Title: Blocking EphB1 Receptor Forward Signaling in Spinal Cord Relieves Bone Cancer Pain and Rescues Analgesic Effect of Morphine Treatment in Rodents

Abbreviated title: EphB1 receptor is critical to bone cancer pain

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Number of figures and tables: 7/0
Contents of supplemental material: Methods and 9 figures and their legends.
Number of pages: 27
Number of words for Abstract, introduction, and Discussion: 249/415/973
Six keywords: EphB1 receptor, cancer pain, morphine tolerance, NMDARs, MMP-2, MMP-9.

Grant Support: Parker Research Foundation (PCCRF-BSR0890405), Natural Science Foundation of China (30628027 and 81000475) and National Institute of Health (R0166332).

Disclosure of Potential Conflicts of Interest: No potential conflicts of interest were disclosed.
Abstract

Treating bone cancer pain continues to be a clinical challenge and underlying mechanisms of bone cancer pain remain elusive. Here, we report that EphB1 receptor forward signaling in the spinal cord is critical to the development of bone cancer pain and morphine tolerance in treating bone cancer pain. Tibia bone cavity tumor cell implantation (TCI) produces bone cancer-related thermal hyperalgesia, mechanical allodynia, spontaneous and movement-evoked pain behaviors as well as bone destruction. Production and persistence of these pain behaviors are well correlated with TCI-induced upregulation of EphB1 receptor and its ligand ephrinB2 in the dorsal horn and primary sensory neurons. Spinal administration of an EphB1 receptor blocking reagent EphB2-Fc prevents and reverses bone cancer-pain behaviors and the associated induction of c-Fos and activation of astrocytes and microglial cells, NR1 and NR2B receptors, Src within the NMDAR complex, and the subsequent Ca$^{2+}$-dependent signals. The exogenous ligand ephrinB2-Fc upregulates level of phosphorylation of NR1 and NR2B receptors depending on the activation of EphB1 receptor. Spinal administration of EphB2-Fc and ephrinB2-Fc induces downregulation of EphB1 and ephrinB2, respectively, accompanied with increased activity of matrix metalloproteinase (MMP)-2/9. Blocking MMP-2 or MMP-9 reverses EphB1-Fc treatment-induced downregulation of EphB1 receptor. In addition, Spinal blocking or targeted mutation of EphB1 receptor reverses morphine tolerance in treating bone cancer pain in rats and, in mice, defensive pain. These findings demonstrate a critical mechanism underlying the pathogenesis of bone cancer pain and suggest a potential target for treating bone cancer pain and improving analgesic effect of morphine clinically.
Introduction

Bone cancer pain is one of the most common symptoms presented by patients with primary bone sarcomas and predominantly occurs as distant metastases of non-bone primary tumors, notably those in breast, prostate and lung (1, 2). Tumor cells act to cause pain in many ways. Production of prostaglandin and other molecules by tumors, tumor-associated macrophages and other host cells stimulate osteoclast-mediated bone resorption. Nociceptors in bone are stimulated via activation of transient receptors potential vanilloid type-1, endothelin A and TrkA receptor. Activation is directed by acid microenvironment, endothelin-1, and nerve growth factor, respectively (2). Mechanisms of bone cancer pain are complex and may involve a combination of inflammatory and neuropathic pain (2, 3) with unique characteristics. For instance, bone cancer pain markedly increases expression of glial fibrillary acidic protein (GFAP) (4), but not substance P and calcitonin gene-regulated peptide (CGRP) in the dorsal horn (DH) of the spinal cord or galanin and neuropeptides Y in dorsal root ganglion (DRG) neurons (2). Despite decades of thorough study, the specific cellular and molecular mechanisms underlying bone cancer pain remain elusive and the clinical approaches for treating bone cancer pain are limited.

Eph receptors, named for its expression in an erythropoietin-producing human hepatocellular carcinoma cell line, consist of the largest family of receptor tyrosine kinases, which play vital roles in transmitting external signals to the interior of many types of cells. EphB receptors and their ligands ephrinBs are critical cues in regulating cell shape, adhesion/repulsion, migration and positioning during developmental processes (5-7). Alteration of mechanisms controlling adhesion and motility has a central role in promoting tumor invasion and angiogenesis (8-10).
Altered expression of ephrins and/or Eph receptors has been implicated in tumor progression in a number of human malignancies (11-13). Recently, we have found that ephrinB-EphB receptor signaling is involved in pain after nerve injury or opiate withdrawal (14-16). We hypothesized that the ephrinB-EphB receptor signaling might play an important yet unknown role in development of bone cancer pain. EphrinB-EphB interaction is well known to lead bidirectional signals, the forward- and the reverse- signaling (17). There is no clear evidence if either the forward- or the reverse- signaling or both is necessary for production and/or persistence of any pain status. Here, we demonstrate that the forward-, but not the reverse-, signaling of EphB1 receptor in the spinal cord is critical to development of bone cancer pain. Spinal inhibition of EphB1 receptor can relieve bone cancer pain and rescue analgesic effect of morphine in treating bone cancer pain.
Materials and Methods

Animals, anesthesia, drugs, and administration

All animals were used in accordance with the regulations of the ethics committee of the International Association for the Study of Pain and all protocols approved by the Institutional Animal Care and Use Committees. Adult, male, Sprague–Dawley rats, adult, male and female CD-1 mice (Charles River Laboratories) and the EphB1+/− and EphB1+/+ mice (Henkemeyer Laboratory at University of Texas Southwestern Medical center) were used in this study. All surgeries were done under anesthesia with pentobarbital (Sigma, 50mg/kg, i.p.). An EphB1 receptor blocking reagent EphB2-Fc and an EphB1 receptor activator ephrinB2-Fc were used to determine the possible roles of EphB1 receptor in TCI-induced pain-like behaviors and the associated neurochemical alterations. EphB2-Fc can combine with the endogenous ephrinBs and thus EphB1 is substituted and cleaved. This may result in inhibition of the downstream signals of EphB1. EphrinB2-Fc can combine with EphB1 receptor activate EphB1 receptor and the downstream signals. Inhibitors for MMP-2 (MMP2i) and MMP-9 (FN439) were used to determine possible roles of MMP-2/9 in modulation of EphB1. Each of the drugs used was dissolved in PBS or DMSO and then injected intrathecally (i.t. 10µl for mice and 20µl for rats) by means of lumbar puncture at the intervertebral space of L4−5 and L5−6 for multiple injections. These drugs and their final concentrations were: EphB2-Fc (2 or 5µg), ephrinB2-Fc (2µg), FN439 (100µg), MMP2i (10µg), DMSO (1%)(Sigma-Aldrich) and IgG-Fc (2 or 5µg, Jackson laboratories).

Model of bone cancer pain

Tumor cells were extracted from ascitic fluid of rats that received Walker-256 rat
mammary gland carcinoma cells. Tumor cells implantation (TCI) was mimicked by injecting the cells (1×10^5 cells/µl, 5 µl) into the intra-medullary space of the right tibia to induce bone cancer in rats (18).

**Assessment of bone cancer-related pain behaviors**

Thermal hyperalgesia was determined by the significant shortened latency of foot withdrawal in response to heat stimulation. Mechanical allodynia was indicated by a significant decrease in the threshold of paw withdrawal to mechanical indentation of the plantar surface of each hindpaw. The protocols were similar to those described previously (19, 20).

**Western blotting**

Protein precipitation procedures in conjunction with Western blots were employed to identify temporal expression of EphB1, ephrinB2 protein and their phosphorylation. Protocols were similar to those described previously (14, 21). EphB1 and ephrinB2 were immunoprecipitated from 2 mg total protein/ml tissue lysate using an anti-EphB1 antibody (2 µg; Santa Cruz Technology, SCT) complex with protine G-agarose (Invitrogen) and wheat germ agglutinin (WGA) linked to agarose (Sigma), respectively. EphrinB2 and its phosphorylation were detected using anti-ephrinB2 (C20, SCT) and anti-phosphotyrosine antibody PY99 (SCT), respectively (22). EphB1 and its phosphorylation were detected using anti-EphB1 antibody (Q20, SCT) and phosphotyrosine antibody 4G10 (Upstate Biotechnology), respectively (23). NR2A immunoprecipitation was performed using anti-NR2A antibody followed by adsorption to protein G-agarose. Whole cell protein extracts lysates were used to identify temporal expression of the phosphorylated protein levels.
of NR1 (pNR1), NR2B (pNR2B), Src (pSrc), ERK (pERK), CaMKII (pCaMKII), CREB (pCREB) and GAPDH. The primary antibodies were used include pNR1 (Ser897), 1:800 (Millipore); pNR2B (Tyr1472), 1:300 (Chemicon); pSrc (Tyr418), 1:800 (Abcam, Cambridge, UK); pERK1/2 (Thr202/Tyr204), 1:500; pCaMKII (Thr286), 1:1000 (Cell Signaling Technology); GAPDH, 1:1000 (Sigma); GFAP and pCREB (Ser133) (SCT). The filters were then developed using ECL reagents (Perkinelmer) with secondary antibodies (Chemicon). Data was analyzed with a Molecular Imager (Gel DocTM XR, 170-8170) and the associated software Quantity One-4.6.5 (Bio-Rad Laboratories).

**Immunohistochemistry**

Sections of the spinal cord or DRG tissues were incubated with polyclonal rabbit antibodies of anti-EphB1 (1:20) and anti-ephrinB2 (1:20) (SCT) and mouse monoclonal anti-neuronal nuclear protein (NeuN) (1:100) (Alexa Fluor-488 conjugated, MAB377X) (Chemicon). Rabbit IgG (1:200) (Vector Laboratories) was used as an isotype control. Morphologic details were examined with a confocal microscope (Leica TCS SP2, Germany). The protocols were similar to those described previously (14, 21).

**Morphine tolerance tests**

Each of CD-1, EphB1+/+ or EphB1−/− mice was placed on a 55°C hot plate apparatus and the latency to lick a paw was measured following subcutaneous morphine injection. Protocol for testing acute tolerance (Mor100-10): the latency to lick a paw was measured at 0.5, 1, 1.5, and 2 h following the second dose (10mg/kg, i.p.) administrated 24 h after the first dose of morphine (100mg/kg). Protocol for
testing chronic tolerance (Mor10): morphine (10mg/kg) was injected daily for 7 days, and the analgesic effect was measured 30min after each injection. To examine the role of EphB receptor in morphine tolerance, EphB2-Fc (2µg, i.t.) or its Fc control was administrated 30 min prior to the morphine at 100mg/kg in acute tolerance or each of the morphine injection at 10mg/kg in the chronic tolerance in CD-1 mice.

**Statistical analyses**

SPSS Rel 15 was used to conduct all the statistical analyses. Alteration of expression of the proteins detected and the behavioral responses to thermal and mechanical stimuli over time among groups were tested with one-way and two-way ANOVA with repeated measures followed by Bonferroni post hoc tests, respectively. All data are presented as means±SEM. Statistical results are considered significant if $p<0.05$. 
Results

Activity and distribution of EphB1 and ephrinB2 in the spinal cord following TCI

Level of protein expression and phosphorylation of EphB1 receptor and its ligand ephrinB2 in the spinal cord was increased significantly in a time-dependent manner after TCI, while they were at low levels in naïve rats (Fig.1A,B). Phosphorylation of EphB1 was slightly decreased on the postoperative 21 days, but not altered during postoperative 3-14 days since ratio of EphB1 and pEphB1 was remained the same. The increased EphB1 and ephrinB2 immunoreactivity were distributed predominately in the superficial dorsal horn (DH) ipsilateral to TCI (Fig.1C). EphB1 immunoreactivity was colocalized primarily with neurons (Fig.1D) and astrocytes, while only a small amount with microglia (Fig.S1A). EphrinB2 immunoreactivity was colocalized primarily with neurons (Fig.1D) and astrocytes, but not with microglia (Fig.S1B). In primary afferent terminals within the DH and the nociceptors within the DRG, ephrinB2 immunoreactivity was colocalized with peptidergic CGRP-, but not the non-peptidergic IB4-fibers and cells (Fig.S1C, D). These results demonstrate that TCI can cause an upregulation of EphB1 and ephrinB2 expression in the neurons and astrocytes within the DH; the ephrinB2 may originate, at least partly, from the peptidergic CGRP-nociceptive neurons.

Blocking EphB1 receptor activation prevents and suppresses behavioral and neurochemical signs of bone cancer pain

Rats that received TCI exhibited thermal hyperalgesia and mechanical allodynia (Fig.2). TCI rats also exhibited spontaneous pain manifested as guarding and
movement-evoked pain manifested as guarding, flinching and reduced limb use (Fig.S2). Production and persistence of these pain-related behaviors were greatly delayed and suppressed by an EphB1 receptor inhibitor EphB2-Fc administrated (each 5µg, i.t., once a day) at postoperative 3, 4 and 5 day (Fig.2A,C) and postoperative 7, 8 and 9 day (Fig.2B,D and Fig.S2), respectively. Normal pain sensation was not altered. In addition, TCI caused induction of c-Fos and activation of astrocytes and microglial cell in the DH ipsilateral to TCI. These alterations were inhibited by repetitive treatments with EphB2-Fc (Fig.S3). These results suggest that ephrinB-EphB receptor signaling may play an important role in production and persistence of bone cancer pain. In addition, pathological observations (X-ray and hematoxylin-eosin staining) showed that bone destruction associated with tumor growth seemed to be slightly improved following such transient EphB2-Fc treatment (Fig.S4).

**Blocking EphB1 receptor activation suppresses TCI-induced increase of level of pNR1, pNR2B, pSrc, pERK, pCaMKII and pCREB in the spinal cord**

NMDARs have a well-developed role in neural plasticity and in various pain states. Src-family kinases can enhance NMDAR function (24-26) and ephrinB2 may activate NR2B receptor via Src during inflammatory hyperalgesia (27). TCI treatment significantly increased levels of pNR1, pNR2B, pSrc (Tyr418) (Fig.3A), pERK, pCaMKII and pCREB (Fig.3B). Timing patterns of upregulation of these phosphorylated molecules were well matched with that of bone cancer pain-related behaviors (see Fig.2). Repetitive treatment with EphB2-Fc (each 5µg, i.t., once a day on postoperative 7, 8 and 9 day, respectively) inhibited the expression of these molecules. (Fig.3). Expression of pNR2A was neither altered by TCI nor EphB2-Fc
treatment (Fig.3A). These results suggest that TCI-induced activation of NR1 and NR2B receptors, Src and the subsequent Ca\(^{2+}\)-dependent signals may be secondary to activation of the ephrinB-EphB receptor signaling.

**EphB2-Fc inhibits TCI-induced increase in expression of EphB1, but not ephrinB2**

EphrinB-EphB interaction is well known to lead bidirectional signals, the forward- and the reverse- signaling (17). There is no evidence if either the forward- or the reverse-signaling or both is necessary for production and maintenance of any pain status. Here, we showed that TCI-induced upregulation of expression of EphB1 receptor was cancelled by EphB2-Fc (5µg, i.t.) in a single dose on the postoperative 7 day (Fig.4A) or repetitive doses on postoperative 7, 8 and 9 day, respectively (Fig.4B). Inhibition occurred within 2h and persisted for at least a week. EphB2-Fc treatment did not affect TCI-induced increase of expression of ephrinB2 and PY99 (Fig.4C). These results, together with those shown in Fig.3, indicate that EphB1 downregulation may contribute to inhibition of pNR1, pNR2B, pSrc(Tyr418), pERK1/2, pCaMKII and pCREB as well as reduction of bone cancer pain behaviors following EphB2-Fc treatment; while continuous activation of the ephrinB2 is unable to maintain activation of these signals and the pain status.

**EphrinB2-Fc activates NR1 and NR2B and induces thermal hyperalgesia on the condition of EphB1 receptor activation in naïve rats**

Spinal administration of an exogenous EphB1 receptor activator ephrinB2-Fc (2µg) increased the level of pEphB1, but not the overall receptor expression of EphB1 (Fig.5A), and upregulated levels of pNR1 and pNR2B (Fig.5B). It also decreased
ephrinB2 expression, but not its phosphorylation PY99, since the ratio ephrinB2/PY99 was maintained at the same level (Fig.S5A), and resulted in thermal hypersensitivity (Fig.S5B). Repetitive ephrinB2-Fc (2µg, i.t., once a day for 3 consecutive days) produced similar effects on phosphorylation or expression of these molecules and thermal hypersensitivity (Fig.S6). We further examined if ephrinB2-Fc-induced activation of NR1 and NR2B and thermal hypersensitivity would be dependent on activation of the EphB1 receptor. TCI-induced upregulation of EphB1 was inhibited by EphB2-Fc treatment (see Fig.4). In naïve rats, EphB2-Fc treatment (5µg, for 3 consecutive days) greatly inhibited expression of EphB1 and its phosphorylation (Fig.5C). Under this condition, ephrinB2-Fc (2µg) failed to activate or rescue EphB1 (Fig.5C), NR1 and NR2B (Fig.5D), but continued to down-regulate ephrinB2 (Fig.S7A). Additionally, ephrinB2-Fc treatment caused thermal hypersensitivity in naïve rats, but failed to do so when co-administrated with EphB2-Fc (Fig.S7B). These results indicate that activation of EphB1 receptor is required for ephrinB2-Fc to activate NR1 and NR2B and further induce thermal hypersensitivity, suggesting that EphB1 and NR1/2 activation is necessary for the development of thermal hyperalgesia. Downregulation of ephrinB2 is not sufficient or unnecessary for ephrinB2-Fc-induced thermal hypersensitivity.

**Inhibition of MMP-2 or MMP-9 activation prevents EphB2-Fc-induced down-regulation of EphB1 in naïve and TCI rats**

Spinal administration of EphB2-Fc results in downregulation of EphB1 protein, in addition to inhibition of TCI-induced pain-like behaviors and neurochemical alterations. Since EphB-ephrinB interactions can be broken by MMPs, which cleaves EphB receptors (28-30), we examined possible roles of spinal MMP-9/2 in
EphB2-Fc-induced EphB1 down-regulation. With EphB2-Fc treatment, levels of EphB1 and pEphB1 were further downregulated from already low levels in naïve rats (Fig.6A) and, in TCI rats, back to control (Sham) levels from greatly increased levels (Fig.6B), respectively. The EphB2-Fc-induced downregulation of EphB1 was completely prevented by blocking MMP-2 with MMP2i (10µg) or MMP-9 with FN439 (100µg) (Fig.6A,B) and the level of EphB1 was brought back to levels originally seen in naïve (A) and TCI (B) rats, respectively. However, without EphB2-Fc treatment, blocking MMP-2 or MMP-9 inhibited TCI-induced upregulation of EphB1 and brought the upregulated EphB1 back to control (Sham) levels (Fig.6C, left), while keeping EphB1 at its physiological levels in naïve rats (Fig.6C, right). Administration of exogenous MMP-2 or MMP-9 caused an increase rather than a decline in the activation (phosphorylation) of EphB1 without altering the expression of EphB1 protein in naïve rats (Fig.6D, left) and did not significantly alter increased levels of EphB1 in TCI rats (Fig.6D, right). Gelatin zymography showed that activity of both MMP-2 and MMP-2 was greatly increased following EphB2-Fc or ephrinB2-Fc treatment (Fig.S8). These results indicate that MMP-2/9 have different roles, with and without EphB2-Fc treatment, in the modulation of EphB1. With EphB2-Fc treatment, they held EphB1 back to the reference levels which are relatively low in naïve rats and greatly increased in TCI rats. Without EphB2-Fc treatment, they may upregulate the EphB1 level.

**Blocking EphB1 receptor activation rescues analgesic effect of morphine in treating bone cancer pain**

Morphine has been used in reducing cancer pain, particularly in advanced and terminal patients, but its tolerance following repeated use has limited its clinical use.
Given that EphB1 receptor may be a potential target for treating bone cancer pain, we extended our experiments to examine whether morphine tolerance in treating bone cancer pain would be involved in the activation of ephrinB-EphB signaling. Injection of morphine (10 mg/kg, s.c., twice a day, 12 h intervals) significantly suppressed thermal hyperalgesia in TCI rats. Such an analgesic effect decreased and then disappeared (tolerance) 6-7 days after repeated use of morphine (Fig.7A). Co-administration of EphB2-Fc at lower doses (each 2 μg accompanied with morphine), which alone was not enough to reduce the hyperalgesia, successfully rescued the analgesic effect of morphine (prevented morphine tolerance) in treating bone cancer hyperalgesia (Fig.7A). Meanwhile, increased level of phosphorylation and expression of EphB1 following TCI and morphine treatment were inhibited by repetitive EphB2-Fc treatment (Fig.7B).

We further investigated the role of EphB1 receptor in morphine tolerance using standard protocols for testing acute and chronic morphine tolerance in naïve and EphB1 receptor knock out (EphB1⁻/) mice. Both acute (Fig.7C) and chronic (Fig.7D) morphine tolerance were reduced by co-administration of EphB2-Fc (each 2μg, i.t.) with morphine or by targeted mutation of EphB1. Meanwhile, acute (Fig.S9A,B) and chronic (Fig.S9C) morphine treatment significantly increased expression of EphB1 and its phosphorylation, and this effect was inhibited by EphB2-Fc (Fig.S9B,C). These results indicate that the EphB1 receptor is involved in development of morphine tolerance.
Discussion

Our study reveals a critical role for the ephrinB-EphB receptor signaling in bone cancer pain and morphine tolerance in treating bone cancer pain. TCI greatly activates spinal ephrinB2-EphB1 receptor signaling, which then induces activation of NR1 and NR2B receptors and the downstream pathways, resulting in bone cancer pain. EphB1 is required for the production and persistence of bone cancer pain and the functional link between the ephrinB-EphB receptor signaling, NR1 and NR2B receptors and downstream responses. Cleavage of EphB1 receptor may be mediated by MMP-2 and MMP-9. These findings provide a novel mechanism underlying the pathogenesis of bone cancer pain. EphB1 receptor is also required for the development of morphine tolerance. Thus, EphB1 receptor may be a potential target for treating bone cancer pain and reducing opioid tolerance in treating bone cancer pain clinically.

EphB receptors and their ligands, ephrinBs, are critical cues in regulating cell shape, adhesion/repulsion, migration and positioning during developmental processes (5-7, 31). Alteration of mechanisms controlling adhesion and motility has a central role in promoting tumor invasion and angiogenesis (8-10). Here, we show that TCI-induced activation of EphB1 receptor and its ligand ephrinB2 are well correlated with the timing patterns of TCI-induced bone cancer pain behaviors. The increased EphB1 receptor and ephrinB2 are predominantly localized with neurons and some astrocytes in the DH, an essential area for the generation and processing of pain signals. EphrinB2 in the peripheral nociceptors may be transported and redistributed along the CGRP-fibers, to the pre-synaptic membranes of their central terminals, where it triggers a series of postsynaptic effects. EphB receptors regulate synaptic plasticity by interaction with NMDARs (32-34). NMDARs have a well-developed role in neural plasticity and in various pain states. Within the NMDAR complex, the
non-receptor tyrosine kinase, Src, provides dynamic-gain-control of NMDAR function via balancing its activity with striatal enriched tyrosine (24-27, 35). We also show that blocking EphB1 receptor activation largely diminished TCI-induced phosphorylation of NR1 and NR2B receptors, Src(Tyr418), and subsequent activation of various Ca^{2+}-dependent signaling enzymes, CREB, induction of c-Fos and bone cancer-related pain behaviors. Therefore, we hypothesize that peripheral nerve damage due to cancer cell invasion leads to upregulation of the presynaptic ephrinB2 ligand and postsynaptic EphB1 receptor molecules in primary neurons and the DH. Such an increased expression of ephrinB-EphB signaling then leads to postsynaptic EphB1 receptor forward signaling clustering and reciprocal phosphorylation on multiple tyrosine residues (32, 36). Thus, downstream signaling proteins containing SH2 domains, including Src family kinases (36, 37), which are physically associated with EphB receptors and NMDARs and phosphorylate NMDARs on tyrosines (38) may be recruited. This results in the subsequent activation of Ca^{2+}-dependent signaling pathways and behavioral manifestations.

We noticed the fact that ephrinB2-Fc treatment and TCI induced thermal hyperalgesia may involve EphB1 receptor, probably via two different mechanisms. EphrinB2-Fc treatment may induce true EphB1 receptor activation, since it does not affect overall receptor expression, but increases the level of phosphorylation of EphB1. EphrinB2-Fc treatment also downregulates expression of both ephrinB2 and PY99. On the contrary, TCI induces an upregulation of both EphB1 and pEphB1, and ephrinB2 and PY99.

EphrinB-EphB interaction is well known to lead bidirectional signals which are propagated into the ephrinB-expressing cells (reverse signaling) and the EphB-expressing cells (forward signaling). It is unknown if either of the forward- or
reverse-signaling or both are important in pain processing. Our findings in this bone cancer model may support that EphB1 receptor forward signaling is critical to the development and maintenance of pain and activation of NR1 and NR2B receptors as well as subsequent Ca\(^{2+}\)-dependent signals. When EphB1 is inhibited, pain behaviors and NMDARs activation are inhibited even though the ephrinB2 continues to be active. When EphB1 receptor is activated, NMDARs are activated and pain is induced even though ephrinB2 is down-regulated. A recent study, by deleting ephrinB2 in Nav1.8\(^{+}\) nociceptive sensory neurons, indicates that presynaptic ephrinB2 expression may play a role in regulating inflammatory pain and some types of neuropathic pain through the regulation of synaptic plasticity in the dorsal horn (39).

Another interesting phenomenon is that EphB2-Fc treatment results in downregulation of EphB1 protein accompanied with increased activity of MMP-2 and MMP-9 in the spinal cord. Such EphB1 downregulation can be prevented or rescued by blocking MMP-2 or MMP-9. We hypothesize that, during EphB2-Fc treatment, EphB2-Fc combines with endogenous ephrinB2 and thus EphB1 is substituted and cleaved (downregulation) mediated by MMP-2 and/or MMP-9. This results in inhibition of the downstream signals of the EphB1. In contrast, without EphB2-Fc treatment, exogenous ephrinB2-Fc or TCI-induced increased ephrinB2 combines with EphB1 and results in upregulation of EphB1 and activation of the downstream signals. In this process, neither ephrinB2-Fc nor ephrinB2 causes downregulation of EphB1 although it induces activation of MMP-2 and MMP-9, thus, blocking MMP-2 or MMP-9 does not cause downregulation of EphB1. In summary, MMP-2/9 may mediate EphB1 cleavage during EphB-Fc treatment and thus enhance the inhibitory effect of EphB2-Fc on EphB1 signaling; while MMP-2/9 alone may activate rather than cleave EphB1. Interactions between EphB1 and MMPs need to be further
examined and they are beyond the scope of this article.

Cancer pain poses a major challenge clinically. Opioid drugs, with or without coanalgesics/adjuvant analgesics, are used to treat moderate to severe pain. However, the effective use of morphine as a treatment on such pain is limited by morphine tolerance and concerns related to addiction and opioid administration-associated severe, sometimes debilitating side effects (40-43). This study demonstrates that pharmacological blockade or genetic deletion of EphB1 receptor can prevent morphine tolerance in treating bone cancer pain and defensive pain, respectively. In addition, we have recently found that inhibition of EphB1 receptor can prevent or largely diminish morphine physical dependence (14). Thus, blocking EphB1 receptor may be a potential pharmaceutical therapy that can not only relieve bone cancer pain, but also facilitate the clinical utility of opioid drugs in treating bone cancer pain.
References


Figure legends

Figure 1. Upregulation of EphB1 and ephrinB2 in the spinal cord following TCI. A,B, Examples of Western blot analysis and data summary show protein expression and phosphorylation of EphB1 (A) and ephrinB2 and PY99 (B) in a time-dependent manner following TCI. Four samples were included in each group. *P<0.05, **P<0.01 indicate significant differences compared to the group of sham. C,D, Confocal images of immunostaining for EphB1 (green) and ephrinB2 (green) and their colocalization with neurons (NeuN, red) ipsilateral to TCI. Magnification: 100 × (C); 200 × (D).

Figure 2. Spinal blocking EphB receptor activation prevents and attenuates pain-related behaviors after TCI. Thermal hyperalgesia (A,B) and mechanical allodynia (C,D) are shown in the feet ipsilateral to TCI. Administration of EphB2-Fc or its Fc control (each 5µg, i.t.) on the postoperative 3,4 and 5 days (A,C) or 7,8 and 9 days (B,D) are indicated by the arrows. TCI was performed on day 0 (▲). Eight rats were included in each group. *P<0.05, **P<0.01 indicate significant differences compared to the group of control (Sham+Fc). #P<0.05, ##P<0.01 indicate significant differences compared to the corresponding TCI group.

Figure 3. Blocking EphB receptor activation suppresses TCI-induced upregulation of pNR1, pNR2B, pSrc(Tyr418), pERK1/2, pCaMKII, and pCREB in the spinal cord. EphB2-Fc (5µg, i.t., daily) was administrated at postoperative 7, 8 and 9 day,
respectively. Four samples were included in each of pNR1, pNR2B, and pSrc(Tyr418) groups and three in the other groups. *P<0.05, **P<0.01 indicate significant differences compared to the control (Sham and Sham+Fc). #P<0.05, ##P<0.01 indicate significant differences compared to the corresponding group of TCI+Fc on the specific day indicated.

**Figure 4.** Spinal administration of EphB2-Fc inhibits TCI-induced upregulation of expression of EphB1, but not ephrinB2. A, A single dose of EphB2-Fc or its Fc control (each 5 μg) was injected on the 7th day after TCI. B, C, Repetitive EphB2-Fc (EphB2-Fc×3, each 5 μg, once a day) were injected on postoperative 7, 8, and 9 day, respectively. Tissues were collected 2 h after the last treatment with EphB2-Fc or Fc. **P<0.01 indicates significant differences compared to the corresponding group of Sham+Fc. #P<0.05, ##P<0.01 indicate significant differences compared to the corresponding group of TCI+Fc.

**Figure 5.** EphB1 receptor activation is required for ephrinB2-Fc in activating NR1 and NR2B receptors in naïve rat spinal cord. A, B, Spinal administration of ephrinB2-Fc (2μg) activated EphB1 (A), NR1 and NR2B (B). C, D, Pretreatment with EphB2-Fc (5μg, i.t., daily for 3 consecutive days) blocked ephrinB2-Fc-induced activation of EphB1 (C), NR1 and NR2B (D). Four samples were included in each of the groups. Tissues were taken 4h after the last dose of EphB2-Fc and ephrinB2-Fc, respectively, in C and D. **P<0.01 indicates significant differences compared to the
corresponding control group (both Naïve and Fc).

Figure 6. Effects of MMP-2 and MMP-9 on expression of EphB1. A, B, MMP2i and FN439 reversed EphB2-Fc-induced downregulation of EphB1 in naïve (A) and TCI rats (B). C, Effects of MMP2i and FN439 on EphB1 in lack of EphB2-Fc treatment in naïve (left) and TCI (right) rats. D, Effects of MMP-2 and MMP-9 on EphB1 without EphB2-Fc treatment in naïve (left) and TCI (right) rats. Four samples were included in each group. Tissues were taken 4h after the last treatment. *P<0.05, **P<0.01 indicate significant differences compared to the corresponding control group (Naïve or Sham). #P<0.05, ##P<0.01 indicate significant differences compared to the corresponding control group (PBS in A and TCI in B).

Figure 7. Spinal administration of EphB2-Fc rescues analgesic effect of morphine (Mor) in treating TCI-induced thermal hyperalgesia in rats and, in mice, defensive pain. A, Effects of EphB2-Fc and Mor on thermal hyperalgesia. Mor: 10 mg/kg, s.c., twice a day. EphB2-Fc: 2 μg, i.t., once a day. Eight rats were included in each group. B, Effects of EphB2-Fc and Mor on expression of EphB1. Five samples were included in each group. C,D, Effects of EphB2-Fc or targeted mutation of EphB1 on acute (Mor100-10)(C) and chronic (Mor10)(D) Mor tolerance. Data were calculated as percentage of maximal possible effect [MPE (%)]. Ten mice were included in each group. EphB2-Fc or its Fc control (each 2 μg) was administrated 30 min before the first dose of Mor in acute tolerance or each of Mor injection in chronic tolerance model. *p<0.05, **p<0.01 indicate significant differences compared to the corresponding control group, PBS (A), Naïve (B), Mor or EphB1+/+ (C,D). #p<0.05,
##p<0.01 indicate significant differences compared to the corresponding group of Mor.
Fig. 1
Fig 2
Fig 3
Fig 4
Fig. 5
Fig 7
Blocking EphB1 Receptor Forward Signaling in Spinal Cord Relieves Bone Cancer Pain and Rescues Analgesic Effect of Morphine Treatment in Rodents

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Cancer Res  Published OnlineFirst May 9, 2011.

Updated version
Access the most recent version of this article at:
doi:10.1158/0008-5472.CAN-10-3870

Supplementary Material
Access the most recent supplemental material at:
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