〈Regular Article〉

Development of a novel analgesic for cancer pain targeting brain-derived neurotrophic factor

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ABSTRACT Brain-derived neurotrophic factor (BDNF) is necessary for nerve growth. BDNF is expressed in the dorsal root ganglion (DRG) and modulates pain transduction from peripheral nociceptors. TrkB, which is a BDNF receptor with a tyrosine kinase domain, acts as a pain modulator on the cell membrane of second neuron. If an exogenous truncated TrkB lacking a tyrosine kinase domain can competitively block the binding of BDNF to endogenous TrkB, inhibitory effects on pain are expected. We constructed two expression vectors coding truncated TrkB-GFP fusion proteins, lacking intracellular tyrosine kinase domain, with and without the transmembrane domain. By transfection of the vectors to HEK293 cells, the expression and localization of the modified receptor proteins were confirmed. The truncated TrkB with the transmembrane domain, TM (+), was localized on cell membrane surface of the transfected cells, and capable of BDNF binding on cell surface. TM (−) without the transmembrane domain was secreted from the transfected cells, and the secreted TrkB protein was confirmed the capability for binding with BDNF by pull-down assay. Furthermore, we developed a rat model of cancerous osteocopic pain for evaluating an analgesic effect of the modified *TrkB* vectors on cancer pain. Pain-related behavior, as assessed by von Frey tests, indicated hyperalgesia after cancer cell administration. *BDNF* expression was higher on the affected side of the DRG at the third lumbar vertebra L3 than on the unaffected side. When the modified *TrkB* vectors were administrated to the cancer pain model rats, both the TM (+) and TM (−) vector administration groups exhibited an analgesic effect. These results suggest that the modified TrkB receptors and their vectors are applicable as molecular targeted drugs for pain control in cancer patients.

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INTRODUCTION

The incidence of cancer is rising because of the increasing elderly population in Japan, and approximately one-third of this population dies from cancer[1]. Approximately 70–80% of patients report severe pain associated with terminal cancer. Bone metastases cause decreased quality of life, including pain and pathological fractures[2]. The treatment of cancer-related bone pain is challenging[3] and is an important component of medical care[4]. Analgesia is often insufficient and the opioid quantity required for treatment may be limited by side effects[5]. It is necessary to establish new sedative methods; accordingly, we developed a new approach to alleviate cancer pain.

Brain-derived neurotrophic factor (BDNF) is associated with pain. BDNF expression is increased in various pain models, including acute pain and neuropathic pain models[6–8], and in the dorsal root ganglion (DRG) of the third lumbar vertebra L3 in a tibia cancer pain model[9].

BDNF is a member of the neurotrophins family, proteins necessary for nervous system development, growth, and maintenance[10], which function by binding to specific receptors[11]. BDNF binds to TrkB receptors and may be involved in the sensory nervous system as a pain modulator[12].

The mechanism by which BDNF transmits pain information is unknown; however, it is thought to involve Na ion channels[13]. When BDNF binds to TrkB, the Nav1.9 channel opens, which induces the depolarization of cells and signal transmission[13]. In a neuropathic pain model, BDNF expression increases. When BDNF acts on TrkB of the spinal cord, it inhibits K^+-Cl^- cotransporter 2, egests Cl^- extracellularly, and the intracellular potential shifts to the anion side. This results in allodynia via the depolarization of cells after the release of γ-aminobutyric acid by tactile stimulation from an inhibitory connector neuron[14]. In BDNF's association with pain, its binding to a specific TrkB receptor is important.

BDNF is generated within a cell body of the DRG in response to pain in the first sensory neuron and is transported in an orthodromic direction to the second neuron. Upon BDNF binding to TrkB on the second neuronal cell membrane, autophosphorylation occurs in the intracellular tyrosine kinase domain of TrkB, which opens the Na ion channel directly or indirectly. An inflow of Na^+ occurs, the membrane potential changes, and pain information is transmitted[15].

We postulated that an analgesic effect might be achieved by the competitive inhibition of the interaction between BDNF and TrkB. Therefore, we constructed vectors encoding truncated TrkB lacking the intracellular tyrosine kinase domain, confirmed their expression and the binding of BDNF to the truncated TrkB, and examined their analgesic effects on a cancer pain.

MATERIALS AND METHODS

Cell culture

The rat breast cancer cell line MRMT-1 was provided by the Cell Resource Center for Biomedical Research, Tohoku University (Miyagi, Japan). The human normal embryonic kidney cell line HEK293 was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). MRMT-1 and HEK293 cells were cultured in RPMI 1640 and DMEM medium, respectively, supplemented with 10% fetal bovine serum, 200 U/mL penicillin, 200 μg/mL streptomycin, and 300 mg/mL L-glutamine, and incubated at 37 ℃ in a humidified atmosphere of 5% CO_2.

Plasmids

Based on the rat TrkB gene (GenBank Accession, M55291), two truncated TrkB cDNAs were obtained using polymerase chain reaction (PCR) as described below. Using the sense primer TrkB-FW1 (5′-GGA TCC GCC ATG TCG CCC TGG CCG AGG TG-3′,

including the restriction endonuclease *Bam*HI site) targeting the protein synthesis initiation region and the antisense primer TrkB-RV1 (5′-GAA TTC ATG CTC CCG ATT GGT TTG GTC-3′, including an *Eco*RI site) targeting the region upstream of transmembrane region, the extracellular region of TrkB without the transmembrane domain was amplified by PCR from the rat cDNA. The extracellular region of TrkB containing the transmembrane domain was amplified by PCR using the sense primer TrkB-FW1 and the antisense primer TrkB-RV2 (5′-GAA TTC TGG GCC TTT CAT GCC AAA CTT G-3′, including an *Eco*RI site) targeting the downstream region of the transmembrane domain. Each PCR product was cloned into the *Eco*RV site of pBluescript KS, and confirmed by DNA sequencing. Subsequently, each TrkB gene was digested using *Bam*HI and *Eco*RI, and the resultant fragment inserted into the pCMVscript-EGFP vector digested with *Bam*HI and *Eco*RI. The pCMVscript-EGFP vector codes a fused protein of enhanced green fluorescent protein (EGFP), FLAG-tag, and S-protein tag downstream of CMV promoter. Sequencing analysis was used to confirm the amino acid frame of the TrkB gene fused with the EGFP, FLAG-tag, and S-protein tag. A TM (−) vector that lacked the transmembrane domain, and a TM (+) vector with the transmembrane domain were created. The pCMVscript-EGFP was used as an empty vector in the following experiments.

Transfection and BDNF binding assay

The empty vector, the TM (−) vector, and the TM (+) vector were introduced into HEK293 cells, using Lipofectamine 2000 (Invitrogen, Tokyo, Japan). Twenty-four hours after transfection, the expression levels of each modified receptor-EGFP fusion protein were observed using an Olympus IXL71 (Olympus, Tokyo, Japan) fluorescence microscope. Localization of TM (+) protein on cell membrane was measured by confocal microscope system LSM780 (Carl Teiss, Jena, Germany) and imaging software ZEN2011 (Carl Teiss).

After the TM (+) vector or empty vector was transfected into HEK293 cells on an 8-well culture slide (BD Falcon, Franklin Lakes, NJ, USA), the cells were cultured for 24 h, and BDNF was added (ab9794, mature type; Abcam, Cambridge, UK). After 1 h, the cells were washed and cultured in OPTI-MEM culture media (Invitrogen) with an anti-BDNF rabbit antibody (ab9793; Abcam), Cy3-labeled anti-rabbit antibody, and 10% fetal bovine serum for 3 h. Fluorescence observations of the BDNF binding capability of TM (+) were conducted using an Olympus IXL71 fluorescence microscope.

Pull-down assay and western blotting analysis

The TM (−) vector or the empty vector was introduced into HEK293 cells, and the supernatant was collected from the culture medium after 72 h. Protease inhibitors (10 μL; Sigma, St. Louis, MO, USA) and 500 ng of BDNF protein (ab9794, mature type; Abcam) were added to 1 mL of the supernatant and mixed for 16 h at 4 ℃. After mixing with S-protein agarose beads (50 μL; Novagen, Madison, WI, USA) for 3 h at 4 ℃, the solution was centrifuged at 5,000 rpm for 3 min, the S-protein agarose complex was electrophoresed on an SDS-polyacrylamide gel, and blotted onto a polyvinylidene fluoride membrane. Anti-FLAG mouse antibody (Sigma; F3165) for detecting TM (−) protein, and anti-BDNF rabbit antibody (ab9793; Abcam) for detecting BDNF were added. Anti-mouse antibody or anti-rabbit antibody (Vectastain ABC-AP Kit; Vector Laboratories, Burlingame, CA, USA), and CDP-star (New England BioLabs, Ipswich, MA, USA), a substrate of alkaline phosphatase, were reacted. Chemiluminescence was detected using the LAS-1000 (FUJIFILM, Tokyo, Japan).

Animals

All experiments in this study were performed according to the protocol approved by the Institutional Animal Care and Use Committee of Kawasaki Medical School (approval number 14-087).

A cancerous osteocopic model was created according to previously described methods[9, 16]. Male Wistar rats, at 7 weeks of age and weighing 190 - 210 g, were used in the study. The tibia of the left leg was exposed to light under general anesthesia with 3% sevoflurane, and the epiphyseal growth plate was punctured 5 mm on the tip side from the knee with a 23-G needle. Then, 3.0×10^3/10 μL MRMT-1 rat breast cancer cells were injected at the site. The injection site was sealed with bone wax, and the wound was closed.

Radiological analysis

Cancer cell growth was observed by simple X-rays; laterality was confirmed by photographing both proximal tibias using X-ray irradiation apparatus (ALOKA, Hitachi Healthcare, Tokyo, Japan). X-ray irradiation was conducted under general anesthesia with sevoflurane after von Frey tests at days 0, 7, and 14. All procedures, including MRMT-1 inoculation and vector administration, were performed after scanning.

Pain evaluation

Pain was evaluated using the von Frey test with von Frey filaments (TACTILE TEST AESTHSIO, Muromachi Kikai Co., Tokyo, Japan). Filaments of 0.4, 0.6, 1.0, 1.4, 2.0, 4.0, 6.0, 8.0, and 15.0 g were used. The 50% paw withdrawal threshold (PWT) of pain was calculated as an indicator of pain irritation[17]. The von Frey tests were performed in a plastic cage ($10 \times 13 \times 15$ cm^3), on which a wire mesh was installed to facilitate access to the plantar paw surface. After a rat rested quietly in the cage in a dark room for more than 30 min, measurements were obtained. Cancer cell inoculation day was considered as day 0 and measurements were obtained on days 0, 2, 5, 7, 9, 12, and 14. The researcher was blinded to the presence or absence of vector administration and the precise vector that was used.

Vector administration

A dorsal skin incision from L3 to L6 was made under general anesthesia on day 7, and the lumbar level was identified from the vertebra spinous process and pelvis. A 23-G needle was inserted into the spinal cord subarachnoid space from the muscular fasciae of L5/6 and a sterilized catheter (polyethylene tube, inner diameter 0.2 mm, outer diameter 0.5 mm) was inserted from the intussuscipiens. The catheter tip was maintained in the vicinity of L3 (6 cm from the puncture site of the muscular fasciae) and the vector (1 μg/μL, 10 μL) was administered with transfection reagent GenomONE (HVJ Envelope VECTOR KIT, 1 μg/μL, 10 μL; ISHIHARA SANGYO KAISHA, LTD., Osaka, Japan) to the site. The three vector-administered groups were the TM (−) group, the TM (+) group, and the empty vector group. An additional group, the vector non-administration group, was subjected to reagent transfection without a vector.

RNA extraction and quantitative real-time reverse transcription-PCR (qRT-PCR)

After pain was evaluated, the rats were decapitated under general anesthesia with sevoflurane and the bilateral L3 DRG and the L3 spinal cord were resected. RNA was extracted from the tissue samples, using an RNeasy Lipid Tissue Mini Kit (Qiagen, Hilden, Germany) and reverse transcribed to obtain cDNA using the Ready-to-Go T-Primed First-Stand Kit (Amersham Biosciences, Little Chalfont, UK). The cDNA was used as a template in the StepOnePlus Real-Time PCR System (Applied

Biosystems, Foster City, CA, USA), with the *BDNF* (GenBank Accession Number NM_012513) FW-primer 5′-GCG GCA GAT AAA AAG ACT GC-3′ and RV-primer 5′-GCA GCC TTC CTT CGT GTA AC-3′, which generated a product of 141 bp, and the ribosomal protein L27 *RPL-27* (GenBank Accession Number NM_022514) FW-primer 5′-GAA TTG ACC GCT ATC CCA GA-3′ and RV-primer 5′-TCG CTC CTC AAA CTT GAC CT-3′, which generated a product of 230 bp as an internal control.

Primers were designed to target EGFP and the S-Tag, which were the common parts of each vector, to detect the mRNA expression of the *GFP*-fusion gene in the tissues of the vector-administered rats; the primers were GFP FW-primer 5′-CGA CAA CCA CTA CCT GAG CA-3′ and RV-primer 5′-GAA TTT AGC AGC AGC GGT TT-3′, which generated a product of 178 bp. QRT-PCR was performed, and the expression levels of *GFP* and *RPL-27* were compared between the vector administration groups and the vector non-administration group.

Immunohistochemistry

To confirm that the gene in the administered vector was expressed in rats, TrkB-GFP protein expression was evaluated. Perfusion fixation of the TM (+) group rats on days 7 and 9 was performed using 4% paraformaldehyde. Subsequently, the spinal cord was resected in sections from T10 to L5. The expression of TrkB-GFP in each specimen was observed with anti-GFP antibody mouse monoclonal IgG2a (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and FITC-labeled goat anti-mouse IgG2a (Santa Cruz Biotechnology) as a secondary antibody. Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI, DOJINDO LABORATORIES, Kumamoto, Japan) to examine the localization of the cells.

Statistical analysis

Statistical analyses of the results were performed using GraphPad Prism6. The von Frey test results were analyzed by repeated measures two-way analysis of variance (ANOVA) and Sidak's post-hoc tests. Gene expression results were evaluated by Student's *t*-tests. Experts reviewed the statistics, and the data were checked, confirming them for normality. A value of $p < 0.05$ was considered statistically significant.

RESULTS

Expression of the modified TrkB receptors in HEK293 cells

We constructed two GFP-expression vectors expressing truncated TrkB lacking the intracellular tyrosine kinase domain (Fig. 1), and transfected them into HEK293 cells to confirm gene expression. In cells treated with the empty vector, fluorescent protein expression was observed in the whole cell (Fig. 2A). In cells treated with the TM (+) vector (with a transmembrane domain), the fluorescent protein was expressed in the vicinity of the cell membrane (Fig. 2B), indicating that the modified TrkB protein with its transmembrane domain localized on the cell membrane. However, in cells treated with the TM (−) vector (without a transmembrane domain), GFP expression was weakly observed in the cytoplasm (Fig. 2C), suggesting a possibility that the modified TrkB protein without a transmembrane domain is secreted into the culture medium.

To verify the possibility, we performed a pull-down assay with S-tag protein agarose beads and western blotting with anti-FLAG antibody using the culture medium. As the modified TrkB protein is a fused protein with S-tag and FLAG-tag, it was expected that we can detect the protein by pull-down assay and western blotting, if the protein is secreted into the culture medium. Secreted TM (−) protein was confirmed in culture medium of cells transfected with the TM (−) vector by a pull-down assay with S-tag protein agarose beads and western

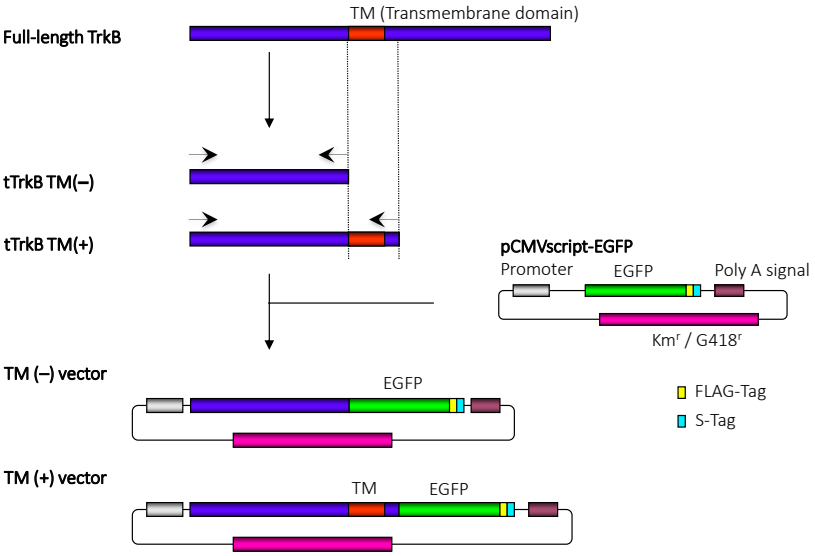

Fig. 1. Construction of the truncated *TrkB* expression vectors.
Based on the rat *TrkB* gene (GenBank Accession No. M55291), two extracellular regions of the *TrkB* cDNA were amplified by PCR and fused with the *GFP* cDNA in-frame in the expression vectors. The truncated *TrkB* that lacked a transmembrane domain was referred to as TM (−), the truncated *TrkB* with a transmembrane domain was referred to as TM (+). EGFP, enhanced green fluorescence protein; TM, transmembrane.

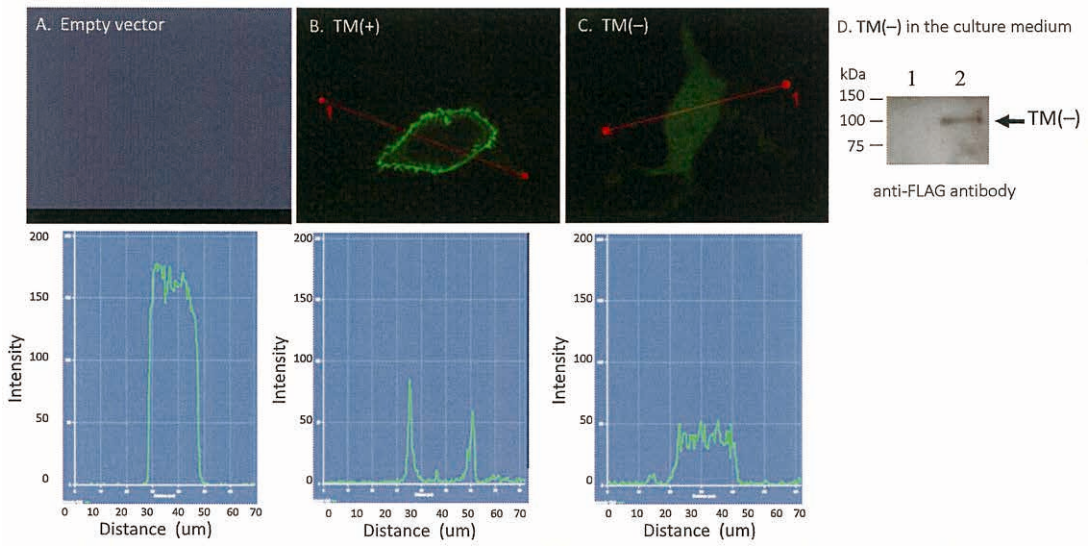

Fig. 2. Confirmation of the expression of modified receptor proteins.
The empty vector, TM (+) vector, and TM (−) vector were transfected into the human kidney cell line HEK293. (A) Fluorescence protein expression was observed in the whole cell in cells treated with the empty vector. (B) Fluorescent protein expression was observed in the membranes of cells treated with the TM (+) vector. (C) Cells treated with the TM (−) vector showed weak fluorescent protein expression. *Upper*, photograph of cellular localization of EGFP-fused protein; *lower*, Intensity of EGFP-fused protein on cross section of a cell. (D) The presence of TM (−) protein secreted in culture medium of cells transfected with the TM (−) vector was confirmed by western blotting with anti-FLAG antibody after pull-down assay with S-tag protein agarose beads. Lane 1, empty vector; lane 2, TM (−) vector.

blotting with an anti-FLAG antibody (Fig. 2D, lane 2), but not in culture medium of the empty vector-transfected cells (Fig. 2D, lane 1).

Confirmation of the BDNF-binding ability of the modified TrkB proteins

After confirming that the modified TrkB proteins were expressed in HEK293 cells, we examined the BDNF-binding ability of the TrkB proteins, using the transfected HEK293 cells. For TM (+), the HEK293 cells transfected with the vectors were co-cultured with BDNF, and BDNF interactions with the cell membrane were detected using an anti-BDNF antibody. Cells treated with the empty vector did not show BDNF binding (Fig. 3, A1, A2, B1, and B2), while cells treated with the TM (+) vector showed localization of GFP (green) and BDNF (red) at the cell membrane surface (Fig. 3, C1, C2, D1, and D2), suggesting that the TM (+) protein is able to bind to BDNF at the cell membrane surface.

For TM (−), the supernatant was collected from the culture medium of the vector-transfected cells, BDNF was added and mixed. After TM (−) protein was pulled down with S-tag protein agarose beads, BDNF was detected by western blotting using an anti-BDNF antibody. It was expected that we can detect BDNF by pull-down assay and western blotting, if TM (−) protein secreted from the transfected cells can bind with BDNF. BDNF was detected using the culture medium of the TM (−) transfected cells (Fig. 3E, lane 2), but was not detected using the culture medium of the empty vector-transfected cells (Fig. 3E, lane 1), suggesting that the TM (−) protein secreted from cells is able to bind with BDNF.

Development of a cancer pain rat model

After confirming that the modified TrkB receptor proteins could bind to BDNF, we developed a cancer pain rat model to examine the analgesic effect of the

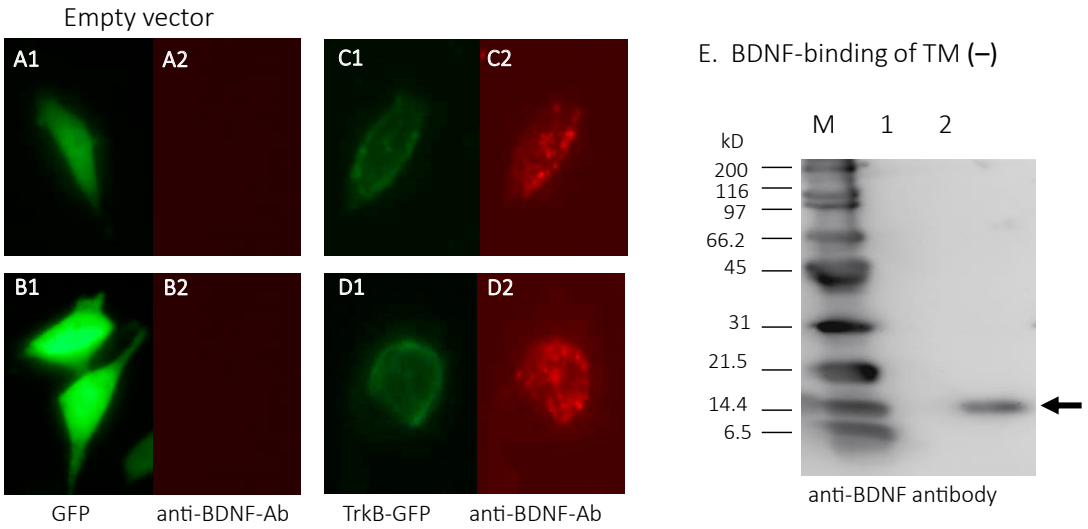

Fig. 3. Confirmation of the BDNF binding capacity of the modified receptor proteins
For TM (+), HEK293 cells transfected with the vectors were co-cultured with BDNF, washed and reacted with anti-BDNF rabbit antibody/Cy3-labeled anti-rabbit antibody. A and B show the cells transfected with empty vector, and C to D show the cells transfected with the TM (+) vector. Number 1 indicates the localisation of GFP (green), and number 2 indicates the localisation of BDNF (red). Localization of BDNF was detected on the cells transfected with TM (+) (C2 and D2), but not on the cells transfected with the empty vector (A2 and B2). For TM (−), after adding BDNF to the culture medium collected from cells transfected with the vectors, BDNF-binding of TM (−) protein was detected by pull-down assay with S-tag protein agarose beads and western blotting with anti-BDNF antibody. E lane 1, empty vector; lane 2, TM (−) vector; M, molecular weight marker.

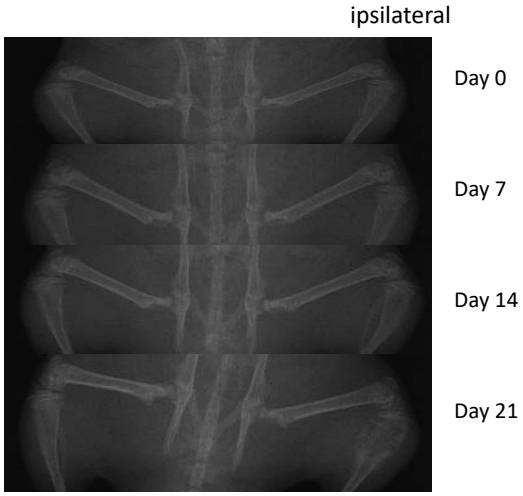

Fig. 4. Radiographical analysis of the rat tibia.
We evaluated the presence of bone fusion using X-rays to confirm MRMT-1 cell colonization. We punctured the epiphyseal growth plate 5 mm at the tip side from the knee of the left leg with a 23-G needle to the marrow and injected the cancer cells (3.0×10^3 cells/10 μL). Day 0 was normal, before cancer cell inoculation. The bone fusion gradually progressed from day 7 and laterality in the permeability of bones was clearly recognized after day 14. We kept only one rat until day 21; however, bone fusion was remarkable, and the continuity of the bone tissue was lacking.

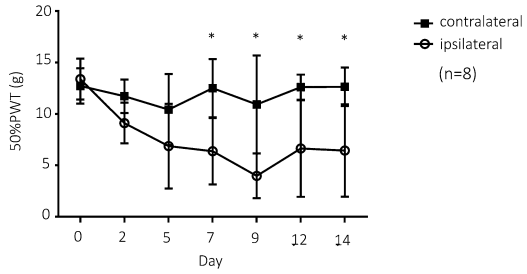

Fig. 5. Pain evaluation for the vector non-administration group.
Cancer cells were inoculated on day 0, and the vector non-administration group rats were administrated by transfection reagent without vector DNAs at day 7. After each rat rested quietly in a cage in a dark room for at least 30 min, the 50% PWT was measured using von Frey tests with filaments of 0.4, 0.6, 1.0, 1.4, 2.0, 4.0, 6.0, 8.0, and 15.0 g. The 50% PWT of the ipsilateral side gradually decreased and was significantly different from that of the contralateral side after day 7. The threshold for the ipsilateral side did not increase spontaneously, and there was no tendency for that of the contralateral side to decrease. Pain irritation only occurred on the ipsilateral side, which was injected with the cancer cells. A statistical analysis was performed using repeated measures two-way analysis of variance (ANOVA) and Sidak's post-hoc tests implemented in GraphPad Prism6. Data are represented as the mean ± standard deviation. 50% PWT, 50% paw withdrawal threshold. *, $p < 0.05$ between the ipsilateral and the contralateral sides.

modified TrkB, TM (+), and TM (−), on cancer pain. A model rat was created by injecting MRMT-1 rat breast cancer cells into the intramedullary cavity of the tibia of the left leg of male Wistar rats. We then evaluated the presence of bone fusion using X-rays to confirm MRMT-1 cell colonization. We punctured the epiphyseal growth plate 5 mm at the tip side from the knee of the left leg with a 23-G needle to the marrow and injected the cancer cells (3.0×10^3 cells/10 μL). Day 0 was normal, before cancer cell inoculation. Bone fusion gradually progressed from day 7 and laterality in the permeability of the bones was recognized clearly after day 14. We kept only one rat until day 21; however, bone fusion was remarkable, and the continuity of the bone tissue was lacking. Plain X-rays were obtained on days 0, 7, and 14, and from one rat on day 21 (Fig. 4). We observed clear bone fusion on the ipsilateral side at day 14; by day 21, we detected a colony of cancer cells.

Analgesic effect of the modified TrkB vectors on cancer pain rats

The growth of inoculated cancer cells was confirmed successfully on the ipsilateral side of the model rats. The rats were then administered a transfection reagent with/without vector DNAs into the spinal cord at day 7, and pain was examined by von Frey tests from day 0. The results of the von Frey tests for the vector non-administration group (by transfection reagent without vector DNA) are summarized in Fig. 5. There was a significant decrease ($p < 0.05$) in the 50% paw withdrawal threshold (PWT) on the ipsilateral side after day 7, which indicated a state of pain irritation. This decrease in the 50% PWT did not improve

spontaneously by day 14. Additionally, a reduction in the 50% PWT on the contralateral side was not found.

For the vector-administered groups, in the empty vector group, the 50% PWT of the ipsilateral side decreased significantly ($p < 0.05$), similar to the vector non-administration group (Fig. 6A). The TM (+) group showed pain irritation before vector administration; however, the threshold on the ipsilateral side increased to the same extent as that on the contralateral side by day 9 (Fig. 6B), suggesting that TM (+) vector administration had an analgesic effect on cancer pain, to a level comparable to a painless state on day 2 after vector administration. The thresholds decreased and a significant difference was observed at day 14 (1 week after vector administration). In the TM (−) group, the 50% PWT level was significantly lower than that of the contralateral side on day 5 and day 7 (Fig. 6C). However, the 50% PWT increased to the approximate value of the contralateral side at day 9. The 50% PWT decreased at day 12 and day 14, but a higher 50% PWT than those of day 5 and day 7 was maintained.

Confirmation of BDNF expression in the DRG of cancer pain rats

To assess the increased expression of the *BDNF* gene induced by pain signaling in cancer pain rats, we evaluated *BDNF* mRNA expression in the L3 DRG and spinal cord, normalized against *RPL-27* mRNA expression at the same sites. We calculated the ratio of ipsilateral *BDNF* mRNA to *RPL-27* mRNA when expression on the contralateral side in the DRG was set to 1. The expression of *BDNF* mRNA was significantly higher on the ipsilateral side than in the contralateral side in both the vector non-administration group (n = 8, $p < 0.0001$, one-tailed Student's *t*-test) and the vector administered groups (n = 34, $p < 0.0001$) (Fig. 7), suggesting that *BDNF* expression is induced in the DRG by pain signaling in our cancer pain model rats, which

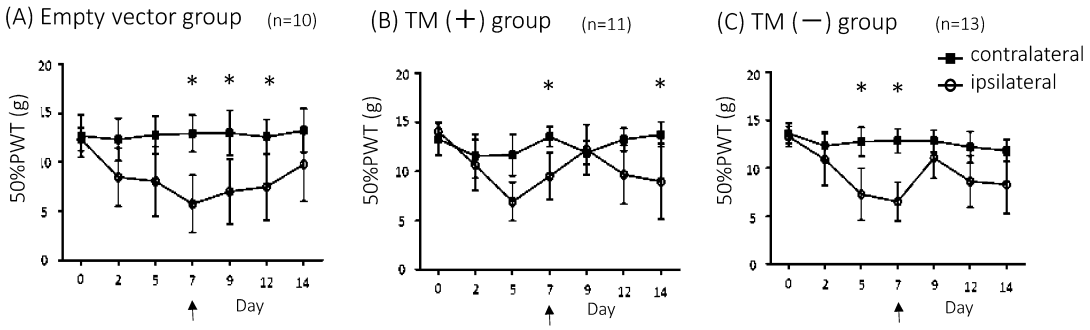

Fig. 6. Pain evaluation for the vector-administered group.
Von Frey tests were performed to evaluate pain in each vector-administered group. (A) In the empty vector group, the state of pain irritation was significant and was similar to the vector non-administration group; the threshold on the contralateral side did not decrease. (B) Plot of the von Frey test results for the TM (+) group. The 50% PWT of the ipsilateral side gradually decreased, similar to the vector non-administration group. After the vector administration (on day 7), the threshold of the ipsilateral side increased at day 9 to a level similar to that of the contralateral side. However, the threshold decreased, with a significant difference between sides at day 14, and a state of pain recurred. (C) Plot of von Frey test results for the TM (−) group. The 50% PWT of the ipsilateral side gradually decreased, similar to the vector non-administration group, and we observed a state of pain irritation from day 5 to day 7. By the vector administration (on day 7), the 50% PWT of the ipsilateral side increased and there was no significant difference between sides. The 50% PWT tended to decrease again gently at day 12 and day 14. A significant difference between sides was not observed on day 12 and day 14. Arrows on day 7 show vector administration. 50% PWT, paw withdrawal threshold; *, $p < 0.05$ between the ipsilateral and the contralateral sides.

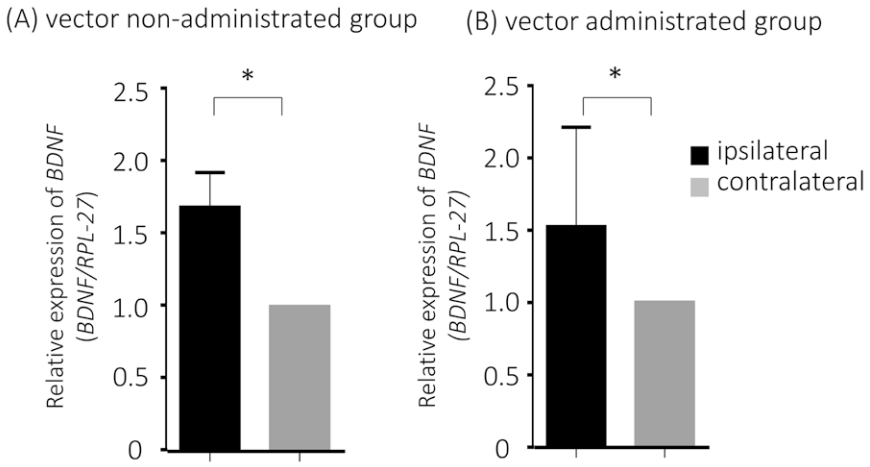

Fig. 7. Expression of *BDNF* mRNA in the L3 DRG of cancer pain rats.
BDNF mRNA expression in the dorsal root ganglion (DRG) of L3 on the contralateral and ipsilateral sides at day 14 was analyzed by quantitative real-time reverse transcription PCR (qRT-PCR), normalizing against *RPL-27* mRNA expression. The expression ratio on the contralateral side was set at 1. *BDNF* mRNA expression was significantly higher in the vector non-administration group (n = 8) (A), and in the vector administration group (n = 34) (B), on the ipsilateral side than in the contralateral side. *BDNF* mRNA expression at the ipsilateral side of the DRG in the vector non-administration group was similar to that of the vector administrated group. RPL-27, ribosomal protein L27: *, $p < 0.05$ between the ipsilateral and the contralateral sides.

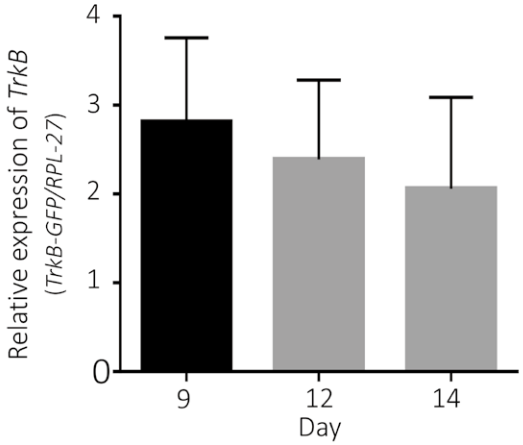

Fig. 8. Detection of *TrkB-GFP* mRNA in the L3 spinal cord of TM (+) rats
Relative expression of the *TrkB-GFP* mRNA in TM (+) rats after TM (+) administration on day 7. The exogenous *TrkB-GFP* mRNA expression in the L3 spinal cord of TM (+) group on days 9, 12, and 14 was examined by quantitative real-time reverse transcription PCR (qRT-PCR) with *GFP* primers, to eliminate the endogenous *TrkB* mRNA, and normalized by *RPL-27* mRNA as an internal control. *RPL-27*; ribosomal protein L27.

was consistent with previous results[9]. The *BDNF* mRNA expression level in the spinal cord did not differ between the two sides (data not shown).

Confirmation of mRNA and protein expression of the modified TrkB in treated rats

The mRNA expression levels of *TrkB-GFP* from the vectors administered to the L3 spinal cord of TM (+) rats were examined by qRT-PCR. Using PCR primers targeting *GFP*, we detected the expression of *TrkB-GFP* in the L3 spinal cord on the ipsilateral site of TM (+) rats on days 9, 12, and 14; data are shown as relative values to *RPL-27* mRNA expression levels as an internal control (Fig. 8).

Protein expression of TrkB-GFP after vector administration to the spinal cords of rats was examined by immunohistochemistry. We performed perfusion fixation of TM (+) group rats on day 9 with 4% paraformaldehyde, and the spinal cord tissue specimens, which were resected in sections from T10 to L5, were reacted with a mouse anti-

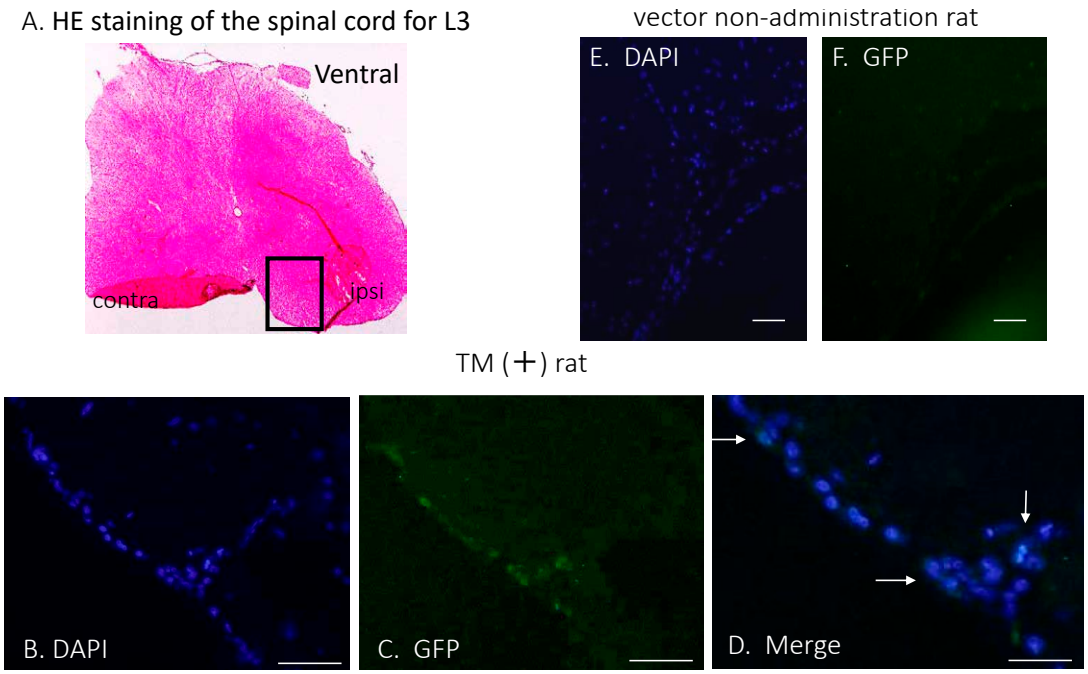

Fig. 9. Detection of TrkB-GFP protein in the L3 spinal cord of TM (+) rats.
(A) HE staining of the spinal cord for L3. Other panels show an extended image of the dorsal spinal cord surface at ipsilateral side. (B, C) In the L3 spinal cord two days (on day 9) after TM (+) administration, the expression of GFP proteins was detected, similar to the site marked by DAPI staining. (D) Merged image of GFP and DAPI staining two days after TM (+) administration. (E, F) DAPI and GFP imaging in rats of the vector non-administration group on day 9. We observed scattered DAPI staining, but did not detect GFP proteins around the cells, even when we increased the brightness. HE, Hematoxylin-Eosin; contra, contralateral; ipsi, ipsilateral; DAPI, 4′,6-diamidino-2-phenylindole. Scale bar, 50 μm.

GFP antibody and FITC-labeled anti-mouse antibodies (Fig. 9). We detected the GFP-fusion protein along the surface cells of the L3 spinal cord at the ipsilateral side (Fig. 9A) of TM (+) rats (Fig. 9B–D), but not in rats of the vector non-administration group (Fig. 9E–F).

DISCUSSION

Based on the *BDNF* expression in the DRG and the von Frey test results using the vector non-administration group, our cancer pain model rats were in a state of pain irritation after the injection of MRMT-1 cells. The TM (−) and TM (+) groups escaped the state of pain irritation, while the empty vector group showed similar von Frey test results to those of the vector non-administration group. These results indicated that an analgesic effect was obtained by the administration of the modified *TrkB* vectors. Complications, such as paralysis, mechanical complications by catheterization, or chemical complications related to vector administration, did not appear to influence the von Frey test results or the analgesic effects. On the basis of a radiological analysis and von Frey tests, 14 days was sufficient to confirm the outcomes of the pain irritation model and vector administration. Accordingly, extending the cancer pain model to beyond 14 days was deemed unethical.

In a transfection assay with HEK293 cells, the secreted TM (−) protein was detected in the culture medium, while some was detected in cells. On the basis of a luciferase assay using a fusion gene

of luciferase with the N-terminal signal peptide sequences of TrkB, we confirmed that the fusion protein was secreted outside of the cells (data not shown). We speculated that TM (−) proteins were secreted from cells under the arachnoid cells of the spinal cord, and bound to BDNF in the subarachnoid space of the model rats. TM (+) protein with its transmembrane domain was detected in the vicinity of the cell membrane. The TM (+) protein might localize to the cell membrane via its transmembrane domain. TM (+) protein may function as a local analgesic agent and TM (−) protein may function as a humoral analgesic agent. Further large-scale studies are required to clarify the duration of the analgesic effect, the stability of the proteins, and the location and frequency of BDNF binding in the presence or absence of the transmembrane domain.

Full-length TrkB has an intracellular tyrosine kinase domain, while one isoform, a truncated TrkB lacking the intracellular tyrosine kinase domain, has also been reported[18]. There are various opinions regarding the roles of truncated TrkB. Eide *et al.*[19] reported that truncated TrkB functions as a dominant negative receptor for BDNF and that full-length TrkB is involved in PLC-γ-dependent nerve communication. Truncated TrkB might conflict with pain communication mediated by BDNF and full-length TrkB, which would be consistent with the results of the present study. Associations of BDNF with neuropathic pain, inflammatory pain, and various other pain models have been reported[7]. Obata *et al.*[20] reported that neuropathic pain could be inhibited by downregulating the expression of BDNF. Thus, similar analgesic effects might be obtained in other pain models by the administration of our truncated *TrkB* vectors.

BDNF is essential for the maintenance of neurological function, including nervous system maturation and cell survival[21, 22]. Additionally, BDNF is associated with various diseases and organs, e.g., neurodegenerative diseases, such as Alzheimer disease[23] or Parkinson's disease[24]. Associations with myocardial contractility[25] or cardiac microvessel stabilization[26] have also been reported. Recently, an association between BDNF and depression was reported. Levels of the neurotrophic factor decrease mainly in response to long-term exposure to stress, and neuropoiesis in the hippocampus is inhibited by a decrease in BDNF. The hippocampus plays an important role in the control of emotions, and depressive symptoms are thought to result from its functional inhibition[27]. BDNF exhibits clear associations with systemic organs. Thus, it may not be suitable to treat the whole body by the transvenous administration of truncated *TrkB* vectors because of potential side effects. Furthermore, long-term administration may cause neurological complications; therefore, limited, short-term use, such as for the advanced stages of cancer pain, or local application may be preferred.

We assessed the analgesic effects based on rat behavior and confirmed the presence of the exogenous modified *TrkB* mRNA in the treated rats. Furthermore, we confirmed the presence of the modified TrkB protein immunohistologically in the L3 spinal cord of treated rats. The modified TrkB protein was detected mostly in the spinal dorsum, which might receive the vectors, and was minimal around the intraspinal cells. The present study had a few limitations. We did not confirm whether the exogenous modified TrkB receptors inhibited endogenous TrkB-BDNF binding competitively, and we did not evaluate histological markers and physiological analgesic effects. Further studies are necessary to examine the analgesic effects and their underlying mechanisms comprehensively.

In summary, we administered two truncated *TrkB* vectors lacking intracellular tyrosine kinase domain. We observed an analgesic effect in a bone cancer pain rat model. This is the first report of an analgesic effect on cancerous bone pain using this method, and a similar strategy might induce analgesic effects

in other pain models. Additional studies are needed to assess the clinical applications of this method.

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CONFLICT OF INTEREST

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