

## ORIGINAL ARTICLE

# Enhanced function of TRPV1 via up-regulation by insulin-like growth factor-1 in a rat model of bone cancer pain

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**Conflicts of interest**

Authors declare no conflicts of interest.

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**Abstract**

**Background:** Up-regulation of transient receptor potential vanilloid subfamily, member 1 (TRPV1) is associated with the development and maintenance of cancer pain. The present study aimed to investigate the electrophysiological function of the up-regulated TRPV1 and the potential regulatory effects of insulin-like growth factor-1 (IGF-1) on TRPV1 expression in peripheral nerves in a rat model of bone cancer pain.

**Methods:** A bone cancer pain model of rats was established by injecting MRMT-1 (rat mammary gland carcinoma cells) breast cancer cells into the tibia bone cavity. Thermal hyperalgesia was assessed by paw-withdrawal latency to a thermal stimulus, and mechanical allodynia was measured with *von* Frey monofilaments. TRPV1 and IGF-1 expression were examined with immunohistochemical staining and Western blot. TRPV1 current density of dorsal root ganglion (DRG) neurons was measured with whole-cell patch clamping recording technique.

**Results:** Rats showed thermal hyperalgesia and mechanical allodynia 14–21 days after MRMT-1 inoculation into the tibia bone marrow. TRPV1 protein expression and its current density increased in DRG neurons. At the same time, IGF-1 expression increased in tibia bone cavity, and IGF-1 incubation increased total or membrane TRPV1 protein expression and TRPV1 current in primary cultured DRG neurons. Inhibition of IGF-1 receptors *in vivo* reversed mechanical allodynia and thermal hyperalgesia in rats with bone cancer pain.

**Conclusion:** Our results provide novel evidence for the increase of IGF-1 in tibia bone marrow, which is responsible for the up-regulation of TRPV1 expression and function in the peripheral nerves of bone cancer pain rats.

## 1. Introduction

Tumour metastasis to bone is one of the most common reasons for cancer pain (Mercadante, 1997). Clinical data show that 70% of breast cancer patients and 90% of advanced breast cancer patients have bone metastases (Mundy and Yoneda, 1995). Patients with lung cancer, ovarian cancer and adenocarcinoma also have high incidence of bone metastases.

Transient receptor potential vanilloid subfamily, member 1 (TRPV1) has been detected primarily in unmyelinated (C-fibres) or thinly myelinated (A $\delta$ -fibres) peripheral sensory neurons (Holzer, 1988). The expression of TRPV1 receptors is up-regulated in inflammation- or nerve injury-induced thermal hyperalgesia (Rashid et al., 2003b; Huang et al., 2006) and in diabetic neuropathy (Rashid et al., 2003a). TRPV1 is also up-regulated and involved in

**What's already known about this topic?**

- Peripheral transient receptor potential vanilloid subfamily, member 1 (TRPV1) protein expression is increased in rats with bone cancer pain after MRMT-1 (rat mammary gland carcinoma cells) bone cancer pain.

**What does this study add?**

- With electrophysiological method – patch clamp recordings, we found that this increased TRPV1 receptors are functionally enhanced.
- Local inoculation of MRMT-1 (rat mammary gland carcinoma cells) cells to tibial bone results in an increase of insulin-like growth factor-1 (IGF-1) in the bone marrow. IGF-1 up-regulated and enhanced the function of TRPV1.
- Inhibition of IGF-1 receptors *in vivo* reversed mechanical allodynia and thermal hyperalgesia in rats with bone cancer pain.

cancer pain (Ghilardi et al., 2005; Niiyama et al., 2007).

The role of TRPV1 receptors in cancer pain is of great interest. Cancer cells metastasized to bone marrow grow rapidly, and bone tissue is progressively destroyed. At the same time, bone tissue reconstruction is initiated as a coordinated process of bone formation and absorption. Many growth factors have been reported to participate in bone formation by activating or increasing the number of bone-formation cells (Canalis, 2009). For example, platelet-derived growth factor (PDGF) and fibroblast growth factor (FGF) show mitogenic function in osteoblast cells (Mazziotti et al., 2007).

In osseous metabolism processes, insulin-like growth factor-1 (IGF-1) promotes mitosis, osteoblast differentiation and bone construction (Bogdanos et al., 2003). Skeletal IGF-1 maintains trabecular bone (Zhao et al., 2000). On the other hand, IGF-1 has neurotrophic effects (Fernihough et al., 1993) after nerve injury. When cancer cells grow in bone marrow after metastasis, nerve regeneration is concurrent with bone destruction and reconstruction (Spencer, 1987). The regeneration of nerves may induce pain. For example, local administration of IGF-1 induces thermal hyperalgesia and mechanical allodynia through activation of IGF-1 receptors (IGF-1R; Spencer, 1987).

The fact that insulin plays a role in diabetic pain through its interaction with TRPV1 may suggest a role of IGF-1 in pain. Diabetic patients with reduced insulin sensitivity experience hypoalgesia and TRPV1

down-regulation (Migdalís et al., 1995; Hong and Wiley, 2005), while type II diabetic patients in early-stage with high insulin levels and insulin resistance often experience hyperalgesia and TRPV1 over-expression (Kamei et al., 2001).

In this study, we aimed to investigate whether the function of TRPV1 receptors is enhanced, and whether IGF-1 is responsible for the up-regulation of TRPV1 expression after bone cancer metastasis. We put forward the following hypothesis: IGF-1 secretion is increased in local skeletal tissue following cancer cell invasion into bone marrow and subsequent bone destruction and reconstruction. The IGF-1 up-regulates TRPV1 expression in the nerve fibre endings innervating the bone membrane and enhances its function.

## 2. Methods

### 2.1 Animals

Female Sprague-Dawley rats weighing 250–300 g were used to establish the bone cancer pain model, and young rats less than 2 weeks old were used for primary culture of sensory neurons. Rats were provided by the Department of Experimental Animal Sciences of our university, and housed in a temperature- and light-controlled (a 12-h light/dark cycle) environment with food and water available *ad libitum*. All experimental protocols were approved by the Animal Use and Care Committee of our university which followed the Guidelines of Animal Use and Protection adopted from the National Institutes of Health, USA.

### 2.2 Preparation of MRMT-1 rat mammary gland carcinoma cells

MRMT-1 rat mammary gland carcinoma cells were cultured in RPMI-1640 medium (Hyclone, Thermo Fisher Scientific Inc. Hyclone, Logan, UT, USA) containing 10% foetal bovine serum (heat-inactivated). Cells were released from the culture dish by 0.25% (weight/volume) trypsin (Hyclone), collected by centrifugation and then washed and re-suspended with phosphate-buffered saline (PBS). These cells were kept on ice until injection into the tibia. In the heat-killed control group, MRMT-1 cells were boiled in water for 20 min prior to injection. In the PBS control group, only PBS was injected.

### 2.3 Surgical procedure for MRMT-1 cancer cells inoculation into tibia bone marrow of rat

As described previously (Medhurst et al., 2002; Tong et al., 2010), after anaesthesia, a 1-cm incision was made in the skin over the top half of the left tibia. A 23-G needle was used to pierce the bone, and was then replaced with a long thin blunt needle. A volume of 3  $\mu$ L containing  $3 \times 10^4$

MRMT-1 live cells, or heat-killed MRMT-1 cells or PBS vehicle was injected into the bone marrow cavity. Following injection, the site was sealed using bone wax. The wound was closed with suture silk using sterile operating procedures and dusted with penicillin antibiotic powder.

## 2.4 Behavioural test for mechanical allodynia and thermal hyperalgesia

For mechanical allodynia, the 50% paw withdrawal threshold (PWT) in response to a series of *von Frey* monofilaments was examined by the up-down method. Eight hairs with approximately equally logarithmic incremental (0.224) bending forces were chosen (0.41–15.10 g). For thermal hyperalgesia, a radiant heat was used for assessing paw-withdrawal latency (PWL) to a thermal stimulus. Only those rats with mechanical allodynia and thermal hyperalgesia were used in the subsequent experiments.

## 2.5 Haematoxylin–eosin (HE) staining of bone marrow

The tibia was fixed in 4% formaldehyde at 4°C overnight, followed by decalcification in 10% EDTA (pH 7.2) for 4 weeks, and embedded in paraffin. Sections 6- $\mu$ m thick were cut in the frontal plane and stained with HE to visualize histological features of the bone marrow in normal rats and tumour-inoculated rats.

## 2.6 Immunohistochemical staining for IGF-1 or TRPV1

Dorsal root ganglion (DRG) sections were cut in 8- $\mu$ m thick serial longitudinal sections and mounted on glass slides coated with gelatin (type II, Sigma-Aldrich Chemicals, St Louis, MO, USA). After blocking with normal goat serum, the tibial sections were incubated overnight at 4°C with IGF-1 primary antibody (1:300; sc-9013, Santa Cruz Biotechnology, Inc., Santa Cruz, TX, USA) and DRG sections were incubated over two nights at 4°C with TRPV1 primary antibody (1:1000; Abcam, ab-10296), followed by incubation with goat anti-rabbit IgG and were finally visualized with diaminobenzidine (DAB). The Image-Pro Plus software (Media Cybernetics Company) was used for image capture and quantification.

## 2.7 Double immunofluorescence staining for IGF-1R and TRPV1: sequential protocol

After blocking in normal goat serum, the sections were incubated with the first primary antibody (TRPV1 antibody, ab-10296 Abcam Ltd., Cambridge, UK) and washed three times in PBS, 5 min for each. All following operations were performed in a dark room. Sections were incubated with first secondary antibody (labelled with Fluorochrome-1) in 1% bovine serum albumin (BSA) in PBS for 1 h. After washing with PBS, sections were incubated with the second serum (10% serum from the species that the secondary antibody

was raised in) for 30 min to block non-specific binding. Sections were sequentially incubated with the second primary antibody (IGF-1R $\beta$ , Santa Cruz, sc-713) in 1% BSA in PBS in a humid chamber overnight at 4°C, then after washing, with a second secondary antibody (labelled with Fluorochrome-2) in 1% BSA for 1 h.

## 2.8 Western blot analysis of TRPV1 protein

Lumbar 4 (L4) and L5 DRGs or primary cultured DRG neurons were disrupted in lysis buffer (50 mM Tris-HCl, 40 mM NaF, 2 mM EDTA, 1 mM dithiothreitol with protease inhibitor, pH 7.6). Lysates were separated on sodium dodecyl sulphate polyacrylamide gel electrophoresis gels and transferred to PVDF membranes. After blocking with 5% non-fat, dried milk in tris buffered saline with Tween (TBST; 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Tween 20), membranes were incubated with rabbit anti-TRPV1 antibody (1:1000, Abcam, ab-10296) in 5% non-fat, dried milk in TBST overnight at 4°C. After washing with TBST, membrane were incubated with goat anti-rabbit antibody (horseradish peroxidase-labelled) diluted with 5% non-fat dried milk in TBST and detected with enhanced chemiluminescence reagents (Amersham Biosciences, Arlington Heights, IL, USA). Blots were scanned with Spot Advanced and Adobe Photoshop 7.0 (Adobe, Inc., San Jose, CA, USA), and the band densities were detected and compared with TotalLAB 2.01 software (Bio-Rad, Totalab, Newcastle, UK). Membrane protein was extracted following the method as described previously (O'Neil et al., 2003). The following is a brief description: The tissues were lysed by sonication. Unlysed tissues and nuclei were removed by centrifugation. The supernatant was diluted with ice-cold 0.1 M Na<sub>2</sub>CO<sub>3</sub> and agitated on ice for 1 h, and then ultra-centrifuged at 150,000 g for 1 h at 4°C. The supernatant was discarded and the membrane pellet was resolved with 1 mL of membrane solubilization buffer containing 7 M urea, 2 M thiourea, 4% (w/v) n-octyl- $\beta$ -D-glucopyranoside, 2% (w/v) 3-(3-cholamidopropyl)dimethylammonio-1-propanesulfonic acid, 2% (w/v) N-decyl-N,N-dimethyl-3-ammonio-1-propane-sulfonate (SB3-10), 10 mM tris (2-carboxyethyl) phosphine and 40 mM Tris.

## 2.9 Primary culture of DRG neurons

Two-week-old rats were decapitated after anaesthesia. The spinal column was removed and bisected. DRGs were dissected free. All ganglia of both sides were removed from cervical, thoracic and lumbar levels. Ganglia were placed in type I collagenase (3 mg/mL, Sigma-Aldrich) in a heated (37°C) chamber for 45 min. After removing the collagenase with PBS washing, 1 mL trypsin (0.25%) was added for 10 min in the same heated chamber. Digestion was inhibited by foetal bovine serum and the cells were spun at 500 rpm for 5 min, replacing with fresh DMEM containing foetal bovine serum. The cells were dissociated by a polished Pasteur pipette and plated on poly-L-lysine coated 35-mm plastic dish or glass sheet for culture.

## 2.10 Acute dissociation of DRG neurons for whole-cell patch clamp recording

Ipsilateral L4 and L5 ganglia were acutely dissociated. The ganglia were placed in a culture dish containing 1 mL trypsin (0.125 mg/mL, Hyclone) and 1 mL type I collagenase (2 mg/mL, Sigma-Aldrich). The dish was shaken for 50 min in a heated (37°C) chamber. After washing and mechanical dispersion with a polishing Pasteur pipette, the cells were plated on poly-L-lysine-coated glass sheets in a culture dish. All patch clamp recordings on the dissociated DRG neurons were completed within 6 h after plating.

## 2.11 Drug administration

IGF-1 (4326-RG, R&D Systems, Inc. Minneapolis, MN, USA) was dissolved and diluted in sterile PBS solution to the desired concentration (3, 30 and 100 ng/mL). IGF-1R inhibitor picropodophyllotoxin (PPP; catalogue #2956, Tocris, Tocris Bioscience, Bristol, UK) at 20 mg/kg/12 h was injected (*i.p.*) for 3 consecutive days from days 15 to 17 after MRMT-1 live cell inoculation into tibia bone marrow.

## 2.12 Whole-cell patch recording of TRPV1 current in acutely dissociated DRG neurons

Whole-cell patch recording was performed as previously described in our lab (Tu et al., 2004). Two hours after plating, the dissociated DRG neurons were perfused with extracellular solution containing (in mM) NaCl 140, KCl 5, MgCl<sub>2</sub> 2, CaCl<sub>2</sub> 2, glucose 10, and N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) 10, adjusted to pH 7.4 with NaOH. The recording electrode was filled with a solution containing (in mM) KCl 120, Na<sub>2</sub>-ATP 5, Na<sub>2</sub>GTP 0.4, ethylene glycol tetraacetic acid 5, CaCl<sub>2</sub> 2.25, MgCl<sub>2</sub> 5 and HEPES 20. The solution was adjusted to pH 7.4 with KOH, and to an osmolarity of 315–325 mOsm. Capsaicin was prepared from 10 mM stock solution (in 100% ethanol) to a final concentration of 1 μM. Capsazepine (CPZ), a TRPV1 blocker, was used to confirm the capsaicin-induced current which was due to TRPV1 activation. All reagents were purchased from Sigma-Aldrich Chemicals.

Glass pipettes (6–8 MΩ) were prepared. Whole-cell recordings were made with a HEKA amplifier (HEKA Instruments). Stimuli were controlled and digital recordings were captured with Patchmaster software. Only neurons with a resting membrane potential of at least –40 mV, stable baseline recordings, and evoked spikes that overshoot 0 mV were used for further experiment and analysis. Series resistance (R<sub>s</sub>) was compensated to above 70% for the recorded DRG neurons. All recordings were made at room temperature.

## 2.13 Data analysis

All data are expressed as mean ± standard error of the mean and were analysed with one-way analysis of variance

(ANOVA) or two-way ANOVA followed by Bonferroni post test. A value of  $p < 0.05$  was taken as statistically significant.

## 3. Results

### 3.1 Bone cancer pain in rats: thermal hyperalgesia, mechanical allodynia and bone destruction

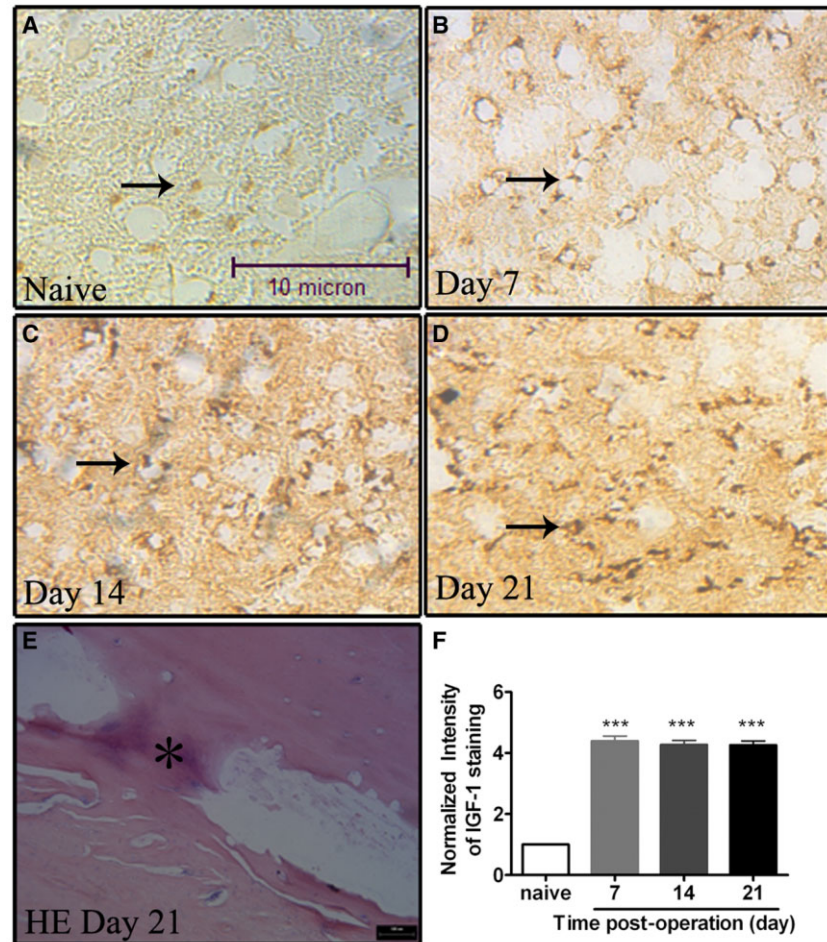
Compared with rats in the PBS group or the heat-killed MRMT-1 cell group, rats in the MRMT-1 live cell group showed thermal hyperalgesia (Supporting Information Fig. S1A) and mechanical allodynia (Supporting Information Fig. S1B) 14–21 days after MRMT-1 inoculation. Similarly, compared with the contralateral hind paw, the ipsilateral hind paw showed thermal hyperalgesia and mechanical allodynia (data not shown).

As previously reported in our lab (Tong et al., 2010), the tibia bone showed significant bone destruction in the MRMT-1 live cell group after 14 days at the injection site. However, tibia bone underwent no obvious morphological changes in the PBS group as well as in the heat-killed MRMT-1 cell group.

Further, histological examination found that MRMT-1 breast cancer cells gradually grew into peripheral tissues in the MRMT-1 live cell group (data not shown). At days 1, 3 and 7 after inoculation, cancer cells gradually grew within the bone marrow cavity and then completely filled the marrow cavity. Normal bone structure disappeared, but bone cortex was still complete. At day 14, cancer cells invaded the bone cortex. At day 21, cancer cells filled the bone marrow cavity completely, bone structure was further damaged. Eventually, the bone cortex was completely destroyed and the normal structure lost. On the contrary, in the PBS group and in the heat-killed cancer cell group, no obvious destruction of bone structure was observed.

### 3.2 Bone regeneration and IGF-1 expression increase in MRMT-1 bone cancer pain rats

We then investigated the expression of IGF-1 in tibia bone marrow after cancer cell inoculation. Figure 1 shows the IGF-1 expression in tibia bone marrow with immunohistochemical staining. The basal level of expression in naïve rats is shown in Fig. 1A. At days 7, 14 and 21 after MRMT-1 live cell inoculation (Fig. 1, B–D); statistical analysis showed that IGF-1 expression increased significantly (Fig. 1F). At day 21, histological HE staining of bone marrow tissues showed apparent



**Figure 1** Immunohistochemical staining of insulin-like growth factor-1 (IGF-1) in rat tibia bone marrow. A, Naïve rat; B, C and D represent cancer pain rats at days 7, 14, 21 after MRMT-1 live cell inoculation, respectively. (E) Haematoxylin–eosin (HE) staining of tibia bone at day 21. Apparent bone regeneration was observed. '\*' represents bone regeneration. (F) Intensity statistics of IGF-1 immunohistochemical staining. The arrows indicate IGF-1-positive staining. Scale bar = 10  $\mu$ m (For HE staining in 'E', scale bar = 100  $\mu$ m).

bone regeneration (Fig. 1E), very different from the normal trabecular structure.

### 3.3 TRPV1 current density increase in acutely dissociated DRG neurons in MRMT-1 bone cancer pain rats

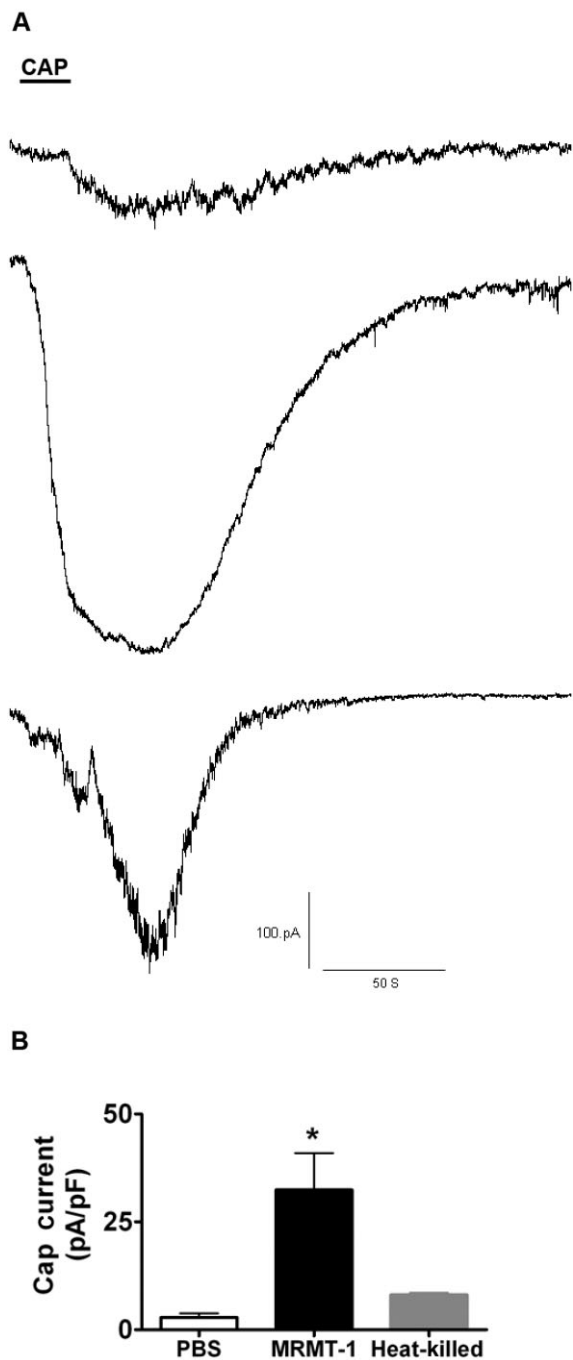
Only one neuron was recorded in each culture dish perfused with capsaicin in order to avoid capsaicin contamination. The sensitivity of a DRG neuron to capsaicin was detected by capsaicin-induced current with patch clamp recordings.

The membrane potential of the neuron was held at  $-60$  mV and the neuron was perfused with capsaicin for 30 s. The peak amplitude of the capsaicin-induced current was used to calculate the current density. Perfusion with 1  $\mu$ M capsaicin produced an inward current in a neuron. As shown in Fig. 2, the current density was significantly increased in DRG neurons from MRMT-1 bone cancer pain rats, while the vehicle (0.01% ethanol) had no obvious effect. Small DRG

neurons from MRMT-1 cancer pain rats were much more sensitive to capsaicin and the increased density of TRPV1 current was observed.

### 3.4 TRPV1 protein expression increase in small DRG neurons in MRMT-1 bone cancer pain rats: immunohistochemistry analysis

We first examined the TRPV1 expression in the DRG neurons with immunohistochemical staining both in the control groups (PBS or heat-killed cancer cell inoculation) and in the MRMT-1 live cell group. Results showed that TRPV1 protein was expressed mainly in small DRG neurons (Supporting Information Fig. S2A). The percentage of neurons expressing TRPV1 was significantly higher on the ipsilateral side at days 7, 14 and 21 after MRMT-1 live cell inoculation (Supporting Information Fig. S2B). In addition, the majority of neurons with increased TRPV1 expression had an area of 300–400  $\mu$ m<sup>2</sup>, which is within the area range of small C-fibre neurons (data not shown).



**Figure 2** Transient receptor potential vanilloid subfamily, member 1 (TRPV1) current density increase in acutely isolated dorsal root ganglion (DRG) neurons from cancer pain rats. Ipsilateral DRG neurons were isolated from MRMT-1 cancer pain rats 14–21 days after inoculation of MRMT-1 cells. Recordings were made by whole-cell patch-clamp. TRPV1 currents were evoked by perfusion with 1  $\mu$ M capsaicin (CAP) for 30 s. A representative current is shown in (A); the statistical results are shown in (B). \* $p < 0.05$  compared with the phosphate-buffered saline (PBS) or heat-killed controls.

### 3.5 TRPV1 protein expression increase in DRG in MRMT-1 bone cancer pain rats: Western blot analysis

TRPV1 protein expression increased in L4–L5 DRGs at days 14 and 21 after MRMT-1 live cell inoculation. A representative result of Western blot detection and the densities normalized to beta-actin control bands are shown in Supporting Information Fig. S3. The densities of TRPV1 protein bands in the MRMT-1 live cell group increased significantly at days 14 and 21. These results confirm that TRPV1 protein expression increased in DRG after MRMT-1 live cell inoculation.

### 3.6 IGF-1 incubation increased total and membrane TRPV1 protein expression in primary cultured DRG neurons: Western blot analysis

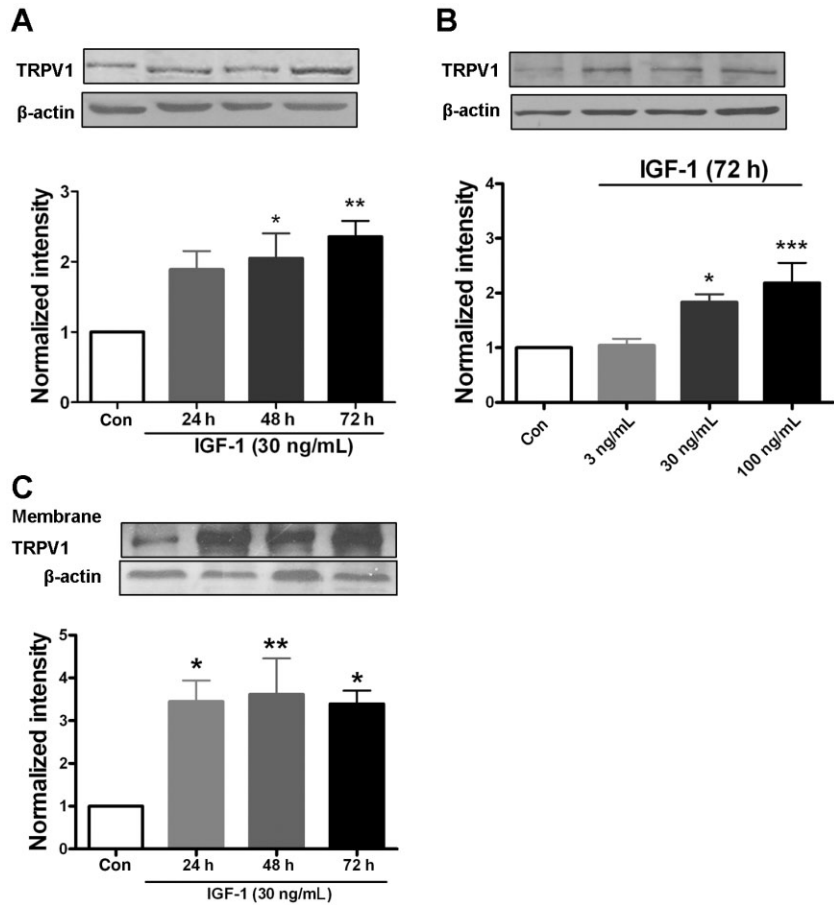
This *in vitro* experiment aimed to answer the question whether the increased IGF-1 expression in the tibia bone marrow could induce up-regulation of TRPV1 expression. The effect of IGF-1 was tested on the primary cultured DRG neurons. First, we analysed the time course of IGF-1-induced TRPV1 expression. After incubation with IGF-1 at 30 ng/mL, the total TRPV1 expression increased significantly at 48 and 72 h in the IGF-1 incubation group as compared with that in the control group (Fig. 3A). This IGF-1-induced up-regulation of TRPV1 protein expression could be observed even at 96 h (data not shown).

We then analysed the dose-effect of IGF-1. After incubation for 72 h with IGF-1 at 3, 30 and 100 ng/mL, DRG neurons were harvested to examine the TRPV1 protein expression. It was found that TRPV1 expression increased significantly at IGF-1 concentrations of 30 and 100 ng/mL as compared with PBS control (Fig. 3B).

Considering the functional importance of membrane TRPV1, membrane TRPV1 protein was further examined after incubation with IGF-1 at 30 ng/mL. As shown in Fig. 3C, membrane TRPV1 protein increased significantly at all time points.

### 3.7 Co-localization of IGF-1R and TRPV1 in DRG neurons: immunofluorescent double staining

Existence of IGF-1Rs on the membrane of TRPV1-expressing DRG neurons is the basis for IGF-1 up-regulation of TRPV1. Therefore, we examined the co-localization of TRPV1 receptors and IGF-1Rs in one DRG neuron. As shown in Fig. 4, immunofluorescent



**Figure 3** Insulin-like growth factor-1 (IGF-1) up-regulation on transient receptor potential vanilloid subfamily, member 1 (TRPV1) protein expression in primary cultured dorsal root ganglion (DRG) neurons. (A) Time-course of IGF-1 incubation at 30 ng/mL on total TRPV1 protein expression; (B) dose-effect of IGF-1 incubation (at 72 h) on total TRPV1 protein expression; C, Time-course of IGF-1 incubation at 30 ng/mL on membrane TRPV1 expression. \* $p < 0.05$ , \*\* $p < 0.01$ .

double staining showed that IGF-1R co-localized with TRPV1 in small DRG neurons.

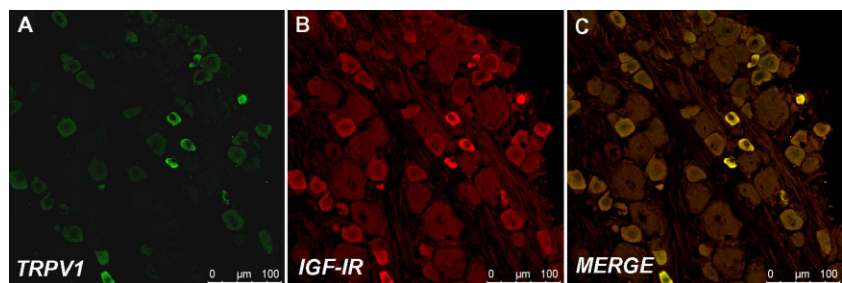
### 3.8 IGF-1 incubation increased TRPV1 current density in primary cultured DRG neurons

We further tested whether the up-regulated membrane TRPV1 by IGF-1 incubation in the primary cultured DRG neurons was functional. With whole-cell patch clamp recording technique, the capsaicin-induced currents were measured in primary cultured DRG neurons. Interestingly, incubation with IGF-1 at

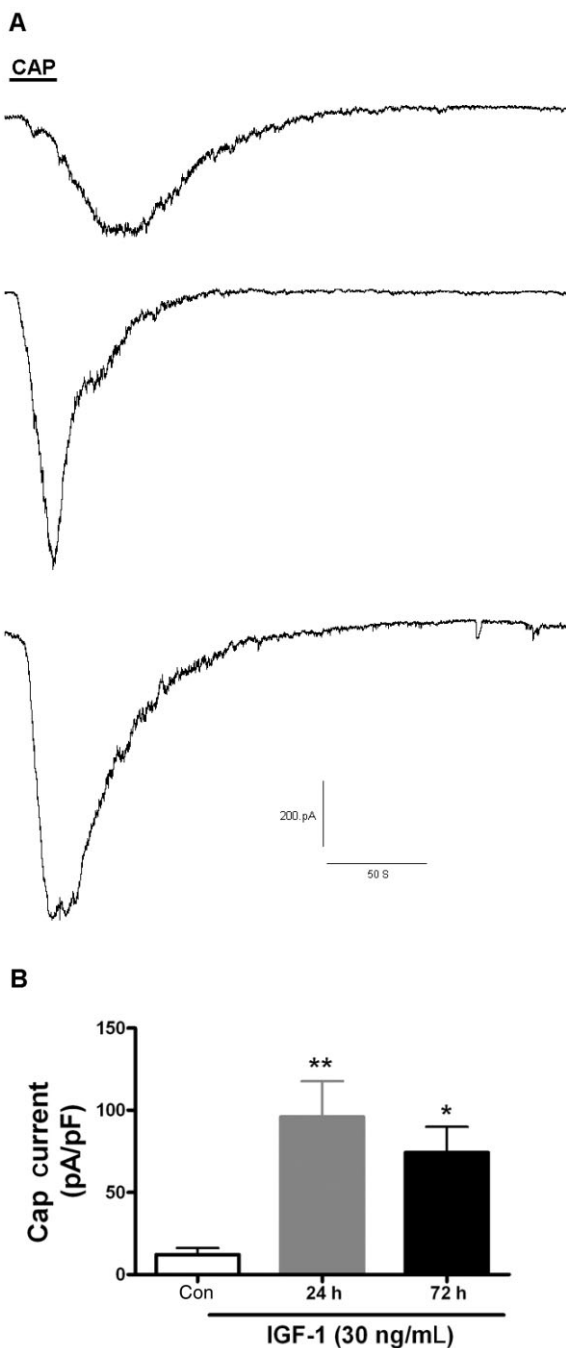
30 ng/mL for 24 and 72 h significantly increased the capsaicin-induced currents, suggesting that the up-regulated TRPV1 by IGF-1 is functional (Fig. 5). In addition, IGF-1 incubation did not change neuronal excitability (data not shown).

### 3.9 IGF-1R inhibitor reversal on pain behaviour in bone cancer pain rats

To detect the contribution of IGF-1 in bone cancer pain *in vivo*, the effect of PPP, an IGF-1R inhibitor, was examined on pain behaviour. PPP was injected intra-



**Figure 4** Co-localization of transient receptor potential vanilloid subfamily, member 1 (TRPV1) and insulin-like growth factor-1 receptor (IGF-1R) in dorsal root ganglion (DRG) neurons by immunofluorescent double staining. (A) TRPV1; (B) IGF-1R; (C) Merge. Bar = 100 μm.



**Figure 5** Transient receptor potential vanilloid subfamily, member 1 (TRPV1) current density increase in primary cultured dorsal root ganglion (DRG) neurons after Insulin-like growth factor-1 (IGF-1) incubation. Recordings were made by whole-cell patch-clamp at holding voltage of  $-60$  mV. TRPV1 currents were evoked by perfusion with  $1 \mu\text{M}$  capsaicin (30 s). (A) Representative currents. (B) Statistical results.  $*p < 0.05$  compared with control group. CAP, capsaicin.

peritoneally for 3 consecutive days from days 15 to 17 after the MRMT-1 live cell inoculation when thermal hyperalgesia and mechanical allodynia were apparent. An equal volume of vehicle (PBS) was used as a control. As shown in Fig. 6, before PPP application at day 14 after MRMT-1 live cell inoculation, both the PPP group and the PBS group showed thermal hyperalgesia and mechanical allodynia consistent with the establishment of pain behaviour. During days 15–17, when PPP was administrated, pain behaviours were alleviated significantly, suggesting that IGF-1R inhibition could reverse bone cancer pain in rats.

#### 4. Discussion

The present study showed that both IGF-1 in local bone marrow and TRPV1 in DRG neurons increased in a rat model of bone cancer pain induced by MRMT-1 cancer cell inoculation into tibia bone marrow. IGF-1 incubation of primary cultured DRG neurons *in vitro* produced increased expression and enhanced function of TRPV1, and inhibition of IGF-1R *in vivo* reversed mechanical allodynia and thermal hyperalgesia in rats with bone cancer pain. These results suggest an enhanced TRPV1 function via IGF-1 up-regulation in metastasized bone cancer pain.

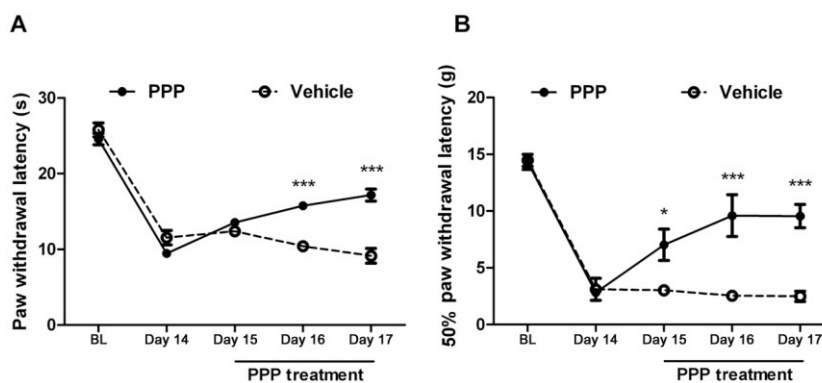
##### 4.1 TRPV1 expression increased as well as functionally enhanced in bone cancer pain rats

Many studies have reported an increase of TRPV1 protein expression in prostate, colorectal, pancreatic and bladder cancer patients with cancer pain (Lazzeri et al., 2005; Sanchez et al., 2005; Hartel et al., 2006; Menendez et al., 2006). TRPV1 antagonists had analgesic effects in models of bone cancer pain (Ghilardi et al., 2005). In the present study, we also found an increase in the TRPV1 expression in DRG neurons at day 7 after inoculation of MRMT-1 live cells into tibia bone marrow of rats (Supporting Information Fig. S3). TRPV1 expression increase occurred mainly in small- and medium-sized DRG neurons ( $300\text{--}400 \mu\text{m}^2$  in area) as determined by immunohistochemical staining (data not shown). The tibia bone receives rich sensory innervations by TRPV1-positive nerve fibres. It is widely accepted that nociceptive transmission takes place mainly through unmyelinated C-fibres as well as thinly myelinated  $A\delta$ -fibres. This increase of TRPV1-positive neurons suggests that more nociceptors are activated and TRPV1 participates in bone cancer pain.

More importantly, we found that the increased TRPV1 protein is also functionally enhanced. Whole-cell patch clamp recording technique showed that the



**Figure 6** Inhibition of insulin-like growth factor-1 receptor (IGF-1R) with picropodophyllotoxin (PPP) reversed thermal hyperalgesia (A) and mechanical allodynia (B) in bone cancer pain rats. Thermal hyperalgesia and mechanical allodynia were measured and confirmed at day 14 after the MRMT-1 live cell inoculation into tibia bone. PPP was injected intraperitoneally at 20 mg/kg/12 h for three consecutive days. \* $p < 0.05$ , \*\*\* $p < 0.001$  compared with the phosphate-buffered saline (PBS) vehicle group.  $n = 9$ .



TRPV1 current density was significantly increased in the acutely isolated DRG neurons from bone cancer pain rats. Small DRG neurons were the most sensitive to capsaicin. The density of membrane capsaicin current was also increased (Fig. 2). These results suggest that not only TRPV1 expression was increased, but also TRPV1 function was enhanced.

#### 4.2 IGF-1 up-regulates TRPV1 expression and enhances its function in bone cancer pain rats

When cancer cells metastasize to bone marrow cavity, cancer cell proliferation triggers the activation of osteoblasts and osteoclasts. Our results showed that the activated osteoblasts could synthesize and secrete IGF-1 from days 7 to 21 (Fig. 1). In cultured DRG neurons, we further found that IGF-1 incubation *in vitro* could up-regulate total and membrane TRPV1 protein expression in a dose- and time-dependent manner (Fig. 3). With electrophysiological patch clamp recordings on the acutely isolated DRG neurons or IGF-1-incubated DRG neurons, the capsaicin-induced currents were increased, suggesting an enhanced function of these TRPV1 receptors.

To connect the *in vitro* results of IGF-1 up-regulation on TRPV1 with *in vivo* pain behaviour, we observed the effect of PPP, an IGF-1R inhibitor, on mechanical hyperalgesia and thermal allodynia in bone cancer pain rats. As shown in Fig. 6, PPP inhibition of IGF-1R successfully reversed mechanical allodynia and thermal hyperalgesia in bone cancer pain rats. This result confirmed that IGF-1 up-regulation on TRPV1 through IGF-1R contributes to bone cancer pain *in vivo*. On the basis that TRPV1 antagonists can attenuate pain behaviour in bone cancer pain rats, as in many previous reports (Ghilardi et al., 2005), the present study suggested that IGF-1 up-regulates the amount of TRPV1 receptors, and more importantly, the function of TRPV1 receptors.

As a potent and selective inhibitor of IGF-1R, picropodophyllotoxin (PPP) inhibits malignant cell growth with low toxicity on normal cells (Feng et al., 2012). It is currently tested in phase II clinical studies against advanced, solid tumours that have progressed despite several lines of treatment. About the mechanisms of PPP (Girmita et al., 2004; Vasilcanu et al., 2008), it is reported that PPP inhibits IGF-1R phosphorylation and Protein Kinase B (PKB)/extracellular-signal-regulated kinases signalling pathway (Girmita et al., 2004). Further investigation is needed of the mechanisms by which PPP reversed mechanical allodynia and thermal hyperalgesia, and whether or not PPP has an intrinsic analgesic effect.

For the first few days following metastasis, secreted IGF-1 is limited to the bone marrow cavity because bone cortex is complete and cannot make contact with nerve fibre endings innervating the bone. In our experiment, IGF-1 was also believed to be limited to the bone marrow cavity for the first 14 days because bone cortex was not destroyed by cancer cells until 14 days after inoculation. Only when cancer cell growth destroyed the bone cortex, the increased IGF-1 could reach the nerve fibre endings and induce up-regulation of TRPV1 expression. This possibly explains the reason that TRPV1 expression in DRG neurons and peripheral nerve fibres lags behind the increase in IGF-1 secretion in the bone marrow cavity in our model of metastasized bone cancer pain. Further study is required to investigate other factors released in response to bone formation that may play roles in the up-regulation of TRPV1 in nociceptors.

#### 4.3 Mechanisms of bone cancer pain rather than IGF-1 up-regulation on TRPV1

In cancer pain, many receptors and channels are activated in addition to TRPV1. For example, growing cancer cells in bone marrow produced an increase in

the number of protons (Cheng and Ji, 2008), which activated acid-sensing ionic channels on nociceptors (Krishtal, 2003). Similarly, in addition to IGF-1, other locally secreted factors such as tumour necrosis factor- $\alpha$ , endothelins, interleukins-1 and -6, epidermal growth factor, transforming growth factor- $\alpha$ , PDGF and nerve growth factor (NGF) could regulate the expression of TRPV1 or other receptors (Joyce and Pollard, 2009). For example, NGF increased TRPV1 expression (Ji et al., 2002), and antagonizing NGF could significantly reduce bone cancer pain.

In summary, the present study provides further evidence for an important role of TRPV1 function in bone cancer pain. It is reported that IGF-1 is important in the development of cancer itself in previous studies. Our results suggest for the first time that IGF-1 also plays an important role in cancer pain via its up-regulation of TRPV1 receptors. TRPV1 as a potential target for cancer pain management is a well-accepted concept. Our study suggests that IGF-1 may be an up-stream regulator of TRPV1, especially in metastasized bone cancer pain. An obvious advantage of targeting IGF-1 may be a combination of cancer pain relief (via inhibition of TRPV1) and suppression of cancer cell growth.

## 5. Author contributions

Yan Li designed and performed all experiments, prepared data and statistical analysis, and contributed to the writing and editing of the paper; Jie Cai participated in electrophysiological experiments and behavioural tests; Ying Han participated in the Western blot experiment, and provided feedback on the paper; Xing Xiao participated in cell culture, immunofluorescent double staining and Western blot experiments; Xiang-Ling Meng participated in behavioural tests, immunofluorescent double staining; Li Su participated in cell culture, immunofluorescent double staining and Western blot experiments; Feng-Yu Liu participated in cell culture, immunofluorescent double staining and Western blot experiments; Guo-Gang Xing carried out electrophysiological experiments and analysed data; You Wan conceived, designed and supervised all experiments, wrote the paper and assimilated all comments from contributing authors and prepared all figures.

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## Supporting Information

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

**Figure S1.** Behavioural test for thermal hyperalgesia (A) and mechanical allodynia (B). \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  compared with the phosphate-buffered saline (PBS) or heat-killed controls.

**Figure S2.** Transient receptor potential vanilloid subfamily, member 1 (TRPV1) protein expression in ipsilateral L4/L5 DRG neurons. (A) Immunohistochemical staining. (B) Statistical results. The percentage of TRPV1 positive neurons increased significantly 7, 14 and 21 days after MRMT-1 live cell inoculation. \*\* $p < 0.01$ , \*\*\* $p < 0.001$  compared with control groups. Scale bar = 100  $\mu\text{m}$ .

**Figure S3.** Expression of total transient receptor potential vanilloid subfamily, member 1 (TRPV1) protein in dorsal root ganglion (DRG) by Western blot. \* $p < 0.05$ , \*\* $p < 0.01$ .