# Upregulation of bone morphogenetic protein 2 (Bmp2) in dorsal root ganglion in a rat model of bone cancer pain

Wei Wang[1], Qiliang Jiang[1], Jingxiang Wu[1], Wei Tang[1], and Meiying Xu[1]

## Abstract

Bone cancer pain is one of the most severe and intractable complications in patients suffering from primary or metastatic bone cancer and profoundly compromises the quality of life. Emerging evidence indicates that the dorsal root ganglion play an integral role in the modulation of pain hypersensitivity. However, the underlying molecular mechanisms during dorsal root ganglion-mediated bone cancer pain remain elusive. In this study, RNA-sequencing was used to detect the differentially expressed genes in dorsal root ganglion neurons of a rat bone cancer pain model established by intratibial inoculation of Walker 256 breast cancer cells. Gene ontology and Kyoto Encyclopedia of Genes and Genomes analysis showed that the differentially expressed genes (fold change $> 1.5$; false discovery rate $< 0.05$) were enriched in the bone morphogenetic protein (BMP) signaling pathway, transforming growth factor-$\beta$ signaling pathway, and positive regulation of cartilage development. Importantly, serum deprivation-response protein (Sdpr), hephaestin (Heph), transthyretin (Ttr), insulin receptor substrate 1 (Irs1), connective tissue growth factor (Ctgf), and Bmp2 genes were associated with bone pain and degeneration. Of note, Bmp2, a pleiotropic and secreted molecule mediating pain and inflammation, was one of the most significantly upregulated genes in dorsal root ganglion neurons in this bone cancer pain model. Consistent with these data, upregulation of Bmp2 in the bone cancer pain model was validated by immunohistochemistry, real-time quantitative polymerase chain reaction, and western blotting. Importantly, intrathecal administration of siRNA significantly reduced Bmp2 transcription and ameliorated bone cancer pain in rat as shown by paw withdrawal mechanical threshold and spontaneous and movement-evoked pain-like behaviors. In conclusion, we have characterized the comprehensive gene expression profile of dorsal root ganglion from a bone cancer pain rat model by RNA-sequencing and identified Bmp2 as a potential therapeutic target for bone cancer pain treatment.

## Introduction

Various tumor types including breast, prostate, and lung carcinomas preferentially metastasize to the skeleton, where they induce significant bone remodeling, destruction, and bone cancer pain (BCP).[1–3] BCP is often severe and intractable and is a significant contributing factor to morbidity and loss of quality of life.[4] Most of the current pharmacological treatments provide nonselective relief for pain syndromes. Even for palliative radiotherapy, the rate of complete pain relief remains low.[5] Therefore, it is pivotal to investigate the pathogenesis of BCP and identify novel therapeutic targets for translational medicine.

Inflammatory, neuropathic, and/or ischemic components have been reported to be involved in cancer-induced bone pain.[6] In rodent BCP models, bone metastasis of cancers alters the structure, physiology, and biochemistry of the bone and concurrently

[1]Department of Anesthesiology, Shanghai Chest Hospital, Shanghai Jiao Tong University, Shanghai, China

**Corresponding Author:**
Jingxiang Wu, Department of Anesthesiology, Shanghai Chest Hospital, Shanghai Jiao Tong University, No. 241 Huaihai West Road, Shanghai 200000, China.
Email: wu_jingxiang@sjtu.edu.cn

induces mechanical and inflammatory hyperalgesia.[7] In particular, hyperexcitability of nociceptive dorsal root ganglion (DRG) neurons is associated with inflammatory pain in rodent models.[8] Emerging evidence indicates that alterations in gene expression[9–15] and signaling pathways[16–18] contribute to induction and maintenance of BCP. For example, upregulation of P2X3 receptors,[9] sodium channels Nav1.8[13] and Nav1.9,[10] brain-derived neurotrophic factor,[11,19] acid-sensing ion channel 3,[12] protease-activated receptor 2 and 4,[14] and activation of cAMP-PKA,[16] PI3K/Akt,[17] and Hedgehog signaling[18] were involved in DRG-mediated BCP in a rat model. Moreover, several studies have shown attenuation of BCP in rats. These studies included overexpression of suppressor of cytokine signaling 3 in DRG,[15] suppression of asparaginyl endopeptidase in cancer cells, intrathecal injection of genetically engineered human bone marrow stem cells expressing the proenkephalin gene,[20] and intraperitoneal administration of a somatostatin receptor type 4 selective agonist, J-2156. However, comprehensive gene expression profiling of DRGs in a BCP model has not been reported. Such profiling could help identify novel therapeutic targets for the relief of BCP.

In this study, next-generation RNA-sequencing (RNA-seq) was performed to detect the differentially expressed genes (DEGs) in DRGs from a rat BCP model. The results showed that DEGs were enriched in signaling pathways associated with bone pain, inflammation, and degeneration. In particular, *Bmp2* was significantly increased in DRG neurons in a BCP rat model, and targeting of *Bmp2* by siRNA ameliorated BCP in vivo, suggesting that *Bmp2* may be an attractive therapeutic target for BCP.

## Materials and methods

### Animal husbandry

Female Sprague-Dawley rats (200 g–250 g, B&K Universal Group Limited) were used for this study. Animals were housed in separate cages at a temperature of $24 \pm 1°C$ under a 12-h/12-h light-dark cycle and had free access to food and water. All animal procedures were carried out in accordance with the guidelines of the International Association for the Study of Pain[21] and were approved by the Animal Care and Use Committee of Shanghai Chest Hospital, Shanghai Jiao Tong University (Permission No. KS (Y)1616).

### BCP model

The BCP model was established as previously described.[22] Briefly, three female Sprague-Dawley rats weighing 70 g to 80 g received an intraperitoneal inoculation of Walker 256 cells. After one week, cells in the ascites were collected and resuspended in saline to a final concentration of $2 \times 10^7$ cells/mL. Then, female Sprague-Dawley rats weighing 200 g to 250 g were anesthetized with chloral hydrate (300 mg/kg, i.p.). A 23-gauge needle was inserted into the intramedullary canal of the left tibia, in the lower one third, to create a cavity for the injection of the cells, and 10 μL vehicle (sham group) or Walker 256 cells ($2 \times 10^5$ cells) (BCP group) were injected into the bone cavity. The cavity was sealed using bone wax prior to closure of the incision. The rats were returned to their home cages upon waking. Rats were sacrificed 21 days after tumor inoculation, and tibias were collected for gross examination.

### Pain-related behaviors

Mechanical allodynia was determined by a significant decrease in the threshold of hind paw withdrawal to mechanical stimulation applied to the plantar surface of each hind paw.[23] Briefly, the animals were individually placed beneath an inverted ventilated cage with a metal-mesh floor. Von Frey monofilaments (0.6-, 1.0-, 1.4-, 2-, 4-, 6-, 8-, 10-, and 15-g), starting with 1 g and ending with 15 g in ascending order, were applied perpendicular to the plantar surface of each hind paw from beneath until the paw was withdrawn. The duration of each stimulus was approximately 2 s. Each monofilament was applied five times at 5-min intervals. Quick withdrawal or paw flinching was considered a positive response. The paw withdrawal mechanical frequency (PWMF) to each monofilament was calculated based on five applications. The paw withdrawal mechanical threshold (PWMT) was determined by the force at which $PWMF \geq 60\%$; 15 g was recorded as the PWMT if $PWMF < 60\%$ to all filaments.

Movement-evoked pain was assessed by limb use score. Rats were permitted to move spontaneously on a smooth plastic table (50 cm × 50 cm). The limb use during spontaneous ambulation was scored on a scale of 4 to 0 (4. normal use; 3. slightly limping; 2. clearly limping; 1. no use of the limbs (partial); and 0. no use of the limbs (complete)).

### RNA-seq

Rats were promptly sacrificed by cervical dislocation. L3-L5 DRGs were quickly harvested from sham and BCP rats ($n = 3$). Total RNA was extracted from samples using TRIzol reagent (Invitrogen, Carlsbad, CA) and the integrity verified (Agilent 2100 bioanalyzer) prior to library construction. RNA-seq was performed using the Illumina HiSeq2000 platform. Sequencing reads were first filtered for adapter sequence, low-quality sequence and rRNA. Reads were then mapped

to Human Genome GRCh38 (downloaded form GENCODE) using STAR Version 2.5.2. Gene expression was quantified by RSEM Version 1.2.31 and differentially expressed transcripts (false discovery rate < 0.05) were identified by EBSeq Version 1.10.0. Partek Genomics Suite and Gene Ontology Consortium (http://geneontology.org) were used for pathway and gene ontology enrichment analysis, respectively.

## Western blotting

The expression of *Bmp2* from sham and BCP mice was measured by western blot analysis. Rats were rapidly sacrificed by cervical dislocation. L3-L5 DRGs were quickly harvested and lysed; lysates were rotated for 2 h at 4°C, followed by centrifugation at 12,000 r/min for 15 min at 4°C. Supernatants were collected, and total protein concentration was titrated using a bicinchoninic acid kit. Equivalent amounts of protein (20 μg) were fractionated on 10% polyacrylamide gels. Proteins were transferred to nitrocellulose membranes (Millipore) at 100 mA for 90 min. Membranes were blocked with 5% nonfat milk in Tris-buffered saline (TBS) (50 mM Tris-HCl, 150 mM NaCl, pH 7.5) for 1 h at room temperature and incubated overnight at 4°C with primary antibody (anti-*Bmp2* at 1:1000, Abcam) in TBS containing 1% milk. Following three washes with TBST, membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (1:500, Abcam) in TBS containing 1% milk for 2 h at room temperature. Immunoreactive proteins were visualized using the enhanced chemiluminescence western blotting detection system (Santa Cruz). Membranes were subsequently stripped and reprobed for anti-β-actin antibody (1: 1000, Sigma). The chemiluminescent signal from the membranes was quantified by a GeneGenome HR scanner using GeneTools software (SynGene). *Bmp2* protein expression was normalized to β-actin.

## Real-time quantitative polymerase chain reaction

Rats were anesthetized with sodium pentobarbital (50 mg/kg i.p.), and the L3-L5 DRGs were quickly removed at the defined timepoint for each group. Total RNA was extracted using a total RNA Kit (invitrogen), and complementary DNA (cDNA) was generated using a cDNA Synthesis Kit (Promega, Madison, WI), according to the manufacturer's instructions. Real-time quantitative polymerase chain reaction (RT-qPCR) was carried out using a SYBR Green assay (Roche Ltd, Basel, Switzerland) and Rotor-Gene 3000 system (Corbett Research, Australia) based on the $2^{-\triangle\triangle Ct}$ method. β-actin was used as a housekeeping gene. The primer sequences for *Bmp2* and β-actin were as follows: *Bmp2*: Sense: 5′-AAGCCAGGTGTCTCCA AG -3′; Antisense: 5′-AAGTCCACATACAAAGGGT G-3′; β-actin: Sense: 5′-ATGGTGGTATGGGTCAGA AGG-3′; Antisense: 5′-TGGCTGGGGTGTTGAAG GTC-3′.

## Immunohistochemistry

Animals were deeply anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and underwent sternotomy, followed by intracardiac perfusion with 200 mL saline and 200 mL 4% ice-cold paraformaldehyde in 0.1 M phosphate-buffered saline. The spinal cord and L3-L5 DRGs were removed, postfixed in 4% paraformaldehyde for 4 h, and subsequently allowed to equilibrate in 30% sucrose in phosphate-buffered saline overnight at 4°C. Immunohistochemical analyses were performed with the Histostain-SP kit (Zymed, San Francisco, CA). After rehydration, inhibition of endogenous peroxidase with 3% hydrogen peroxide, and blocking (10% rabbit serum) for 30 min, the tissue sections were incubated with a specific antibody against *Bmp2* (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) (1:500). Normal goat IgG was used as a negative control. The expression of *Bmp2* was detected by a biotin-streptavidin-peroxidase system using diaminobenzidine as a chromogen. Counterstaining was performed with hematoxylin.

## Targeting Bmp2 by siRNA in BCP model

*Bmp2* was targeted by siRNA (sequence: Sense: 5′-GA AGCCAUCGAGGAACUUUTT-3′, Antisense: 5′-A AAGUUCCUCGAUGGCUUCTT-3′; GenePharma, Shanghai, China). Intrathecal administration of *Bmp2*-siRNA (2 μg siRNA-bone morphogenetic protein 2 (*BMP2*) or siRNA-negative control with in vivo-jet PEI (Invitrogen)) was performed seven days postinoculation of Walker 256 cells. In vivo *Bmp2* knockdown efficiency from L4-L5 DRGs was examined by RT-qPCR. PWMT and movement-evoked pain from sham and BCP rats were monitored on day 4, 6, 8, 10, and 12 postintrathecal administration.

## Statistics

Data are expressed as the mean ± SD for behavioral and molecular assays. Statistical analyses were conducted using SPSS statistics 18.0 software and included SNK and LSD test for two-sample data and analysis of variance for multiple comparisons where appropriate. A *P* value less than 0.05 was set as the level of statistical significance. Data and figures were organized using GraphPad 5.0 software.

## Results

### Pain behaviors of the rat BCP model

Rats inoculated with Walker 256 tumor cells displayed a gradual increase in sensitivity to Von Frey filament stimulation. On day 21 postinoculation of Walker 256 cancer cells, the PWMT from the BCP group decreased from the baseline level of $14.3 \pm 1.4$ g to $0.9 \pm 0.3$ g (Figure 1(a)). The limb use score for the BCP group decreased from the baseline level ($4.0 \pm 0.0$) to $0.3 \pm 0.23$ (Figure 1(b)). The anatomic structure of the tibia appeared thicker, and palpable tumors were noted outside the bone upon gross examination of BCP rats 21 days postinoculation of Walker 256 cancer cells (Figure 1(c)).

### Gene expression profiling of DRG neurons from BCP rats by RNA-seq

RNA-seq of rat DRG neurons was performed after confirming the BCP phenotype by radiographic imaging and pain-related behavior measurement at day 21 postinoculation of Walker 256 cancer cells. Eighty DEGs (fold change (FC) > 1.5; false discovery rate < 0.05) were identified in the BCP group ($n = 3$) compared to the sham group (45 upregulated and 35 downregulated). In silico functional analysis showed that 9 of these genes were associated with bone pain and 11 were associated with cancer development (Table 1 and Supplementary Table 1). Gene ontology analysis showed that the DEGs were enriched in Golgi cisternae membrane and synaptic cleft (cellular component), SMAD binding and protein kinase C binding (molecular function), BMP signaling pathway, and cartilage condensation (biological process) (Figure 2(a)). Kyoto Encyclopedia of Genes and Genomes analysis indicated that the Hippo, mTOR, and transforming growth factor (TGF)-β signaling pathways were activated, while steroid biosynthesis, vitamin digestion and absorption, and histidine metabolism were suppressed in DRG from the BCP group compared to the sham group (Figure 2(b) and (c)). In particular, *Bmp2* (FC = 2.09), BmP15 (FC = 2.99), and Bmpr1b (FC = 0.64) from the TGF-β superfamily were significantly increased in the BCP group.

### Upregulation of Bmp2 in DRG is associated with BCP

*Bmp2*, similarly to other BMP family members, plays an important role in the development of bone and cartilage. Consistent with the RNA-seq data, upregulation of *Bmp2* in BCP rats was further validated by immunohistochemistry (Figure 3(a)), RT-qPCR (Figure 3(b)), ELISA (Figure 3(c)), and western blotting (Figure 3(d)).
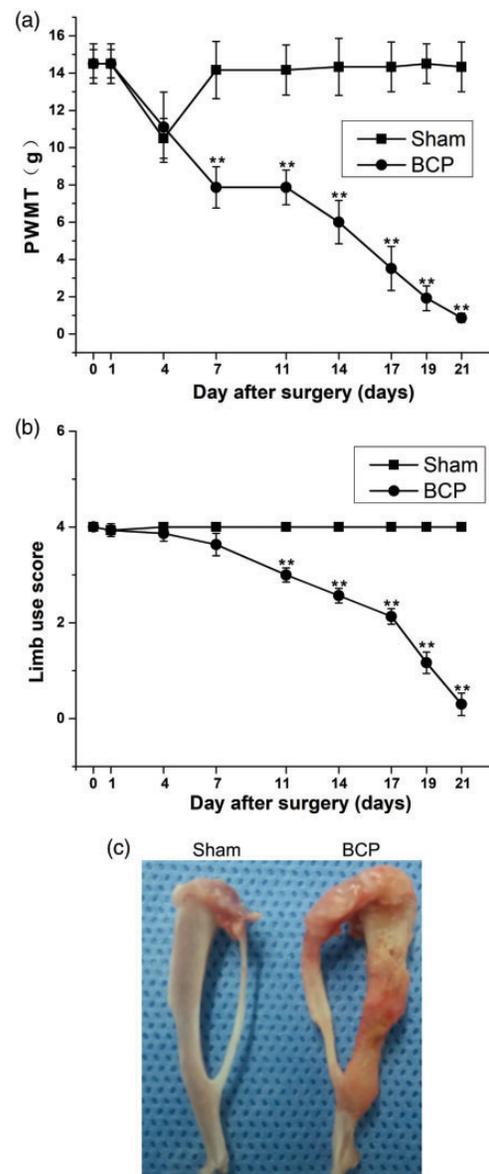


**Figure 1.** The establishment of BCP model by Walker 256 cancer cells inoculation in rats. Rats were inoculated with live or heat-killed Walker 256 cells as described in Materials and method section. (a) Paw withdrawal mechanical threshold (PWMT) and (b) paw withdrawal thermal latency for pain behavior test in sham and BCP group. (c) Anatomic structure of tibia bone from sham and BCP mice on 21 days postinoculation of Walker 256 cancer cells in rats. Data (a and b) are presented as mean $\pm$ SD ($n = 6$ rats per group), and inter-group differences are assessed using Student's *t* test. *$P < 0.05$, **$P < 0.01$, compared with the sham control.
BCP: bone cancer pain.

### Targeting Bmp2 ameliorates BCP in vivo

Information arising from RNA-seq data and subsequent validation prompted us to further investigate the role of *Bmp2* upregulation during BCP hypersensitivity. *Bmp2* was targeted by siRNAs in BCP rats. Targeting by

**Table 1.** Differentially expressed genes (false discovery rate < 0.05) in DRG neurons (BCP vs. Sham) by RNA-seq.

| AccID | Description | **Role in pain** and *cancer* | Fold change | P | Expression |
|---|---|---|---|---|---|
| Prss12 | Neurotrypsin | Unknown | 0.65817 | 1.47E-11 | Down |
| Asphd2 | Aspartate beta-hydroxylase domain-containing protein 2 | Unknown | 0.644274 | 5.51E-09 | Down |
| Clnk | cytokine-dependent hematopoietic cell linker | Unknown | 5.108594 | 1.1E-08 | Up |
| Sdpr | Serum deprivation-response protein | **Increased in peripheral inflammatory pain,**[1]*inhibit breast cancer progression.*[2,3] | 1.515262 | 2.89E-07 | Up |
| Krt2 | Keratin, type II cytoskeletal 2 epidermal | Unknown | 0.459607 | 2.95E-07 | Down |
| Heph | Hephaestin | **Play a role in migraine pathogenesis.**[4,5] | 1.692449 | 3.55E-07 | Up |
| LOC685048 | similar to paired immunoglobin-like type 2 receptor beta | Unknown | 3.77884 | 7.93E-07 | Up |
| Cd300le | Cd300 molecule-like family member E | unknown | 1.946738 | 1.42E-06 | Up |
| Arpc5l | Actin-related protein 2/3 complex subunit 5-like protein | *Play a role in breast cancer induced nociceptor aberrant growth*[6] | 1.519523 | 1.78E-06 | Up |
| Adarb2 | Double-stranded RNA-specific editase B2 | Unknown | 0.441258 | 3.6E-06 | Down |
| Dpysl5 | Dihydropyrimidinase-related protein 5 | Unknown | 0.62157 | 5.99E-06 | Down |
| LOC304396 | Protein LOC304396 | Unknown | 0.662713 | 7.2E-06 | Down |
| Phldb3 | Protein Phldb3 | *Support cancer growth via a negative feedback loop involving p53*[7] | 1.973821 | 8.39E-06 | Up |
| Kcnj13 | Inward rectifier potassium channel 13 | Unknown | 1.914467 | 2.52E-05 | Up |
| Trim14 | Trim14 protein | Unknown | 2.198957 | 3.48E-05 | Up |
| Arpc5l-ps1 | actin related protein 2/3 complex, subunit 5-like, pseudogene 1 | *Play a role in breast cancer induced nociceptor aberrant growth*[8] | 0.16867 | 3.62E-05 | Down |
| Ccdc17 | coiled-coil domain containing 17 | Unknown | 1.66459 | 5.05E-05 | Up |
| Ms4a6c | Protein Ms4a6c | Unknown | 3.541317 | 6.78E-05 | Up |
| Slc25a34 | Solute carrier family 25 member 34 | Unknown | 2.206727 | 8.09E-05 | Up |
| Ttr | Transthyretin | **Transthyretin mutation induced familial amyloid polyneuropathy ongoing refractory neuropathic pain**[9,10] | 7.784136 | 8.44E-05 | Up |
| Cldn16 | Claudin-16 | Unknown | 2.682996 | 8.46E-05 | Up |
| Cubn | Cubilin | Unknown | 1.83827 | 9.16E-05 | Up |
| Lmx1a | Protein Lmx1a | Unknown | 0.560506 | 9.72E-05 | Down |
| Bmp15 | Protein Bmp15 | Unknown | 2.999657 | 9.87E-05 | Up |
| Neto1 | Similar to Neuropilin-and tolloid-like protein 1 (Predicted) | Unknown | 0.559382 | 9.94E-05 | Down |
| Alpk2 | Protein Alpk2 | Unknown | 1.650776 | 0.000106 | Up |
| Fam179a | family with sequence similarity 179, member A | Unknown | 1.995452 | 0.000108 | Up |
| Casq2 | Calsequestrin-2 | Unknown | 1.927822 | 0.000113 | Up |
| Aldh1b1 | RCG55098 | **Aldehyde dehydrogenase-2 regulates nociception in rodent models of acute inflammatory pain.**[11] | 0.643453 | 0.000121 | Down |

**Table 1.** Continued.

| AccID | Description | **Role in pain** and *cancer* | Fold change | *P* | Expression |
|---|---|---|---|---|---|
| Pmel | Silver homolog (Mouse) (Predicted), isoform CRA_a | Unknown | 0.517713 | 0.000138 | Down |
| Bmpr1b | Receptor protein serine/threonine kinase | **BMP signaling regulates nociceptive sensitization**[12–14] | 0.640617 | 0.000154 | Down |
| Hmgn5 | High mobility group nucleosome-binding domain-containing protein 5 | Unknown | 0.383264 | 0.000181 | Down |
| Depdc1 | Protein Depdc1 | *DEPDC1 promotes tumorprostate cancer growth.*[15,16] | 3.357028 | 0.000203 | Up |
| Cyp2r1 | Uncharacterized protein | Unknown | 1.606352 | 0.000204 | Up |
| Fam43a | family with sequence similarity 43, member A | Unknown | 1.931784 | 0.000238 | Up |
| Irs1 | Insulin receptor substrate 1 | **IRS-1 plays important role in mTORC1 inhibition induces pain.**[17] | 0.601025 | 0.000332 | Down |
| B4galnt3 | Protein B4galnt3 | *B4galnt3 modulates the development of colon cancer, epithelial ovarian cancer, thyroid carcinoma, and neuroblastoma.*[18–21] | 0.4967 | 0.000337 | Down |
| Fut4 | Alpha-(1,3)-fucosyltransferase 4 | *FUT4 as an effective biomarker for the diagnosis of breast cancer.* | 1.530915 | 0.000351 | Up |
| Capsl | Protein Capsl | Unknown | 4.464927 | 0.00041 | Up |
| Has2 | Hyaluronan synthase 2 | Unknown | 0.577088 | 0.000422 | Down |
| Fut10 | Alpha-(1,3)-fucosyltransferase 10 | *FUT4 as an effective biomarker for the diagnosis of breast cancer.*[22] | 0.615564 | 0.000423 | Down |
| Rad2lll | Protein Rad2lll | Unknown | 1.617961 | 0.000423 | Up |
| Sytl4 | Synaptotagmin-like 4, isoform CRA_b | Unknown | 1.840074 | 0.000426 | Up |
| Mpv17l2 | Similar to FKSG24 (Predicted), isoform CRA_b | Unknown | 1.528021 | 0.000459 | Up |
| Bmp2 | Bone morphogenetic protein 2 | **Bone morphogenetic protein-2-mediated pain and inflammation in a rat model of posterolateral arthrodesis.**[23] | 2.043089 | 0.000466 | Up |
| Slc6a11 | Sodium- and chloride-dependent GABA transporter 3 | Unknown | 0.491062 | 0.000489 | Down |
| Ddit4 | DNA damage-inducible transcript 4 protein | *DDIT4 plays roles ofgastric cancer and proliferation.*[24,25] | 1.695812 | 0.00053 | Up |
| Dab2 | Disabled homolog 2 | *Dab2 is required for migration and invasion of prostate cancer.*[26] | 1.550524 | 0.000539 | Up |
| LMCD1 | LIM and cysteine-rich domains 1 | *LMCD1 mutations promoted cell migration and tumor metastasis in hepatocellular carcinoma.*[27] | 2.180509 | 0.000542 | Up |
| Slc10a6 | Solute carrier family 10 member 6 | Unknown | 3.655645 | 0.00059 | Up |
| CTGF | CTGF protein | **Expression of CTGF increases in painful disc fibrosis and degeneration.**[28,29] | 3.048432 | 0.00062 | Up |
| Arntl | Aryl hydrocarbon receptor nuclear translocator-like, isoform CRA_d | Unknown | 1.689748 | 0.00064 | Up |

**Table 1.** Continued.

| AccID | Description | **Role in pain** and *cancer* | Fold change | P | Expression |
|---|---|---|---|---|---|
| Rsph1 | Radial spoke head 1 homolog (Chlamydomonas) | Unknown | 0.63951 | 0.00065 | Down |
| Clca4l | chloride channel calcium activated 4-like | Unknown | 0.115282 | 0.000733 | Down |
| Rhox5 | Homeobox protein Rhox5 | Unknown | 4.463283 | 0.000766 | Up |
| Ccl24 | Protein Ccl24 | Unknown | 0.348753 | 0.000835 | Down |

Note: The references cited in this table are shown in Supplementary Table 1.
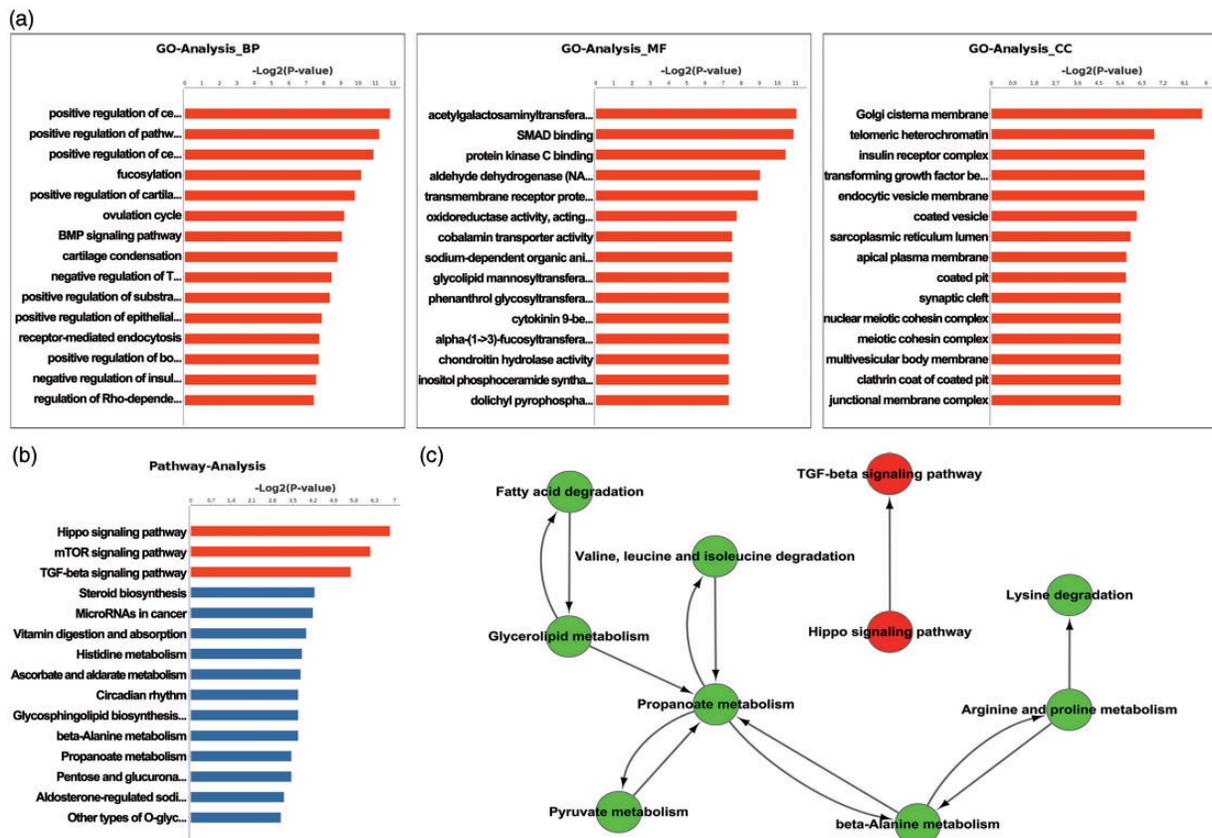


**Figure 2.** GO and KEGG analysis of DEGs in DRGs (BCP vs. Sham). (a) Gene ontology showed the enrichment of DEGs (BCP vs. Sham) in cellular component, molecular function, and biological process. (b) and (c) KEGG pathway analysis of DEGs (BCP vs. Sham). GO: gene ontology.

siRNA resulted in significantly reduced *Bmp2* transcription (Figure 4(a)) and ameliorated BCP in rats as indicated by increased PWMT levels (Figure 4(b)) at day 4, 6, 8, 10, and 12 postintrathecal siRNA administration. Consistently, limb use scores were also increased in BCP rats at day 6, 8, and 10 postintrathecal *Bmp2*-siRNA administration (Figure 4(c)). These data indicated that *Bmp2* antagonists may be effective for BCP treatment in vivo.

## Discussion

Primary and metastatic cancers that affect the bone are frequently associated with severe and intractable BCP including nociceptive, neuropathic, and inflammatory pain.[1,24] Although the etiology of BCP remains unclear, animal models of BCP make it possible to unravel neuropathological processes that occur in the region of tumor growth. In recent years, next-generation
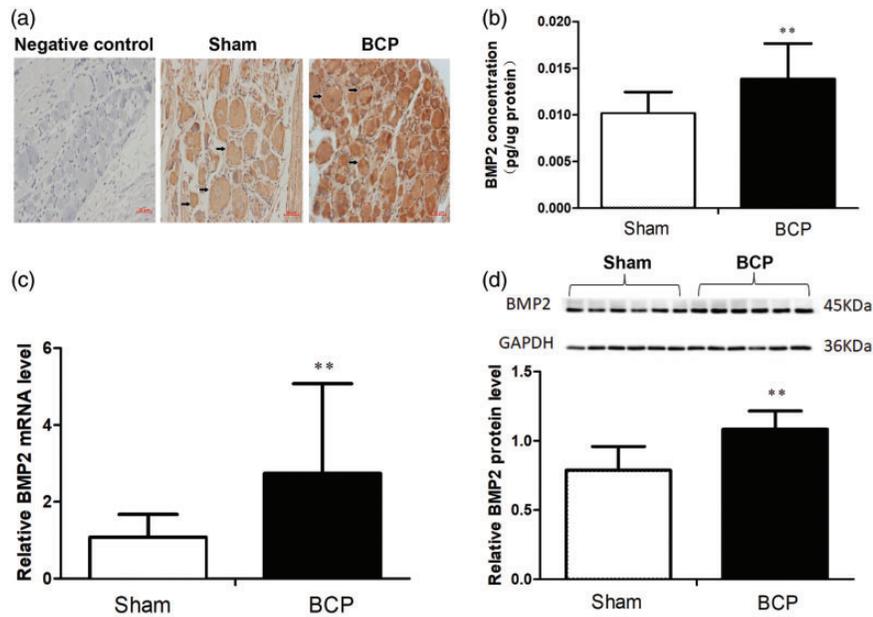
**Figure 3.** Upregulation of the expression of Bmp2 in DRGs of BCP rats. The expression of Bmp2 in DRGs from BCP rats was validated by immunohistochemistry (a), RT-qPCR (b), ELISA (c), and western blotting (d). Immunofluorescent microscopy was done using anti-BMP2 antibody as described in Materials and method section. The mRNA level of BMP2 was measured by real-time RT-PCR and normalized by $\beta$-actin. The protein level of BMP2 in DRG samples was measured by ELISA and western blotting signals. Representative protein bands are presented on the top of the responding histogram. Data (b and c) are presented as mean $\pm$ SD ($n=12$ rats per group), and inter-group differences are assessed using Student's $t$ test. *$P<0.05$, **$P<0.01$, compared with the sham control.
BCP: bone cancer pain; BMP2: bone morphogenetic protein 2.

sequencing including RNA-seq has been used to analyze the cellular transcriptome for mechanistic study and translational medicine. However, gene expression profiling by RNA-seq of DRGs during BCP and peripheral hypersensitivity has not been reported. Here, we made use of RNA-seq and identified eighty DEGs in DRGs from BCP compared to sham rats. Functional annotation showed that nine of the DEGs were associated with bone pain. For example, *Ttr* mutation was associated with familial amyloid polyneuropathy and refractory neuropathic pain.[25,26] Sdpr was reported to be increased in peripheral inflammatory pain.[27] In addition, eleven DEGs were associated with cancer development, suggesting that the alterations in these genes may play important roles in cancer metastasis. Gene ontology and Kyoto Encyclopedia of Genes and Genomes analysis showed that the DEGs were enriched in the Hippo, mTOR, and TGF-β signaling pathways and associated with inflammation, enzyme activity, synaptic cleft, etc. In previous studies of BCP,[28] dorsal horn activation of mTOR was shown to participate in N-methyl-D-aspartate receptor-triggered dorsal central sensitization. In addition, previous studies[29] have found that TGF-β1 is highly expressed in tumor-bearing bone, where it contributes to BCP via the upregulation and sensitization of transient receptor potential vanilloid type 1 in primary sensory neurons.

BMPs are a group of growth and morphogenetic factors originally discovered by their ability to induce the formation of bone and cartilage. Among the BMP family members, BMP2-7 belongs to the TGF-β superfamily of proteins. BMP2, 4, 6, and 7 are commonly referred to as the osteogenic BMPs, based on their potent bone-inducing activity.[30] BMP3 is a negative regulator of bone density.[31] BMP4 is an important factor in early nervous system development and regeneration after injury and a critical mediator of adult physiological plasticity.[32] In this study, we found that *Bmp2* was significantly increased in DRGs from a rat BCP model. Notably, *Bmp2* is involved in the Hedgehog and TGF-β signaling pathways and in cytokine-cytokine receptor interactions important for cell proliferation, adhesion, migration, and apoptosis. BMP targeting, either through deletion of BMP coreceptor RGMb or BMP antagonist Noggin, retarded early axonal regeneration after sciatic nerve crush injury.[33] Moreover, implantation of recombinant human BMP2 in lumbar spine triggered potent neuroinflammatory responses in the DRGs.[34] However, the role of *Bmp2* in DRGs during peripheral hypersensitivity of BCP was unknown.

There are several limitations for the current study. First, we did not include double labeling of the nociceptive neurons and *Bmp2* in the immunohistochemical analyses. Therefore, it is unknown whether *Bmp2* is
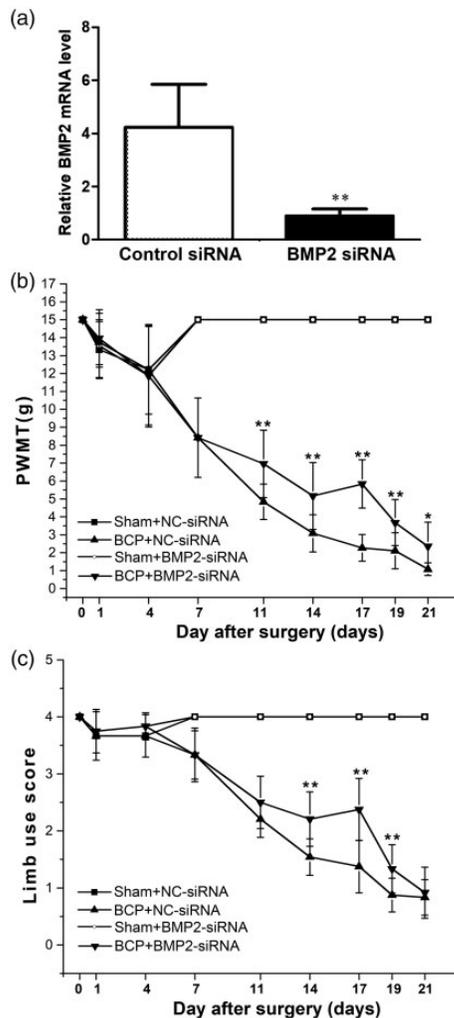
**Figure 4.** Targeting Bmp2 by intrathecal siRNA administration in BCP rats. (a) The knockdown effect of Bmp2 siRNA was tested by RT-qPCR ($n=8$). (b) and (c) PWMT and PWTL were measured after Bmp2 siRNA administration in BCP rats at different time points. Data (b and c) are presented as mean $\pm$ SD ($n=12$ rats per group), and inter-group differences are assessed using Student's t test. *$P<0.05$, **$P<0.01$, compared with the BCP+NC-siRNA group.
BCP: bone cancer pain; BMP2: bone morphogenetic protein 2.

specifically expressed in nociceptive DRG neurons. Second, our assessment of siRNA efficiency by mRNA expression analysis would be improved by further confirmation using western blot analysis.

Despite their limitations, our results confirm the importance of *Bmp2* in the mechanism of peripheral sensitization of BCP. In this study, breast cancer cell injection led to upregulation of *Bmp2* expression in DRG neurons, which was correlated with enhanced neuronal excitability and pain hypersensitivity. In vivo targeting of *Bmp2* by siRNA attenuated pain hypersensitivity and ameliorated BCP in rats. Our data suggest that BMP2

antagonists may be effective for treatment of BCP hypersensitivity.

## Author Contributions

WJX designed and supervised the research project and wrote the paper. WW executed the rat bone cancer model and performed western blotting and data analysis. JQL performed the paw withdrawal threshold and flinching behavior tests. TW assisted with the rat bone cancer model. XMY aided in data analysis.

## Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

## Supplemental Material

Supplemental Material is available for this article online.

## References

1. Clohisy DR and Mantyh PW. Bone cancer pain. *Clin Orthop Relat Res* 2003;(415 suppl): S279–S288.
2. Schmidt BL, Hamamoto DT, Simone DA and Wilcox GL. Mechanism of cancer pain. *Mol Interv* 2010; 10: 164–178.
3. Schwei MJ, Honore P, Rogers SD, Salak-Johnson JL, Finke MP, Ramnaraine ML, Clohisy DR and Mantyh PW. Neurochemical and cellular reorganization of the spinal cord in a murine model of bone cancer pain. *J Neurosci* 1999; 19: 10886–10897.
4. Mercadante S. Malignant bone pain: pathophysiology and treatment. *Pain* 1997; 69: 1–18.
5. Lutz S, Balboni T, Jones J, Lo S, Petit J, Rich SE, Wong R and Hahn C. Palliative radiation therapy for bone metastases: update of an ASTRO Evidence-Based Guideline. *Pract Radiat Oncol* 2017; 7: 4–12.
6. Urch CE and Dickenson AH. Neuropathic pain in cancer. *Eur J Cancer* 2008; 44: 1091–1096.
7. Zheng Q, Fang D, Cai J, Wan Y, Han JS and Xing GG. Enhanced excitability of small dorsal root ganglion neurons in rats with bone cancer pain. *Mol Pain* 2012; 8: 24.
8. Khasabov SG, Hamamoto DT, Harding-Rose C and Simone DA. Tumor-evoked hyperalgesia and sensitization of nociceptive dorsal horn neurons in a murine model of cancer pain. *Brain Res* 2007; 1180: 7–19.

9. Wu JX, Xu MY, Miao XR, Lu ZJ, Yuan XM, Li XQ and Yu WF. Functional up-regulation of P2X3 receptors in dorsal root ganglion in a rat model of bone cancer pain. *Eur J Pain* 2012; 16: 1378–1388.

10. Qiu F, Jiang Y, Zhang H, Liu Y and Mi W. Increased expression of tetrodotoxin-resistant sodium channels Nav1.8 and Nav1.9 within dorsal root ganglia in a rat model of bone cancer pain. *Neurosci Lett* 2012; 512: 61–66.

11. Tomotsuka N, Kaku R, Obata N, Matsuoka Y, Kanzaki H, Taniguchi A, Muto N, Omiya H, Itano Y, Sato T, Ichikawa H, Mizobuchi S and Morimatsu H. Up-regulation of brain-derived neurotrophic factor in the dorsal root ganglion of the rat bone cancer pain model. *J Pain Res* 2014; 7: 415–423.

12. Qiu F, Wei X, Zhang S, Yuan W and Mi W. Increased expression of acid-sensing ion channel 3 within dorsal root ganglia in a rat model of bone cancer pain. *Neuroreport* 2014; 25: 887–893.

13. Liu XD, Yang JJ, Fang D, Cai J, Wan Y and Xing GG. Functional upregulation of nav1.8 sodium channels on the membrane of dorsal root ganglia neurons contributes to the development of cancer-induced bone pain. *PLoS One* 2014; 9: e114623.

14. Bao Y, Hou W, Yang L, Liu R, Gao Y, Kong X, Shi Z, Li W, Zheng H, Jiang S and Hua B. Increased expression of protease-activated receptor 2 and 4 within dorsal root ganglia in a rat model of bone cancer pain. *J Mol Neurosci* 2015; 55: 706–714.

15. Wei J, Li M, Wang D, Zhu H, Kong X, Wang S, Zhou YL, Ju Z, Xu GY and Jiang GQ. Overexpression of suppressor of cytokine signaling 3 in dorsal root ganglion attenuates cancer-induced pain in rats. *Mol Pain* 2017; 13: 1744806916688901.

16. Zhu GQ, Liu S, He DD, Liu YP and Song XJ. Activation of the cAMP-PKA signaling pathway in rat dorsal root ganglion and spinal cord contributes toward induction and maintenance of bone cancer pain. *Behav Pharmacol* 2014; 25: 267–276.

17. Guan X, Fu Q, Xiong B, Song Z, Shu B, Bu H, Xu B, Manyande A, Cao F and Tian Y. Activation of PI3Kgamma/Akt pathway mediates bone cancer pain in rats. *J Neurochem* 2015; 134: 590–600.

18. Liu S, Lv Y, Wan XX, Song ZJ, Liu YP, Miao S, Wang GL and Liu GJ. Hedgehog signaling contributes to bone cancer pain by regulating sensory neuron excitability in rats. *Mol Pain* 2018; 14: 1744806918767560.

19. Huang Y. Expression of BDNF in dorsal root ganglion of rats with bone cancer pain and its effect on pain behavior. *J Musculoskelet Neuronal Interact* 2018; 18: 42–46.

20. Sun Y, Tian Y, Li H, Zhang D and Sun Q. Antinociceptive effect of intrathecal injection of genetically engineered human bone marrow stem cells expressing the human pro-enkephalin gene in a rat model of bone cancer pain. *Pain Res Manag* 2017; 2017: 1–11.

21. Zimmermann M. Ethical guidelines for investigations of experimental pain in conscious animals. *Pain* 1983; 16: 109–110.

22. Zhang RX, Liu B, Li A, Wang L, Ren K, Qiao JT, Berman BM and Lao L. Interleukin 1beta facilitates bone cancer pain in rats by enhancing NMDA receptor NR-1 subunit phosphorylation. *Neuroscience* 2008; 154: 1533–1538.

23. Pogatzki EM and Raja SN. A mouse model of incisional pain. *Anesthesiology* 2003; 99: 1023–1027.

24. Colvin L and Fallon M. Challenges in cancer pain management–Bone pain. *Eur J Cancer* 2008; 44: 1083–1090.

25. Mazzeo A, Russo M, Di Bella G, Minutoli F, Stancanelli C, Gentile L, Baldari S, Carerj S, Toscano A and Vita G. Transthyretin-related familial amyloid polyneuropathy (TTR-FAP): a single-center experience in sicily, an Italian endemic area. *J Neuromuscul Dis* 2015; 2: S39–S48.

26. Plante-Bordeneuve V and Said G. Transthyretin related familial amyloid polyneuropathy. *Curr Opin Neurol* 2000; 13: 569–573.

27. Tome ME, Herndon JM, Schaefer CP, Jacobs LM, Zhang Y, Jarvis CK and Davis TP. P-glycoprotein traffics from the nucleus to the plasma membrane in rat brain endothelium during inflammatory pain. *J Cereb Blood Flow Metab* 2016; 36: 1913–1928.

28. Shih MH, Kao SC, Wang W, Yaster M and Tao YX. Spinal cord NMDA receptor-mediated activation of mammalian target of rapamycin is required for the development and maintenance of bone cancer-induced pain hypersensitivities in rats. *J Pain* 2012; 13: 338–349.

29. Xu Q, Zhang XM, Duan KZ, Gu XY, Han M, Liu BL, Zhao ZQ and Zhang YQ. Peripheral TGF-beta1 signaling is a critical event in bone cancer-induced hyperalgesia in rodents. *J Neurosci* 2013; 33: 19099–19111.

30. Luu HH, Song WX, Luo X, Manning D, Luo J, Deng ZL, Sharff KA, Montag AG, Haydon RC and He TC. Distinct roles of bone morphogenetic proteins in osteogenic differentiation of mesenchymal stem cells. *J Orthop Res* 2007; 25: 665–677.

31. Daluiski A, Engstrand T, Bahamonde ME, Gamer LW, Agius E, Stevenson SL, Cox K, Rosen V and Lyons KM. Bone morphogenetic protein-3 is a negative regulator of bone density. *Nat Genet* 2001; 27: 84–88.

32. Bhattacherjee A, Rumi MA, Staecker H and Smith PG. Bone morphogenetic protein 4 mediates estrogen-regulated sensory axon plasticity in the adult female reproductive tract. *J Neurosci* 2013; 33: 1050–1061a.

33. Ma CH, Brenner GJ, Omura T, Samad OA, Costigan M, Inquimbert P, Niederkofler V, Salie R, Sun CC, Lin HY, Arber S, Coppola G, Woolf CJ and Samad TA. The BMP coreceptor RGMb promotes while the endogenous BMP antagonist noggin reduces neurite outgrowth and peripheral nerve regeneration by modulating BMP signaling. *J Neurosci* 2011; 31: 18391–18400.

34. Mitchell K, Shah JP, Dalgard CL, Tsytsikova LV, Tipton AC, Dmitriev AE and Symes AJ. Bone morphogenetic protein-2-mediated pain and inflammation in a rat model of posterolateral arthrodesis. *BMC Neurosci* 2016; 17: 80.