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Hematopoietic colony–stimulating factors mediate tumor-nerve interactions and bone cancer pain

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Pain is one of the most severe and debilitating symptoms associated with several forms of cancer[1,2]. Various types of carcinomas and sarcomas metastasize to skeletal bones and cause spontaneous bone pain and hyperalgesia, which is accompanied by bone degradation and remodeling of peripheral nerves[2]. Despite recent advances, the molecular mechanisms underlying the development and maintenance of cancer-evoked pain are not well understood[2]. Several types of non-hematopoietic tumors secrete hematopoietic colony-stimulating factors that act on myeloid cells[3] and tumor cells[4]. Here we report that receptors and signaling mediators of granulocyte- and granulocyte-macrophage colony-stimulating factors (G-CSF and GM-CSF)[3] are also functionally expressed on sensory nerves. GM-CSF sensitized nerves to mechanical stimuli *in vitro* and *in vivo*, potentiated CGRP release and caused sprouting of sensory nerve endings in the skin. Interruption of G-CSF and GM-CSF signaling *in vivo* led to reduced tumor growth and nerve remodeling, and abrogated bone cancer pain. The key significance of GM-CSF signaling in sensory neurons was revealed by an attenuation of tumor-evoked pain following a sensory nerve–specific knockdown of GM-CSF receptors. These results show that G-CSF and GM-CSF are important in tumor-nerve interactions and suggest that their receptors on primary afferent nerve fibers constitute potential therapeutic targets in cancer pain.

Pancreatic biopsy samples from patients with pancreatic adenocarcinoma showed significantly higher levels of G-CSF and GM-CSF than were seen in pancreatic samples from healthy donors (**Fig. 1a**; $P < 0.05$). The transmembrane receptors for G-CSF (G-CSFR) and GM-CSF (GM-CSFRα) are known to be expressed on hematopoietic cells, where these CSFs promote the survival, proliferation, differentiation and recruitment of myeloid-lineage cells[3], as well as on certain types of tumor cells, where autocrine regulation of proliferation has been reported *in vitro*[4]. Unexpectedly, our immunohistochemical analysis revealed that G-CSFR and GM-CSFRα are also expressed (i) on pancreatic nerves in biopsy samples in healthy individuals and (ii) in the large hypertrophic nerves (a hallmark feature of human pancreatic carcinoma) in the vicinity of tumors in individuals with carcinoma[5] (**Fig. 1b** and **Supplementary Fig. 1a**). Nerve fibers positive for G-CSFR and GM-CSFRα showed coimmunoreactivity for nerve markers, such as PGP9.5 (**Fig. 1b**), peripherin (**Supplementary Fig. 1b**) and calcitonin gene–related peptide (CGRP) (**Fig. 1c**). Incubation of antibodies with the corresponding blocking peptides abrogated staining (**Fig. 1c**).

In a mouse sarcoma model of bone tumor–induced pain, which has been previously shown to closely mimic pain induced by human bone metastases[6], increased levels of GM-CSF and G-CSF were detected in lysates of bone marrow and the adjoining connective tissue as compared to corresponding healthy tissue (**Fig. 1d,e**). A few studies have reported expression of hematopoietic growth factor receptors in neurons of the central nervous system in rodents[7]. We observed immunoreactivity for G-CSFR and GM-CSFRα in peripheral nerves dispersed in the periosteum and throughout the bone matrix (**Fig. 1f**) and in sections of dorsal root ganglia (DRG) (**Supplementary Fig. 1c**). Nearly all isolectin-B4–binding and ~28 ± 4% of CGRP-positive peptidergic nociceptive neurons in the DRG expressed G-CSFR, whereas GM-CSFRα was found in about 41 ± 4% of peptidergic neurons, but only very rarely in nonpeptidergic nociceptive neurons (**Fig. 1g**). Western blot analysis on mouse DRG lysates and lysates of mouse DRG cultures enriched for sensory neurons revealed expression of the expected glycosylation variants of GM-CSFRα[8–10] (**Fig. 1h**). Finally, mRNA *in situ* hybridization using specific riboprobes (**Fig. 1i**) and reverse-transcriptase PCR (**Supplementary Fig. 1d**) further confirmed expression of G-CSFR and GM-CSFRα in sensory neurons.

Receptors for G-CSF and GM-CSF signal directly via the Janus kinase–signal transducer and activator of transcription (JAK-STAT) pathway[3,11] and exhibit cross-talk with the ERK–mitogen activated kinase pathway and the phophatidylinositol-3 (PI(3)) kinase–Akt

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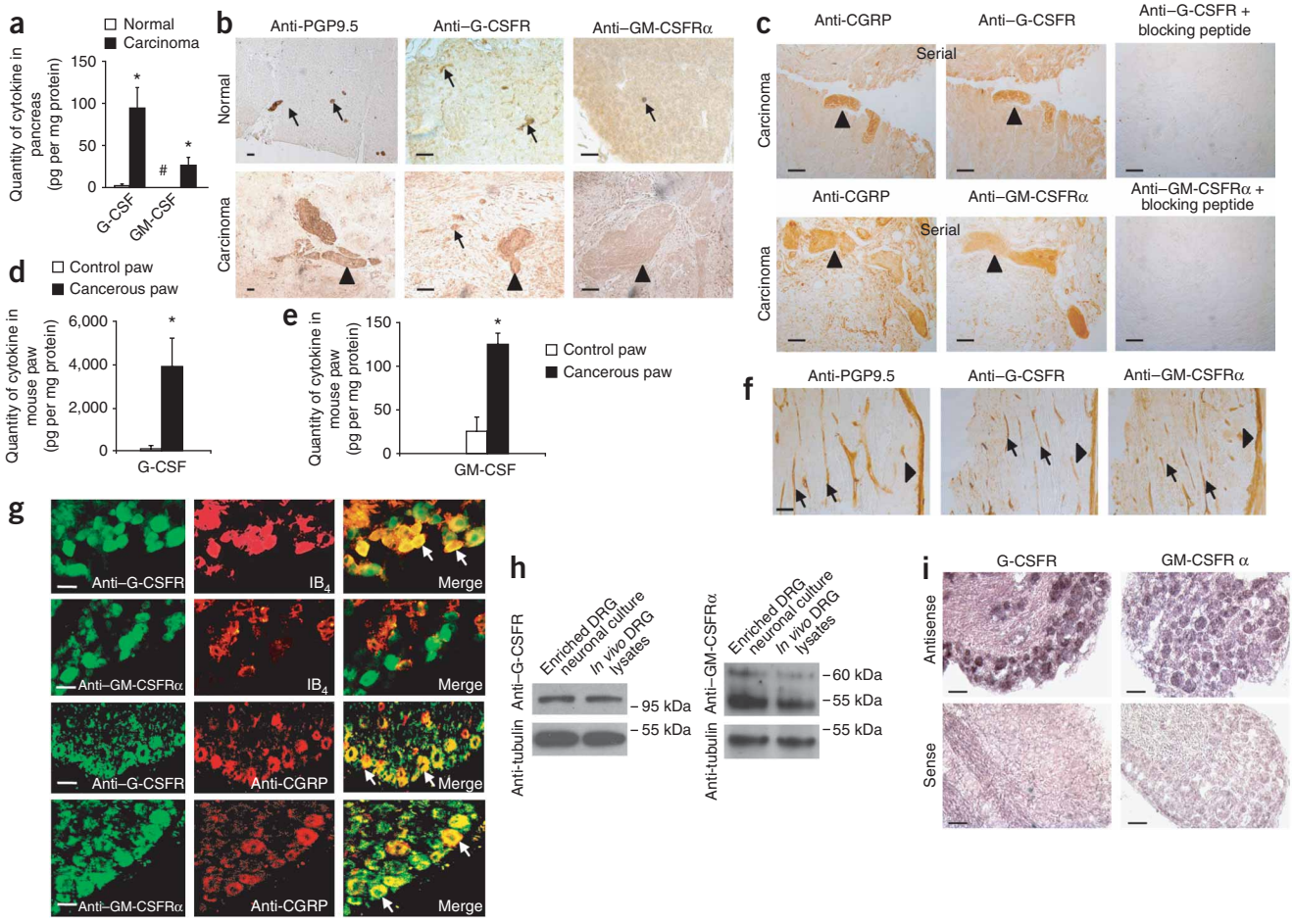

Figure 1 Evidence for release of G-CSF and GM-CSF from tumor-affected tissues and expression of their receptors (G-CSFR and GM-CSFRα) on sensory nerve fibers and cell bodies. (**a**) Analysis of release of G-CSF and GM-CSF from human pancreatic carcinoma. # indicates values below detection limits. (**b**,**c**) Immunoreactivity for G-CSFR and GM-CSFRα on normal nerves (arrows) in healthy human pancreas and in hypertrophic nerves (arrowheads) in individuals with pancreatic carcinoma, identified via the nerve marker proteins PGP9.5 (**b**) and CGRP (**c**; serial sections). Panels at right show specificity controls with the corresponding blocking peptides. (**d**,**e**) Levels of G-CSF (**d**) and GM-CSF (**e**) in paw lysates of naive mice (control) and mice bearing calcaneus bone tumors. (**f**,**g**) Immunoreactivity for G-CSFR and GM-CSFRα on PGP.5-positive sensory nerves (arrows) in mouse tibial bone and adjoining periosteal nerves (arrowheads) and in nociceptive neurons binding isolectin-B4 (IB4) or expressing CGRP in the DRG. (**h**) Western blot analysis with antibodies against G-CSFR and GM-CSFRα on lysates of whole mouse DRG or enriched DRG neuronal cultures. (**i**) mRNA *in situ* hybridization using digoxigenin-labeled antisense riboprobes showing expression of G-CSFR and GM-CSFRα in neuronal somata in mouse DRG sections. The corresponding sense probes serve as negative controls. $^*P < 0.05$, ANOVA followed by *post hoc* Fisher's test. Scale bars: 100 μm (**b**,**f**), 40 μm (**g**), 50 μm (**i**).

pathway[11]. G-CSF and GM-CSF rapidly produced phosphorylation of STAT3, a prominent transcription factor[3,11], and caused phosphorylation and nuclear translocation of ERK1/2 in cultured DRG neurons, thereby showing that G-CSF and GM-CSF receptors on sensory neurons are functional (**Fig. 2a**,**b**; **Supplementary Fig. 2a**,**b**). Both effects were blocked to a significant degree by pretreatment with neutralizing antibodies against G-CSFR[12] and GM-CSFRα[13] and by inhibition of the JAK1/2 and MEK pathways, in comparison to the respective controls ($P < 0.05$, ANOVA; **Fig. 2b** and **Supplementary Fig. 2b**), whereas PI(3) kinase inhibition consistently blocked only G-CSF– and GM-CSF–induced ERK1/2 phosphorylation (**Fig. 2b**; **Supplementary Fig. 2b**).

In line with the ability of activated STAT3 to activate and regulate gene transcription[3,11], we observed in western blot analysis on cultured DRG cells that exposure to G-CSF led within 4 h to a significant upregulation of the sodium channel $Na_v1.8$ ($P < 0.003$; Student's *t*-test) as well as the heat-activated channel TRPV1 ($P < 0.001$), but not $Na_v1.7$ ($P = 0.73$)—all three channels being linked to nociception and hyperalgesia[14] (**Fig. 2c**). However, we also observed induction of the expression of potassium channels, such as $K_v4.2$ and TREK-1 ($P < 0.006$), which might be expected to contribute to reduction of neuronal excitability[15] (**Fig. 2c**). Furthermore, we observed that release of CGRP, a major pain-related peptide, in response to nociceptive stimulation via topical application of capsaicin *in vivo*, was strongly potentiated in skin areas that had been injected subcutaneously with GM-CSF as compared to areas injected with the vehicle as a control (**Fig. 2d**; $P < 0.05$ at 20–60 min after capsaicin treatment). These results suggest a marked peripheral sensitization of nociceptive nerves upon exposure to GM-CSF.

Indeed, by performing single-fiber recordings from the mouse saphenous nerve in the mouse skin-nerve preparation, we found direct evidence for a modulation of peripheral nerve activity by GM-CSF. Responses evoked by controlled mechanical stimuli applied to the receptive field via displacement of a nanomotor (represented in μm) were analyzed in low-threshold (Aβ) and high-threshold (Aδ) mechanoreceptive fibers before and 30 min after application of either

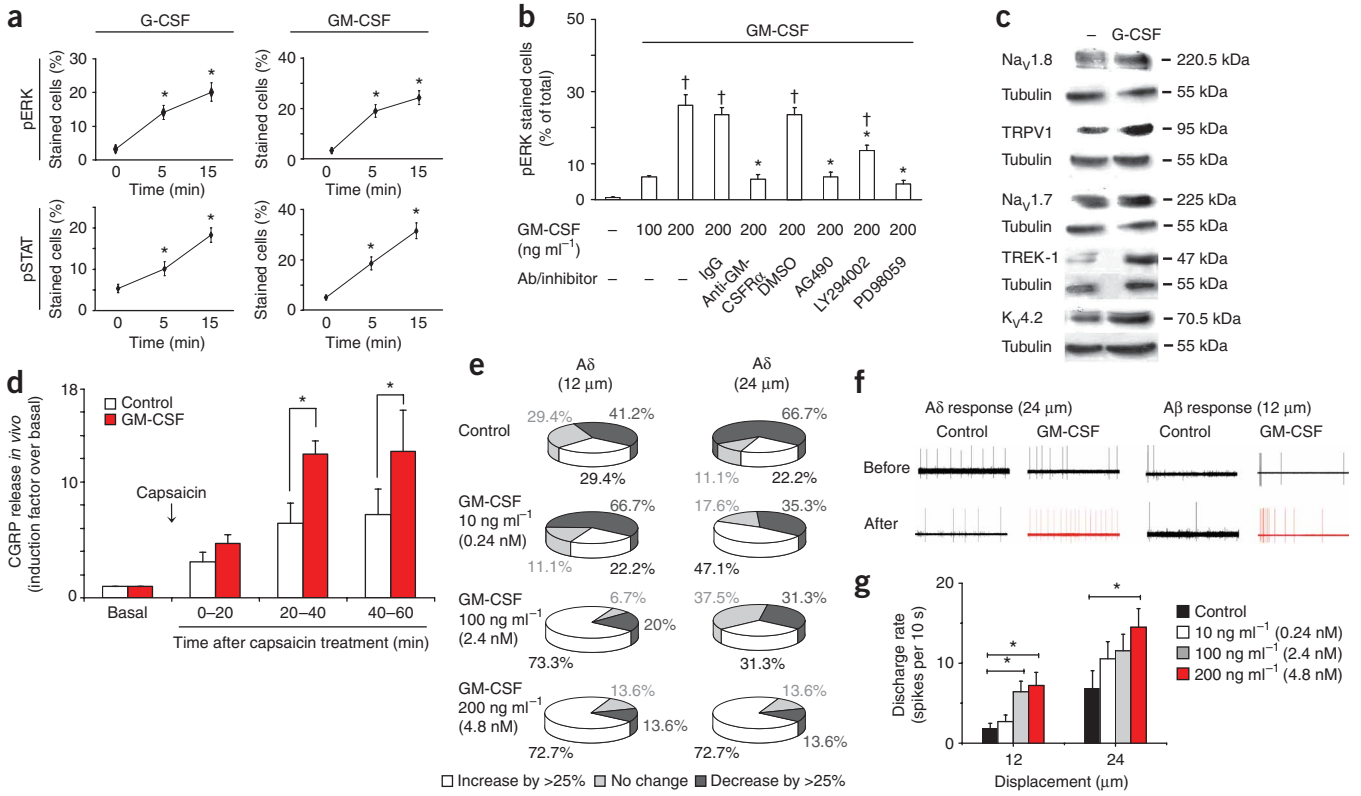

Figure 2 Evidence for mouse G-CSF- and GM-CSF-evoked functions in sensory neurons with respect to molecular signaling, gene expression, CGRP release and nerve activity. (**a**) Time course of phosphorylation of ERK1/2 and STAT3 in cultured DRG cells after application of G-CSF (200 ng ml^{-1}) or GM-CSF (200 ng ml^{-1}). (**b**) Effects of blockade of GM-CSFRα via a neutralizing antibody (4 μg ml^{-1}) or inhibitors of signaling pathways (see text for details) in cultured DRG cells. †$P < 0.05$ as compared to the vehicle PBS (–);*$P < 0.05$ with respect to the corresponding cytokine alone; ANOVA followed by *post hoc* Fisher's test. (**c**) Western blot analysis of expression levels of pain-related proteins in cultured DRG cells treated with vehicle (–) or G-CSF (100 ng ml^{-1}) for 4 h. (**d**) Potentiation of CGRP release evoked by capsaicin (1%) in rat skin *in vivo* upon exposure to GM-CSF (100 μl of 5 ng μl^{-1}) as compared to vehicle (control). *$P < 0.05$ with respect to the corresponding control value; ANOVA followed by *post hoc* Fisher's test. (**e**–**g**) Electrophysiological analysis of single fiber activity before and 30 min after application of GM-CSF (10, 100 or 200 ng ml^{-1}) or vehicle (0.9% NaCl; control) to the receptive field in the skin-nerve preparation. (**e**–**g**) Population analysis of responsive fibers as a fraction of total fibers tested (**e**), typical examples (**f**) and summary of mean firing rates of the recorded fibers (**g**) are shown. *$P < 0.01$; ANOVA followed by *post hoc* Fisher's test; $n = 15$–20 fibers per group.

the vehicle (saline) or GM-CSF (10 ng ml^{-1}–200 ng ml^{-1}) (**Fig. 2e–g**). Population analyses revealed that GM-CSF treatment led to a marked recruitment of Aδ fibers (**Fig. 2e,f**) and evoked an increase in the mean discharge rates of Aδ fibers (**Fig. 2g** and **Supplementary Fig. 2c**; $P < 0.01$, ANOVA) and of Aβ fibers in response to mechanical stimuli (**Supplementary Fig. 2d**; $P < 0.01$, ANOVA, data not shown), suggesting sensitization of peripheral sensory nerves. In support, in behavioral experiments, G-CSF and GM-CSF induced significant dose-dependent thermal hyperalgesia and mechanical hyperalgesia, respectively, as compared to vehicle-treated mice (**Fig. 3a**).

Having thus found morphological, electrophysiological and behavioral evidence for G-CSFR– and GM-CSFRα–induced signaling in nociceptor sensitization, we examined whether these processes contribute to cancer-evoked pain in the mouse sarcoma model. Consistent with previous reports[6,16], mice with tumors in the calcaneus bone showed increased withdrawal responses to plantar tactile stimulation—stimulation with 0.07*g* force using a von Frey monofilament—as compared to sham-treated mice (**Supplementary Fig. 3a**). A similar degree of hyperalgesia was observed in mice that received control IgG injections in the vicinity of the calcaneus bone at 1, 3, 5, 7 and 9 d after implantation of tumor cells (**Fig. 3b**). In marked contrast, mice that received local injections of the neutralizing antisera against either G-CSFR or GM-CSFRα at 1, 3, 5, 7 and 9 d after implantation of tumor cells in the calcaneus bone did not show hyperalgesia throughout the 16-d period of daily testing (**Fig. 3b**; *$P < 0.001$ as compared to control IgG group). Injection of the JAK inhibitor AG490 or the MEK inhibitor PD98059 in the vicinity of the calcaneus bone at 1, 3, 5, 7 and 9 d after tumor induction also abrogated hyperalgesia, whereas the PI(3) kinase inhibitor LY294002 did not significantly modulate tumor-evoked hyperalgesia ($P < 0.001$ for AG490 and PD98059 and $P = 0.669$ for LY294002) (**Fig. 3c**). Tissue punches derived from the paw skin overlaying the growing tumor after the termination of the experiment revealed that in contrast to the unaffected contralateral paw, the IgG-treated tumor-affected paw showed increased branching and sprouting of PGP9.5-positive nerve fibers and occasional hypertrophy of nerves in the dermis, which did not occur in mice treated with antibodies against G-CSFR or GM-CSFRα (**Fig. 3d**). Furthermore, histopathological analysis revealed that tumor size was significantly reduced in mice that received antisera against either G-CSFR or GM-CSFRα, or that received AG490, but not in those that received PD98059, in comparison with the corresponding controls (**Fig. 3e,f**).

In the above setting, it was not feasible to rule out the possibility that the reduction in pain and the structural changes might have resulted at least partially from a reduction in tumor growth owing to neutralization of G-CSF and GM-CSF receptors or JAK inhibition in tumor cells.

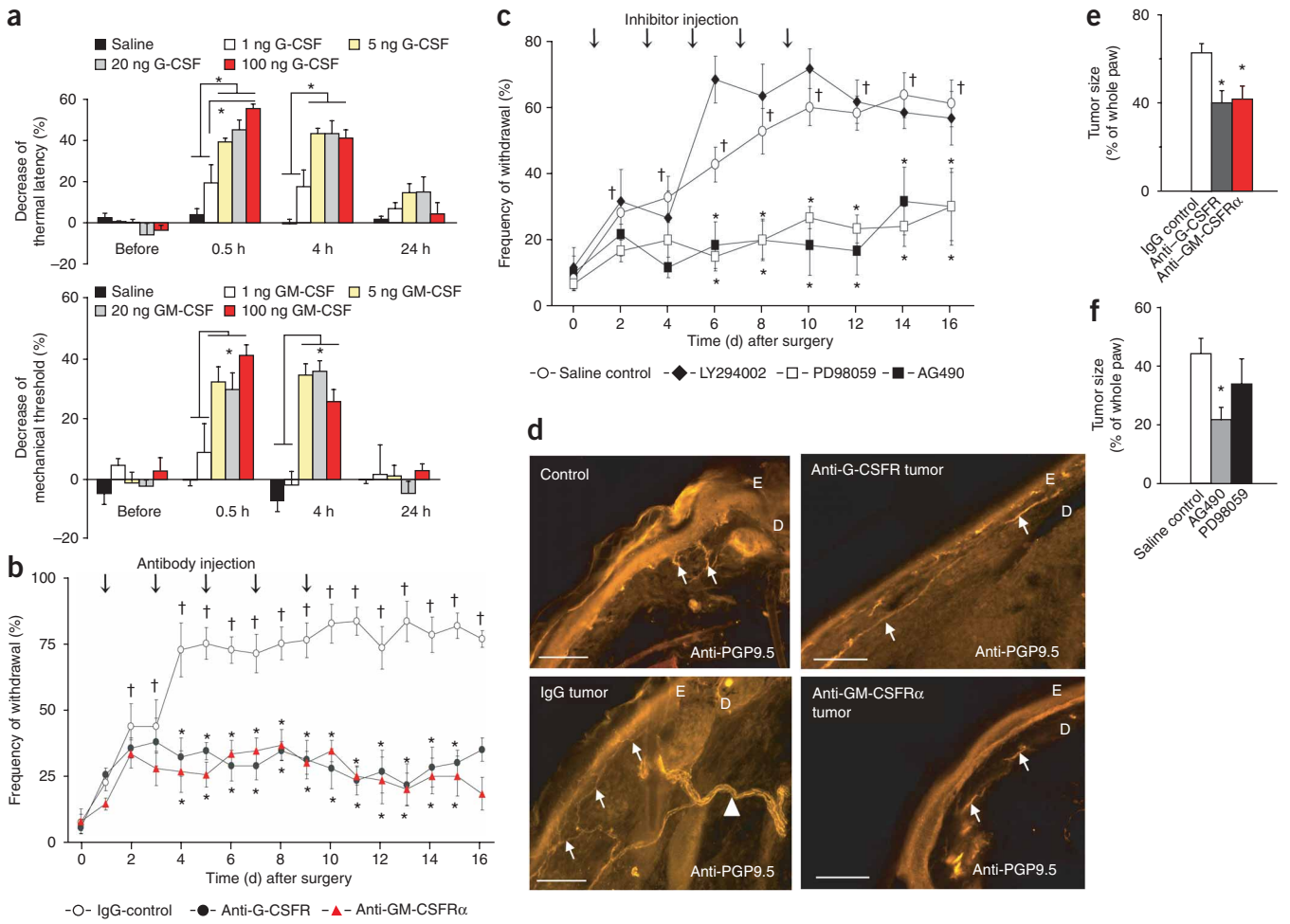

Figure 3 Functional role of G-CSF and GM-CSF signaling in nociceptive sensitization, tumor-induced hyperalgesia and hypertrophy of cutaneous nerve fibers *in vivo*. (**a**) Dose-dependent effects of G-CSF (above) or GM-CSF (below) on paw withdrawal latency to noxious heat or paw withdrawal threshold to mechanical pressure applied via a Dynamic aesthesiometer at 30 min, 4 h and 24 h following intraplantar injection in the hindpaw (n = at least 8 mice each). *y* axes show percentage decreases in the injected paw over the uninjected, contralateral hindpaw. (**b**,**c**) Frequency of paw withdrawal in response to plantar von Frey filament application before and after induction of calcaneus bone tumors in mice treated with neutralizing antibodies against G-CSFR or GM-CSFRα (**b**) or with pharmacological inhibitors (**c**) (see text for details), which were injected in the vicinity of the calcaneus bone (injection days indicated by arrows; n = at least 8 mice in each treatment group). In **b**,**c**, †$P < 0.01$ as compared to basal values; *$P < 0.05$ as compared to IgG or NaCl controls, respectively. (**d**) Labeling of PGP9.5-expressing nerve fibers (arrows) in skin punches derived from control paws or tumor-bearing paws treated with either IgG (control) or neutralizing antibodies against G-CSFR or GM-CSFRα. Arrowhead indicates a hypertrophic nerve fiber. E, epidermis; D, dermis. Scale bar, 50 μm. (**e**,**f**) Histopathological analysis of the area of tumor expressed as percent of the area of the paw.

However, the following experiments revealed that receptors for hematopoietic growth factors expressed on nerve fibers indeed contributed to tumor-induced pain independently of their role in tumor growth. We generated lentivirions expressing short hairpin RNAs (shRNAs)[17] against GM-CSFRα (Lenti-GM-CSFRα shRNA) and a control lentivirus expressing enhanced green fluorescent protein (EGFP) (Lenti-GFP) (**Fig. 4a**) and directly injected them into L4 and L5 DRG unilaterally in mice *in vivo* as described previously for adenoviruses[18] without eliciting any obvious toxicity (see **Supplementary Fig. 3b** for example). When calcaneus bone tumors were induced in these mice 3 weeks later, mice expressing Lenti-GFP in DRG developed hyperalgesia to a similar extent as saline-injected mice with tumors did (**Supplementary Fig. 3c**). In contrast, hyperalgesia developed to a significantly lesser degree in mice expressing Lenti-GM-CSFRα shRNA (**Fig. 4b**), as revealed by comparing the groups in repeated-measures ANOVA as well as ANOVA of random measures and via comparison of integral functions as revealed by Student's *t*-test (**Fig. 4c**, $P < 0.001$; Student's *t*-test). Western blot analysis on DRG lysates at the termination of the above experiment revealed a specific knockdown of GM-CSFRα in L4-L5 DRG of mice injected with Lenti-GM-CSFRα shRNA mice as compared to those injected with Lenti-GFP (**Fig. 4d**). Notably, tumor growth was similar between mice expressing Lenti-GFP and Lenti-GM-CSFRα shRNA (**Fig. 4e**; $P = 0.32$, Student's *t*-test). Thus, a specific loss of GM-CSFRα in sensory neurons led to a reduction in bone tumor–evoked pain, suggesting that GM-CSF signaling in peripheral nerves contributes substantially to cancer pain.

In tissue punches derived from the skin adjacent to tumor growth, we frequently observed an increase in the branching of nerve fibers and a hypertrophy of nerve endings in the paw skin of Lenti-GFP-expressing mice, but not in paws of Lenti-GM-CSFRα shRNA–expressing mice or control paws (**Fig. 4f**). Furthermore, live imaging of neurite outgrowth from cultured DRG cells indicated that GM-CSF–treated DRG neurons showed a significant increase in the number of secondary branches sprouting from primary axons as

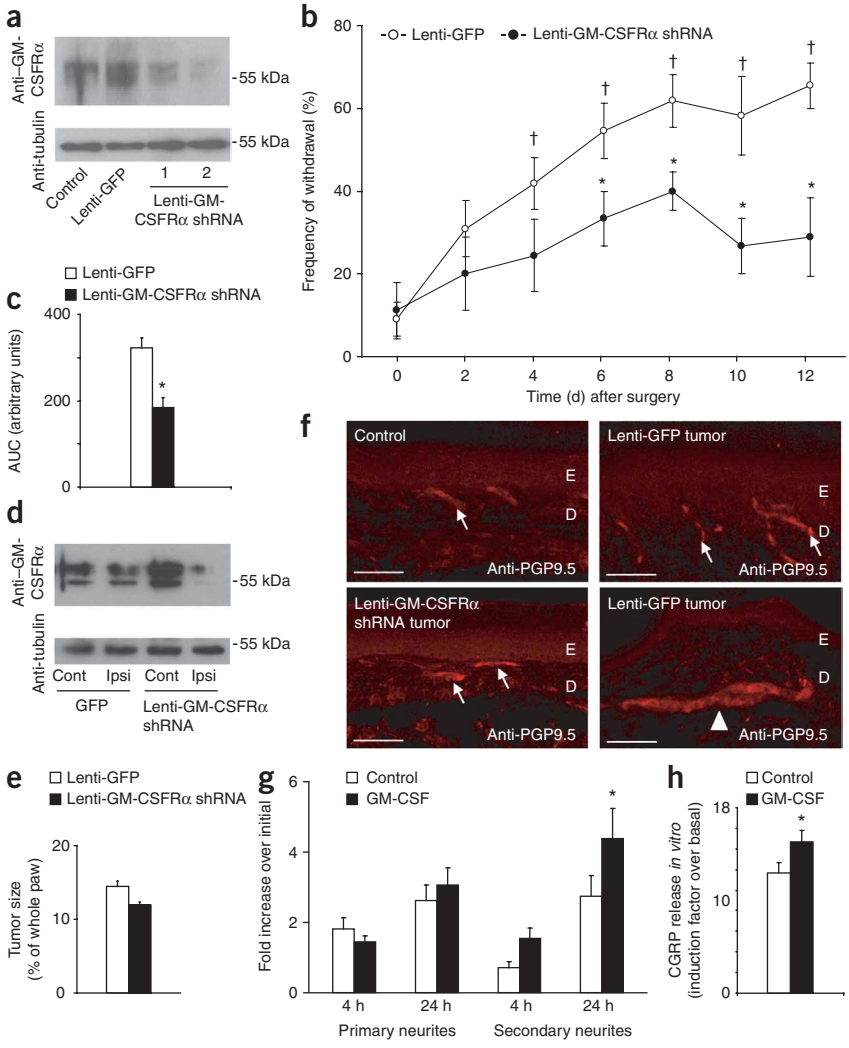

Figure 4 Effects of RNA interference-induced knockdown of GM-CSFRα specifically in sensory neurons on tumor growth and tumor-induced hyperalgesia and nerve remodeling. (**a**) Screening of lentiviral shRNA constructs for GM-CSFRα knockdown in B16 melanoma cells. (**b**) Significant reduction of tumor-evoked hyperalgesia in response to plantar von Frey stimulation with 0.07*g* force in mice injected with lentivirions expressing GM-CSFRα shRNA ($n = 10$ mice) unilaterally in L4-L5 DRG as compared to mice injected with lentivirions expressing GFP ($n = 12$ mice) *in vivo*. †$P < 0.05$, ANOVA for repeated measures; *$P < 0.05$, ANOVA for random measure followed by *post hoc* Fisher's test. (**c**) Reduced area under the curve for tumor-induced hyperalgesia in mice expressing lentiviral GM-CSFRα shRNA in comparison with mice expressing lentiviral GFP. *$P < 0.001$, Student's *t*-test. (**d**) Western blot analysis of GM-CSFRα expression in the injected (ipsi) and the non-injected (contra) L4-L5 DRG in mice from the above groups. (**e**) Comparable tumor growth in mice expressing GFP or GM-CSFRα shRNA specifically in the DRG. (**f**) PGP9.5-positive nerve fibers (arrows) in skin punches derived from control paws or tumor-affected paws in mice from the above groups. Arrowhead indicates a hypertrophic nerve. Scale bars, 50 μm. E, epidermis; D, dermis. (**g**,**h**) Time-lapse imaging of nerve outgrowth (*$P < 0.05$, ANOVA followed by *post hoc* Fisher's test) and capsaicin-evoked release of CGRP (*$P < 0.05$, Student's *t*-test) from mouse DRG neurons cultured in the presence of GM-CSF (200 ng ml^{-1}) or control medium for 24 h.

compared to vehicle-treated cultures (control) after 24 h ($P = 0.03$), although the number of primary neurites (axons) was similar (**Fig. 4g**). Because this test system was free of any tumor cells, we conclude that GM-CSF receptors in the DRG directly mediated remodeling of nerve structure. Furthermore, in DRG cultures cultivated in the presence of GM-CSF for 24 h, CGRP released in response to stimulation with capsaicin was greater than in corresponding control cultures (**Fig. 4h**; $P = 0.01$, Student's *t*-test).

Taken together, these results point to G-CSF and GM-CSF as mediators of tumor-nerve interactions and bone cancer pain. Reciprocal interactions between tumor cells and sensory neurons represent an emerging and promising aspect in the pathophysiology of cancer pain[2,6,16,19]. In this regard, G-CSF and GM-CSF may contribute to autocrine and paracrine mechanisms involving proliferation and migration of tumor cells with subsequent osteoclast activation and recruitment of inflammatory macrophages and monocytes[3,8], respectively. Furthermore, cross-talk between known paracrine mediators of tumor nerve interactions, such as endothelins[20], TNFα[21] and CCL2 (or MCP-1)[22], and hematopoietic growth factors via reciprocal modulation of release[23–25] may contribute to the pathogenesis of cancer-induced pain. Finally, and importantly, the morphological and functional findings of this study strongly suggest that G-CSF and GM-CSF directly activate receptors located on primary afferent nerve fibers to produce peripheral sensitization, which is recognized as a key component of cancer pain[2,6,16,26]. In terms of underlying molecular pathways, our results point to a role for the JAK-STAT3 pathway and ERK1/2 signaling in mediating the sensitization of nociceptors following exposure to CSFs.

The results of this study bear direct clinical relevance in humans, not only from the therapeutic point of view in regard to the management of cancer pain but also from the perspective of clinical usage of hematopoietic growth factors in hematopoietic stem cell donors[27,28] and patients undergoing myelosuppressive chemotherapy or radiotherapy[27,28], for whom intense bone pain is reported to be an important side-effect[29,30]. Our unexpected findings of a role for G-CSF and GM-CSF receptors expressed on sensory neurons in nociceptor sensitization can directly account for this unexplained clinical observation. Furthermore, G-CSF is released in human skin after inflammation[31] or operative injury[32] and in rheumatoid arthritis[33], suggesting that G-CSF and GM-CSF signaling is indeed functionally linked to exaggerated pain in humans.

The most noteworthy point from a therapeutic perspective is the observation that local inhibition of G-CSF and GM-CSF receptors via neutralizing antibodies in the vicinity of the tumor diminished bone cancer pain and also reduced tumor growth *in vivo*. This may make it possible to develop a locally directed therapeutic intervention devoid of systemic side effects. In this regard, the recent elucidation of the crystal structure of the GM-CSFR complex[34] offers further perspectives in regard to the development of interventional small-molecule drugs to therapeutically exploit the functions of hematopoietic growth factor receptors in cancer growth and cancer pain.

METHODS

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

Note: Supplementary information is available on the Nature Medicine website.

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AUTHOR CONTRIBUTIONS

M. Schweizerhof performed the largest portion of the experiments and analyzed data; S.S., M.K. and C.N. performed several experiments and analyzed data; V.G., N.A., M. Schmelz, C.W.M., K.K.B. and S.B. performed some experiments and analyzed data; A.D. contributed knowledge of mouse models; D.A.S. contributed knowledge of mouse models and helped with some experiments and with the writing of the manuscript; R.K. designed and supervised the study, analyzed data and wrote the manuscript.

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1. Portenoy, R.K. & Lesage, P. Management of cancer pain. *Lancet* **353**, 1695–1700 (1999).
2. Honoré, P. & Mantyh, P.W. Bone cancer pain: from mechanism to model to therapy. *Pain Med.* **1**, 303–309 (2000).
3. Hamilton, J.A. Colony-stimulating factors in inflammation and autoimmunity. *Nat. Rev. Immunol.* **8**, 533–544 (2008).
4. Tachibana, M. & Murai, M. G-CSF production in human bladder cancer and its ability to promote autocrine growth: a review. *Cytokines Cell. Mol. Ther.* **4**, 113–120 (1998).
5. Zhu, Z. *et al.* Nerve growth factor expression correlates with perineural invasion and pain in human pancreatic cancer. *J. Clin. Oncol.* **17**, 2419–2428 (1999).
6. Wacnik, P.W. *et al.* Functional interactions between tumor and peripheral nerve: morphology, algogen identification, and behavioral characterization of a new murine model of cancer pain. *J. Neurosci.* **21**, 9355–9366 (2001).
7. Schäbitz, W.R. *et al.* Neuroprotective effect of granulocyte colony-stimulating factor after focal cerebral ischemia. *Stroke* **34**, 745–751 (2003).
8. Kasper, B. *et al.* Severe congenital neutropenia patients with point mutations in the granulocyte colony-stimulating factor (G-CSF) receptor mRNA express a normal G-CSF receptor protein. *Blood* **90**, 2839–2841 (1997).
9. Lehtonen, A., Matikainen, S., Miettinen, M. & Julkunen, I. Granulocyte-macrophage colony-stimulating factor (GM-CSF)-induced STAT5 activation and target-gene expression during human monocyte/macrophage differentiation. *J. Leukoc. Biol.* **71**, 511–519 (2002).
10. Monfardini, C. *et al.* Construction and binding kinetics of a soluble granulocyte-macrophage colony-stimulating factor receptor alpha-chain-Fc fusion protein. *J. Biol. Chem.* **273**, 7657–7667 (1998).
11. de Groot, R.P., Coffer, P.J. & Koenderman, L. Regulation of proliferation, differentiation and survival by the IL-3/IL-5/GM-CSF receptor family. *Cell. Signal.* **10**, 619–628 (1998).
12. Schneider, A. *et al.* The hematopoietic factor G-CSF is a neuronal ligand that counteracts programmed cell death and drives neurogenesis. *J. Clin. Invest.* **115**, 2083–2098 (2005).
13. Giron-Michel, J. *et al.* Detection of a functional hybrid receptor γc/GM-CSFRβ in human hematopoietic CD34$^+$ cells. *J. Exp. Med.* **197**, 763–775 (2003).
14. Julius, D. & Basbaum, A.I. Molecular mechanisms of nociception. *Nature* **413**, 203–210 (2001).
15. Honoré, E. The neuronal background K2P channels: focus on TREK1. *Nat. Rev. Neurosci.* **8**, 251–261 (2007).
16. Cain, D.M. *et al.* Functional interactions between tumor and peripheral nerve: changes in excitability and morphology of primary afferent fibers in a murine model of cancer pain. *J. Neurosci.* **21**, 9367–9376 (2001).
17. McIntyre, G.J. & Fanning, G.C. Design and cloning strategies for constructing shRNA expression vectors. *BMC Biotechnol.* **6**, 1 (2006).
18. Glatzel, M. *et al.* Adenoviral and adeno-associated viral transfer of genes to the peripheral nervous system. *Proc. Natl. Acad. Sci. USA* **97**, 442–447 (2000).
19. Lindsay, T.H. *et al.* Pancreatic cancer pain and its correlation with changes in tumor vasculature, macrophage infiltration, neuronal innervation, body weight and disease progression. *Pain* **119**, 233–246 (2005).
20. Davar, G. Endothelin-1 and metastatic cancer pain. *Pain Med.* **2**, 24–27 (2001).
21. Wacnik, P.W., Eikmeier, L.J., Simone, D.A., Wilcox, G.L. & Beitz, A.J. Nociceptive characteristics of tumor necrosis factor-α in naive and tumor-bearing mice. *Neuroscience* **132**, 479–491 (2005).
22. Owen, J.L. *et al.* The expression of CCL2 by T lymphocytes of mammary tumor bearers: role of tumor-derived factors. *Cell. Immunol.* **235**, 122–135 (2005).
23. Dedhar, S., Gaboury, L., Galloway, P. & Eaves, C. Human granulocyte-macrophage colony-stimulating factor is a growth factor active on a variety of cell types of nonhemopoietic origin. *Proc. Natl. Acad. Sci. USA* **85**, 9253–9257 (1988).
24. Danis, V.A., Franic, G.M., Rathjen, D.A. & Brooks, P.M. Effects of granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-2, interferon-γ (IFN-γ), tumour necrosis factor-α (TNF-α) and IL-6 on the production of immunoreactive IL-1 and TNF-α by human monocytes. *Clin. Exp. Immunol.* **85**, 143–150 (1991).
25. Salh, B., Hoeflick, K., Kwan, W. & Pelech, S. Granulocyte-macrophage colony-stimulating factor and interleukin-3 potentiate interferon-γ-mediated endothelin production by human monocytes: role of protein kinase C. *Immunology* **95**, 473–479 (1998).
26. Khasabova, I.A. *et al.* Chemical interactions between fibrosarcoma cancer cells and sensory neurons contribute to cancer pain. *J. Neurosci.* **27**, 10289–10298 (2007).
27. Tigue, C.C. *et al.* Granulocyte-colony stimulating factor administration to healthy individuals and persons with chronic neutropenia or cancer: an overview of safety considerations from the Research on Adverse Drug Events and Reports project. *Bone Marrow Transplant.* **40**, 185–192 (2007).
28. Waller, E.K. The role of sargramostim (rhGM-CSF) as immunotherapy. *Oncologist* **12** Suppl 2, 22–26 (2007).
29. Lindemann, A. *et al.* Hematologic effects of recombinant human granulocyte colony-stimulating factor in patients with malignancy. *Blood* **74**, 2644–2651 (1989).
30. Vadhan-Raj, S. *et al.* Effects of recombinant human granulocyte-macrophage colony-stimulating factor in patients with myelodysplastic syndromes. *N. Engl. J. Med.* **317**, 1545–1552 (1987).
31. Angst, M.S. *et al.* Cytokine profile in human skin in response to experimental inflammation, noxious stimulation, and administration of a COX-inhibitor: a microdialysis study. *Pain* **139**, 15–27 (2008).
32. Carvalho, B., Clark, D.J. & Angst, M.S. Local and systemic release of cytokines, nerve growth factor, prostaglandin E2, and substance P in incisional wounds and serum following cesarean delivery. *J. Pain* **9**, 650–657 (2008).
33. Cook, A.D., Braine, E.L., Campbell, I.K., Rich, M.J. & Hamilton, J.A. Blockade of collagen-induced arthritis post-onset by antibody to granulocyte-macrophage colony-stimulating factor (GM-CSF): requirement for GM-CSF in the effector phase of disease. *Arthritis Res.* **3**, 293–298 (2001).
34. Hansen, G. *et al.* The structure of the GM-CSF receptor complex reveals a distinct mode of cytokine receptor activation. *Cell* **134**, 496–507 (2008).

ONLINE METHODS

Mouse model of tumor-evoked pain. All animal usage procedures were in accordance with ethical guidelines and were approved by the local governing body (Regierungspräsidium Karlsruhe). All behavioral measurements were done in awake, unrestrained, age-matched adult (more than 3 months of age) male C3H/HeNCrl mice. National Collection of Type Cultures (NCTC) clone 2472 fibrosarcoma cells (ATCC) were cultured and injected unilaterally into and around the calcaneus bone as described previously[6,16]. Receptor-blocking antibodies and inhibitors were injected intraplantarly in the vicinity of the calcaneus bone into the hindpaw ipsilateral to tumor induction. We injected the following reagents at days 1, 3, 5, 7 and 9 after fibrosarcoma cell implantation in the vicinity of the tumor: antibodies against the G-CSF receptor (10 μg diluted in 50 μl; sc-9173; Santa Cruz Biotechnology) or against the GM-CSF receptor α subunit (10 μg diluted in 50 μl; sc-25472; Santa Cruz Biotechnology), normal rabbit IgG (10 μg diluted in 50 μl; sc-2027; Santa Cruz Biotechnology), the PI(3) kinase inhibitor LY 294002, the Jak family tyrosine kinase inhibitor AG 490, the MEK inhibitor PD 98059 (10 μg inhibitor diluted in 20 μl normal saline; Invitrogen) or the corresponding vehicle (4% DMSO in 0.9% NaCl). Mechanical hyperalgesia was measured using a von Frey monofilament with the bending force of 0.07*g*[6,16] applied ten times onto the plantar skin overlying the calcaneus bone. Mice were acclimatized to the experimental setups several times before the analysis. The identity of the animals being analyzed was masked for the experimenter.

Immunohistochemistry on epidermal nerve fibers, pancreas and DRG and western blotting. Plantar punch biopsies of the skin overlaying the tumerous calcaneus bone were taken; 30-μm cryotome sections were stained with antibody against PGP9.5 (1:1,000, Chemicon) as described before[6,16]. Human pancreatic samples were cut at 3 μm using a microtome and stained with antibodies recognizing the following: (i) PGP9.5 (a marker for all peripheral nerves; 1:1,000; AB1761, Chemicon/Millipore); (ii) CGRP (recognizing rat and human CGRP; 1:200; cat. no. 24112, Immunostar); (iii) G-CSFR (recognizing mouse, rat and human G-CSFR; 1:100; sc-694 and sc-9173; Santa Cruz Biotechnology); in some experiments specificity controls were established by incubating with blocking peptide (sc-694P; Santa Cruz Biotechnology); and (iv) GM-CSFRα (recognizing human GM-CSFRα; 1:100; sc-690, Santa Cruz Biotechnology); in some experiments specificity controls were established by incubating with blocking peptide (sc-690P, Santa Cruz Biotechnology).

Cryotome sections (16 μm) of wild-type mouse DRG were stained using standard protocols with the following antibodies: (i) anti-G-CSFR (which recognizes mouse, rat and human G-CSFR; 1:100; sc-9173, Santa Cruz Biotechnology); (ii) antibody recognizing mouse GM-CSFRα (1:100; sc-25472, Santa Cruz Biotechnology); and (iii) antibody recognizing mouse and rat CGRP (1:300; BP022, Acris). Biotinylated IB_4 (1:125; B-1205, Vector) was used to detect nonpeptidergic sensory neurons. Please see **Supplementary Methods** for details. Western blots were performed on lysates of melanoma cells, enriched DRG neuronal cultures or mouse DRG with antibodies recognizing mouse G-CSFR (1:1,000; sc-9173, Santa Cruz Biotechnology) and mouse GM-CSFRα (1:1,000; sc-25472, Santa Cruz Biotechnology) or α-tubulin (1:2,500; T9026, Sigma-Aldrich) using standard protocols.

Generation and injection of lentivirions. The following lentiviral constructs expressing shRNAs designed against mouse GM-CSFRα mRNA was obtained from Open Biosystems Inc. (pLKO.1 lentiviral vector), represented as sense-loop-antisense in the 5′→3′ direction: shRNA#1, CCGGCATCACACAGTGCATTGCGAACTCGAGTTCGCAATGCACTGTGTGATGTTTTTG;shRNA#2, CCGGGCCCTGCTCTTCTCCACGCTACTCGAGTAGCGTGGAGAAGAGCAGGGCTTTTTG. Similarly, a control lentiviral construct expressing EGFP from System Biosciences (cat. no. LV500A-1) was used to generate lentivirions expressing EGFP, which served as a negative control for potential damaging effects of lentiviral injection in DRG *in vivo*. Lentivirions were generated using the pPACKH1 packaging system manufacturer's directions (System Biosciences). Approximately 3×10^6 transfection units were added per ml of the culture medium to B16 melanoma cells. Cell lysates were prepared 4 d later and analyzed for expression of GM-CSFRα via western blotting. Lentivirions expressing either EGFP or shRNA#2 against GM-CSFRα were injected into DRG *in vivo* using a modified version of a published method[18]. Briefly, lentiviral preparations ($\sim 9 \times 10^9$ transfection units per ml) were diluted 1:2 with 20% mannitol and injected unilaterally into L4 and L5 DRGs (2 μl per DRG, or 6×10^6 transfection units per DRG) using a 35G needle with a microinjection pump (WPI) at a rate of 500 nl min^{-1} in adult mice deeply anesthetized using fentanyl/domitor/dormicum (4:6:16 vol/vol/vol; 0.7 μl g^{-1}, intraperitoneally). At 3 weeks after viral infection, mice were subjected to tumor induction in the calcaneus bone as described above. Mice were killed on day 13 after tumor induction and the injected L4-L5 DRGs were rapidly isolated and subjected to western blot analysis for GM-CSFRα and tubulin (control).

Data analysis and statistics. All data are presented as mean ± s.e.m. Student's *t*-test or analysis of variance (ANOVA) for random measures was performed followed by *post hoc* Fisher's test to determine statistically significant differences. $P < 0.05$ was considered significant.

Additional methods. Detailed methodology is described in the **Supplementary Methods**.

Hematopoietic colony stimulating factors mediate tumor-nerve interactions and bone cancer pain

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Supplementary online information

Supplementary methods:

Materials from human tissues: Tissue samples were collected from patients following pancreatic resections for pancreatic carcinoma (n = 16). Normal pancreatic tissue samples were obtained through an organ donor procurement program whenever there was no suitable recipient for pancreas transplantation (n = 8). The use of human tissue for the analysis was approved by local ethical committees (University of Heidelberg, Germany) and written informed consent was obtained from the patients prior to the operation.

ENF, pancreas and DRG immunohistochemistry: Punch biopsies of the plantar surface were taken on day 12 and 21 to determine the nerval innervation of the skin overlaying the tumor, as described previously[1]. After perfusion of the animals with Zamboni's solution (2% paraformaldehyde, 0.2% picric acid in PBS, pH 7.6), 4 mm punches were taken and postfixed in Zamboni's solution for 24 h at 4°C and cryoprotected in 30% sucrose in 0.05 M PBS at 4 °C. Punches were cut at 30 µm

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using a cryotome (CM3050 S, Leica Microsystems). After blocking with 5% normal donkey serum in 0.1 M PBS and 0.3% Triton-X-100, sections were incubated with an antibody recognising PGP9.5 (1:1000; Chemicon), which is used as a phenotypic marker for peripheral neurons, in 0.1% normal donkey serum in 0.1 M PBS, 0.3% Triton-X-100 over night. After 3 washes for 1 h with 1% normal donkey serum in PBS, sections were incubated with TRITC-labelled anti-rabbit-antibody (1:200; Dianova,) over night. After 3 washes for 1 h with 1% normal donkey serum in PBS, sections were rinsed with 0.1 M PBS for 1 h and mounted with 1.35% noble agar solution at 60 °C onto coverslips, dehydrated in 95% and 100% Ethanol for 30 min, cleared in methyl salicylate for 30 min and mounted with DPX mounting medium.

Immunofluorescence for PGP9.5 in paw sections was imaged on a confocal laser-scanning microscope (TCS SP2 AOBS, Leica) and maximal projections were created.

Human paraffin embedded pancreatic tissue was cut in 3 µm sections using a microtome (HM 350 S, Microm). The sections were treated with Roticlear (Roth) for 30 min, rehydrated using 100%, 95%, 70%, 50% Ethanol for 5 min subsequentially and kept in warm citrate buffer (200 mM citric acid, pH 6.0) for 20 min. After washing with H2O and PBS for 20 min each, sections were permeabilised using 3% H_2O_2 in Methanol for 20 min, washed again with PBS for 10 min and blocked with 5% normal goat serum and 0.05% Tween-20 in PBS for 30 min. Primary antibodies were applied overnight in 5% normal goat serum, 0.05% Tween-20 in PBS at 4°C. After treatment with 2% normal goat serum, 0.05% Tween-20 in PBS for 30 min, the biotinylated secondary antibody (1:200, Vectastain ABC kit, Vector) in 2.5% normal goat serum in PBS was applied for 30 min. After washing with 1% normal goat serum in PBS and PBS for 20 min, the avidin biotinylated enzyme complex was added for 30 min. After washing with PBS, the sections were stained with diaminobenzidine as substrate for

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the enzyme complex according to the manufacturer's instructions (DAB Substrate kit for peroxidase, Vector) and embedded with mowiol. DRGs were dissected from wildtype mice and cut at 16 µm using a cryotome. Sections were washed with 50 mM glycine in PBS and PBST for 10 min each, blocked with 7% normal horse serum in PBS for 40 min and inoculated with primary antibody against ither the G-CSF receptor (1:100; sc-9173, SCBT) or the GM-CSF receptor alpha subunit (1:100; sc-25472, SCBT) combined with antibody recognising either Isolectin B4, IB4 (1:125; B-1205, Vector) or the Calcitonin Gene Related Peptide, CGRP (1:300; BP022, Acris) overnight. After washing with 7% normal horse serum in PBS, the secondary antibodies anti-rabbit-FITC (1:200), anti-goat-TRITC (1:200) and Streptavidin-TRITC respectively (1:200; against the biotinylated IB4 antibody) were applied for 30 min. After washing with PBS for 20 min, and treatment with 10 mM TRIS-HCl for 10 min, sections were embedded with mowiol and stored at 4°C in the dark.

Culturing and analysis of DRG neurons: Cells were seeded on Poly-L-Lysine coated coverslips and maintained in F12 Media (Sigma) supplemented with 15% Amino Acids (Gibco), 10% horse serum (Invitrogen), 1% Penicillin/Streptomycin (Gibco), 0.5% L-Glutamine (Gibco) and Nerve Growth Factor (100mg ml^{-1}; Roche). Cells were treated with media containing 200 ng ml^{-1} murine G-CSF (sc-4584, SCBT) or murine GM-CSF (sc-4622, SCBT), fixed at given time points and stained with antibodies against phospho-ERK1/2 (1:150; Cell Signalling, Danvers, MA, USA) and phospho-STAT (1:100; Cell Signalling). In some experiments, pharmacological inhibitors, such as AG490 (10 µM), PD98059 (50 µM), LY 294002(50 µM) (all in 4% DMSO) or neutralising antibodies against GM-CSFRα or G-CSFR (4 µg ml^{-1}) or the respective controls (4% DMSO or rabbit IgG) were added to cells 15 min prior to and concurrently with G-CSF or GM-CSF treatment. Cultured cells were fixed with 4%

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PFA, washed with PBS, permeabilised with PBST, blocked with 4% Normal Goat Serum in PBST and inoculated overnight with the primary antibody against pERK1/2 (1:150; Cell Signalling) and pSTAT (1:100; Cell Signalling). After washing with 1% normal serum in PBS the secondary antibody (1:200; anti-rabbit-FITC) and DAPI (1:20.000) were applied for 30 min. After washing with 1% Normal Goat Serum in PBS, PBS and 10mM Tris-HCl, cells were mounted with Mowiol and stored in the dark at 4°C. Positive cells were counted using a fluorescence microscope (DM4000 B, Leica Microsystems) and normalised to the total amount of cells.

Live cell imaging: In live cell imaging experiments, cultured DRG neurons were visualised using a time-lapse imaging system (Leica) in a chamber maintained at 37 ºC at 5% CO2 in culture medium described above. GM-CSF (200 ng ml^{-1}) or the vehicle (PBS) was added to the medium and images were taken every 5 minutes for 24 h. The number of primary and secondary neurites at the specified time points was counted using ImageJ software (National Institute of Health) and normalised to the corresponding initial number of neurites in the same neurons. For analysis of CGRP release DRG cultures were incubated with GM-CSF (200 ng ml^{-1}) or the vehicle for 24 h as described above. 250 µl of supernatants were taken for analysis of basal levels of CGRP, capsaicin (0.1 µM) was added for 30 min. and the remaining 250 µl supernatants were taken for analysis of induced CGRP release. CGRP concentrations were measured using an EIA Kit (A05482, Spibio) according to manufacturer's instructions.

ELISA determination of G-/GM-CSF levels in tissue lysates: Mice with calcaneus bone tumors were killed using CO_2; the heel region around the ipsilateral calcaneus

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bone was dissected and immediately frozen at –80 °C. Contralateral tissue was taken as a control. Tissue was homogenized in PBS containing 0.4 mM NaCl, 0.05% Tween-20, 0.5% normal goat serum, 0.1 M phenylmethylsulfonyl fluoride, 0.1 mM benzethonium chloride, 10 mM EDTA and protease inhibitors (Roche Diagnostics). Pancreatic tissue was pulverized using a mortar and pestle cooled with liquid nitrogen, collected in precooled tubes (Precellys Keramik-Kit 1,4 mm, Peqlab) containing 300 µl lysis buffer (200 mM NaCl, 10 mM TRIS, 5 mM EDTA) with 10% glycine and protease inhibitors (Roche Diagnostics).

Paw and pancreatic homogenates were centrifuged at 13.000 rpm for 10 min at 4°C, the supernatants were collected and subjected to Bradford protein estimation. The levels of G-CSF and GM-CSF were examined with Quantikine ELISA-kits (R&D Systems) according to the manufacturer's instructions and normalised to pg/µg protein per sample.

In-Situ Hybridisation: *In Situ* mRNA Hybridisation with sense and anti-sense digoxigenin-labeled riboprobes detecting G-CSFR and GM-CSFRα was performed on wildtype mouse DRGs using protocols as described before[2]. Sense and anti-sense riboprobes were synthesised corresponding to 124-3054 bp of mouse G-CSFR cDNA (accession number BC116635) and 1004-1813 bp of mouse GM-CSFRα cDNA (Image ID 6492843).

Microdialysis analysis of CGRP release in vivo: Adult male Wistar rats were injected s.c. with GM-CSF (100µl of 5 ng μl^{-1} rat GM-CSF, G0792, Sigma Aldrich) and vehicle on 2 cm^2 of the depilated abdominal skin under deep anaesthesia and five microdialysis membranes were inserted intradermally as described before[3]. Basal microdialysates were collected for 60 min. before capsaicin (100 µl of 1% solution in

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ethanol) was applied to the skin overlaying the membranes. Microdialysates were collected for 60 min. and CGRP concentration was analysed using an EIA Kit (A05482, Spibio) according to manufacturer's instructions.

Single nerve electrophysiological recordings in the skin-nerve preparation: An in vitro skin nerve preparation[4] was used to study the properties of the afferent fibers in the saphenous nerve in control conditions and 30 minutes after the application of 10, 100 and 200 ng ml^{-1} concentration of GM-CSF or the vehicle, 0.9% saline. Animals were killed under CO_2; the saphenous nerve was dissected with the innervated skin attached and placed in organ bath chorium side up. The skin was placed in the oxygen-saturated modified synthetic interstitial fluid solution (123 NaCl, 3.5 KCl, 0.7 $MgSO_4$, 1.7 NaH_2PO_4, 2.0 $CaCl_2$, 9.5 sodium gluconate, 5.5 glucose, 7.5 sucrose, and 10 HEPES, in mM) at temperature of 32 ± 1°C and pH 7.4 ± 0.05, and the nerve was desheathed and teased to enable single-unit recording. Units were classified according to their conduction velocities, von Frey thresholds, and firing properties. Electrical stimulation of the nerve trunk was employed to calculate conduction velocities of individual nerve fibers. Fibers which conducted > 10 m s^{-1} and fibers conducting between 1-10 m s^{-1} were considered to be myelinated A-β fibers and A-δ fibers, respectively. Receptive fields were found using mechanical stimulation with a glass rod. A computer-controlled linear stepping motor (Nanomotor Kleindiek Nanotechnik)[4] was used to apply standardized mechanical stimuli. A hollow metal cylinder was placed above the unit and the unit was tested with ascending series of displacement mechanical stimuli ranging from 6 to 96 μm. The same test was used after the application of either GM-CSF or saline applied directly to the receptive field within the metal cylinder. Electrophysiological data were collected with Powerlab 4.0 system and analyzed off-line with the spike histogram extension of the software.

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Analysis of responses to noxious heat and mechanical pressure: Mice were acclimatised and the latency in response to heat and the threshold to mechanical pressure were measured as described before[5]. After injection of 1, 5, 20 and 100 ng of murine G-CSF or murine GM-CSF dissolved in saline (0.9% NaCl) into the plantar surface, withdrawal latency to heat was measured at 30 min, 4 h and 24 h according to the Hargreaves method using a Plantar test apparatus (Ugo Basile Inc.). Paw withdrawal threshold to mechanical stimuli was determined using a Dynamic aesthesiometer[5] (Ugo Basile Inc.). Saline was given to control animals. Changes in withdrawal latencies or thresholds at the injected paw were represented as percent change over the contralateral, uninjected paw.

Histological analysis of tumor growth: Mice were transcardially perfused with 4% paraformaldehyde in PBS under deep anesthesia, paws were dissected and decalcified by shaking in 10% EDTA in 0.3 M TRIS buffer for at least 21 days. The decalcified paws were dehydrated in ethanol and xylene, embedded in paraffin. Hematoxylin and Eosin staining was performed on microtome sections (10 µm) and tumor area was evaluated in microscopic images using Cell explorer software (BioSciTec) and normalized to the total paw area measured in the same sections.

RT-PCR: Total RNA was isolated from enriched neuronal DRG cultures and in vivo DRG lysates and cDNA was synthesized from 1µg total RNA. Primer sequences:

murine GM-CSFRα[6] GAGGTCACAAGGTCAAGGTG (forward) and GATTGACAGTGGCAGGCTTC (reverse) amplicon size 598 bp

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murine G-CSFR[6] CTCAAACCTATCCTGCCTCATG (forward) and TCCAGGCAGAGATGAGCGAATG (reverse) amplicon size 573 bp

murine Gα12 GGAGTTCCGCGACACCATCTTCG (forward) and GCACTGGAACCACTTCTGGCGC (reverse) amplicon size 451 bp.

References:

1. Cain, D.M.*, et al.* Functional interactions between tumor and peripheral nerve: changes in excitability and morphology of primary afferent fibers in a murine model of cancer pain. *J Neurosci* **21**, 9367-9376 (2001).
2. Agarwal, N.*, et al.* Cannabinoids mediate analgesia largely via peripheral type 1 cannabinoid receptors in nociceptors. *Nat Neurosci* **10**, 870-879 (2007).
3. Obreja, O., Schmelz, M., Poole, S. & Kress, M. Interleukin-6 in combination with its soluble IL-6 receptor sensitises rat skin nociceptors to heat, in vivo. *Pain* **96**, 57-62 (2002).
4. Wetzel, C.*, et al.* A stomatin-domain protein essential for touch sensation in the mouse. *Nature* **445**, 206-209 (2007).
5. Hartmann, B.*, et al.* The AMPA receptor subunits GluR-A and GluR-B reciprocally modulate spinal synaptic plasticity and inflammatory pain. *Neuron* **44**, 637-650 (2004).
6. Olson, M.C.*, et al.* PU. 1 is not essential for early myeloid gene expression but is required for terminal myeloid differentiation. *Immunity* **3**, 703-714 (1995).

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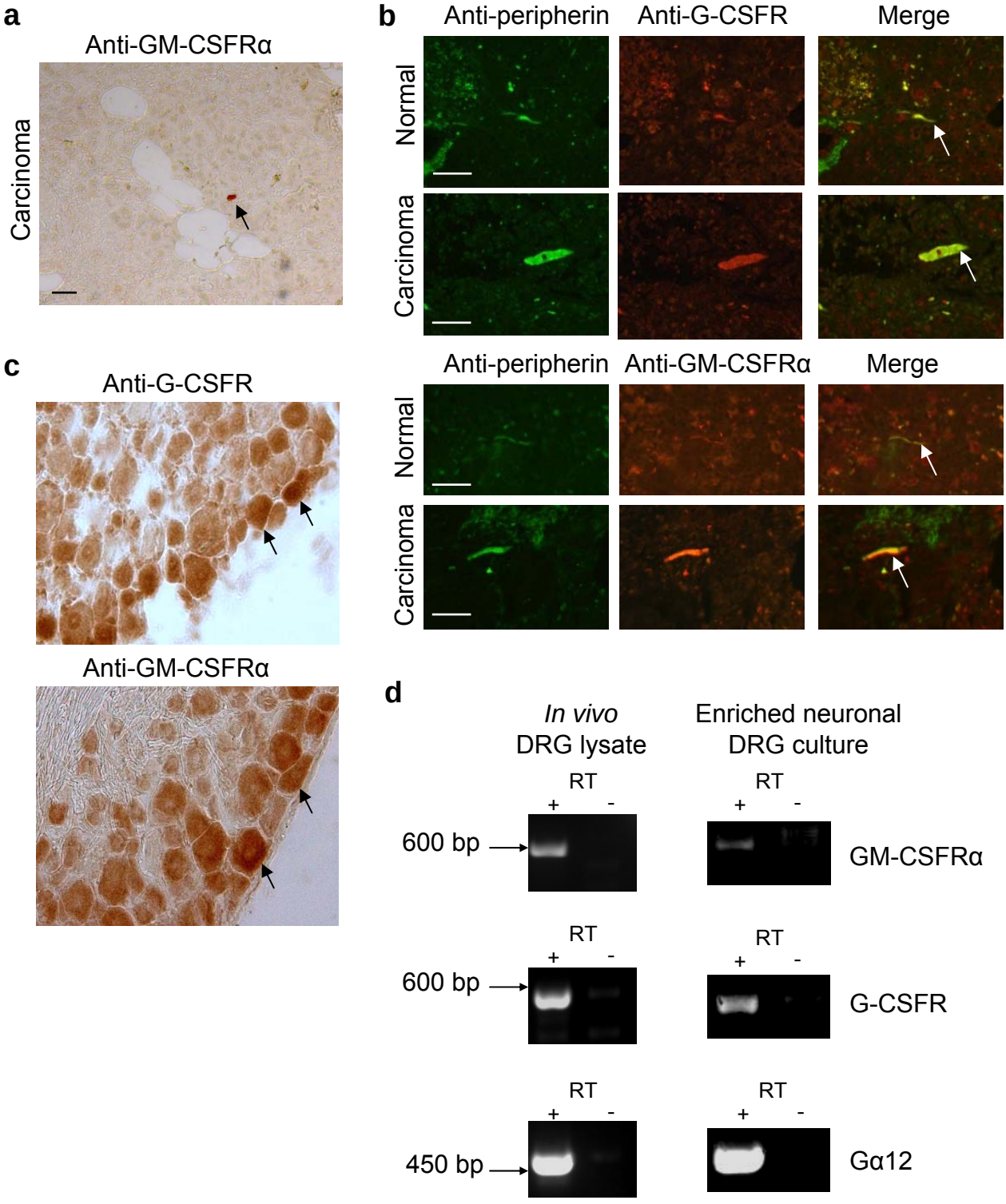
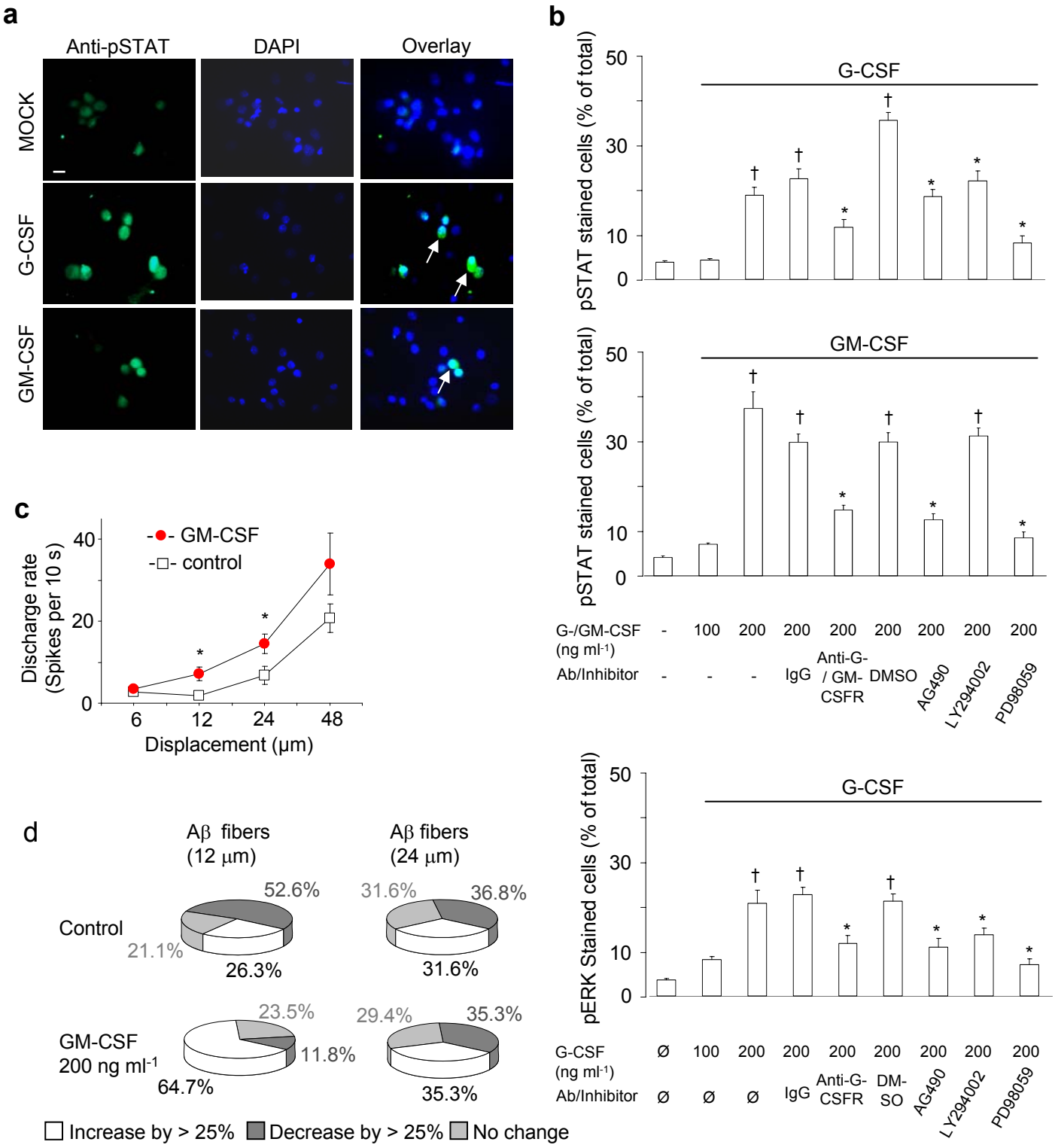
Supplementary Figure 1



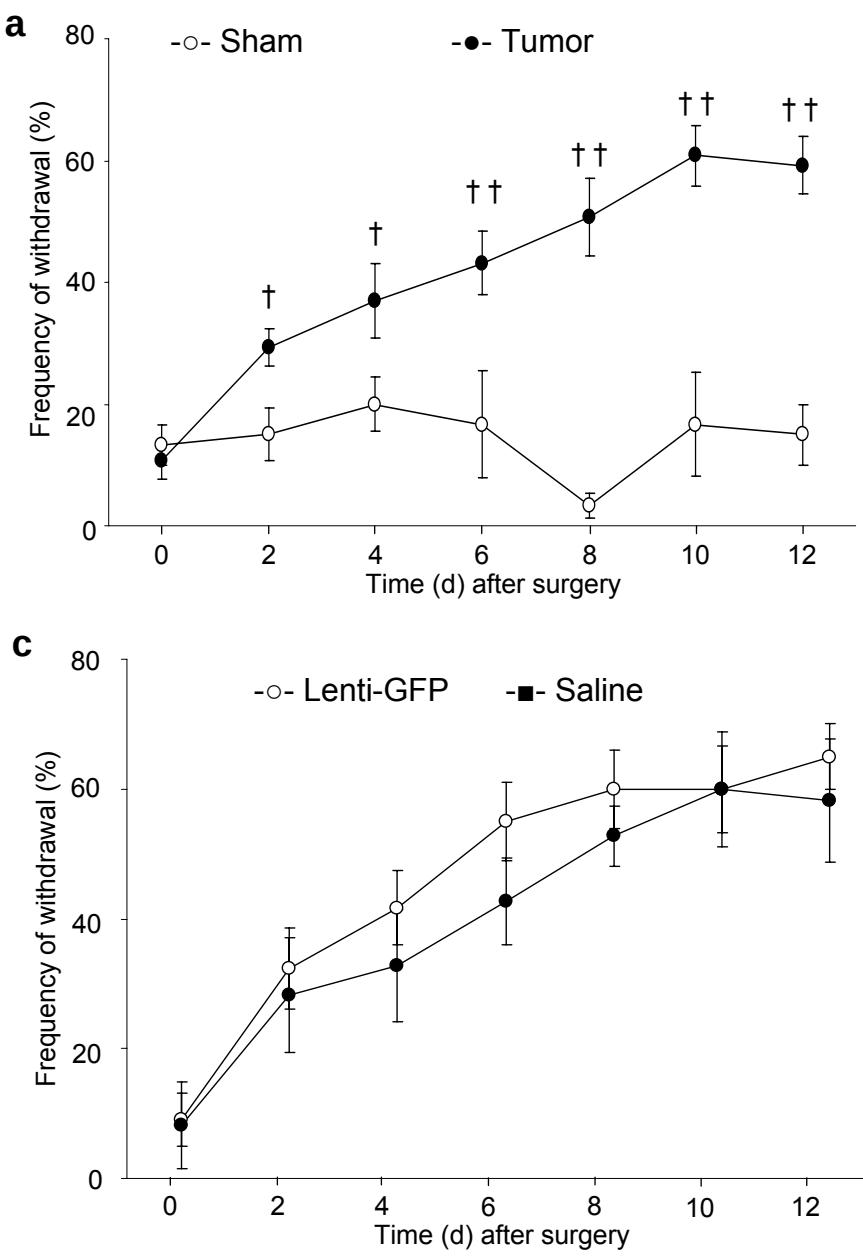
Supplementary Figure 1 Expression of G-CSFR and GM-CSFRα in pancreatic sensory nerves and their cell bodies in the dorsal root ganglia (DRG). (**a**) Analysis of expression of GM-CSFRα in sensory nerves (arrow) in sections from human pancreatic carcinoma biopsies via immunohistochemistry. (**b**) Co-immunofluorescence analysis with the nerve marker protein, peripherin, confirms expression of G-CSFR and GM-CSFRα sensory nerves (arrows) in biopsy sections from human pancreatic carcinoma. (**c**) Immunohistochemistry shows expression of G-CSFR and GM-CSFRα in populations of DRG neurons (arrows). (**d**) Reverse transcriptase (RT)-PCR analysis showing expression of G-CSFR, GM-CSFRα and Gα12 (positive control) in cDNA generated from enriched DRG neuronal cultures and *in vivo* DRG lysates. Scale bars indicate 100 μm in panel a and 30 μm in panel b.

Supplementary Figure 2



Supplementary Figure 2 Activation of STAT3 and ERK1/2 by G-CSF and GM-CSF in cultured DRG neurons. (**a**) Typical examples of phosphorylation and nuclear translocation of STAT3 (arrows) in DRG neurons in culture within 10 minutes after stimulation with GCSF or GM-CSF (200 ng each per ml of medium). Cell nuclei were identified via DAPI fluorescence. (**b**) Effects of blockade of G-CSFR and GM-CSFRα via their respective neutralising antibodies (4 μg ml^{-1}) or of the JAK inhibitor (AG490, 10 μM), PI-3-kinase inhibitor (LY294002, 50 μM) and MEK inhibitor (PD98059, 50 μM) on phosphorylation and STAT3 evoked by G-CSF (100 or 200 ng ml^{-1}, upper panel) and GM-CSF (100 or 200 ng ml^{-1}, middle panel) and of ERK1/2 by G-CSF (100 or 200 ng ml^{-1}, lower panel) in cultured DRG cells. Comparable concentrations of rabbit serum (IgG) and DMSO functioned as controls. † indicates $p < 0.05$ as compared to the vehicle PBS and * indicates $p < 0.05$ with respect to the corresponding cytokine alone; ANOVA followed by post-hoc Fisher's test. (**c**) Stimulus-response curves showing mean changes in spiking rate of Aδ fibers upon treatment with vehicle (control) or GM-CSF (200 ng ml^{-1}). (**d**) Distribution of low-threshold (Aβ) mechanosensitive fibers which either exhibited no change, an increase of > 25% or a decrease of > 25% in responses to displacement (12 or 24 μm) following treatment with GM-CSF or the vehicle, expressed as a fraction of total fibers tested. Scale bar indicates 25 μm in panel a.

Supplementary Figure 3



Supplementary Figure 3 Tumor-induced allodynia and lentiviral injection into mouse DRG *in vivo.* (**a**) Mice with tumors induced in the calcaneus bone of the heel demonstrate increased frequency of responses to innocuous mechanical stimulation (0.07 g force) applied via von Frey hair application to the plantar paw surface in comparison to sham-operated mice. † indicates $p < 0.01$ and †† indicates $p < 0.001$, as compared to values prior to tumor induction or sham treatment, respectively; ANOVA followed by post-hoc Fisher's test. (**b**) Typical fluorescence images of DRG sections observed using a standard FITC filter set from mice injected with GFP-expressing lentivirions in the L4-L5 DRGs *in vivo* or from uninjected control mice. (**c**) Mice injected with lentiviral GFP in L4-L5 DRGs develop comparable hyperalgesia (above) to mice injected with saline following tumor induction in the calcaneus bone in the heel.

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