3F, mesenchymal progenitors lacking HIF factors showed decreased capacity to differentiate to mesenchymal lineages. Specifically, only a few colonies generated by mesenchymal progenitors lacking HIF factors differentiated into adipocytes, whereas the great majority maintained a fibroblastic-like morphology (Figure 3D). Similarly, only few cells lacking expression of HIF factors generated mature alkaline phosphatase-positive osteoblasts (Figure 3E), although cell concentrations were similar to control cells (as reported by crystal violet staining in Figure 3E on the right). Finally, upon induction of chondrogenesis, mesenchymal progenitors lacking HIF factors expressed normal levels of *Col9* (collagenase 9) compared to control cells but failed to express *Col10* (collagenase 10), thus indicating that they may generate immature chondrocytes but not terminally differentiated chondrocytes (Figure 3F).

In conclusion, these results demonstrate that in PαS$^+$ cells HIF factors are required ex vivo to maintain a mesenchymal progenitor state characterized by proliferation, colony-forming capacity, and competence to differentiate to multiple mesenchymal lineages.

Expression of HIF-1α and HIF-2α in Mesenchymal Progenitors Regulates Hematopoiesis

PαS$^+$ cells have been shown to promote HSC proliferation and maintenance (Morikawa et al., 2009; Nakamura et al., 2010). Given the profound changes caused by reduced expression of HIF factors on cell-autonomous properties of PαS$^+$ cells, we asked whether expression of HIF factors also modified non-cell-autonomous functions toward hematopoietic cells. PαS$^+$ mesenchymal progenitors were cocultured in vitro with sorted hematopoietic stem/progenitor cells (C-KIT$^+$Lin$^-$SCA-1$^+$ cells, KLS) in hypoxic conditions for 2 and 4 days (Figure S3A). By first using control PαS$^+$ cells, we observed that hematopoietic cells increase in number in the 4 days coculture period (Figure 4A), and this expansion is accompanied by a gradual decline in the percentage of KLS cells and hematopoietic progenitors (C-KIT$^+$ hematopoietic cells; Figure 4B), concomitantly with an increase in differentiated hematopoietic cells (Lin$^+$ cells; Figure 4B). Upon silencing of HIF-1α or HIF-2α in PαS$^+$ cells, the total number of hematopoietic cells (CD45$^+$) recovered after 2 and 4 days in coculture increased about 2-fold compared to cocultures with control PαS$^+$ cells (Figure 4C). When analyzing the relative representation of hematopoietic cells in the cocultures, we found that at day 2 there was no significant difference in the percentage of KLS, C-KIT$^+$, and Lin$^+$ cells recovered after silencing of HIF factors (Figures 4B and 4D), thus indicating a 1.5-fold expansion of all hematopoietic cells (Figure 4C). Because 90% of hematopoietic cells in coculture at day 2 are Lin$^-$C-KIT$^+$ cells (Figures 4B and 4D), this indicates an expansion of hematopoietic progenitors. Conversely, at day 4 a significant increase in the percentage of differentiated Lin$^+$ hematopoietic cells was observed where HIF factors had been silenced in PαS$^+$ cells

(C) Morphology of CFU-F colonies of PαS$^+$ cells cultured for 7 days at 20% or 1% oxygen (one representative experiment out of three independent experiments is shown).
(D) Relative mRNA expression of *Nanog*, *Oct-4*, and *Sox2* in PαS$^+$ cells cultured for 7 days at 20% or 1% oxygen (data show one representative experiment out of two independent experiments performed in triplicate with similar results ±SD).
(E) SCA-1 and PDGFRα expression in PαS$^-$ and PαS$^+$ cells (graph on the left) after a 7 day culture at 1% oxygen. Representative pictures of colonies are shown on the right (colonies from one out of two independent experiments are shown).
(F) Number of colonies recovered from PαS$^-$ cells or PαS$^+$ cells cultured at 1% oxygen for 1 week (data are pooled from two independent experiments, mean ± SEM).
(G) RT-PCR of *Oct-4*, *Sox2*, and *Pdgf* mRNA levels in PαS$^-$ and PαS$^+$ cells cultured for 7 days at 1% oxygen (data show one representative experiment out of two independent experiments performed in triplicate with similar results ±SD).

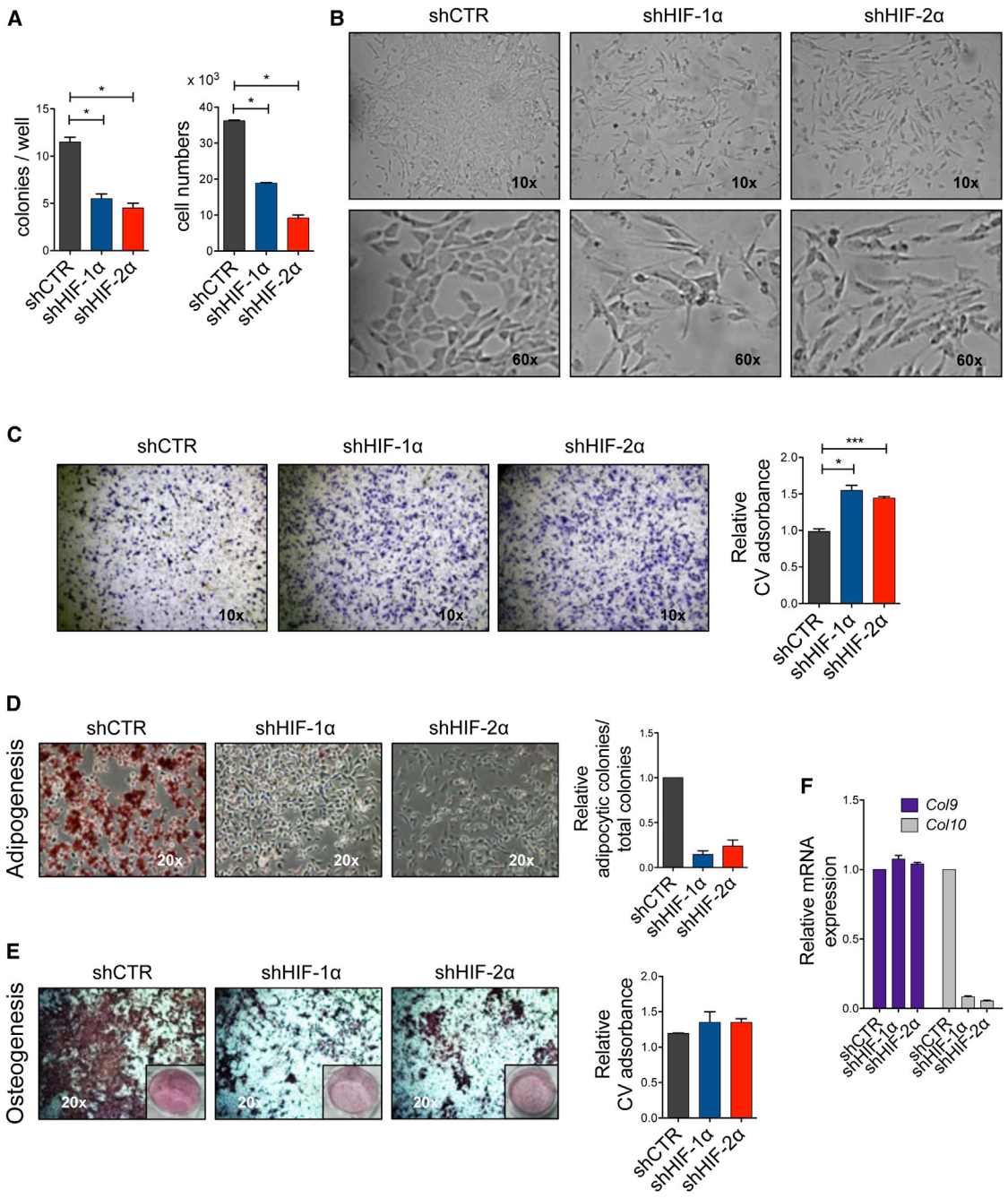

Figure 3. HIF-1α and HIF-2α Regulate the Biology of Mesenchymal Progenitors

(A) Quantification of CFU-F colonies (left graph) and cell numbers (right graph) from PαS$^+$ cells transduced with shCTR, shHIF-1α, or shHIF-2α and cultured at 1% oxygen for 7 days (data are pooled from three independent experiments, mean ± SEM).

(B) Morphology of CFU-F colonies generated by PαS$^+$ cells transduced with shCTR, shHIF-1α, or shHIF-2α and cultured at 1% oxygen for 7 days (representative colonies of one out of three independent experiments are shown).

(C) Migration properties of PαS$^+$ cells transduced with shCTR, shHIF-1α, or shHIF-2α and cultured at 1% oxygen; representative pictures of crystal violet staining are shown on the left, relative quantification is shown on the right (one representative experiment is shown on the left, whereas data pooled from three independent experiments are shown on the right, mean ± SEM).

(D) Adipogenesis by PαS$^+$ cells transduced with shCTR, shHIF-1α, or shHIF-2α. Representative pictures of adipogenic colonies are shown on the left; quantification of the adipogenic colonies among the total colonies generated by PαS$^+$ cells is shown on the right (data are pooled from two independent experiments, mean ± SEM).

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(Figure 4E). More specifically, percentages of myeloid ($Gr1^+CD11b^+$ cells) and $CD3^+$ lymphoid cells (Figures 4F and S3B) increased, whereas B220 percentages did not change (data not shown).

In summary, our data indicate that silencing of HIF factors in mesenchymal progenitors favors early expansion of hematopoietic progenitors followed by increased differentiation. Interestingly, similar results were obtained when freshly isolated KLS cells were cultured with media collected from $P\alpha S^+$ cells transduced with shCTR, shHIF-1α or shHIF-2α: total numbers of $CD45^+$ hematopoietic cells increased (Figure S3C), and Lin^+ cells were more abundant in percentage as well as in total numbers when KLS were exposed for 4 days to soluble factors released by $P\alpha S^+$ cells lacking HIF expression (Figure S3D).

To further validate these data, stable forms of HIF-1α and HIF-2α (mut HIF-1α and mut HIF-2α) were overexpressed in $P\alpha S^+$ cells and the fate of KLS cells was analyzed after a 4 days coculture. Although the total number of hematopoietic cells did not change upon overexpression of HIF factors (Figure 4G), the percentage of cells that maintained a hematopoietic stem/progenitor expression profile (KLS cells) was significantly higher upon overexpression HIF factors in mesenchymal progenitors (Figure 4H), thus denoting higher maintenance and/or delayed commitment of hematopoietic progenitors.

Taken together, these data indicate that HIF-1α and HIF-2α non-cell-autonomously regulate hematopoiesis in mesenchymal progenitors by inhibiting the expansion and differentiation of hematopoietic stem/progenitor cells.

HIF-1α and HIF-2α Inhibit STAT1 Activation in Mesenchymal Progenitors

To get insights into the molecular circuitry regulated by HIF factors in $P\alpha S^+$ cells, gene expression profiling was performed upon ex vivo hypoxic culture and HIF-1α or HIF-2α silencing. Gene set enrichment analysis (GSEA) (Subramanian et al., 2005) was performed using 3,648 pathway-associated gene sets included in the C2 curated genes sets of the Molecular Signatures Database (MSigDB v4.0). Remarkably, the gene sets more enriched upon downregulation of either gene included interferon-responding genes (false discovery rate <0.0001) (Figures 5A and S4A), comprising the transcription factor *Stat1*, Interferon Regulatory Factors (*Irf*), and the cytokine *Cxcl10* (Figures 5B, S4B, and S4C). Consistently, secretion of CXCL10 into the supernatant was significantly higher upon silencing of HIF factors (Figure 5C).

HIF-1α was previously reported to inhibit STAT1 expression through activation of the transcriptional repressor DEC1/STRA13 in renal carcinoma cells (Ivanov et al., 2007). Accordingly, silencing of both HIF-1α and HIF-2α caused reduced *Stra13* expression also in mesenchymal progenitors (Figure 5B), whereas expression of stable forms of HIF-1α or HIF-2α increased the levels of *Stra13* and decreased those of *Cxcl10* (Figure 5D).

In conclusion, the pathway that is most significantly deregulated by HIF factors in mesenchymal progenitors is a STAT-interferon pathway that leads to a significant overproduction of cytokines like CXCL10.

STAT1 Expression Downstream HIF Blockade Induces Hematopoietic Cell Proliferation and Differentiation

To investigate if STAT1 regulated hematopoiesis downstream HIF in $P\alpha S^+$ mesenchymal progenitors, STAT1 was silenced along with HIF factors, and $P\alpha S^+$ cells were cocultured with KLS cells for 4 days. Increased expression of *Stat1* and *Cxcl10* was abolished by cosilencing of STAT1 (Figures 6A and 6B), as was the increase in hematopoietic cell numbers in coculture observed upon silencing of HIF factors (Figures 6C and 4A). Furthermore, silencing of STAT1 abrogated hematopoietic differentiation induced by HIF downregulation in $P\alpha S^+$ cells (Figure 6D). These data indicate that HIF factors block expression of STAT1 in $P\alpha S^+$ mesenchymal progenitors to prevent proliferation and differentiation of hematopoietic progenitors in a non-cell-autonomous manner.

The interferon/STAT pathway has been implicated in regulating stress-induced hematopoiesis at multiple levels upon infectious stimuli, by acting directly or indirectly on HSC to promote demand-driven hematopoiesis (Baldridge et al., 2011; King and Goodell, 2011). Recently, indications that this pathway may also impact on hematopoiesis through non-cell-autonomous regulation of the HSC niche are beginning to emerge (Takizawa et al., 2012). Because of the involvement of STAT1 downstream HIF factors in mesenchymal progenitors in vitro, we asked whether regulation of HIF factors was also occurring in vivo, in conditions of hematopoietic expansion upon infection. Mice were treated with poly(I:C) to simulate viral infection (Sato et al., 2009), and HIF-1α expression was analyzed in hematopoietic stem/progenitors (KLS cells) and in $P\alpha S^+$ mesenchymal progenitors. Poly(I:C) treatment

(E) Osteogenesis by $P\alpha S^+$ cells transduced with shCTR, shHIF-1α, or shHIF-2α. Representative pictures of ALP^+ cells are shown on the left; quantification of crystal violet staining is shown on the right (data are pooled from two independent experiments, mean ± SEM).
(F) Chondrogenic potential of $P\alpha S^+$ cells transduced with shCTR, shHIF-1α, or shHIF-2α. Expression levels of *Col9* and *Col10* were analyzed by RT-PCR (data are pooled from two independent experiments, mean ± SEM).
See also Figure S2.

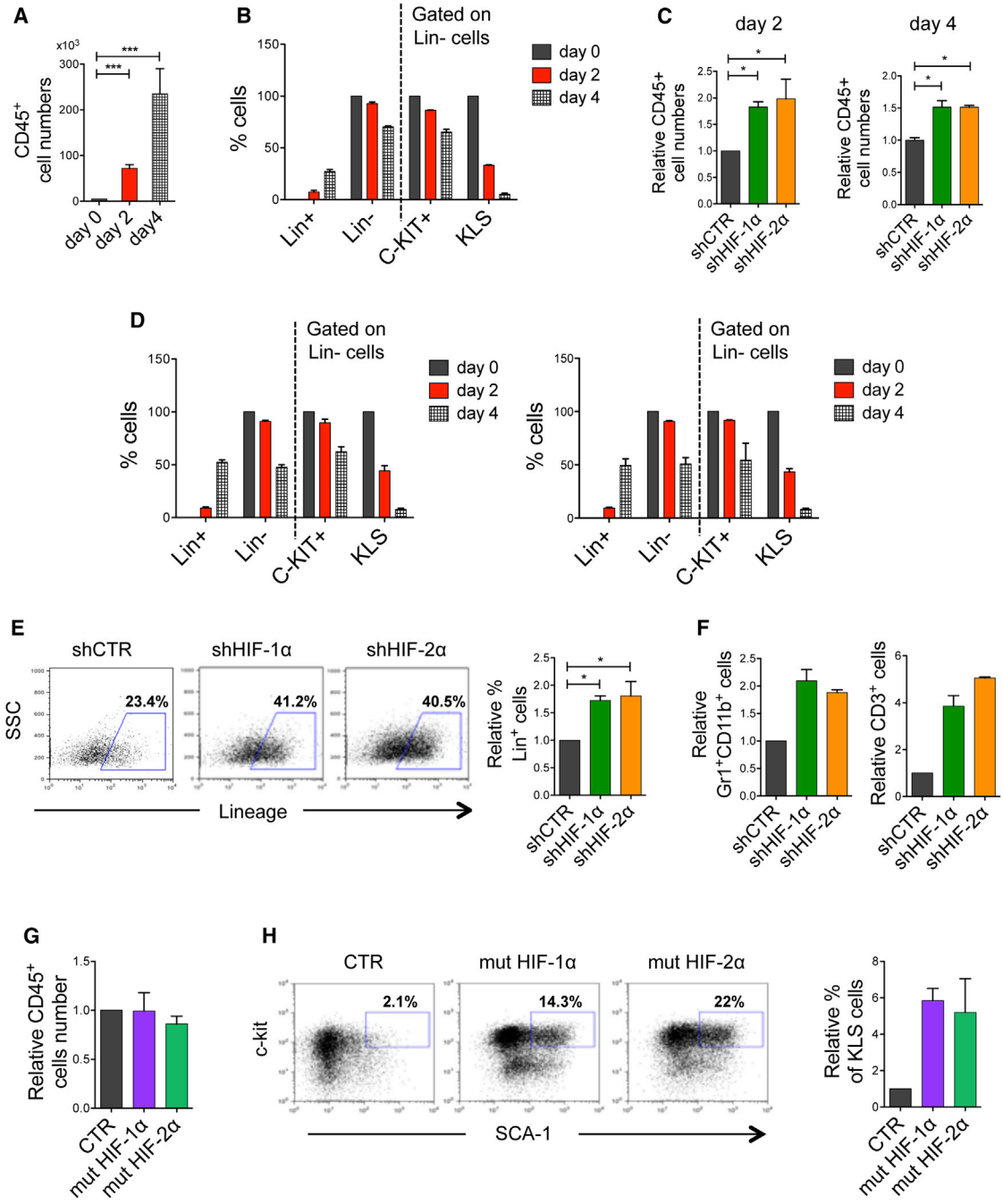

Figure 4. HIF-1α and HIF-2α in Mesenchymal Progenitors Non-Cell-Autonomously Regulate HSC Maintenance

(A) $CD45^+$ cell numbers at day 2 and day 4 upon coculture of KLS ($C\text{-}KIT^+Lin^-SCA\text{-}1^+$) cells with $P\alpha S^+$ cells (data are pooled from three independent experiments, mean ± SEM).

(B) Percentage of Lin^+, Lin^-, $Lin^-C\text{-}KIT^+$, or KLS cells at day 2 and day 4 upon coculture of KLS cells with $P\alpha S^+$ cells (representative data of one out of three independent experiments are shown ±SD).

(C) Coculture of KLS cells with $P\alpha S^+$ cells transduced with shCTR, shHIF-1α, or shHIF-2α at day 2 (left) and at day 4 (right). $CD45^+$ cell numbers are normalized to $CD45^+$ cells obtained from control cocultures (shCTR) (data are pooled from three independent experiments, mean ± SEM).

(D) Percentage of Lin^+, Lin^-, $Lin^-C\text{-}KIT^+$, or KLS cells at day 2 and day 4 upon coculture of KLS cells with $P\alpha S^+$ cells (after HIF-1α silencing on the left and HIF-2α silencing on the right; representative data of one out of three independent experiments are shown ±SD).

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led to increased KLS cell numbers in the BM (Figure 6E, left panel) and decreased KLS quiescence, as measured by pyroninY staining (Figure 6E, right panel). Interestingly, although HIF-1α expression did not change in KLS cells (Figure 6F), HIF-1α protein levels were significantly reduced in PαS$^+$ cells upon poly(I:C) (Figure 6G). To understand whether the reduction of HIF-1α levels was directly caused by poly(I:C) treatment, isolated PαS$^+$ mesenchymal progenitors were treated with poly(I:C) in vitro. As shown in Figure 6H, mRNA levels of *Hif-1α*, *Hif-2α* and HIF-target genes (*Ca9*, *Glut-1*, and *Vegf*) were significantly reduced upon treatment with poly(I:C), wheras *Stat1* levels increased, thus suggesting that HIF factors and HIF-induced pathways can be directly regulated by infection-like stimuli in conditions that demand hematopoietic expansion.

DISCUSSION

A number of reports have suggested that hypoxia-regulated pathways participate at various levels in the regulation of HSC maintenance (Keith and Simon, 2007; Mohyeldin et al., 2010). It was first demonstrated that the BM is a hypoxic environment and primitive HSC reside in hypoxic niches adjacent to endosteal BM regions (Parmar et al., 2007; Suda et al., 2011). More recently, it was suggested that HSC are characterized by an intrinsic HIF-dependent "hypoxic status" that does not solely depend on oxygen concentrations but may be initiated by oxygen-independent regulation of HIF-1α (Nombela-Arrieta et al., 2013). Importantly, genetic evidence points to a very important role of HIF-1α in promoting HSC maintenance (Ramírez-Bergeron et al., 2004; Takubo et al., 2010), which it exerts by implementing a glycolytic metabolic checkpoint that promotes cell quiescence and stem cell capacity (Takubo et al., 2013).

We investigated the possibility that a "hypoxic status" may characterize not only HSC but also mesenchymal progenitors residing close to HSC in BM niches and exposed to the same environmental conditions. It was recently shown that low oxygen concentrations promote the expansion of different types of mesenchymal cells derived from BM (Haque et al., 2013; Tsai et al., 2012b; Cicione et al., 2013). However, HIF factors have been proposed to play different and sometimes opposite roles in the maintenance and differentiation of mesenchymal stem cells, ranging from block of differentiation to induction of differentiation to specific mesenchymal lineages (Hsu et al., 2013; Hung et al., 2012; Shimba et al., 2004; Wagegg et al., 2012; Yun et al., 2002). This controversy may derive from the misleading use of the term mesenchymal stem cells to define very different cell populations, varying from cells that simply adhere to the culture dish upon BM crushing, to very specific cell populations identified by expression of specific markers (Bianco et al., 2013; Frenette et al., 2013). We analyzed the role of HIF factors in mesenchymal progenitors expressing SCA-1 and PDGFRα because these cells reside in periendosteal locations and regulate HSC maintenance (Morikawa et al., 2009; Nakamura et al., 2010). We found that PαS$^+$ endosteal mesenchymal progenitors incorporate pimonidazole to higher levels than other periendosteal stromal cells and express higher levels of both HIF factors *Hif-1α* and *Hif-2α* not only ex vivo, but also when exposed to similar oxygen levels in vitro. Also, we demonstrate that PαS$^+$ cells proliferate and form colonies more efficiently in hypoxic conditions, which indicates that, although a hypoxic profile may be intrinsically wired within these cells, low oxygen cooperates with intrinsic molecular programs to maintain their identity. These results may have important implications for optimizing current protocols aimed at expanding mesenchymal progenitors ex vivo for therapeutic purposes.

Importantly, we found that both HIF factors nonredundantly regulate the morphology and behavior of primary mesenchymal progenitors: in the absence of HIFα expression PαS$^+$ mesenchymal progenitors show impaired proliferation, colony forming ability, and differentiation and acquire an elongated and motile phenotype. In addition, HIF factors non-cell-autonomously regulate hematopoiesis in mesenchymal progenitors by suppressing the

(E) Expression of lineage markers at day 2 and day 4 of KLS cells coculture with PαS$^+$ cells upon HIF-1α or HIF-2α silencing. Representative data of one out of three independent experiments are shown on the left. Graph on the right represents pooled data from three independent experiments, mean ± SEM; cell numbers are normalized over control cocultures (shCTR).
(F) Gr1, CD11b, and CD3 expression at day 4 of KLS cells coculture with PαS$^+$ cells upon HIF-1α or HIF-2α silencing. Percentages are normalized over control coculture (shCTR); representative data of one out of two independent experiments are shown ±SD.
(G) Coculture of PαS$^+$ cells transduced with stable HIF factors (mut HIF-1α or mut HIF-2α) and KLS cells for 4 days. CD45$^+$ cell numbers are normalized over CD45$^+$ cells obtained from control cocultures (shCTR) (data are pooled from two independent experiments, mean ± SEM).
(H) Expression of lineage markers at day 4 upon coculture of KLS cells with PαS$^+$ cells expressing mutant HIF factors. One representative experiment out of two independent experiments is shown on the left. Graph on the right represents data normalized over control coculture (shCTR) and pooled from two independent experiments, mean ± SEM.
See also Figure S3.

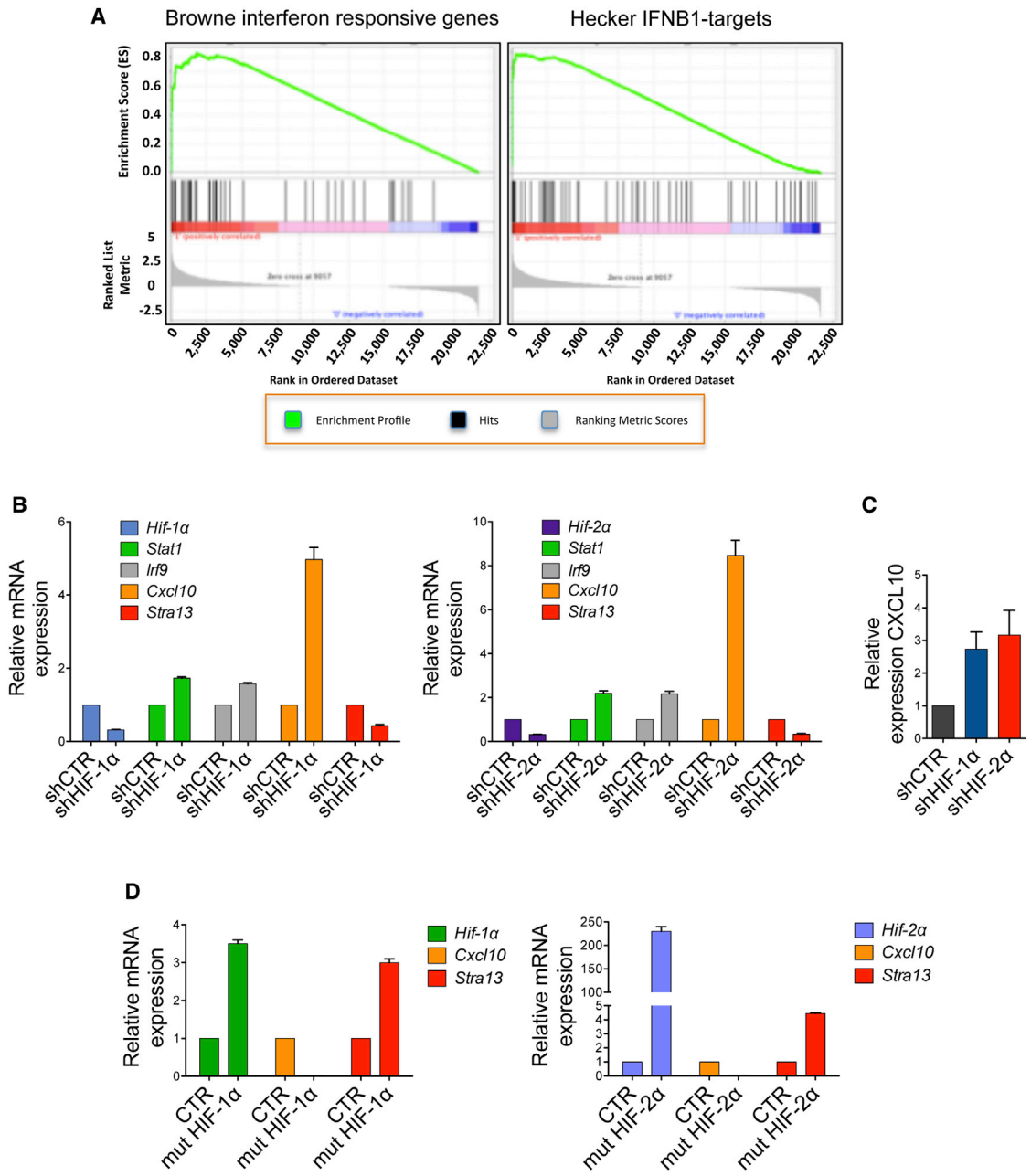

Figure 5. HIF-1α and HIF-2 α Inhibit Stat1 Activation in Mesenchymal Progenitors

(A) GSEA reveals a significant enrichment in interferon-responsive gene sets in PαS$^+$ cells transduced with shHIF-1α compared to control cells (shCTR). Browne_interferon_responsive_genes (left panel) and Hecker_IFNB1_targets (right panel) gene sets from MSigDB are shown upon HIF-1α silencing. The barcode plot indicates the position of the genes belonging to each gene set on the expression data rank sorted by its association with *Hif-1α* knockdown, with red and blue colors indicating over- and underexpression in the HIF-1α-silenced group. The gray scale bar indicates the t statistics, used as weighting score for GSEA analysis.

(B) Relative mRNA expression of *Hif-1α*, *Hif-2α*, *Stat1*, *Irf9*, *Cxcl10*, and *Stra13* in PαS$^+$ cells transduced with shCTR and shHIF-1α (left graph) or shCTR and shHIF-2α (right graph). Data show one representative experiment out of two independent experiments performed in triplicate with similar results ±SD.

(C) Quantification of CXCL10 secretion in the supernatant of PαS$^+$ cells transduced with shHIF-1α or shHIF-2α normalized to CXCL10 released from shCTR PαS$^+$ cells (data are pooled from two independent experiments, mean ± SEM).

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production of secreted factors that induce hematopoietic progenitors' proliferation and differentiation. In line with our findings, HIF-dependent non-cell-autonomous regulation of HSC and hematopoiesis had been recently suggested both for HIF-1α and HIF-2α. For example, stromal cells residing at the endosteal surface of the BM were shown to induce HSC maintenance by secreting the HIF-1α-regulated soluble factor CRIPTO (Miharada et al., 2011). Moreover, *Hif-2α*$^{-/-}$ mice have hematopoietic defects also when irradiated and transplanted with wild-type BM cells, thus underpinning a non-cell-autonomous role of HIF-2α in the regulation of hematopoiesis (Scortegagna et al., 2003).

Mechanistically, we found that HIFα factors regulate hematopoiesis by limiting activation of interferon/STAT1 responses in mesenchymal progenitors, possibly through STRA13-induced inhibition of STAT1. Interestingly, it is recently being shown that in pathological conditions such as systemic infections, inflammatory signals including those generated by interferons promote proliferation and differentiation of HSC at multiple levels, through both direct and indirect mechanisms (Schuettpelz and Link, 2013; Baldridge et al., 2010; King and Goodell, 2011) that include modifying the function of cells composing the hematopoietic stem cell niche (Takizawa et al., 2012). In this context, we found that stimuli that mimic systemic infections and cause HSC expansion suppress expression of HIFα factors specifically in mesenchymal progenitors, thus providing further indication that demand-driven hematopoiesis may be mediated by different cell types within the HSC niche and suggesting that HIF factors may participate in this regulation.

In summary, our results raise a number of interesting points: first, they suggest that expression of HIF factors characterizes specific HSC microenvironments rather than single cell types, and in these microenvironments convergent HIF-regulated functions may cooperate to regulate HSC maintenance and differentiation. Second, they reinforce the idea that not only hypoxia conditioning, but also additional factors present in hematopoietic stem cell niches induce the expression of *Hif* genes (Nombela-Arrieta et al., 2013) and indicate that HIF factors may be importantly regulated at the transcriptional level, although through mechanisms not yet identified. Finally, they reveal that both HIFα subunits are expressed and play similar and yet nonredundant functions in mesenchymal progenitors. It will be interesting in the future to understand whether HIF factors play specific roles in regulating other aspects of the biology of mesenchymal progenitors that we have not currently addressed.

EXPERIMENTAL PROCEDURES

Mice

C57BL/6J mice were used for all experiments. Animal experiments were performed in accordance with the guidelines of Beth Israel Deaconess Medical Center Institutional Animal Care and Use Committee.

Endosteal Mesenchymal Progenitors Isolation

Endosteal bone surfaces were digested with collagenase II, and mesenchymal progenitors were isolated and expanded ex vivo as indicated in Supplemental Experimental Procedures. In selected experiments, cells were treated with 500 ng/ml poly(I:C) for 16 hr before RNA extraction and analysis.

Pimonidazole Incorporation and Detection

Pimonidazole (HP2-100 Kit) (1.5 mg/mouse) was injected intraperitoneum, and mice were sacrificed 2 hr after injection (PBS was injected in control mice). For flow-cytometry detection, cells were stained for surface markers (CD45, Ter119, SCA-1, and PDGFRα) and then fixed and permeabilized (DB Pharmingen) to allow intracellular pimonidazole detection using antipimonidazole fluorescein-isothiocyanate-conjugated antibody.

Lentiviral Vectors

pGIPZ shRNA against HIF-1α and HIF-2α or scrambled shRNA plasmids and pDLKO.1 shRNA against Stat1 were purchased from Open Biosystems. mut HIF-1α, mut HIF-2α vectors were cloned in third-generation bidirectional lentiviral vector generated in the laboratory of Dr. Naldini at San Raffaele Scientific Institute (Amendola et al., 2005).

Isolation of HSC and Coculture with Mesenchymal Progenitors

Upon crushing of long bones, hematopoietic cells were collected, filtered, and subjected to red blood cells lysis in ACK lysis buffer (Gibco); recovered cells were then washed and stained for fluorescence-activated cell sorting with anti-CD3, anti-CD8, anti-CD4, anti-B220, anti-CD11b, anti-Gr1, anti-Ter119 (all biotinylated), anti-SCA1-PE, and anti-C-KIT-APC (BioLegend). Freshly sorted HSC cells were seeded on a layer of confluent PαS^{+} cells in StemSpan (STEMCELL Technologies) supplemented with 50 ng TPO and 50 ng SCF at 37°C in 1% oxygen concentration. When cultured with conditioned media derived from PαS^{+} cells, HSC cells were maintained in 70% StemSpan supplemented with 50 ng TPO and 50 ng SCF fresh medium and 30% conditioned medium. All coculture experiments and all cultures with

(D) Relative mRNA expression of *Hif-1α*, *Hif-2α*, *Cxcl10*, and *Stra13* in PαS^{+} cells transduced with CTR plasmid and mutant stable forms of HIF-1α (left graph) or HIF-2α (right graph). Data show one representative experiment out of two independent experiments performed in triplicate with similar results ±SD.
See also Figures S4–S6.

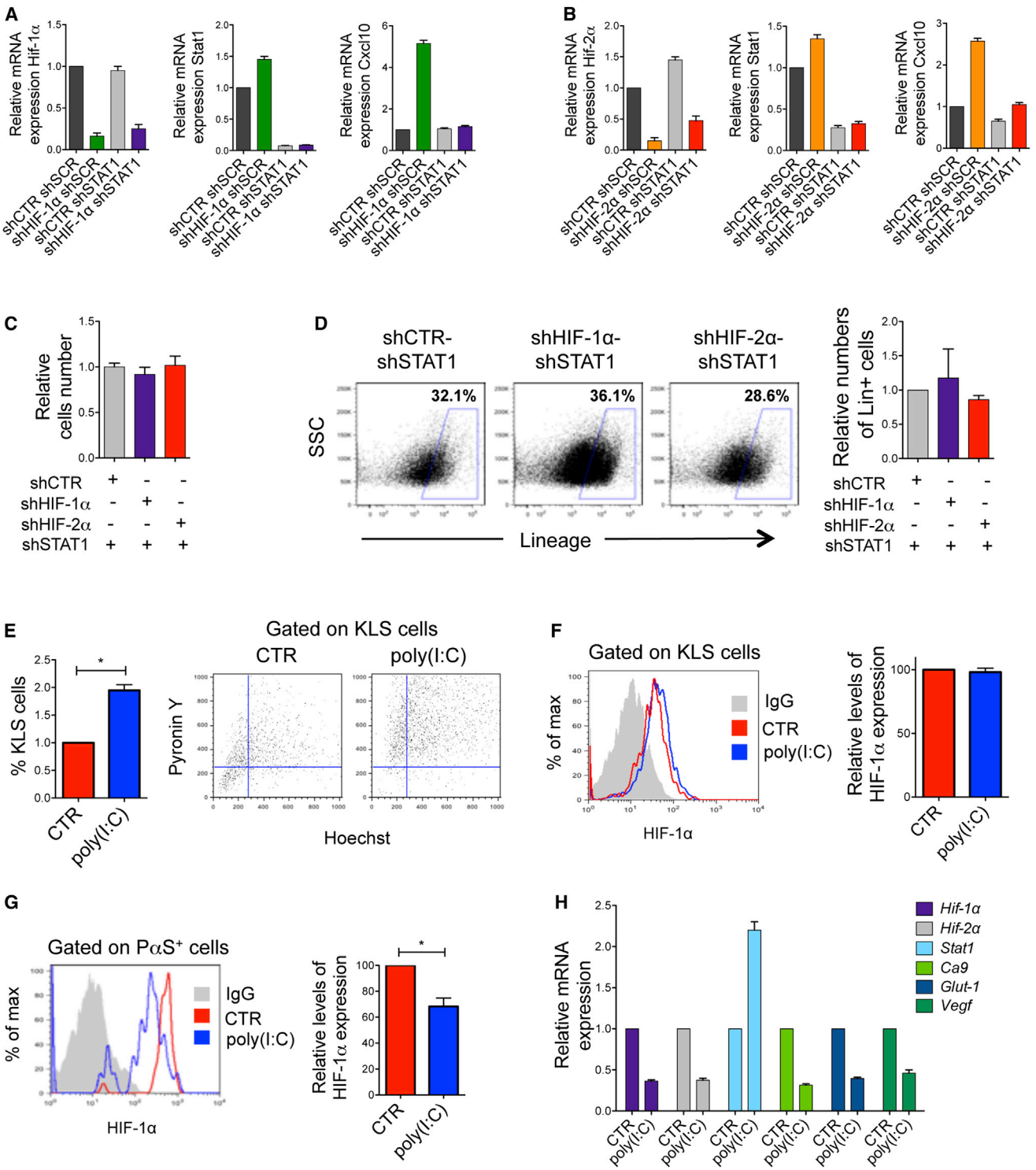

Figure 6. HIF-1α and HIF-2α Expression in Mesenchymal Progenitors Non-Cell-Autonomously Regulate HSC Maintenance through STAT1 Inhibition

(A) Relative mRNA expression of *Hif-1α* (left graph), *Stat1* (middle graph), and *Cxcl10* (right graph) in PαS$^+$ cells transduced with shCTR or shHIF-1α either with scrambled shSCR or shSTAT1 (data are pooled from two independent experiments, mean ± SEM).

(B) Relative mRNA expression of *Hif-2α* (left graph), *Stat1* (middle graph), and *Cxcl10* (right graph) in PαS$^+$ cells transduced with shCTR or shHIF-2α either with scrambled shSCR or shSTAT1 (data are pooled from two independent experiments, mean ± SEM).

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conditioned media from PαS$^+$ cells have been performed in at least three independent experiments. Results have been normalized compared to control conditions and a pool of experiments is shown in the figures.

Statistic

Significance calculation was obtained by Student's t test: n.s. if not significant, *p < 0.05, **p < 0.01, ***p < 0.001.

ACCESSION NUMBERS

The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (Edgar et al., 2002) and are accessible through GEO Series accession number GSE56141.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and six figures and can be found with this article online at http://dx.doi.org/10.1016/j.stemcr.2014.04.002.

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(C) Coculture of KLS cells and PαS$^+$ cells transduced with shCTR, shHIF-1α or shHIF-2α, and shSTAT1. CD45$^+$ cell numbers are normalized over cells obtained from control cocultures (shCTR, shSTAT1) (data are pooled from two independent experiments, mean ± SEM).
(D) Expression of lineage markers at day 4 upon coculture of KLS cells with PαS$^+$ cells transduced with shCTR, shHIF-1α or shHIF-2α, and shSTAT1. Plots on the left show Lin$^+$ cell percentages; graph on the right shows relative quantification of Lin$^+$ cells (one of two independent experiments is shown on the left; data on the right are pooled from two independent experiments, mean ± SEM).
(E) Percentage of KLS cells (left) and representative plots of pyroninY-DAPI staining of KLS cells (right) 24 hr after mice injection with poly(I:C) (n = 5, mean ± SEM).
(F) HIF-1α protein expression in KLS cells 24 hr after mice injection with poly(I:C) (n = 5, mean ± SEM). Representative analysis of two mice is shown on the left.
(G) HIF-1α protein expression in PαS$^+$ cells 24 hr after mice injection with poly(I:C) (n = 5, mean ± SEM). Representative analysis of two mice is shown on the left.
(H) Relative mRNA expression of *Hif-1α*, *Hif-2α*, *Stat1*, *Ca9*, *Glut-1*, and *Vegf* in PαS$^+$ cells treated with poly(I:C) for 12 hr. Data show one representative experiment out of two independent experiments performed in triplicate with similar results ±SD.

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