

Intrathecal injection of lentivirus-mediated glial cell line-derived neurotrophic factor RNA interference relieves bone cancer-induced pain in rats

Fu-fen Meng,^{1,8} Yang Xu,^{2,8} Qi-qin Dan,^{2,3} La Wei,¹ Ying-jie Deng,⁴ Jia Liu,³ Mu He,⁵ Wei Liu,² Qing-jie Xia,⁵ Fiona H. Zhou,⁶ Ting-hua Wang^{2,3} and Xi-yan Wang⁷

¹Department of Anesthesia, Xinjiang Tumor Hospital, Urumqi; ²Department of Anesthesia, Translational Neuroscience Center, West China Hospital, Sichuan University, Chengdu; ³Institute of Neuroscience, Kunming Medical University, Kunming; ⁴The Second Department of Orthopedics, Xinjiang Traditional Chinese Medicine Hospital, Urumqi; ⁵Translational Neuroscience Center, West China Hospital, Sichuan University, Chengdu, China; ⁶Sansom Institute of Health, University of South Australia, Adelaide, South Australia, Australia; ⁷Department of Hepatopancreatobiliary Surgery, Xinjiang Tumor Hospital, Urumqi, China

Key words

Bone cancer pain, GDNF, lentivirus, RNA interference, spinal cord

Correspondence

Ting-hua Wang, 17#, Section 3, Ren Min Nan Lu, Chengdu, Sichuan 610041, China.
Tel/Fax: +86-28-8550-1036;
E-mail: tinghua_neuron@263.net
and
Xi-yan Wang, 789#, SuZhou East Street, Urumqi, China.
Tel: +86-99-1796-8111;
Fax: +86-99-1796-8002;
E-mail: xjwangxiyan@foxmail.com

⁸These authors contributed equally to this work.

Funding information

This project was supported by the Natural Science Foundation of the Xinjiang Uygur Autonomous Region for young researchers (project no. 2010211B13; project name, The effect of analgesia in bone cancer pain induced by HSV-1 mediated the expression of HPPE).

Received May 1, 2014; Revised December 30, 2014;
Accepted January 11, 2015

Cancer Sci 106 (2015) 430–437

doi: 10.1111/cas.12609

Cancer-induced pain is incapacitating and the most common condition in cancer patients, which can seriously affect their quality of life.^(1,2) Overall, the experience of pain is increased in 75–95% of patients with cancer, and in 30–50% of these who suffered advanced or terminal disease.^(3,4) In addition, 80% of patients also developed chronic pain caused by bone metastases. Due to the relative ineffectiveness and untoward effects of current available therapies, cancer-induced pain is difficult to manage.⁽⁵⁾ Therefore, it is important to find new therapies for pain relief and to clarify the mechanism of bone cancer-induced pain (BCIP).⁽⁶⁾

It has been reported that glial cell line-derived neurotrophic factor (GDNF), a member of the neurotrophic factor family, can sensitize nociceptors and induce behavioral hyperalgesia in an inflammatory pain model.⁽⁷⁾ Some neuromuscular diseases

are associated with both increased release of GDNF and intense muscle pain. Intramuscularly injected GDNF induced a dose-dependent persistent mechanical hyperalgesia in rat models,⁽⁸⁾ and intrathecal injection of anti-GDNF reduced the delayed bilateral hyperalgesia in a Freund's adjuvant-induced chronic pain in a rat model.⁽⁹⁾ These data suggest that GDNF participates in the production of inflammatory pain. However, interestingly, in this study, we found the expression of GDNF mRNA and protein levels were decreased in a bone cancer pain rat model. Therefore, it is important to identify the role of GDNF in bone cancer pain model. RNA interference is a tool to silence expression of target genes by triggering post-transcriptional degradation of homologous transcripts through a multistep reaction involving double-stranded siRNA and the positive feedback amplification effect.⁽¹⁰⁾ Lentiviral (LV)

are associated with both increased release of GDNF and intense muscle pain. Intramuscularly injected GDNF induced a dose-dependent persistent mechanical hyperalgesia in rat models,⁽⁸⁾ and intrathecal injection of anti-GDNF reduced the delayed bilateral hyperalgesia in a Freund's adjuvant-induced chronic pain in a rat model.⁽⁹⁾ These data suggest that GDNF participates in the production of inflammatory pain. However, interestingly, in this study, we found the expression of GDNF mRNA and protein levels were decreased in a bone cancer pain rat model. Therefore, it is important to identify the role of GDNF in bone cancer pain model. RNA interference is a tool to silence expression of target genes by triggering post-transcriptional degradation of homologous transcripts through a multistep reaction involving double-stranded siRNA and the positive feedback amplification effect.⁽¹⁰⁾ Lentiviral (LV)

vectors have emerged as powerful tools in many fields like neuroscience, hematology, developmental biology, stem cell biology, and transgenics.⁽¹¹⁾ Lentiviral vectors used in gene therapy are known as real therapeutic alternatives for many inherited monogenic diseases like Parkinson's disease.⁽¹²⁾ Moreover, LV vectors are currently being explored in human clinical trials.⁽¹³⁾ Lentiviral vectors with a self-inactivating architecture enhance their safety properties.^(14,15) In addition, LV vectors have been inserted into host genomes in the gene coding regions rather than in the promoter regions, which also reduces the risk of proto-oncogene upregulation.^(16–19)

In the present study, we used an LV vector containing an artificial GDNF siRNA to further downregulate the expression of GDNF in the lumbar spinal cord of rats with bone cancer. We found that intrathecal injection of LV-mediated GDNF RNAi (Lvs-siGDNF) may be a novel strategy to attenuate pain-related behaviors by inhibiting the level of substance P (SP) and blocking the pain-related ERK signaling pathway.

Materials and Methods

Complete materials and methods, excluding packaging of the lentivirus, intrathecal catheterization, drug administration, and behavioral analysis, are described in Document S1.

Animals. All animal protocols were carried out in accordance with the guidelines of the International Association for the Study of Pain.⁽²⁰⁾

Cell cultures. Mammary rat metastasis tumor (MRMT-1) cells were cultured in flasks as previously described.⁽²¹⁾

Bone cancer pain model. The model of rat bone cancer pain induced by cancer was established by intratibial injection of MRMT-1 rat mammary gland carcinoma cells as previously described.^(22,23,24)

Lentivirus package containing artificial siGDNF-specific gene (Lvs-siGDNF) or empty psiHIV-U6 vector (Lvs-NC). The vector containing the GDNF siRNA was provided by GeneCopoeia gene company (Rockville, MD, USA): vector size, 9027 bp (backbone only, insert not counted); stable selection marker, puromycin; reporter gene, mcherryFP; hairpin loop sequence, TCAAGAG. The GDNF gene expression cassette vector and Lenti-Pac HIV Expression Packaging Kits (GeneCopoeia, Rockville, MD, USA), containing an HIV packaging mix of the three packaging plasmids, were cotransfected using EndoFectin Lenti transfection reagent into 293Ta cells to package capable lentiviruses, and were defined as pseudovirion siGDNF. Then the viral supernatant was collected into sterile capped tubes 48 h post-transfection and centrifuged at 2000 g for 10 min to get rid of cell debris. Following centrifugation, LV supernatants were harvested and mixed with Lenti-Pac Concentration Solution (GeneCopoeia) at the ratio of 5:1, then centrifuged at 3500 g for 25 min at 4°C after incubate at 4°C for 2 h. The supernatant was then discarded carefully to avoid disturbing the virus pellet. The virus pellet at the bottom of the tube was resuspended using PBS at 1/100 of the original sample volume by gently pipetting up and down. The concentrated viral samples were titrated by infecting HT-1080 cells, and they were stored at –80°C in single-use aliquots until use. The average titer was 3×10^7 infectious units/mL. Lvs-NC was packaged in the same way.

Application of immunosuppressant. Cyclosporine was injected i.p.

Intrathecal catheterization. The operation procedures were modified based on Fang *et al.*⁽²⁵⁾ Briefly, the fourth coccygeal spinous process was removed and the dura mater exposed after

each rat was anesthetized. The dura was punctured by a needle, resulting in some leakage of cerebrospinal fluid. A polyethylene catheter (PE-10, 15 cm) filled with normal saline prior to the procedure was immediately inserted 2 cm through the dura slit into the subarachnoid space. Subsequently, a segment of the catheter near the dura opening was fixed with the surrounding tissues. The rest of the catheter was buried under the skin and the tip of the catheter was punctured through the skin, at the nape of the neck and tightened with silk threads. The catheter orifice was connected to the needle of a microsyringe (50 μ L) that was used for intrathecal injection. The dead space of the catheter lumen was approximately 10 μ L.

All animals were checked the next day for any neurological abnormalities. Lidocaine, 2% (10 μ L) was injected through the catheter to temporarily paralyze the rats' hind limbs to confirm the correct intrathecal localization.

Drug administration. The Lvs-siGDNF and Lvs-NC aliquots were dissolved in PBS (0.01 M) for subarachnoid administration (injection dose, 20 μ L). Salt morphine (10 mg/mL) was dissolved in sterile normal saline at the final concentration (1 μ g/mL) for subarachnoid administration in the same volume.

Behavioral analysis. For each rat, the behavioral tests were carried out pre-surgery to determine the baseline and on days 7, 14, 21, and 28 after tumor injection. After subarachnoid catheter and gene treatment, the tests were measured on days 7 and 14. For the morphine-treated group, the tests carried out pre-injection and 10 min post-morphine application at each time point. For both tests, mean data were established by averaging the records of four tests with a 10-min interval between each animal.

Tail-flick latency (TL) test. Thermal hyperalgesia tests were determined by measuring the time of the rats' tail responded to a radiating thermal stimulus. An automatic tail-flick analgesiometer (7360; Ugo Basile, Varese, Italy) was used in this test. Parameter settings were: intensity, 80; cut-off time, 20 s, which was used to prevent tissue damage. The TL was not recorded automatically from the onset of the test, when the tail was withdrawn, until an abrupt flick of the tail was sensed.

Paw withdrawal threshold (PWT) test. Mechanical nociceptive withdrawal responses were measured using the Randall-Selitto paw pressure device (Bioseb, Chaville, France). The mechanical hyperalgesic threshold was recorded when a rat withdrew its paw. The data were recorded in grams $\times 20$ g.

Statistical analyses. Data were recorded as mean \pm SD; the mean difference was significant at the 0.05 level.

Results

Induction of MRMT-1-induced cancer bone cancer pain model.

Two parameters for hyperalgesia, relative PWT ($\times 20$ g) and TL (s), of survived rats were used to detect the pain induced by bone cancer (Fig. 1a). Reduced bone mineral density was confirmed by X-ray 28 days after MRMT-1 injection into the tibia (Fig. 1b), and increased tumor cells and osteoclasts at the carcinoma injection site stained with H&E were observed 14 days after treatment (Fig. 1c). Consequently, implantation of MRMT-1 cells into the tibia of bilateral hind limbs of rats induced mechanical and thermal hyperalgesia as indicated by decreased PWT and TL (Fig. 1a). X-ray films showed engrafted MRMT-1 cells invaded into the tibia medullary canal (Fig. 1b1, black arrow). Bone destruction characterized as decreased bone mineral density irregularly could be seen in the injection area (Fig. 1b2) 28 days later. A large number of

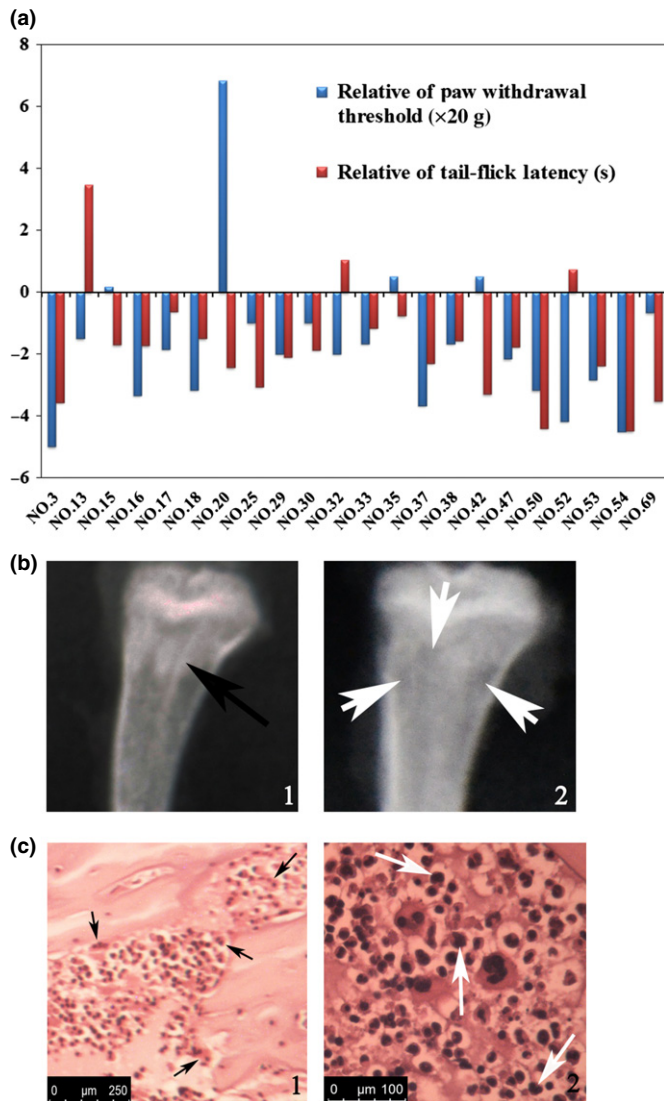


Fig. 1. Establishment of bone cancer pain model. (a) Rats recruited were defined as data from 28 days after injection with MRMT-1 cells minus pre-operation data for the two parameters, paw withdrawal threshold and tail-flick latency tests, which were mostly negative. (b) Radiological confirmation of tumor development in the tibia of rats injected with MRMT-1 cells. Reduced bone mineral density in bone X-rays indicated that the cancerous cells induced bone destruction (b2, arrow). (c) The number of MRMT-1 cells (white arrows) and osteoclasts (black arrows) increased in the tibia section stained with H&E.

MRMT-1 cells, osteoclasts, and macrophages appeared in the marrow (Fig. 1c).

Localization and changes of GDNF in the spinal dorsal horn of BCIP models. In BCIP rats, the lumbar spinal cords showed a significant reduction in the level of GDNF mRNA expression (*vs* normal, $P = 0.024$; Fig. 2a) and protein expression (*vs* normal, $P = 0.000$; Fig. 2b), as detected by quantitative PCR and Western blot, respectively. GDNF positive staining in the spinal cord was mainly found in nerve varicosities located in the superficial layer (Fig. 2c). Some neurons in gray matter were also positively stained.

Construction of recombinant siGDNF and package of Lvs-siGDNF. After transduction with four GDNF siRNA target sequences (Fig. 3a), the PC12 cells showed a significant reduction in the level of GDNF mRNA expression only in F2, F3, and F4 transfected groups ($P = 0.022, 0.018, \text{ and } 0.025$, respec-

tively). Sequence 3 was the most effective interference segment confirmed by RT-PCR (Fig. 3b). Therefore, plasmid containing F3 was used to be packaged in 293T α cells (Fig. 3c). In order to detect the titer of Lvs-siGDNF, the pseudovirus was infected into HT1080 cells (Fig. 3d), then “R” (defined as the percentage of fluorescent cells to the bright field) was 0.87 and “M” (the total number of HT1080 cells) was $4 \times 10^4/\text{mL}$, $T_{\text{Lvs-siGDNF}} = 10^4 \text{RM titer}/\text{mL} = 3.48 \times 10^8/\text{mL}$.

Mechanical and thermal hyperalgesia significantly improved after virus injection. For analysis of the mechanical and thermal hyperalgesia, we used PWT and TL tests (Fig. 4a,b). Bone cancer pain model treated with Lvs-siGDNF showed a significant extension in TL (Lvs-siGDNF *vs* normal, control, Nor+Lvs-siGDNF, saline, and NC, $P = 0.003, 0.000, 0.000, 0.005, \text{ and } 0.000$, respectively) 7 days after intrathecal injection. In this study, morphine, an opioid agonist commonly used to ease pain, was used as a positive control. The effect of Lvs-siGDNF to relieve thermal hyperalgesia was similar to morphine (morphine *vs* control group, $P = 0.002$). Although Lvs-siGDNF treatment showed a trend in the increase in mechanical PWT similar to the significant effect of the morphine-treated group (morphine *vs* normal, Nor+Lvs-siGDNF, control, and saline group, $P = 0.026, 0.000, 0.000, \text{ and } 0.000$, respectively), no statistical significance between Lvs-siGDNF and NC-treated groups was found on day 7 after treatment. However, by day 14 after intrathecal injection, treatment with Lvs-siGDNF significantly abolished both thermal and mechanical hyperalgesia (TL: Lvs-siGDNF *vs* normal, control, Nor+Lvs-siGDNF, saline, and NC, $P = 0.009, 0.000, 0.000, 0.006, \text{ and } 0.000$, respectively; PWT: Lvs-siGDNF *vs* control, saline, and NC, $P = 0.003, 0.016, \text{ and } 0.046$, respectively; Fig. 4b). In addition, treatment with morphine continued to extend the TLs and PWTs 14 days after treatment (TL: morphine *vs* normal, control, Nor+Lvs-siGDNF, and saline, $P = 0.019, 0.000, 0.001, \text{ and } 0.013$, respectively; PWT: morphine *vs* control, Nor+Lvs-siGDNF, and saline, $P = 0.002, 0.000, \text{ and } 0.000$, respectively). Treatment with saline or nothing resulted in significant reduction in PWT compared to normal rats without bone cancer 7 and 14 days after treatment: $P = 0.002, 0.008$ at 7 days; $P = 0.001, 0.006$ at 14 days, respectively. Furthermore, we assessed the translation level of GDNF in lumbar spinal cords of the BCIP model (Fig. 4c). Treatment with Lvs-siGDNF significantly reduced the translation of GDNF (0.07 ± 0.003) compared to saline (0.43 ± 0.08), morphine (0.38 ± 0.11) and vehicle (0.28 ± 0.10) treatment groups ($P = 0.000, 0.002, 0.005, \text{ and } 0.036$, respectively).

Lentivirus-mediated siGDNF downregulated expression of SP. Before investigating the chemical effect of Lvs-siGDNF treatment in our bone cancer model, we examined the expression of SP, a nociceptor in the dorsal horn of rat’s spinal cord. Our finding showed that SP staining existed in laminae I-II of the dorsal horn of normal rats’ spinal cord (Fig. 5a). However, rats with bone cancer showed significant enhanced levels of SP (Fig. 5b–e). Notably, 14 days after intrathecal injection, Lvs-siGDNF treatment significantly reduced SP immune-intensity to 56.7 ± 8.9 -positive spots compared to saline and NC-treated groups ($P = 0.026, 0.015$, respectively) (Fig. 5f). Although a similar trend in reduction of SP staining was found in the morphine treatment group (66.4 ± 15.2 -positive spots), no statistical significance was found ($P > 0.05$) (Fig. 5f). These data indicated that Lvs-siGDNF can further exert analgesic effects by reducing the expression of SP, whereas morphine treatment did not.

Changes in pERK/ERK ratio in each group. The phosphorylation state of MAPK (ERK1/2) was evaluated using

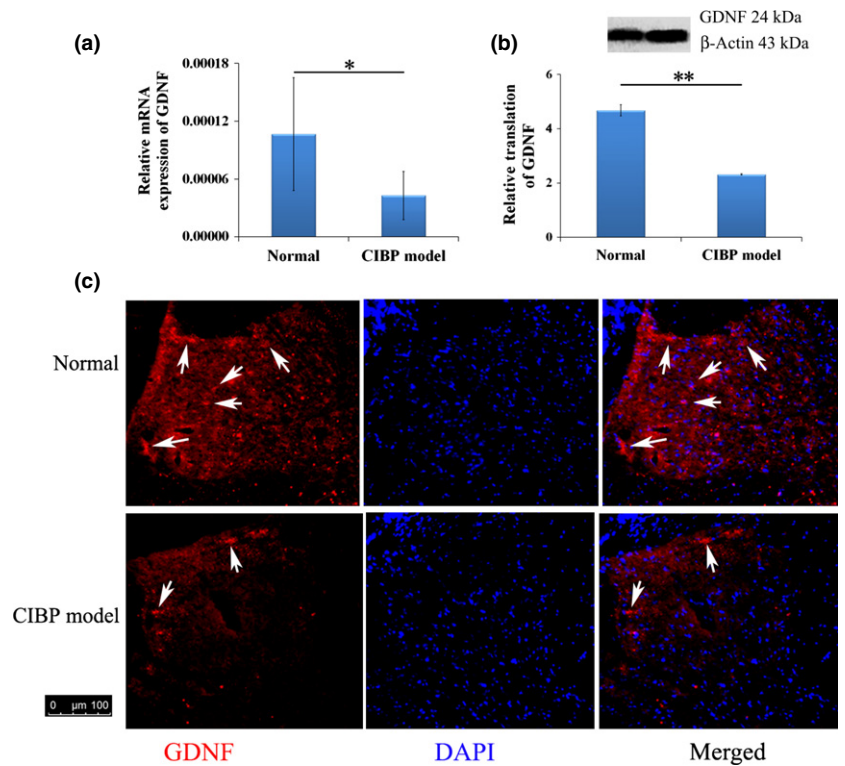


Fig. 2. Changes in glial cell line-derived neurotrophic factor (GDNF) expression in the lumbar spinal cords of rats with bone cancer. (a) Rats with bone cancer-induced hyperalgesia significantly reduced the transcriptional expression of GDNF mRNA. (b) This pain model also reduced GDNF translational protein expression compared with untreated normal rats. (c) Photomicrographs show the location of GDNF (red) in the dorsal horn of the spinal cord. DAPI indicates cell nucleus marker (blue). * $P < 0.05$, ** $P < 0.001$, one-way ANOVA, Dunnett's T3 *post hoc* test. BCIP, Bone Cancer-induced pain.

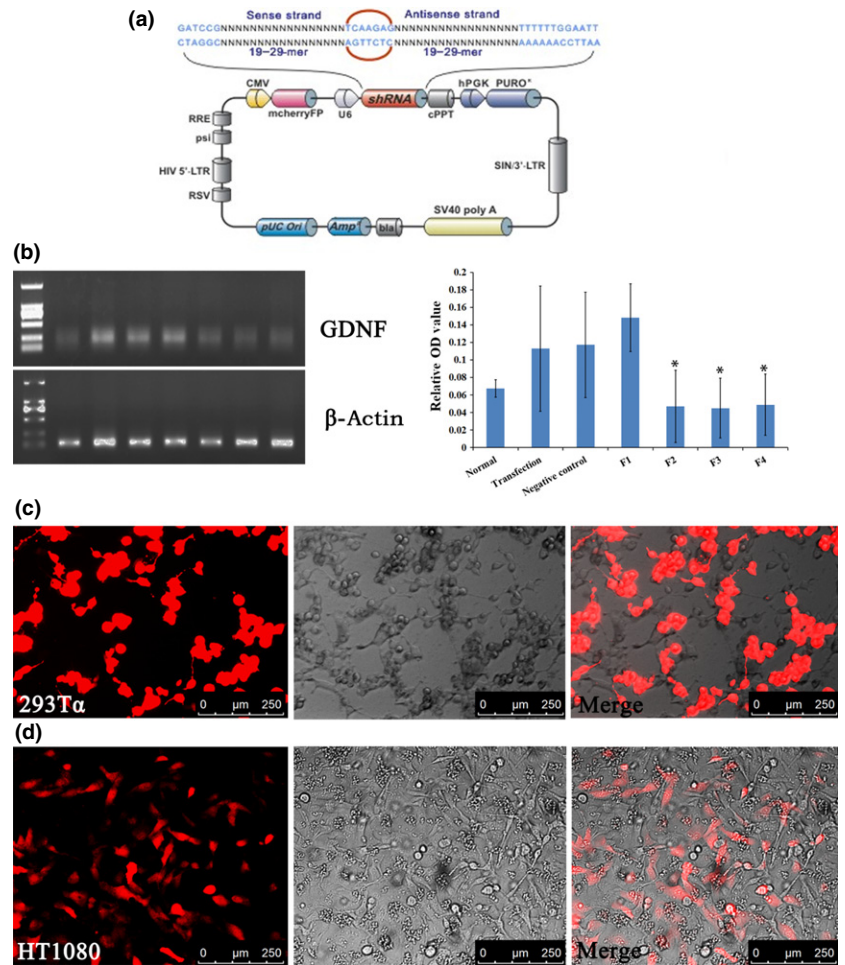


Fig. 3. Construction of recombinant lentivirus-mediated glial cell line-derived neurotrophic factor RNAi (Lvs-siGDNF). (a) Recombinant information for Lvs-siGDNF. (b) Recombinant 3 was selected as the most effective interference plasmid from four GDNF shRNA recombinants. Left panel, electrophoresis gel picture. Unlabeled lanes from left to right: DL2000 marker; normal; transfection buffer; negative control (lentivirus control); fragment 1 (F1); F2; F3; and F4. Right panel, quantitative analysis. (c) Pseudovirion containing the siGDNF-F3 vector was produced by virus packaging with 293T α cells as indicated by transfected cells (red). (d) The pseudovirion was transfected into HT1080 cells as indicated by transfected cells (red). Red fluorescent protein is a marker protein encoded by a gene segment named mcherryFP (a). * $P < 0.05$, one-way ANOVA, least significant difference *post hoc* test.

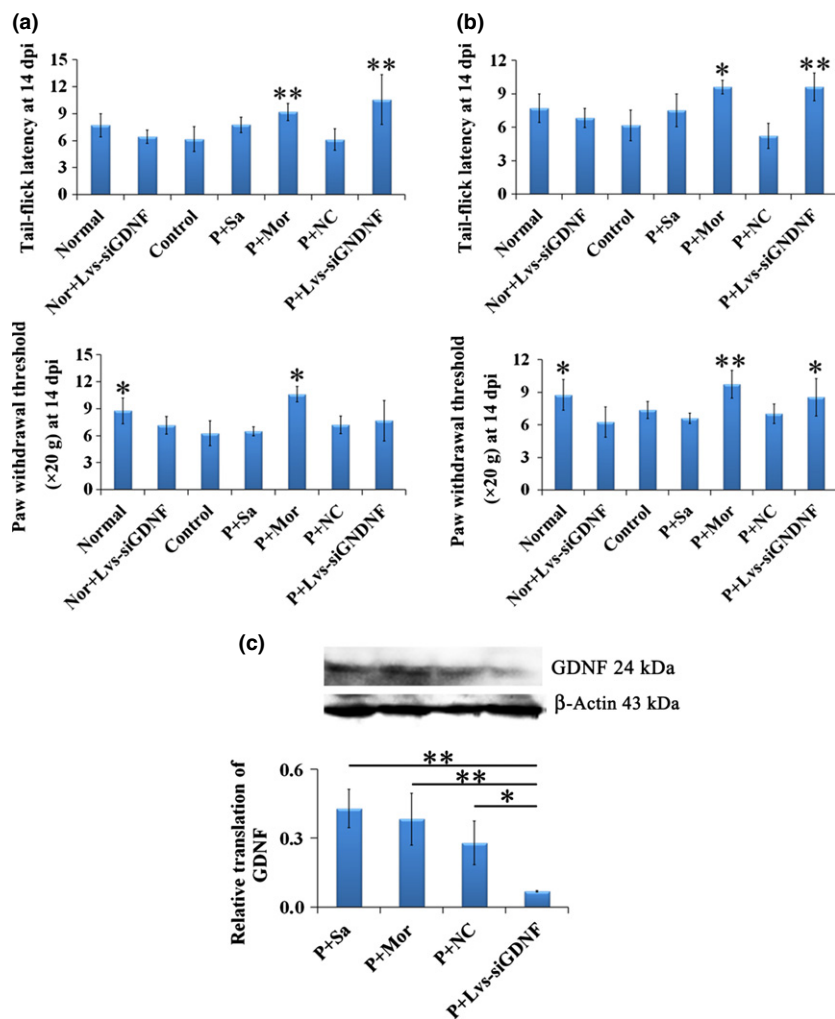


Fig. 4. Morphine and lentivirus-mediated glial cell line-derived neurotrophic factor RNAi (Lvs-siGDNF) alleviated thermal and mechanical hyperalgesia in a bone cancer-induced pain model. (a) Tail-flick latencies significantly increased in rats treated with Lvs-siGDNF or morphine. Only morphine treatment extended the paw withdrawal threshold compared to saline and vehicle (NC) treatment 7 days after intrathecal injection (7 days post injection [dpi]). (b) Both tail-flick latencies and paw withdrawal thresholds were significantly extended in bone cancer rats treated with Lvs-siGDNF or morphine compared to saline and NC treatments 14 days after intrathecal injection (14 dpi). (c) Treatment with Lvs-siGDNF significantly downregulated the protein expression of GDNF; morphine did not change GDNF protein levels compared to control treatments of rats with bone cancer. * $P < 0.05$; ** $P < 0.01$, one-way ANOVA, least significant difference *post hoc* test. Control, rats contain bone lesion without any treatment; Nor+Lvs-siGDNF, normal rats treated with lentiviral vector containing the GDNF interference RNA; P+Lvs-siGDNF, bone cancer pain rats treated with lentiviral vector containing the GDNF interference RNA; P+Mor, bone cancer pain rats treated with morphine; P+NC, bone cancer pain rats treated with negative control virus; P+Sa, bone cancer pain rats treated with saline.

immunofluorescence and Western blot analysis at day 14 after intrathecal injection. There was a significant increase in the ratio of pERK/ERK of 7.82 ± 2.23 in our bone cancer model ($P = 0.035$, compared to normal rats). While Lvs-siGDNF treatment showed a trend in reduced pERK and ERK immunoreactive cells (Fig. 6a–j), Western blot analysis showed that Lvs-siGDNF treatment significantly decreased the ratio of pERK/ERK in spinal cord segment L4–6 compared to the vehicle (NC)-treated group (8.02 ± 2.41 in vehicle treated groups; 0.94 ± 0.48 in Lvs-siGDNF treated group; $P = 0.044$) (Fig. 6k). Furthermore, treatment with morphine also significantly reduced the ratio of pERK/ERK compared to saline and vehicle (NC)-treated groups (1.10 ± 0.43 in morphine treated group; $P = 0.041, 0.048$, respectively) (Fig. 6k).

Discussion

Many previous researchers showed that carcinoma-evoked osteoclast activity is involved in bone destruction, which leads to bone cancer pain.^(22,26,27) Currently there are few effective therapies with zero adverse effects available to fight against the severity or frequency of intermittent episodes of incapacitating pain caused by bone destruction.^(28,29) Over the past decades, genetic engineering technology has been widely used in pain research, based on a biological understanding of the mechanisms involved in the generation and maintenance of pain states.⁽³⁰⁾ However, its use as a tool for treatment of pain

is still a novelty. As increased expression of GDNF was found in the spinal cord of some pain models,⁽⁹⁾ afferent pain pathways could become a treatment target in our therapeutic approach using LV vector technology. Due to the superiority of LV vectors as previously described, we constructed a recombinant lentivirus vector (Lvs) containing siRNA of GDNF (siGDNF) and administered the Lvs-siGDNF by intrathecal catheterization of the spinal cord. This is because the lumbar enlargement of the spinal cord (segments L4–6) is the most important transfer station between the encephalon and the peripheral nerves that control the hind limbs.

Although our bone cancer model induced bone destruction accompanied by both thermal and mechanical hyperalgesia systemically, the levels of GDNF gene and protein expression were downregulated in the spinal cord. This observation is similar to a previous study showing that levels of aquaporin (AQP)-4, a molecule involved in edema, was also downregulated at the early stage of edema in the brain following ischemia.⁽³¹⁾ The decrease observed in both cases might be due to an internal defense mechanism to control the pain or edema, as both GDNF and AQP-4 are positive mediators of pain and edema, respectively. Fu *et al.*⁽³¹⁾ further confirmed that downregulating endogenous AQP-4 using siRNA interference plays a protective role against brain edema *in vivo* and *in vitro*. Therefore, in our current study, we injected siGDNF LV vector into spinal cords to examine the role of GDNF in bone cancer-induced hyperalgesia in rats.

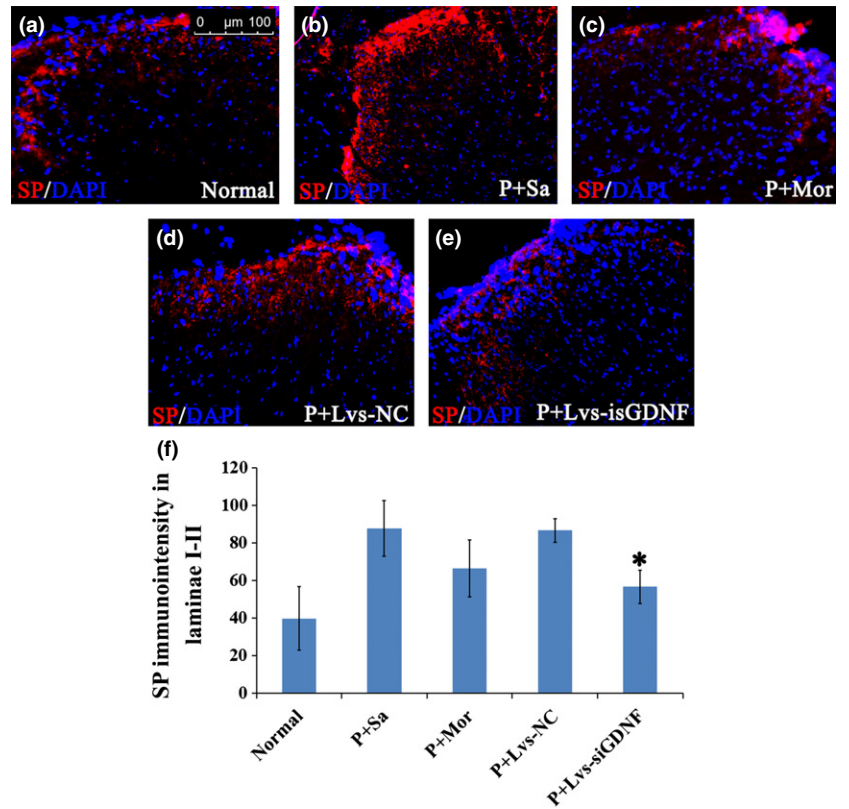


Fig. 5. Lentivirus-mediated glial cell line-derived neurotrophic factor RNAi (Lvs-siGDNF) treatment attenuated the expression of substance P (SP) in the dorsal horn of spinal cord of bone cancer rats. Immunofluorescence for SP in the dorsal horn of normal (a) and bone cancer model (b–e) spinal cords in the intumescentia lumbalis is depicted. (f) At 14 days after gene therapy, quantification of SP immunointensity in laminae I–II revealed an increased level in all bone cancer rats (P) regardless of their treatment compared with normal rats, but only the increase in SP staining from saline (Sa) and vehicle (NC) treatments of bone cancer groups were statistically significant. Moreover, treatment with Lvs-siGDNF significantly attenuated the level of SP immunointensity compared to saline and NC among the treated groups. * $P < 0.05$, one-way ANOVA, Dunnett's T3 post hoc test. Mor, morphine; Normal, rats without bone cancer.

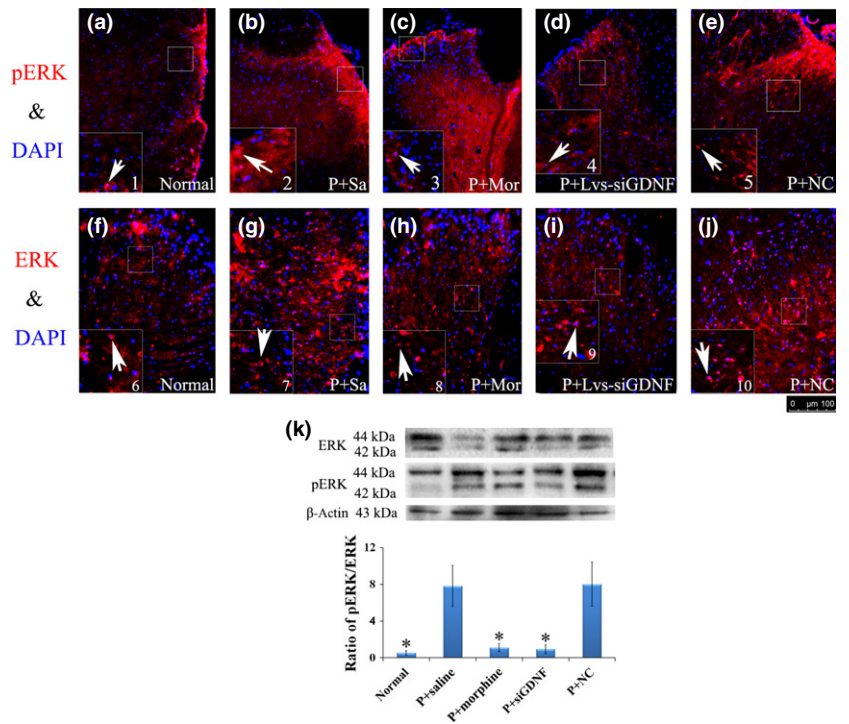


Fig. 6. Treatment with lentivirus-mediated glial cell line-derived neurotrophic factor RNAi (Lvs-siGDNF) or morphine reduced the ratio of pERK/ERK at 14 days post injection. (a–j) Representative pERK and ERK immunofluorescence images of lumbar enlargement spinal cord dorsal horn sections from normal rats and saline (Sa), morphine (Mor), vehicle (NC), and Lvs-siGDNF treated bone cancer rats (P) on day 14. Insets 1–10 at the left bottom of (a–j) are magnified views of an area in laminae I–II within the white box. (k) Representative Western blot bands of pERK and ERK and quantification of changes of the ratio of pERK/ERK normalized to β -actin in lumbar enlargement from normal and all treatment groups. Data represent mean \pm SEM. * $P < 0.05$, one-way ANOVA, least significant difference post hoc test for two isoforms of pERK. Normal, rats without bone cancer.

Morphine, a classical opioid agonist is defined as one of the main analgesics used to alleviate pain of patients with bone cancer.⁽³²⁾ However, the tolerance to this narcotic analgesia and the number of adverse effects it produces may limit its effectiveness during long-term use.⁽³³⁾ Morphine was used as a positive control to evaluate the efficiency of Lvs-siGDNF gene

therapy. Tail-flick latency of thermal hyperalgesia was a well-characterized and simple model with suitable parameters for predicting analgesia in humans,^(33–35) so we recorded the value of TL instead of paw withdrawal latency. From our results, intrathecal injection of Lvs-siGDNF further suppressed translation of GDNF protein and subsequently attenuated the

mechanical and thermal hyperalgesia of rats induced by MRMT-1 cells in their tibial bone, whereas the reduction in hyperalgesia by morphine treatment did not involve the down-regulation of GDNF protein expression. This suggests that the analgesic properties of morphine may not be through down-regulation of GDNF, whereas directly silencing GDNF expression played an analgesic role in this bone cancer model. Noticeably, we also investigated the effects of Lvs-siGDNF in normal rats. Consequently, it looks like there is no positive effect on sensory behavior in normal rats after treatment with Lvs-siGDNF. This indicated that normal animals may be different from animals subjected to pathological lesions. Moreover, siGDNF LV vector injected into the spinal cord could reverse hyperalgesia in bone cancer rats compared to controls, but this reversion surpassed the level of normal and Lvs-siGDNF treated normal animals, indicated by the TL test. These results confirmed that administration of Lvs-siGDNF RNA in bone cancer patients is effective and it may be made available for the treatment of bone cancer patients in future clinic practice.

In addition, along with pain behaviors, increase in the expression of SP immunoreactive varicosities in the dorsal horn was observed in our bone cancer model. The stimulation of this nociceptor may indicate that central sensitization within the spinal cord appeared following persistent pain states,^(36,37) and the analgesic properties of GDNF interference may be accompanied by reduced SP expression. Furthermore, as the expression of pERK1/2 had been regarded as a marker for central sensitization,^(38,39) we showed that bone cancer increased activation of ERK (pERK) compared with the

non-phosphorylated ERK. This result is supported by previous reports showing pERK was enhanced in the spinal cord under inflammatory and neuropathic pain conditions.^(38,40–42) As previously described, the activation of MAPK pathways has been considered to contribute to peripheral and central sensitization.^(43–45) Comparatively, Lvs-siGDNF treatment reversed the increased levels of SP immunostaining and pERK protein expression within lumbar enlargement of spinal cord in rats with bone cancer, whereas morphine treatment was only able to significantly inhibit the increase in activation of ERK. This suggests that GDNF may contribute to cancer-induced central sensitization, through regulating both levels of SP release and ERK phosphorylation. Thus, targeting GDNF may be more effective than morphine in inhibiting the neurochemicals involved in central sensation resulting in sensory function amelioration. Previously, we have primarily reported information on the knockdown of BDNF in bone cancer,⁽⁴⁶⁾ this study provided more detailed evidence to address the role of GDNF and its effect on the treatment of pain from bone cancer. In conclusion, the downregulation of GDNF expression using Lvs-siGDNF reversed bone cancer-induced hyperalgesia in rats. The possible mechanism may be through inhibiting SP release and pERK activity. Therefore, intrathecal injection with Lvs-siGDNF may be a useful therapy to relieve pain induced by bone cancer in future clinical trials.

Disclosure Statement

The authors have no conflict of interest.

References

- Li X, Wang XW, Feng XM. Stage-dependent anti-allodynic effects of intrathecal Toll-like receptor 4 antagonists in a rat model of cancer induced bone pain. *J Physiol Sci* 2013; **63**: 203–9.
- Mercadante S. Malignant bone pain: pathophysiology and treatment. *Pain* 1997; **69** (1): 1–18.
- Portenoy RK, Lesage P. Management of cancer pain. *Lancet* 1999; **353**: 1695–700.
- Mantyh PW. Cancer pain and its impact on diagnosis, survival and quality of life. *Nat Rev Neurosci* 2006; **7**: 797–809.
- de Wit R, van Dam F, Loonstra S. The Amsterdam Pain Management Index compared to eight frequently used outcome measures to evaluate the adequacy of pain treatment in cancer patients with chronic pain. *Pain* 2001; **91**: 339–49.
- Hu S, Mao-Ying QL, Wang J. Lipoxins and aspirin-triggered lipoxin alleviate bone cancer pain in association with suppressing expression of spinal proinflammatory cytokines. *J Neuroinflammation* 2012; **9** (1): 278.
- Malin SA, Molliver DC, Koerber HR. Glial cell line-derived neurotrophic factor family members sensitize nociceptors in vitro and produce thermal hyperalgesia in vivo. *J Neurosci* 2006; **26**: 8588–99.
- Alvarez P, Chen X, Bogen O. IB4 (+) nociceptors mediate persistent muscle pain induced by GDNF. *J Neurophysiol* 2012; **108**: 2545–25537.
- Zhang M, Wang Y, He QH. Glial cell line-derived neurotrophic factor contributes to delayed inflammatory hyperalgesia in adjuvant rat pain model. *Neuroscience* 2003; **117**: 503–12.
- Yang L, Hong Q, Zhang M. The role of Homer 1a in increasing locomotor activity and non-selective attention, and impairing learning and memory abilities. *Brain Res* 2013; **1515**: 39–47.
- Papanikolaou E, Kontostathi G, Drakopoulou E. Characterization and comparative performance of lentiviral vector preparations concentrated by either one-step ultrafiltration or ultracentrifugation. *Virus Res* 2013; **175** (1): 1–11.
- Zhang Y, Zhang ZJ. Transfer the lentiviral vectors containing rat GDNF and TH bi-gene into brain to have a protective effect in Parkinson's disease. *Zhong Yao Tong Bao* 2011; **27**: 234–9.
- Cavazzana-Calvo M, Payen E, Negre O. Transfusion independence and hmg2 activation after gene therapy of human [bgr]-thalassaemia. *Nature* 2010; **467**: 318–22.
- Montini E, Cesana D, Schmidt M. The genotoxic potential of retroviral vectors is strongly modulated by vector design and integration site selection in a mouse model of HSC gene therapy. *J Clin Invest* 2009; **119**: 964.
- Modlich U, Navarro S, Zychlinski D. Insertional transformation of hematopoietic cells by self-inactivating lentiviral and gammaretroviral vectors. *Mol Ther* 2009; **17**: 1919–28.
- Rittelmeyer I, Rothe M, Brugman MH. Hepatic lentiviral gene transfer is associated with clonal selection, but not with tumor formation in serially transplanted rodents. *Hepatology* 2013; **58** (1): 397–408.
- Mitchell RS, Beitzel BF, Schroder ARW. Retroviral DNA integration: ASLV, HIV, and MLV show distinct target site preferences. *PLoS Biol* 2004; **2**: e234.
- Beard BC, Dickerson D, Beebe K. Comparison of HIV-derived lentiviral and MLV-based gammaretroviral vector integration sites in primate repopulating cells. *Mol Ther* 2007; **15**: 1356–65.
- Hematti P, Hong BK, Ferguson C. Distinct genomic integration of MLV and SIV vectors in primate hematopoietic stem and progenitor cells. *PLoS Biol* 2004; **2**: e423.
- Zimmermann M. Ethical guidelines for investigations of experimental pain in conscious animals. *Pain* 1983; **16**: 109–10.
- Salamanna F, Martini L, Pagani S. MRMT-1 rat breast carcinoma cells and models of bone metastases: improvement of an in vitro system to mimic the in vivo condition. *Acta Histochem* 2013; **115** (1): 76–85.
- Medhurst SJ, Walker K, Bowes M. A rat model of bone cancer pain. *Pain* 2002; **96** (1): 129–40.
- Luger NM, Honore P, Sabino MAC. Osteoprotegerin diminishes advanced bone cancer pain. *Cancer Res* 2001; **61**: 4038–47.
- Doré-Savard L, Otis V, Belleville K. Behavioral, medical imaging and histopathological features of a new rat model of bone cancer pain. *PLoS One* 2010; **5**: e13774.
- Fang M, Lü TM, De Ma A. Comparative pharmacokinetics of continuous and conventional intrathecal amphotericin B in rabbits. *Antimicrob Agents Chemother* 2012; **56**: 5253–7.
- Honore P, Luger NM, Sabino MAC. Osteoprotegerin blocks bone cancer-induced skeletal destruction, skeletal pain and pain-related neurochemical reorganization of the spinal cord. *Nat Med* 2000; **6**: 521–8.
- Wang J, Zhang R, Dong C. Topical treatment with Tong-Luo-San-Jie Gel alleviates bone cancer pain in rats. *J Ethnopharmacol* 2012; **143**: 905–13.

- 28 Fulfaro F, Casuccio A, Ticozzi C. The role of bisphosphonates in the treatment of painful metastatic bone disease: a review of phase III trials. *Pain* 1998; **78** (3): 157–69.
- 29 Lipton A. Bisphosphonates and breast carcinoma. *Cancer* 1997; **80** (S8): 1668–73.
- 30 Woolf CJ, Bennett GJ, Doherty M. Towards a mechanism-based classification of pain. *Pain* 1998; **77**: 227–9.
- 31 Fu X, Li Q, Feng Z. The roles of aquaporin-4 in brain edema following neonatal hypoxia ischemia and reoxygenation in a cultured rat astrocyte model. *Glia* 2007; **55**: 935–41.
- 32 Urch CE, Donovan-Rodríguez T, Gordon-Williams R. Efficacy of chronic morphine in a rat model of cancer-induced bone pain: behavior and in dorsal horn pathophysiology. *J Pain* 2005; **6**: 837–45.
- 33 Vidal-Torres A, de la Puente B, Rocasalbas M. Sigma-1 receptor antagonism as opioid adjuvant strategy: enhancement of opioid antinociception without increasing adverse effects. *Eur J Pharmacol* 2013; **711** (1–3): 63–72.
- 34 Le Bars D, Gozariu M, Cadden SW. Animal models of nociception. *Pharmacol Rev* 2001; **53**: 597–652.
- 35 Colburn RW, Rickman AJ, DeLeo JA. The effect of site and type of nerve injury on spinal glial activation and neuropathic pain behavior. *Exp Neurol* 1999; **157**: 289–304.
- 36 Hunt SP, Pini A, Evan G. Induction of c-fos-like protein in spinal cord neurons following sensory stimulation. *Nature* 1986; **328**: 632–4.
- 37 Doyle CA, Hunt SP. Substance P receptor (neurokinin-1)-expressing neurons in lamina I of the spinal cord encode for the intensity of noxious stimulation: a c-Fos study in rat. *Neuroscience* 1999; **89** (1): 17–28.
- 38 Wang XW, Li TT, Zhao J. Extracellular signal-regulated kinase activation in spinal astrocytes and microglia contributes to cancer-induced bone cancer pain in rats. *Neuroscience* 2012; **217**: 172–81.
- 39 Gao YJ, Ji RR. c-Fos and pERK, which is a better marker for neuronal activation and central sensitization after noxious stimulation and tissue injury? *Open Pain J* 2009; **2**: 11.
- 40 Zhuang ZY, Xu H, Clapham DE. Phosphatidylinositol 3-kinase activates ERK in primary sensory neurons and mediates inflammatory heat hyperalgesia through TRPV1 sensitization. *J Neurosci* 2004; **24**: 8300–9.
- 41 Zhuang ZY, Gerner P, Woolf CJ. ERK is sequentially activated in neurons, microglia, and astrocytes by spinal nerve ligation and contributes to mechanical allodynia in this neuropathic pain model. *Pain* 2005; **114** (1): 149–59.
- 42 Zhang Y, Cai G, Ni X. The role of ERK activation in the neuronal excitability in the chronically compressed dorsal root ganglia. *Neurosci Lett* 2007; **419**: 153–7.
- 43 Horvath RJ, Landry RP, Romero-Sandoval EA. Morphine tolerance attenuates the resolution of postoperative pain and enhances spinal microglial p38 and extracellular receptor kinase phosphorylation. *Neuroscience* 2010; **169**: 843–54.
- 44 Obata K, Noguchi K. MAPK activation in nociceptive neurons and pain hypersensitivity. *Life Sci* 2004; **74**: 2643–53.
- 45 Ji RR, Gereau RW IV, Malcangio M. MAP kinase and pain. *Brain Res Rev* 2009; **60** (1): 135–48.
- 46 Fufen M, La W, Yang X, Tinghua W. Analgesic effect of lentivirus-mediated RNA interference of the expression of Glia derived neurotrophic factor gene on bone cancer pain. *Zhonghua Shi Yan Wai Ke Za Zhi* 2014; **31**: 956–9.

Supporting Information

Additional supporting information may be found in the online version of this article:

Doc. S1. Complete materials and methods excluding packaging of lentivirus, intrathecal catheterization, drug administration, and behavioral analysis.