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CREB-regulated transcription coactivator 1 enhances CREB-dependent gene expression in spinal cord to maintain the bone cancer pain in mice

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Abstract

Background: cAMP response element binding protein (CREB)-dependent gene expression plays an important role in central sensitization. CREB-regulated transcription coactivator 1 (CRTC1) dramatically increases CREB-mediated transcriptional activity. Brain-derived neurotrophic factor, N-methyl-D-aspartate receptor subunit 2B, and miRNA-212/132, which are highly CREB responsive, function downstream from CREB/CRTC1 to mediate activity-dependent synaptic plasticity and in turn loops back to amplify CREB/CRTC1 signaling. This study aimed to investigate the role of spinal CRTC1 in the maintenance of bone cancer pain using an RNA interference method.

Results: Osteosarcoma cells were implanted into the intramedullary space of the right femurs of C3H/HeNCrlVr mice to induce bone cancer pain. Western blotting was applied to examine the expression of spinal phospho-Ser133 CREB and CRTC1. We further investigated effects of repeated intrathecal administration with Adenoviruses expressing CRTC1-small interfering RNA (siRNA) on nociceptive behaviors and on the upregulation of CREB/CRTC1-target genes associated with bone cancer pain. Inoculation of osteosarcoma cells induced progressive mechanical allodynia and spontaneous pain, and resulted in upregulation of spinal p-CREB and CRTC1. Repeated intrathecal administration with Adenoviruses expressing CRTC1-siRNA attenuated bone cancer–evoked pain behaviors, and reduced CREB/CRTC1-target genes expression in spinal cord, including BDNF, NR2B, and miR-212/132.

Conclusions: Upregulation of CRTC1 enhancing CREB-dependent gene transcription in spinal cord may play an important role in bone cancer pain. Inhibition of spinal CRTC1 expression reduced bone cancer pain. Interruption to the positive feedback circuit between CREB/CRTC1 and its targets may contribute to the analgesic effects. These findings may provide further insight into the mechanisms and treatment of bone cancer pain.

Keywords
Bone cancer pain, cAMP response element binding protein, CREB-regulated transcription coactivator 1, RNA interference, Adenoviruse

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Background

Cancer pain is usually cited as most critical and profoundly impacts all dimensions of patients' quality of life (QOL).[1,2] Patients with bone cancer experience more frequent and severe pain.[3,4] However, it has been reported that half of cancer patients still have inadequate or undermanaged pain control.[5] Therefore, it is essential to investigate the mechanisms of bone cancer pain in order to develop novel and efficacious therapies.

N-methyl-D-aspartate receptor (NMDAR), specifically NR2B subunit-dependent central sensitization,

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contributes to the central mechanisms of bone cancer pain.[6,7] Nociception-induced Ca^{2+} influx via NMDARs increases the phosphorylation of cyclic adenosine monophosphate (cAMP) response element binding protein (CREB) at Serine 133 (p-CREB), which serves as a biomarker for the activation of CREB, is considered to be an important response that mediates the initiation and maintenance of central sensitization.[8–12] Activation of the transcription factor CREB couples synaptic activity to long-term changes in neuronal plasticity, which is thought to underlie central sensitization, via interacting with various cofactors as well as components of the basal transcription complex and mediating the transcription of downstream genes which have been linked to nociceptive pathways, including c-fos,[13,14] brain-derived neurotrophic factor (BDNF),[15] and many neuropeptides (such as somatostatin, enkephalin, and corticotropin-releasing hormone).[16,17] However, recent studies revealed that phosphorylation of CREB is not sufficient to initiate and regulate the transcription of downstream genes in response to stimuli. A new protein family of CREB coactivators called CREB-regulated transcription coactivators (CRTCs), which are activated and translocated into the nucleus in response to increased intracellular calcium and cAMP,[18] has been demonstrated to dramatically increase CREB-mediated transcriptional activity independently of the phosphorylation status of CREB.[19,20] Among CRTC family members, CRTC1 is primarily expressed in central nervous system and plays an important role in activity-dependent gene transcription mediated by CREB and in regulating long-term potentiation (LTP) and neuronal plasticity.[21–23]

Moreover, BDNF was recently shown to upregulate NR2B via transcription-dependent mechanisms,[24,25] and increase the activity of NMDAR[26] through tyrosine phosphorylation of NR1 and NR2B.[27] These studies suggested that BDNF in turn loops back to amplify CREB/CRTC1 signaling via upregulation and activation of NMDAR. In addition to BDNF, CREB activates expression of a gene locus that produces two microRNAs (miRNAs), miR-132 and miR-212,[28,29] which have been involved in activity-dependent dendritic growth, spine formation[30] synaptic plasticity,[31,32] and long-term potentiation,[33] and are likely to play a role in the central sensitization. miR-212 and miR-132 also enhance cAMP-responsive gene expression and amplify the canonical CREB/CRTC1 signaling cascade by sensitizing cAMP production, increasing phosphorylation of CREB and increasing expression of CRTC1.[31]

As mentioned earlier, we hypothesized that CREB/CRTC1 signaling, which may form a positive feedback signaling circuit with its targets, plays an important role in the development and maintenance of central sensitization, and thereby results in persistent and progressive bone cancer pain. In the present study, we detected the expression of CREB and CRTC1 in spinal cord in response to bone cancer pain in mice, and further examined whether inhibition of CRTC1 attenuated bone cancer pain via blockage of this signaling circuit.

Materials and methods

Animals

The experiments were approved by the Animal Care and Use Committee at the Medical School of Nanjing University and were in accordance with the guidelines for the use of laboratory animals.[34] All efforts were made to minimize the mice suffering and to reduce the number of mice used. Experiments were performed on male C3H/HeNCrlVr mice (20–25 g, 4–6 weeks old; Vital River Laboratory Animal Technology Co., Ltd., Beijing, China; SCXK JING 2012-0001). The mice were housed in groups of six per cage and fed with food pellets and water was provided ad libitum. All animals were maintained in a temperature-controlled ($21 \pm 1^{\circ}C$) room with 12-h alternating dark/light cycles.

Cell culture and implantation

Osteosarcoma NCTC 2472 cells (American Type Culture Collection, Manassas, VA, USA, ATCC 2087787) were incubated and subcultured in NCTC 135 medium (Sigma–Aldrich, St. Louis, MO, USA) with 10% horse serum (Gibco, Carlsbad, CA, USA) at 37°C in an atmosphere of 5% CO_2 and 95% air (Thermo Forma, OH, USA), and passaged twice a week according to the recommendations provided by ATCC.

The mouse model of bone cancer pain was established as previously described by Schwei et al.[35] On day 0, the mice were anesthetized with an intraperitoneal injection of 50 mg/kg pentobarbital sodium (1% in normal saline), and a superficial incision was made in the skin above the right articulatio genus using eye scissors. Gonarthrotomy was performed, which exposed the femur condyles. A light depression was made using a dental bur. A 30-gauge needle was used to perforate the cortex, and a 25-µl microsyringe was used to inject a volume of 20-µl α-minimum essential medium (α-MEM) containing no or 2×10^5 NCTC 2472 cells into the intramedullary space of the femur, which corresponded to sham or tumor-bearing mice, respectively. Subsequently, the injection hole was sealed using dental amalgam, followed by copious irrigation with normal saline. The wound was then sutured closed.

Construction of recombinant adenoviruses

The small interfering RNA (siRNA) targeting the gene sequence of the mouse CRTC1 (GeneBank accession

no. NM_001004062) was designed and subjected to a Blast-Search (http://blast.ncbi.nlm.nih.gov) to ensure that only one gene was being targeted. The target-specific siRNA duplexes were utilized as follows: 5′-TGC TGCATCATGGCAGCCTGGGAATAGTTTTGGC CACTGACTGACTATTCCCACTGCCATGATG-3′ (Forward), 5′-CCTGCATCATGGCAGTGGGAATAG TCAGTCAGTGGCCAAAACTATTCCCAGGCTGC CATGATGC-3′ (Reverse). The interfering target sequence was 5′-TATTCCCAGGCTGCCATGATG-3′ for CRTC1. The plasmids expressing CRTC1-siRNA (pcDNA6.2-EGFP-CRTC1) were constructed and cotransfected into human embryonic kidney (HEK) 293 cells (ATCC, Manassas, VA, USA) with the corresponding gene eukaryotic expression vectors (pIRES-EGFP-Crtc1) by Shanghai R&S Biotechnology Co., Ltd (Shanghai, China). The interference efficiency was confirmed using real-time quantitative PCR. Through homologous recombination in *Escherichia coli* DH5α competent cells, E1A-deleted adenoviral vectors expressing enhanced green fluorescent protein (EGFP) and CRTC1-siRNA were generated (Ad-EGFP-CRTC1). Adenoviruses expressing EGFP only were used as negative control (Ad-EGFP). The sequences of the constructs were confirmed by sequencing. The replication-deficient viruses were plaque-purified twice, propagated in HEK 293 cells, concentrated, and purified by double cesium chloride (CsCl) gradient centrifugation. The titers of Ad-CRTC1 or Ad-EGFP were determined by cytopathic effect (CPE) assay using HEK 293 cells before storage at −80°C. Virus was diluted in 0.9% saline immediately before injection.

Drugs preparation and intrathecal injection

Ad-CRTC1 and Ad-EGFP were dissolved in 0.9% saline to a concentration of 10^{10} TCID50/ml. For vehicle treatment, 0.9% saline was used. Ad-CRTC1, Ad-EGFP, and vehicle were injected intrathecally in a volume of 5 μl in each group, respectively, once a day for three consecutive days starting from day 14 after inoculation of tumor cells.

Intrathecal injections (i.t.) were performed manually between the L5 and L6 lumbar space in unanesthetized mice according to a previous method described by Hylden and Wilcox.[36] The injection was performed using a 25-gauge needle attached to a glass microsyringe. Each mouse was injected with a volume of 5 μl. The accurate placement of the needle was confirmed by a quick ''flick'' of the mouse's tail.

Assessment of bone cancer pain

All tests were performed during the light phase. Prior to each test, the mice were allowed to acclimatize for at least 30 min. All behavioral responses were measured by the experimenters who were blind to the treatment groups.

Mechanical allodynia

Mechanical allodynia was assessed using von Frey filaments (Stoelting, Wood Dale, IL, USA) as previously described.[37] The mice were placed into individual transparent plexiglass compartments (10 cm × 10 cm × 15 cm) onto a metal mesh floor (graticule: 0.5 cm × 0.5 cm). Paw withdrawal mechanical threshold (PWMT) was measured using a set of von Frey filaments (0.16 g, 0.4 g, 0.6 g, 1.0 g, 1.4 g, and 2.0 g). The filaments were pressed vertically against the plantar surface of the right hind paw with such sufficient force as to cause a slight bending against the paw and were held for 6–8 s with a 10-min interval between two stimulations. Brisk withdrawal or paw flinching were considered positive responses. Each mouse was tested five times per stimulus strength. The lowest von Frey filament, which had three or more positive responses, was regarded as the PWMT.

Spontaneous lifting behavior

The mice were placed into individual plexiglass compartments (10 cm × 10 cm × 15 cm) for 30 min and observed for 2 min to quantify the number of spontaneous flinches (NSF) of the right hind paw. Every lift of the right hind limb that was not related to walking or grooming was considered as one flinch. Each mouse was tested five times. The data were presented as the mean ± SD.[38]

Western blotting analyses

The mice were deeply anesthetized with pentobarbital sodium (1% in normal saline, 50 mg/kg, i.p.) and sacrificed by decapitation. The spinal cord L3–L5 segments were removed rapidly and stored in liquid nitrogen. Tissue samples were homogenized in 1 ml of 0.1 M phosphate buffered saline (PBS). The homogenate was transferred to a fresh tube and spun at 13,000 r/min for 5 min at 4°C. The supernatant was removed and the pellet was washed twice in ice-cold PBS and spun at 13,000 r/min for 5 min at 4°C. The PBS was aspirated, and the pellet was resuspended in ice-cold lysis buffer (1% Nonidet P-40; 0.5% sodium deoxycholate, 0.1% sodium lauryl sulfate in PBS) with freshly added protease inhibitors (Roche Diagnostics, Shanghai, China), and incubated for 30 min on ice. The tubes were centrifuged at 13,000 r/min for 20 min at 4°C. Supernatants containing the protein lysates were collected and stored as aliquots at −70°C. The protein concentration was determined using the bicinchoninic acid method according to the kit's instructions (Kaiji Biotechnology, Nanjing,

China). Protein lysates (50 μg) and a protein molecular weight marker, PageRuler Prestained Standard (10–170 kDa, Multiscience Biotechnology, Hangzhou, China) were separated using SDS-PAGE (8%), and for 90 min at 120 V. The proteins were transferred onto polyvinylidene difluoride membranes (Millipore Corporation, MA, USA) at 200 mA for 2 h using a wet blotting system. The membranes were blocked in PBS/5% skim milk/0.1% Tween 20 for 2 h at room temperature, followed by an overnight incubation at 4°C with the following primary antibodies: rabbit anti-t-CREB (1:800; Abcam, UK, ab32515), anti-p-CREB (phospho Ser133, 1:800; Abcam, UK, ab32096), anti-CRTC1 (1:1000; Cell Signaling Tech, USA, 2501), anti-NR2B (1:1000; Abcam, UK, ab65783), anti-BDNF (1:1000; Abcam, UK, ab108383), and anti-β-actin (1:10000; Bioworld Tech, USA, BSAP0060) diluted in blocking buffer. The next day, the membranes were washed six times with tris-buffered saline-tween 20 buffer for 1 h and incubated with the goat anti-rabbit secondary antibody conjugated with horseradish peroxidase for 2 h at room temperature (1:10,000; Abcam, UK, ab97200). Immunoblots were detected using the ECL system (Millipore Corporation, MA, USA) and visualized on Kodak BIOMAX MR X-ray film (Sigma–Aldrich). β-actin was used as a loading control for total protein. Images of the Western blotting protein bands were collected and analyzed using Quantity One V4.40 (Bio-Rad, USA). The data were presented as the mean ± SD.

Quantitative real-time polymerase chain reaction (real-time PCR)

The L3–L5 spinal cord segments of sacrificed mice were dissected and frozen in liquid nitrogen and stored at −70°C. Total RNA was isolated with Trizol reagent (Invitrogen, Carlsbad, CA). RNA concentration was measured using a NanoDrop ND-1000 Spectrometer (NanoDrop Technologies, Wilmington, DE). The purity of the RNA was assessed by the ratio of absorbance at 260 nm and 280 nm. RNA was stored as aliquots at −70°C. RNA samples were reversely transcribed using the reverse transcription-polymerase chain reaction (PCR) kit (Takara, Dalian, China), and quantitative real-time reverse transcription-PCR was performed using the SYBR PrimeScript quantitative real-time reverse transcription-PCR kit (Takara) according to the manufacturer's protocol. Information about the reverse transcription-PCR primers: miR-212 RT primer (5′-GTCGTATCCAGTGCAGGGTCCGAGGTATTC GCACTGGATACGACTGGCCG-3′), miR-132 RT primer (5′-GTCGTATCCAGTGCAGGGTCCGA GGTATTCGCACTGGATACGACCGACCA-3′), and U6 RT primer (5′-AACGCTTCACGAATTTGCGT-3′). Information about the quantitative real-time reverse transcription-PCR analysis primers: miR-212 primers (upstream primer, 5′-TAACAGTCTCCAGTCA-3′, and downstream primer, 5′-GTGCAGGGTCCGAGGT-3′), miR-132 primers (upstream primer, 5′-TAAC AGTCTACAGCCA-3′, and downstream primer, 5′-GT GCAGGGTCCGAGGT-3′), U6 primers (upstream primer, 5′-CTCGCTTCGGCAGCACA-3′, and downstream primer, 5′-AACGCTTCACGAATTTGCGT-3′). Reaction mix was aliquoted to the wells on a real-time PCR plate. Each sample was made in triplicate. A volume of 2 μl complementary DNA (cDNA) was added to each well. A no-template control sample contained water instead of complementary DNA. Quantitative real-time reverse transcription-PCR was run on ABI PRISM 7500 (Applied Biosystems, Carlsbad, CA) using standard conditions. Expression of miR-132 was normalized to RNA loading for each sample using the reference gene U6 as an internal standard. The quantity of RNA was given as $2^{-\Delta\Delta ct}$. Δct and ΔΔct were calculated as follows: $\Delta ct = ct$ (gene of interest) $- ct$ (reference gene); $\Delta\Delta ct = \Delta ct$ (group of interest) $- \Delta ct$ (control group). Data were presented as mean ± SD.

Statistical analyses

Data were expressed as the mean ± SD. Mice were assigned to different treatment groups in a randomized manner. Repeated measures analysis of variance (ANOVA) was performed to determine the overall differences at each time point for pain behaviors. One-way ANOVA was used to determine differences in the expression of proteins or miRNAs among all of the experimental groups. In both cases, when significant main effects were observed, the least significant difference (LSD) *post-hoc* tests were performed to determine the source(s) of these differences. A P value < 0.05 was considered statistically significant.

Results

Bone cancer-induced pain behaviors over time

There was no significant difference in PWMT and the NSF between groups prior to the operation. The ipsilateral hind paw in response to the operation in both tumor-bearing and sham mice showed a decrease in the PWMT in response to von Frey filaments stimulation at day 4 ($p < 0.05$). The PWMT of sham mice recovered to the baseline level at day 7 and the tumor-bearing mice subsequently showed a decrease in the PWMT ($p < 0.05$), which was 0.44 ± 0.15 g at day 14 and 0.36 ± 0.14 g at day 21 (Figure 1(a)).

At day 4 after inoculation, both tumor-bearing and sham mice displayed increased spontaneous lifting

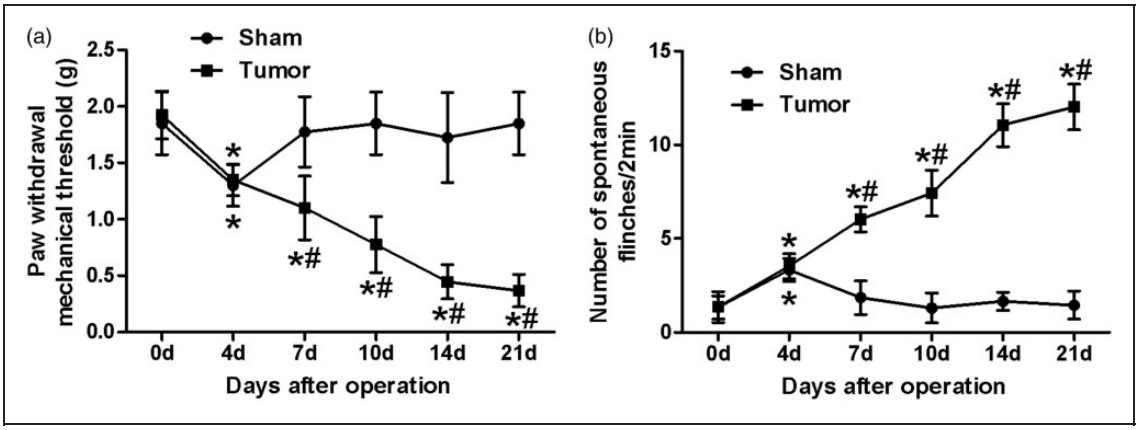

Figure 1. Changes in pain behaviors of the right hind limb over time in tumor-bearing mice and sham mice. (a) The PWMT in response to von Frey filaments in tumor-bearing mice decreased over time after day 7. (b) The NSF over 2 min in tumor-bearing mice began to increase at day 7 and increased with time. 0 d, 4 d, 7 d, 10 d, 14 d, and 21 d indicate the days after NCTC2472 cell inoculation in the right femur. Each group consisted of eight mice. The data were expressed as the mean ± SD. $^{*}p < 0.05$ versus day 0, $^{\#}p < 0.05$ versus sham mice. NSF: number of spontaneous flinches; PWMT: paw withdrawal mechanism threshold.

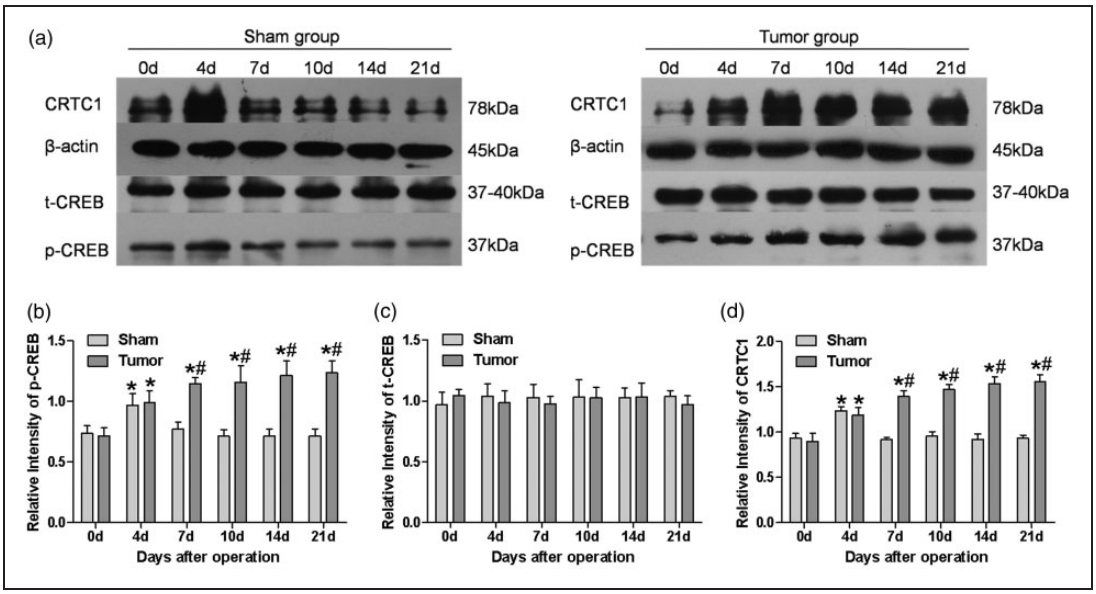

Figure 2. Changes in the spinal p-CREB, t-CREB, and CRTC1 protein expression in tumor-bearing mice and sham mice. Tumors induced the up-regulation of spinal p-CREB and CRTC1 expression from day 7 post-inoculation. (a) Western blot for CRTC1, β-actin, t-CREB, and p-CREB resulted in products of 78, 45, 37–40, and 37 kDa, as expected (markers show predicted band sizes). (b–d) Densitometric quantification of β-actin, p-CREB, t-CREB, and CRTC1 immunoreactivity on Western blots. β-actin was used as a loading control. Each group consisted of five mice. The data were expressed as the mean ± SD. $^{*}p < 0.05$ versus day 0. $^{\#}p < 0.05$ versus sham mice. CREB: cAMP response element binding protein; CRTC1: CREB-regulated transcription coactivator 1; EGFP: enhanced green fluorescent protein.

behavior of the ipsilateral hind paw ($p < 0.05$), and the NSF of the sham mice recovered to the baseline at day 7. However, the tumor-bearing mice displayed a gradual increase in the NSF over time ($p < 0.05$). The NSF was 11.05 ± 1.14 during a 2-min period at day 14 and 12.02 ± 1.22 at day 21, when only occasional flinches of the right hind paw was observed in the sham mice (1.45 ± 0.75) (Figure 1(b)).

Upregulation of spinal p-CREB and CRTC1 in response to bone cancer pain

Western blotting was performed to quantify the protein expression levels of p-CREB, t-CREB, and CRTC1 in the lumbar spinal cord. The p-CREB, t-CREB, CRTC1, and β-actin antibody labeled a band at 37 kDa, 37–40 kDa, 78 kDa, and 45 kDa, respectively (Figure 2(a)).

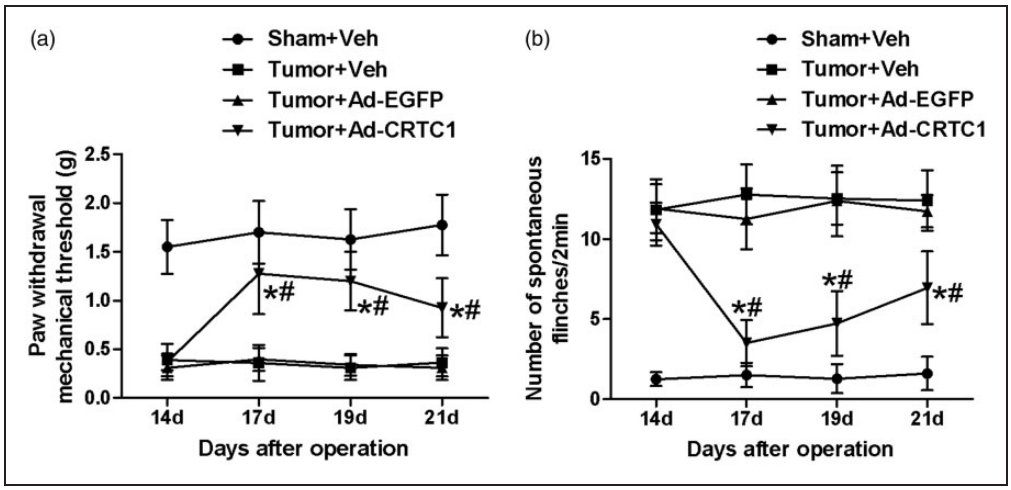

Figure 3. The effect of repeated intrathecal administration of Ad-CRTC1 on bone cancer pain–related behaviors. (a) The PWMT in response to von Frey filaments and (b) the number of spontaneous flinches over 2 min. Mice were treated with once-daily injections on days 14–16 after inoculation with Adenoviruses or vehicle. Pain behaviors were analyzed at 14, 17, 19, and 21 days after inoculation. The pain behaviors did not change after intrathecal administration of vehicle or Ad-EGFP. The anti-hyperalgesic effect of Ad-CRTC1 was maintained for at least 6 days. Each group consisted of eight mice. All data were expressed as the mean ± SD. $^{*}p < 0.05$ versus Tumor + vehicle mice. $^{\#}p < 0.05$ versus baseline before administration. CRTC: CREB-regulated transcription coactivator; EGFP: enhanced green fluorescent protein; PWMT: paw withdrawal mechanical threshold.

Results were expressed as integrated optical density ratio (interest protein vs. β-actin) (Figure 2(b) to (d)). The expression level of p-CREB and CRTC1 in spinal cord from tumor-bearing mice increased gradually over time compared with sham mice. At day 4, there was no significant difference in the expression level of p-CREB and CRTC1 between tumor-bearing mice and sham mice. At day 7, the level of p-CREB and CRTC1 of sham mice recovered to the baseline level before inoculation. However, the expression level of total CREB in spinal cord did not change after inoculation in either tumor-bearing mice or sham mice.

Effects of intrathecal administration of Ad-CRTC1 on bone cancer pain

Inhibition the expression of spinal CRTC1 by intrathecal administration of Ad-EGFP-CRTC1 attenuated bone cancer–related pain behaviors of the ipsilateral hind limb in response to the inoculation (Figure 3). The vehicle saline did not affect the pain behaviors in tumor-bearing or sham mice.

At day 14 after the inoculation, the PWMT of the tumor-bearing mice was decreased dramatically compared with that of sham mice ($p < 0.05$). There was no significant difference in the PWMT between tumor-bearing mice at any time point after administration of Ad-EGFP and the baseline prior to administration. The mechanical allodynia of tumor-bearing mice was ameliorated by intrathecal administration of Ad-EGFP-CRTC1. Compared with the baseline prior to administration of Ad-EGFP-CRTC1 or the PWMT of tumor-bearing mice receiving saline at the same time point, the increase in PWMT was observed from day 17 to day 21 after inoculation (Figure 3(a)).

The NSF of the tumor-bearing mice profoundly increased at day 14, compared with that of sham mice ($p < 0.05$). There was no significant difference in the NSF between the mice treated with Ad-EGFP at any time point and the baseline prior to administration ($P > 0.05$). After administration of Ad-EGFP-CRTC1, the NSF of tumor-bearing mice decreased significantly ($p < 0.05$), and this effect was maintained until day 21 (Figure 3(b)).

Downregulation of spinal CRTC1 mediated by intrathecal administration of Ad-CRTC1

Compared with sham mice receiving vehicle, the expression level of CRTC1 in tumor-bearing mice receiving vehicle or Ad-EGFP was upregulated significantly after the last intrathecal administration. Compared with tumor-bearing mice receiving vehicle, the expression level of CRTC1 was downregulated in tumor-bearing mice receiving Ad-EGFP-CRTC1 ($p < 0.0001$). However, the expression level of CRTC1 in group Tumor + Ad-EGFP-CRTC1 was still higher than that in group Sham + vehicle after administration ($p = 0.03$) (Figure 4(a, d)).

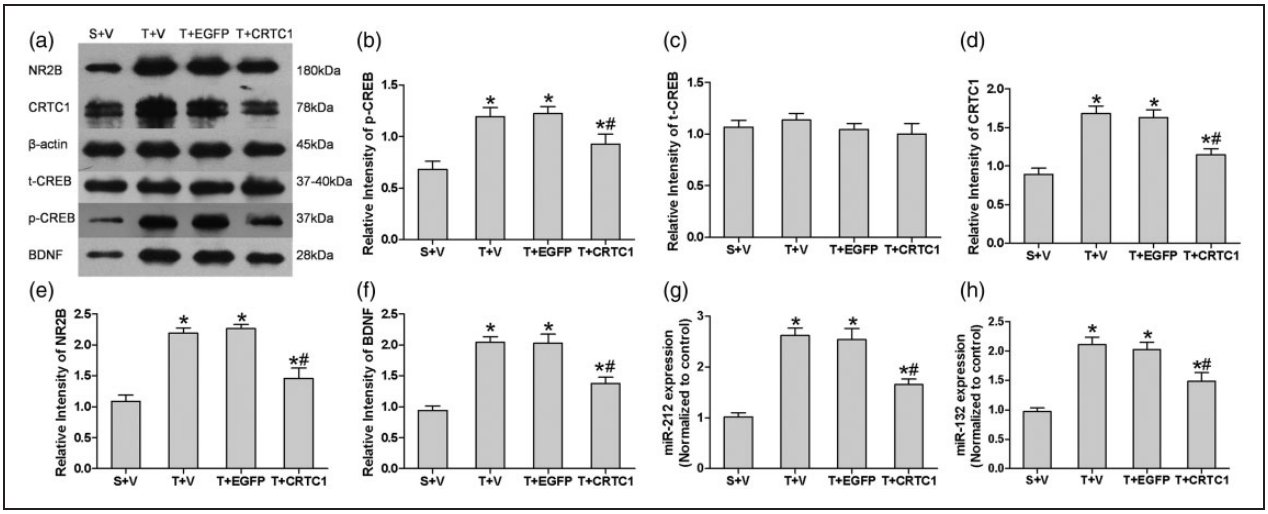

Figure 4. Effects of intrathecal administration of Ad-CRTC1, Ad-EGFP, and vehicle on spinal p-CREB, t-CREB, CRTC1, NR2B, and BDNF protein expression and miRNA-212/132 expression. Mice were treated with once-daily injections of Adenoviruses or vehicle on days 14–16 after inoculation. The spinal cord was removed on day 17 after inoculation. Treatment with Ad-CRTC1 downregulated spinal p-CREB, CRTC1, NR2B, BDNF, and miRNA-212/132 expression in tumor-bearing mice. (a) Western blot for NR2B, CRTC1, β-actin, t-CREB, p-CREB, and BDNF resulted in products of 180, 78, 45, 37–40, 37, and 28 kDa, as expected (markers show predicted band sizes). (b–f) Densitometric quantification of β-actin, p-CREB, t-CREB, CRTC1, NR2B, and BDNF immunoreactivity on Western blots. β-actin was used as a loading control. (g and h) Quantitative real-time PCR analysis of changes of spinal miRNA-212 and miRNA-132 after intrathecal administration. U6 was used as a reference gene. Each group consisted of five mice. The data were expressed as the mean ± SD. $^{*}p < 0.05$ versus Sham + vehicle mice, $^{\#}p < 0.01$ versus Tumor + vehicle mice. BDNF: brain-derived neurotrophic factor; CREB: cAMP response element binding protein; CRTC: CREB-regulated transcription coactivator; EGFP: enhanced green fluorescent protein.

Effects of intrathecal administration of Ad-CRTC1 on the expression of spinal miR-212/132, CREB, p-CREB, BDNF, and NR2B

After the last intrathecal administration, compared with sham mice receiving vehicle, the expression levels of miRNA-212/132, p-CREB, BDNF, and NR2B in tumor-bearing mice receiving vehicle or Ad-EGFP after administration were upregulated significantly at day 17 ($p < 0.0001$). Treatment with Ad-CRTC1 downregulated the expression of miRNA-212/132, p-CREB, BDNF, and NR2B in spinal cord in tumor-bearing mice compared with the tumor-bearing mice receiving vehicle. However, the expression levels of miRNA-212/132, p-CREB, BDNF, and NR2B in group Tumor + Ad-CRTC1 were still higher than that in group Sham + vehicle after administration. There was no significant difference in the expression of t-CREB among groups (Figure 4(b) to (f)).

Discussion

Bone cancer pain remains a clinically challenging problem and the mechanisms are still unclear. The mouse model of bone cancer pain provides an appropriate system for preclinical study of pain. The present study demonstrated that inoculation of NCTC 2472 sarcoma cells into the right femur of male C3H/HeNCrlVr mice produced progressive spontaneous flinches and mechanical allodynia. These results were similar with our previous studies[39] and the clinical spontaneous and evoked pain that bone cancer patients suffer from, indicating the successful establishment of a mouse model of bone cancer pain.

Central sensitization plays an important role in the development of bone cancer pain. Long-lasting changes in synaptic plasticity, which underlying central sensitization, require activity-dependent transcription of CREB-driven genes in the central nervous system. The transcriptional factor CREB has been proposed as a molecular switch in hippocampal synaptic plasticity.[40] Significant evidence of recent animal experiments links the increase in phosphorylation of CREB at Serine 133, which is triggered by calcium influx through activated NMDAR and leads transcriptional activation in spinal dorsal horn, with the development of pain hypersensitivity following formalin injection,[8] partial sciatic nerve ligation,[9,10] chronic constriction injury (CCI) of the sciatic nerve,[11,41] spared nerve injury,[42] and intramedullary inoculation of Walker 256 cells.[43,44] Inhibition of CREB expression via intrathecal administration with CREB antisense oligonucleotide effectively alleviated

the mechanical allodynia and thermal hyperalgesia in animal models of inflammatory[45] or neuropathic pain.[41,42] Recent evidence indicates that the essential CREB coactivator CRTC1, which is necessary for CREB-mediated transcription in neurons, can potently activate CREB-dependent gene expression in the absence of extracellular stimuli,[19,20] and thus play a critical role in facilitating hippocampal plasticity[21,22] and long-term memory formation.[46] CRTC1 is maintained in an inactive state in the cytoplasm as a result of phosphorylation. Stimuli lead to the activation of NMDA receptors by glutamate, and the concomitant activation of calcium and cAMP signaling pathways, finally result in CRTC1 dephosphorylation and subsequent nuclear accumulation. Functionally, the translocation of CRTC1 from the cytoplasm to the nucleus of hippocampal neurons enhances CREB-dependent gene transcription via interaction with the bZIP domain of CREB.[21] Although the role of CRTC1 in pain is unexplored, previous results show that knockdown of CRTC1 with RNA interference prevented the transcription of CREB-driven gene and disrupted late-phase LTP (L-LTP) maintenance, while overexpression of CRTC1 lowered the threshold for L-LTP induction in hippocampal slices.[21,22] In the present study, results of Western blotting suggested that inoculation of sarcoma cells induced a significant increase in the expression of p-CREB and CRTC1 in spinal cord, which corresponded to the same time-course as the pain behaviors observed. Thus, we speculated that upregulation of p-CREB and CRTC1 in spinal cord was most likely involved in spontaneous pain and mechanical allodynia induced by bone cancer. Our study further found that the repeated intrathecal administration of the Adenoviruses expressing siRNA targeting CRTC1 from 14 d to 16 d after inoculation not only effectively attenuated the development of spontaneous flinching and mechanical allodynia of the ipsilateral hind limb, but also significantly inhibited the upregulation of CREB-target genes (BDNF, NR2B, and miRNA-212/132) in tumor-bearing mice. The anti-hyperalgesia effects on bone cancer pain induced by Ad-CRTC1 maintained for at least one week after administration.

Among the various CREB/CRTC1 downstream genes regulated via CREB binding site, we detected the expression of BDNF,[21] NR2B,[47] and miRNA-212/132,[31] which have been linked to nociceptive pathways. The results showed that BDNF, NR2B, and miRNA-212/132 were all upregulated in tumor-bearing mice receiving vehicle or Ad-EGFP, while knockdown of CRTC1 via intrathecal administration of Ad-CRTC1 significantly inhibited the upregulation of the three downstream genes. Previous studies demonstrated that BDNF in spinal cord, which is involved in synaptic plasticity and central sensitization, is essential for development of neuropathic pain[48,49] and bone cancer pain.[15] Compelling evidence suggested that BDNF is regarded as a linker molecule between neurons and glia, which plays an important role in the initiation and maintenance phase of nerve injury induced neuropathic pain or inflammatory pain, through its receptor tyrosine kinase receptor B (TrkB).[50] Moreover, in addition to CREB-mediated direct regulation of NR2B transcription, BDNF also increases the expression of NMDAR subunits in hippocampal neurons via transcription-dependent mechanisms.[24,25] In addition, BDNF increases NMDA single-channel open probability,[26] presumably through tyrosine phosphorylation of the NMDAR subunits NR1 and NR2B in cortical and hippocampal postsynaptic densities.[27] These studies, in combination with the present study, suggested that NR2B-containing NMDAR-Ca^{2+}/calmodulin (CaM)-cAMP-CREB/CRTC1-BDNF-NR2B might form a positive feedback to reinforce the activity of NMDAR in neurons, thus, may further enhance neuronal excitability and contribute to central sensitization of chronic pain.

In addition to BDNF and NR2B, miRNA-212 and miRNA-132, the two closely related miRNAs encoded in the same intron of a small noncoding gene, are highly CREB responsive.[28] Recent studies suggested that both miRNA-212 and miRNA-132, via regulating their target gene expression post-transcriptionally, function downstream from CREB to mediate activity-dependent dendritic growth, spine formation,[30] synaptic plasticity,[31,32] and long-term potentiation (LTP)[33] in response to a variety of signaling pathways. Moreover, the microarray-based screening approach revealed that the expression of miRNA-132 and miRNA-212 was upregulated in rat hippocampus in the model of neuropathic pain induced by CCI.[51] The expression of miRNA-212 was also upregulated in dorsal root ganglia (DRG) following rat sciatic nerve injury.[52,53] Furthermore, miRNA-212 and miRNA-132 in turn loop back to amplify CREB/CRTC1 signaling in the dorsal striatum via sensitizing cAMP production, increasing phosphorylation of CREB and increasing expression of CRTC1, and that striatal CREB/CRTC1-miRNA-212/132 circuit contributes to the neuroplasticity and cocaine addiction in rats.[31] In agreement with previous studies, we observed that miRNA-212 and miRNA-132 were both upregulated significantly in spinal cord after inoculation of sarcoma cells, and intrathecal administration of Ad-CRTC1 significantly inhibited the upregulation of miRNA-212/132 and attenuated pain behaviors in tumor-bearing mice accordingly.

We found that the expression levels of miRNA-212/132, p-CREB, BDNF, and NR2B in group Tumor + Ad-CRTC1 were still higher than that in group Sham + vehicle after administration. This indicates that the contribution of CRTC1 in the cancer pain may be partial. Studies have demonstrated that the mechanisms

for bone cancer pain may involve a combination of inflammatory and neuropathic pain.[54] Perhaps, NR2B contributes to bone cancer pain through interacting with EphrinB-EphB receptor signaling, which participate in the development process of the synaptic plasticity of adult nervous systems.[55] Studies have also demonstrated that astrocytes and microglial cells become active in the status of bone cancer and release various substances which activate the excitatory amino acid receptor including mGluR5.[34] Moreover, previous studies demonstrated that BDNF is synthesized in the primary sensory neurons and the upregulated BDNF acts as an important modulator of synaptic plasticity in neuropathic and inflammatory pain.[56,57] In agreement with previous studies, we observed that the expression levels of BDNF in spinal cord of tumor-bearing mice increased significantly, and administration of Ad-CRTC1 significantly inhibited the upregulation of BDNF. Therefore, it is reasonable to assume that BDNF, a CREB-target gene, is regulated by p-CREB/CRTC1 signaling in neurons considering the present results in combination with previous studies. However, BDNF is also found in astrocytes[58] and microglia.[59,60] These two cellular origins of BDNF may also contribute to bone cancer pain. Although the contribution of CRTC1 in the cancer pain is partial, inhibiting CREB/CRTC1 signaling via CRTC1 RNA interference still resulted in significant analgesia.

Conclusions

This study demonstrated that bone cancer pain induced an upregulation of p-CREB and CRTC1 in spinal cord. The central inhibition of CRTC1 via intrathecal administration with Adenoviruses expressing CRTC1-siRNA effectively relieved the bone cancer–induced mechanical allodynia and spontaneous pain, and the anti-hypersensitivity effects were parallel with the inhibition of spinal BDNF, NR2B, and miRNA-212/132 expression. As CREB/CRTC1-dependent gene transcription contributes to neuronal plasticity, and activation of spinal CREB/CRTC1 signaling forms a positive feedback circuit with its target genes, upregulation of CRTC1 enhancing CREB-dependent gene transcription in spinal cord is likely to play an important role in the maintenance of central sensitization during bone cancer pain, which is characterized by increased excitability within the pain circuitry. The present study may provide further insight into the mechanisms of cancer pain, and contribute to the development of therapeutic strategy for clinical treatment of bone cancer pain.

Author contributions

Liang Y and Liu Y carried out the surgical procedure, administration of drugs, pain behaviors measurement, Western blotting studies and drafted the manuscript. Hou B and Zhang W participated in the real-time PCR studies. Liu M and Sun Y participated in the statistical analysis. Gu X and Ma Z conceived of the study, and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript. Ying Liang and Yue Liu contributed equally to this paper.

Declaration of Conflicting Interests

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