Supplemental Digital Content

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

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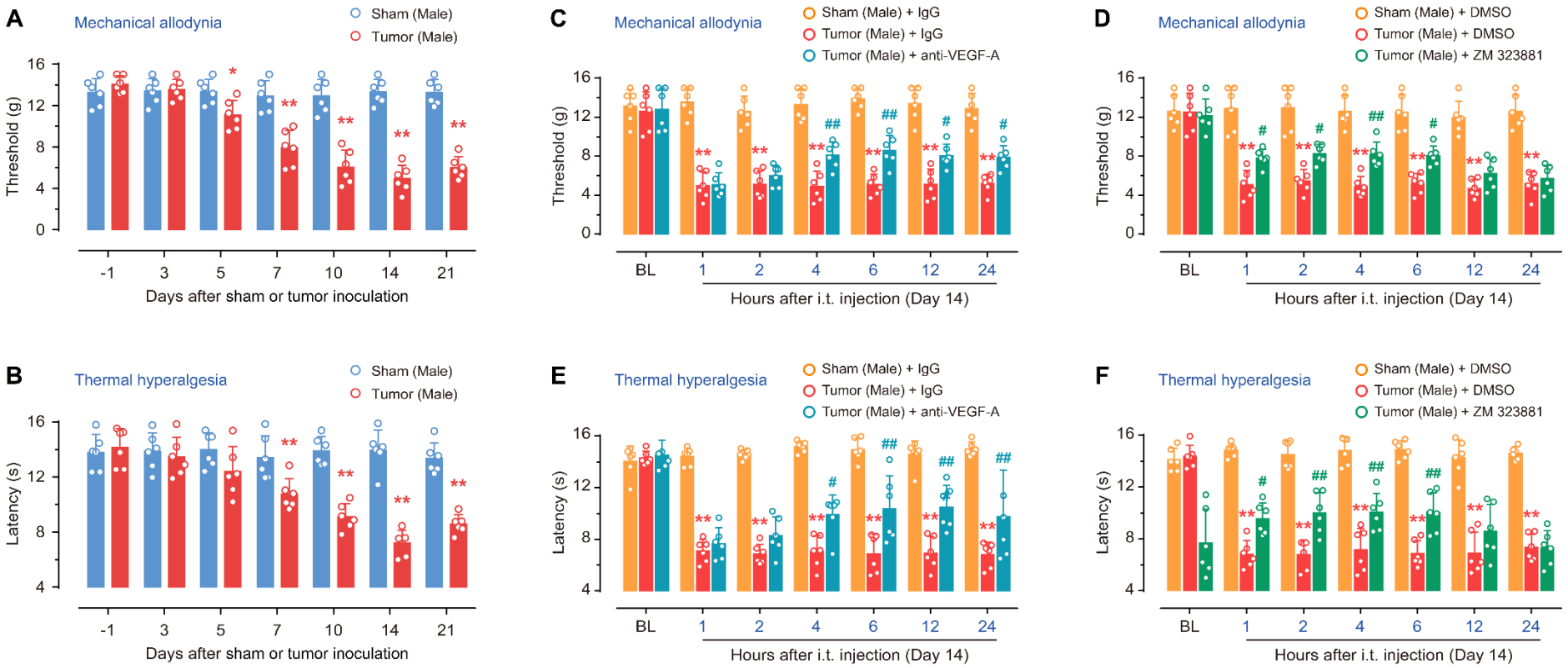
A
Mechanical allodynia
Sham (Male)
Tumor (Male)
Threshold (g)
Days after sham or tumor inoculation
B
Thermal hyperalgesia
Latency (s)
C
Sham (Male) + IgG
Tumor (Male) + IgG
Tumor (Male) + anti-VEGF-A
Hours after i.t. injection (Day 14)
D
Sham (Male) + DMSO
Tumor (Male) + DMSO
Tumor (Male) + ZM 323881
E
F

Fig. S1. Spinal blockade of VEGF-A/VEGFR2 attenuates tumor-induced pain behaviors in male rats. (*A, B*) Behavioral analysis shows that tumor inoculation induced a time-dependent reduction in threshold (*A*) and latency (*B*) in the affected hindpaw. Behavioral tests were performed 1 day before or 3, 5, 7, 10, 14, and 21 days after sham or tumor inoculation. Data are expressed as mean ± SD. **P* < 0.05, ***P* < 0.01 *versus* sham group, N = 6 for each group, two-way repeated-measures ANOVA with *post hoc* Bonferroni's test. (*C-F*) Spinal administration of VEGF-A neutralizing antibody or VEGFR2 inhibitor ZM 323881 significantly reversed tumor-induced reduction in the threshold (*C, D*) and latency (*E, F*) in tumor-bearing rats. (*C, E*) VEGF-A neutralizing antibody (anti-VEGF-A, 2 μg, i.t.) or IgG (vehicle control, 2 μg, i.t.) was injected on day 14 post-tumor inoculation. Behavioral tests were performed 1, 2, 4, 6, 12 and 24 hours after injection. Data are expressed as mean ± SD. ***P* < 0.01 *versus* sham + IgG group; $^{\#}P$ < 0.05, $^{\#\#}P$ < 0.01 *versus* tumor + IgG group, N = 6 for each group, two-way repeated-measures ANOVA with *post hoc* Bonferroni's test. (*D, F*) ZM 323881 (100 nM, i.t.) or DMSO (vehicle control, 1%, i.t.) was injected once daily on day 14 post-tumor inoculation. Behavioral tests were performed 1, 2, 4, 6, 12 and 24 hours after injection. Data are expressed as mean ± SD. ***P* < 0.01 *versus* sham + DMSO group; $^{\#}P$ < 0.05, $^{\#\#}P$ < 0.01 *versus* tumor + DMSO group, N = 6 for each group, two-way repeated-measures ANOVA with *post hoc* Bonferroni's test.

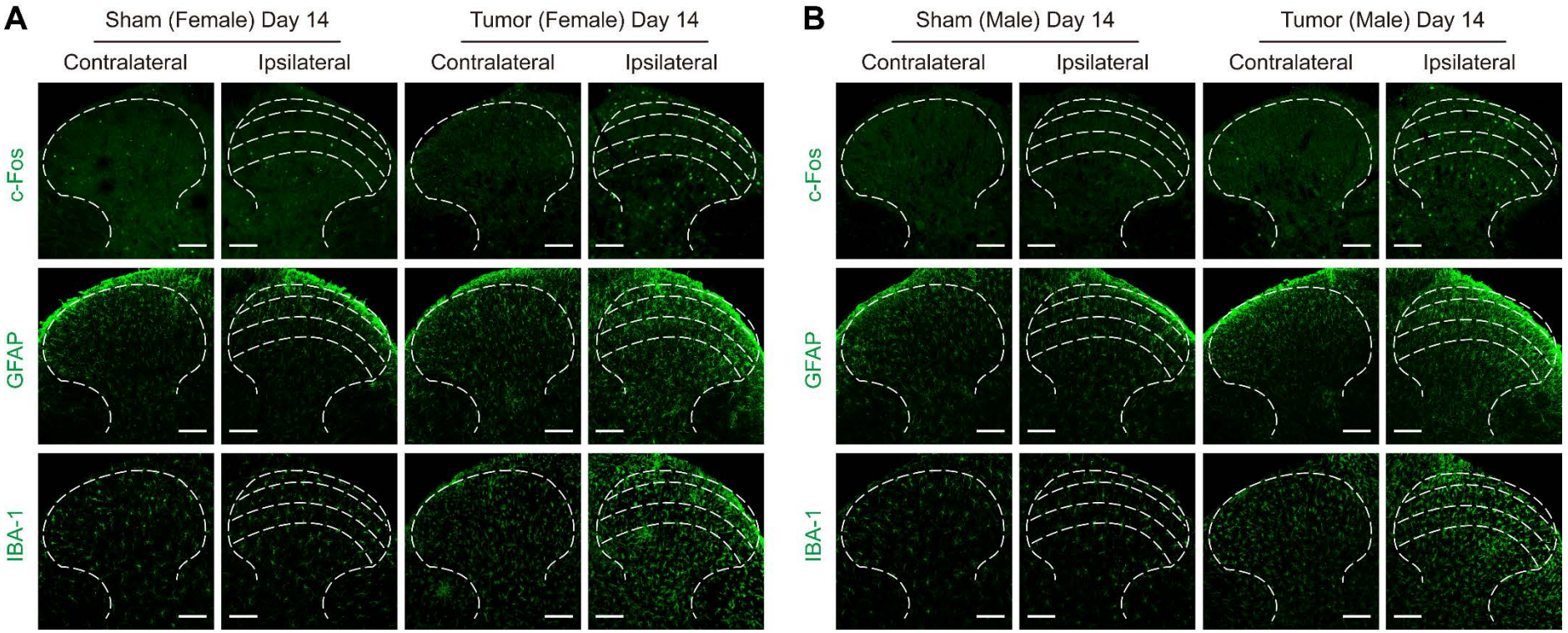

Fig. S2. The spinal central sensitization following tumor inoculation in female and male rats. (*A, B*) Immunofluorescence shows that the expression of c-Fos, GFAP, and IBA-1 was low in both sides of the spinal dorsal horn in sham rats, but increased in the ipsilateral side of the spinal dorsal horn compared with the contralateral side of spinal dorsal horn in both female (*A*) and male (*B*) tumor-bearing rats. Tissues were collected on day 14 after sham or tumor inoculation. Scale bars: 100 μm.

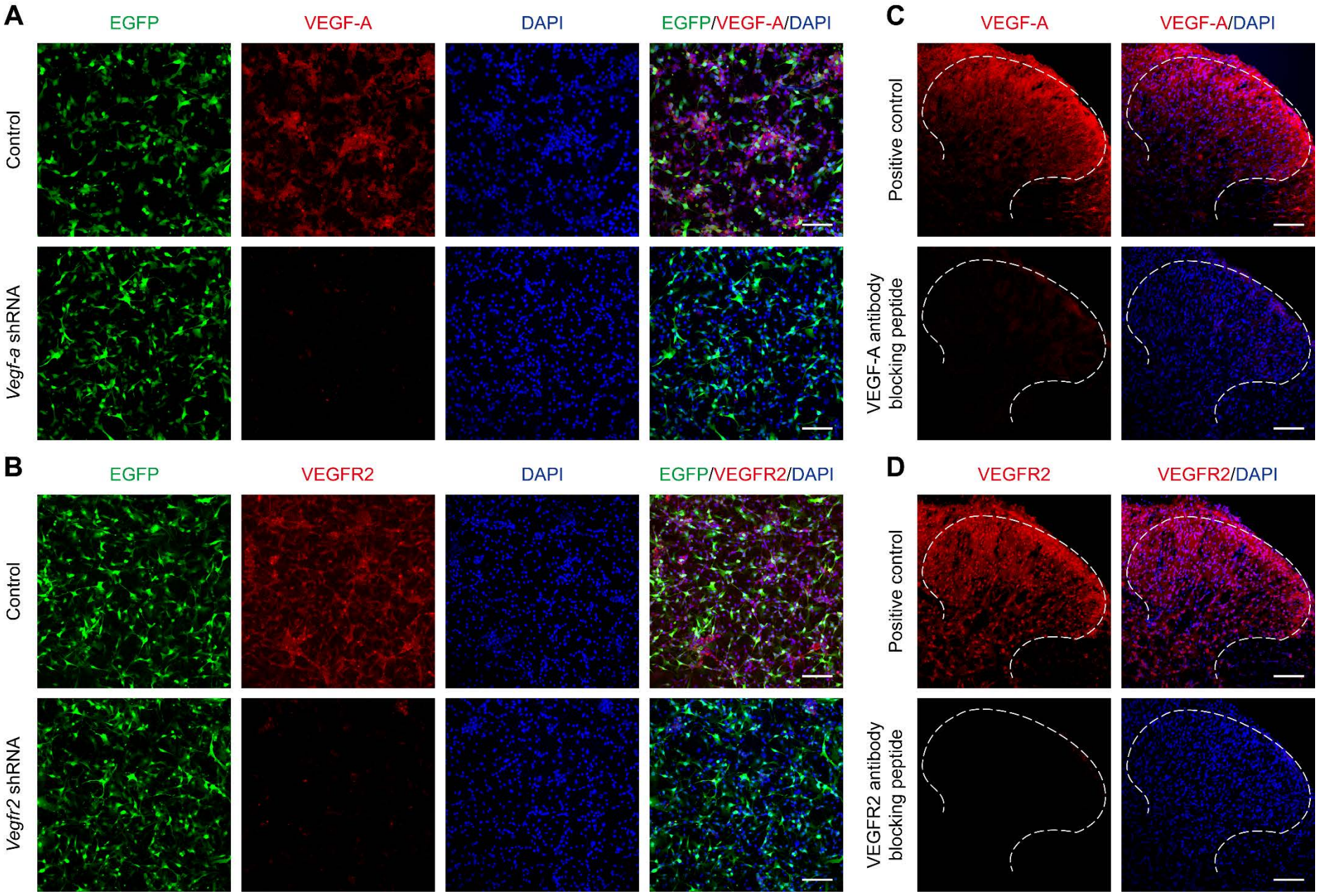
A
EGFP
VEGF-A
DAPI
EGFP/VEGF-A/DAPI
Control
Vegf-a shRNA
B
EGFP
VEGFR2
DAPI
EGFP/VEGFR2/DAPI
Control
Vegfr2 shRNA
C
VEGF-A
VEGF-A/DAPI
Positive control
VEGF-A antibody blocking peptide
D
VEGFR2
VEGFR2/DAPI
Positive control
VEGFR2 antibody blocking peptide

Fig. S3. Specificity validation of VEGF-A and VEGFR2 antibody in B16-BL6 cells and rat spinal cord. (*A, B*) The specificity of VEGF-A (*A*) and VEGFR2 (*B*) antibody was verified by the corresponding shRNA plasmid transfection experiment in B16-BL6 cells with immunocytochemistry method. Scale bars: 100 μm. (*C, D*) The specificity of VEGF-A (*C*) and VEGFR2 (*D*) antibody were verified by the corresponding blocking peptide pre-absorption experiment in rat spinal cord with immunofluorescence method. Tissues were collected on day 14 post-tumor inoculation in female rats. Scale bars: 100 μm.

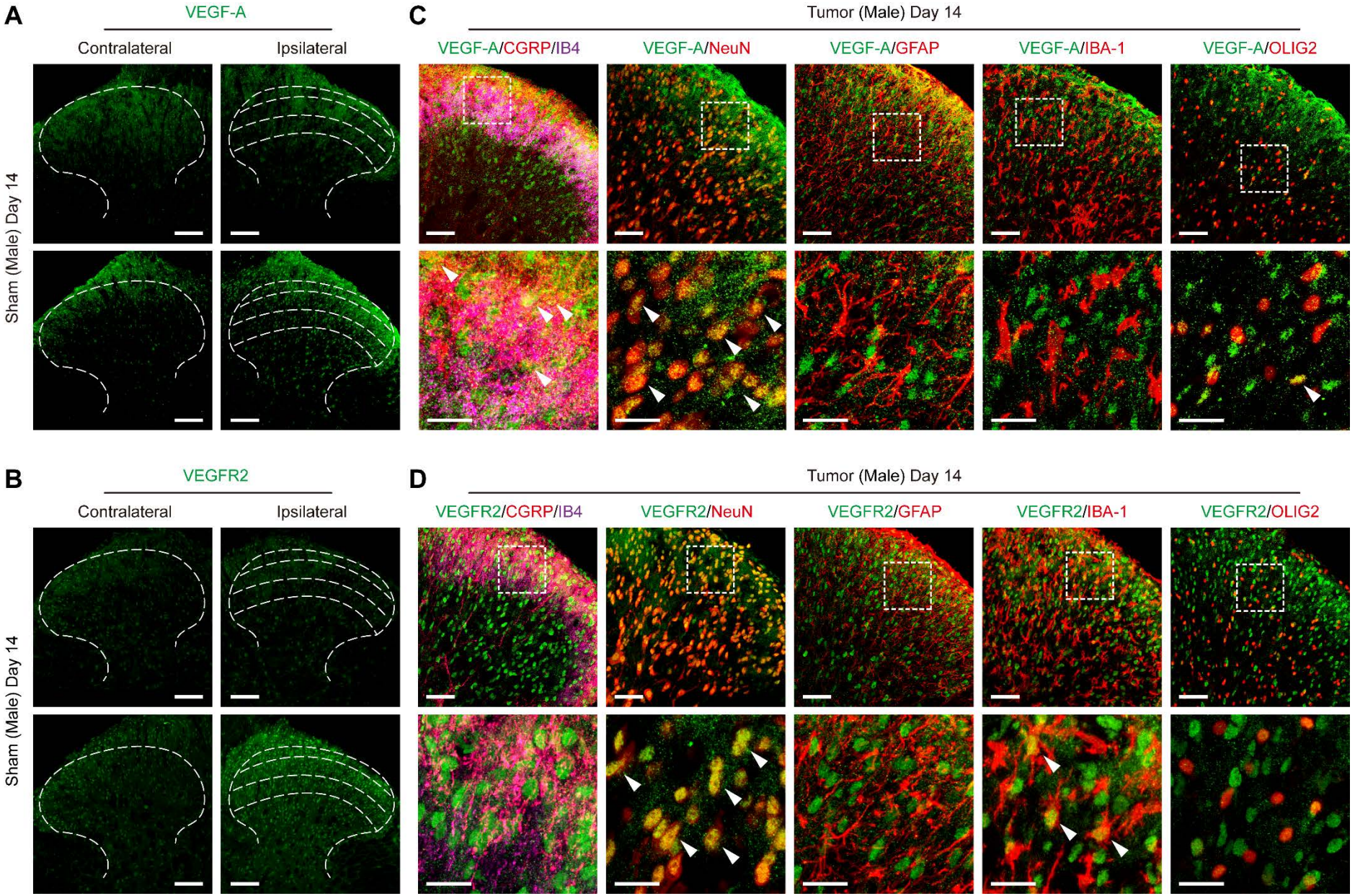
A
VEGF-A
Contralateral
Ipsilateral
Sham (Male) Day 14
C
Tumor (Male) Day 14
VEGF-A/CGRP/IB4
VEGF-A/NeuN
VEGF-A/GFAP
VEGF-A/IBA-1
VEGF-A/OLIG2
B
VEGFR2
Contralateral
Ipsilateral
Sham (Male) Day 14
D
Tumor (Male) Day 14
VEGFR2/CGRP/IB4
VEGFR2/NeuN
VEGFR2/GFAP
VEGFR2/IBA-1
VEGFR2/OLIG2

Fig. S4. Cellular localization of VEGF-A and VEGFR2 in the spinal cord following tumor inoculation in male rats. (*A, B*) Immunofluorescence shows that VEGF-A (*A*) and VEGFR2 (*B*) was 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. (*C, D*) Immunofluorescence staining for VEGF-A and VEGFR2 combined with cell markers in the spinal dorsal horn: CGRP (peptidergic primary afferent terminals), IB4 (non-peptidergic primary afferent terminals), NeuN (neurons), GFAP (astrocytes), IBA-1 (microglia), and OLIG2 (oligodendrocytes). (*C*) VEGF-A immunoreactivity primarily colocalized with CGRP/IB4, NeuN and OLIG2 in the spinal dorsal horn; (*D*) VEGFR2 immunoreactivity primarily colocalized with NeuN and IBA-1 in the spinal dorsal horn. Arrowheads indicate co-expression of VEGF-A/VEGFR2 and cell markers. Tissues were collected on day 14 post-tumor inoculation. Scale bars: 50 μm and 20 μm (zoom).

Table S1. Primer sequences for RT-qPCR

Gene	Primer forward (5'-3')	Primer reverse (5'-3')
Vegf-a	5' TGG TCT TTC GTC CTT CTT AG	5' GAT GGG TTT GTC GTG TTT C
Vegfr2	5' GCG AAT CAC TCA CAC CAG TT	5' ATG CCA CAA TCA AGA TGT CG
Tnf-a	5' CCC AAT CTG TGT CCT TCT AA	5' CAC TAC TTC AGC GTC TCG TG
Il-1β	5' AGT GTG TGA TGT TCC CAT TA	5' GGC TTA TGT TCT GTC CAT TG
Il-6	5' CAA CTT CCA ATG CTC TCC T	5' GTT TGC CGA GTA GAC CTC AT
Il-18	5' GCT GTG ACC CTA TCT GTG AAG	5' ATT CCA TTT TGT TGT GTC CTG
Gapdh	5' GTG CCA GCC TCG TCT CAT AG	5' GAA CTT GCC GTG GGT AGA GT

Table S2. Information for primary antibodies in western blot

Name	Molecular Weight	Cat. No.	Manufacturer	Dilution
Rabbit anti-VEGF-A	45 kDa	ab46154	abcam	1: 1000
Rabbit anti-VEGFR2	210 kDa	#9698	CST	1: 1000
Rabbit anti-VEGF Receptor 2 (phospho-Y951)	151 kDa	ab38473	abcam	1: 1000
Rabbit anti-VEGF Receptor 2 (phospho-Y1175)	152 kda	ab194806	abcam	1: 1000
Rabbit anti-c-Fos	62 kDa	sc-52	Santa Cruz	1: 1000
Goat anti-IBA1	17 kDa	ab5076	abcam	1: 1000
Mouse anti-phospho-PKCα (Ser 657)	80 kDa	sc-377565	Santa Cruz	1: 1000
Rabbit anti-phospho-Src Family (Tyr 416)	60 kDa	#6943	CST	1: 2000
Rabbit anti-phospho-NMDAR 2B (Tyr 1472)	190 kDa	#4208	CST	1: 1000
Rabbit anti-phospho-ERK1/2 (Thr 202/Tyr 204)	42, 44 kDa	#9101	CST	1: 2000
Rabbit anti-phospho-CaMKIIα (Thr 286)	50 kDa	#12716	CST	1: 2000
Rabbit anti-phospho-CREB (Ser 133)	43 kDa	#9198	CST	1: 2000
HRP-Conjugated GAPDH Antibody	36 kDa	HRP-60004	Proteintech	1: 10000
HRP-Conjugated Beta Actin Antibody	42 kDa	HRP-60008	Proteintech	1: 10000

Table S3. Information for primary antibodies in immunofluorescence

Name	Cat. No.	Manufacturer	Dilution
Rabbit anti-VEGF-A	ab46154	abcam	1: 200
Rabbit anti-VEGFR2	sc-505	Santa Cruz	1: 500
Mouse anti-c-Fos	ab208942	abcam	1: 500
Mouse anti-NeuN	MAB377	Millipore	1: 1000
Mouse anti-GFAP	#3670	CST	1: 1000
Goat anti-IBA1	NB100-1028	Novus	1: 500
Mouse anti-OLIG2	MABN50	Millipore	1: 500
Goat anti-CGRP	ab36001	abcam	1: 500
Isolectin GS-IB4	I32450	Invitrogen	1: 500
Mouse anti-phospho-PKCα (Ser 657)	sc-377565	Santa Cruz	1: 500
Rabbit anti-phospho-Src Family (Tyr416)	#6943	CST	1: 500

Table S4. Primer sequences for shRNA

Gene	Target	Primer forward (5'-3')	primer reverse (5'-3')
Vegf-a	GCGGAGAAAGCATTTGTTT	GATCCCCGCGGAGAAAGCATTTGTTTcTCA AGAGAAAACAAATGCTTTCTCCGCTTTTT	tcgaaaaaGCGGAGAAAGCATTTGTTTTCTCT TGAgAAACAAATGCTTTCTCCGCGGG
Vegfr2	GCACGAAACATTCTCCTAT	GATCCCCGCACGAAACATTCTCCTATTTCA AGAGAATAGGAGAATGTTTCGTGCTTTTT	tcgaaaaaGCACGAAACATTCTCCTATTCTCTT GAAATAGGAGAATGTTTCGTGCGGG