Identification of Adiponectin as a Novel Hemopoietic Stem Cell Growth Factor[1]

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The hemopoietic microenvironment consists of a diverse repertoire of cells capable of providing signals that influence hemopoietic stem cell function. Although the role of osteoblasts and vascular endothelial cells has recently been characterized, the function of the most abundant cell type in the bone marrow, the adipocyte, is less defined. Given the emergence of a growing number of adipokines, it is possible that these factors may also play a role in regulating hematopoiesis. Here, we investigated the role of adiponectin, a secreted molecule derived from adipocytes, in hemopoietic stem cell (HSC) function. We show that adiponectin is expressed by components of the HSC niche and its' receptors AdipoR1 and AdipoR2 are expressed by HSCs. At a functional level, adiponectin influences HSCs by increasing their proliferation, while retaining the cells in a functionally immature state as determined by in vitro and in vivo assays. We also demonstrate that adiponectin signaling is required for optimal HSC proliferation both in vitro and in long term hemopoietic reconstitution in vivo. Finally we show that adiponectin stimulation activates p38 MAPK, and that inhibition of this pathway abrogates adiponectin's proliferative effect on HSCs. These studies collectively identify adiponectin as a novel regulator of HSC function and suggest that it acts through a p38 dependent pathway. *The Journal of Immunology,* **2007, 178: 3511–3520.**

All stem cells have the remarkable ability to develop into multiple cell types while maintaining a constant reserve of undifferentiated cells for the lifetime of an organism. Hemopoietic stem cells (HSCs)[3] in particular, are capable of generating all of the blood forming and immune cells found in the body both during homeostatic tissue maintenance and during regeneration in response to injury (1). HSCs exist primarily in a quiescent state, infrequently entering cycle and choosing between a fate of self-renewal or commitment. The outcome of this choice is dictated by signals present in the bone marrow microenvironment. Elucidation of the signals present in the stem cell niche and their specific roles will allow a clearer view of how HSC function is regulated and is thus of critical importance to both basic stem cell biology and transplantation based therapy.

The cells of the hemopoietic microenvironment include osteoblasts, endothelial cells, adipocytes and fibroblasts. These cells are likely to provide support for hemopoietic cells through secretion of soluble factors as well as cell-cell contact. Indeed, several studies have identified osteoblasts as a primary component of the HSC niche (2–4). These bone producing cells have been shown to produce multiple factors involved in HSC quiescence and maintenance, including angiopoietin (4), N-cadherin (5), and the Notch ligand, Jagged1(2). Recently, HSCs have also been shown to associate with the sinusoidal endothelium in the bone marrow (6–8), indicating that endothelial cells may also form an important supportive niche for HSCs.

The adipocyte is the most abundant stromal cell type found in the marrow (9); however, the role of this cell in hematopoiesis has not been clearly defined. Mature adipocytes are present in long-term bone marrow cultures (10) and are capable of supporting both lymphopoiesis (11) and granulopoiesis (12). Additionally, following irradiation injury, adipocytes first appear after 7 days, a time point corresponding to the initiation of hemopoietic proliferation (13). These fat-containing cells are known to secrete a number of proteins, or adipokines, that play a role in hematopoiesis. IL-6 (14–16) and IL-8 (16), two growth factors derived from adipocytes, have well established roles in the proliferation and differentiation of hemopoietic cells. Prostaglandin, another adipokine, has been demonstrated to have an inhibitory influence on HSCs through induction of apoptosis (17, 18). Additionally, leptin has been shown to be required for normal lymphopoiesis (19) by differentially regulating the proliferation of naive and memory T cells (20) and is capable of stimulating the proliferation of myelocytic progenitors (21).

The role of another adipokine, adiponectin, in hemopoietic stem cell function is less well defined. Adiponectin was originally identified as being produced exclusively by differentiated adipocytes (22–24). It has since been shown to be expressed by multiple other cell types including fibroblasts and osteoblasts (25, 26). It has three known receptors, AdipoR1, AdipoR2 (27), and T-cadherin (28). At the cellular level, stimulation with adiponectin increases insulin sensitivity (29), glucose uptake (30), and fatty acid oxidation (31). Furthermore, mice deficient for adiponectin exhibit delayed clearance of free fatty acids from the plasma, severe diet-induced insulin resistance (32), and impaired angiogenic (33) and myocardial (34) repair following ischemic injury. Previous studies have examined the effect of adiponectin on myeloid (35) and lymphoid (18) cells, showing that adiponectin can suppress the growth of these mature cell types in

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[3] Abbreviations used in this paper: HSC, hemopoietic stem cell; AMPK, AMP-activated protein kinase; DAPI, 4′,6′-diamidino-2-phenylindole.

vitro; however, its effects on HSC development are unknown. Here, we sought to investigate the role of adiponectin in hemopoietic stem cells. We find that adiponectin can enhance HSC proliferation in vitro, and that cells expanded in this manner are more efficient at reconstituting lethally irradiated hosts. Adiponectin signaling is also essential for normal HSC function as knockdown of AdipoR1 impairs HSC proliferation in vitro and reconstitution in vivo. Finally we provide a potential mechanism through which adiponectin influences HSCs by demonstrating that p38 MAPK is activated by adiponectin and that its inhibition leads to abrogation of adiponectin mediated HSC proliferation. Our data provides the first evidence that adiponectin is a growth factor for HSCs and that AdipoR1 mediated signaling plays a physiological role in hemopoietic reconstitution.

Materials and Methods

Mice

C57BL/Ka CD45.1, Thy1.1, C57BL/Ka CD45.2, Thy1.1, and AKR/J mice were used at 6–10 wk of age. Mice used as transplant recipients were greater than 10 wk of age. Cyclophosphamide (Sigma-Aldrich) was injected i.p. on day 1 at 200 mg/kg in PBS. G-CSF (Amgen) was injected s.c. on day 2 at 250 mg/kg/day in 0.1% BSA in PBS as described previously (36). HSCs were harvested on day 3, 24 h after G-CSF treatment. All animal experiments were performed according to protocols approved by the Duke University Institutional Animal Care and Use Committee.

Primary stroma isolation

Whole bone marrow was isolated from C57BL/Ka mice, seeded in a flat-bottom 96-well plate, and cultured for 48 h. At this time all nonadherent cells were removed from culture and the remaining adherent cells were either trypsonized and removed to isolate RNA, synthesize cDNA and perform RT-PCR or used in coculture experiments with HSCs.

Whole bone marrow serum isolation and adiponectin protein quantification

Bone marrow serum was isolated by flushing 4 long bones into 350 μl of X-Vivo15 with 50 μM 2-ME and penicillin streptomycin. Sera were spun for 10 min at 4°C at 13,200 rpm and the soluble fraction aspirated off. Adiponectin and SCF levels in bone marrow serum were then quantified by ELISA (R&D Systems).

HSC isolation

HSCs were isolated from bone marrow as described (37) and sorted by flow cytometry on the basis of c-Kit, Sca-1, low levels of Thy 1.1, and low to negative levels of lineage markers (KTLS cells). The combination of the following Abs defined the lineage markers 145-2C11 (anti-CD3e), 53-7.3 (anti-CD5), GK1.5 (anti-CD4), 53-6.7 (anti-CD8), RB6-8C5 Ly-6G (anti-Gr-1), M1/70 (anti- CD11b, Mac-1), Ter119 (anti-erythrocyte-specific Ag), 6B2 (anti-B220). Other Abs used included clones 2B8 (anti-CD117, c-Kit), D7 (anti-Ly-6A/E, Sca-1). All Abs were purchased from BD Pharmingen or eBioscience. Analysis and cell sorting were conducted on a FACSVantage (BD Biosciences) at the Duke Cancer Center FACS facility.

Viral production and infection

Virus was produced by triple transfection of 293T cells with LentiTriplX2 or lentiviral FG12 siRNA along with gag-pol and VSVG constructs. Viral supernatant was collected for two days and concentrated 100-fold by ultracentrifugation at 50,000 $\times$ *g*. For viral infection, 20,000 HSCs were cultured in a 96-well U-bottom plate in the presence of 50 μl HSC media (X-Vivo15, 5×10^{-5} M 2-ME, 4 μg/ml polybrene) with 30 ng/ml SCF 30 ng/ml Flt3 ligand and 2% FCS (for siRNA infection).

Real-time PCR and RT-PCR analysis

RNA from untreated, Cy/G treated, or retrovirally infected HSCs was isolated using RNAqueous-Micro (Ambion) and converted to cDNA using Superscript II (Invitrogen Life Technologies). PCR was performed on cDNA samples using Platinum *Taq* (Invitrogen Life Technologies) on a PTC-200 Peltier Thermo Cycle (MJ Research). For qPCR, cDNA concentrations were measured with a fluorometer (Turner Designs) using RiboGreen reagent (Molecular Probes). Quantitative real-time PCR was performed using an iCycler (Bio-Rad) by mixing equal amounts of cDNAs, iQ SYBR Green Supermix (Bio-Rad) and gene-specific primers.

Adiponectin forward: 5′-TGA GCC TCT TCA AGA AGG ACA AGG-3′; adiponectin reverse: 5′-TCT GCA TAG AGT CCA TTG TGG TCC-3′; adiponectin receptor 1 forward: 5′-ACG TTG GAG AGT CAT CCC GTA T-3′; adiponectin receptor 1 reverse: 5′-CTC TGT GTG GAT GCG GAA GAT-3′; adiponectin receptor 2 forward: 5′-TGC CAG GAA GAT GAA GGG TTT AT-3′; adiponectin receptor 2 reverse: 5′-TTC CAT TCG TTC GAT AGC ATG A-3′.

In vitro HSC assays

Freshly purified KTLS cells were plated at 10 cells/well in 60-well Nunc Terasaki plates. Cells were sorted into wells containing serum-free medium (X-Vivo15; BioWhittaker) supplemented with 5×10^{-5} M 2-ME, penicillin/streptomycin, gentamicin, 0.5% FCS, Tpo (5 ng/ml), SCF (10 ng/ml) in the absence or presence of globular or full-length adiponectin (100–600 ng/ml) (R&D Systems, Phoenix Pharmaceuticals, or a gift from Dr. H. Lodish, Whitehead Institute for Biomedical Research, Cambridge, MA), or 15 μM SB202190 or SB203580. For AMP-activated protein kinase (AMPK) experiments cells were first infected with either wild type of dominant-negative AMPK and then cultured as above. Proliferation was monitored by counting the number of cells in 30 wells per condition at defined intervals.

To assess colony-forming capacity of adiponectin cultured cells, KTLS cells were cultured in the presence or absence of adiponectin for 3 days after which cells were sorted at a density of one cell per well in 96-well plates and cultured in complete methylcellulose medium (Methocult GF M3434 from StemCell Technologies). Colonies were assigned scores after 8 days of culture and were identified based on morphological criteria as being single lineage (M/G/E) or mixed lineage (GEMM/GM).

In vivo analysis of HSC function

After 4 days of culture (using same culture medium as in in vitro experments), 1500 or 500 adiponectin-treated and control (PBS plus 0.5% FBS) treated HSCs (or LacZ and AdipoR1 siRNA infected KTLS cells) were injected retro-orbitally into groups of 4–7 recipient mice along with 300,000 rescuing host total bone marrow. Host mice were lethally irradiated with 9.5 Gy using a cesium irradiator 4–24 h before transplantation, and subsequently maintained on antibiotic water. Transplanted mice were bled every 2–3 wk to determine the percentage chimerism. Donor and host cells were distinguished by allelic expression of CD45.1/CD45.2.

Immunofluorescence analysis

Freshly isolated KTLS cells were stimulated with 1 μg/ml adiponectin or vehicle control for 30 min in a 96-well plate. Following stimulation, cells were removed from culture, cytospins were prepared, and cells were fixed on the slides in 4% paraformaldehyde for 20 min. Fixed slides were then washed in PBS plus 1% Tween and blocked with 20% normal goat serum. The slides were stained with anti-P-AMPK, anti-P-JNK, or anti-P-p38 Ab (Cell Signaling) or a corresponding isotype control followed by anti-Rabbit IgG-Cy3 (Jackson ImmunoResearch Laboratories) and DAPI. Samples were mounted using fluorescent mounting medium (Fluoromount-G; SouthernBiotech) and viewed by confocal microscopy.

Lentiviral RNA interference assays

Two complementary DNA oligomers were chemically synthesized (by IDT), annealed and ligated into the FG12 lentiviral vector (expressing GFP) as described previously (19). The sequence of the mouse AdipoR1 sense siRNA used was 3′ GAGACUGGCAACAUCUGGACATT 5′ (27). Virus was produced as described above.

For in vitro assays KTLS cells were transduced with lentiviral LacZ or AdipoR1 siRNA in the presence of 4 μg/ml polybrene in X-Vivo15, 5×10^{-5} M 2-ME, 30 ng/ml Flt3 ligand, and 30 ng/ml SCF. Equal numbers of GFP^+ cells were resorted 48 h after infection directly onto primary stroma cells in X-Vivo15 and cultured for three to four days in a 96-well plate. At the end of the culture period all cells were removed from culture, stained for CD45 expression, and sorted by flow cytometry to determine the absolute hemopoietic cell count. Transplants were conducted as described above. Briefly, 48 h after infection of KTLS cells with siRNA, GFP^+ cells were re-sorted and 4000 GFP^+ cells were transplanted along with 300,000 rescuing host whole bone marrow.

Results

Expression analysis of adiponectin receptor and adiponectin

Previous studies conducted using cell lines or cells from nonhemopoietic tissues have shown that adiponectin is produced by mature adipocytes (22–24), fibroblasts (25) and osteoblasts (26).

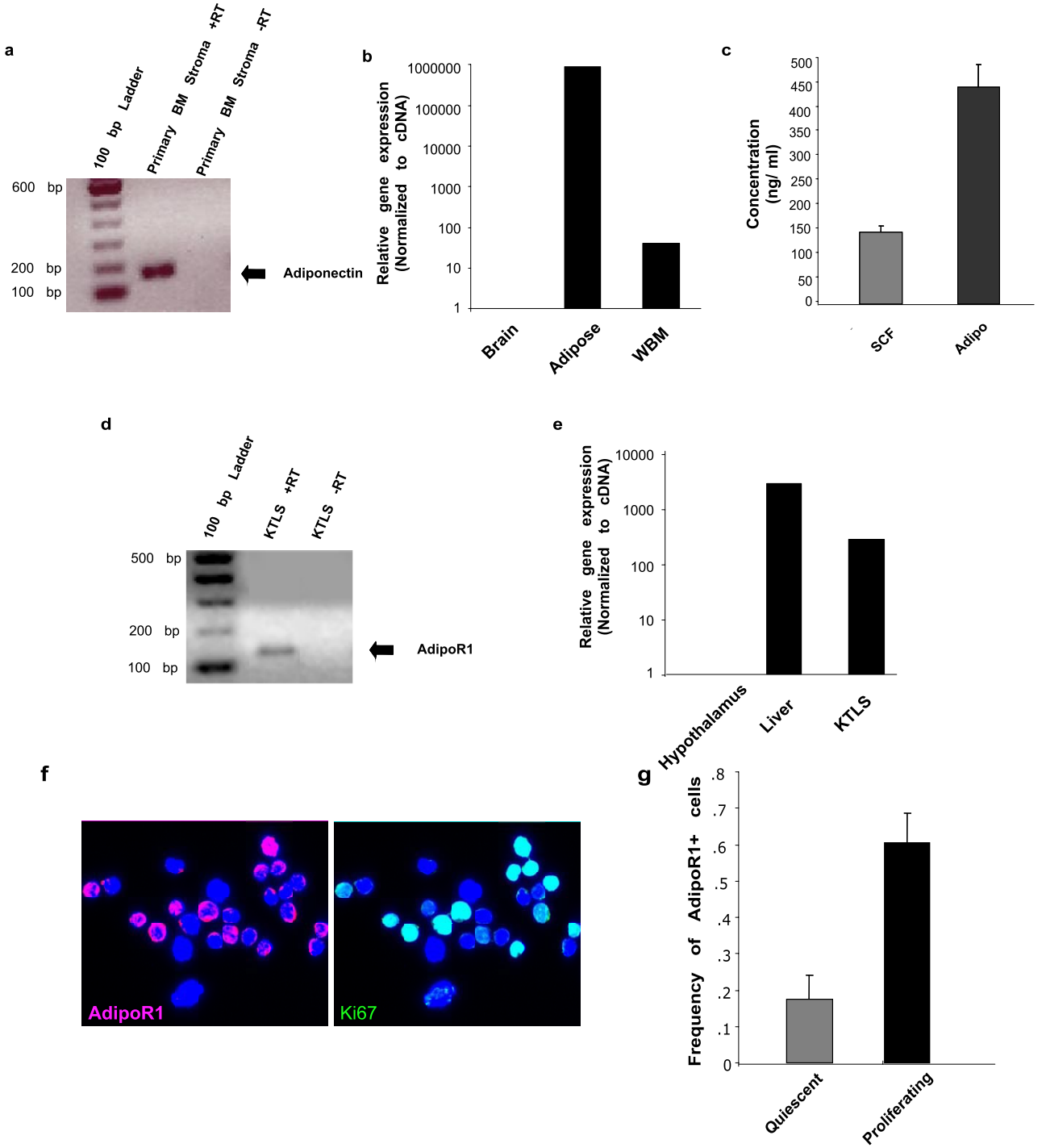

FIGURE 1. Adiponectin is expressed in the HSC microenvironment and adiponectin receptor expression is increased in proliferating HSCs. *a*, RNA was isolated and cDNA synthesized from primary whole bone marrow stromal cell populations. PCR was performed using primers specific for adiponectin (amplicon size 180 bp) on cDNA generated in the presence or absence of reverse transcriptase (RT), to control for genomic contamination. Results shown are representative of two experiments. *b*, Adiponectin expression was examined in cDNA from Adipose tissue, brain and whole bone marrow by real time quantitative PCR and results were normalized to total cDNA concentration. *c*, Whole bone marrow serum was isolated and adiponectin protein levels were quantified relative to SCF by ELISA. Results shown are the average of four independent mice $p = 0.002$. *d*, AdipoR1 expression was examined in cDNA from freshly isolated KTLS cells by RT-PCR. PCR was performed using AdipoR1-specific primers (amplicon size 150 bp) on positive or negative reverse transcriptase (RT) reactions. Results shown are representative of three experiments. *e*, AdipoR1 expression was examined in cDNA isolated from liver, hypothalamus, and KTLS cells by real time quantitative PCR and results normalized to total cDNA concentration. *f*, Freshly isolated KTLS cells were stained with Abs specific for AdipoR1 and Ki67 or a corresponding isotype control and nuclei were counterstained with DAPI. Cells were then visualized with a fluorescent microscope. *g*, Quantification of AdipoR1 expression in $Ki67^{+/-}$ fractions. Results shown are the average of three independent experiments. $p = 0.01$.

To specifically determine whether adiponectin signaling could influence HSCs physiologically we first examined whether adiponectin was expressed within the bone marrow microenvironment. Using RT-PCR, we found that whole bone marrow stromal cell populations did indeed express adiponectin (Fig. 1*a*). We next compared adiponectin levels in the bone marrow to levels in

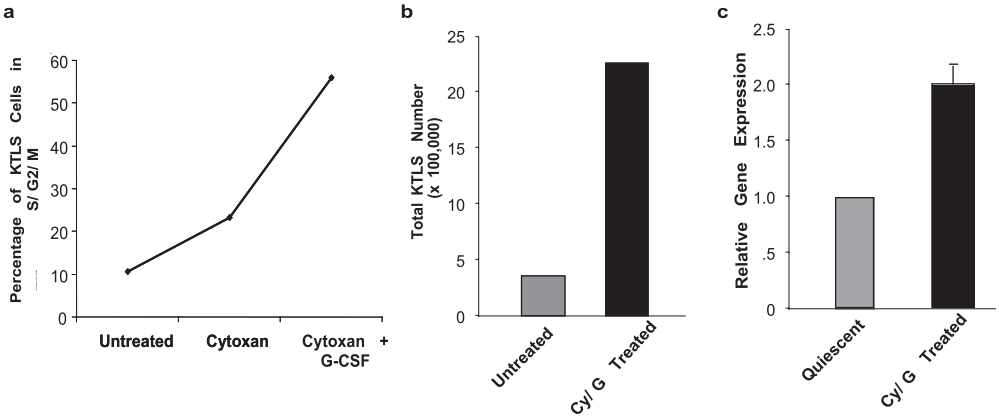

FIGURE 2. Effect of cytoxan/G-CSF treatment on HSCs. Mice were treated with 200 mg/kg of Cytoxan followed 24 h later by treatment with 250 mg/kg of G-CSF (Cy/G treatment). *a*, The cell cycle status of bone marrow KTLS cells was evaluated before treatment, following cytoxan treatment alone, and following Cy/G treatment. *b*, The absolute number of KTLS cells present in the bone marrow was determined following Cy/G treatment relative to untreated mice. *c*, KTLS cells were isolated from untreated mice (gray) or mice treated systemically with Cy/G (black). AdipoR1 expression was examined in each group by real-time PCR and normalized to total cDNA. Results were similar when normalized to β_2-microglobulin. Results are representative of six independent samples ± SEM. $p = 0.03$.

Adipose tissue where adiponectin is abundantly expressed, and to brain tissue where adiponectin is expressed at low levels. Using real time PCR we found that the expression of adiponectin in the bone marrow is higher by ~40-fold than in the brain which was found to express adiponectin at the lowest levels of all tissues tested (Fig. 1*b*, data not shown). In addition it was expressed at levels similar to that found in the liver, heart and hypothalamus (data not shown) To further quantitate adiponectin levels within the microenvironment we examined the protein concentration of adiponectin in bone marrow serum relative to the well characterized HSC growth factor, SCF (Fig. 1*c*). We found that compared with SCF which is present at 150 ng/ml, adiponectin is present at ~450 ng/ml. These concentrations are above the EC_{50} values of both SCF and adiponectin which are 5–10 ng/ml (R&D Systems) and 100 ng/ml (as determined in our experiments), respectively. These data cumulatively suggest that adiponectin is present in the bone marrow microenvironment at levels that are physiologically relevant for use by HSCs.

To determine whether HSCs have the ability to respond to adiponectin, we examined whether HSCs express receptors for this adipokine. Expression of AdipoR1, AdipoR2, and T-cadherin by bone marrow derived KTLS cells (c-kit$^+$ Thy1.1^{low} lin$^{low/-}$ Sca-1$^+$), a population highly enriched for HSCs, was determined by RT-PCR. We found that AdipoR1 (Fig. 1*d*) and to a lesser extent AdipoR2 (data not shown) are expressed by HSCs. However, T cadherin expression was undetectable (data not shown). We also compared the expression levels of adiponectin receptor in KLSF (c-kit$^+$Lin$^{-/low}$Sca-1$^+$Flk$^-$) cells to that of other tissues and found that it is expressed at levels similar to that of the liver which has the highest expression of all tissues tested (data not shown). Additionally, expression of AdipoR1 in KLSF cells was over a hundred fold greater than the expression in the hypothalamus where the receptor was expressed at the lowest level (Fig. 1*e*, data not shown) We next wanted to determine whether expression of this receptor is modulated during HSC proliferation in vivo. To this end, we examined AdipoR1 expression in freshly isolated HSCs in active vs resting phases of the cell cycle, as identified by Ki67 expression. Actively cycling HSCs expressed three fold higher levels of AdipoR1 relative to cells that were not in cycle (Fig. 1, *f* and *g*), suggesting that AdipoR1 levels increase during homeostatic proliferation. To determine whether a similar up-regulation occurs when HSCs rapidly proliferate in response to damage, we examined HSCs undergoing regeneration following treatment with the chemotherapeutic drug cyclophosphamide (Cy). This treatment acts by killing mature, cycling cells in the bone marrow compartment through DNA cross linking (38), and induces the rapid proliferation of HSCs required to regenerate the damaged hemopoietic system (36, 39–41). When cyclophosphamide treatment is combined with administration of the growth factor, G-CSF, a 5- to 6-fold increase in the number of cells in cycle is seen (Fig. 2*a*). This increased cycling results in a corresponding synergistic expansion in the absolute number of bone marrow stem cells (36, 39–41) (Fig. 2*b*). Using this approach we found that AdipoR1 expression is two fold higher in HSCs after Cy/G-CSF treatment compared with control conditions (Fig. 2*c*), indicating that AdipoR1 is up-regulated during HSC regeneration in vivo. Thus, the increased expression of adiponectin receptor as HSCs enter cycle suggests that adiponectin may be differentially used by proliferating HSCs.

Adiponectin enhances HSC proliferation and in vivo reconstitution

To test whether adiponectin influences HSCs functionally we examined the effect of recombinant adiponectin on HSCs in vitro. Adiponectin contains an N-terminal signal sequence and collagenous domain, along with a C-terminal globular domain (22), and can circulate as both the full length form, having a higher affinity for AdipoR2 and in lower concentrations as a protease generated globular domain with a higher affinity for AdipoR1 (31). Freshly isolated KTLS cells were cultured in serum free conditions in the presence or absence of either recombinant full length or globular domain adiponectin along with limiting doses of the cytokines SCF and Tpo, which serve as early acting growth factors for HSCs. Although treatment with full length adiponectin led to only a minimal increase in proliferation (Fig. 3, *a* and *b*), stimulation with the globular domain of adiponectin led to a two to four fold increase in proliferation of KTLS cells compared with control conditions (Fig. 3, *c* and *d*). These data demonstrate that globular adiponectin signaling can induce proliferation of KTLS cells in vitro.

To define whether HSCs that proliferate in response to adiponectin retain progenitor cell function, in vitro methylcellulose colony-forming assays were performed. Cells cultured in the presence of adiponectin were better able to maintain mixed-lineage

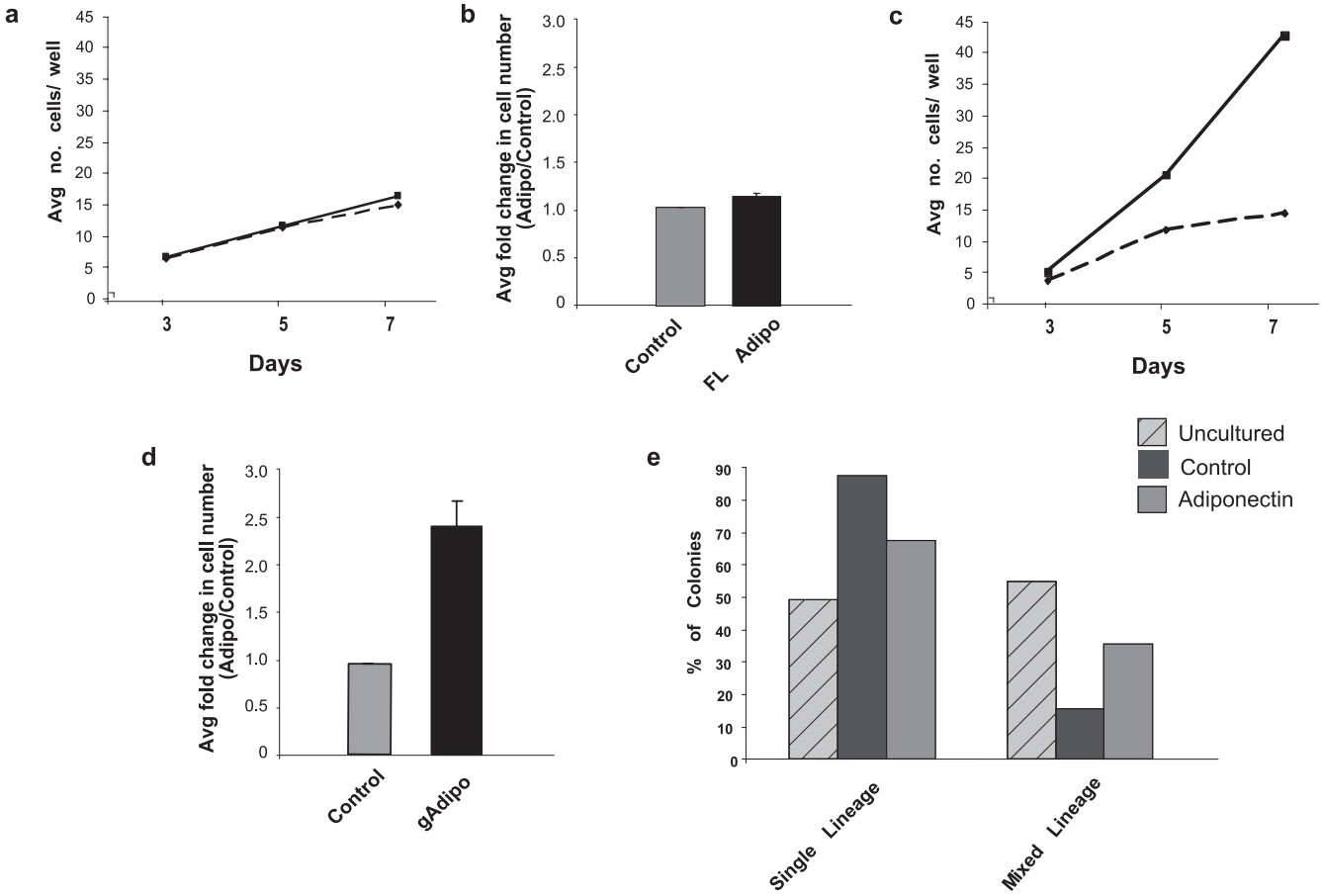

FIGURE 3. Adiponectin enhances the proliferation of KTLS cells in vitro. *a* and *c*, KTLS cells were sorted into Terasaki wells at 10–20 cells/well and cultured in the presence (solid line, black bar) or absence (dashed line, gray bar) of full length or globular adiponectin for 7 days. Cultures contained SCF and Tpo as synergizing factors. *a* and *c*, Representative experiments. *b* and *d*, The average fold change in cell number over three independent experiments ± SEM. $p = 0.1$ and $p = 0.02$. *e*, The colony-forming potential of freshly isolated (striped) vs control (black) or adiponectin cultured (gray) cells was assayed by plating singles cells in methylcellulose medium. The percentage of single lineage (M/G/E) vs mixed lineage (GM/GEMM,) colonies was assessed in two independent experiments.

colony production than their control counterparts (Fig. 3*e*), indicating that adiponectin-treated cells retain a more multipotent, immature state.

To address whether these cells retained enhanced stem cell capacity in vivo, we conducted long-term, limiting dilution transplantation experiments. KTLS cells were cultured in serum free media in the presence or absence of adiponectin for four days. Subsequently, equal numbers of cells were transplanted in a serial dilution series into two cohorts of lethally irradiated mice and the degree of chimerism analyzed. The average peripheral blood chimerism 12 wk after transplantation (Fig. 4*a*) was 2- to 3-fold higher in mice transplanted with limiting numbers of cells cultured with adiponectin (Fig. 4*b*). At 28 wk post-transplant, peripheral blood chimerism was ~9-fold higher in mice injected with adiponectin cultured cells relative to control (Fig. 4*c*). Additionally, there was no significant difference in myeloid and lymphoid lineage distribution following transplantation with control-treated and adiponectin-treated cells, suggesting that adiponectin stimulation of HSCs does not influence commitment to a particular hemopoietic lineage (Table I). Collectively, these results suggest that adiponectin can enhance the proliferation of HSCs from the quiescent state while maintaining enhanced reconstitution capacity relative to control cultured cells.

HSCs require adiponectin receptor signaling for in vitro growth and in vivo repopulation

To determine whether the adiponectin pathway is required for HSC proliferation, we knocked down AdipoR1 expression via siRNA delivery. We specifically chose to inhibit AdipoR1 because it has a higher affinity for the globular domain of adiponectin, with which we saw the greatest proliferative effect in vitro (Fig. 3, *c* and *d*). We used a viral siRNA system in which expression of the siRNA target sequence is driven by a U6 promoter from a lentiviral vector containing an independently driven GFP sequence (42), allowing infected cells to be visualized and analyzed based on fluorescence. We infected primary KTLS cells with AdipoR1 siRNA or an unrelated LacZ control siRNA. 48 h after infection, GFP^+ cells were sorted by FACS and AdipoR1 expression was analyzed by real time PCR. Infection with AdipoR1 siRNA resulted in a 90% knockdown of AdipoR1 expression relative to cells carrying LacZ siRNA (Fig. 5*a*). To test the effect of adiponectin receptor knockdown on HSC growth and function in vitro, HSCs were cocultured with primary stromal cell cultures composed of osteoblasts and adipocytes, which we have found can support HSC proliferation. These cells express adiponectin (data not shown) and provide a culture system similar to the HSC microenvironment, allowing us to test the effect of inhibition of the

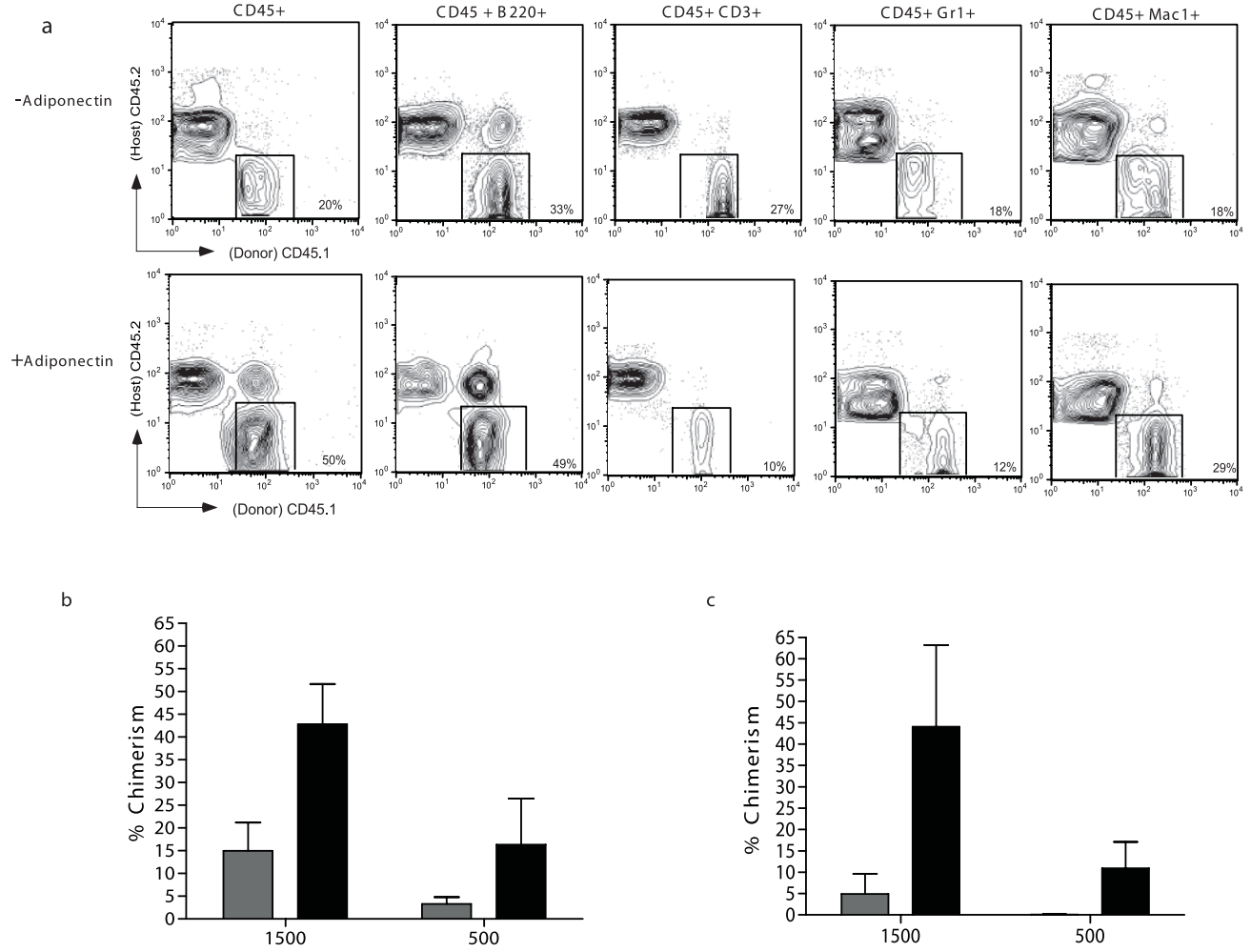

FIGURE 4. HSCs cultured with adiponectin can reconstitute lethally irradiated mice in vivo. Freshly isolated CD45.2$^+$ KTLS cells were cultured with 5 ng/ml Tpo and 10 ng/ml SCF in the presence of globular adiponectin or vehicle control for four days after which equal numbers of cells (1500 or 500) were transplanted into two cohorts of lethally irradiated mice (CD45.1$^+$), respectively. Mice were bled at 2–3 wk intervals and PBMC were stained for donor and host specific CD45 alleles and appropriate lineage markers to determine chimerism and lineage composition. *a*, Representative FACS plots at 12 wk show total peripheral blood chimerism or lineage specific contribution of mice transplanted with 1500 cells cultured in the presence or absence of adiponectin. *b*, Peripheral blood chimerism of mice transplanted with cells cultured in the presence (black) or absence (gray) of adiponectin at 12 wk ($p = 0.01$). *c*, Peripheral blood chimerism of mice transplanted with cells cultured in the presence (black) or absence (gray) of adiponectin at 28 wk ($p = 0.04$). Data represent average percent chimerism ± SEM. Percentages indicate the frequency of donor-derived cells (CD45.2) in each of the outlined populations; percentage was determined after gating out contribution from RBC (CD45 cells).

AdipoR1 pathway. Control LacZ siRNA infected KTLS cells exhibited a 2–3-fold increase in total cell number over AdipoR1 siRNA infected cultures (Fig. 5*b*), suggesting that adiponectin signaling is necessary for optimal growth of HSCs in vitro. In addition, we compared the effect of AdipoR1 knockdown to the effect of knocking down another gene expressed in hemopoietic stem and progenitor cell. To this end we used MAFK, a transcription factor which is expressed in HSCs (U. Sankar and A. R. Means, unpublished observations) with no observed function. Similar to knocking down LacZ, knock down of MAFK (data not shown) had no effect on cell growth in contrast to knocking down AdipoR1 (Fig. 5*c*). We also tested whether intact AdipoR1 was required for HSC growth and function in vivo. A total of 4000 KTLS cells infected with either LacZ siRNA or R1 siRNA were transplanted into lethally irradiated mice, in the presence of competing wild-type bone marrow cells and reconstitution monitored from 4 to 12 weeks. Mice reconstituted with AdipoR1 siRNA KTLS cells displayed a 3-fold reduction in donor chimerism compared with those mice transplanted with KTLS cells carrying the unrelated LacZ siRNA construct (Fig. 5, *d* and *e*). Furthermore the lineage distribution was essentially similar suggesting that inhibition of AdipoR1 did not result in specific defects in lineage commitment and differentiation (Table II), but rather a more specific growth defect. These data not only suggest that the loss of AdipoR1 has a specific impact on HSC growth in vivo, but also demonstrate that lentivirally delivered siRNA can be successfully used to test the requirement of specific signals for HSC function in vivo. These data together

Table I. *Lineage distribution of transplantations from HSCs cultured in the presence or absence of adiponectin*

	−Adipo		+Adipo	
	Average PB Chimerism (%)	Range (%)	Average PB Chimerism (%)	Range (%)
B220	24	2.8–51.3	40	10.6–62.7
CD3	22	6.6–53.5	25	11.2–44.4
Gr1	15	1.7–43.2	8	5.8–11.5
Mac1	38	3.7–72.7	16	11.1–20.9

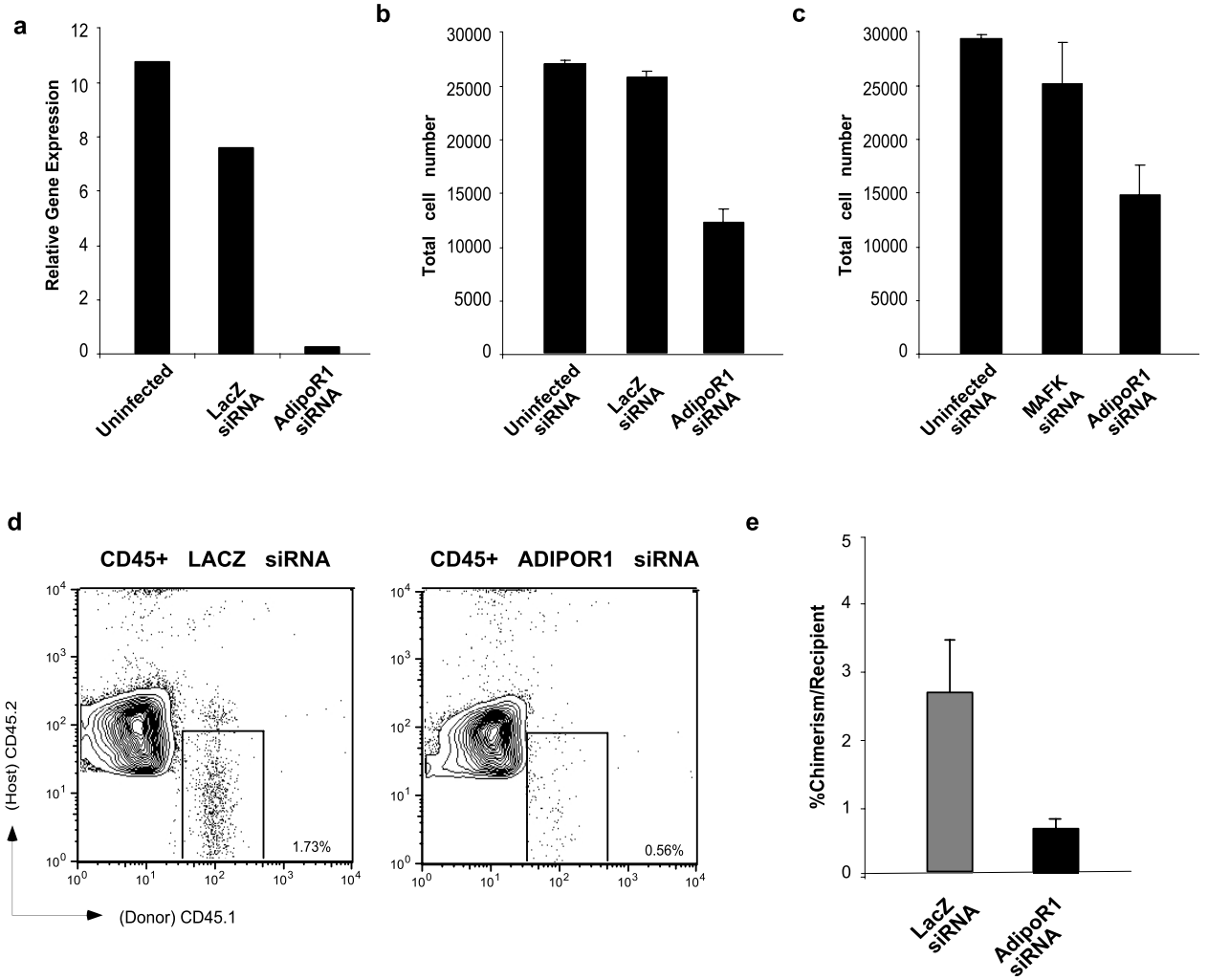

FIGURE 5. Inhibition of adiponectin signaling impairs HSC proliferation in vitro and reduces reconstitution in vivo. siRNA was designed against AdipoR1 and an unrelated control in a lentiviral vector expressing GFP. KTLS cells were infected with lentiviruses expressing either LacZ siRNA, or AdipoR1 siRNA for 48 h and subsequently resorted for GFP expression. *a*, AdipoR1 expression was examined in GFP^+ cells by real time PCR. Results were normalized to total cDNA levels and are representative of two independent experiments. *b*, Uninfected or GFP^+ LacZ or AdipoR1 siRNA infected KTLS cells were cocultured with primary stromal cells in X-Vivo medium alone for 4 days. Subsequently the absolute number of $CD45^+$, live cells was determined by flow cytometry. Results are shown as the total cell number in culture after 4 days, ± SEM. Results are representative of three independent experiments $p = 0.046$. *c*, Uninfected or GFP^+ MAFK siRNA or AdipoR1 siRNA infected KTLS cells were cocultured with primary stromal cells and analyzed as shown in *b*. Results are representative of three independent experiments $p = 0.049$. *d*, Equal cell numbers (4000 cells/mouse) of LacZ or AdipoR1 siRNA infected KTLS cells were transplanted into two cohorts of 6 lethally irradiated mice. Representative FACS plots showing reconstitution at 4 wk after transplantation. *e*, Average percentage chimerism of mice transplanted with LacZ (gray) or R1 siRNA (black) infected cells 4 wk after transplantation. $p = 0.04$. Similar relative levels of chimerism were seen at 12 wk. $p = 0.008$. Results are representative of two independent experiments.

with the results from the previous in vitro experiments suggest that an intact cellular response to adiponectin is required for optimal HSC growth in vitro and reconstitution in vivo.

Table II. *Lineage distribution of transplantations from AdipoR1 knockdown or control HSCs*

	lacZ		AdipoR1	
	Average PB Chimerism (%)	Range (%)	Average PB Chimerism (%)	Range (%)
B220	73	55–84	42	25.6–63.6
CD3	3	1.2–6.1	2	1.3–7.0
Gr1	2	1.0–2.6	3	1.2–5.88
Mac1	9	7.4–12.1	12	5.4–21.1

Adiponectin mediated HSC proliferation is dependent on p38 MAPK activation

Next we wanted to investigate the mechanism behind adiponectin's effect on HSCs. Several downstream mediators of adiponectin signaling have been identified in other cell types. In the liver and skeletal muscle, adiponectin is known to activate AMPK (27, 30, 43), JNK (44), and p38 MAPK (27) (45). Therefore, we tested whether adiponectin may activate these downstream targets in HSCs by treating KTLS cells with adiponectin and monitoring the phosphorylation status of each target. Although no increase in JNK activation was observed (data not shown), we found that AMPK was activated and p38 phosphorylation was enhanced in response to adiponectin stimulation (Fig. 6, *a*, *c*, and *d*).

To determine whether AMPK or p38 activation plays a functional role in adiponectin mediated proliferation we inhibited the

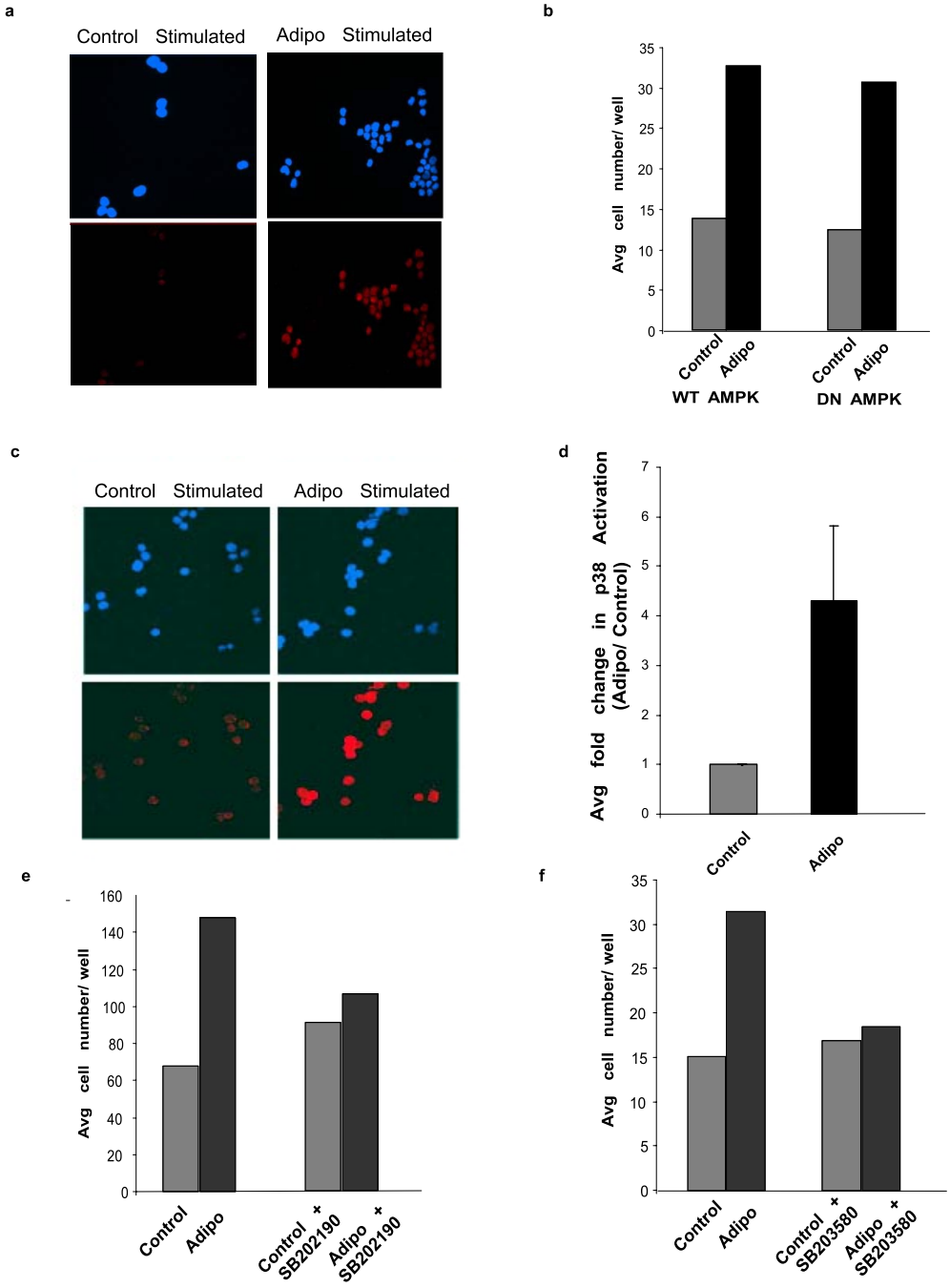

FIGURE 6. Adiponectin stimulation activates p38 in HSCs. *a* and *c*, Freshly isolated KTLS cells were stimulated for 30 min with vehicle control or 500 ng/ml to 1 μg/ml adiponectin and subsequently stained with anti-P-AMPK anti-P-p38 or isotype control and counterstained with DAPI to visualize all nuclei. Cells were then viewed by confocal microscopy. *b*, KTLS cells were sorted into Terasaki wells at 10 cells/well and cultured in the presence (black bar) or absence (gray bar) of adiponectin and infected with DN-AMPK for 7 days. Cultures contained SCF and Tpo as synergizing factors. Results are representative of three independent experiments. *d*, Frequency of activation was quantified by counting the number of cells with detectable P-p38 staining in each condition over isotype background. Cells stained with isotype control Abs were below detection levels. Average activation in response to adiponectin is shown. Results are representative of seven independent experiments. Activation ranged from 1.5- to 12-fold depending on the source and the concentration of adiponectin. *e* and *f*, KTLS cells were sorted into Terasaki wells at 10 cells/well and cultured in the presence (black bar) or absence (gray bar) of adiponectin and cultured with or without the p38 inhibitors SB202190 or SB203580 for 7 days. Cultures contained SCF and Tpo as synergizing factors. Results are from three representative experiments.

function of both targets. First, we cultured HSCs infected with either a wild-type or dominant-negative form of AMPK in the presence or absence of adiponectin. HSCs infected with the dominant-negative form of AMPK showed no change in levels of adiponectin mediated proliferation relative to wild-type infected cells, suggesting that AMPK is not required for this effect (Fig. 5*b*). To inhibit p38 function we cultured cells in the presence or absence of SB202190 or SB203580, small molecule inhibitors which have been demonstrated to be specific for p38 (46–50). When HSCs were cultured in the presence of either inhibitor, adiponectin mediated proliferation was abrogated, indicating that p38 activity is necessary for adiponectin's effect on HSCs (Fig. 6, *e* and *f*).

Discussion

In this report we identify adiponectin signaling as a novel regulator of stem cell function. We find that adiponectin is expressed by cells in the HSC microenvironment and that HSCs express the adiponectin receptors AdipoR1 and AdipoR2, with AdipoR1 being up-regulated when stem cells enter cycle. This up-regulation of AdipoR1 corresponds with our observation that stimulating HSCs with the globular form of adiponectin in vitro can enhance proliferation. The functional capacity of these cells was confirmed by in vitro colony forming assay experiments where HSCs cultured with adiponectin yielded increased numbers of mixed lineage colonies which are indicative of a more immature, progenitor cell type. Consistent with this, limiting numbers of cells expanded in culture with adiponectin were more efficient in reconstituting lethally irradiated hosts in long term transplantation assays in vivo. Our data also suggests that adiponectin signaling is essential for stem cell function because inhibition of the pathway reduced growth and reconstitution. Finally, we identify a potential mechanism of action for adiponectin by demonstrating that inhibition of p38 inhibits adiponectin mediated proliferation.

Although previous experiments have demonstrated an inhibitory effect of adiponectin on lymphoid and myeloid-committed cell proliferation (18, 35) and experiments in which progenitor KLS cells were cultured with adiponectin resulted in increased myeloid cell production with reduced lymphoid cell growth in vitro (18), the effect of adiponectin stimulation or inhibition on HSCs (51, 52) has not been examined in in vivo transplantation assays. Additionally, previous experiments conducted on the KLS population used the full length recombinant form of adiponectin which only slightly increased proliferation in our experiments when compared to stimulation with the globular domain of adiponectin. Our data show that adiponectin favors proliferation of a population enriched for the most primitive HSCs, suggesting the possibility that the effect of adiponectin on more multipotent cell populations may be distinct from that on more committed progenitors. This is also consistent with other studies which show that the effect of adiponectin differs depending on the cell type examined, even between closely related cells (43, 53, 54).

Because our results suggest that adiponectin is sufficient to enhance HSC proliferation while maintaining these cells in a more undifferentiated state, we also wanted to determine whether adiponectin signaling is required for HSC function. To test this we inhibited AdipoR1 by a lentiviral knockdown strategy. This led to impaired HSC proliferation in vitro and reconstitution in vivo, suggesting that adiponectin is required for optimal proliferation of HSCs. Based on the current literature in which adiponectin is the only ligand identified for AdipoR1 to date (27) we interpret these results to indicate that adiponectin is required for optimal HSC proliferation and reconstitution. However, the possible existence of unknown ligands for this receptor cannot be ruled out. Engagement of AdipoR1 by (as yet unidentified) ligands other than adiponectin could therefore prove to be in part responsible for the decreased reconstitution seen with AdipoR1 knockdown cells compared with control cells in our experiments.

To determine the downstream mechanism by which adiponectin is acting in HSCs we looked to other cell types for known components of the AdipoR1 signaling pathway. Based on activation of AMPK, JNK, and p38 by adiponectin in several cell types, we examined whether adiponectin may also be acting through these targets in HSCs. Indeed, we found that AMPK and p38 were activated in response to adiponectin stimulation. Although adiponectin mediated proliferation was unaffected in the presence of a dominant-negative form of AMPK, inhibition of p38 led to abrogation of adiponectin's proliferative effect, indicating that p38 activation is functionally required for adiponectin mediated proliferation. Interestingly p38 has an established role in hematopoiesis. The hemopoietic cytokines erythropoietin, IL-3, and G-CSF are all known to activate p38 (55, 56). IL-3 induced p38 activation leads to increased expression of the anti-apoptotic gene mcl-1 (57) and the proliferative response of hemopoietic progenitor cells to GCSF is mediated by p38 (56). Additionally, thrombopoietin (Tpo) an important factor in HSC expansion, acts via p38 to increase HoxB4 expression (58), which can in turn enhance HSC expansion (59, 60).

p38 has been associated with both enhanced cell survival and apoptosis in some cell types. It has been shown that both the duration of activation (61), and the presence and/or activation status of other downstream factors (62) are responsible for determining the final cellular response, indicating that p38's function is context dependent. p38 has been implicated in the homeostatic proliferation of cells, including smooth muscle (63), thyroid (64), and Sertoli cells (65) and in malignant transformation of prostate (66) and breast cancer cells, (67) however, the mechanism of p38 mediated proliferation has not been well characterized. In thyroid cells, p38 activity is required for DNA synthesis, the G_1-S phase transition, and an increase in Cdk2 levels (64). Additionally, in breast cancer cells, p38 activation results in increased expression of cyclin D1 (67). It is possible that in response to adiponectin, a similar phenomenon occurs, where activated p38 directly affects cell cycle regulatory components. Alternatively, adiponectin mediated p38 activation may be analogous to p38 activated in response to Tpo or IL-3 stimulation in hemopoietic cells, thus leading to HoxB4 mediated proliferation or diminished apoptosis. In the future, further characterization of the pathways by which adiponectin influences HSC proliferation will provide exciting new insight into the regulation of HSC growth and development.

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Disclosures

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