Abstract—For many patients, pain is the first sign of cancer and, while pain can be present at any time, the frequency and intensity of pain tend to increase with advancing stages of the disease. Thus, between 75 and 90% of patients with metastatic or advanced-stage cancer will experience significant cancer-induced pain. One major unanswered question is why cancer pain increases and frequently becomes more difficult to fully control with disease progression. To gain insight into this question we used a mouse model of bone cancer pain to demonstrate that as tumor growth progresses within bone, tropomyosin receptor kinase A (TrkA)-expressing sensory and sympathetic nerve fibers undergo profuse sprouting and form neuroma-like structures. To address what is driving the pathological nerve reorganization we administered an antibody to nerve growth factor (anti-NGF). Early sustained administration of anti-NGF, whose cognate receptor is TrkA, blocks the pathological sprouting of sensory and sympathetic nerve fibers, the formation of neuroma-like structures, and inhibits the development of cancer pain. These results suggest that cancer cells and their associated stromal cells release nerve growth factor (NGF), which induces a pathological remodeling of sensory and sympathetic nerve fibers. This pathological remodeling of the peripheral nervous system then participates in driving cancer pain. Similar to therapies that target the cancer itself, the data presented here suggest that, the earlier therapies blocking this pathological nerve reorganization are initiated, the more effective the control of cancer pain. © 2010 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: periosteum, breakthrough pain, preventive analgesia.

In 2010, it is projected that twelve million individuals will be diagnosed with cancer and eight million will die from this disease. For many patients, pain is the first sign of cancer and most individuals will experience moderate to severe pain during the course of their disease (van den Beuken-van Everdingen et al., 2007). As such, cancer pain not only causes significant suffering but contributes to a decreased quality of life, functional status, and greatly increases health care utilization.

Cancer pain is commonly divided into three categories: ongoing pain, spontaneous breakthrough pain, and movement-evoked breakthrough pain (Portenoy and Hagen, 1990; Mercadante and Arcuri, 1998). Ongoing pain, which is the most common form of cancer pain and is frequently the first sign of cancer, usually begins as a dull, aching pain that increases in intensity with time (Portenoy and Lesage, 1999). With disease progression, intermittent episodes of breakthrough pain can occur either spontaneously or with movement of the tumor-bearing organ (Mercadante, 1997; Portenoy and Lesage, 1999; Mercadante et al., 2004). This pain is referred to as breakthrough pain as it “breaks through” the analgesic regimen controlling the ongoing cancer pain. Of all cancers, breakthrough pain is generally the most difficult to fully control, as this pain can be severe, sudden in onset (seconds—minutes), and can occur several times per day. Additionally, the dose of opioids required to fully control this pain is generally significantly higher than that required to control ongoing pain, and administration of high doses of opioids is often accompanied by unwanted side effects such as sedation, somnolence, depression, cognitive impairment, respiratory depression, and constipation (Mercadante, 1997; Portenoy, 1999).

In the past decade there has been progress in understanding some of the mechanisms that drive ongoing cancer pain, which include sensitization of nociceptors by algogenic products released from tumor and associated stromal cells, acidosis, injury, and destruction of nerve fibers by tumor cells and hypoxia (Schwei et al., 1999; Ghilardi et al., 2005; Peters et al., 2005; Sevcik et al., 2005a,b; Mantyh, 2006). What remains largely unknown is why cancer pain usually increases with time and what may drive spontaneous and/or movement evoked breakthrough cancer pain. One largely unexplored possible mechanism that could drive cancer pain is that nerve fibers are not merely static structures that simply respond to the changing tumor environment, but rather can undergo active and pathological remodeling. This pathological reorganization of nerve fibers would then set in place a neuroanatomical
substrate that would not only be highly sensitive to movement of the tumor-bearing organ but also create an "ectopic generator" which could spontaneously discharge with accompanying pain.

Here we provide evidence, using an established model of cancer pain, that sensory and sympathetic nerve fibers can undergo a profound and pathological reorganization which, in other pain states, is known to give rise to severe movement evoked and spontaneous chronic pain. This pathological sprouting and neuroma formation appears to be driven by nerve growth factor (NGF), as sequestration of NGF using an anti-NGF antibody largely blocks this pathological reorganization and inhibits the development of severe cancer pain.

**EXPERIMENTAL PROCEDURES**

**Culture and injection of tumor cells**

All procedures were approved by the Institutional Animal Care and Use Committee at the Minneapolis VA Medical Center (Minneapolis, MN, USA) and the University of Arizona (Tucson, AZ, USA). All efforts were made to minimize the suffering and number of animals used. A total of 70 mice were evaluated in this study, 43 of which were injected with osteolytic murine sarcoma cells (NCTC 2472, ATCC, Rockville, MD, USA) stably transfected with green fluorescent protein (GFP). These cancer cells were injected into the femoral intramedullary space (left femur) of male C3He/H11001 mice (8 weeks old, Jackson) according to previously described protocol (Sevcik et al., 2004, 2005b). Following induction of general anesthesia with ketamine (Ketaset, Fort Dodge Animal Health, Fort Dodge, IA, USA), xylazine (AnaSed, Lloyd Laboratories, Shenandoah, IA, USA) (100 mg/kg:5 mg/kg, i.p.), an arthrotomy was performed exposing the condyles of the distal femur. The bone was cored with a 30 gauge needle inserted at the level of the intercondyral notch. The coring needle was then replaced with a 29 gauge hypodermic needle used to inject either Hank’s buffered saline (HBSS) as the control (Sigma, 2029 gauge hypodermic needle used to inject either Hank’s buffered saline (HBSS) as the control (Sigma, 2000). The anti-NGF antibody possesses a plasma half-life of approximately 5–6 days in the mouse and it does not appreciably change, Shenandoah, IA, USA) (100 mg/kg:5 mg/kg, i.p.), an arthro-omy was performed exposing the condyles of the distal femur. The bone was cored with a 30 gauge needle inserted at the level of the intercondyral notch. The coring needle was then replaced with a 29 gauge hypodermic needle used to inject either Hank’s buffered saline (HBSS) as the control (Sigma, 20 µl) or HBSS containing 10^5 sarcoma cells (20 µl) into the intramedullary space. In order to prevent cell reflux following injection, the injection site was sealed with dental grade amalgam (Dentsply, Dentsply, Milford, DE, USA) using an endodontic messaging gun (Union Broach, Emigsville, PA, USA), followed by copious irrigation with sterile filtered water (hypotonic solution). Wound closure was achieved using a single 7 mm auto wound clip (Becton Dickinson, Sparks, MD, USA).

**Anti-NGF treatment**

The anti-NGF sequestering antibody (mAb 911, Rinat/Pfizer), is effective in blocking the binding of NGF to both TrkA and p75 NGF receptors and inhibiting TrkA autophosphorylation (Hongo et al., 2000). The anti-NGF antibody possesses a plasma half-life of 6.9 at 4 °C. Unlike our previous work involving therapeutic administration of anti-NGF (Sevcik et al., 2005b) in which the mice were sacrificed at day 14 post-cancer injection, mice were sacrificed at day 20 following cancer cell injection in the present study. This change was incorporated because of the observation that although at day 12 the osteolytic sarcoma cells began to migrate from the marrow space via nutrient foramens, inducing microfractures and cortical lesions, an extended experimental period allowed a more comprehensive examination of nerve fiber sprouting and neuroma formation.

After sacrifice and perfusion, mouse femurs were removed, post-fixed for 4 h in the perfusion fixative and placed in PBS solution at 4 °C.

**Radiographic analysis of tumor-induced bone destruction**

After behavioral analysis, mice were lightly anesthetized (2% isoflurane) and digital radiographs (MX20 DC12, Faxitron XRay, Lincolnshire, IL, USA) of lower extremities were obtained. Radiograph images of the medial-lateral plane of both bones were used to evaluate tumor-induced bone destruction as previously performed (Honore et al., 2000). Radiographs of tumor-bearing femora were used to evaluate bone destruction and were assigned scores of 0–4: 0, normal bone with no signs of destruction; 1, small radiolucent lesions indicative of bone destruction (1–3 lesions); 2, increased number of lesions (3–6 lesions) and loss of medullary bone; 3, loss of medullary bone and erosion of cortical bone; 4, full-thickness unicortical bone loss. Analysis was performed in a blinded fashion.

**Euthanasia**

At day 20 post sarcoma cell injection, mice were sacrificed using CO2 delivered from a compressed gas cylinder and perfused intracardially with 20 ml of 0.1M phosphate buffered saline (PBS, pH=7.4 at 4 °C) followed by 20 ml of 4% formaldehyde/12.5% picric acid solution in 0.1 M PBS (pH=6.9 at 4 °C). Unlike our previous work involving therapeutic administration of anti-NGF (Sevcik et al., 2005b) in which the mice were sacrificed at day 14 post-cancer injection, mice were sacrificed at day 20 following cancer cell injection in the present study. This change was incorporated because of the observation that although at day 12 the osteolytic sarcoma cells began to migrate from the marrow space via nutrient foramens, inducing microfractures and cortical lesions, an extended experimental period allowed a more comprehensive examination of nerve fiber sprouting and neuroma formation.

After sacrifice and perfusion, mouse femurs were removed, post-fixed for 4 h in the perfusion fixative and placed in PBS solution at 4 °C.

**Micro-computed tomography (µCT) analysis**

In order to characterize disease progression and cancer-induced changes in mineralized bone micro-architecture, femurs were analyzed with an eXplore Locus SP micro-computed tomograph (µCT, GE Healthcare, London, ON, Canada). This cone beam µCT scanner uses a 2300×2300 CCD detector with current and voltage set at 80 µA and 80 kVp, respectively. Specimens were scanned in 1080 views through 360° with a 2100 ms integration time. Scans were then reconstructed at 16-µm3 resolution using Recontruction Utility software (GE Healthcare, London, ON, Canada). µCT parameters used to assess disease progression and cancer-induced bone deterioration included cortical thickness, bone mineral density, trabecular number, and Bone Volume/Tissue Volume (BV/TV).
Immunohistochemistry

Femurs were processed for immunohistochemistry, and were visualized either as periosteal whole mounts (bird’s eye view of the periosteum) or as frozen sections (lateral/cross-sectional view). Of the 70 total mice evaluated in the present report, 37 were used for whole mount preparations. For whole mount preparations, periosteum from the diaphyseal shaft was removed and processed for immunohistochemistry according to the following procedure adapted from previous studies (Mach et al., 2002). Briefly, excess muscle was carefully removed from the femur using surgical scissors without disturbing the bone and attached periosteum. Periosteum was harvested from the distal growth plate region to immediately below the third trochanter. Periosteum was removed from the bone by tracing the lower and upper limits of the desired area with a micro scalpel blade and a vertical cut was then performed along the posterior surface of the bone. Under a dissecting microscope, the periosteum was removed by gently scraping against the bone using the edge of forceps (Brownlow et al., 2000). In our hands, the technique described above resulted in maximal preservation and minimal damage to both the cambium and fibrous layers of the periosteum. During periosteum removal, femurs were continually irrigated with PBS to prevent tissue dehydration. The size of the periosteal whole mount preparation and its attached thin muscle layer used for immunohistochemistry was approximately: width=6 mm; length=6 mm; thickness=0.5 mm.

Preparations were blocked with 3% normal donkey serum for 1 h and incubated with primary antibodies overnight. Peptide-rich sensory nerve fibers were labeled with rabbit anti-calcinogen gene related peptide (CGRP, 1:12,000; Sigma, St. Louis, MO, USA) (Lawson et al., 1993). Myelinated primary sensory nerve fibers were labeled with chicken anti-NF200 (200 kD neurofilament H, 1:1000; Chemicon, Billerica, MA, USA) (Lawson and Waddell, 1991). Post-ganglionic sympathetic nerve fibers were labeled with rabbit anti-tyrosine hydroxylase (TH, 1:1000; Chemicon). Nerve fibers positive for tropomyosin receptor kinase A (TrkA) were labeled with goat anti-NGF or late/acute anti-NGF (Yen et al., 2006). For each femur, we obtained approximately 32 separate frozen sections, each section being cut at 20 μm intervals with a 40× objective. For double immunofluorescence, preparations were incubated with a mixture of primary antibodies followed by a Cy3 and Cy5-conjugated secondary antibodies (1:600; Jackson ImmunoResearch, West Grove, PA, USA) for 1 h. For double immunofluorescence, preparations were incubated with a mixture of primary antibodies followed by a Cy3 and Cy5-conjugated secondary antibodies.

Preparations were then washed with PBS 3 times and counterstained with DAPI (4′,6-diamidino-2-phenyl-indole, dihydrochloride, 1:30,000, Molecular Probes, OR, USA) for 5 min and washed with PBS. Finally, tissue was dehydrated through an alcohol gradient (70, 80, 90, and 100%, cleared in xylene, mounted (with attached muscle layer in contact with the slide) on gelatin-coated slides, and coverslipped with di-n-butyl phthalate-xylene (Sigma). Preparations were allowed to dry at room temperature for 12 h before imaging.

To view the lateral/cross-sectional areas of periosteum attached to the bone, decalcified frozen sections of the left femur were obtained. Of the total 70 mice, 33 were used for frozen sections. Mouse femurs were gently decalcified in 10% EDTA, for ~2 weeks, at which point, as evaluated by radiographic analysis, the femur reaches the minimum decalcification necessary for cryostat sectioning. Femurs were then cryoprotected by immersion in a 30% sucrose solution for 48 h, and then serially sectioned along the longitudinal axis at a thickness of 30 μm. Frozen cross-sections were stained with the same methodology and antibodies used for whole mount preparations.

Laser confocal microscopy

The Olympus Fluoview FV1000 system (Olympus, Melville, NY, USA) used in the present study is equipped with different lasers (Multiline argon (458, 488, 515 nm), Green HeNe (543 nm), Red HeNe (633 nm), and Blue Diode (405 nm)) and multiple excitation and emission fluorescence filters. The endogenous GFP signal emitted by the sarcoma cells did not require amplification for analysis. The GFP expression was visualized by using an excitation laser beam of 488 nm wavelength, and emissions were detected using a 522 nm emission filter. Sequential acquisition mode was used to reduce bleed-through.

For whole mounts, confocal images were obtained from the mid-diaphysis. Images were projected from 280 optical sections at 0.25 μm intervals with a 40× objective. For frozen sections, images were acquired in the proximal metaphyseal periosteum (~2 mm below the growth plate). Confocal images of frozen sections were acquired from sections 30 μm in thickness and were projected from 120 optical sections at 0.25 μm intervals with a 40× objective.

The 3D renderings of bone with overlaid nerve fibers were assembled and created with the use of Amira software (Visage Imaging, Inc., San Diego, CA, USA). For each 3D rendering, four 40× confocal images were acquired and overlaid, to scale, on top of the image’s corresponding bone, which was rendered using μCT slices compiled with Amira software (Visage Imaging, Inc., San Diego, CA, USA).

Sprouting and neuroma quantification

For quantification, frozen sections were used, as the cross sections allow visualization of the bone’s landmarks (such as the growth plate), which enable the observer to locate the same anatomical area when quantifying different animals. The number of animals used for frozen section analysis was: n=8 for sham + vehicle, n=9 for sarcoma + vehicle, n=9 for sarcoma + early/sustained anti-NGF, and n=7 for sarcoma + late/acute anti-NGF.

For each femur, we obtained approximately 32 separate frozen sections, each section being cut at 20 μm. For each given marker, 3 images were obtained. Each image was acquired 2 mm from the proximal growth plate, with images taken from different sections at least 0.1 mm apart. The area of periosteum that was analyzed was an average 620 μm (length), 70 μm (width), and 20 μm (depth). The Z-stacked images were analyzed with Image-Pro Plus v. 6.0 (Media Cybernetics, Bethesda, MD, USA) and nerve fibers were manually traced to determine the length of nerve fibers. In each image the area of the periosteum was determined using the built-in area tool. Nerve sprouting was reported as total length of nerve fibers per volume of periosteum (Yen et al., 2006).

To quantify the extent of formation of neuroma-like structures, frozen sections were examined with a fluorescent microscope and these structures were manually counted and totaled from the entire 30 μm-thick section. Three different sections, each at least 0.1 mm apart, were evaluated per animal. A neuroma-like structure was defined as satisfying all three of the following characteristics: (i) a disordered mass of blind ending axons (CGRP or TH) that has an interlacing and/or whirling morphology, (ii) a structure with a size of more than 10 individual axons as assembled and created with the use of Amira software (Visage Imaging, Inc., San Diego, CA, USA) and nerve fibers were manually traced to determine the length of nerve fibers. In each image the area of the periosteum was determined using the built-in area tool. Nerve sprouting was reported as total length of nerve fibers per volume of periosteum (Yen et al., 2006).

Statistical analysis

Data are presented as mean ± SEM. Immunohistochemical and pain behavioral data were analyzed across treatment groups using a Kruskal–Wallis nonparametric analysis of variance. Significant main effects of groups were followed by Mann–Whitney nonparametric t-tests with Bonferroni adjustment for multiple comparisons.
RESULTS

Tumor growth induces exuberant sprouting of sensory and sympathetic nerve fibers and the formation of neuroma-like structures

In the present report, tumor-induced changes were examined in the periosteum, as this bone compartment is richly innervated by sensory and sympathetic nerve fibers (Mach et al., 2002) and appears to be pivotally involved in detecting injury to the skeleton (Inman and Saunders, 1944; Greenfield, 2006). Additionally, the periosteum is the only bone tissue that can be immunohistochemically analyzed in both decalcified frozen sections and non-decalcified whole mount preparations (Jimenez-Andrade et al., 2010). In comparing the organization and density of CGRP$^+$ (Figs. 1A and 2A), NF200$^+$ (Fig. 3A), or TH$^+$ (Fig. 3D) periosteal nerve fibers across the naïve and sham operated groups, there did not appear to be any difference in naïve versus sham operated mice (data not shown). However, it is clear that in both naïve and sham operated mice, the organization and density of CGRP$^+$ and NF200$^+$ nerve fibers is very different from TH$^+$ sympathetic nerve fibers. Thus, whereas CGRP$^+$ and NF200$^+$ sensory nerve fibers have a net-like organization and are not closely associated with blood vessels, TH$^+$ sympathetic nerve fibers innervate and wrap blood vessels in a tight corkscrew-like fashion.

Six days following tumor injection into the intramedullary space of the mouse femur, we observed significant osteoclast-mediated bone resorption, the first signs of osteolytic lesions, and significant bone cancer pain. By 12 days post-tumor injection, GFP$^+$ tumor cells had completely filled the intramedullary space and, either via...
growth through the nutrient foramen or tumor induced micro-fracture of the bone, GFP\(^+\) tumor cells had begun to grow between the outside of the cortical bone and the overlying periosteum as revealed by immunohistochemical analysis (data not shown). By 20 days post-tumor injection, we observed significant sprouting and formation of neuroma-like structures by CGRP\(^+\) (Figs. 1B and 2B), NF200\(^+\) (Fig. 3B), and TH\(^+\) (Fig. 3E) nerve fibers. Sprouting CGRP\(^+\),

Fig. 2. Preventive sequestration of NGF reduces CGRP\(^+\) and TrkA\(^+\) nerve fiber sprouting and the formation of neuroma-like structures in the periosteum of tumor-injected mice. Representative confocal images of femoral sections from sham + vehicle (A, D), sarcoma + vehicle (B, E), and sarcoma + early/sustained anti-NGF (C, F) mice. Decalcified bone sections were double-immunostained with an antibody against CGRP (orange in A–C) and an antibody against TrkA (cognate receptor for NGF, red in D–F). Confocal images were acquired in the proximal metaphyseal periosteum (~2 mm below the growth plate) using a sequential acquisition mode to reduce bleed-through. Note that at day 20 post-injection there is sprouting by CGRP\(^+\) nerve fibers in sarcoma + vehicle mice (B) and that nearly all the sprouted CGRP\(^+\) nerve fibers also express TrkA (E). Nerve fibers that undergo sprouting have a pathological and disorganized morphology as compared with nerve fibers innervating sham bones. Preventive sequestration of NGF (10 mg/kg i.p., given at days 6, 12, and 18 post cell injection) significantly reduces the pathological tumor-induced reorganization of sensory CGRP\(^+\) (C) and TrkA\(^+\) (F) nerve fibers. Confocal images were acquired from bone sections (20 \(\mu\)m in thickness) and were projected from 80 optical sections at 0.25 \(\mu\)m intervals with a 40× objective.

Fig. 3. Preventive sequestration of NGF inhibits tumor-induced nerve fiber sprouting of NF200\(^+\) sensory nerve fibers and TH\(^+\) sympathetic nerve fibers. Representative confocal images of decalcified bone sections from sham + vehicle (A, D), sarcoma + vehicle (B, E), and sarcoma + early/sustained anti-NGF treated mice (C, F). Decalcified bone sections were separately stained with an antibody against 200 kD Neurofilament (NF200, a marker for myelinated sensory nerve fibers, white in A–C) and an antibody against Tyrosine Hydroxylase (TH, a marker for sympathetic nerve fibers, white in D–F). Note that at day 20 post cell injection, GFP\(^+\) tumor cells (green) induce ectopic sprouting and formation of neuroma-like structures by NF200\(^+\) and TH\(^+\) nerve fibers in the periosteum (B, E). Previous data has shown that nearly all sympathetic TH\(^+\) nerve fibers and a significant population of NF200\(^+\) sensory nerve fibers express TrkA (Averill et al., 1995; Schmidt et al., 1998) Early/sustained anti-NGF therapy (10 mg/kg i.p., given at days 6, 12, and 18 post cell injection) largely blocks the tumor-induced remodeling of TrkA-expressing NF200\(^+\) and TH\(^+\) nerve fibers (C, F). In all cases, images were acquired in the proximal metaphyseal periosteum (~2 mm below the growth plate). Confocal images were acquired from bone sections (30 \(\mu\)m in thickness) and were projected from 80 optical sections at 0.25 \(\mu\)m intervals with a 40× objective.
NF200<sup>+</sup>, and TH<sup>+</sup> nerve fibers appeared to be intermingled among themselves, the GFP<sup>+</sup> tumor cells, and CD68<sup>+</sup> tumor-associated macrophages. It should be noted that in the tumor-bearing tissue not only was there an increased density of CGRP<sup>+</sup>, NF200<sup>+</sup>, and TH<sup>+</sup> nerve fibers, but also a highly pathological and disorganized pattern of innervation that is never observed in the periosteum of naïve or sham operated mice.

Whereas 100% of GFP<sup>+</sup> sarcoma injected mice treated with vehicle showed significant sprouting of CGRP<sup>+</sup>, NF200<sup>+</sup>, and TH<sup>+</sup> nerve fibers, approximately 50% of these mice had 1–2 neuroma-like structures in the periosteum (Fig. 1B, D). These neuroma-like structures appear as a disordered mass of blind ending axons that have an interlacing or whirling morphology and are never observed in sham or naïve animals. Previous studies have shown that neuromas frequently form after nerve injury and are comprised of both sensory and sympathetic nerve fibers (Small et al., 1990; Lindqvist et al., 2000). Given that our antibodies for sympathetic nerve fibers (TH) and sensory nerve fibers (CGRP) are raised in the same species, co-localization experiments were not possible. However, in serial sections of tumor-bearing periosteum consecutively stained for CGRP<sup>+</sup> (Fig. 4A), NF200<sup>+</sup> (Fig. 4B), and TH<sup>+</sup> (Fig. 4C), all 3 populations of nerve fibers seem to be present in the same neuroma-like structure (Fig. 4D). In order to rule out the possibility that the overlap between myelinated and unmyelinated fibers in this model is because of hypertrophy of sympathetic fibers, which is accompanied by a myelination of peripheral fibers, we performed colocalization experiments of TH and NF200 in both normal and tumor bearing bones. Although both antibodies provide robust staining, we have never observed NF200 staining in TH<sup>+</sup> nerve fibers or TH staining in NF200<sup>+</sup> nerve fibers.

**Early sequestration of NGF attenuates tumor-induced nerve sprouting and formation of neuroma-like structures**

To begin to define what factor(s) might drive the sprouting and neuroma formation of CGRP<sup>+</sup>, NF200<sup>+</sup>, and TH<sup>+</sup> nerve fibers, we used double-label immunohistochemistry with an additional antibody to TrkA. While the majority of CGRP<sup>+</sup> and TH<sup>+</sup> cell bodies, as well as some NF200<sup>+</sup> cell bodies, have been shown to colocalize with TrkA (Averill et al., 1995; Schmidt et al., 1998), nearly all the sprouting CGRP<sup>+</sup> (Fig. 2B, E), NF200<sup>+</sup> and TH<sup>+</sup> (data not shown) nerve fibers expressed TrkA (Fig. 2D–F).

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**Fig. 4.** Sensory and sympathetic nerve fibers are observed in the same neuroma-like structures in tumor-bearing mice. Confocal images of serial sections of bone (each 30 µm apart) immunostained with CGRP (serial section 1, red, A), NF200 (serial section 2, blue, B), and TH (serial section 3, yellow, C) from a tumor-bearing mouse. At 20 day post-tumor cell injection, GFP<sup>+</sup> cancer cells (green, A–C) had induced dramatic sprouting and formation of neuroma-like structures by CGRP<sup>+</sup>, NF200<sup>+</sup>, and TH<sup>+</sup> nerve fibers in the periosteum/cortical bone interface. Note that when the confocal images of nerve fibers are merged (D), all three fiber types appear to be present in the same neuroma-like structure, which appears white in (D). The GFP background in (A–C) was acquired from the middle section (serial section 2). The merged image is a Z-stack of confocal images of the 3 types of nerve fibers projected from 80 optical sections at 0.25 µm intervals with a 40× objective.
In light of these findings, and given that TrkA is the cognate receptor for NGF (Pezet and McMahon, 2006), we treated sarcoma-injected mice with a highly specific NGF sequestering therapy (anti-NGF) (Hongo et al., 2000). Data from these studies demonstrated that early/sustained treatment with anti-NGF (given at 6, 12 and 18 days post tumor injection) significantly attenuated the sprouting of CGRP+ (Fig. 2C), NF200+ (Fig. 3C), and TH+ (Fig. 3F) nerve fibers. Interestingly, significant attenuation of this sprouting was only observed with the early/sustained anti-NGF administration but not with late/acute administration (given once at day 18 post tumor injection) (Fig. 5A–C).

Early/sustained administration of anti-NGF similarly resulted in a marked decrease in the formation of neuroma-like structures whereas in the late/acute administration group, as many as 50% of the animals examined had CGRP+, NF200+, and TH+ neuroma-like structures. Importantly, early/sustained administration of anti-NGF did not affect the organization or density of CGRP+, NF200+, or TH+ fibers in the contralateral, non-tumor bearing bones compared to sham mice (Table 1). Additionally, in agreement with previous in vivo studies, anti-NGF therapy had no effect on disease progression as measured by tumor growth within or outside the marrow space, tumor-induced bone destruction/remodeling, or tumor metastasis (Halvorson et al., 2005; Sevcik et al., 2005b).

**Early, but not late sequestration of NGF attenuates tumor-induced pain**

To assess whether the observed aberrant nerve growth correlates with increasing cancer pain, and to determine whether anti-NGF therapy attenuates this pain, pain behaviors were analyzed in tumor-bearing mice treated with early/acute anti-NGF (anti-NGF administered once at day 6), early/sustained anti-NGF (anti-NGF administered at day 6, 12, and 18), and late/acute anti-NGF (anti-NGF administered once at day 18), and compared with sham animals treated with vehicle. These behavioral analyses showed that at early time-points (days 8–12 post tumor cell injection), pain-related behaviors gradually increased with time (Fig. 6A), and correlate with tumor growth in the intramedullary space of the femur, as well as progressive tumor-induced bone destruction. Interestingly, pain behaviors appear to escalate more rapidly upon the escape of sarcoma cells from the intramedullary space (days 12–20 post tumor injection) (Fig. 6A), which correlates with tumor-induced sprouting of CGRP+, NF200+, and TH+ nerve fibers (Figs. 1B, 2B, and 3B, D). Behavioral analysis revealed that when anti-NGF was given at day 6 post tumor injection, pain behaviors are reduced by ~40% by day 8, whereas early/sustained administration of anti-NGF from days 6–18 reduced pain behaviors by ~60% at day 20. In contrast, when anti-NGF was administered late (on day 18), it did not produce a statistically significant reduction in cancer pain behaviors at day 20 (Fig. 6B).

**Table 1. Anti-NGF does not affect the density of nerve fibers in the non-tumor bearing bone**

<table>
<thead>
<tr>
<th>Marker</th>
<th>Sham + vehicle density of nerve fibers (mm/mm²)</th>
<th>Contralateral + early/sustained anti-NGF density of nerve fibers (mm/mm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CGRP</td>
<td>1669±288 (n=8)</td>
<td>1594±86 (n=6)</td>
</tr>
<tr>
<td>NF200</td>
<td>1535±215 (n=8)</td>
<td>1620±81 (n=6)</td>
</tr>
<tr>
<td>TH</td>
<td>1053±200 (n=8)</td>
<td>1112±96 (n=6)</td>
</tr>
</tbody>
</table>

Mean values ± SEM.
Non significant differences following Mann–Whitney non-parametric test.
P-values are: CGRP $P = .945$; NF200 $P = 1.00$; TH $P = .662$.
late/acute administration of anti-NGF.

... which corresponds to the invasion and growth of cells into the intramedullary space of the femur results in significantly escalated pain behaviors in part results from significant sprout-... at day 14 there is a rapid escalation of the pain behaviors in part results from significant sprout-

**Discussion**

In the present report we use a mouse model of bone cancer pain (Schwei et al., 1999; Brainin-Mattos et al., 2006; King et al., 2007) to show that sensory and sympathetic nerve fibers innervating bone undergo a remarkable and pathological reorganization that appears to significantly contribute to cancer pain. In particular, we have shown that when GFP<sup>+</sup> tumor cells growing within the bone marrow escape and invade the periosteum, a rapid and ectopic sprouting of CGRP<sup>+</sup> and NF200<sup>+</sup> sensory, and TH<sup>+</sup> sympathetic nerve fibers occurs in the periosteum. These newly sprouted nerve fibers are intermingled among themselves, the tumor cells, and their associated stromal, inflammatory, and immune cells. Interestingly, this dense and disorganized appearance of sensory and sympathetic nerve fibers is never observed in normal bone. These data are supported by previous reports that show that when provided with the appropriate trophic factor, even adult sensory and sympathetic nerve fibers can grow at a remarkable pace, sprouting several millimeters a day (Cohen et al., 1954; Madduri et al., 2009). In addition to the exuberant sprouting of CGRP<sup>+</sup> nerve fibers, in approximately one out of two tumor-bearing, vehicle treated bones we observed the appearance of occasional but highly recognizable neuroma-like structures. In all cases the neuroma formation occurred when the tumor cells had reached the periosteum. Whether the mechanisms that induce nerve sprouting versus neuroma formation are similar or different is unknown, and future studies are clearly needed to answer this question. As previously described in both animals and humans, these neuroma-like structures appear as a disordered mass of blind ending axons that have an interlacing or whirling morphology (Devor and Wall, 1976; Sung and Mastri, 1983) and are not observed in the sham + vehicle-treated or naïve bone. Previous studies have shown that injury to peripheral nerves associated with trauma, amputation, compression, or surgery can lead to painful neuromas (Devor and Govrin-Lippmann, 1983; Lindqvist et al., 2000; Kryger et al., 2001; Black et al., 2008), which have a morphology similar to the neuroma-like structures observed in the present tumor-bearing mouse bones. In humans, these non-malignant neuromas frequently cause chronic and severe pain (Lindqvist et al., 2000; Devor, 2001; Black et al., 2008), produce spontaneous ectopic discharges (Nystrom and Hagbarth, 1981; Devor and Govrin-Lippmann, 1983; Devor et al., 1990) in part by up-regulation of sodium channels (Devor et al., 1993; England et al., 1996; Black et al., 2008), and are largely refractory to medical treatment (Black et al., 2008). Whether these neuroma-like structures in the tumor-bearing bone also show an up-regulation of sodium channels and produce spontaneous discharges is unknown, but these structures could partially explain the phenomenon of spontaneous breakthrough cancer pain, as movement would not be required for these spontaneous ectopic and painful discharges to occur.

In the normal bone neither unmethylated and thinly myelinated (CGRP<sup>+</sup>) (Lawson et al., 1993) nor myelinated (NF200<sup>+</sup>) (Lawson and Waddell, 1991) nerve fibers are usually found in close contact with TH<sup>+</sup> sympathetic nerve fibers. Thus, whereas the sensory nerve fibers are generally not closely associated with blood vessels that vascularize bone, sympathetic TH<sup>+</sup> fibers show a striking innervation of blood vessels by tightly ensheathing blood ves-
sels in a “corkscrew”-like pattern. In contrast, in areas where there is extensive tumor induced nerve sprouting or neuroma formation, CGRP\(^+\), NF200\(^+\), and TH\(^+\) nerve fibers all appear to have a similar disorganized pattern and appear to be intermingled among each other. While we do not yet know whether areas of intense nerve sprouting eventually become neuromas, in the few cases where we have been able to obtain consecutive serial sections of CGRP\(^+\), TH\(^+\), and NF200\(^+\) from the same neuroma, all three populations of nerve fibers appear to be present. This is of significant interest, as one mechanism that is thought to generate and maintain pain in complex regional pain syndrome (CRPS) is an aberrant sprouting of sympathetic nerve fibers, such that noradrenaline released from sympathetic nerve fibers now excites nearby sensory nerve fibers (Janig and Baron, 2003). As we observe a similar intermingling of sensory and sympathetic nerve fibers in areas of dense sprouting and neuroma-like formation in tumor-bearing bones, this pathological intermingling of sensory and sympathetic nerve fibers may provide an anatomical substrate that drives a sympathetically maintained cancer pain.

To address the question of what factors might be driving the sprouting and formation of neuroma-like structures, we focused on the NGF/TrkA axis, as in the tumor-bearing mice the great majority of CGRP\(^+\) and NF200\(^+\) nerve fibers that undergo sprouting also express TrkA. Previous reports have exhaustively demonstrated that in both the developing and adult animal, NGF can induce marked sprouting of TrkA\(^+\) sensory and sympathetic nerve fibers (Pezet and McMahon, 2006). Using a mouse monoclonal antibody against NGF (anti-NGF) (Hong et al., 2000), we show that while early and sustained administration of anti-NGF results in a marked reduction of sprouting and neuroma-like formation by CGRP\(^+\), TH\(^+\), and NF200\(^+\) nerve fibers in the tumor-bearing bone, this treatment does not reduce the density of normal nerve fibers in the contralateral, non-tumor-bearing bone. Whether NGF drives this sprouting through binding to the TrkA or p75 receptor is as yet unknown, but other studies suggest that TrkA is more involved in driving sprouting (Gallo et al., 1997) while p75 is more involved in apoptosis (Bamji et al., 1998). The precise contributions of these two receptors in these processes, however, remain unclear.

A key unknown is the source of the NGF that appears to be driving the sprouting of sensory nerve fibers and ultimately the pain in the tumor-bearing tissue. Sarcoma cells of the type used here produce small but significant amounts of NGF (Cohen et al., 1954; Sevcik et al., 2005b), suggesting that the tumor may be the primary source of NGF. However, a canine prostate cell line (ACE-1) that does not express any identifiable NGF mRNA (Halvorson et al., 2005) also induces significant bone cancer pain and nerve sprouting that is reduced by treatment with anti-NGF (unpublished data). These data, together with published data suggesting that macrophages, neutrophils, endothelial cells, T-lymphocytes, and fibroblasts can all express significant levels of NGF, suggest that most of the NGF produced in cancers arises from tumor-associated inflamma-tory, immune, and stromal cells rather than from the tumor itself (Lindsay et al., 2005; Pezet and McMahon, 2006). This is not surprising in light of the fact that in most cancers, 10–60% of the tumor mass is composed of these tumor-associated stromal cells (Normann, 1985; Bingle et al., 2002).

To evaluate the effect of acute or chronic dosing of anti-NGF on pain-related behaviors, these behaviors were evaluated in early stages of disease progression at day 8 (after anti-NGF was begun at day 6) and at late stages of disease progression at day 20 (after anti-NGF administration was begun at day 6 or day 18). When anti-NGF administration was begun at day 6 post-tumor injection there was a 40 and 60% reduction when pain behaviors were assessed at days 8 and 20, respectively. In contrast, when anti-NGF was administered late in disease progression at day 18 and pain behaviors assessed at day 20 (when ectopic spouting and neuroma formation had already occurred) there was no significant reduction in pain-related behaviors. It should be noted that a mouse fracture pain model showed that when the anti-NGF therapy was administered at day 1 following fracture, when the pain was severe but nerve sprouting had yet to occur, anti-NGF achieved full analgesic efficacy (a 50% reduction in pain) one day following initial administration (Jimenez-Andrade et al., 2007). Together, these data suggest that once robust sprouting and neuroma formation has occurred, the same dose of anti-NGF that effectively reduced severe pain before the pathological reorganization no longer has potency after the reorganization has occurred. Interestingly, the present data fits well with previous data suggesting that whereas anti-NGF is highly efficacious at inhibiting the regeneration of sensory nerve fibers, anti-NGF showed little if any efficacy at “trimming back” or “pruning” nerve fibers once regeneration had occurred (Diamond et al., 1992). It should be noted that previous studies in our laboratory have shown that anti-NGF therapy is effective at reducing the pain-related behaviors even before the appearance of nerve sprouting and neuroma-like structures. Furthermore, it is important to note that in the present study, animals treated with anti-NGF still display some, albeit reduced, nociceptive behaviors. In light of this, we believe that NGF is involved in sensitizing TrkA\(^+\) sensory nerve fibers and driving nerve sprouting and neuroma formation. However, even in the absence of NGF, tumor-induced acidosis, mechanical distortion of sensory nerve fibers, release of algogenic substances such as bradykinin, endothelin, prostaglandins and proteases all contribute to driving bone cancer pain (Mantyh, 2006). Thus, while NGF is an important player in driving bone cancer pain, it is certainly not the only player.

The present data suggest that at least in bone cancer pain, anti-NGF produces optimal analgesic efficacy when administered early, before the development of a chronic pain state, a concept known as preventive analgesia. Although the concept of preventive analgesia is both intuitive and appealing, its scientific basis is largely unknown. For example, whereas preventive analgesia has been demonstrated in animal models, with experiments showing that...
blockade of early pain reduces synaptic strengthening of
pain circuits in the spinal cord and brain (Woolf and Chong,
1993), human clinical trials have been equivocal (Pogatzki-
Zahn and Zahn, 2006). It remains unclear whether this
failure is caused by problems with the definition of preventiv
analgesia, the design of the studies, the specific types of
pain that have been targeted, or whether there really is
no clinical benefit to preventive analgesia (Ballantyne,
2001). However, the present study identifies a potential
anatomical substrate by which blockade of the NGF/TrkA
axis could produce a clear preventive analgesic effect.
Previous studies have shown that inappropriate remodel-
ing of sensory and sympathetic nerve fibers, whether it be
sprouting or neuroma-like formation, can give rise to hy-
peralgesia, allodynia, and spontaneous ectopic discharges
that are perceived as highly painful in humans (Lindqvist et
al., 2000; Janig and Baron, 2003; Black et al., 2008; Cey-
han et al., 2009).

CONCLUSION
The present data suggest that preventive analgesia by
administration of therapies that block the NGF/TrkA axis
may be significantly more effective than late administration
in reducing the ectopic nerve sprouting as well as pain in a
mouse model of bone cancer pain. Whether the pathologi-
sical sprouting and neuroma-like formation by sensory and
sympathetic nerve fibers is pivotally involved in driving
human cancer pain remains unknown but amenable to
testing in clinical trials with the several NGF/TrkA blocking
strategies that are being developed or are already in hu-
man clinical trials. Understanding whether similar patho-
logical reorganization of sensory and sympathetic nerve
fibers occurs in other malignant and non-malignant chronic
pain states is an open and fertile area for both preclinical
and clinical research.

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