Cholinergic signals preserve haematopoietic stem cell quiescence during regenerative haematopoiesis

Claire Fielding1,2,3, Andrés García-García1,2,3, Claudia Korn1,2,3, Stephen Gadomski1,2,3,4,5, Zijian Fang1,2,3, Juan L. Reguera6, José A. Pérez-Simón5,6, Berthold Göttgens1,2 & Simón Méndez-Ferrer1,2,3,7,8✉

The sympathetic nervous system has been evolutionarily selected to respond to stress and activates haematopoietic stem cells via noradrenergic signals. However, the pathways preserving haematopoietic stem cell quiescence and maintenance under proliferative stress remain largely unknown. Here we found that cholinergic signals preserve haematopoietic stem cell quiescence in bone-associated (endosteal) bone marrow niches. Bone marrow cholinergic neural signals increase during stress haematopoiesis and are amplified through cholinergic osteoprogenitors. Lack of cholinergic innervation impairs balanced responses to chemotherapy or irradiation and reduces haematopoietic stem cell quiescence and self-renewal. Cholinergic signals activate α7 nicotinic receptor in bone marrow mesenchymal stromal cells leading to increased CXCL12 expression and haematopoietic stem cell quiescence. Consequently, nicotine exposure increases endosteal haematopoietic stem cell quiescence in vivo and impairs hematopoietic regeneration after haematopoietic stem cell transplantation in mice. In humans, smoking history is associated with delayed normalisation of platelet counts after allogeneic haematopoietic stem cell transplantation. These results suggest that cholinergic signals preserve stem cell quiescence under proliferative stress.
Hematopoietic stem cell transplantation (HSCT) is routinely performed to regenerate the hematopoietic and immune systems of patients with cancer, immune or metabolic disorders. However, hematopoietic stem cell (HSC) responses are heterogeneous, but the underlying mechanisms are not yet clear. HSCs have reduced quiescence and cannot self-renew in peripheral blood, compared with bone marrow (BM). Sympathetic noradrenergic fibers innervate the BM and regulate traffic and activation of HSCs and leukocytes. Additionally, stress-induced noradrenergic activity increases HSC proliferation. However, preserving HSC quiescence is critical to prevent HSC attrition, but the mechanisms regulating HSC quiescence under stress are understudied. Cholinergic signals cooperate with noradrenergic signals to regulate HSC and leucocyte migration. However, whether cholinergic signals regulate HSC proliferation has not been described. Noradrenergic signaling and regulatory T cells (Tregs) are generally postganglionic neurons in the sympathetic nervous system (SNS) and (cholinergic) parasympathetic nervous system, respectively. However, some sympathetic neurons convert to cholinergic phenotype during postnatal development in sweat glands and the periosteum. Furthermore, the function of sympathetic cholinergic fibers in bone has remained unknown.

Here, we show the source and function of cholinergic fibers during regenerative hematopoiesis. Cholinergic-neural signals activate bone-associated nestin+ BM mesenchymal stem cells (BMSCs) and regulate HSC quiescence locally in endosteal BM niches. These results illustrate the regulation of stem cell quiescence by the cholinergic system. This mechanism seems to allow stem cells to meet physiological demands and respond to stress, without losing potency.

**Results**

**Cholinergic expansion during stress hematopoiesis.** The autonomic nervous system has been evolutionarily selected to efficiently respond to stress. Therefore, we have studied the role of sympathetic cholinergic signals during stress hematopoiesis. WT mice underwent BM transplantation (BMT) following lethal irradiation. BM ACh concentration transiently increased 2 weeks after BMT (Fig. 1a), suggesting a role for cholinergic signals during stress hematopoiesis. To investigate the source of BM ACh, we performed genetic lineage tracing of neural cells in Wnt1-Cre; mice and cholinergic cells using ChAT-IRES-Cre and ChAT-Gfp mice. Matching the transient ACh increase, cholinergic nerve fibers peaked 2 weeks after transplantation (Fig. 1b and Supplementary Fig. 1a). Signalling through the GDNF family receptor alpha-2 (GFRα2) promotes the survival of cholinergic neurons. Matching the transient increase in cholinergic innervation, BM GFRα2 mRNA expression increased 9-fold 2 weeks after transplantation (Fig. 1c). In sharp contrast, BM tyrosine hydroxylase (TH)+ noradrenergic fibers decreased steadily over 4 weeks (Fig. 1d and Supplementary Fig. 1b). In a separate study, we found that cholinergic neural signals are transmitted in the skeletal system through cholinergic osteoprogenitors. Therefore, we measured ChAT+ osteoprogenitors and found them similarly expanded 2 weeks after irradiation (Fig. 1e–h and Supplementary Fig. 1c–e). These results suggest that both neural and non-neuronal BM cholinergic signals increase during hematopoietic regeneration.

**Cholinergic regulation of HSC quiescence.** Since GFRα2 is required for the survival of cholinergic neurons, we used Gfra2−/− mice as a model to study the cholinergic regulation of

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**Fig. 1 Cholinergic expansion during hematopoietic regeneration.** a, c Acetylcholine (ACh) content (a, N = 5,9,14; P = 0.03) and Gfra2 mRNA expression (c, N = 5,4,7; P = 0.002,0.0008) in endosteal BM before or 2-4 weeks after transplantation. b, d Density of (b) cholinergic (GFRα2+; N = 4,5,9; P = 0.003,0.02) or (d) noradrenergic (tyrosine hydroxylase, TH+; N = 4,8,6; P = 0.01) genetically marked nerve fibers in Wnt1-Cre;AlI4D mice. e, f Representative genetic tracing showing the expansion of cholinergic stromal cells (red) adjacent to Nes-GFP+ BMSCs (green) associated with CD31+ or endomucin (EMCN)+ blood vessels (white) near the growth plate of ChAT-IRES-Cre;AlI4D;Nes-GFP tibias (N = 3). Nuclei are counterstained with DAPI (blue). Scale, 50 µm. g, h Transient expansion of endosteal BM cholinergic PDGFRα+;Scal− skeletal stem cells (SSC), PDGFRα+;Scal−POPs, PDGFRα+;CD51+Scal− bone-lining osteoprogenitors (OPCs) or PDGFRα+;CD51+Scal− osteoblast precursors (OBPs) genetically traced in (g) ChAT-Gfp mice (0 weeks, SSC, PaS, N = 9, OPC, OBP, N = 4; 2 weeks, SSC, PaS, N = 8, OPC, N = 7, OBP, N = 8; 4 weeks, SSC, PaS, OPC, OBP, N = 8) or (h) ChAT-Ires-Cre mice (0 and 4 weeks, N = 2; weeks, N = 5). a, c, g, h Each dot is a mouse. Data are mean of biological replicates ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. ANOVA and Tukey’s multiple comparisons test.
HSCs. First, we analysed HSC proliferation separately in the endosteal or central BM of Gfra2−/− mice and WT mice (Supplementary Fig. 2a). In agreement with a previous study14, BM lin−/scai1−/ckit+ (LSK) CD150+/CD48− HSCs were >5-fold more abundant in the central WT BM, which contained 7-fold more nucleated cells. However, the frequency of quiescent HSCs was 4-fold higher in the endosteal WT BM. Notably, Gfra2−/− mice showed 5-fold-reduced frequency of quiescent HSCs in the endosteal BM, whereas HSC proliferation remained unchanged in the central BM (Fig. 2a, b). An identical result was obtained using a different marker combination (LSK Flt3−) to label HSCs (Fig. 2c).

We have previously shown that decreased parasympathetic activity in Gfra2−/− mice derepresses sympathetic activity and causes abnormal BM egress of HSCs and leukocytes via sympathetic activation of the β3-adrenergic receptor9 (encoded by the Adrb3 gene). To investigate the possible contribution of the noradrenergic system to decreased HSC quiescence in the endosteal BM of Gfra2−/− mice, we intercrossed these mice with Adrb3−/− mice. Unlike HSC mobilisation9, increased endosteal HSC proliferation was not the consequence of derepressed noradrenergic activity in Gfra2−/− BM because it was not rescued in Gfra2−/−/Adrb3−/− compound mice (Fig. 2c). These results suggest that cholinergic signals inhibit HSC proliferation locally in the endosteal BM niche.

**Niche α7nAChR promotes HSC quiescence.** GFRα2 has been shown to promote HSC self-renewal and ex vivo expansion through its co-receptor RET expressed in HSCs15,16, but its role during regenerative haematopoiesis is unclear. Furthermore, GFRα2 protein was detected on the membrane surface of some (~5%) mature haematopoietic cells but was not detectable in immunophenotypically defined HSCs (Supplementary Fig. 2b, c). To clearly dissect haematopoietic-cell-autonomous and HSC-extrinsic regulation in Gfra2−/− mice, we generated chimeric mice through long-term transplantsations of BM cells into lethally irradiated recipients. The decreased quiescence of endosteal HSCs in Gfra2−/− mice (see Fig. 2c) was not observed in WT mice carrying Gfra2−/− haematopoietic cells (Supplementary Fig. 2d, e), suggesting that HSC quiescence is extrinsically regulated by cholinergic signals through the microenvironment.

The chemokine CXCL12/SDF1α produced by non-haematopoietic niche cells regulates HSC quiescence17,18. We measured CXCL12 concentration in long-term BM chimeras using Gfra2−/− or control Gfra2+/− mice as donors or recipients to discriminate haematopoietic-cell-autonomous from niche regulation. In agreement with disrupted cholinergic regulation of the BM microenvironment in Gfra2−/− mice, only Gfra2−/− recipient mice (but not WT recipients of Gfra2−/− donor haematopoietic cells) exhibited a 35% reduction in BM CXCL12 concentration (Fig. 3a).

CXCL12 is highly produced by HSC niche-forming BMSCs marked by the regulatory elements of Nestin (Nes)19. Therefore, we intercrossed Gfra2−/− mice with Nes-gfp mice and isolated endosteal and central (see Supplementary Fig. 2a) BM CD45−/Ter119−/CD31−/Nes-GFP+ cells to investigate their possible regulation by cholinergic signals. Cxcl12 mRNA expression was 2.5-fold higher in endosteal (vs. central) Nes-GFP+ cells to investigate their possible regulation by cholinergic signals. Cxcl12 mRNA expression was 2.5-fold higher in endosteal (vs. central) Nes-GFP+ cells, but not in Gfra2−/− mice. This was due to 3-fold-reduced Cxcl12 mRNA expression in endosteal Nes-GFP+ cells from Gfra2−/− mice (Fig. 3b). In Gfra2−/− mice, Cxcl12 expression was specifically deregulated in Nes-GFP+ cells (Fig. 3c), which reside in the endosteal BM9 and promote HSC quiescence20.

To further investigate how cholinergic signals regulate CXCL12, we treated WT mice with cholinergic antagonists selective for nicotinic or muscarinic receptors. Only the nicotinic antagonists decreased BM CXCL12 content by 40% (Fig. 3d), suggesting that cholinergic signals are transduced by nicotinic receptors in the BM microenvironment. Therefore, we treated different BM stromal cells with acetylcholine or nicotine (Supplementary Fig. 3a). CXCL12 production was highest in MS-5 cells (resembling nestin+ BMSCs) and was equally induced by ACh and nicotine, but was not affected by the muscarinic receptor antagonist atropine (Fig. 3e; Supplementary Fig. 3b). These results are consistent with more abundant expression of nicotinic receptors than muscarinic receptors in Nes-GFP+ BMSCs19. Among nicotinic receptors, mRNA expression of α7 (Chnra7) was high in Nes-GFP+ cells (Supplementary Fig. 3c). Moreover, CXCL12 secretion was 5-fold lower in primary BM cultures from Gfra2−/− mice or Chnra7−/− mice (Fig. 3f), suggesting that cholinergic signals regulate BM CXCL12 expression through the α7 nicotinic ACh receptor (α7nAChR). Supporting this possibility, CXCL12 was 3-fold lower in the BM extracellular fluid from Chnra7−/− mice (Fig. 3g). Together, these results suggest that cholinergic signals induce CXCL12 expression via α7nAChR in niche cells.
Preserved HSC quiescence after chemotherapy. Since CXCL12 is essential to preserve HSC quiescence\textsuperscript{17,18}, we asked whether its regulation by cholinergic signals might affect HSC quiescence and activation during emergency haematopoiesis. 5-Fluorouracil (5-FU) is commonly used to treat cancer since it triggers apoptosis of proliferative cells but spares quiescent cells\textsuperscript{21}. BM Gfra2 and its ligand neurturin (Nrtn) mRNA expression increased 7 days after 5-FU (Fig. 3h, i), resembling the NRTN-GFR\textalpha\textsubscript{2} induction after BMT. Gfra2\textsuperscript{−/−} mice and Chrna7\textsuperscript{−/−} mice exhibited a decreased ability to regulate haematopoietic recovery and fine-tune immune responses acutely after 5-FU (14–18d); in contrast, haematopoiesis normalised in these mice after 20d, when stress haematopoiesis reverted to homeostasis (Fig. 3j), underscoring the role of cholinergic signals during stress haematopoiesis.

To confirm that cholinergic niche regulation is required to preserve HSC quiescence under proliferative stress, Gfra2\textsuperscript{−/−} or control donor/recipient mice were transplanted with 500 endosteal HSCs and 10\textsuperscript{5} congenic BM helper cells; 4 weeks after transplantation, HSC quiescence was compromised only in the cholinergic-neural-deficient endosteal BM (Fig. 3k and Supplementary Fig. 4).
Furthermore, reduced CXCL12 in Chrna7−/− BM (Fig. 3f, g) correlated with reduced endosteal HSC quiescence in Chrna7−/− BM (Fig. 3i), phenocopying Gfra2−/− mice (Fig. 2c). Importantly, α7nAChR deletion in Leptin-receptor-Cre-targeted HSC niche cells22, which overlap with Nes-GFP+ BMSCs23,24, or in Nes-CreERT2 targeted HSC niche cells, similarly increased endosteal (not central) BM HSC proliferation (Fig. 3m and Supplementary Fig. 5). These results suggest that ACh limits HSC proliferation by inducing CXCL12 expression in endosteal BMSCs via α7nAChR.

HSCs cycling in cholinergic-deficient niche. To evaluate the functional consequences of decreased HSC quiescence in the cholinergic-neural-deficient niche, we performed HSC long-term competitive repopulation assays using Gfra2−/− or control mice as donors or recipients. Consistent with increased WT HSC proliferation in cholinergic-deficient niches and with its normalisation in WT niches (see Fig. 3k), multilineage haematopoietic reconstitution from Gfra2−/− HSCs was unchanged, and that from WT HSCs remained high, 4–16 weeks after transplantation into Gfra2−/− mice (Fig. 4 and Supplementary Fig. 6). This was opposite to, and not explained by, the competitive disadvantage of CD45.1+ (compared with CD45.2+) cells25 (Fig. 4, blue lines).

Reduced HSC self-renewal in cholinergic-deficient niche. To determine whether decreased HSC quiescence in Gfra2−/− mice compromises the HSC self-renewing programme, we compared the gene expression profiles (RNA-Seq) of endosteal and central HSCs isolated from WT mice and Gfra2−/− mice. Notably, gene set enrichment analysis (GSEA) showed that central (compared with endosteal) BM WT HSCs exhibited increased ribosomal, mitochondriand and GFRα1-related pathways (Fig. 5a, Supplementary Fig. 7a–d and Supplementary Data 1), suggesting a higher activation and different response to GFR signalling. Furthermore, the expression of target genes of the GFRα1/2 co-receptor RET, which promotes HSC self-renewal and expansion15,16, was reduced in HSCs from Gfra2−/− mice (Fig. 5b). In contrast, myc-related glycolysis and Notch1-dependent pathways, which regulate HSC maintenance26,27 and GFRα1/2-dependent, but RET-independent, maintenance of cardiac progenitors38, were enriched in endosteal (compared with central) WT HSCs (Supplementary Fig. 7e, f). Similarly, neurite outgrowth-related pathways and interleukin-6 (IL-6)-dependent transcription were reduced in HSCs from Gfra2−/− mice (Supplementary Fig. 7g, h). In contrast, targets co-activated by Notch1 and Myc39 were enriched in endosteal (compared with central) HSCs from WT, but not in Gfra2−/− mice (Fig. 5c and Supplementary Data 2). Matching cell cycle, GSEA revealed an abnormal upregulation of proliferation-associated gene sets in HSCs from endosteal Gfra2−/− BM. Similarly, mRNA levels of genes that are highly expressed in mobilized HSCs30 were increased in the endosteal HSCs from Gfra2−/− mice. In contrast, gene signatures associated with the most primitive long-term- (LT-) HSCs30 and the HSC-fingerprint31 were decreased in endosteal HSCs, compared with HSCs from central Gfra2−/− BM (Fig. 5d, Supplementary Fig. 7i and Supplementary Data 1–2). To investigate the impact of the cholinergic regulation of HSCs on their self-renewal ability, 500 donor-derived HSCs were isolated from the primary recipient mice and were retransplanted (together with congenic helper BM cells) into lethally irradiated secondary recipients of the same genetic background as the primary recipient mice (Fig. 6a). Contrasting their normal reconstitution capacity in primary recipients (see Fig. 4b–g) and consistent with their reduced self-renewing gene programme (see Fig. 5d), endosteal HSCs from Gfra2−/− mice failed to reconstitute secondary recipients (Fig. 6b–g). Notably, WT HSCs continued to yield increased reconstitution shortly (4w) after secondary transplantation into Gfra2−/− mice; however, by 8 weeks their reconstitution capacity dropped sharply as a sign of decreased self-renewal or premature exhaustion (Fig. 6b–g). Altogether, these results suggest that cholinergic signals preserve HSC quiescence and self-renewal in the endosteal BM niche under proliferative stress.

Nicotine impairs haematopoietic regeneration. Since the cholinergic HSC regulation is transduced by nicotinic receptors in the niche (Fig. 3d–m and Supplementary Fig. 5), to complement the loss-of-function models, we treated mice with nicotine, as gain-of-function. A 3-day nicotine treatment increased quiescent endosteal BM HSCs (Supplementary Fig. 8). Therefore, transplanted mice were treated with nicotine or vehicle over 7 weeks (Fig. 7a). Decreased blood multilineage reconstitution in nicotine-treated mice (Supplementary Fig. 9a–h) was explained by decreased donor-derived chimerism in peripheral blood, persisting 7 weeks after transplantation (Fig. 7b–d). This was likely caused by increased quiescence of transplanted HSCs (Fig. 7e and Supplementary Fig. 9i). As a complementary loss-of-function model, transplanted mice were treated with a nicotinic receptor antagonist (Fig. 8a). Nicotinic receptor blockade increased BM HSC proliferation after transplantation (Fig. 8b, c). These results suggest that nicotinic signalling reduces HSC proliferation and haematopoietic reconstitution after transplantation.
significantly delayed in previous smokers (Fig. 9), independently of the type of donor (matched family vs. alternative donor) and the number of CD34+ cells infused, which also influenced platelet reconstitution kinetics, as expected. Since increased transplant-related mortality has been noted in smokers treated for chronic myeloid leukaemia, these results suggest that cholinergic nicotinic signalling might impact clinical haematopoietic recovery after transplantation.

**Discussion**

This study shows that cholinergic signals contribute to preserve HSC quiescence in endosteal BM niches. Cholinergic signalling increases in these niches upon haematopoietic stress (myeloablation, irradiation) and preserves HSC quiescence under proliferative stress, thereby helping protect HSCs from exhaustion.

Daily release of noradrenaline by sympathetic fibres innervating the BM activates the β3-adrenergic receptor and reduces CXCL12 expression, permitting HSC egress to the circulation. Moreover, the SNS-β3-adrenergic-receptor-CXCL12 axis causes HSC mobilisation and correlates with HSC hyperproliferation under chronic stress. However, preserving HSC quiescence under stress is critical to prevent HSC attrition, but the underlying mechanisms remain largely unknown. Besides noradrenergic fibres, cholinergic nerve fibres have been found in periosteal regions and inside the BM during postnatal stages, but their function had remained elusive. We recently demonstrated that dual cholinergic signals (acting centrally and peripherally) regulate HSC traffic between the BM and the bloodstream, but whether cholinergic signals regulate HSC quiescence, which is essential to preserve HSC self-renewal, was unknown. Non-myelinating Schwann cells associated with peripheral nerves reduce HSC proliferation, but it remained unclear whether and how different neurotransmitters released by these nerve fibres, and particularly acetylcholine (ACh), regulate HSC quiescence.

The current study expands the cholinergic regulation of HSC and leucocyte traffic to the preservation of HSC quiescence and self-renewal under proliferative stress. The results suggest that the sympathetic nervous system (SNS) can simultaneously promote two opposing processes (stem cell activation/migration and induction of quiescence) through different neurotransmitters in separate BM niches. In this model, a stress-response system (SNS) could simultaneously trigger migration of activated stem cells in one niche (through noradrenaline), but at the same time protect the stem pool from exhaustion by promoting quiescence in another niche (through acetylcholine). This data illustrates a gatekeeper mechanism that can simultaneously trigger migration of activated stem cells in one niche, but at the same time protect
The observed endosteal and central HSC distribution is in agreement with previous studies showing that the majority of HSCs are located in the perisinusoidal (central) niche. However, consistently with our work, many studies highlight the presence of a smaller subset of HSCs associated with bone surfaces (endosteal niches), which were initially proposed to harbour quiescent HSCs that expand to regenerate the damaged BM. Our results support these contentions and more recent descriptions of low-permeability blood vessels (including endosteal arterioles and capillaries named “transition zone vessels”) that serve as specialised niches for quiescent HSCs, whilst highly permeable sinusoids allow for HSC activation and trafficking. We speculate that dynamic regulations in these two niches might regulate the reversible switch between dormant and activated HSCs based on physiological requirements (homeostasis vs stress). Our findings reveal Nes-GFP LepR-Cre-targeted BMSCs as a target for cholinergic HSC regulation in the endosteal BM niche. CXCL12 levels in Nes-GFP cells associated with decreased HSC quiescence in the endosteal BM of cholinergic-neuronal-deficient mice. Since CXCL12 is essential to preserve HSC quiescence and our data shows that nicotine induces CXCL12 secretion by BM stromal cells, this appears to be one mechanism of cholinergic regulation of HSC quiescence through the niche. Nes-GFP cells have been previously associated with HSC quiescence in arteriolar niches and this might also be the case for endosteal transition zone vessels. It is possible that other stromal cells expressing cholinergic receptors, such as endothelial cells, and monocytes/macrophages might be targeted by this cholinergic regulation. Therefore, future studies should elucidate the possible regulation of other HSC niche cells by cholinergic signals.

Previous studies have suggested the expression of cholinergic nicotinic receptors in HSPCs and their possible activation by nicotine. Specifically, α7nAChR has been proposed to mark HSPCs with a prominent role during inflammatory responses. We have shown that cholinergic signals activate α7nAChR and induce CXCL12 expression in endosteal BMSCs as one mechanism promoting HSC quiescence. Indeed, our results show that α7nAChR deletion in LepR-Cre-targeted SSC-enriched cells, which overlap with Nes-GFP cells, or in Nes-CreERT2, targeted HSC niche cells both increase endosteal (not central) BM HSC proliferation. Consequently, nicotine exposure increases endosteal HSC quiescence in vivo and impairs haematopoietic regeneration after HSC transplantation in mice. Our retrospective analysis of platelet recovery after clinical allogeneic HSC transplantation shows a significant delay in the normalisation of platelets in smokers, compared with non-smokers, independently of the stem cell dose and type of donor. Although other factors could contribute to delayed haematopoietic reconstitution in smokers, our data suggest that the cholinergic nicotinic signalling might affect clinical haematopoietic recovery after transplantation. These data are consistent with the reportedly inhibitory effects of nicotine during in vivo haematopoiesis and in vitro megakaryopoiesis, and suggest that the cholinergic nicotinic signalling might affect clinical haematopoietic recovery after transplantation. Our data might relate to the increased transplant-related mortality previously noted in high-dose smokers who received HSC transplantation for the treatment of chronic myeloid leukaemia.

In summary, these results suggest that the cholinergic nicotinic regulation of the endosteal HSC niche preserves HSC quiescence under haematopoietic proliferative stress. They also reveal how adult stem cells can be regulated by different signals from the autonomic nervous system to meet physiological demands at various sites and time, and to efficiently respond to stress without being exhausted.

The stem pool from exhaustion by promoting quiescence in another niche (Fig. 10).

Importantly, the expression of Gfra2 and its ligand Nrtnt, and the presence of cholinergic (but not noradrenergic) fibres increases in the BM under haematopoietic stress induced by myeloablation with 5-FU or after irradiation. Therefore, increased cholinergic nicotinic signalling in endosteal BM niches preserves HSC quiescence during emergency haematopoiesis.

Three independent experimental paradigms of our study (acute myelosuppression in two independent cholinergic-deficient lines, Gfra2−/− and Chrna7−/− mice, and BM reconstitution following lethal irradiation) convey the same message—increased HSC proliferation when cholinergic signals are impaired in the BM niche. However, this decreased HSC quiescence is associated with reduced self-renewing gene programme and diminished self-renewal in serial HSC transplantations. Decreased HSC quiescence in Gfra2−/− mice can be transiently reverted when the haematopoietic cells are transplanted in a WT microenvironment, although HSCs from Gfra2−/− mice fail to reconstitute secondary recipients. Conversely, the Gfra2−/− microenvironment decreases WT HSC quiescence and self-renewal in serial HSC transplantations. Altogether, these data points towards the cholinergic regulation of the HSC niche—rather than HSCs themselves—as a gatekeeper mechanism to protect HSC quiescence under stress.
Methods

Human study design. Patients provided written consent before transplant for the collection and analysis of anonymised data, which were maintained in the Hospital Animals. Age and sex-matched Gfra2−/−REF2, Nes-gfp61 (generously provided by G.E. Enikolopov), FVB/N-Adh6tm1Low/J (Stock number 086402), B6.129 x 1-NetteGma1Bos/J (Stock number 012238), B6.12957-Ctmm1BosJf1 (Stock number 003323), ChatBAC-eGFP (Stock number 007902), a7mAChRflx (Stock number 026965), B6.129(Gt)-Lprm2(cre)Rck/J (Stock number 088320), NescreERT2(REF12) (generously provided by G. Fishell), B6.Cg-Comm1D10Tg(Vav1-icre)IRES-Cre2 (generously provided by G. Fishell), B6.129S-Chattm1(cre)Lowl/MwarJ (mouse number 031661), B6.129S7-Chrna7tm1Bay/J (mouse number 007902), and congenic CD45.1 and CD45.2 C57BL/6 J mice (Charles River) were used in this study. For genetic lineage tracing, B6.C57Gt(ROSA)26Sortm14(CAG-tdTomato)Hze/J (Al24ID) reporter mice (Stock number 008610) (Jackson Laboratories), androgenic CD45.1 and CD45.2 C57BL/6 J mice (Charles River) were used in this study. For genetic lineage tracing, mice were housed in specific pathogen-free facilities. All experiments using mice followed protocols approved by the Animal Welfare Ethics Committees at the University of Cambridge (PPL 70/8406 and PPL 02428783). All experiments were compliant with EU recommendations.

Cell culture. MS-5 cells (DSMZ, ACC 441), a stromal cell line established by irradiation of adherent cells in long-term bone marrow cultures63, were grown in monolayers in α-MEM supplemented with 10% FBS, 2 mM l-glutamine and 2 mM sodium pyruvate (Invitrogen). ST-2 cells, another stromal cell line established from Whitlock-Witte type long-term bone marrow cultures64, were grown in RPMI 1640 medium containing 10% FBS. MC3T3-E1 cells, fibroblastic cell line established from the skull of an embryo/foetus C57BL/6 mouse65, were purchased from ATCC. MLO-Y4 cells, a broblastic cell line established from the skull of an embryo/foetus C57BL/6 mouse65, were purchased from ATCC (generously provided by G. Fishell), B6.Cg-Comm1D10Tg(Vav1-icre)IRES-Cre2 (generously provided by G. Fishell), B6.129S-Chattm1(cre)Lowl/MwarJ (mouse number 031661), B6.129S7-Chrna7tm1Bay/J (mouse number 007902), and congenic CD45.1 and CD45.2 C57BL/6 J mice (Charles River) were used in this study. For genetic lineage tracing, B6.C57Gt(ROSA)26Sortm14(CAG-tdTomato)Hze/J (Al24ID) reporter mice (Stock number 008610) (Jackson Laboratories), androgenic CD45.1 and CD45.2 C57BL/6 J mice (Charles River) were used in this study. For genetic lineage tracing, mice were housed in specific pathogen-free facilities. All experiments using mice followed protocols approved by the Animal Welfare Ethics Committees at the University of Cambridge (PPL 70/8406 and PPL 02428783). All experiments were compliant with EU recommendations.

BM extraction, flow cytometry and fluorescence-activated cell sorting. For BM hematopoietic cell isolation, bones were crushed in a mortar and filtered through a 40-μm strainer to obtain single-cell suspensions. Tissues were depleted of red blood cells by commercial lysis (Biolegend, 420301) for 8 min at 4 °C. Blood samples were directly lysed.

Cells were incubated with the appropriate dilution (2–5 μg/ml) of fluorescent antibody conjugates and 4,6-diamidino-2-phenylindole (DAPI, 1:2000) for dead cell exclusion, and analysed on LSRFortessa flow cytometer (BD Biosciences, Franklin Lakes, NJ) equipped with FACSDiva Software (BD Biosciences). The following antibodies were used: Rat Anti-Mouse CD45R/B220 (Clone RA3-6B2, BD Biosciences, 553088), Ckit (Clone 2B8, BioLegend, 105825), Biotin Mouse Human/Mouse GFR alpha-2/GDNF R alpha-2 Antibody (Bio-Techne, AF429), CD45.1 (Clone A20, InSight Biotechnology, 60-0453-U100), CD45.2 (Clone 104, BioLegend UK Ltd, 109823), Sca1 (Clone D7, Biolegend, 108114), Goat anti-rabbit

Cell culture. MS-5 cells (DSMZ, ACC 441), a stromal cell line established by irradiation of adherent cells in long-term bone marrow cultures63, were grown in monolayers in α-MEM supplemented with 10% FBS, 2 mM l-glutamine and 2 mM sodium pyruvate (Invitrogen). ST-2 cells, another stromal cell line established from Whitlock-Witte type long-term bone marrow cultures64, were grown in RPMI 1640 medium containing 10% FBS. MC3T3-E1 cells, fibroblastic cell line established from the skull of an embryo/foetus C57BL/6 mouse65, were purchased from ATCC and grown in α-MEM medium supplemented with 10% FBS. MLO-Y4 cells, a broblastic cell line established from the skull of an embryo/foetus C57BL/6 mouse65, were purchased from ATCC.

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AF488 (Thermo Fisher Scientific, A-11008), Scagliola 1 (Clone E13-161.7, BioLegend UK Ltd, 108113), CD34 (Clone HM34, BioLegend UK Ltd, 128603), CD350 (Clone TC15-12F12.2, BioLegend UK Ltd, 518989), CD48 (Clone HM48-1, BioLegend UK Ltd, 103439), CD41 (Clone MWReg30, BD Biosciences, 561850), CD49b (Clone HMa2, BD Biosciences, 558759), CD150 (Clone TC15-12F12.2, BioLegend UK Ltd, 115909), CD48 (Clone HM48-1, BioLegend UK Ltd, 103439), CD41 (Clone MWReg30, BD Biosciences, 561850), CD49b (Clone HMa2, BD Biosciences, 558759), CD135 (Clone A2F10, BioLegend UK Ltd, 103307), Flt3 (Clone A2F10.1, BD Biosciences, 560718), Rabbit anti-kbeck67 (Abcam, AB15580), CD31 (Clone MEC 13.3, BD Biosciences, 553371), Hoechst 33342 Solution (20 mM) (Thermo Fisher Scientific, 62249), Dylight 650 Donkey anti-rat (Thermo Fisher Scientific, SA5-10029), Ly-6G/Ly-6C (Gr1) (Clone HK1.4, BioLegend, 108411), CD11b (Clone M1/70, Biolegend, 101207), CD3e (Clone 145-2C11, Biolegend, 100349). Biotinylated antibodies were detected with fluorochrome-conjugated streptavidin (BD Biosciences, 554061). All antibodies were used at 1:200 except for biotinylated lineage antibody mix and rabbit anti-kbeck67 at 1:100, cells incubated in 300 ul for staining.

We isolated HSPCs from bone-associated and non-associated marrow fractions as previously described45. Briefly, long bones were flushed gently to obtain hematopoietic cells of the endosteal compartment. Cells were stained with the above-mentioned antibodies and analysed by flow cytometry or sorted (FACS Aria cell sorter, BD Biosciences). HSPCs were immunophenotypically defined as lin− c-kit+/Sca1− CD34− CD48− CD49b− CD150−. Cells were then enriched by using a FACS Aria cell sorter, BD Biosciences. HSPCs were immunophenotypically defined as lin− c-kit+/Sca1− CD34− CD48− CD49b− CD150− cells.

To isolate nestin− cells, bones were cleaned off surrounding tissue, crushed in a mortar with a pestle, and digested with collagenase (catalogue number C2674, Sigma; 0.25% collagenase in PBS supplemented with 20% foetal bovine serum) in water bath at 37 °C for 30 min with agitation. Cells were filtered through a 40-µm strainer and erythrocytes were lysed as described above. The resulting BM-enriched cell suspensions were pelleted, washed and resuspended in PBS containing 2% FCS for further analyses. BM stromal CD45− (30-F11) CD31− (MEC 13.3) Ter119− were further purified according to GFP fluorescence using an LSRFortessa flow cytometer (BD Biosciences) for immunophenotypic analysis, or a BD FACS Aria or BD Influx Sorter (BD Biosciences) for cell sorting.

To separate endosteal and non-endosteal nestin− cells, we gently flushed the long bones as described above. We digested both the flushed fraction and the remaining bone samples with collagenase (catalogue number C2674, Sigma; 0.25% collagenase in PBS supplemented with 20% foetal bovine serum) in a water bath at 37 °C for 30 min with agitation.

Cell cycle analysis was performed through Hoechst 33342 (H42)/ PironinY (PY) staining as described or through Hoescht 33342 (H42)/ ki67. Briefly, for Hoescht 33342 (H42)/ PironinY (PY) staining, sorted lin− c-kit+/Sca1−/fltuorochrome− cells were collected in a-MEM medium supplemented with 2% foetal calf serum and 10 mM Hepes. Cells were incubated with H42 (5 µg/ml) 35 min in a-MEM medium supplemented with 2% foetal calf serum, 10 mM Hepes and 50 µM verapamil at 37 °C. PY (1 µg/ml) was then added and cells were incubated for 20 min at 37 °C. Finally, cells were resuspended in a medium with 50 µM verapamil and analysed by flow cytometry. For Hoescht 33342 (H42)/ki67 method, single cell suspensions were obtained and stained for cell surface markers and fixed Cytofix/Cytoperm (BD Biosciences, 554714) for 10 min at RT. Cells were then washed in Perm/Wash (BD Biosciences, 554714) and resuspended in ki67 Abs (Abcam, AB15580, 1:100) for a minimum of 45 mins at 4 °C or overnight. Cells were washed and resuspended in goat anti-rabbit AF488 (Thermo Fisher Scientific, A-11008) at 20 mins on ice and subsequently in Hoescht 1:2000 for 5 mins at 4 °C. A final wash was carried out and cells were resuspended in 300 µl Perm/Wash for analysis.

**Long-term competitive repopulation assay.** To assess endosteal HSCs, 500 sorted HSCs (Lin− Sca1− c-kit+/CD34− CD150− /Gfra2−/−, Gfra2−/− (CD45.2) or congenic CD45.1 mice isolated from flushed-flushed bones as donor cells together with either 105 CD45.1− or CD45.2− competitor bone marrow nucleated cells. In secondary transplantations, 500 sorted HSCs (Lin− Sca1− c-kit+/CD34− CD150− /Gfra2−/− isolated from flushed-flushed bones from the primary recipients and sorted and used as donor cells along with either 105 CD45.1− or CD45.2− competitor bone marrow nucleated cells. All recipient mice were split dose irradiated with 12 Gy. Hematopoietic reconstitution was assessed in the peripheral blood of recipient mice several times after transplantation by measuring blood CD45.2 or CD45.1 chimerism in the different mature hematopoietic lineages: B cells (B220+), T cells (CD3+), monocytes (CD11b+ Gr1−), eosinophils (CD11b+ Gr1+), and neutrophils (CD11b+ Gr1+).
Fig. 8 Nicotinic receptor blockade reduces HSC quiescence after transplantation. 

a Scheme illustrating the treatment of sub-lethally irradiated WT CD45.2 recipients of $2 \times 10^6$ BM nucleated cells from CD45.1 mice, treated with Hexamethonium bromide (20 mg/kg/d over 30d, i.p.) or vehicle. 

b Cell cycle profiles of donor-derived (CD45.1+) lin<sup>-</sup>scAl<sup>+</sup>ckit<sup>+</sup> (LSK) CD34<sup>-</sup>flt3<sup>-</sup> HSCs isolated from the endosteal or central BM 30 days after transplantation and chronic treatment with hexamethonium bromide or vehicle ($N = 5; p = 0.002,005$). Data are mean of biological replicates ± SEM. **P < 0.01. ANOVA and Sidak’s comparisons. 

c Representative flow cytometry plots showing HSCs in different phases of the cell cycle. The frequencies of the gated populations are indicated.
enriched in HSC niche-forming mesenchymal stem cells (MSCs). For pharmacological cholinergic blockade experiments, mice were i.p. injected with nicotinic antagonists (mecamylamine, 3 mg/kg; and hexamethonium, 20 mg/kg), muscarinic antagonists (scopolamine, 3 mg/kg; and methylatropine nitrate, 3 mg/kg) or vehicle at Zeitgeber time (ZT) 5 (5 h after light onset). Mice were culled and analysed at ZT13.

For the nicotinic agonist and antagonist experiments, CD45.2 C57BL/6 J mice were treated with 100 µg/ml nicotine (Sigma, N3876-5ML) in 1.5% saccharin (Sigma) via their drinking water. To assess in the transplantation setting, CD45.2 C57BL/6 J recipient mice were split dose irradiated with 9.5 Gy and transplanted with 2 million CD45.1 bone marrow nucleated cells. Mice were administered with 65µg/ml nicotine 24 h later via their drinking water for the remainder of the study, changed every 2–3 days. For the antigenic experiments, CD45.2 C57BL/6 J recipient mice were split dose irradiated with 12 Gy and transplanted with 2 million CD45.1 bone marrow nucleated cells. Mice were treated with 20 µg/kg/day Hexamethonium bromide (i.p.) daily for 30 days.

Immunofluorescence. Immunofluorescence staining of cryostat sections was performed as previously described. Briefly, tissues were permeabilized with 0.1% Triton X-100 (Sigma) for 10 min at RT and blocked with TNB buffer (0.1 M Tris–HCl, pH 7.5, 0.15 M NaCl, 0.5% blocking reagent, Perkin Elmer) for 1 h at RT. Primary antibody incubations were conducted overnight at 4 °C. Secondary antibody incubations were conducted for 2 h at RT. Repetitive washes were performed with sections were counter-stained for 5 min with 5 µM DAPI and rinsed with PBS. Slides were mounted in Vectashield Hardset mounting medium (Vector Labs). CHAT-Ires-Cre-positive nerve fibre staining was performed on half-bones according to previous reports (Acar et al., 2015), whereby bones were longitudinally bisected using a cryostat, blocked O/N in staining buffer (5% donkey serum, 0.5% (gePal, 10X DMSO) supplemented with 1% BlokHen (Aves Labs, Cat. No. BH-1001) and stained and primary and secondary antibodies for 3 days in staining buffer with daily intervening washes in PBS at RT. We used the following primary antibodies: tyrosine hydroxylase (1:1000, rabbit polyclonal antibody, Millipore), Gfap (1:200, goat polyclonal antibody, R&D), Chicken anti-GFP (Aves Labs, Cat. No. GFP-1020), Rat anti-CD31 (1:100, BD Biosciences, Cat. No. 550274, Clone MEC 13.3), Rat anti-Endomucin (1:200, Santa Cruz, Cat. No. sc-65495, clone V.7C7) and anti-DSRed polyclonal antibody (Takara Cat. No. 632496). The following antibodies were used for secondary staining: Alexa Fluor 488 donkey anti-chicken (Jackson Immuno, Cat. No. 703-545-155), Alexa Fluor 647 Donkey anti-rat (Abcam, Cat. No. ab510155), Alexa Fluor 647 Donkey anti-goat and Alexa Fluor 647 Donkey anti-Rabbit anti-sheep. Sections were scanned with Zeiss Axioscan Z1 slide scanning microscope. For all stainings, control and experimental samples were processed simultaneously and were blindly analysed using Zen lite software.

ELISA. CXCL12 protein levels were measured by conventional ELISA. Briefly, 96-well plates were coated overnight at 4 °C with 2 µg/ml of monoclonal CXCL12/ SDF-1 antibody (MAB350, R&D Systems). After blocking, BM extracellular fluids were incubated with the antibody for 2h at room temperature, followed by addition of biotinylated anti-human and mouse CXCL12/SDF-1 antibody (BAF310, R&D), Streptavidin-horseradish peroxidase conjugate (RNPH2131, DAKO) was used to detect the signal and the reaction was developed with horseradish peroxidase substrate (TMB, ES001-500ML, Chemicon, Millipore). Standard curve was performed with recombinant SDF-1 alpha (500-NS, R&D).

ELISA for norepinephrine/epinephrine (Bi-CAT ELISA ALPCO) was performed according to the manufacturers’ recommendations. Acetylcholinesterase measures were performed with Choline/Acetylcholine Assay Kit, Fluorometric protocol (Abcam, Cat. No. ab65345).

RNA isolation, reverse transcription and quantitative real-time PCR (qPCR). Total RNA extraction was performed with TRIzol (Invitrogen), followed by treatment with DNase to eliminate contaminating genomic DNA with RNase-free DNase Set (Qiagen) and RNA clean-up with RNaseasy mini kit (Qiagen). Alternative methods for small cell numbers, RNA was isolated using the Dynabeads® mRNA DIRECT™ Micro Kit (Invitrogen). Reverse transcription was performed using the Reverse Transcription System (Promega), following the manufacturer's recommendations. The expression level of each gene was determined by the relative standard curve method, using a standard curve prepared from serial dilutions of a mouse or human reference total RNA (C57Bl6). The expression level of each gene was calculated by interpolation from the standard curve. All values were normalised to GAPDH as the endogenous control. The sequence of oligonucleotides used for quantitative real-time RT-PCR are available in Supplementary Data 4.

RNAseq sample preparation. Pools of 30 viable LSK CD48^-CD150^+ cells were sorted (BD Influx cell sorter) into 4 µl lysing buffer (0.5 U/µl Superase In RNase Inhibitor in 0.2 % (v/v) Triton X-100) containing 12.5 mM DTT and 2.5 mM dNTP. RNAseq was performed following Smart-seq2 protocols33. Briefly, RNA was primed with oligo-dT primers and reverse transcribed using SuperScript II Reverse Transcriptase (200 U/µl, Thermo Fisher 18060-070), KAPA HiFi Hotstart

**Fig. 9** Smoking history is associated with delayed platelet normalisation after transplantation. Cumulative incidence of time (t) until normalisation of circulating platelets in patients undergoing allogeneic HSC transplantation, taking into account their smoking history (N = 248). Multivariate Cox Analysis included the type of donor (matched family vs. alternative donor) and the number of CD34+ cells infused as independent variables. Omnibus tests of model coefficients.
Bioinformatic analysis of RNAseq. RNAseq reads were aligned to Mus musculus genome (Ensembl version 38.81) using GSNAP (version 2015-09-29) with parameters (-B 5 -24 -n 1 -Q 1). Reads in features were counted with htseq-count (HTSeq version 0.3.3p3) with the parameter (–s no). Quality control was performed with the following cut-offs: more than one and a half million uniquely mappable reads, <20% of reads mapping to mitochondrial genes over mitochondrial genes, <3000 genes with 500 reads, <8500 high coverage genes identified. Counts were normalized using size factors as calculated by DESeq2 using a 10% FDR, and then log10 transformed. Highly variable genes were selected using the method described by Brennecke et al. PCA was then performed in R using the pcorpc function.

Gene set enrichment analyses were performed as described (24) (http://www.broadinstitute.org/gsea/index.jsp), using a weighted statistic, ranking by signal to noise ratio, 1000 gene-set permutations, and a custom gene set database gene lists manually compiled from the literature.

GEO accession number—GSE94078

Statistical analysis and reproducibility. We used similar response variables for the calculation of sample size. For each hypothesis, the total cell number and the calculation of sample size. For each hypothesis, the total cell number and

Statistical analyses and graphics were carried out with GraphPad Prism software and Microsoft Excel.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability. The RNAseq data generated in this study has been deposited in the GEO database under accession code GSE94078. Source data are provided with this paper.

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Author contributions
C.F., A.G.-G., C.K. and S.M.-F. performed experiments and analyses and prepared figures, J.L.R. and J.A.P.-S. retrospectively analysed human data. Z.F. and B.G. helped with RNAseq and its analysis. S.M.-F. designed and supervised the study and wrote the manuscript. All authors edited the manuscript.

Competing interests
The authors declare no competing interests.

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Correspondence and requests for materials should be addressed to Simón Méndez-Ferrer.
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