ANESTHESIOLOGY

Vascular Endothelial Growth Factor A Signaling Promotes Spinal Central Sensitization and Pain-related Behaviors in Female Rats with Bone Cancer

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EDITOR'S PERSPECTIVE

What We Already Know about This Topic

- Pain resulting from cancer metastatic to bone is a major clinical problem
- Vascular endothelial growth factor receptors are involved in tumor angiogenesis and are felt to be analgesic targets

What This Article Tells Us That Is New

- In a female rat model of metastatic breast cancer, expression of vascular endothelial growth factor A and its receptor vascular endothelial growth factor receptor 2 were upregulated in spinal tissue
- Blocking vascular endothelial growth factor signaling improved several measures of nociception and function in this model suggesting a role for vascular endothelial growth factor antagonists in reducing cancer-related pain

ABSTRACT

Background: Cancer pain is a pervasive clinical symptom impairing life quality. Vascular endothelial growth factor A has been well studied in tumor angiogenesis and is recognized as a therapeutic target for anti-cancer treatment. This study tested the hypothesis that vascular endothelial growth factor A and vascular endothelial growth factor receptor 2 contribute to bone cancer pain regulation associated with spinal central sensitization.

Methods: This study was performed on female rats using a metastatic breast cancer bone pain model. Nociceptive behaviors were evaluated by mechanical allodynia, thermal hyperalgesia, spontaneous pain, and CatWalk gait analysis. Expression levels were measured by real-time quantitative polymerase chain reaction, western blot, and immunofluorescence analysis. Excitatory synaptic transmission was detected by whole-cell patch-clamp recordings. The primary outcome was the effect of pharmacologic intervention of spinal vascular endothelial growth factor A/vascular endothelial growth factor receptor 2–signaling on bone cancer pain behaviors.

Results: The mRNA and protein expression of vascular endothelial growth factor A and vascular endothelial growth factor receptor 2 were upregulated in tumor-bearing rats. Spinal blocking vascular endothelial growth factor A or vascular endothelial growth factor receptor 2 significantly attenuated tumor-induced mechanical allodynia (mean ± SD: vascular endothelial growth factor A, 7.6 ± 2.6 g *vs.* 5.3 ± 3.3 g; vascular endothelial growth factor receptor 2, 7.8 ± 3.0 g *vs.* 5.2 ± 3.4 g; n = 6; $P < 0.0001$) and thermal hyperalgesia (mean ± SD: vascular endothelial growth factor A, 9.0 ± 2.4 s *vs.* 7.4 ± 2.7 s; vascular endothelial growth factor receptor 2, 9.3 ± 2.5 s *vs.* 7.5 ± 3.1 s; n = 6; $P < 0.0001$), as well as spontaneous pain and abnormal gaits. Exogenous vascular endothelial growth factor A enhanced excitatory synaptic transmission in a vascular endothelial growth factor receptor 2–dependent manner, and spinal injection of exogenous vascular endothelial growth factor A was sufficient to cause pain hypersensitivity *via* vascular endothelial growth factor receptor 2–mediated activation of protein kinase C and Src family kinase in naïve rats. Moreover, spinal blocking vascular endothelial growth factor A/vascular endothelial growth factor receptor 2 pathways suppressed protein kinase C-mediated *N*-methyl-D-aspartate receptor activation and Src family kinase-mediated proinflammatory cytokine production.

Conclusions: Vascular endothelial growth factor A/vascular endothelial growth factor receptor 2 contributes to central sensitization and bone cancer pain *via* activation of neuronal protein kinase C and microglial Src family kinase pathways in the spinal cord.

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Bone cancer pain is the most common and intractable type of cancer pain symptoms and is associated with metastatic breast, lung, and prostate cancer in the clinic, all of which generally consist of ongoing pathologic and breakthrough pain.[1] Mounting oncologic evidence demonstrates that effective pain control is not only an essential aspect of comprehensive cancer management, but is also linked to prolonged survival.[2] Unfortunately, conventional pharmacologic therapies for bone cancer pain management are inadequate due to either limited analgesic effects or inevitable side effects. Thus, the exact cellular and molecular mechanisms underlying bone cancer pain should be examined within preclinical pain research for active pharmacologic

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development. Our and other laboratories have established rat or mouse models of bone cancer pain *via* local injection of tumor cells into the femur, tibia, or calcaneus in order to elicit long-lasting pain hypersensitivity associated with apparent tumor growth and bone destruction.[3,4] Of interest, in these models pain behaviors were detected at the affected hind paw, rather than at the tumor-injected area, which was not directly exposed to tumor infiltration. This phenomenon suggests that although the pathogenic site is derived from the cancer microenvironment and implicated in peripheral sensitization, the bone cancer pain, as a form of secondary hypersensitivity in this context, is particularly dependent on central sensitization, which refers to enhanced nociceptive process *via* a state of neuronal hyperexcitability and glial activation in the spinal dorsal horn.[5]

Vascular endothelial growth factor A is a key mediator of angiogenesis that promotes tumor neovascularization *via* binding to vascular endothelial growth factor receptor 2, which is recognized as a potential target for anti-cancer therapeutics.[6,7] Given that vascular endothelial growth factor A, as a multifunctional cytokine, also plays an important role in the excitability and plasticity of primary sensory neurons in the dorsal root ganglion,[8–10] we hypothesized that vascular endothelial growth factor A in the spinal cord may also be involved in bone cancer pain regulation.

In this study, using a Walker 256 tumor–induced bone cancer pain model in female rats, we determined the expression and localization of vascular endothelial growth factor A and vascular endothelial growth factor receptor 2 in the spinal dorsal horn after tumor inoculation. We also investigated the effect of pharmacologic intervention of vascular endothelial growth factor A and vascular endothelial growth factor receptor 2 in neuronal protein kinase C–mediated *N*-methyl-D-aspartate (NMDA) receptor activation and microglial Src family kinase–mediated proinflammatory cytokine production, as well as bone cancer pain behaviors induced by tumor inoculation. These new findings extend our understanding of the nonangiogenic roles of vascular endothelial growth factor A/vascular endothelial growth factor receptor 2 signaling pathways in the spinal cord concerning central sensitization and bone cancer pain hypersensitivity.

Materials and Methods

Animals

Adult (8 to 12 weeks; 180 to 220 g) and young (4 to 6 weeks, for electrophysiologic recordings only) Sprague–Dawley rats were purchased from Shanghai Experimental Animal Center of Chinese Academy of Sciences (China), and were housed under a standard 12-h light/dark cycle (light from 8:00 AM to 8:00 PM) at constant room temperature (23 ± 0.5°C) with food and water available *ad libitum*. Animals were numbered and randomly assigned to different experimental groups by use of a random number table, then tested in sequential order. All experimental protocols were approved by Medical Experimental Animal Administrative Committee of Fudan University (Shanghai, China) and were conducted in accordance with the ethical guidelines of the National Institutes of Health and the International Association for the Study of Pain for care and use of laboratory animals under pain research.[11] All efforts were made to minimize the number of animals used as well as their suffering. After the experiments rats were euthanized *via* carbon dioxide inhalation. Researchers were blinded to model condition and drug treatment during behavioral tests. All experiments were conducted between 9:00 AM and 6:00 PM. The primary outcome was the effect of pharmacologic intervention of spinal vascular endothelial growth factor A/vascular endothelial growth factor receptor 2 signaling on bone cancer pain behaviors.

Metastatic Breast Cancer Bone Pain Model

Tumor cells were extracted from the ascitic fluid of rats having received Walker 256 mammary gland carcinoma cells (Institute for Biomedical Research of Shanghai, China); then a cell suspension of 1×10^5 cells/μl in sterile phosphate-buffered saline was prepared. The operating procedure was performed as described in our previous study with some modifications. Briefly, rats were anesthetized with sodium pentobarbital (50 mg/kg, intraperitoneal). The superficial skin of the right hindlimb was shaved and disinfected with 75% (v/v) ethanol, and 5 μl of tumor cells (1×10^5 cells/μl) were slowly injected into the tibia cavity using a 10 μl microinjection syringe. After withdrawing the needle, the injection site was sealed with bone wax to prevent leakage of the tumor cells outside of the bone injection site. For sham control, 5 μl of phosphate-buffered saline was injected into rat right tibia.

Drugs and Administration

Rat vascular endothelial growth factor A neutralizing antibody (AF564) and control antibody (normal goat immunoglobulin G, AB-108-C) were purchased from R&D Systems (USA). Vascular endothelial growth factor receptor 2 inhibitor ZM 323881 (S2896), protein kinase C inhibitor GF109203X (S7208), and Src family kinase inhibitor PP2 (S7008) were purchased from Selleck Chemicals (China). Rat recombinant vascular endothelial growth factor A was purchased from ProSpec (CYT-392; USA). Vascular endothelial growth factor A neutralizing antibody, control immunoglobulin G, and recombinant vascular endothelial growth factor A were dissolved in phosphate-buffered saline. ZM 323881, GF109203X, and PP2 were initially dissolved in dimethyl sulfoxide, then diluted in phosphate-buffered saline (with a final concentration of 1% dimethyl sulfoxide). All reagents were delivered at a volume of 10 μl into the cerebral spinal fluid *via* intrathecal injection, with doses determined by preliminary experiments. For intrathecal

injection, lumbar puncture was performed using a 25-μl Hamilton syringe with a 30-gauge needle in the L5-6 interspace. Reagent doses and treatment time points are described in the figure 2, 3, 5, and 6–9 legends.

Bone Histology

After demineralization in 10% EDTA for 2 weeks, the ipsilateral tibia was embedded in paraffin and cut into 5-mm–thick sections using a microtome with a tungsten carbide blade. Sections were then stained with hematoxylin and eosin to visualize the extent of tumor infiltration and bone destruction, or stained with tartrate-resistant acid phosphatase to visualize activated osteoclasts. Histochemistry images were captured using a digital slide scanner (NanoZoomer S210; Hamamatsu, Japan).

Mechanical Allodynia

Paw withdrawal threshold (threshold) in response to von Frey filament (Aesthesio; Danmic Global, USA) stimulation was measured to represent mechanical allodynia. In brief, after 3 consecutive days for habituation, the rat was placed in a plexiglass chamber on a wire net floor and allowed 10 to 15 min to habituate before experiment. A series of filaments (0.4, 0.6, 1.4, 2, 4, 6, 8, 10, and 15 g) were applied to the mid-plantar surface of the hind paw with sustaining pressure to bend the filament for 5 s or elicit a paw withdrawal reflex within 5 s. Each filament was applied five times, and the 50% threshold (g) was calculated using the following formula: maximum bending force value − [(maximum bending force value − minimum bending force value)/(positive rate of the maximum bending force − positive rate of the minimum bending force)] × (positive rate of the maximum bending force − 50%).

Thermal Hyperalgesia

Paw withdrawal latency (latency) in response to noxious thermal stimulation generated as by a Plantar Analgesia Meter (Model 390, Series 8; IITC Life Science, USA) was measured to represent thermal hyperalgesia. In brief, after 3 consecutive days for habituation, the rat was placed in a plexiglass chamber on a glass plate and allowed 10 to 15 min to habituate before the experiment. The duration from the onset of radiant heat stimulus to the withdrawal of the hind paw was defined as the latency (s), and a 20-s cut-off was set to avoid potential tissue damage. The heat stimulus was repeated three times to determine the latency with a 10-min interval.

Spontaneous Pain

The rat was placed in a transparent plastic cylinder and allowed to habituate for 20 min. After acclimatization, the time spent in spontaneous pain behaviors (s) was measured during a 5-min observation period.[10] Spontaneous pain behaviors were defined as follows: (1) spontaneous flinching (*i.e.,* lifting the affected limb); (2) spontaneous guarding (*i.e.,* holding aloft the affected limb); and (3) sporadic hopping or limping (*i.e.,* intermittent jumping without using the affected limb during movement).

Limb Use Score

The rat was placed on a glass plate, observed during spontaneous ambulation, and scored on a scale of 0 to 4 as follows: 0 = complete loss of limb usage; 1 = partial loss of limb usage; 2 = clear limping and guarding; 3 = slight limping and guarding; and 4 = normal walking.[12]

CatWalk Automated Gait Analysis

Gait analysis was performed using a CatWalk XT system (Noldus, The Netherlands) based on the voluntary movement of rodents in an enclosed walkway, which has proven to be a reliable method for measuring pain-associated behaviors.[13] The rat was first placed in the open end of the enclosed glass platform under a red ceiling light-emitting diode light and allowed to walk voluntarily through the walkway. While the rat walked across the glass floor, a high-speed camera positioned underneath the apparatus captured images of the illuminated area of each paw and transferred the data to the gait analysis software (CatWalk XT, version 10.0; Noldus). A minimum of three serial step cycles, or complete passes through the tunnel, were gathered as valid data. In this study, four available parameters were identified to evaluate dynamic behaviors associated with bone cancer pain: (1) "max contact area" as the print area during maximun hind paw contact; (2) "max contact max intensity" as the maximum intensity during maximum hind paw contact; (3) "single stance" as the duration of ground contact of a single hind paw where the contralateral hind paw did not touch the glass plate; and (4) "swing" as the duration of no hind paw contact with the glass plate. Data were calculated as the percentage of ipsilateral (right)/contralateral (left) hind paw.

Real-Time Quantitative Polymerase Chain Reaction

Total RNA from L4-6 spinal dorsal horn tissues was extracted using TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions, and then 1 μg of total RNA was reverse transcribed into cDNA using PrimeScript RT Master Mix (RR036A; Takara, Japan). Each process was carried out in triplicate with a 20-μl reaction mixture (containing 2 μl of cDNA and 10 μM of gene-specific primers) using a SYBR Premix Ex Taq kit (RR420A; Takara) and was run on a 7300 Plus Real-Time PCR system (Applied Biosystems, USA) with thermocycling conditions of 95°C for 30 s followed by 40 amplification cycles (5 s at 95°C and 30 s at 60°C). The mRNA expression levels were analyzed using the $2^{-\Delta\Delta Ct}$ method, in which *glyceraldehyde-3-phosphate dehydrogenase* was used as an endogenous control. The detailed primer sequences (BioTNT, China) are listed in

table S1 (Supplemental Digital Content, http://links.lww.com/ALN/C50).

Western Blot

L4-6 spinal dorsal horn tissues were collected and homogenized in ice-cold radio immunoprecipitation assay lysis buffer containing 1% phenylmethylsulfonyl fluoride and a protease/phosphatase inhibitor cocktail (5872, CST, USA). Protein concentrations were determined by bicinchoninic acid protein assay (23227, Thermo Scientific, USA), and 20 μg in 5 μl of protein per lane was loaded and separated *via* 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The separated proteins were then transferred onto a 0.22-μm polyvinylidene fluoride or polyvinylidene difluoride membrane (Millipore, USA). After blocking with 5% nonfat dry milk in 0.01M tris-buffered saline containing 0.1% Tween-20 for 1 h at room temperature, membranes were incubated overnight at 4°C with primary antibodies (table S2, Supplemental Digital Content, http://links.lww.com/ALN/C50). After washing, membranes were incubated with specific horseradish peroxidase–conjugated secondary antibodies (1:10000, Proteintech, China). Proteins were detected using immobilon western chemiluminescent horseradish peroxidase substrate (WBKLS0500; Millipore) and ImageQuant LAS 4000 mini system (GE Healthcare, USA). Blots were analyzed using Quantity One analysis software (Version 4.6.5; Bio-Rad Laboratories, USA), and protein band values of intensity × area (mm^2) were normalized to those of glyceraldehyde 3-phosphate dehydrogenase or β-actin. The fold change of the control group was set as 1 for quantification.

Immunofluorescence and Image Analysis

Rats were anesthetized and perfused intracardially with saline and 4% paraformaldehyde in 0.1 M phosphate buffer. L4-6 spinal tissues were removed, fixed in 4% paraformaldehyde overnight at 4°C, then transferred to 30% sucrose in 0.1 M phosphate buffer at 4°C. Subsequently, 30-μm-thick serial sections were prepared using a cryostat and blocked with 10% normal donkey serum in 0.01 M phosphate-buffered saline containing 0.3% Triton X-100 for 2 h at room temperature. Spinal sections were then incubated overnight at 4°C with primary antibodies (table S3, Supplemental Digital Content, http://links.lww.com/ALN/C50). After washing, free-floating sections were incubated for 2 h at room temperature with the corresponding Alexa Fluor 488-, 594-, or 647-conjugated secondary antibodies (1:1000; Invitrogen). For blocking peptide preabsorption experiment, five-fold blocking peptide of vascular endothelial growth factor A (ab46160; Abcam, USA) or vascular endothelial growth factor receptor 2 (ab39255; Abcam) was mixed with corresponding antibody by weight and incubated overnight at 4°C, then the staining protocol was performed as previously described. After mounting on slides, sections were sealed in mounting medium with 4′,6-diamidino-2-phenylindole (Fluoromount-G; SouthernBiotech, USA) for storage and visualization. Fluorescence images were captured using a confocal scanning laser microscope (FV1000; Olympus, Japan), and images are shown as merged Z-stack projections consisting of approximately 10 optical slices (1 μm per slice). The number of c-Fos-positive neurons and mean fluorescence intensity of ionized calcium-binding adaptor molecule 1, phospho-protein kinase C, and phospho-Src family kinase within the entire spinal dorsal horn (lamina I-V), including 16 spinal sections from four rats in each group, were measured using ImageJ software (open source software from the National Institutes of Health, USA). For cell counts, images were converted into 16-bit format. The density threshold was adjusted to distinguish cells from background (lower threshold level: approximately 30; upper threshold level: 85), with particles of 10 to 100 pixel units. For mean fluorescence intensity analysis, images were quantified using the default parameters.

Short Hairpin RNA Plasmid Transfection and Immunocytochemistry

Transfection was performed using Lipofectamine 3000 reagent (Invitrogen), following the manufacturer's instruction. In brief, BL6-B6 cells were plated in a six-well plate with 2 ml of Dulbecco Modified Eagle Medium (Invitrogen) containing 10% fetal bovine serum under humidified 95% O_2 and 5% CO_2 at 37°C in for 3 days and allowed to transfect until 70 to 90% confluence. The plasmid DNA-lipid complex was prepared with the two recommended doses of lipid (3.75 μl and 7.5 μl). Transfection was initiated by testing these two concentrations of Lipofectamine 3000 reagent, with selection of the optimum volume of 7.5 μl. Both plasmid DNA and Lipofectamine 3000 reagent were diluted in 125 μl of serum-free Opti-MEM (Invitrogen) medium separately. Then, diluted DNA was added to each tube of the diluted Lipofectamine 3000 Reagent (1:1 ratio) and incubated for 5 min. After incubation, the DNA-lipid complex was mixed gently, added to each well, and incubated for 48 h. The detailed primer sequences (OBiO Technology, China) are listed in table S4 (Supplemental Digital Content, http://links.lww.com/ALN/C50). For immunocytochemistry, the transfected BL6-B6 cells were rinsed for 10 min with 0.01 M phosphate-buffered saline and fixed with 4% paraformaldehyde for 20 min at room temperature. Then, the cells were blocked with 10% normal donkey serum for 2 h at room temperature, and subsequently incubated overnight at 4°C with the primary antibody: vascular endothelial growth factor A (1:500, ab46154; Abcam) or vascular endothelial growth factor receptor 2 (1:500, sc-505; Santa Cruz, USA). After washing, the cells were incubated with Alexa Fluor 594–conjugated secondary antibodies (1:1000, Invitrogen) for 2 h, sealed in mounting medium with 4′,6-diamidino-2-phenylindole.

Spinal Cord Slice Preparation

Female Sprague–Dawley rats (4 to 6 weeks) were anesthetized and transcardially perfused with preoxygenated

ice-cold sucrose-based artificial cerebrospinal fluid containing the following (in mM): 80 NaCl, 2.5 KCl, 1.25 NaH_2PO_4, 0.5 $CaCl_2$, 3.5 $MgCl_2$, 25 $NaHCO_3$, 75 sucrose, 1.3 sodium ascorbate, and 3.0 sodium pyruvate. The lumbar spinal segment (L4-L6) was placed in an agarose block and then cut on a vibratome (VT 1200S; Leica, Germany). Spinal slices (300 μm) were then incubated for approximately 1 h at 32°C in a recording solution saturated by a mixture of 95% O_2 and 5% CO_2 containing the following (in mM): 126 NaCl, 2.5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 25 D-glucose, 1.3 sodium ascorbate, and 3.0 sodium pyruvate. Slices were subsequently maintained at room temperature for at least 30 min and then transferred into a recording chamber and perfused with oxygenated recording solution at 3 ml/min during electrophysiologic recordings.

Whole-cell Patch-clamp Recordings

The spinal lamina IIo neurons were recorded under whole-cell patch-clamp mode. The resistance of the pipette electrode was 5 to 8 MΩ when filled with an internal solution containing the following (in mM): 120 potassium gluconate, 20 KCl, 2.0 $MgCl_2$, 2.0 Na_2-ATP, 0.5 Na_2-GTP, 20 HEPES, and 0.5 EGTA (pH 7.3; 300 to 310 mOsm). The membrane current was processed at 30°C using a MultiClamp 700B amplifier (Axon Instruments, USA). Signals were filtered at 4 kHz (lowpass filter frequency) and digitized at 10 kHz (sampling rate) with a Digidata 1440A digitizer (Axon Instruments). A seal resistance (greater than 2 GΩ) within the patch stage and an access resistance (less than 35 MΩ) within the cell stage were considered to be acceptable. Spontaneous excitatory postsynaptic currents were recorded at a holding potential of −70 mV in the presence of 10.0 μM gabazine (SR95531, abcam) and 1.0 μM strychnine (45661; Sigma-Aldrich, USA) to block γ-aminobutyric acid type A and glycine receptors, respectively. Data were collected for approximately 5 min to obtain at least 200 events as the baseline or treatment values in each neuron with pClamp10.3 software (Axon Instruments) and were then processed with Igor Pro 6.02 software (WaveMetrics, USA).

Statistical Analysis

All data are expressed as the mean ± SD. No statistical power calculation was conducted before the study. The sample sizes were based on our previous knowledge and research. There were no missing data, with the exception of five rats that did not survive before the end of the behavior experiments, and were thus excluded from the analysis. All data from different groups were verified for normality and homogeneity of variance using Kolmogorov–Smirnov and Brown–Forsythe tests before analysis. For pain behavioral experiments, differences between groups for threshold and latency results were determined using two-way repeated-measures ANOVA followed by *post hoc* Bonferroni multiple comparison test, and Pearson correlation test was used for the linear correlation analysis; differences between groups for spontaneous pain, limb use score, and CatWalk gait results were determined using one-way ANOVA followed by *post hoc* Dunnett multiple comparison test. For real-time quantitative polymerase chain reaction, western blot, and immunofluorescence experiments, group differences were determined using one-way ANOVA followed by *post hoc* Dunnett multiple comparison test. For electrophysiologic experiments, group differences were determined using a paired or independent unpaired two-tailed Student's *t* test. Differences were considered statistically significant if $P < 0.05$. Outliers, if any, were not evaluated, and no data were excluded from statistical analyses. All statistical analyses were performed using GraphPad Prism 6.0 software.

Results

Intratibial Tumor Inoculation Induces Bone Destruction, Pain Hypersensitivity, and Spinal Central Sensitization

To verify the establishment of the bone cancer pain model in female rats, we evaluated the microscopic characteristics of tumor growth, osteoclast activation, and bone destruction in the bone microenvironment after tumor inoculation. Hematoxylin and eosin staining showed progressive tumor cell growth in the bone marrow cavity and destruction of the cortical and trabecular bone in a time-dependent manner on days 3, 7, and 14 post–tumor inoculation. In addition, tartrate-resistant acid phosphatase staining also showed gradually progressive bone destruction on days 3, 7, and 14 post–tumor inoculation, with obvious reactive osteoclasts, the principal bone-resorbing cells, on day 14 post–tumor inoculation. However, these microscopic characteristics were not observed in sham rats (fig. 1A). Consistent with our previous studies and others, tumor-bearing rats exhibited progressive nociceptive hypersensitivity after tumor inoculation, which was characterized by mechanical allodynia and thermal hyperalgesia in the affected limb.[4,14] As a sign of mechanical allodynia, the threshold caused by von Frey filament stimulation was decreased significantly on day 5 and maintained until day 21 post–tumor inoculation (fig. 1B). As a sign of thermal hyperalgesia, the latency caused by radiant heat stimulation was also decreased significantly on day 7 and maintained until day 21 post–tumor inoculation (fig. 1C). In addition, male rats suffering from tumor inoculation showed similar behavioral changes (fig. S1, Supplemental Digital Content, http://links.lww.com/ALN/C50).

It is well established that persistent noxious stimulus from the bone cancer microenvironment can enhance spinal central sensitization through primary afferent fibers projecting to the L4-L6 spinal dorsal horn, which is a critical pathogenic mechanism underlying bone cancer pain.[15,16]

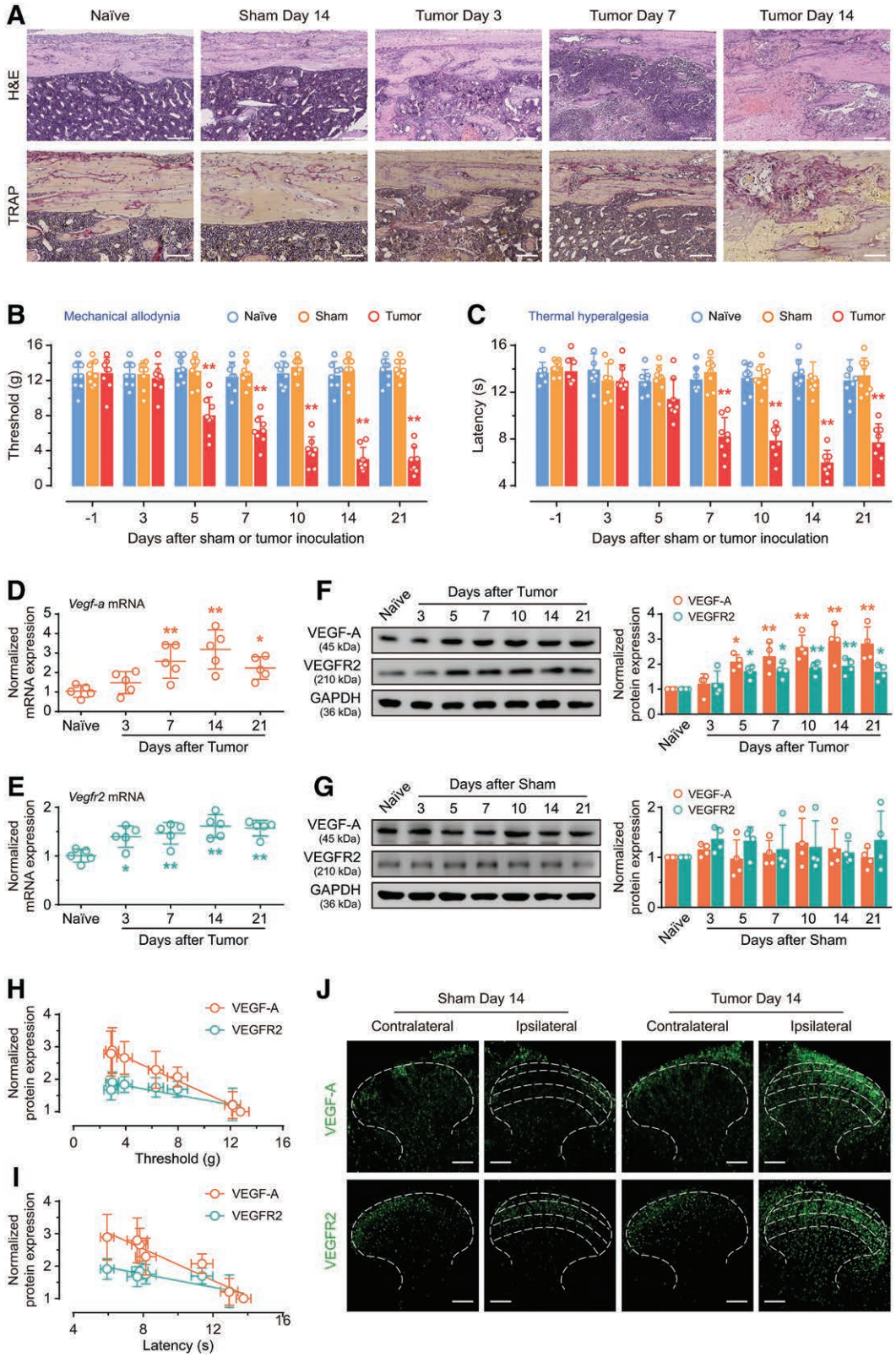

Fig. 1. *(Continued)*

Fig. 1. Expression and distribution of vascular endothelial growth factor A (VEGF-A) and vascular endothelial growth factor receptor 2 (VEGFR2) in the spinal cord after tumor inoculation. (*A*) Hematoxylin and eosin (H&E) staining shows that bone substance and bone marrow were destroyed and infiltrated by cancer cells after tumor inoculation. Tartrate-resistant acid phosphatase (TRAP) staining shows that bone destruction and osteoclasts were increased and activated by cancer cells after tumor inoculation. Scale bars: 200 μm. (*B, C*) Behavioral analysis shows that tumor inoculation induced reduction of threshold (*B*) and latency (*C*) in the affected hind paw. Data are expressed as mean ± SD. $^{**}P < 0.01$ *versus* naïve group, N = 8 for each group, two-way repeated-measures ANOVA with *post hoc* Bonferroni test. (*D, E*) Real-time quantitative polymerase chain reaction analysis shows the time-course expression of *Vegf-a* (*D*) and *Vegfr2* (*E*) mRNA in rats before (naïve) and after tumor inoculation. Data were normalized to the housekeeping gene *Gapdh* (N = 5 for each time point). (*F, G*) Western blot analysis shows the time-course expression of VEGF-A and VEGFR2 protein in rats before (naïve) and after tumor inoculation (*F*) or sham operation (*G*). Data were normalized to the housekeeping protein GAPDH (N = 4 for each time point). (*D* to *G*) Data are expressed as mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$ *versus* naïve group, one-way ANOVA with *post hoc* Dunnett test. (*H, I*) Pearson correlation test shows that the relative levels of VEGF-A and VEGFR2 protein expression were negatively correlated with the threshold (*H*, VEGF-A: $Y = -0.1813 \times X + 3.397$; $R^2 = 0.9912$; $P < 0.0001$; VEGFR2: $Y = -0.07389 \times X + 2.101$; $R^2 = 0.8277$; $P = 0.0045$) or latency (*I*, VEGF-A: $Y = -0.2431 \times X + 4.476$; $R^2 = 0.9229$; $P = 0.0006$; VEGFR2: $Y = -0.1016 \times X + 2.565$; $R^2 = 0.8111$; $P = 0.0057$), respectively. (*J*) Immunofluorescence shows that VEGF-A and VEGFR2 were low in both sides of the spinal dorsal horn in sham rats; but increased in the ipsilateral spinal dorsal horn compared with the contralateral spinal dorsal horn in tumor-bearing rats. Tissues were collected on day 14 after sham or tumor inoculation. Scale bars: 100 μm.

In the present study, we further confirmed that the immunofluorescence expression of c-Fos (marker for neuronal sensitization), glial fibrillary acidic protein (marker for astrocytic activation), and ionized calcium-binding adaptor molecule 1 (marker for microglial activation) in the ipsilateral spinal dorsal horn were all distinctly increased on day 14 post–tumor inoculation in both female and male rats, which demonstrated spinal central sensitization in the bone cancer pain condition (fig. S2, Supplemental Digital Content, http://links.lww.com/ALN/C50).

Vascular Endothelial Growth Factor A/Vascular Endothelial Growth Factor Receptor 2 Is Upregulated in the Spinal Cord after Tumor Inoculation

To investigate the expression and distribution of vascular endothelial growth factor A and vascular endothelial growth factor receptor 2 in the spinal dorsal horn after tumor inoculation, we first analyzed changes in the mRNA expression of *vascular endothelial growth factor A* and *vascular endothelial growth factor receptor 2* in the spinal dorsal horn during the precancerous (day 3), early (day 7), advanced (day 14), and late (day 21) phases of bone cancer pain. Compared with naïve rats, *vascular endothelial growth factor A* mRNA in the spinal dorsal horn was significantly upregulated on day 7 and remained at high levels until day 21 post–tumor inoculation (fig. 1D). Similarly, *vascular endothelial growth factor receptor 2* mRNA in the spinal dorsal horn was persistently upregulated from day 3 to day 21 post–tumor inoculation (fig. 1E), suggesting upregulation of *vascular endothelial growth factor A* and *vascular endothelial growth factor receptor 2* genes *in situ* throughout the course of bone cancer pain. Consistent with real-time quantitative polymerase chain reaction analysis, western blot analysis also showed that tumor inoculation induced rapid-onset and long-lasting expression of vascular endothelial growth factor A and vascular endothelial growth factor receptor 2 proteins in the spinal dorsal horn, which was significantly increased on day 5 and maintained through day 21 (fig. 1F). However, protein expression levels were not changed in sham rats at any detected time point (fig. 1G). Meanwhile, for all time points observed, the correlation analysis showed that the time course of tumor-induced vascular endothelial growth factor A and vascular endothelial growth factor receptor 2 protein upregulation shown in figure 1F was closely related to that of tumor-induced reduction in the threshold shown in figure 1B (fig. 1H) and the latency shown in figure 1C (fig. 1I), respectively. Furthermore, immunofluorescence revealed that the increased expression of vascular endothelial growth factor A and vascular endothelial growth factor receptor 2 was distributed predominately in the ipsilateral, but not contralateral spinal dorsal horn, on day 14 post–tumor inoculation and specifically distributed in the superficial spinal dorsal horn, a region critical for processing nociceptive signal transmission (fig. 1J). According to published proposals,[9,14,17] the vascular endothelial growth factor A and vascular endothelial growth factor receptor 2 antibody specificity was verified by short hairpin RNA plasmid transfection experiment in B16-BL6 cells and by blocking peptide preabsorption experiment in rat spinal cord (fig. S3, Supplemental Digital Content, http://links.lww.com/ALN/C50). Taken together, these results suggest that spinal vascular endothelial growth factor A/vascular endothelial growth factor receptor 2 may be functionally upregulated and is relevant for bone cancer pain behaviors.

Vascular Endothelial Growth Factor A/Vascular Endothelial Growth Factor Receptor 2 Mediates Bone Cancer Pain Behaviors

Next, we employed pharmacologic approaches to examine the regulatory role of spinal vascular endothelial growth factor A/vascular endothelial growth factor receptor 2 in the maintenance of bone cancer pain. A single intrathecal injection of vascular endothelial growth factor A neutralizing antibody or vascular endothelial growth

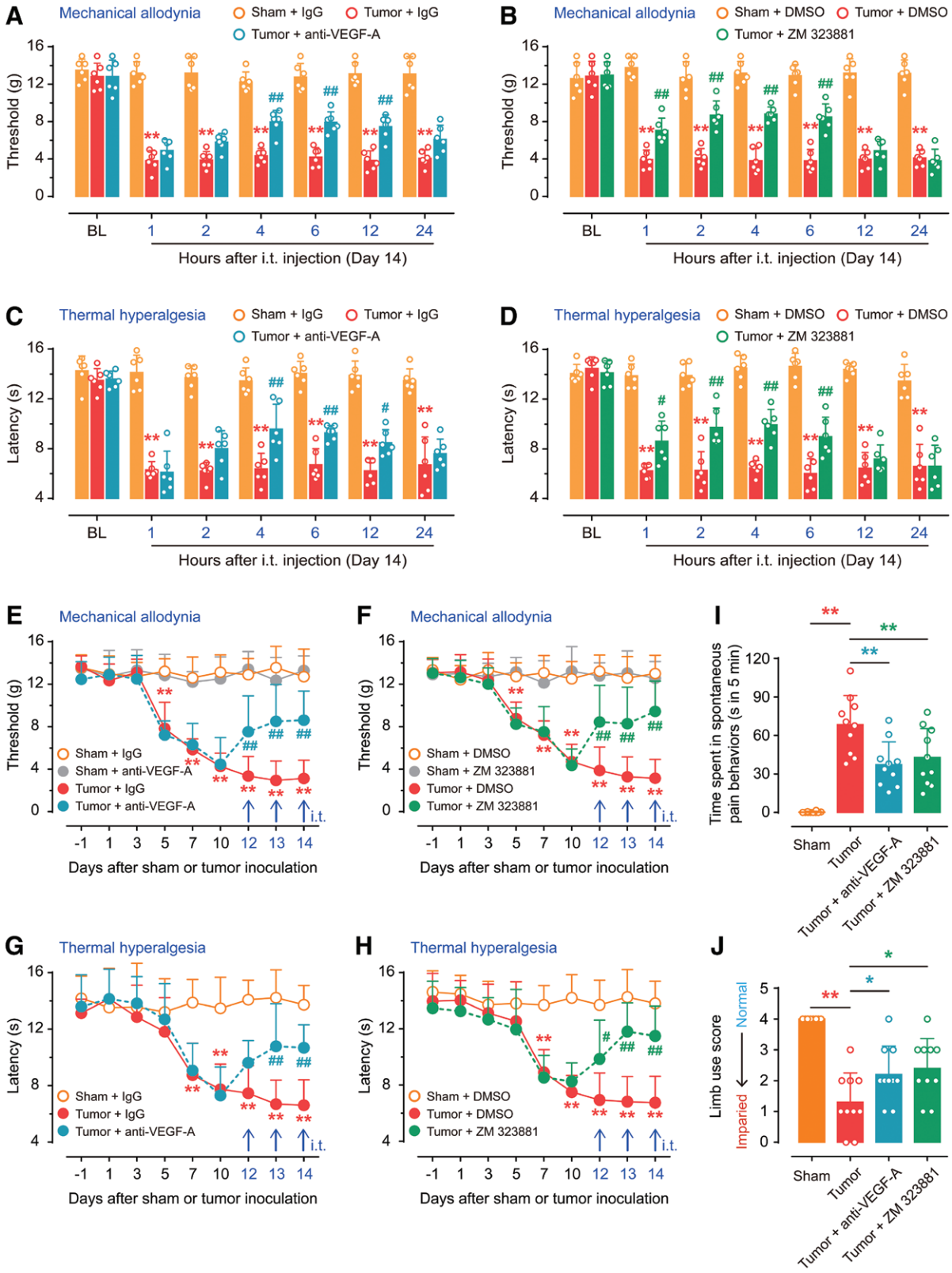

Fig. 2. *(Continued)*

Fig. 2. Spinal blockade of vascular endothelial growth factor A (VEGF-A)/vascular endothelial growth factor receptor 2 (VEGFR2) attenuates mechanical allodynia, thermal hyperalgesia and spontaneous pain in tumor-bearing rats. (*A* to *D*) Single administration of VEGF-A neutralizing antibody or VEGFR2 inhibitor ZM 323881 time-dependently reversed tumor-induced reduction in the threshold (*A*, *B*) and latency (*C*, *D*) in tumor-bearing rats. VEGF-A neutralizing antibody (anti-VEGF-A, 2 μg, intrathecal), immunoglobulin G (IgG; vehicle control for anti-VEGF-A, 2 μg, intrathecal [i.t.]), ZM 323881 (100 nM, i.t.) or dimethyl sulfoxide (DMSO)(vehicle control for ZM 323881, 1%, i.t.) was injected on day 14 post–tumor inoculation. Data are expressed as mean ± SD. $^{**}P < 0.01$ *versus* sham + IgG (or DMSO) group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *versus* tumor + IgG (or DMSO) group, N = 6 for each group, two-way repeated-measures ANOVA with *post hoc* Bonferroni test. (*E* to *H*) Repetitive administration of VEGF-A neutralizing antibody or ZM 323881 significantly attenuated tumor-induced reduction in the threshold (*E*, *F*) and latency (*G*, *H*) in tumor-bearing rats. VEGF-A neutralizing antibody (anti-VEGF-A, 2 μg, i.t.), IgG (2 μg, i.t), ZM 323881 (100 nM, i.t.) or DMSO (1%, i.t.) was injected once daily on days 12, 13, and 14 post–tumor inoculation. Behavioral tests were performed 4 h after each injection. Data are expressed as mean ± SD. $^{**}P < 0.01$ *versus* sham + IgG (or DMSO) group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *versus* tumor + IgG (or DMSO) group, N = 8 for each group, two-way repeated-measures ANOVA with *post hoc* Bonferroni test. (*I, J*) Repetitive administration of VEGF-A neutralizing antibody or ZM 323881 significantly attenuated tumor-induced spontaneous pain behaviors (*I*) and limb uselessness (*J*) in tumor-bearing rats. VEGF-A neutralizing antibody (anti-VEGF-A, 2 μg, i.t.) or ZM 323881 (100 nM, i.t.) was injected once daily on days 12, 13, and 14 post–tumor inoculation. Behavioral tests were performed 4 h after the last injection on day 14. Data are expressed as mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$, N = 10 for each group, one-way ANOVA with *post hoc* Dunnett test.

factor receptor 2–specific inhibitor ZM 323881 on day 14 post–tumor inoculation time-dependently alleviated tumor-induced established mechanical allodynia (fig. 2A: 7.6 ± 2.6 g *vs.* 5.3 ± 3.3 g; fig. 2B: 7.8 ± 3.0 g *vs.* 5.2 ± 3.4 g; n = 6; $P < 0.0001$) and thermal hyperalgesia (fig. 2C: 9.0 ± 2.4 s *vs.* 7.4 ± 2.7 s; fig. 2D: 9.3 ± 2.5 s *vs.* 7.5 ± 3.1 s; n = 6; $P < 0.0001$). Similar results were also observed in male rats on day 14 post–tumor inoculation (fig. S1, Supplemental Digital Content, http://links.lww.com/ALN/C50). Furthermore, repetitive intrathecal injection of vascular endothelial growth factor A neutralizing antibody or ZM 323881 once daily for 3 consecutive days on days 12, 13, and 14 post–tumor inoculation effectively and persistently attenuated tumor-induced mechanical allodynia (fig. 2, E and F) and thermal hyperalgesia (fig. 2, G and H). In addition, tumor-related spontaneous pain (fig. 2I) and limb uselessness (fig. 2J) were also relieved by repeated intrathecal administration of vascular endothelial growth factor A neutralizing antibody or ZM 323881 on day 14 post–tumor inoculation.

CatWalk gait analysis is suggested to be a valuable method for the objective assessment of chronic pain behavior in several neuropathic and inflammatory pain models.[18,19] Here, we employed CatWalk analysis in the context of bone cancer pain research, with consideration of its comprehensive and detailed analysis of the affected hind paw during voluntary movement. We selected four specific parameters that were significantly altered in tumor-bearing rats: (1) "max contact area," considered as the hind paw print area during max contact; (2) "max contact max intensity," considered as the maximum intensity of the maximum hind paw contact; (3) "single stance," considered as the duration of ground contact for a single hind paw during which the contralateral hind paw did not touch the glass plate; and (4) "swing," considered as the duration of no hind paw contact with the glass plate. Percentages of ipsilateral (right)/contralateral (left) hind paw ratio of max contact area, max contact max intensity, single stance, and swing were approximately 100% in sham rats on day 14 (fig. 3A). After tumor inoculation, percentages of max contact area, max contact max intensity, and single stance were decreased significantly, whereas percentage of swing was increased significantly on day 14, which could be representative of bone cancer pain behaviors from different aspects (fig. 3B). However, repeated intrathecal injection of vascular endothelial growth factor A neutralizing antibody (fig. 3C) or ZM 323881 (fig. 3D) partially, but significantly, reversed tumor-induced gait alterations (fig. 3, E to H).

Vascular Endothelial Growth Factor A/Vascular Endothelial Growth Factor Receptor 2 Is Responsible for Neuronal Sensitization and Microglial Activation in Tumor-bearing Rats

The findings above (figs. 2 and 3) indicated a modulatory role of vascular endothelial growth factor A and vascular endothelial growth factor receptor 2 in the spinal processing of bone cancer pain. However, the specific cellular mechanism underlying vascular endothelial growth factor A/vascular endothelial growth factor receptor 2–mediated bone cancer pain remains unclear. Thus, we further defined the cellular localization of vascular endothelial growth factor A and vascular endothelial growth factor receptor 2 *via* immunostaining with various cell markers in the spinal dorsal horn on day 14 post–tumor inoculation. Immunofluorescence demonstrated that vascular endothelial growth factor A predominantly colocalized with the central terminals of primary sensory neurons (calcitonin gene-related peptide and isolectin B4) and spinal neurons (neuronal nuclei), and occasionally colocalized with oligodendrocytes (oligodendrocyte lineage transcription factor 2) (fig. 4A). Meanwhile, vascular endothelial growth factor receptor 2 predominantly colocalized with spinal neurons and microglia (ionized calcium-binding adaptor molecule 1) (fig. 4B). Similar results were also observed in male rats on day 14 post–tumor inoculation (fig. S4, Supplemental Digital Content, http://links.lww.com/ALN/C50).

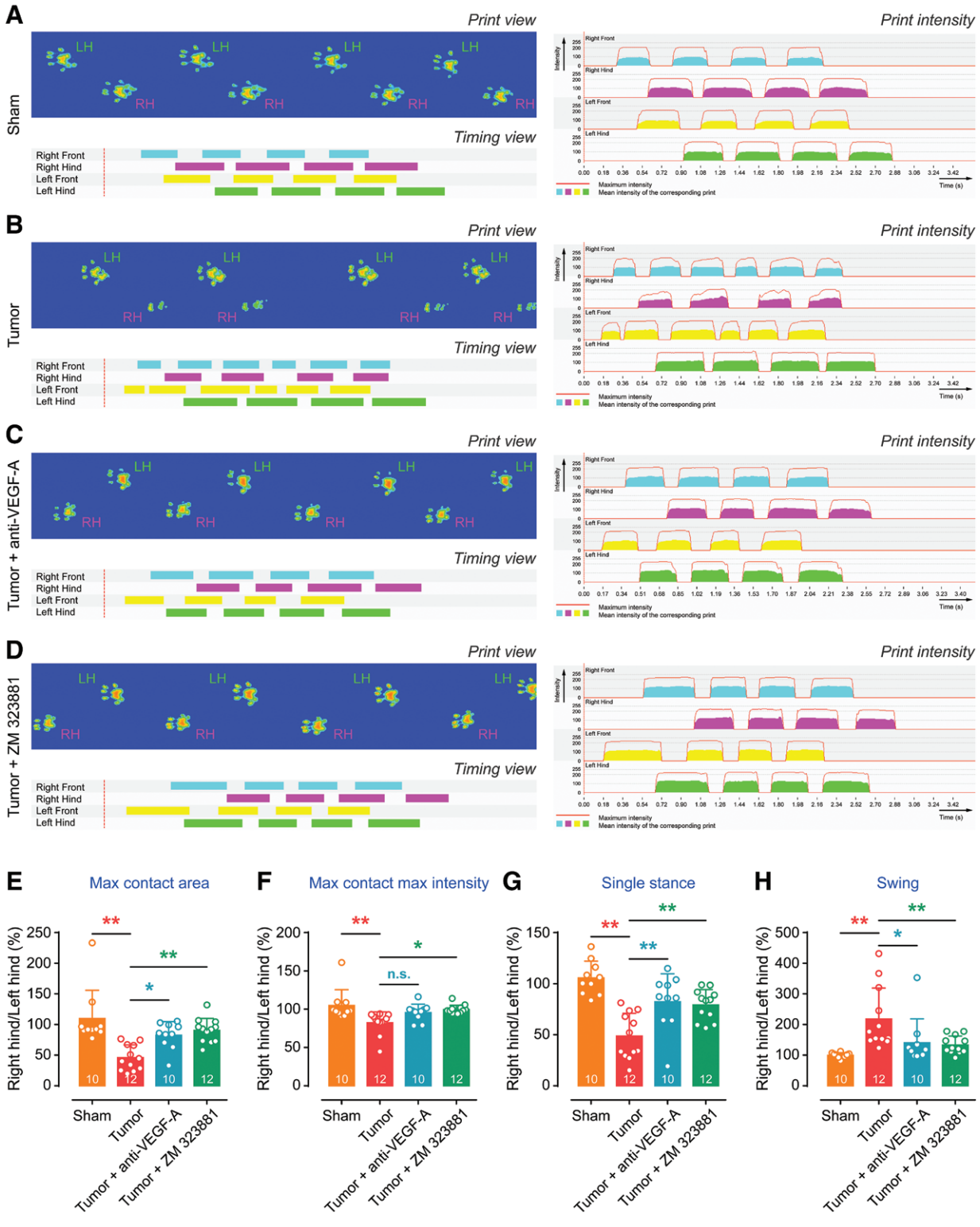

Fig. 3. *(Continued)*

Fig. 3. Spinal blockade of vascular endothelial growth factor A (VEGF-A)/vascular endothelial growth factor receptor 2 (VEGFR2) attenuates abnormal gait in tumor-bearing rats. (*A* to *D*) Representative CatWalk gait, including print view, timing view, and print intensity, in sham (*A*), tumor (*B*), tumor + anti-VEGF-A (*C*), and tumor + ZM 323881 (*D*) group. (*E* to *H*) Spinal administration of VEGF-A neutralizing antibody or VEGFR2 inhibitor ZM 323881 significantly attenuated tumor-induced decrease in max contact area (*E*), max contact max intensity (*F*) and single stance (*G*), as well as increase in swing (*H*) in tumor-bearing rats. VEGF-A neutralizing antibody (anti-VEGF-A, 2 μg, intrathecal [i.t.]) or ZM 323881 (100 nM, i.t.) was injected once daily on days 12, 13, and 14 post–tumor inoculation. Behavioral tests were performed 4 h after the last injection. Data were calculated as percentage of ipsilateral (right)/contralateral (left) hind paw. Data are expressed as mean ± SD. *$P < 0.05$, **$P < 0.01$, one-way ANOVA with *post hoc* Dunnett test. The number of detected animals is indicated inside of each column. LH, left hind; n.s., no statistical significance; RH, right hind.

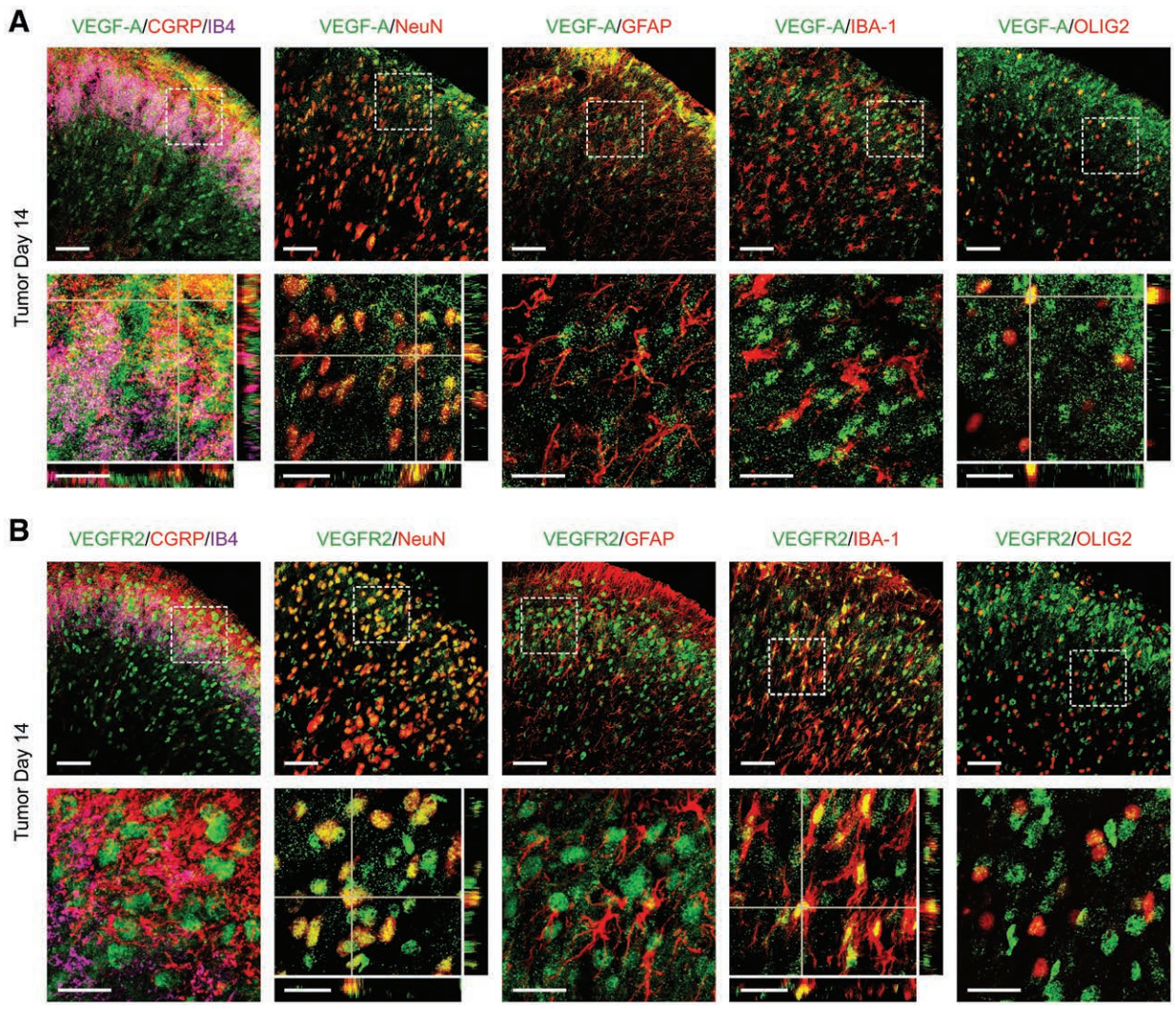

Fig. 4. Cellular localization of vascular endothelial growth factor A (VEGF-A) and vascular endothelial growth factor receptor 2 (VEGFR2) in the spinal cord after tumor inoculation. (*A, B*) Immunofluorescence staining of VEGF-A (*A*) and VEGFR2 (*B*) combined with cell markers in the spinal dorsal horn: calcitonin gene-related peptide (CGRP; peptidergic primary afferent terminals), isolectin B4 (IB4; nonpeptidergic primary afferent terminals), neuronal nuclei (NeuN; neurons), glial fibrillary acidic protein (GFAP; astrocytes), ionized calcium-binding adaptor molecule 1 (IBA-1; microglia), and oligodendrocyte lineage transcription factor 2 (OLIG2; oligodendrocytes). (*A*) VEGF-A immunoreactivity predominantly colocalized with CGRP, IB4 and NeuN, occasionally colocalized with OLIG2; (*B*) VEGFR2 immunoreactivity predominantly colocalized with NeuN and IBA-1. Tissues were collected on day 14 post–tumor inoculation. Scale bars: 50 μm and 20 μm (zoom).

Given that vascular endothelial growth factor receptor 2 was expressed and increased in both spinal neurons and microglia, we next investigated whether vascular endothelial growth factor A/vascular endothelial growth factor receptor 2 mediates neuronal sensitization and microglial activation in bone cancer pain conditions. Western blot analysis showed that tumor inoculation caused a persistent and evident upregulation of c-Fos and ionized calcium-binding adaptor molecule 1, indicating activation of neurons and microglia in the spinal dorsal horn, respectively (fig. 5A). However, this tumor-induced c-Fos and ionized calcium-binding adaptor molecule 1 upregulation was remarkably suppressed by repeated intrathecal injection of vascular endothelial growth factor A neutralizing antibody (fig. 5B) or ZM 323881 (fig. 5C). In addition, we observed that c-Fos and ionized calcium-binding adaptor molecule 1 were clearly coexpressed with vascular endothelial growth factor receptor 2 (fig. 5D), and immunofluorescence analysis showed that tumor-induced increase in number of c-Fos-positive neurons and mean fluorescence intensity of ionized calcium-binding adaptor molecule 1-labeled microglia were both suppressed by vascular endothelial growth factor A neutralizing antibody or ZM 323881 in the spinal dorsal horn (fig. 5, E to G). Taken together, these

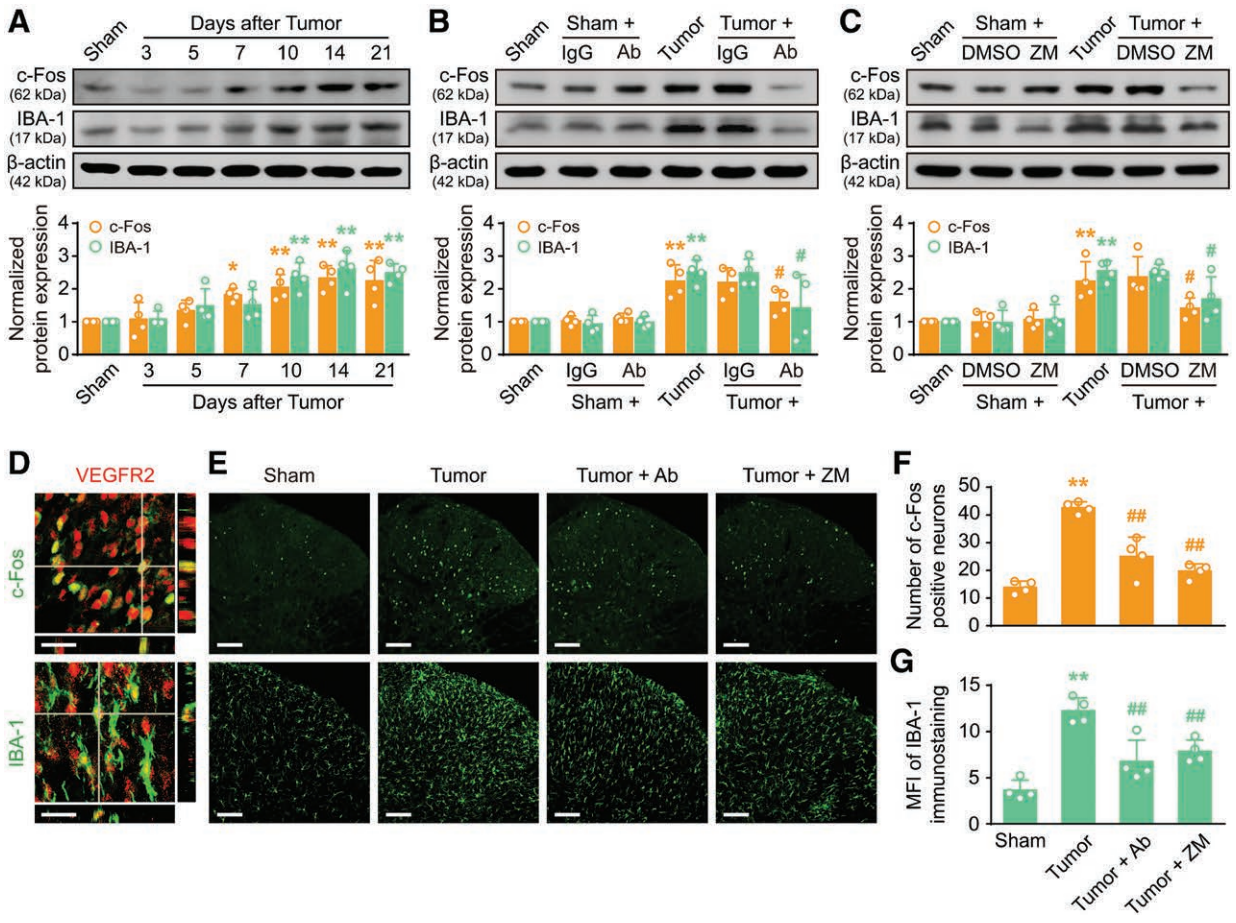

Fig. 5. Spinal blockade of vascular endothelial growth factor A (VEGF-A)/vascular endothelial growth factor receptor 2 (VEGFR2) suppresses tumor-induced neuronal sensitization and microglial activation. (*A*) Tumor inoculation induced upregulation of c-Fos and ionized calcium-binding adaptor molecule 1 (IBA-1) in a time-dependent manner (N = 4 for each time point). Data are expressed as mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$ *versus* sham group, one-way ANOVA with *post hoc* Dunnett test. (*B, C*) Spinal injection of VEGF-A neutralizing antibody (*B*) or VEGFR2 inhibitor ZM 323881 (*C*) significantly suppressed tumor-induced upregulation of c-Fos and IBA-1 expression in the spinal dorsal horn. (*D* to *G*) VEGFR2 immunoreactivity was primarily coexpressed with c-Fos or IBA-1 in the spinal dorsal horn (*D*), and spinal injection of VEGF-A neutralizing antibody or ZM 323881 significantly suppressed tumor-increased number of c-Fos (*E, F*) and mean fluorescence intensity (MFI) of IBA-1 (*E, G*). Scale bars: 20 μm (*D*) and 100 μm (*E*). (*B, C, F, G*) VEGF-A neutralizing antibody (Ab, 2 μg, intrathecal), IgG (2 μg, intrathecal), ZM 323881 (ZM, 100 nM, intrathecal) or DMSO (1%, intrathecal) was injected once daily on days 12, 13, and 14 post–tumor inoculation. Tissues were collected 4 h after the last injection on day 14. Data are expressed as mean ± SD. $^{**}P < 0.01$ *versus* sham group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *versus* tumor group, N = 4 for each group, one-way ANOVA with *post hoc* Dunnett test.

results suggest that spinal vascular endothelial growth factor A/vascular endothelial growth factor receptor 2 may directly impact neuronal sensitization and microglial activation underlying the bone cancer pain state.

Vascular Endothelial Growth Factor A/Vascular Endothelial Growth Factor Receptor 2 Enhances Excitatory Synaptic Transmission in Spinal Neurons

Since vascular endothelial growth factor receptor 2 could regulate neuronal sensitization and microglial activation, we hypothesized that vascular endothelial growth factor receptor 2 could enhance excitatory synaptic transmission *via* activating neurons and microglial cells. Thus, we further examined the effects of vascular endothelial growth factor A/vascular endothelial growth factor receptor 2 on spontaneous excitatory postsynaptic currents in lamina IIo neurons in naïve rats. After whole cell configuration was established, recombinant vascular endothelial growth factor A perfusion induced an evident increase in both the frequency and amplitude of spontaneous excitatory postsynaptic currents (fig. 6, A to C). However, vascular endothelial growth factor A-increased frequency and amplitude of spontaneous excitatory postsynaptic currents were remarkably suppressed by ZM 323881 perfusion (fig. 6, D to H). These results indicate that vascular endothelial growth factor A/vascular endothelial growth factor receptor 2 enhances excitatory synaptic transmission *via* promoting presynaptic glutamate vesicle release (*i.e.*, presynaptic effect) and postsynaptic glutamate receptor expression and function (*i.e.*, postsynaptic effect).[20]

Protein Kinase C and Src Family Kinase Signals Are Functionally Activated in Vascular Endothelial Growth Factor A/Vascular Endothelial Growth Factor Receptor 2–Mediated Pain Hypersensitivity in Naïve Rats

Since exogenous vascular endothelial growth factor A/vascular endothelial growth factor receptor 2 could enhance the excitatory synaptic transmission of superficial spinal neurons, we investigated whether vascular endothelial growth factor A/vascular endothelial growth factor receptor 2 at the spinal level was sufficient to evoke pain hypersensitivity in naïve rats. After recombinant vascular endothelial growth factor A intrathecal administration, rats manifested an obvious mechanical allodynia and thermal hyperalgesia in a time-dependent manner within 1 h, with persistence for at least 12 h. However, intrathecal delivery of ZM

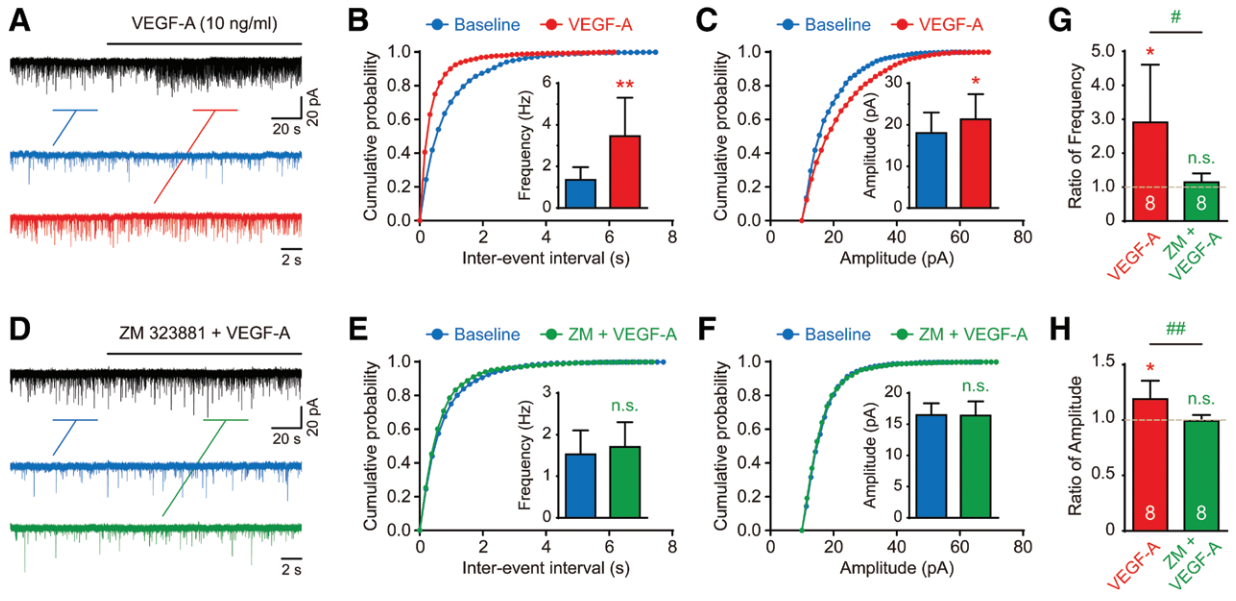

Fig. 6. Vascular endothelial growth factor A (VEGF-A) increases spontaneous excitatory postsynaptic currents in lamina IIo spinal neurons *via* vascular endothelial growth factor receptor 2 (VEGFR2). (*A*) Patch-clamp recording showing the frequency and amplitude of spontaneous excitatory postsynaptic currents after perfusion with VEGF-A (10 ng/ml). (*B, C*) Corresponding cumulative distribution and quantification of spontaneous excitatory postsynaptic current frequency (*B*) and amplitude (*C*). (*D*) Patch-clamp recording showing the frequency and amplitude of spontaneous excitatory postsynaptic currents after perfusion with ZM 323881 (ZM, 10 nM) + VEGF-A (10 ng/ml). (*E, F*) Corresponding cumulative distribution and quantification of spontaneous excitatory postsynaptic current frequency (*E*) and amplitude (*F*). (*G, H*) Ratio of the frequency (*G*) and amplitude (*H*) of spontaneous excitatory postsynaptic currents following treatment with VEGF-A (10 ng/ml) or ZM 323881 (ZM, 10 nM) + VEGF-A (10 ng/ml). *Dashed line* indicates baseline (set as 1 for normalization). Data are expressed as mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$ *versus* baseline; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *versus* VEGF-A group, N = 8 neurons from 4 rats, Student's paired (*B, C, E, F*) or independent unpaired (*G, H*) two-tailed *t* test.

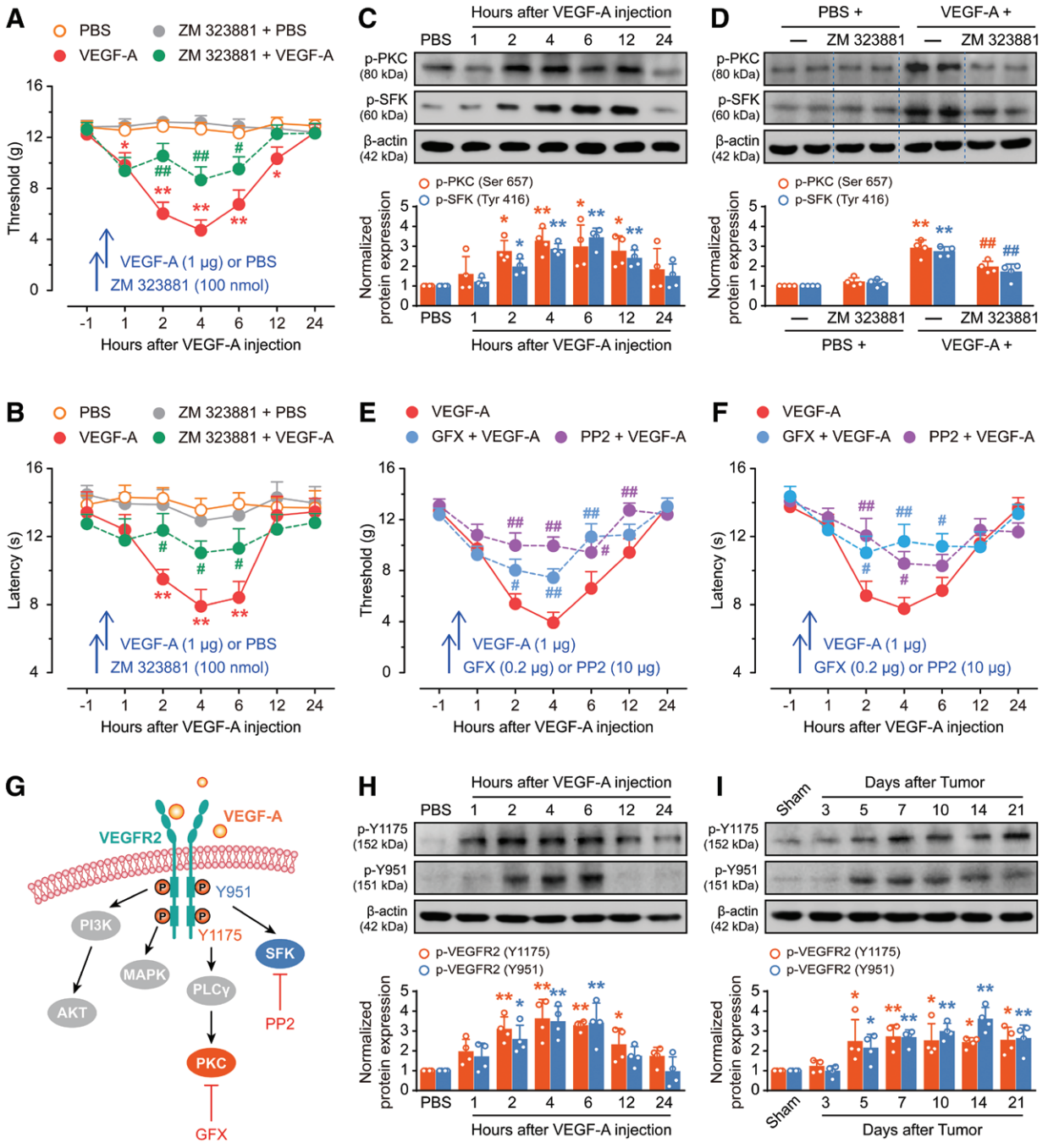

Fig. 7. Vascular endothelial growth factor A (VEGF-A) induces vascular endothelial growth factor receptor 2 (VEGFR2)–dependent pain hypersensitivity in naïve rats *via* activating protein-kinase C (PKC) and Src family kinase (SFK). (*A, B*) Spinal administration of exogenous VEGF-A–induced time-dependent mechanical allodynia (*A*) and thermal hyperalgesia (*B*) in naïve rats, while preadministration of VEGFR2 inhibitor ZM 323881 30 min before VEGF-A injection partially attenuated VEGF-A–induced pain hypersensitivity. Data are expressed as mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$ *versus* phosphate-buffered saline group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *versus* VEGF-A group, N = 8 for each group, two-way repeated-measures ANOVA with *post hoc* Bonferroni test. (*C, D*) Exogenous VEGF-A induced the upregulation of phospho- (p-)-PKC and p-SFK in the spinal dorsal horn (*C*), while preadministration of ZM 323881 30 min before VEGF-A injection significantly suppressed VEGF-A–increased p-PKC and p-SFK expression (*D*). Data are expressed as mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$ *versus* phosphate-buffered saline group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *versus* VEGF-A group, N = 4 for each group, one-way ANOVA with *post hoc* Dunnett test. (*E, F*) Preadministration of PKC inhibitor GF109203X or SFK inhibitor PP2 30-min before VEGF-A injection partially attenuated VEGF-A–induced mechanical allodynia (*E*)

(Continued)

(**Fig. 7.** *Continued*) and thermal hyperalgesia (*F*) in naïve rats. Data are expressed as mean ± SD. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *versus* VEGF-A group, N = 8 for each group, two-way repeated-measures ANOVA with *post hoc* Bonferroni test. (*G*) A schematic overview of VEGF-A–mediated VEGFR2 activation and intracellular signaling pathways. (*H*) Exogenous VEGF-A induced the upregulation of p-Y951 and p-Y1175 in the spinal dorsal horn (N = 4 for each time point). Data are expressed as mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$ *versus* phosphate-buffered saline group, one-way ANOVA with *post hoc* Dunnett test. (*I*) Tumor inoculation induced the upregulation of p-Y951 and p-Y1175 in a time-dependent manner (N = 4 for each time point). $^{*}P < 0.05$, $^{**}P < 0.01$ *versus* sham group, one-way ANOVA with *post hoc* Dunnett test.

323881 30 min before vascular endothelial growth factor A injection partially prevented vascular endothelial growth factor A–induced mechanical allodynia (fig. 7A) and thermal hyperalgesia (fig. 7B). These results indicate that spinal vascular endothelial growth factor A is sufficient to trigger nociceptive behaviors *via* vascular endothelial growth factor receptor 2 in naïve rats.

We then examined the specific molecular signaling mechanisms underlying vascular endothelial growth factor A/vascular endothelial growth factor receptor 2–mediated pain hypersensitivity. As described in previous studies, vascular endothelial growth factor receptor 2 has the ability to activate four major intracellular signaling pathways: phospholipase Cγ-protein kinase C, Src family kinase, phosphatidylinositol 3-kinase–Akt, and mitogen-activated protein kinases. In this study, we primarily focused on protein kinase C and Src family kinase based on the following considerations: (1) protein kinase C is primarily expressed in neurons and involved in membrane translocation and phosphorylation of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, which is the main molecular mechanism for increased spontaneous excitatory postsynaptic currents; and (2) Src family kinase is primarily expressed in microglial cells and involved in activation of extracellular signal-regulated kinase and p38 signaling pathways, which are the major molecular mechanisms for microglia-mediated inflammatory responses. Here, we first examined the activation of protein kinase C and Src family kinase in the spinal dorsal horn through detection of phospho-protein kinase C (Ser657) and phospho-Src family kinase (Tyr416) expression after intrathecal injection of recombinant vascular endothelial growth factor A in naïve rats. As shown by western blot analysis, phospho-protein kinase C and phospho-Src family kinase were increased within 2 h and 4 h, respectively. In addition, this upregulation of phospho-protein kinase C and phospho-Src family kinase persisted for at least 12 h and returned to normal levels at 24 h post–vascular endothelial growth factor A injection (fig. 7C). Furthermore, intrathecal administration of ZM 323881 30 min before vascular endothelial growth factor A injection significantly suppressed vascular endothelial growth factor A-induced upregulated expression of phospho-protein kinase C and phospho-Src family kinase (fig. 7D), suggesting a conceivable link between vascular endothelial growth factor receptor 2–mediated protein kinase C and Src family kinase activation in the spinal dorsal horn and vascular endothelial growth factor A–evoked nociceptive hypersensitivity in naïve rats. To further address these upstream and downstream relationships, we administered protein kinase C inhibitor GF109203X or Src family kinase inhibitor PP2 30 min before vascular endothelial growth factor A injection and observed changes in pain behaviors. Similarly, both GF109203X and PP2 could partially, but significantly, attenuate the vascular endothelial growth factor A–induced mechanical allodynia (fig. 7E) and thermal hyperalgesia (fig. 7F). In addition, previous studies demonstrated that the tyrosine phosphorylation sites of vascular endothelial growth factor receptor 2 at positions Y1175 and Y951 regulate protein kinase C and Src family kinase activity, respectively (fig. 7G), and thereby influences vascular endothelial growth factor A–mediated biologic function.[21–23] Herein, we further confirmed that the phosphorylation levels of phospho-Y1175 and phospho-Y951 in the spinal cord were time-dependently increased after both vascular endothelial growth factor A injection in naïve rats (fig. 7H) and tumor inoculation in tumor-bearing rats (fig. 7I). The above findings demonstrate that spinal protein kinase C and Src family kinase are critical pathways underlying vascular endothelial growth factor A/vascular endothelial growth factor receptor 2–evoked pain hypersensitivity in naïve rats.

Vascular Endothelial Growth Factor A/Vascular Endothelial Growth Factor Receptor 2 Activates Neuronal Protein Kinase C and Microglial Src Family Kinase Pathways in Tumor-bearing Rats

We continued to verify whether spinal protein kinase C and Src family kinase signaling pathways were downstream of vascular endothelial growth factor receptor 2 in tumor-bearing rats. Compared with sham rats, tumor inoculation produced persistent phospho-protein kinase C and phospho-Src family kinase upregulation in a time-dependent manner in the spinal dorsal horn, which commenced on days 7 to 10 and were maintained at a high level until day 21 (fig. 8A). However, the tumor-induced upregulation of phospho-protein kinase C and phospho-Src family kinase was apparently reduced by repeated intrathecal administration of vascular endothelial growth factor A neutralizing antibody (fig. 8B) or ZM 323881 (fig. 8C). In addition, immunofluorescence staining confirmed that phospho-protein kinase C was predominantly expressed in neurons (fig. 8D), but not in astrocytes or microglia (fig. 8E); whereas phospho-Src family kinase was predominantly expressed in microglia (fig. 8F), but not in neurons or astrocytes (fig. 8G), in the spinal dorsal horn on day 14 post–tumor inoculation.

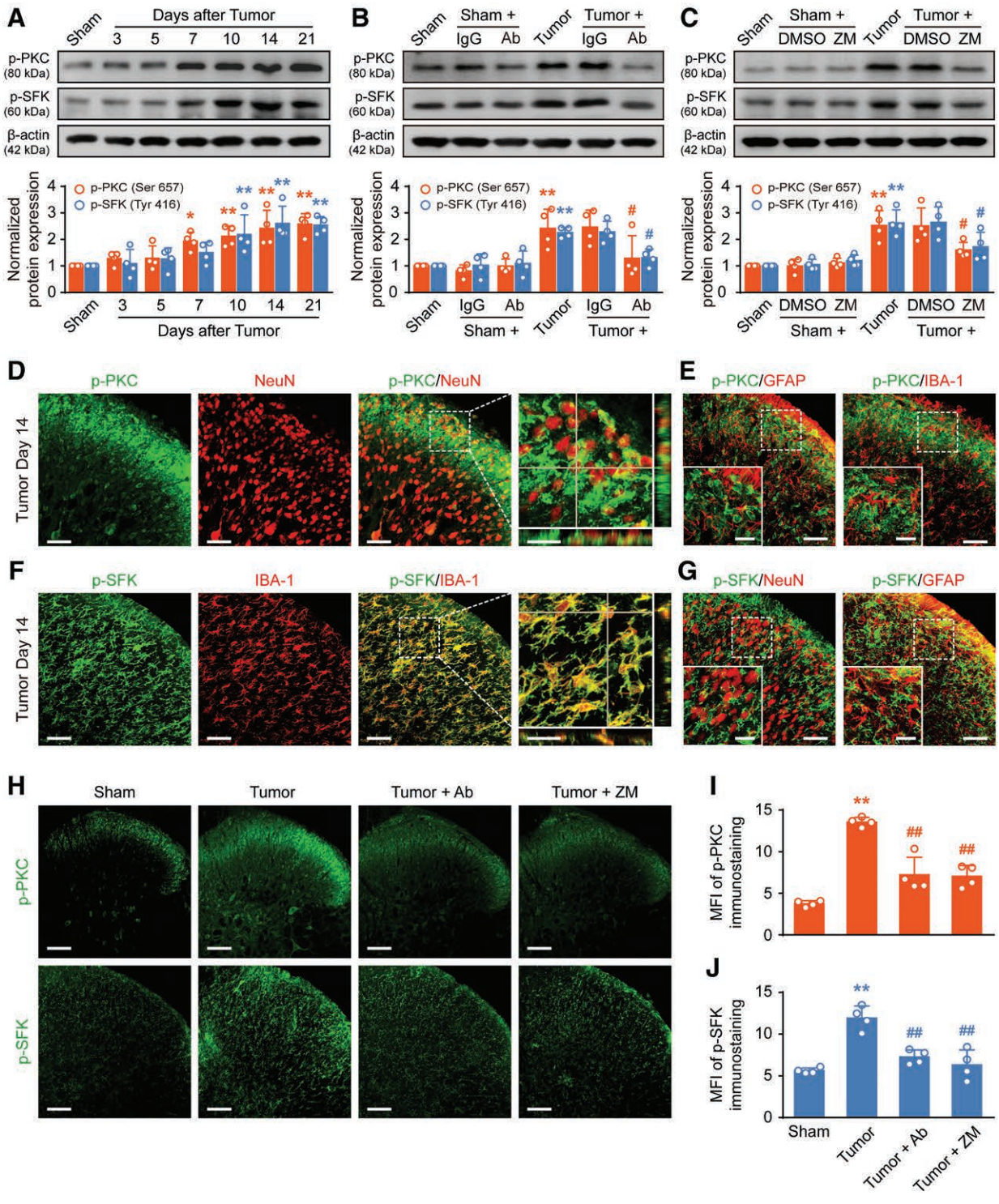

Fig. 8. Protein kinase C (PKC) and Src family kinase (SFK) signals are activated by vascular endothelial growth factor A (VEGF-A)/vascular endothelial growth factor receptor 2 (VEGFR2) in tumor-bearing rats. (*A*) Tumor inoculation induced the upregulation of phosphor- (p-)PKC and p-SFK in a time-dependent manner (N = 4 for each time point). Data are expressed as mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$ *versus* sham group, one-way ANOVA with *post hoc* Dunnett test. (*B, C*) Spinal injection of VEGF-A neutralizing antibody (*B*) or VEGFR2 inhibitor ZM 323881 (*C*) significantly suppressed tumor-induced upregulation of p-PKC and p-SFK expression in the spinal dorsal horn. (*D* to *G*) p-PKC immunoreactivity colocalized with NeuN (*D*), but not with glial fibrillary acidic protein (GFAP) or ionized calcium-binding adapter molecule 1 (IBA-1) *(Continued)*

(**Fig. 8.** *Continued*) (*E*) in the spinal dorsal horn; meanwhile, p-SFK immunoreactivity colocalized with IBA-1 (*F*), but not with NeuN or GFAP (*G*) in the spinal dorsal horn. Scale bars: 50 μm and 20 μm (zoom). (*H* to *J*) Spinal injection of VEGF-A neutralizing antibody or ZM 323881 significantly suppressed tumor-increased mean fluorescence intensity (MFI) of p-PKC (*H, I*) and p-SFK (*H, J*). Scale bars: 100 μm. (*B, C, H* to *J*) VEGF-A neutralizing antibody (Ab; 2 μg, intrathecal), IgG (2 μg, intrathecal), ZM 323881 (ZM, 100 nM, intrathecal) or dimethyl sulfoxide (1%, intrathecal) was injected once daily on days 12, 13, and 14 post–tumor inoculation. Tissues were collected 4 h after the last injection on day 14. Data are expressed as mean ± SD. $^{**}P < 0.01$ *versus* sham group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *versus* tumor group, N = 4 for each group, one-way ANOVA with *post hoc* Dunnett test.

Similarly, the effect of vascular endothelial growth factor A/vascular endothelial growth factor receptor 2 on phospho-protein kinase C and phospho-Src family kinase upregulation was further confirmed by immunofluorescence analysis (fig. 8, H to J).

Vascular Endothelial Growth Factor A/Vascular Endothelial Growth Factor Receptor 2/Protein Kinase C Signaling Enhances NMDA Receptor Activation in Spinal Neurons

Previous reports have shown that in the process of chronic pain formation, NMDA receptors in the postsynaptic neuronal membranes are widely expressed at the spinal cord level. After activation, NMDA receptors can increase the concentrations of intracellular secondary messenger Ca^{2+} and then activate downstream Ca^{2+}-dependent signaling molecules (*e.g.,* extracellular signal-regulated kinase, calcium/calmodulin-dependent protein kinase II, and cyclic-AMP response element-binding protein), which plays a key role in inducing and maintaining central sensitization. Among them, extracellular signal-regulated kinase and calcium/calmodulin-dependent protein kinase II enhance neuronal excitability *via* phosphorylation of AMPA and NMDA receptors, as well as ion channels;[24,25] meanwhile, cyclic-AMP response element-binding protein as a transcription factor promotes the synthesis of NMDA receptor substrates.[26] Previous reports have demonstrated that protein kinase C pathway can not only indirectly activate NMDA receptors by promoting the synergistic effect of AMPA receptors, but also directly activate NMDA receptors *via* phosphorylation of the NR1 subunit.[27–29] Therefore, we speculated that vascular endothelial growth factor A/vascular endothelial growth factor receptor 2 signaling facilitates the activation of NMDA receptors and Ca^{2+}-dependent signals *via* protein kinase C pathway in spinal neurons. To explore this neuronal mechanism of vascular endothelial growth factor A/vascular endothelial growth factor receptor 2/protein kinase C in bone cancer pain, we further examined changes of protein expression of phospho-NMDA receptor subunit 2B (Tyr1472), phospho-extracellular signal-regulated kinase (Thr202/Tyr204), phospho-calcium/calmodulin-dependent protein kinase II (Thr286), and phospho-cyclic-AMP response element-binding protein (Ser133) in the spinal dorsal horn *via* spinal blockade of vascular endothelial growth factor A, vascular endothelial growth factor receptor 2, or protein kinase C. As shown by western blot analysis, the protein expression levels of phospho-NMDA receptor subunit 2B, phospho-extracellular signal-regulated kinase, phospho-calcium/calmodulin-dependent protein kinase II, and phospho-cyclic-AMP response element-binding protein were all pronouncedly increased on day 14 post–tumor inoculation. However, intrathecal administration of vascular endothelial growth factor A neutralizing antibody, vascular endothelial growth factor receptor 2 inhibitor ZM 323881, or protein kinase C inhibitor GF109203X effectively reduced tumor-induced protein expression of phospho-NMDA receptor subunit 2B (fig. 9A), phospho-extracellular signal-regulated kinase (fig. 9B), phospho-calcium/calmodulin-dependent protein kinase II (fig. 9C), and phospho-cyclic-AMP response element-binding protein (fig. 9D). These findings suggest that the neuronal mechanism of vascular endothelial growth factor A/vascular endothelial growth factor receptor 2 in relation to bone cancer pain regulation is dependent on protein kinase C-mediated NMDA receptor activation.

Vascular Endothelial Growth Factor A/Vascular Endothelial Growth Factor Receptor 2/Src Family Kinase Signaling Promotes Proinflammatory Cytokine Production in Spinal Microglia

Reactive microglial cells in the spinal cord promote persistent production of proinflammatory cytokines *via* activation of extracellular signal-regulated kinase and p38 pathways under different pain situations.[30,31] On the one hand, these proinflammatory cytokines promote glia-mediated "inflammation soup" formation *via* acting on the corresponding receptors located in their own, as well as surrounding, microglia and astrocytes in the spinal microenvironment.[32] On the other hand, they directly mediate synaptic plasticity and neuronal excitability *via* acting on the corresponding receptors located in presynaptic and/or postsynaptic membranes.[33] Previous reports have demonstrated that activity of Src family kinase pathway can increase the phosphorylation of p38 or extracellular signal-regulated kinase pathways in spinal microglia in various chronic pain models.[34,35] Thus, we speculated that vascular endothelial growth factor A/vascular endothelial growth factor receptor 2 signaling facilitates proinflammatory cytokine production *via* Src family kinase pathway in spinal microglia. To explore this microglial mechanism of vascular endothelial growth factor A/vascular endothelial growth factor receptor 2/

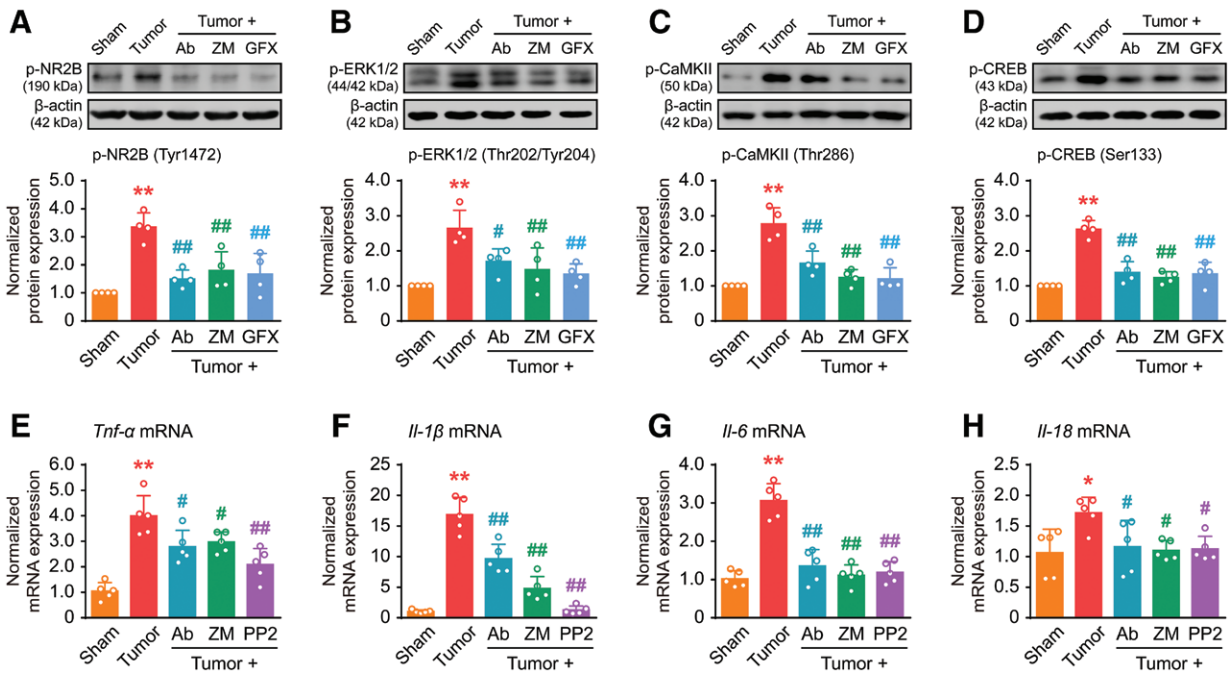

Fig. 9. Spinal blockade of vascular endothelial growth factor A (VEGF-A)/vascular endothelial growth factor receptor 2 (VEGFR2) suppresses tumor-induced NMDA receptor activation and proinflammatory cytokine production *via* protein kinase C (PKC) and Src family kinase (SFK), respectively. (*A* to *D*) Western blot analysis shows that VEGF-A neutralizing antibody, ZM 323881, or GF109203X significantly attenuated tumor-induced upregulation of phospho- (p-)NMDA receptor subunit 2B (NR2B) (*A*), p-extracellular signal-regulated kinase (ERK)1/2 (*B*), p-calcium/calmodulin-dependent protein kinase II (CaMKII) (*C*), and p-cyclic-AMP response element-binding protein (CREB) (*D*) protein expression in the spinal dorsal horn. (*E* to *H*) Real-time quantitative polymerase chain reaction analysis shows that VEGF-A neutralizing antibody, ZM 323881, or PP2 significantly attenuated tumor-induced upregulation of *Tnf-α* (*E*), *Il-1β* (*F*), *Il-6* (*G*), and *Il-18* (*H*) mRNA expression in the spinal dorsal horn. (*A* to *H*) VEGF-A neutralizing antibody (Ab; 2 μg, intrathecal), ZM 323881 (ZM, 100 nM, intrathecal), GF109203X (GFX, 0.2 μg, intrathecal), or PP2 (10 μg, intrathecal) was injected once daily on days 12, 13, and 14 post–tumor inoculation. Tissues were collected 4 h after the last injection on day 14. Data are expressed as mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$ *versus* sham group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *versus* tumor group, N = 4 (*A-D*) or 5 (*E* to *H*) for each group, one-way ANOVA with *post hoc* Dunnett test.

Src family kinase in bone cancer pain, we further examined changes of mRNA expression of proinflammatory cytokines (including TNF-α, IL-1β, IL-6, and IL-18) in the spinal dorsal horn *via* spinal blockade of vascular endothelial growth factor A, vascular endothelial growth factor receptor 2, or Src family kinase. As shown by real-time quantitative polymerase chain reaction analysis, the mRNA expression levels of *Tnf-α*, *Il-1β*, *Il-6*, and *Il-18* were all pronouncedly increased on day 14 post–tumor inoculation. However, intrathecal administration of vascular endothelial growth factor A neutralizing antibody, vascular endothelial growth factor receptor 2 inhibitor ZM 323881, or Src family kinase inhibitor PP2 effectively reduced tumor-induced mRNA expression of *Tnf-α* (fig. 9E), *Il-1β* (fig. 9F), *Il-6* (fig. 9G), and *Il-18* (fig. 9H). These findings suggest that the microglial mechanism of vascular endothelial growth factor A/vascular endothelial growth factor receptor 2 in bone cancer pain regulation is dependent on Src family kinase-mediated proinflammatory cytokine production.

Discussion

Several recent studies have shown that vascular endothelial growth factor A/vascular endothelial growth factor receptor 2 signaling plays an important role in peripheral sensitization in primary sensory neurons *via* activation of $P2X_{2/3}$ receptor or calcineurin in the dorsal root ganglion.[8,9] However, there are no relevant reports concerning whether vascular endothelial growth factor A also modulates bone cancer pain and central sensitization at the spinal cord level. Our findings here revealed that intrathecal injection of vascular endothelial growth factor A neutralizing antibody or vascular endothelial growth factor receptor 2 inhibitor could significantly relieve mechanical allodynia, thermal hyperalgesia, and spontaneous pain behaviors in tumor-bearing rats, as well as neuronal sensitization and microglial activation in the spinal dorsal horn. These results suggest that vascular endothelial growth factor A/vascular endothelial growth factor receptor 2 signaling plays a critical role in the regulation of bone cancer pain at the spinal cord level.

Notably, our current study cannot exclude the possibility that intrathecal injection of neutralizing antibody or inhibitor may also block vascular endothelial growth factor A and vascular endothelial growth factor receptor 2 activity in dorsal root ganglion neurons, thereby partially contributing to the alleviation of bone cancer pain.

In addition, we applied a CatWalk gait analysis system to study abnormal gait features in tumor-bearing rats *via* observation of weight-bearing conditions (*i.e.,* max contact area and max contact max intensity) and usage conditions (*i.e.,* single stance and swing) of the affected limb during voluntary walking. CatWalk gait analysis has been reported in studies of inflammatory and neuropathic pain.[36–38] However, since intratibial tumor inoculation leads to neither hind paw swelling (*e.g.,* inflammatory pain model) nor injury to motor nerve fibers of sciatic nerve (*e.g.,* neuropathic pain model), we believe that CatWalk gait analysis is more reflective of the bone cancer pain state. In the current study, tumor-bearing rats exhibited reductions in max contact area, max contact max intensity and single stance duration, as well as increase in swing duration in the affected limbs, all of which demonstrate a loss of weight-bearing capacity and reduced limb usability. These results are consistent with clinical findings that patients suffering from bone cancer pain exhibit reduced functional abilities in daily life. Our behavioral data further revealed that intrathecal injection of vascular endothelial growth factor A neutralizing antibody or vascular endothelial growth factor receptor 2 inhibitor could significantly reverse these tumor-induced abnormal gait features.

In addition to previous observation that vascular endothelial growth factor A and vascular endothelial growth factor receptor 2 are increased in dorsal root ganglion neurons of cancer rats,[9] we also found that the mRNA and protein expression levels of vascular endothelial growth factor A and vascular endothelial growth factor receptor 2 were increased in the ipsilateral spinal dorsal horn after tumor inoculation in a time-dependent manner. Moreover, we identified that vascular endothelial growth factor A was primarily expressed in the central terminals of primary sensory neurons and spinal neurons, while vascular endothelial growth factor receptor 2 was primarily expressed in spinal neurons and microglia, and spinal blockade of vascular endothelial growth factor A or vascular endothelial growth factor receptor 2 could significantly inhibit tumor-induced upregulation of c-Fos and ionized calcium-binding adapter molecule 1 in the spinal dorsal horn. These results confirm our hypothesis that vascular endothelial growth factor A was released from central terminals of primary sensory neurons and spinal neurons, acts on vascular endothelial growth factor receptor 2 at the surface of spinal neurons and microglia *via* the "ligand-receptor" formation, and mediates neuronal sensitization and microglial activation *via* "neuronal-neuronal" and "neuronal-microglial" cross-talks during the maintenance of bone cancer pain.

Recent evidence demonstrated sex dimorphism in the activation and roles of microglia in inflammatory and neuropathic pain models. For example, spinal TLR4, P2X4R, and p38 mediate inflammatory and/or neuropathic pain in male, but not female mice and/or rats, suggesting the possibility of male-dominant microglial signaling in the spinal dorsal horn.[39–41] However, it is well documented that the sex dimorphism in microglia-mediated pain regulation may be dependent on certain sex-specific signaling activation and pain models.[31] In order to closely mimic the human pathophysiology of breast cancer–induced pain, female rats are the most commonly used animal species for this model with Walker 256 tumor inoculation.[4] Our data provided several lines of evidence to suggest equivalent pain hypersensitivity and spinal central sensitization within male and female rats in response to tumor inoculation, accompanied by similar reactions to vascular endothelial growth factor A/vascular endothelial growth factor receptor 2–mediated bone cancer pain behaviors. First, at the behavioral level, tumor inoculation induced apparent and time-dependent mechanical allodynia and thermal hyperalgesia occurred to a similar degree in both sexes. Second, at the cellular level, tumor inoculation increased expression of c-Fos, glial fibrillary acidic protein, and ionized calcium-binding adapter molecule 1 in the spinal dorsal horn occurred to a similar degree in both sexes. Third, at the molecular level, tumor inoculation elicited upregulation and localization of vascular endothelial growth factor A and vascular endothelial growth factor receptor 2 in the spinal dorsal horn occurred to a similar degree in both sexes, and spinal blockade of vascular endothelial growth factor A or vascular endothelial growth factor receptor 2 could significantly reverse bone cancer pain behaviors in both sexes.

Spinal excitatory synaptic transmission is the material basis of central sensitization.[42,43] In this study, exogenous vascular endothelial growth factor A increased both the frequency and amplitude of spontaneous excitatory postsynaptic currents in spinal lamina IIo neurons in naïve rats, and vascular endothelial growth factor receptor 2 inhibitor largely prevented these changes, suggesting that vascular endothelial growth factor A enhances excitatory synaptic transmission in superficial spinal dorsal horn neurons *via* vascular endothelial growth factor receptor 2. Considering these *in vitro* results, we continued to detect the algogenic effect of vascular endothelial growth factor A/vascular endothelial growth factor receptor 2 on naïve rats *in vivo*. Behavioral data further indicated that intrathecal injection of exogenous vascular endothelial growth factor A simultaneously reduced threshold and latency in naïve rats, but pretreatment with vascular endothelial growth factor receptor 2 inhibitor could inhibit this phenomenon. These findings suggest that vascular endothelial growth factor A is sufficient to induce allodynia and hyperalgesia *via* vascular endothelial growth factor receptor 2, and the underlying causative mechanism may be related to the enhancement of spinal excitatory synaptic transmission.

For patch-clamp recordings, vascular endothelial growth factor A–induced spontaneous excitatory postsynaptic currents increases were primarily mediated by AMPA receptors. In the central nervous system, protein kinase C mediates AMPA receptor membrane translocation and increases AMPA receptor activation *via* binding to serine 831 phosphorylation site on its GluA1 subunit.[44] Therefore, we speculated that protein kinase C might be involved in the regulation of vascular endothelial growth factor A/vascular endothelial growth factor receptor 2–mediated neuronal sensitization and pain behaviors. Our results showed that exogenous vascular endothelial growth factor A upregulated phospho-protein kinase C expression in the spinal dorsal horn *via* vascular endothelial growth factor receptor 2, and that intrathecal injection of protein kinase C inhibitor GF109203X inhibited vascular endothelial growth factor A/vascular endothelial growth factor receptor 2–induced pain sensitization in naïve rats. Moreover, we found that phospho-protein kinase C was upregulated after tumor inoculation and could be significantly inhibited by vascular endothelial growth factor A neutralizing antibody or vascular endothelial growth factor receptor 2 inhibitor. In addition, the central sensitization maintenance is mainly dependent on the synergistic effect of AMPA and NMDA receptors in combination,[45] and protein kinase C also plays a key regulatory role in activation of NMDA receptors *via* binding to two phosphorylation sites of serine 890 and 896 on NMDA receptors, or *via* relief of Mg^{2+} blocking effect on NMDA receptors.[29,46,47] Therefore, we further focused on the effect of vascular endothelial growth factor A/vascular endothelial growth factor receptor 2/protein kinase C signaling pathway on the activation of NMDA receptors, as well as downstream Ca^{2+}-dependent signals in the spinal cord after tumor inoculation. Western blot analysis showed that spinal blockade of vascular endothelial growth factor A, vascular endothelial growth factor receptor 2, or protein kinase C remarkably suppressed tumor-induced protein expression of phospho-NMDA receptor subunit 2B, phospho-extracellular signal-regulated kinase, phospho-calcium/calmodulin-dependent protein kinase II, and phospho-cyclic-AMP response element-binding protein in tumor-bearing rats. These findings suggest that neuronal protein kinase C mediates the role of vascular endothelial growth factor A/vascular endothelial growth factor receptor 2 in bone cancer pain regulation *via* promotion of the activation of NMDA receptor subunit 2B and Ca^{2+}-dependent signals in the spinal cord (fig. 10).

Among the four vascular endothelial growth factor receptor 2–mediated signaling pathways, the role of Src family kinase in microglial activation is clear. Previous studies have shown that Src family kinase is selectively activated in spinal microglia after formalin paw injection or spinal nerve ligation, and intrathecal injection of Src family kinase inhibitor PP2 can significantly attenuate both inflammatory and neuropathic

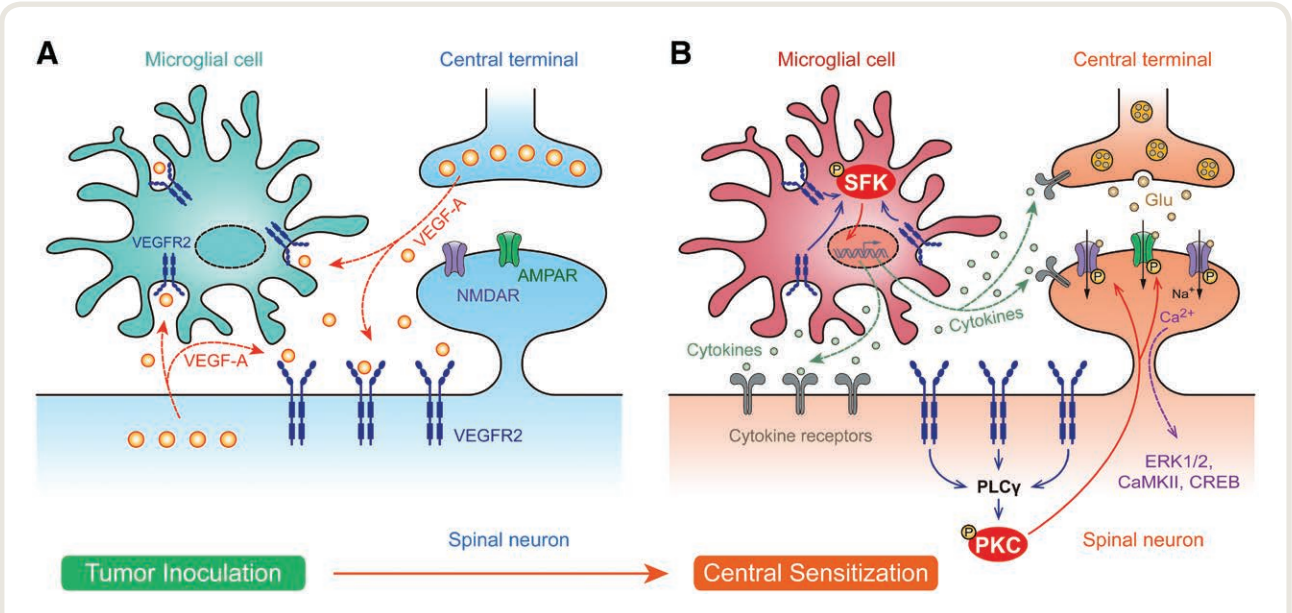

Fig. 10. Schematic illustration of the mechanisms underlying contributions of vascular endothelial growth factor A (VEGF-A)/vascular endothelial growth factor receptor 2 (VEGFR2) to spinal central sensitization in bone cancer pain. (*A*) After tumor inoculation, VEGF-A is primarily produced and released from presynaptic and postsynaptic neurons, and its receptor VEGFR2 is upregulated in spinal neurons and microglial cells. (*B*) After ligand-receptor binding, VEGF-A/VEGFR2 enhances neuronal sensitization *via* protein kinase C (PKC)-mediated NMDA receptor activation, and promotes microglial activation *via* Src family kinase (SFK)-mediated proinflammatory cytokine production. As a result, VEGF-A/VEGFR2 signaling regulates neuron-neuron and neuron–glia interactions in the development of spinal central sensitization, and contributes to bone cancer pain hypersensitivity. *Solid lines* indicate the pathways in this study. Dashed lines indicate possible pathways in other studies.

pain behaviors in rat models.[34,35] Therefore, we speculated that Src family kinase may be involved in vascular endothelial growth factor A/vascular endothelial growth factor receptor 2–regulated pain behaviors. Our results showed that exogenous vascular endothelial growth factor A could upregulate phospho-Src family kinase expression in the spinal dorsal horn *via* vascular endothelial growth factor receptor 2, and that intrathecal injection of PP2 suppressed vascular endothelial growth factor A/vascular endothelial growth factor receptor 2–induced pain hypersensitivity in naïve rats. Moreover, we found that phospho-Src family kinase was upregulated after tumor inoculation and could be significantly inhibited by application of vascular endothelial growth factor A neutralizing antibody or vascular endothelial growth factor receptor 2 inhibitor. In addition, the regulatory role of microglia in spinal neuronal sensitization is primarily dependent on release of proinflammatory cytokines, such as TNF-α, IL-1β, IL-6, and IL-18.[48] Among these, TNF-α, IL-1β, and IL-18 can increase excitatory synaptic transmission, whereas IL-1β and IL-6 can attenuate inhibitory synaptic transmission. Spinal injection of TNF-α, IL-1β, IL-6 or IL-18 can significantly induce mechanical allodynia and/or thermal hyperalgesia in naïve rats.[14,49] Therefore, we further investigated the effect of vascular endothelial growth factor A/vascular endothelial growth factor receptor 2/Src family kinase signaling pathway on the production of proinflammatory cytokines in the spinal cord after tumor inoculation. Real-time quantitative polymerase chain reaction data showed that spinal blockade of vascular endothelial growth factor A, vascular endothelial growth factor receptor 2, or Src family kinase could remarkably suppress tumor-induced mRNA expression of *Tnf-α*, *Il-1β*, *Il-6*, and *Il-18* in tumor-bearing rats. These findings suggest that microglial Src family kinase mediates the role of vascular endothelial growth factor A/vascular endothelial growth factor receptor 2 in bone cancer pain regulation *via* promotion of the inflammatory response in the spinal cord (fig. 10).

In summary, remarkable achievements have been made in both basic and clinical research in the field of oncology on vascular endothelial growth factor A and vascular endothelial growth factor receptor 2. Antiangiogenic drugs against vascular endothelial growth factor A and/or vascular endothelial growth factor receptor 2 have been employed clinically for antitumor therapy.[50] This study reports a novel role for vascular endothelial growth factor A/vascular endothelial growth factor receptor 2 signaling in bone cancer pain regulation and the implicated mechanisms in the spinal cord. Thus, blockade of vascular endothelial growth factor A/vascular endothelial growth factor receptor 2 signaling could be a potential pharmaceutical therapy used not only to treat cancers, but also to relieve cancer pain symptoms within comprehensive cancer treatment.

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Competing Interests

The authors declare no competing interests.

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