ARTICLES

Haematopoietic stem cell release is regulated by circadian oscillations

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Haematopoietic stem cells (HSCs) circulate in the bloodstream under steady-state conditions, but the mechanisms controlling their physiological trafficking are unknown. Here we show that circulating HSCs and their progenitors exhibit robust circadian fluctuations, peaking 5 h after the initiation of light and reaching a nadir 5 h after darkness. Circadian oscillations are markedly altered when mice are subjected to continuous light or to a 'jet lag' (defined as a shift of 12 h). Circulating HSCs and their progenitors fluctuate in antiphase with the expression of the chemokine CXCL12 in the bone marrow microenvironment. The cyclical release of HSCs and expression of *Cxcl12* are regulated by core genes of the molecular clock through circadian noradrenaline secretion by the sympathetic nervous system. These adrenergic signals are locally delivered by nerves in the bone marrow, transmitted to stromal cells by the β_3-adrenergic receptor, leading to a decreased nuclear content of Sp1 transcription factor and the rapid downregulation of *Cxcl12*. These data indicate that a circadian, neurally driven release of HSC during the animal's resting period may promote the regeneration of the stem cell niche and possibly other tissues.

Under homeostasis, small numbers of HSCs are detectable in the bloodstream of mammals[1]. The omnipresence of HSCs and their progenitors in the circulation and their rapid clearance after injection suggest a rapid flux between the blood and bone marrow compartments under homeostasis[2]. Studies in humans have previously revealed conflicting patterns in circadian variations of circulating progenitors[3,4]. Mobilization of HSCs and their progenitors into the circulation represents the basis for modern bone marrow transplantation procedures and can be elicited by multiple structurally distinct compounds with various speeds of action[5–8]. The prototypical agent used in the clinic, granulocyte colony-stimulating factor (G-CSF), mobilizes HSCs and their progenitors to the blood through complex mechanisms, involving notably the induction of proteolytic activity that cleaves CXCL12 (or stromal-derived factor-1; SDF-1)[9,10], and the suppression of osteoblast function[11,12], an integral cellular constituent of the HSC niche[13–15]. This leads to decreases in *Cxcl12* expression in the bone marrow microenvironment[10–12] and to HSC/progenitor egress that depends on an intact peripheral sympathetic nervous system (SNS)[11]. Whereas signals from the β_2-adrenergic receptor (encoded by *Adrβ2*)—the sole β-adrenergic receptor expressed in the osteoblast[16]—enhance HSC/progenitor egress, *Adrβ2*-mediated signals are not sufficient to suppress osteoblast function[11], suggesting the contribution of other adrenergic or non-adrenergic mediators. In addition, recent studies suggest that catecholaminergic pathways can promote human $CD34^+$ cell migration and engraftment by acting directly on HSC/progenitor cells, probably through Adrβ2 (ref. 17). Because the mechanisms mediating HSC release in homeostasis are not known, it is not clear whether the pharmacological induction of stem cell egress usurps the endogenous physiological trafficking machinery.

While investigating further the mechanisms mediating enforced mobilization of HSCs and their progenitors by G-CSF, we noted by chance that continuous exposure to light significantly altered the number of colony-forming units in culture (CFU-C) and HSC-enriched lineage-negative (lin^-)$Sca\text{-}1^+c\text{-}Kit^+$ (LSK) cells mobilized by the administration of G-CSF (Supplementary Fig. 1). These results suggested that photic cues, processed in the central nervous system, could influence the trafficking of HSCs in unperturbed steady-state animals. We therefore investigated the circadian patterns and the mechanisms behind HSC release in homeostasis.

Circadian HSC release is modulated by photic cues

Under steady-state conditions in which mice are exposed to standard cycles of 12 h light/12 h darkness (LD), the number of circulating progenitors oscillated markedly, peaking 5 h after the initiation of light (Zeitgeber time, ZT5) and reaching a low point at ZT17 (Fig. 1a; analysis of variance (ANOVA): $F_{5,53} = 3.840$, $P = 0.005$, with post hoc test for linear trend $P = 0.002$). To determine whether this circadian oscillation affected stem cells, we analysed LSK cells (Fig. 1b) and conducted competitive repopulation analyses with limiting blood dilutions to quantify long-term (four months) reconstituting HSCs at the peak (ZT5) and the trough (ZT17) (Fig. 1c). These analyses revealed that the number of HSCs in the circulation at ZT5 is about twofold to threefold that at ZT17.

Circadian fluctuations of progenitors were entrained and modulated by photic cues, because the pattern was arrhythmic in mice subjected to continuous light (LL) for two weeks (Fig. 1d; ANOVA: $F_{5,17} = 0.7522$; $P = 0.6$). However, rhythmic oscillations were observed in mice maintained in continuous darkness for two weeks (Fig. 1e; ANOVA: $F_{5,29} = 3.003$, $P = 0.03$, with post hoc test for linear trend $P = 0.002$), which is consistent with free-running circadian rhythms in darkness[18,19]. To assess whether changes in light input were sufficient to alter progenitor trafficking, we subjected mice to a 'jet lag' by advancing the LD cycle by 12 h (Fig. 1f, bottom). Sampling of blood progenitors revealed a markedly flattened, arrhythmic pattern of circulating progenitor counts over the following 24 h in jet-lagged mice in comparison with control LD-maintained animals (Fig. 1f; ANOVA: $F_{5,45} = 1.366$; $P = 0.26$). Thus, a simple change in light cycle is sufficient to bring about a marked alteration in HSC/progenitor behaviour.

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Rhythmic *Cxcl12* oscillations in bone marrow

CXCL12 is the only known chemokine capable of directed migration of HSCs[20,21]. The circadian fluctuations in HSCs and their progenitors suggested that CXCL12 might also be subjected to circadian control. To investigate this possibility, we measured the expression of *Cxcl12* in the bone marrow microenvironment by ELISA. Remarkably, CXCL12 protein levels fluctuated in a pattern that mirrored oscillations in circulating HSCs and their progenitors (Fig. 2a; ANOVA: $F_{5,49} = 2.485$, $P = 0.04$, with post hoc test for linear trend $P = 0.006$). Similar rhythmic fluctuations were observed in *Cxcl12* mRNA expression (Fig. 2a), suggesting that CXCL12 is not regulated by proteolytic degradation under homeostasis and that the steady-state circadian oscillations in HSCs and their progenitors are controlled by fluctuations in *Cxcl12* expression. Further, oscillations of *Cxcl12* expression in bone marrow were arrhythmic in mice maintained in constant light (Fig. 2b) or under conditions of jet lag (Fig. 2c), whereas *Cxcl12* expression continued to fluctuate rhythmically in mice maintained in constant darkness (Fig. 2d; ANOVA: $F_{5,18} = 3.664$, $P = 0.02$, with post hoc test for linear trend $P = 0.001$). These results suggest that circadian release of HSCs is triggered by rhythmic expression of *Cxcl12* in the bone marrow.

Neural signals regulate *Cxcl12* through Adrβ3

Because SNS signals affect the G-CSF-induced mobilization of HSCs[11] and modulate osteoblast proliferation through the peripheral expression of core clock genes[22], we next lesioned the SNS of adult mice with 6-hydroxydopamine (6OHDA) to assess the role of SNS signals in the circadian release of HSCs. SNS disruption abolished the normal circadian pattern of circulating progenitors (Fig. 3a). In addition, 6OHDA did not affect the number of CFU-Cs, LSK cells or $CD150^{+}CD48^{-}CD41^{-}$ HSCs[23] in the bone marrow (data not shown), excluding a direct toxicity on HSCs and progenitors. Competitive repopulation assays to evaluate the number of circulating stem cells at ZT5 revealed a marked decrease in long-term repopulating stem cell activity in 6OHDA-lesioned mice compared with control C57BL/6 mice up to 17 weeks after transplantation (Fig. 3b and Supplementary Fig. 2). These results suggested that adrenergic signals are critical for the circadian release of stem cells from the bone marrow.

Because 6OHDA disrupts the SNS systemically, it could be argued that its effect might be mediated either through a loss of soluble catecholamines (for example from the adrenal glands), through other indirect effects to non-haematopoietic organs or through a local delivery of noradrenaline (norepinephrine) from adrenergic nerve terminals in the bone marrow. To distinguish between these possibilities, we surgically sympathectomized the tibiae of mice by unilateral microsection of both the sciatic and femoral nerves. We checked whether the sympathectomy was complete by staining for tyrosine hydroxylase, and found no specific staining for sympathetic fibres in the denervated bone marrow, whereas the contralateral sham-operated side exhibited multiple stained fibres, mostly near blood vessels (Fig. 3c, d). Strikingly, the circadian oscillations of *Cxcl12* expression were severely altered in the denervated tibiae, whereas *Cxcl12* expression showed the characteristic diurnal pattern in the contralateral sham-operated tibiae of the same mice (Fig. 3e, f). These results demonstrate that the rhythmic fluctuations in *Cxcl12* expression and the resulting release of HSCs require adrenergic signals delivered locally in the bone marrow by nerves from the SNS.

To evaluate the exact contribution of adrenergic receptors and the downstream mechanisms, we treated bone marrow stromal cells (MS-5) with selective and non-selective adrenergic agonists and antagonists. Noradrenaline, the natural neurotransmitter of the SNS, and isoprenaline (isoproterenol), a non-selective β-adrenergic agonist, decreased CXCL12 production in a dose-dependent manner (Fig. 4a). A similar decrease in *Cxcl12* mRNA expression was detected with either adrenergic agonist (Supplementary Fig. 3). This effect was mediated by the β_3-adrenergic but not the β_2-adrenergic receptor, because it was induced by a β_3-adrenergic agonist (BRL37344) and inhibited by a selective β_3-adrenergic antagonist (SR59230A), whereas Adrβ2 engagement or blockade had no effect (Fig. 4a, b). Treatment of primary myeloid bone marrow cultures with isoprenaline or BRL37344 markedly suppressed *Cxcl12* expression in stromal

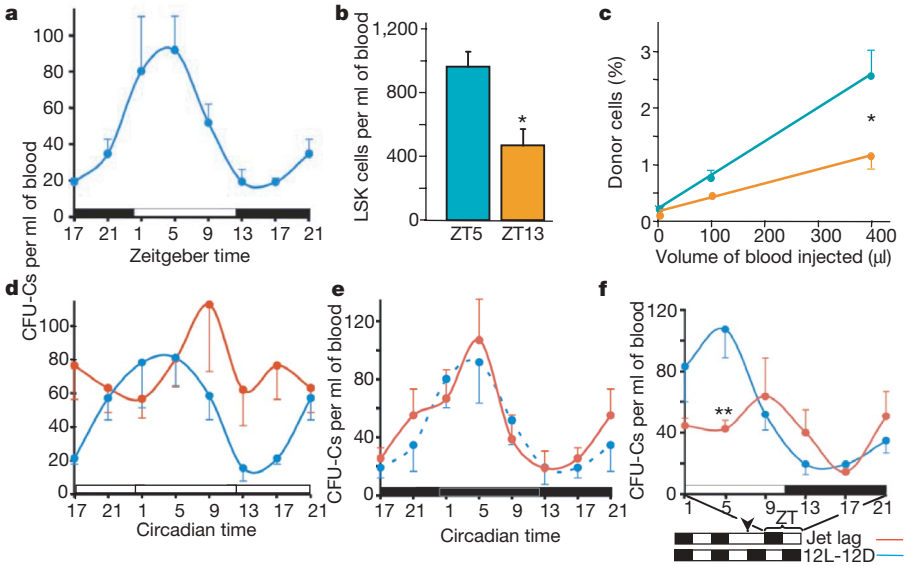

Figure 1 | Circadian traffic of HSCs and their progenitors is entrained by photic input. **a**, **b**, Circadian fluctuations of circulating CFU-Cs (**a**, $n = 10$ mice per time point) and LSK cells (**b**, $n = 6$ or 7) in standard LD conditions. **c**, Stem cell activity using long-term competitive reconstitution assays. Blood harvested at ZT5 (cyan) showed a roughly 2.5-fold increase compared with ZT17 (orange) in competitive repopulation 16 weeks after transplantation ($n = 4$ mice per group). **d**–**f**, Circadian traffic of CFU-Cs was disrupted in mice subjected to altered light cycles and preserved in animals kept in constant darkness: **d**, constant light for 2 weeks (red), control LD (blue) ($n = 4$ mice per time point); **e**, constant darkness for 2 weeks (solid red line), control LD (dashed blue line); $n = 6$ mice per time point; **f**, jet lag (red), control LD (blue). The arrowhead in **f** indicates the initiation of light shift. Circulating progenitors were monitored 12 h later and for the next 24 h ($n = 6$–13 mice per time point). Results are shown as means and s.e.m. Asterisk, $P < 0.05$; two asterisks, $P < 0.01$ (unpaired two-tailed *t*-test). In **a** and **d**–**e**, time points 17 and 21 have been duplicated to facilitate viewing of the time curve. All experiments were performed in male C57BL/6 mice six to nine weeks old.

cells and significantly increased the number of released progenitors in the supernatant, whereas the β_2-adrenergic agonist clenbuterol did not affect *Cxcl12* mRNA levels (Fig. 4c). β_3-Adrenergic signalling was sufficient to alter *Cxcl12* expression, because isoprenaline injection sharply decreased the expression of *Cxcl12* in bone marrow *in vivo* 2 h after administration in wild-type animals (Fig. 4d). However, despite the large decrease in bone marrow CXCL12 induced by isoprenaline, no significant elevation of circulating progenitors was observed (data not shown). This suggested the possibility that β-adrenergic signalling might also enhance homing or trapping in tissues. To circumvent this issue, we evaluated the effect of isoprenaline in mice in which HSC/progenitor homing and adhesion in the vasculature are compromised[24–26]. We found a significant increase in circulating progenitors after the administration of isoprenaline when both endothelial selectins and α_4 integrins were absent or blocked (Fig. 4e), suggesting that physiological, locally delivered β_3-adrenergic signals control HSC/progenitor release, whereas the pharmacological administration of non-selective β-adrenergic agonists may also enhance progenitor clearance. Further, a significant decrease in *Cxcl12* expression was observed in the presence of a selective β_2-adrenergic receptor blocker (data not shown) or in stroma derived from *Adrβ2*$^{-/-}$ mice (Supplementary Fig. 4). In sharp contrast, isoprenaline did not affect *Cxcl12* expression in stroma derived from *Adrβ3*$^{-/-}$ mice (Fig. 4f) and induced a significant mobilization of progenitors in control but not *Adrβ3*$^{-/-}$ mice after blockade of endothelial selectins and α_4 integrins (Fig. 4g). Moreover, the effect of isoprenaline was mimicked by a selective β_3-adrenergic agonist (BRL37344; Fig. 4g). Because adrenergic activity in the murine bone marrow follows a circadian variation[27] that closely mirrors fluctuations in *Cxcl12* expression (Fig. 2), we reasoned that the release of noradrenaline in the bone marrow microenvironment might be responsible for the rapid downregulation of *Cxcl12* and the release of progenitors that follow the onset of light (Fig. 2). To test this possibility, we administered a β_3-adrenergic antagonist (SR59230A) at ZT23 (1 h before the onset of light) and harvested circulating progenitors at ZT1. As shown in Fig. 4h, SR59230A prevented the morning increase in circulating progenitors. Furthermore, circadian fluctuations in bone marrow CXCL12 and circulating progenitors were preserved in *Adrβ2*$^{-/-}$ mice and markedly attenuated in *Adrβ3*$^{-/-}$ animals, and additional blockade of Adrβ1 and Adrβ2 in *Adrβ3*$^{-/-}$ mice did not affect the number of circulating progenitors (Supplementary Fig. 5), suggesting a crucial role for *Adrβ3* but not the other β-adrenergic receptors in circadian CXCL12 regulation.

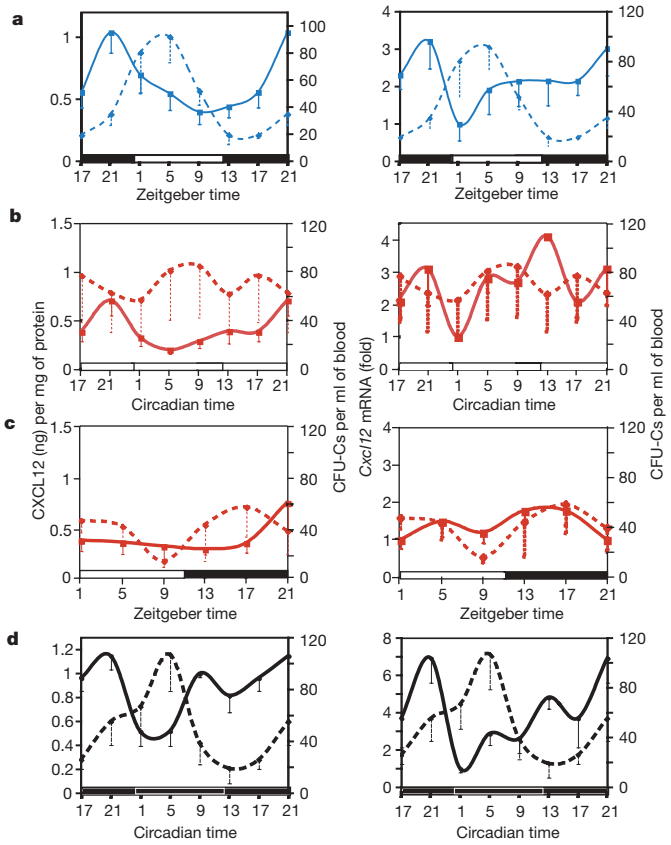

Figure 2 | Bone marrow *Cxcl12* expression oscillates in antiphase with circulating progenitors. CXCL12 content in bone marrow extracellular fluids (solid lines in left panels) and *Cxcl12* mRNA levels in bone marrow (solid lines in right panels) were analysed in mice kept under normal LD cycles (**a**, $n = 10$ mice per time point) or subjected to constant light (**b**), 12 h jet lag (**c**, $n = 4$ mice per time point) or constant darkness (**d**, $n = 6$ mice per time point). CXCL12 levels mirrored fluctuations in circulating progenitors (dashed lines) in all conditions. The decreased amplitude of circulating CFU-C oscillations under constant light and jet lag were also correlated with decreased fluctuations in bone marrow CXCL12 levels. **a**, **b**, **d**, Time points 17 and 21 have been duplicated to facilitate viewing of the time curve. Results are shown as means and s.e.m. Male C57BL/6 mice six to nine weeks old were used.

HSC release is orchestrated by the central clock

The circadian regulation of HSC release suggests a role for the core genes of the molecular clock that orchestrate the expression of numerous target genes centrally from the suprachiasmatic nucleus and/or peripherally in various tissues (reviewed in refs 28, 29). Circulating CFU-C counts and *Cxcl12* expression did not oscillate in a circadian manner in *Bmal-1*$^{-/-}$ mice (Fig. 5a), indicating that clock genes do indeed regulate *Cxcl12*. Because the SNS regulates osteoblast proliferation through the expression of clock genes in osteoblasts[22], we profiled the expression of clock genes in the bone marrow. We observed a trend consistent with the expression patterns

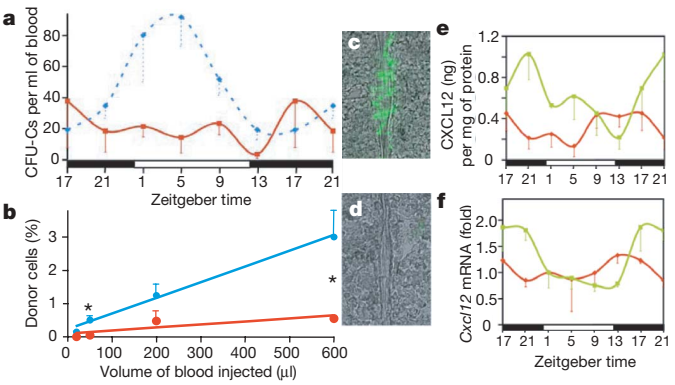

Figure 3 | Adrenergic innervation of the bone marrow regulates oscillations in *Cxcl12* expression and circulating HSCs. **a**, Neurochemical sympathectomy of adult mice with 6OHDA abolished fluctuations in circulating progenitors (solid red lines). The dashed blue line represents control LD ($n = 4$ mice per time point). **b**, Stem cell activity measured with long-term competitive reconstitution assays. Blood harvested at ZT5 from 6OHDA-treated mice (red) showed a marked decrease in competitive repopulation units at 17 weeks. Asterisk, $P < 0.05$ (unpaired two-tailed *t*-test). The estimated number of competitive repopulation units was 1 in 505 μl in 6OHDA-treated mice in comparison with 1 in 42 μl in control mice (Supplementary Fig. 2; 1.5% reconstitution threshold; $n = 4$ mice per dose). **c**–**e**, Surgical denervation of the tibiae of adult mice destroyed catecholaminergic fibres in the bone marrow (**c**, sham-operated bone marrow; **d**, denervated bone marrow; green, immunofluorescence for tyrosine hydroxylase) and abolished fluctuations (red lines) in CXCL12 protein (**e**) and *Cxcl12* mRNA levels (**f**), whereas *Cxcl12* showed the characteristic circadian pattern in the sham-operated contralateral tibiae (**e**, **f**, green lines; $n = 4$ mice per time point). **a**, **e**, **f**, Time points ZT17 and ZT21 have been duplicated to facilitate viewing of the time curve. Results are shown as means and s.e.m. Male C57BL/6 mice six to nine weeks old were used.

reported for *Clock*, *Bmal1*, *Per1*, *Per2*, *Cry1* and *Rev-erbα* in peripheral oscillators under LD[28,29], and apparent alterations under continuous light or conditions of jet lag, but these patterns were not statistically significant (Supplementary Fig. 6). The *Cxcl12* promoter contains several non-canonical E boxes (CGNNAT) that nevertheless suggest the possibility of a direct control of *Cxcl12* by peripheral clock proteins. However, treatment of bone marrow-derived *Bmal-1*$^{-/-}$ or *Per1*$^{-/-}$ *Per2*$^{m/m}$ stromal cells with isoprenaline decreased *Cxcl12* expression to the same extent as that in wild-type control stroma (Fig. 5b). These results therefore suggest that clock genes probably regulate *Cxcl12* and stem cell trafficking indirectly from the central nervous system rather than peripherally in the stromal cells that synthesize CXCL12.

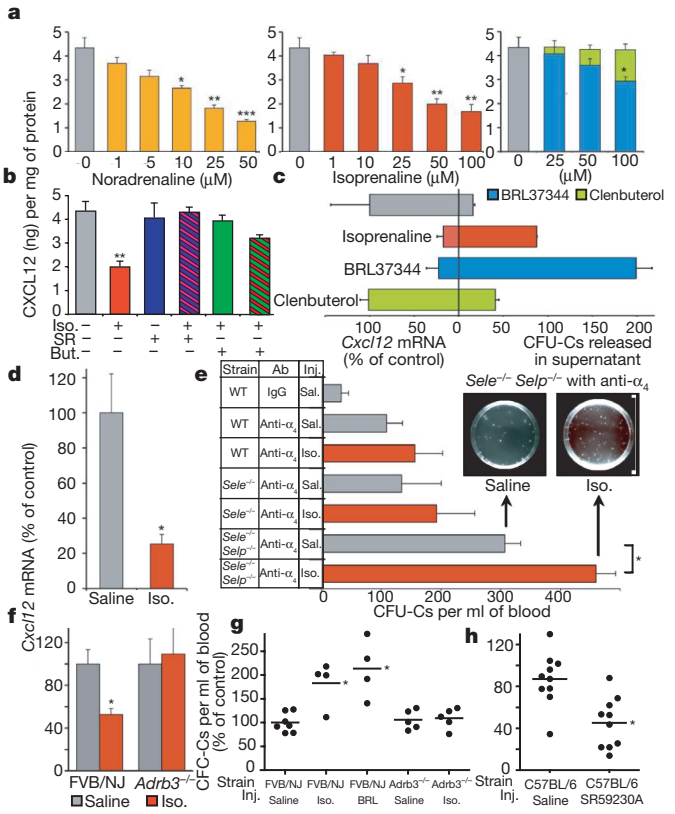

Figure 4 | β$_3$-Adrenergic receptor activation decreases *Cxcl12* mRNA levels in bone marrow stromal cells and triggers HSC/progenitor mobilization. **a**, Dose-dependent decrease in CXCL12 secretion by MS-5 cells 36 h after treatment with noradrenaline was mimicked by the non-selective β-adrenoceptor agonist isoprenaline and a selective β$_3$-adrenergic receptor agonist (BRL37344) but not a β$_2$-adrenergic receptor agonist (clenbuterol); $n = 8$. **b**, The effect of isoprenaline (iso., 50 μM) in MS-5 cells was prevented by a selective β$_3$-adrenergic receptor antagonist (1 μM SR59230A; SR) but not a β$_2$-adrenergic receptor antagonist (1 μM butoxamine; but.); $n = 8$. **c**, Decrease in *Cxcl12* mRNA levels 2 h after treatment of primary myeloid cultures derived from adult C57BL/6 mice with isoprenaline or BRL37344, but not clenbuterol (50 μM, $n = 4$). This decrease correlated with the number of CFU-Cs that were detached from the stromal layer and released in the supernatant. **d**, Isoprenaline (2 mg kg^{-1}, injected intraperitoneally) decreased bone marrow *Cxcl12* mRNA levels by 80% 2 h after its administration to adult C57BL/6 mice ($n = 6$). **e**, Injection of isoprenaline (2 mg kg^{-1}, injected (inj.) intraperitoneally) induced HSC/progenitor mobilization when homing was compromised by injection with anti-α$_4$ integrin antibodies (Ab) in endothelial selectins-deficient mice (of C57BL/6 genetic background; $n = 6$). Sal., saline. **f**, *Cxcl12* mRNA levels were significantly decreased 2 h after 100 μM isoprenaline treatment of primary myeloid cultures from control FVB/NJ mice but not *Adrb3*$^{-/-}$ mice of the same background ($n = 4$). **g**, Injection of isoprenaline or BRL37344 (2 mg kg^{-1}, injected intraperitoneally) induced, 2 h later, HSC/progenitor mobilization in FVB/NJ but not *Adrb3*$^{-/-}$ mice in which homing was blocked by the injection of antibodies against P-selectin, E-selectin and α$_4$ integrins ($n = 4$–7). **h**, Morning (ZT1) physiological increase in circulating progenitors was prevented in C57BL/6 mice ($n = 10$) previously injected (ZT23) with a β$_3$-adrenergic receptor antagonist (SR59230A, 5 mg kg^{-1}, intraperitoneally). Note the smaller numbers of circulating CFU-Cs in C57BL/6 mice than in FVB/NJ mice. Results are shown as means and s.e.m. Asterisk, $P < 0.05$; two asterisks, $P < 0.01$; three asterisks, $P < 0.001$ (unpaired two-tailed *t*-test).

Degradation of Sp1 transcription factor

Previous studies with the human *Cxcl12* promoter have shown the presence of several Sp1 transcription factor-binding sites and significant decreases in promoter activity when only one Sp1-binding motif was mutated[30]. Alignment of the human and mouse *Cxcl12* promoter sequence revealed a remarkable conservation of these Sp1-binding sites. To gain more insight into the mechanisms that regulate the circadian fluctuations of *Cxcl12* expression, we treated MS-5 cells and primary stromal cultures with mithramycin A, an inhibitor of the binding of Sp transcription factors to GC-enriched DNA sequences. Mithramycin A significantly decreased *Cxcl12* expression (Fig. 5c), suggesting a requirement for Sp1 function in efficient *Cxcl12* transcription. Phosphorylation of Sp1 by the cAMP-dependent

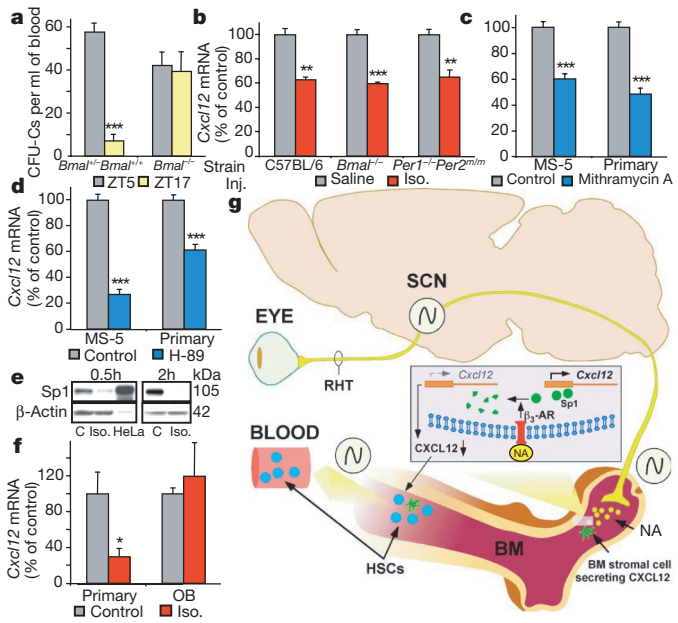

Figure 5 | The central clock regulates *Cxcl12* in bone marrow stromal cells through signals from the sympathetic nervous system. **a**, Circulating progenitors did not oscillate in *Bmal1*$^{-/-}$ mice, compared with *Bmal1*$^{+/+}$ and *Bmal1*$^{+/-}$ controls (C57BL/6 genetic background; $n = 4$). **b**, Decrease in *Cxcl12* mRNA levels 2 h after treatment with 100 μM isoprenaline (iso.) was similar in primary myeloid cultures from C57BL/6, *Bmal1*$^{-/-}$ and *Per1*$^{-/-}$*Per2*$^{m/m}$ mice (of the same genetic background; $n = 4$). **c**, **d**, Treatment of MS-5 cells or myeloid cultures derived from adult C57BL/6 mice for 2 h with either mithramycin A (250 nM), an inhibitor of the binding of Sp transcription factors to DNA (**c**), or the PKA inhibitor H-89 (25 μM) (**d**) significantly decreased *Cxcl12* mRNA levels ($n = 8$). **e**, Sp1 content in nuclear extracts from MS-5 cells after treatment with isoprenaline (100 μM). The left panel shows a marked decrease 30 min after the addition of 100 μM isoprenaline. HeLa, nuclear extracts from HeLa cells. Nuclear Sp1 is greatly decreased 2 h after exposure to isoprenaline (100 μM). Two representative experiments of 12 are shown. **f**, Treatment with isoprenaline (100 μM) for 2 h markedly decreased *Cxcl12* mRNA levels in myeloid cultures derived from adult C57BL/6 mice, but not in osteoblasts differentiated from stromal cells for 4 weeks in L-ascorbic acid 2-phosphate (1 mM; $n = 5$). **g**, Model for circadian regulation of trafficking of HSCs and their progenitors. β$_3$-AR, β$_3$-adrenergic receptor; BM, bone marrow; NA, noradrenaline; RHT, retinal–hypothalamic tract; SCN, suprachiasmatic nucleus. Results are shown as means and s.e.m. Asterisk, $P < 0.05$; two asterisks, $P < 0.01$; three asterisks, $P < 0.001$ (unpaired two-tailed *t*-test).

protein kinase (PKA) has been shown to enhance its DNA binding activity and subsequently to induce the transcription of target genes[31]. Although β_3-adrenergic receptor stimulation induces PKA through a G_s protein, it can also decrease intracellular cAMP levels after coupling to $G_{i/o}$ proteins[32], eventually leading to decreased Sp1 phosphorylation and *Cxcl12* downregulation. Treatment of MS-5 or primary stromal cells with H-89, a selective inhibitor of PKA, significantly decreased *Cxcl12* expression (Fig. 5d). Lipopolysaccharide, a known HSC mobilizing agent, was recently found to induce noradrenaline release by phagocytes[33], raising the possibility that bone marrow macrophage-derived noradrenaline might participate in lipopolysaccharide-induced HSC mobilization. In addition, lipopolysaccharide can induce Sp1 dephosphorylation and degradation[34], indicating that Sp1 degradation might be relevant to HSC trafficking. Consistent with this possibility was our observation that treatment of MS-5 cells with isoprenaline resulted in a marked and rapid (30 min) decrease in Sp1 content in nuclear extracts (Fig. 5e). Further, Sp1 was almost undetectable 2 h after treatment with isoprenaline (Fig. 5e), indicating that Sp1 degradation after β_3-adrenergic receptor stimulation may be responsible for the downregulation of *Cxcl12* and the subsequent egress of HSCs and progenitors from the bone marrow.

The SNS targets a stromal cell distinct from the osteoblast

The role of *Adrβ3* suggests that the cellular target of the SNS is not a mature osteoblast, because previous studies have reported that *Adrβ2* is the only β-adrenergic receptor expressed in osteoblasts[16]. MS-5 (fibroblastic reticular) and ST-2 (pre-osteoblast) cell lines expressed both *Adrβ2* and *Adrβ3* (Supplementary Fig. 7) and also downregulated *Cxcl12* after treatment with isoprenaline, whereas MC3T3-E1 (differentiated osteoblast) did not express *Adrβ3* and did not respond to isoprenaline (data not shown). We generated osteoblasts from mice carrying the *R26R* allele that also express the Cre recombinase under the control of the *Col1a1* promoter (*Col1a1–LacZ*). Total bone marrow or primary stromal cells expressed both *Adrβ2* and *Adrβ3*, whereas *Adrβ2* was the sole β-adrenergic receptor in osteoblasts derived from stromal cells differentiated in L-ascorbic acid 2-phosphate for four weeks and sorted for *Col1a1* expression (Supplementary Fig. 7). Further, isoprenaline did not decrease *Cxcl12* expression in differentiated primary osteoblasts (Fig. 5f). Thus, these results suggest that an adherent stromal cell producing CXCL12, distinct from the osteoblast, is targeted by the SNS in the bone marrow.

DISCUSSION

Our studies show that HSCs do not steadily or randomly circulate under homeostasis, but rather follow a physiologically regulated rhythmic release. Circadian HSC trafficking is orchestrated in the central nervous system by core genes of the molecular clock that regulate HSC attraction to their bone marrow niches by rhythmic secretion of noradrenaline from nerve terminals, activation of the β_3-adrenergic receptor, degradation of Sp1, and downregulation of *Cxcl12* (Fig. 5g). These studies show that the central nervous system can directly regulate the function of a stem cell niche in peripheral tissues.

Recent studies have suggested that under steady state, HSCs are associated with osteoblasts[13,14,35] and sinusoids[23,36] in the bone marrow. In both cases, HSCs have been localized near CXCL12-abundant reticular cells[36]. Further, $CD146^+$ human stromal cells, recently identified as precursors of both sinusoidal adventitial reticular cells and osteoblasts, may share similarities with mouse CXCL12-abundant reticular cells[37]. The term 'neuro-reticular complex' has been coined[38] to describe the rich association of efferent nerve terminals with sinus adventitial reticular and other stromal cells that are inter-connected by gap junctions around sinusoids of the BM. Taken in this context, our studies suggest that the SNS may regulate a stromal cell associated with the vasculature, possibly an osteoblast precursor, thereby controlling HSC release. Enhanced circulating stem cells in the resting period may constitute an essential component of the regenerative programme of the bone marrow niche itself, or potentially of other tissues by, for instance, the extramedullary restoration of specialized haematopoietic cells[39]. The clinical corollary of rhythmic HSC release implies significantly higher yields if stem cells were harvested at the acrophase.

METHODS SUMMARY

Mouse strains. *Bmal1*$^{-/-}$ (ref. 19) (gift from C. A. Bradfield), *Per1*$^{-/-}$*Per2*$^{m/m}$ (ref. 40), *Adrb2*^{tm1Bkk}/J (ref. 41) (gift from G. Karsenty), FVB/N-*Adrb3*^{tm1Lowl}/J (ref. 42) and 129S-*Gt(ROSA)26Sor*^{tm1Sor}/J (Jackson Laboratories) mice, FVB-Tg(*Col1a1-cre*)1Kry/Mmcd (ref. 43) (Mutant Mouse Regional Resource Center) and *Sele*$^{-/-}$*Selp*$^{-/-}$ double knockout mice[44] and the inbred FVB/NJ (Jackson Laboratories) and C57BL/6-CD45.1/2 (Charles River Laboratories) congenic strains were used in this study. Experimental procedures were approved by the Animal Care and Use Committee of Mount Sinai School of Medicine.

Cell culture. MS-5 cells were grown in monolayers in α-MEM medium supplemented with 10% FBS, 2 mM L-glutamine and 2 mM sodium pyruvate. ST-2 cells were grown in RPMI 1640 medium containing 10% FBS. MC3T3-E1 cells were grown in α-MEM medium supplemented with 10% FBS. Cultures were maintained with 1% penicillin–streptomycin (Invitrogen) at 37 °C in a water-jacketed incubator with 5% CO_2 and 1:10 split with 0.05% trypsin-EDTA (Invitrogen) every three or four days, when cells reached about 80% confluence. Cells in passages 3–15 were used in this study.

Flow cytometry. Blood was collected from the retro-orbital sinus and erythrocytes were lysed by incubation in 0.8% NH_4Cl. The number of LSK cells was determined by staining lineage antigens (anti-CD3e, anti-CD11b, anti-CD45R/B220, anti-Ly-6G, anti-Ly-6C and anti-TER119) with the Biotin-Conjugated Mouse Lineage Panel (BD Pharmingen), followed by streptavidin-AMCA (West Grove), Pacific Blue-conjugated anti-Sca1 (Biolegend) and PECy7-conjugated anti-c-Kit (eBioscience). Antibodies against CD45.1 and CD45.2 (eBioscience) were used to determine the percentage of engraftment after competitive bone marrow transplantation. Mobilization of progenitors[11], neurochemical sympathectomy of adult mice[11] and CFU-C assays[24] were performed as described previously. Primary myeloid[45] and osteoclast[46] cultures, CXCL12 ELISA[10], preparation of nuclear extracts[47] and unilateral denervation of the bone marrow[48] were performed as described previously, with some modifications detailed in the full Methods. Conventional western blot analysis was performed with an anti-Sp1 rabbit polyclonal antibody (Upstate) in accordance with the manufacturer's recommendations.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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METHODS

Long-term competitive repopulation assays. HSC activity was assessed by using competitive repopulation assays with the congenic Ly5.1/Ly5.2 system. Various blood volumes (5, 50, 100, 200, 400 and 600 µl) obtained from male B6/SJL (CD45.1) mice six to ten weeks old were mixed with 2×10^5 total bone marrow cells from female C57BL/6 (CD45.2) mice as competitors. Recipient C57BL/6 (CD45.2) mice (six to eight weeks old) were subjected to lethal irradiation (10 Gy) 3–5 h before transplantation. At various times (4, 8, 12 and 16 weeks) after transplantation, peripheral blood nucleated cells were collected from recipients by retro-orbital bleeding. Cells were stained and analysed by fluorescence-activated cell sorting. The percentage of recipients in each experimental group that failed reconstitution was plotted against the number of cells transplanted, and Poisson statistics were applied to estimate the stem cell frequency within the donor cell population.

Unilateral denervation of bone marrow. Under anaesthesia, the femoral and sciatic nerves of adult C57BL/6 mice were exposed and unilaterally sectioned with sterile microscissors close to their exit from the spinal chord, as described previously[48]. Sham surgery was performed on the contralateral side in which the nerves were exposed identically but were not sectioned. After the wound had been sutured, the animals were returned to their cages for eight days before being killed for the study.

***In vivo* injection of antibodies.** Rat monoclonal antibodies against P-selectin (clone RB40.34) and mouse α_4 integrins (clone PS/2) were purified from hybridoma supernatants and subjected to potential endotoxin decontamination with a polymyxin B column (Detoxi-Gel; Pierce Biotechnology Inc.). Rat anti–mouse E-selectin Ab (clone 9A9) was a gift from B. Wolitzky. Control rat IgG was obtained from Sigma. Animals were injected intravenously with 1 mg kg^{-1} antibody.

Differentiation of mouse bone marrow stromal cells into osteoblasts and osteoclasts. For differentiation into osteoblasts, the bone marrow of *Col1a1–LacZ* mice was flushed as described previously[45]. After the nucleated cell fraction had been counted, 5×10^5 and 10^6 bone marrow nucleated cells were seeded in six-well plates in 2 ml of red-phenol-free α-MEM medium supplemented with 20% FBS, 1% penicillin–streptomycin (Invitrogen) and 1 mM L-ascorbic acid 2-phosphate (Sigma) and cultured for four weeks at 37 °C under 5% CO_2 in a water-jacketed incubator. Half-medium changes were performed every five days. Cells were stained with the ImaGene Green C_{12}FDG *LacZ* Gene Expression Kit (Invitrogen) in accordance with the manufacturer's recommendations, and mature osteoblasts were sorted by *Col1a1–LacZ* expression with an automated cell sorter (InFlux). Osteoclast cultures were prepared as described previously[46], with some modifications. In brief, femora and tibiae of eight-week-old C57BL/6 mice were flushed with 15% FBS in α-MEM (Invitrogen), centrifuged and plated for 2 days at 37 °C under 5% CO_2 in a water-jacketed incubator in α-MEM supplemented with 10% FBS (Invitrogen) and 5 ng ml^{-1} recombinant murine macrophage colony-stimulating factor (M-CSF; Peprotech). Non-adherent cells were collected, filtered through 70-µm sterile nylon filters and a gradient centrifugation with Ficoll-Paque PLUS (Amersham Biosciences) was performed by following the manufacturer's recommendations. Mononuclear cells (250,000) were plated in each well of a 24-well BioCoat Osteologic Bone Cell Culture System (BD Biosciences) in α-MEM supplemented with 10% FBS (Invitrogen), 30 ng ml^{-1} recombinant murine tumour necrosis factor-α (TNF-α; Peprotech) and 60 ng ml^{-1} M-CSF and cultured at 37 °C under 5% CO_2 in a water-jacketed incubator. After one day, 100 ng ml^{-1} recombinant murine soluble receptor activator of NF-κB ligand (sRANKL; Peprotech) was added to the culture medium.

Immunohistochemistry. Anaesthetized animals were perfused through the left ventricle with 50 ml of PBS followed by 100 ml of freshly prepared cold 4% paraformaldehyde (Sigma). The femora and tibiae were postfixed for 2 h in 4% paraformaldehyde at 4 °C. After two washes in cold PBS, bones were decalcified for 48 h in 250 mM EDTA pH 7.4 at 4 °C in a shaker. Decalcified bones were cryoprotected, sectioned (10 µm) with a cryostat, mounted onto Poly-prep slides (Sigma) and kept at −80 °C. For immunohistochemistry, the slides were placed in Coplin jars (Sigma) and rinsed twice with PBS. Two additional washes with 0.1% Triton X-100 in PBS (PBTx; Sigma) were followed by incubation for 15 min with 0.3% Triton X-100 in PBS for cell permeabilization. The endogenous tissue peroxidase was blocked by incubating the sections for 2 h at 4 °C with 0.4% H_2O_2 in PBS. Non-specific binding of antibodies was blocked by leaving the sections for 2 h at 4 °C with blocking solution (10% goat serum (Chemicon), 1 mg ml^{-1} BSA (Sigma) in PBTx). The endogenous biotin was blocked by incubating the sections sequentially in avidin for 30 min and biotin (Vector Laboratories) for 30 min. A polyclonal rabbit anti-tyrosine hydroxylase (1:1,000 dilution; Chemicon) in blocking solution was applied overnight at 4 °C. A biotinylated goat anti-rabbit antibody (1:200 dilution; Pierce Biotechnology Inc.) in blocking solution was applied for 2 h. After being blocked for 1 h at 4 °C with 2 mg ml^{-1} BSA (Sigma) in PBS, sections were incubated with the ABC kit (Vector Laboratories) for 2 h. After washes, tissue sections were incubated for 8 min with fluorescein isothiocyanate-tyramide (PerkinElmer Life Sciences, Inc.) diluted 1:100 in amplification reagent, in accordance with the manufacturer's recommendations. Bone marrow autofluorescence was largely quenched by incubating the slides for 30 min at 20 °C with 0.1% Sudan Black B (Sigma) in 70% ethanol. Sections were mounted with Vectashield mounting medium with 4,6-diamidino-2-phenylindole (Vector Laboratories), analysed and photographed with a fluorescence microscope.

Nuclear extracts preparation. Nuclear extracts were prepared as described[47], with modifications. Cells in culture dishes were washed briefly in cold PBS, and detached by scraping. After centrifugation for 15 min at 300*g* and 4 °C, the pellets were resuspended in 100 µl of 1.5 mM $MgCl_2$, 10 mM KCl in 10 mM HEPES pH 7.9 and placed on ice for 5 min. After centrifugation for 40 s at 16,000*g*, the pellet was homogenized in 20 µl of 1.5 mM $MgCl_2$, 420 mM NaCl, 0.2 mM EDTA in 20 mM HEPES pH 7.9 at 4 °C with a Pellet Pestle Motor (Kontes Glass Co.) and placed on ice for 5 min. After centrifugation for 40 s at 16,000*g*, the supernatant was transferred to precooled Eppendorf tubes containing 20 µl of 1.5 mM KCl, 0.2 mM EDTA, 20% glycerol in 20 mM HEPES pH 7.9, and stored at −80 °C. All buffers contained 1 × protease inhibitors cocktail (Sigma).

RNA isolation, reverse transcription and quantitative real-time PCR (Q-PCR). Total RNA extraction with TRIzol (Invitrogen) followed by treatment with DNase to eliminate contaminating genomic DNA with RNase-free DNase Set (Qiagen), RNA clean-up with RNeasy mini kit (Qiagen) and conventional reverse transcription, using the Sprint PowerScript reverse transcriptase (Clontech), were performed in accordance with the manufacturers' instructions. Q-PCR was performed with SYBR GREEN on an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems). Primers were designed with the Primer Express software (Applied Biosystems) and when possible were selected to span introns to prevent the amplification of contaminating genomic DNA. A primer concentration of 300 nM was found to be optimal in all cases. The sequences of the oligonucleotides used are included in Supplementary Table 1. The PCR protocol consisted of one cycle at 95 °C (10 min) followed by 40 cycles of 95 °C (15 s) and 60 °C (1 min). A dissociation curve analysis was included after each experiment to confirm the presence of a single product and the absence of primer dimers. In addition, a standard curve with multiple concentrations of cDNAs was designed for each sample and pair of primers to ensure that the amplification efficiency was similar in the different samples. Expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was generally used as a standard. The average threshold cycle number (C_t) for each tested mRNA was used to quantify the relative expression of each gene: $2^{-[Ct(\text{gene}) - Ct(\text{GAPDH})]}$.