

Connexin 43 Mediates CXCL12 Production from Spinal Dorsal Horn to Maintain Bone Cancer Pain in Rats

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Abstract Tumor metastasis to bone can subsequently lead to bone cancer pain (BCP). Currently, BCP is difficult to conquer due to a poor understanding of the potential mechanisms. Several studies have indicated that astrocyte-specific connexin 43 (Cx43) was involved in the neuropathic pain, and Cx43 induced the release of chemokine CXCL12 in bone marrow stromal cells. However, whether spinal Cx43 mediates the production of CXCL12 to participate in the maintenance of BCP is still unknown. Here we showed that Walker 256 tumor cells inoculation into the tibia induced a significant mechanical allodynia, which was accompanied by upregulation of spinal p-Cx43 and CXCL12 expression levels from day 6 to day 18 after inoculation. Spinal Cx43 was mainly expressed in astrocytes, and intrathecal ⁴³Gap26 (a selective Cx43 blocker) markedly attenuated mechanical allodynia as well as reduced p-Cx43 and CXCL12 expression at day 18 after inoculation. Pre-intrathecal administration of CXCL12 almost abolished the attenuated mechanical allodynia by ⁴³Gap26. Furthermore, intrathecal injection of anti-CXCL12 neutralizing antibody could ameliorate mechanical allodynia with concomitant inhibition of upregulation of CXCL12 expression, but not influence on p-Cx43 expression. Our results indicate that Cx43 mediates

CXCL12 production from spinal dorsal horn in astrocytes to maintain bone cancer pain in rats. These findings may improve our understanding of the underlying mechanisms of BCP and provide a novel target for the treatment of BCP.

Keywords Bone cancer pain · Cx43 · CXCL12 · Chemokine · Spinal dorsal horn

Introduction

Bone metastasis is a common characteristic of many aggressive cancers, such as breast, kidney, lung, and prostate cancer [1, 2]. Tumor metastasis to bone can subsequently lead to bone cancer pain (BCP) [3, 4]. Clinically, BCP is difficult to conquer because response to current pharmacological treatment still remains poor. Therefore, understanding the potential molecular mechanisms of BCP may explore new targets for the therapeutic intervention.

Gap junctions allow cells to communicate directly with one another in the central nervous system (CNS). The main structural constituents of gap junctions are connexins, and the connexin 43 (Cx43) subunit is the most prominent connexin expressed by astrocytes [5]. Increasing evidence demonstrated that activation of astrocytes in the spinal dorsal horn might play a vital role in BCP [6, 7]. Several studies indicated that astrocyte-specific Cx43 was involved in the neuropathic pain in multiple animal models [8–11]. However, the characteristics of BCP are not the same as neuropathic pain, and it has unique molecular mechanisms [1, 4]. Whether spinal Cx43 is involved in BCP remains unclear.

Activated astrocytes may secrete various inflammatory mediators, including cytokines and chemokines, which

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may amplify the nociceptive signals in the CNS. Previous studies have demonstrated that spinal chemokine CXC motif ligand 12 (CXCL12) was upregulated in astrocytes after tumor cell implantation, which contributed to the initiation and maintenance of BCP [6, 12]. Cx43 can induce the production of CXCL12 in bone marrow stromal cells [13]. However, whether spinal Cx43 is able to regulate the secretion of CXCL12 to participate in the maintenance of BCP needs to be investigated.

In the present study, we examined Cx43 expression and distribution in the spinal dorsal horns after injection of Walker 256 carcinoma cells into the tibia. The roles of Cx43 and CXCL12 in pain hypersensitivity after Walker 256 cells inoculation were also being examined.

Materials and Methods

Animals

Female Wistar rats weighing between 150 and 180 g were purchased from the animal center of Shanghai Laboratory and housed individually at a temperature of 22 ± 2 °C with free access to both food and water. All animal procedures in this study were in accordance with the recommendations of the International Association for the Study of Pain [14], and approved by the Animal Care and Use Committee of the Jiangsu University.

Preparation of Tumor Cells

Walker 256 mammary gland carcinoma cells were kindly supplied by the Soochow University (China). The female rats were injected intraperitoneally with tumor cells (0.5 ml, 1×10^7 cells/ml). After 6–7 days, tumor cells were collected from the ascites, rinsed in Hanks' solution and centrifugation for 5 min at 3000 g. Then, the pellet was resuspended in Hanks' solution and the concentration was adjusted to 1×10^5 cells per 10 μ L. The suspension of tumor cells was maintained on ice until injection.

Bone Cancer Pain Model

Our previously described model of rat BCP was used [15, 16]. In brief, under anesthesia with pentobarbital sodium (50 mg/kg, i.p), the animal was placed with the abdominal side up. A small skin incision was made in a disinfected area on the surface of the left tibia and the tibial plateau carefully exposed. A 23-gauge needle was used to drill a hole in the tibia, then, a volume of 10 μ L containing 1×10^5 tumor cells was injected into the intramedullary space of the tibia. Subsequently, the injection hole was sealed with bone wax. Sham rats were injected with same

amount of heat-killed tumor cells. Finally, the rats were placed on a heat pad until fully awake.

Intrathecal Cannulation

Intrathecal catheter implantation was carried out as our previously described [17, 18]. In brief, 3 days before any procedures, under anesthesia with pentobarbital sodium (50 mg/kg, i.p), a PE-10 polyethylene tube was inserted through the space between the L5 and L6 vertebrae. The cannula position was determined by intrathecal injection of 1 % lidocaine (10 μ L). The animals were excluded from the study if insertion of the catheter caused an evidence of neurological damage.

Drug Application

⁴³Gap26 (a selective Cx43 blocker) was purchased from AnaSpec (Fremont, CA, USA) and dissolved in phosphate buffered saline (PBS). Anti-CXCL12 neutralizing antibody, recombinant rat CXCL12, and normal goat IgG (for control) were obtained from Abcam (Cambridge, MA, USA). All drugs were delivered through the intrathecal catheter in a total volume of 10 μ L. The dosage of drugs is mainly relied on the results from pilot experiments.

Radiology

The rats were radiographed at day18 after inoculation to verify cancer development in the tibia. The rats were anesthetized with pentobarbital sodium (50 mg/kg, i.p), the hind limbs were exposed to X-ray for 1/20 s at 40 kVp. The images were taken from sham rats and BCP rats.

Behavioral Analysis

The general health situation of the animals was evaluated daily. The body weights were measured at day 0, 6, 12, 18 in both sham rats and BCP rats. A series of von Frey filaments (Stoelting, Wood Dale, IL, USA) were used to assess the mechanical allodynia, as described previously [19]. In brief, animals were put in separate test cages (25 \times 20 \times 15 cm) with a metal mesh floor, and allowed to adapt to the experimental environment for 30 min. The experimenter applied the tip of hair perpendicular to the plantar surface of the left hind paw for 5 s with enough force to cause a slight bend. A positive response was defined as brisk withdrawal or paw flinching, and the Dixon's up-and-down method was used to determine the 50 % paw withdrawal threshold (PWT) to mechanical stimuli [20]. Behavioral testing was performed before and then at day 6, 12, and 18 post-carcinoma inoculation in a blinded manner.

Immunohistochemistry

Under deep anesthesia with pentobarbital sodium, the rats were transcardially perfused with PBS followed by 4 % paraformaldehyde. The L4 and L5 spinal segments were quickly removed, and post-fixed overnight with the same fixative at 4 °C, then, cut with the vibratome to a thickness of 30 μ m. The sections were first blocked for 1 h using 2 % goat serum at room temperature, then incubated overnight at 4 °C with the following primary antibodies: phospho-Cx43 (Ser368) polyclonal antibody (rabbit, 1:100; Sigma-Aldrich, Shanghai, China), OX-42 monoclonal antibody (mouse, 1:400; Abcam, Cambridge, MA, USA), GFAP monoclonal antibody (mouse, 1:800; Millipore, Billerica, MA, USA), NeuN monoclonal antibody (mouse, 1:1000, Abcam), CXCL12 polyclonal antibody (rabbit, 1:400; Abcam, Cambridge, MA, USA). After three times washing with PBS, the sections were incubated for 1 h at room temperature with the DyLight™ 488 or 594 conjugated secondary antibodies (1:800, Jackson ImmunoResearch Europe Ltd, UK). For double staining, the sections were incubated with a mixture of primary antibodies followed by a mixture of conjugated secondary antibodies. Finally, the sections were detected by a laser scanning confocal microscopy. Image J software was used to analyze the fluorescence intensity of images. The intensities of immunoreactivity were measured in four to six randomly selected sections from each animal in laminae I and II of the spinal dorsal horn. The average intensity from each animal was calculated and these values from at least four animals in each group were averaged and are presented as group data [15].

Western Blot

Under deep anesthesia with pentobarbital sodium, the rats were sacrificed. The spinal dorsal horn segments (L4 and L5) were rapidly dissected and frozen in liquid nitrogen and stored at -70 °C. The spinal segments were homogenized in RIPA buffer plus the inhibitors of phosphatase and protease. Then, the homogenates were centrifuged for 15 min at 13,000g at 4 °C to get the supernatants for Western blot analysis. The concentrations of protein were measured by using the BCA method according to the kit's instructions. Equivalent amounts of protein (50 μ g) were separated on SDS-PAGE gel (10 %) and transferred to the PVDF membrane. Then, the membranes were incubated with p-Cx43 polyclonal antibody (rabbit, 1:1000; Sigma) overnight at 4 °C. After washed with PBST three times, the membranes were incubated with horseradish peroxidase-conjugated secondary antibody. The blots were detected using a chemiluminescence detection system and exposed to film. β -actin (mouse, 1:5000; Sigma) was used as a

loading control. Western blotting bands from four independent experiments were quantified with Image J. Each band density of phosphorylated Cx43 was compared to that of the β -actin and normalized to the intensity of the sham sample.

ELISA

Protein samples were prepared in the same way as for Western blot analysis. The expression level of CXCL12 was examined using a rat CXCL12 ELISA kit (Abnova Ltd, Cambridge, UK) following the manufacturer's protocol.

Statistical Analysis

Data were presented as the mean \pm SEM and analyzed using SPSS16.0 software. The molecular data were analyzed by using one-way ANOVA followed by Bonferroni test, and the behavioral data were analyzed by using repeated-measures (RM) ANOVA followed by Bonferroni test. $P < 0.05$ was regarded statistically significant.

Results

Intramedullary Inoculation of Walker 256 Tumor Cells Induces Bone Cancer Pain

After Walker 256 tumor cells were injected into the tibia, the rats showed general good health and gradual increase in body weight during an 18-day observation period (Fig. 1c). Eighteen days after inoculation, major cortical destruction and severe loss of medullary bone were distinctly detected in the proximal epiphysis of the left tibia. However, there was no radiological change in the contralateral tibia or bilateral tibia of sham rats (Fig. 1a, b). The ipsilateral PWT to von Frey filaments was decreased from 8.0 ± 0.9 g before inoculation to 5.1 ± 0.8 g at day 6, 3.1 ± 0.7 g at day 12, and 2.3 ± 0.6 g at day 18. Neither the contralateral paw of BCP rats nor bilateral paws of sham rats showed a significant change in pain sensitivity (Fig. 1d). Pain behavioral results showed that injection of Walker 256 cells into the tibia led to the development of mechanical allodynia.

Upregulation of p-Cx43 and CXCL12 Expression Levels in Spinal Dorsal Horn in BCP Rats

We first examined the expression and distribution of p-Cx43 in the spinal dorsal horn after Walker 256 tumor cells inoculation. The result of immunostaining showed

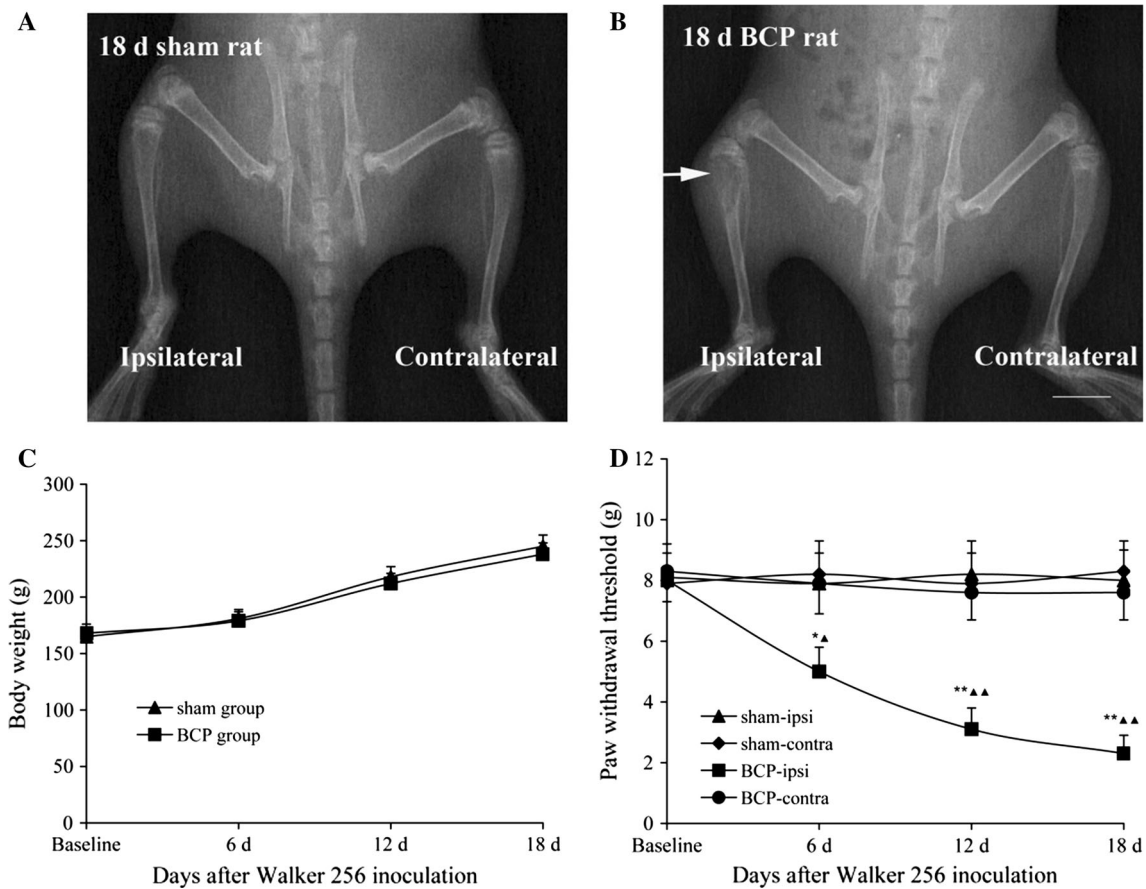


Fig. 1 Intramedullary inoculation of Walker 256 tumor cells induces bone cancer pain. **a, b** Radiography showed severe loss of medullary bone and major cortical destruction of the left tibia (arrow) at day 18 after inoculation. No radiological changes were found in the contralateral tibia or bilateral tibia of sham rats. **c** The body weight was gradually increased in both sham rats and BCP rats during an 18-day observation period. **d** The ipsilateral PWT progressively

decreased from day 6 to day 18 in BCP rats. Neither the contralateral paw of BCP rats nor bilateral paws of sham rats showed a significant change in pain sensitivity. Data were expressed as mean \pm SEM. $n = 8$ rats in each group [$*P < 0.05$, $**P < 0.01$ vs. sham-ipsi., $\blacktriangle P < 0.05$, $\blacktriangle\blacktriangle P < 0.01$ vs. baseline using repeated-measure (RM) ANOVA followed by Bonferroni test]. BCP bone cancer pain. Scale bar 1 mm (**a, b**)

that a significant increase of p-Cx43 protein expression in the ipsilateral spinal dorsal horn at day 6, 12, 18 after inoculation (Fig. 2a–d). Compare with sham rats, the intensity of p-Cx43 immunoreactivity in BCP rats was gradually increased from day 6 to day 18 ($P < 0.01$, Fig. 2h). In addition, Western blot analysis also revealed that tibia inoculation of Walker 256 cells induced a progressively increased expression of p-Cx43 protein from day 6 to day 18 after tumor cells inoculation ($P < 0.01$, Fig. 2i). To detect the cellular distribution of p-Cx43 in spinal dorsal horn after inoculation, we carried out double immunostaining of p-Cx43 with different cell markers: GFAP (for astrocytes), OX-42 (for microglia), and NeuN (for neurons). Confocal images indicated that p-Cx43 was extensively colocalized with GFAP (Fig. 2e), but not with OX-42 (Fig. 2f) or NeuN (Fig. 2g), which suggested that the expression of p-Cx43 by astrocytes, but not by microglia or neurons in BCP rats.

We then used ELISA analysis to measure CXCL12 produced in spinal dorsal horn in BCP rats. As shown in Fig. 2j, the concentration of CXCL12 significantly increased from day 6 to day 18 in BCP rats. The CXCL12 expression peaked at day 12, and increased expression was sustained up to day 18 in BCP rats. These results suggested that spinal CXCL12 was upregulated in inoculated rats.

Intrathecal Administration of ⁴³Gap26 Attenuated Pain Behavior and Reduced p-Cx43 and CXCL12 Expression in BCP Rats

To investigate the exact role of Cx43 in bone cancer pain, ⁴³Gap26, a selective Cx43 blocker, was administered intrathecally at day 18 after tumor cells inoculation. As shown in Fig. 3, intrathecal injection of ⁴³Gap26 reversed mechanical allodynia for about 1.5 h, in a dose-dependent

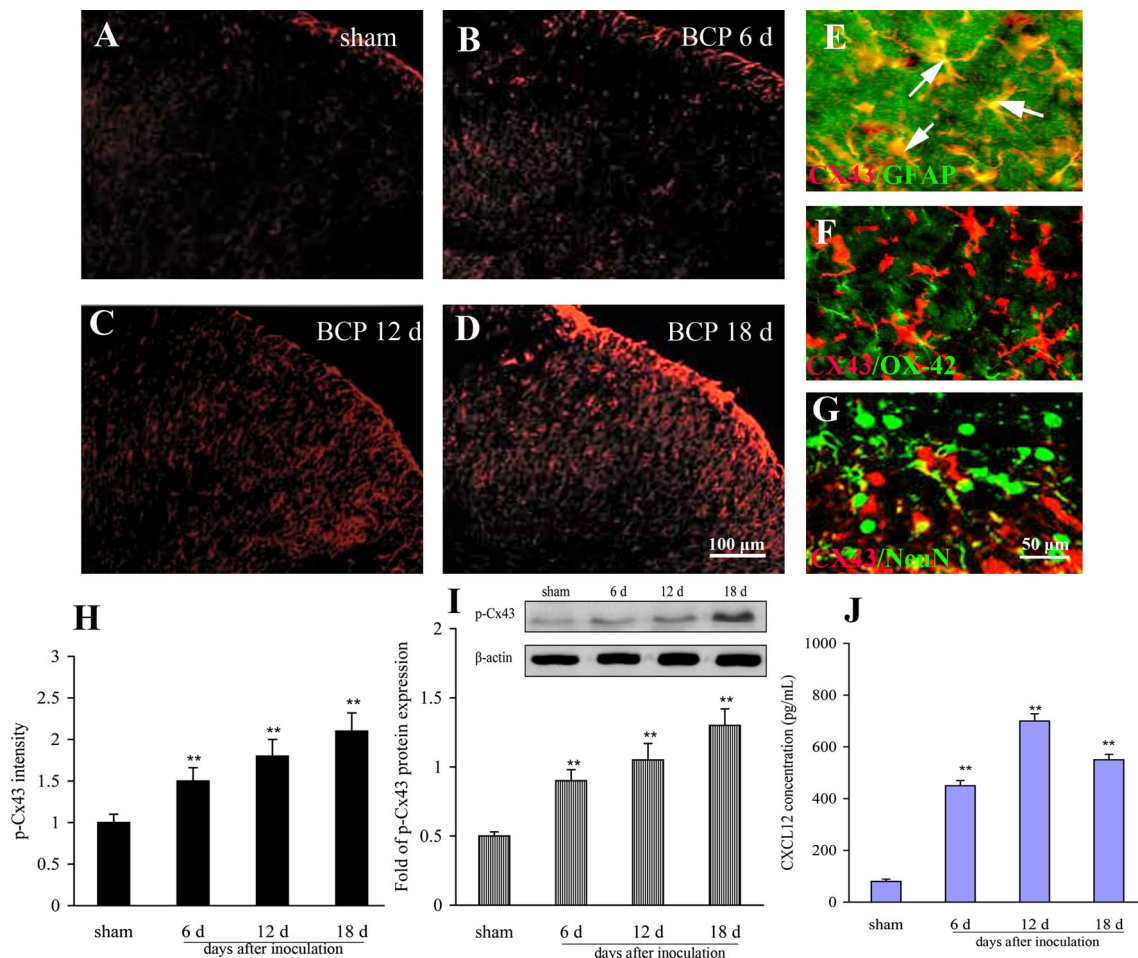


Fig. 2 Upregulation of p-Cx43 and CXCL12 expression in spinal dorsal horn in BCP rats. **a–d** Immunostaining indicated that a significant increase of p-Cx43 expression in the ipsilateral spinal dorsal horn at day 6, 12, 18 after inoculation. *Scale bar* 100 μ m. **e–g** Double immunostaining showed that p-Cx43 (red) was colocalized with GFAP (**e**, arrows indicate double-labeled, yellow), but not with OX-42 (green, **f**) or NeuN (green, **g**). Tissues were collected at day 18 after inoculation. *Scale bar* 50 μ m. **h** Compare with sham rats, the intensity of p-Cx43 immunoreactivity in BCP rats was gradually

increased from day 6 to day 18 after tumor cells inoculation. **i** Each image shows representative western blotting bands from four independent experiments. Western blot analysis showed that a progressively increased expression of p-Cx43 protein from day 6 to day 18 after inoculation. **j** The ELISA analysis showed that the concentration of CXCL12 significantly increased from day 6 to day 18 in BCP rats. All data were expressed as mean \pm SEM. $n = 4$ rats in each group (** $P < 0.01$ vs. sham rats using one-way ANOVA followed by Bonferroni test) (Color figure online)

manner ($P < 0.05$ or 0.01). Intrathecal dose (10 μ g) of 43 Gap26 almost completely reversed mechanical allodynia at the 1.5 h time point after intrathecal injection ($P < 0.01$, Fig. 3).

To explore the roles of Cx43-CXCL12 interaction in modulation of BCP, the recombinant rat CXCL12 (0.1 μ g) was intrathecally administered 1.5 h before 43 Gap26 administration. As shown in Fig. 3, intrathecal CXCL12 (0.1 μ g) exhibited no effects on the PWT compared with PBS group, however, in CXCL12 0.1 μ g + 43 Gap26 10 μ g group, pre-intrathecal injection of CXCL12 almost completely abolished the attenuated mechanical allodynia by 43 Gap26 administration.

To examine whether intrathecal 43 Gap26 would affect the spinal p-Cx43 and CXCL12 expression, the spinal dorsal horn segments were collected at 1.5 h after intrathecal administration. Western blot analysis indicated that intrathecal 43 Gap26 (10 μ g) could markedly attenuate spinal p-Cx43 expression. The p-Cx43 expression in the PBS group was significant higher than that of sham rats ($P < 0.01$, Fig. 4a). ELISA analysis and immunostaining indicated that intrathecal 43 Gap26 (10 μ g) could markedly decrease CXCL12 expression in BCP rats ($P < 0.01$). The CXCL12 expression level in the PBS group was significant higher than that of Gap26-treated rats or sham rats ($P < 0.01$, Fig. 4b–f).

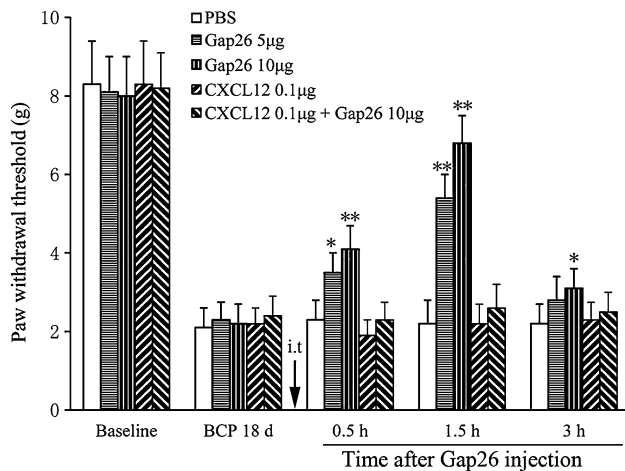


Fig. 3 Intrathecal administration of $^{43}\text{Gap26}$ attenuated pain behavior in BCP rats. Intrathecal injection of $^{43}\text{Gap26}$ reversed mechanical allodynia for about 1.5 h, in a dose-dependent manner. Intrathecal dose (10 μg) of $^{43}\text{Gap26}$ almost completely reversed mechanical allodynia at the 1.5 h time point after intrathecal injection. In CXCL12 0.1 μg + $^{43}\text{Gap26}$ 10 μg group, CXCL12 was intrathecally administered 1.5 h before $^{43}\text{Gap26}$ administration. Pre-intrathecal injection of CXCL12 almost completely abolished the attenuated mechanical allodynia by $^{43}\text{Gap26}$. Data were expressed as mean \pm SEM. $n = 8$ rats in each group [$*P < 0.05$, $**P < 0.01$ vs. PBS group using repeated-measure (RM) ANOVA followed by Bonferroni test]

Taken together, these results indicated that inhibition of spinal p-Cx43 upregulation resulted in attenuation of pain behavior as well as decrease of CXCL12 production from spinal dorsal horn in BCP rats.

Intrathecal Administration of Anti-CXCL12 Neutralizing Antibody Attenuated Pain Behavior and Decreased CXCL12 Expression in BCP Rats

To further verify Cx43 is an upstream molecule of CXCL12 for maintenance of bone cancer pain, anti-CXCL12 neutralizing antibody was used to confirm this hypothesis. Eighteen days after inoculation, intrathecal anti-CXCL12 neutralizing antibody (4 and 8 μg) could markedly reverse mechanical allodynia in a dose-dependent manner ($P < 0.05$ or 0.01, Fig. 5a). The spinal dorsal horn segments were collected at 6 h after intrathecal administration, and ELISA analysis showed that intrathecal anti-CXCL12 neutralizing antibody (8 μg) could significantly reduce spinal CXCL12 expression in BCP rats ($P < 0.01$, Fig. 5b). However, Western blot analysis showed that there was no influence on spinal p-Cx43 expression by intrathecal injection of anti-CXCL12 neutralizing antibody (Fig. 5c). Taken together, these findings further indicate that Cx43 mediates CXCL12 production to maintain bone cancer pain in rats.

Discussion

Bone cancer pain is a complex pain state including background pain, spontaneous pain, and incident pain (movement-evoked) [1, 21]. To investigate the potential mechanisms underlying BCP, animal models have been developed using direct inoculation of tumor cells (Walker 256 mammary gland carcinoma cells, osteolytic 2472 murine osteosarcoma tumor cells, and prostate tumor cells) into the intramedullary space of long bones (tibia, femur, and calcaneus) [22, 23]. These models can well mimic the clinical conditions, and enable systematic examination of the molecular and cellular mechanisms of BCP. After inoculation of Walker 256 cells into the tibia, pain behavioral and radiological results indicated that the site-specific BCP model was well established. An interesting ‘mirror-image pain’ phenomenon was described in some BCP studies [24]. However, we found there was no marked change in the contralateral pain sensitivity in BCP rats. Studies have revealed that inoculation of different types tumor cells could lead to different types of pain behaviors [1, 25]. Female rats are more sensitive to Walker 256 mammary gland cells, therefore, only female rats were chosen in the present study.

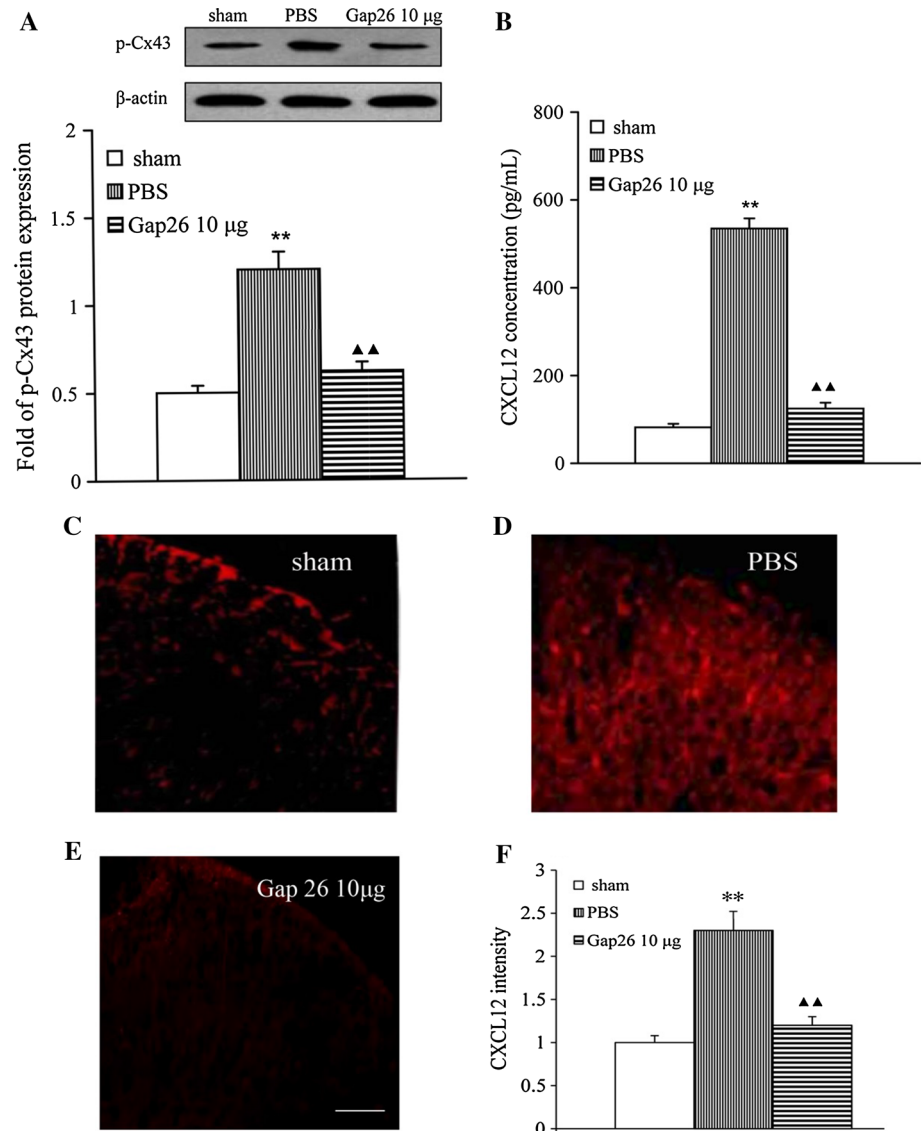
Cx43, an integral membrane protein also known as gap junction alpha-1 protein, is broadly expressed in different tissues including the central nervous system (CNS), and especially expressed in astrocytes [5, 26]. In the current study, we found that spinal Cx43 was mainly expressed in astrocytes in the BCP model. The localization of Cx43 in astrocytes was also confirmed by other investigators in the different neuropathic pain models [10, 11]. Growing evidence indicates that gap junctions in astrocytes are involved in pain modulation. Spinal cord injury-induced heat hyperalgesia and mechanical allodynia were abolished in Cx43/Cx30 double-deletion mice, but not in only Cx30 deletion mice, suggesting a crucial role of Cx43 in the chronic neuropathic pain [27]. Consistently, inhibition of Cx43 expression by using antisense oligodeoxynucleotide led to decrease neuroinflammation and ameliorate pain hypersensitivity in two spinal cord injury models [28]. Intrathecal Cx43 siRNA alleviated mechanical allodynia in a rat model of L5 spinal nerve ligation [8]. Similarly, we found that intrathecal $^{43}\text{Gap26}$ (a selective Cx43 blocker) could attenuate established BCP at day 18 after inoculation, suggesting Cx43 may participate in the maintenance of BCP. Our data indicate that targeting spinal Cx43 may lead to a potential effective therapy for the treatment of the maintenance phase of BCP in clinical practice.

CXCL12, also known as stromal cell-derived factor 1 (SDF-1), is expressed in many kinds of cells including nervous cells, and plays a vital role in nociception

Fig. 4 Intrathecal administration of $^{43}\text{Gap26}$ reduced p-Cx43 and CXCL12 expression in BCP rats.

a Western blot analysis indicated that intrathecal $^{43}\text{Gap26}$ (10 μg) could markedly attenuate spinal p-Cx43 expression. The p-Cx43 expression in the PBS group was significant higher than that of sham rats ($P < 0.01$). Each image shows representative western blotting bands from four independent experiments.

b ELISA and **c–f** immunostaining indicated that intrathecal $^{43}\text{Gap26}$ (10 μg) could markedly decrease CXCL12 expression in BCP rats ($P < 0.01$). The CXCL12 expression level in the PBS group was significant higher than that of Gap26-treated rats or sham rats ($P < 0.01$). Scale bar 100 μm . Tissues were harvested at 1.5 h after intrathecal injection. All data were expressed as mean \pm SEM. $n = 4$ rats in each group (** $P < 0.01$ vs. sham rats, $\blacktriangle\blacktriangle P < 0.01$ vs. PBS group using one-way ANOVA followed by Bonferroni test)



regulation. Studies have shown that intrathecal or intraplantar injection of CXCL12 could induce mechanical hyperalgesia in naive rats [29, 30]. Inhibition of CXCR4, a major receptor of CXCL12, could attenuate pain-related behaviors [31–33]. In the current study, we found that intrathecal anti-CXCL12 neutralizing antibody could attenuate mechanical allodynia in BCP rats. Similar results were reported by Shen et al. [6], who found that CXCL12/CXCR4 contributed to the development and maintenance of BCP.

Cx43 assembles to constitute hemichannels and gap junction channels, which plays a vital physiological role in the behavior of astrocytes as cellular networks [34]. According to the earlier report, Cx43 and Cx45 gap junctions could regulate CXCL12 secretion in bone marrow stromal cells [13]. Increasing evidence suggests that astrocytes can produce proinflammatory cytokines, such as

CXCL12 and CXCL1, which play a vital role in the maintenance of BCP [6, 7, 12]. The results from our present study showed that a causal relationship between Cx43 expression and secretion of CXCL12 in the maintenance of BCP. Astrocytic gap junctions may mediate the intercellular transmission of Ca^{2+} waves to activate neighboring cells [35]. So, we speculate that upregulating Cx43 may activate astrocytes, via intracellular Ca^{2+} -cAMP-PKA signaling pathway, to induce CXCL12 secretion [13]. However, upstream factors for Cx43 activation are still not well documented. Activation of TLR4 via proinflammatory molecules was able to increase the expression of Cx43 in astrocytes [36]. Chen et al. [10] found that TNF- α -induced upregulation of Cx43 led to the chemokine CXCL1 release, which contributed to maintain late-phase neuropathic pain in mice. CXCR4 (a major receptor of CXCL12) expression has been detected in spinal neurons [6, 32]. So, Cx43–

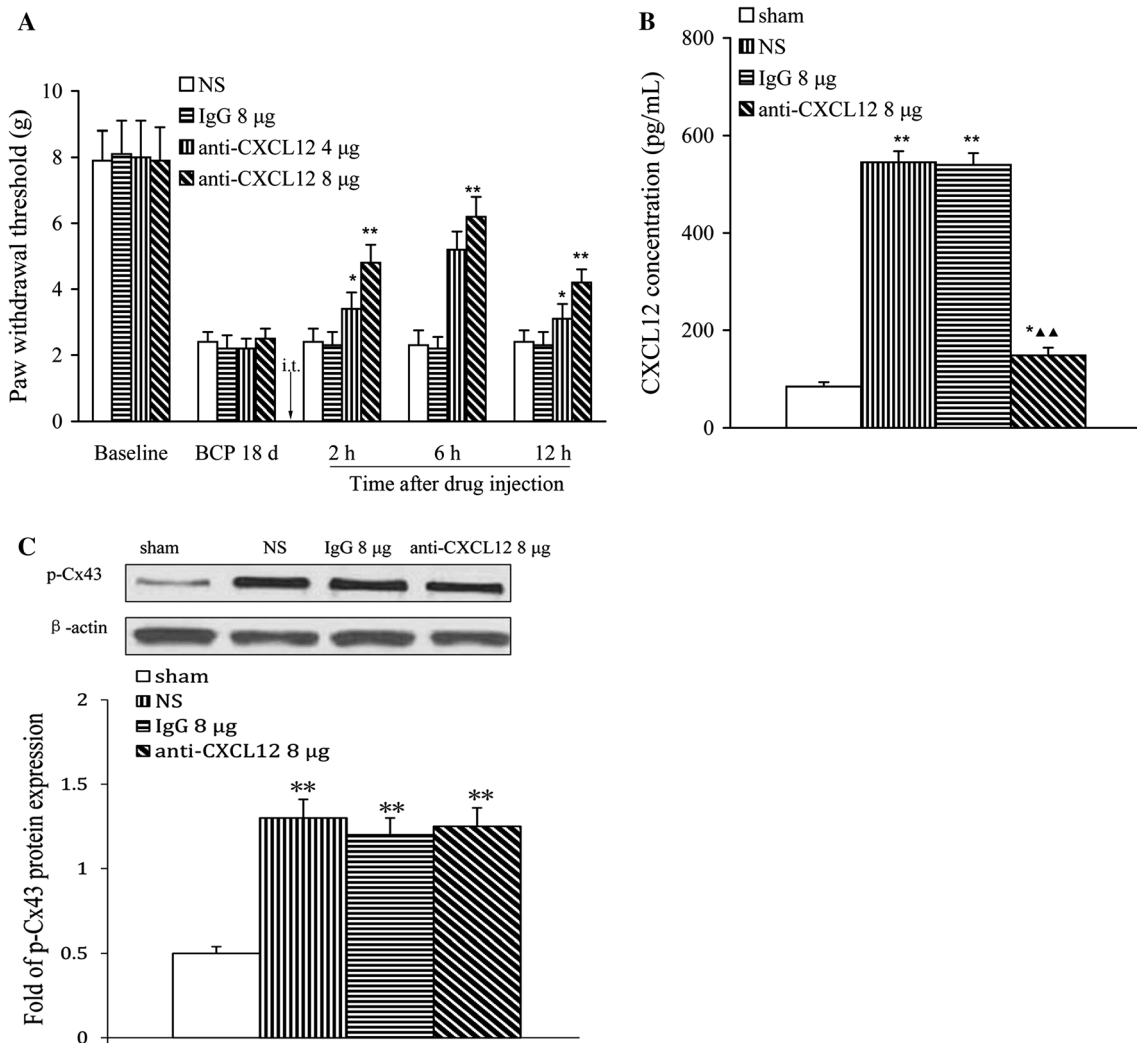


Fig. 5 Intrathecal administration of anti-CXCL12 neutralizing antibody attenuated pain behavior and decreased CXCL12 expression, but not influence on p-Cx43 expression in BCP rats. **a** At day 18 after inoculation, intrathecal anti-CXCL12 neutralizing antibody (4 and 8 µg) could markedly reverse mechanical allodynia in a dose-dependent manner ($P < 0.05$ or 0.01). Data were expressed as mean \pm SEM. $n = 8$ rats in each group [$*P < 0.05$, $**P < 0.01$ vs. NS group using repeated-measure (RM) ANOVA followed by Bonferroni test]. NS normal saline. **b** ELISA analysis showed that intrathecal anti-CXCL12 neutralizing antibody (8 µg) could

significantly reduce spinal CXCL12 expression in BCP rats ($P < 0.01$). **c** Western blot analysis showed that there was no influence on spinal p-Cx43 expression by intrathecal injection of anti-CXCL12 neutralizing antibody (8 µg). Tissues were collected at 6 h after intrathecal injection. Each image shows representative western blotting bands from four independent experiments. Data were expressed as mean \pm SEM. $n = 4$ rats in each group ($*P < 0.05$, $**P < 0.01$ vs. sham rats, $\blacktriangle P < 0.01$ vs. NS group using one-way ANOVA followed by Bonferroni test)

CXCL12–CXCR4 signaling might mediate astroglial–neuronal interaction in the maintenance of central sensitization in the BCP. Future studies are needed to verify this hypothesis.

In conclusion, our results suggest Cx43 mediates CXCL12 production from spinal dorsal horn in astrocytes to maintain bone cancer pain in rats. The findings may improve our understanding of the underlying mechanisms of BCP and provide a novel target for the treatment of BCP.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no competing interests.

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