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Substance P Signaling Mediates BMP-Dependent Heterotopic Ossification

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ABSTRACT

Heterotopic ossification (HO) is a disabling condition associated with neurologic injury, inflammation, and overactive bone morphogenetic protein (BMP) signaling. The inductive factors involved in lesion formation are unknown. We found that the expression of the neuro-inflammatory factor Substance P (SP) is dramatically increased in early lesional tissue in patients who have either fibrodysplasia ossificans progressiva (FOP) or acquired HO, and in three independent mouse models of HO. In Nse-BMP4, a mouse model of HO, robust HO forms in response to tissue injury; however, null mutations of the preprotachykinin (PPT) gene encoding SP prevent HO. Importantly, ablation of SP^+ sensory neurons, treatment with an antagonist of SP receptor NK1r, deletion of NK1r gene, or genetic down-regulation of NK1r-expressing mast cells also profoundly inhibit injury-induced HO. These observations establish a potent neuro-inflammatory induction and amplification circuit for BMP-dependent HO lesion formation, and identify novel molecular targets for prevention of HO. J. Cell. Biochem. 112: 2759–2772, 2011. © 2011 Wiley-Liss, Inc.

KEY WORDS: HETEROTOPIC OSSIFICATION (HO); FIBRODYSPLASIA OSSIFICANS PROGRESSIVA (FOP); BONE MORPHOGENETIC PROTEIN (BMP); SUBSTANCE P (SP); TACHYKININ RECEPTOR 1 (NK1R); NK1R ANTAGONIST; MAST CELLS

Heterotopic ossification (HO), the formation of extraskeletal bone, is a common and serious complication of soft tissue trauma [Cullen and Perera, 2009; Forsberg and Potter, 2010; Salisbury et al., 2010]. In fibrodysplasia ossificans progressiva (FOP), a rare, life-threatening condition of progressive and episodic HO, mutations in a bone morphogenetic protein (BMP) type I receptor, ACVR1/ALK2, cause dysregulated BMP signaling [Shore et al., 2006]. Despite advances in understanding the genetics of HO, the cellular and molecular triggers of HO remain unclear.

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A fundamental feature of all forms of HO is the requirement for an inflammatory trigger [Sawyer et al., 1991; Kaplan et al., 2007, 2008; Cullen and Perera, 2009]. In a previous study, we found that injury-induced inflammation triggers HO in a unique transgenic mouse model with features of both sporadic HO and FOP in which BMP4 is driven by neuron-specific enolase (Nse) promoter [Forss-Petter et al., 1990]. Detailed studies found that the transgene is expressed not only in neurons (including DRG neurons), but also in macrophages [Forss-Petter et al., 1990; Kan et al., 2009; Mukhopadhyay et al., 2009]. The robust injury-induced phenotype and the unique transgene expression pattern make this an attractive animal model for injury-induced sporadic HO as well as FOP [Kan and Kessler, 2011], and suggest that HO might be prevented if the inflammatory trigger could be inhibited. However, it is challenging to identify the specific loci that we can efficiently regulate the inflammation triggers, because it is well known that the inflammatory response is regulated by multiple cytokines [Nathan, 2002] and also under complex neuro-endocrine control [Rowley et al., 1980; Savastano et al., 1994; Tracey, 2002; Czura and Tracey, 2005; Marques-Deak et al., 2005].

In this study, we reasoned that inflammatory neuropeptides might be ideal candidates to trigger inflammation and the HO, because they mediate neuro-inflammatory feedback loops both in physiological and pathophysiological conditions and are often dysregulated in trauma [Bergstrom et al., 2006; Lerner and Persson, 2008; Allison et al., 2009]. Among pro-inflammatory neuropeptides, substance P (SP) [McGillis et al., 1987; Yano et al., 1989; Mantyh, 1991] was identified in active areas of bone regeneration following fracture [Edoff et al., 1997; Li et al., 2010]. Moreover, the SP receptor, neurokinin 1 (NK1r), was demonstrated on chondrocytes [Millward-Sadler et al., 2003], osteocytes [Goto et al., 1998], osteoblasts [Goto et al., 2001], osteoclasts [Wang et al., 2009], and mast cells [Okada et al., 1999]. SP is an undecapeptide expressed by subsets of neurons in the central and peripheral nervous systems [Barbut et al., 1981; Zubrzycka and Janecka, 2000; Datar et al., 2004] and also by non-neuronal cells including macrophages and T lymphocytes, cells involved at the earliest stages of pre-osseous fracture repair [Nelson and Bost, 2004; Pinto et al., 2004]. SP enhances lymphocyte proliferation and immunoglobulin production as well as cytokine secretion from lymphocytes, monocytes, macrophages, and mast cells [Nakaya et al., 1994; Quartara and Maggi, 1998; Goto and Tanaka, 2002]. By promoting vasodilatation, leukocyte chemotaxis, and leukocyte/endothelial cell adhesion, SP promotes the extravasation, migration, and accumulation of leukocytes at sites of tissue injury [McGillis et al., 1987; Mantyh, 1991; Donkin et al., 2009]. In addition to immune modulation, SP also participates in injury-inducible mobilization of $CD29^{+}$ mesenchymal stem/progenitor cells, a cell type that is involved in HO formation [Hong et al., 2009]. Clinical studies indicate that SP is dysregulated, at least transiently, after traumatic brain or spinal cord injury (SCI) [Sharma et al., 1990; Donkin et al., 2007, 2009], pre-conditions that frequently lead to acquired HO [Sawyer et al., 1991].

Here, we studied the role of SP in patients with sporadic, post-traumatic, and neurologically associated HO as well as FOP, and in three independent mouse models of post-traumatic and FOP-like HO [Kan et al., 2004, 2009]. We found that SP expression was up-regulated in early pre-osseous sporadic HO and FOP lesions, and that blocking SP secretion or function in the animal models prevented HO. We further determined that mast cells, which robustly express NK1r [Okada et al., 1999], are required to mediate the downstream events of SP-mediated BMP-dependent HO. These observations identify SP as a critical regulatory factor in the induction of HO, and suggest that blocking SP signaling or the downstream amplification circuit of SP-mediated inflammation could be a novel therapeutic approach to prevent BMP-mediated HO.

MATERIALS AND METHODS

PATIENTS' TISSUE SAMPLES AND PROCESSING

Collection of specimens was approved by the Office of Regulatory Affairs and the Institutional Review Board of the University of Pennsylvania (Federal wide Assurance #00004028). Specimens from six FOP patients who underwent biopsy for presumptive neoplasm were obtained from superficial and deep back masses later determined to be early FOP lesions. Tissue samples from 10 patients who were diagnosed with post-traumatic or neurologically associated HO by clinical and radiographic criteria, were divided into the following groups, according to the specific predisposing medical conditions: two from SCI, five from traumatic brain injury (TBI), one from non-neurologic trauma (NNT), and two from total hip arthroplasty (THA). Normal muscle tissue was obtained from the backs of four unaffected males. Tissue samples were fixed in neutral buffered formalin, decalcified, infiltrated, embedded in paraffin, and sectioned at a thickness of five micrometers. Samples were deparaffinized, stained with Harris hematoxylin solution and counterstained with hematoxylin and eosin (H&E) by standard procedures. Prepared specimens were examined under light microscopy for histological characteristics of HO lesion formation. Findings were confirmed by two independent examiners (R.J.P. and F.S.K.).

TRANSGENIC AND MOUSE MODELS

Previously generated Nse-BMP4 and NK1r−/− mice were used in this study [Bozic et al., 1996; Kan et al., 2004]. Floxed caALK2 transgenic mice were a gift from Dr. Yuji Mishina (University of Michigan). All other lines were from the Jackson Laboratory (Bar Harbor, Maine), unless otherwise specified. All animal experiments were approved by the Animal Care and Use Committee at Northwestern University, or by the Institutional Animal Care and Use Committee at the University of Pennsylvania.

Superficial and deep muscle injury model. The superficial and deep muscle injury was preformed according to previous description [Kan et al., 2009]. Briefly, deep skin incision caused disruption of skin and subcutaneous tissue as well as injury of superficial panniculous carnosus muscle. Deep intramuscular injection of 100 μl of 10 μM cardiotoxin (CTX, Calbiochem) caused deep muscle injury [Kan et al., 2009].

caALK2 mouse model. To induce expression of caALK2, Adenovirus-Cre (Penn Vector Core; 1×10^{11} particles per mouse) together with cardiotoxin (100 μl of 10 μM solution) was injected into the left hindlimb musculatures of mice at 3 weeks of age. Tissues were recovered at 4 and 8 days after injections. Tissues were fixed in

4% PFA and frozen in isopentane (2-methylbutane) and cryosections were cut at 10 mm for further studies.

BMP4 matrigel injection model. Growth factor-reduced Matrigel (BD Biosciences, Bedford, MA) was impregnated with recombinant human BMP4 (rhBMP4, R&D) at a concentration of 2.5 μg/50 μl and injected intramuscularly into the mid-belly of the tibialis anterior muscle of adult mice, with or without 10 μM cardiotoxin (CTX, Calbiochem). Mice injected with Matrigel only served as controls. Lesional tissue was recovered 7 days to 3 weeks following implantation for histochemical and immunohistochemical analysis.

Colchicine treatment. Control and Nse-BMP4 mice were treated with colchicine (intraperitoneal, 5 mg/kg of body weight) for 24 h, and then the lumbar dorsal root ganglia (DRG) were fixed and harvested for immunohistochemistry (IHC).

NK1r antagonist treatment. Young (1–2 months old) Nse-BMP4 mice were treated with the tachykinin NK1 receptor antagonist, RP-67580 (Tocris Bioscience) at a dose of 2 mg/kg body weight, intraperitoneally, twice daily for 4 weeks [Kennedy et al., 1997]. Age- and sex-matched mice treated with PBS were used as controls. RP-67580 treatment was started 4 h after superficial or deep muscle injury. The same treatment was also administrated to Nse-BMP4 mice that already developed HO to test if these mice could benefit from RP-67580 treatment.

Radiographic evaluation. Radiographs were taken weekly to monitor the progression of HO after superficial or deep muscle injury. HO formation was assayed by whole body X-ray imaging, using the TruDR Digital Radiography System (Sound Technologies, Carlsbad, CA) according to previous description [Kan et al., 2009]. To further quantify the HO after muscle injury and the effect of the SP antagonists, microComputed Tomography (micro-CT) was performed with a MicroCT-40 system (Scanco Medical AG, Bruettisellen, Switzerland). A total of 460 contiguous slices were collected of each tibia (~17 mm length) at 45 kV, 88 μA, 300 ms integration per projection, and 500 projections of 1024 samples. Reconstruction was with 37 μm isotropic volume elements (voxels) on a 1,024 × 1,024 grid. The volume of heterotopic-ossified tissue was quantified from the micro-CT data sets using the Scanco software suite [Stock et al., 2004].

Neonatal capsaicin treatment. We permanently ablated SP^+-positive neurons in DRG by treating neonatal (1–2 days old) mice with a single subcutaneous injection of capsaicin (50 mg/kg) or vehicle (10% ethanol and 10% Tween 80 in sterile saline) into mouse back, as previously reported [Sugimoto et al., 1998]. Capsaicin- and vehicle-treated animals were group housed and weaned the same way. These animals were subjected to similar injury at 1–2 months of age. Injury-induced SP up-regulation in skin, subcutaneous tissues and muscles were determined by IHC.

DISSOCIATED SENSORY NEURON CULTURE

Lumbar DRGs from 1- to 2-month-old Nse-BMP4 and sex- and age-matched control mice were harvested, dissociated, and cultured according to the published protocol [Malin et al., 2007], with or without BMP4/Noggin treatment on 24-well cover slips. Cells were fixed and supernatants were collected 3 days after culture. The SP concentration in the cell supernatant was determined by Substance P EIA Kit (Cayman Chemical) and the SP-positive neurons were determined by counting the SP/NF200 double positive neurons on the cover slips.

IMMUNOHISTOCHEMISTRY

Immunostaining was performed using standard protocols. Briefly, sections were fixed with 4% paraformaldehyde in PBS. Non-specific binding was blocked with 10% normal serum diluted in 1% bovine serum albumin (BSA, Jackson Lab) and 0.25% Triton X-100 for 1 h in room temperature. The sections were incubated with primary antibodies diluted with 1% BSA + 0.25% Triton X-100 at 4°C overnight, then incubated with appropriate secondary antibodies (Cy3 or Cy2 conjugated antibodies; Jackson Lab), diluted with 1% BSA + 0.25% Triton X-100 or Alexa Fluor 488, Alexa Fluor 594, and Alexa 647 (1:1,000; Invitrogen) in the dark at room temperature for 2 h, and Counterstained with DAPI (1:5,000). Mouse anti-SP antibody (R&D Systems), Rabbit anti-P75 (Promega), and Rabbit Anti-Neurofilament 200 (Sigma), chicken Anti-Neurofilament 200 (Millipore), rabbit anti-GFP (Invitrogen), and rabbit anti-LAMP2 (LifeSpan Biosciences), were used in this study. Fluorescent images were processed by Adobe Photoshop and quantified by ImagJ.

Peptide-blocking experiment. SP, NKA, and NKB peptides (from AnaSpec) were used in the blocking experiment: SP: RPKPQQFFGLM, NKA: HKTDSFVGLM, and NKB: DMHDFFVGLM were all diluted to 1 mM final concentration and incubated with equal amount of antibody (1:1,000) in blocking buffer 4°C overnight, and then control (without blocking peptide) and blocked Ab were used to stain the neighboring mouse sections from muscle injury model.

RESULTS

SP IS UP-REGULATED IN FOP LESIONS AND ACQUIRED HO AND IS NEURONAL IN ORIGIN

SP is a potent pro-inflammatory factor [McGillis et al., 1987; Yano et al., 1989; Mantyh, 1991; Thornton et al., 2010] and has been identified in the most active areas of physiological and pathological postnatal osteogenesis [Edoff et al., 1997; Li et al., 2010; Salisbury et al., 2010]. To examine whether SP expression is elevated in HO lesions, immunocytochemistry was used to detect SP protein in both mouse and human pre-osseous lesions. The specificity of the antibody was confirmed by comparing antibody binding of tissues from SP precursor gene (PPT-A) knockout mice [Cao et al., 1998] with that of WT and Nse-BMP4 mice. We found specific SP staining in the skin, subcutaneous connective tissues, CNS, DRG, and other tissues from WT and Nse-BMP4 mice. By contrast, this staining was totally absent in the same tissues of SP precursor gene (PPT) knockout mice (Supplementary Fig. 1 and data not shown) demonstrating that the antibody is specific and sensitive to probe expression of SP in target tissues. The peptide blocking experiment further confirmed the specificity and excluded the cross-reactivity of this antibody with highly conserved mammalian homologs, such as NKA and NKB (Supplementary Fig. 1). Since this antibody is specifically targeted against the mature SP peptide, and the mature peptide is identical across all mammalian species, we also used this antibody to examine SP expression in surgically removed lesional tissues samples from patients with FOP and acquired HO.

High levels of SP expression were detected in early pre-osseous FOP lesions (Fig. 1A). These early lesions were located in muscle tissue, which showed signs of fiber degeneration in phase images (Supplementary Fig. 2B) and inflammatory cell infiltration in H&E staining (Supplementary Fig. 3). In contrast, minimum SP expression was observed in normal muscle tissue (Figs. 1E and 2E). Two staining patterns can be recognized in these samples: strong punctate and weak diffuse staining. We speculated that the diffuse staining could be an artifact of dying/degenerating muscle fibers but further study with specific blocking peptide excluded that possibility (Fig. 2A,B). In fact, both punctate and diffuse staining were blocked by SP peptide. Further double staining and morphologic examination confirmed that majority of the diffuse staining was in muscle fibers (data not shown).

We also tested SP expression in samples of heterotopic bone from patients with four types of acquired HO: SCI, TBI, NNT, and THA. Due to the maturity of lesions at the time of collection, early lesional stages were found only in small portions of these samples (Fig. 1E and Supplementary Fig. 4). Consistently, SP was up-regulated in early lesions in acquired HO, but less dramatically compared to FOP early lesions. However, no appreciable SP expression was observed in later stage lesions from acquired HO samples (Fig. 2D).

To help clarify whether neurons or non-neuronal cells contributed to the observed SP up-regulation, we double-stained sections with SP and NF-200, a heavy neurofilament protein that is commonly used as a biomarker of neurons [Kaku et al., 1993], and we found extensive co-localization of SP and NF-200 both in the

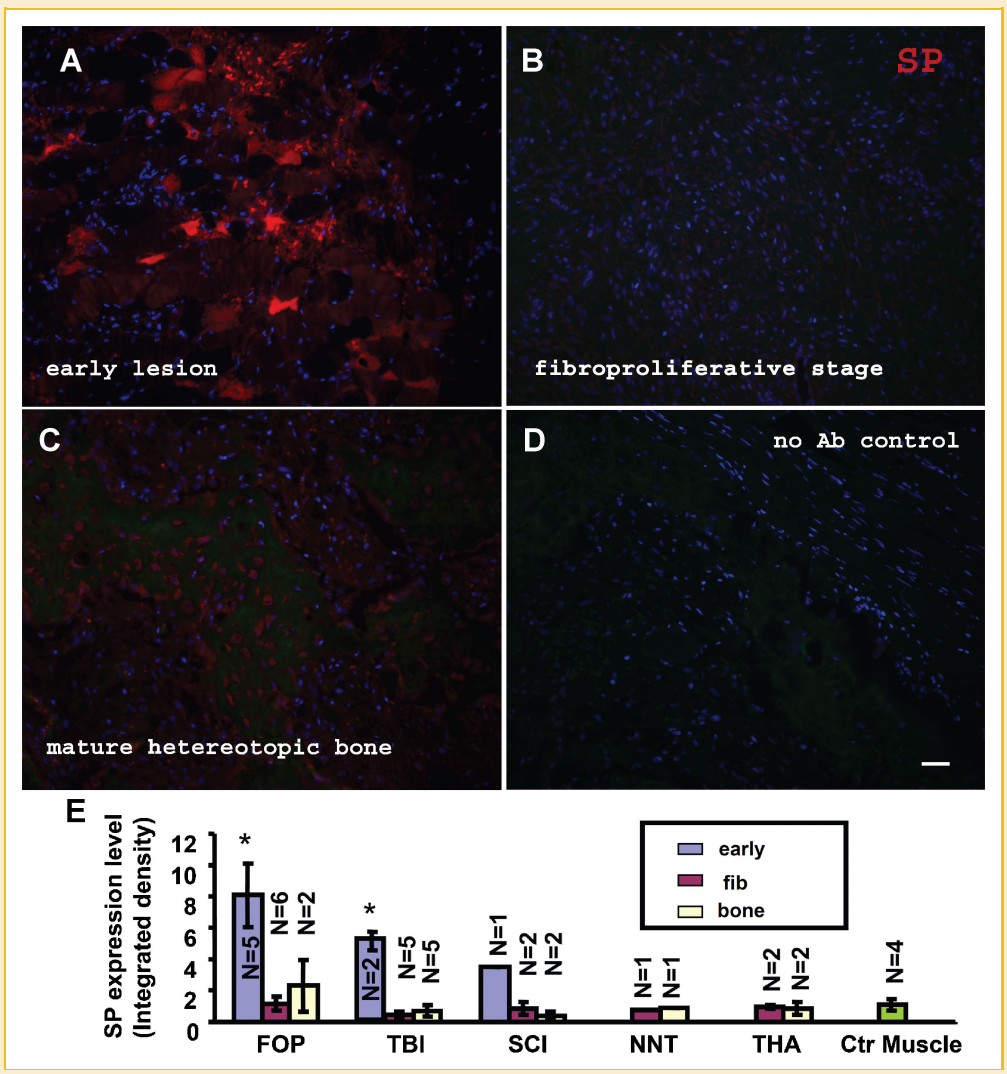

Fig. 1. SP is up-regulated in early FOP lesions. A: SP up-regulation in an early FOP lesion. Note the widespread, but not universal, SP staining. Two patterns can be recognized: the strong punctate and semi-diffuse staining. B: No appreciable level of SP staining was observed in the fibroproliferative (pre-cartilage) stage. C: SP staining of the mature HO from a FOP patient. D: Negative control (no primary antibody). Bar = 40 μm. E: Summary of relative SP expression levels in FOP, acquired-HO patients, and normal controls. Relative SP expression levels are graphed based on signal intensity of fluorescence images. fibrodysplasia ossificans progressiva (FOP), spinal cord injury (SCI), traumatic brain injury (TBI), non-neurologic trauma (NNT) and total hip arthroplasty (THA). Note significant SP up-regulation was observed only in early lesions. ($^*P < 0.01$ vs. control muscles by ANOVA). Error bars represent SD early: early lesion, fib: fibrotic lesion, bone: mature bone.

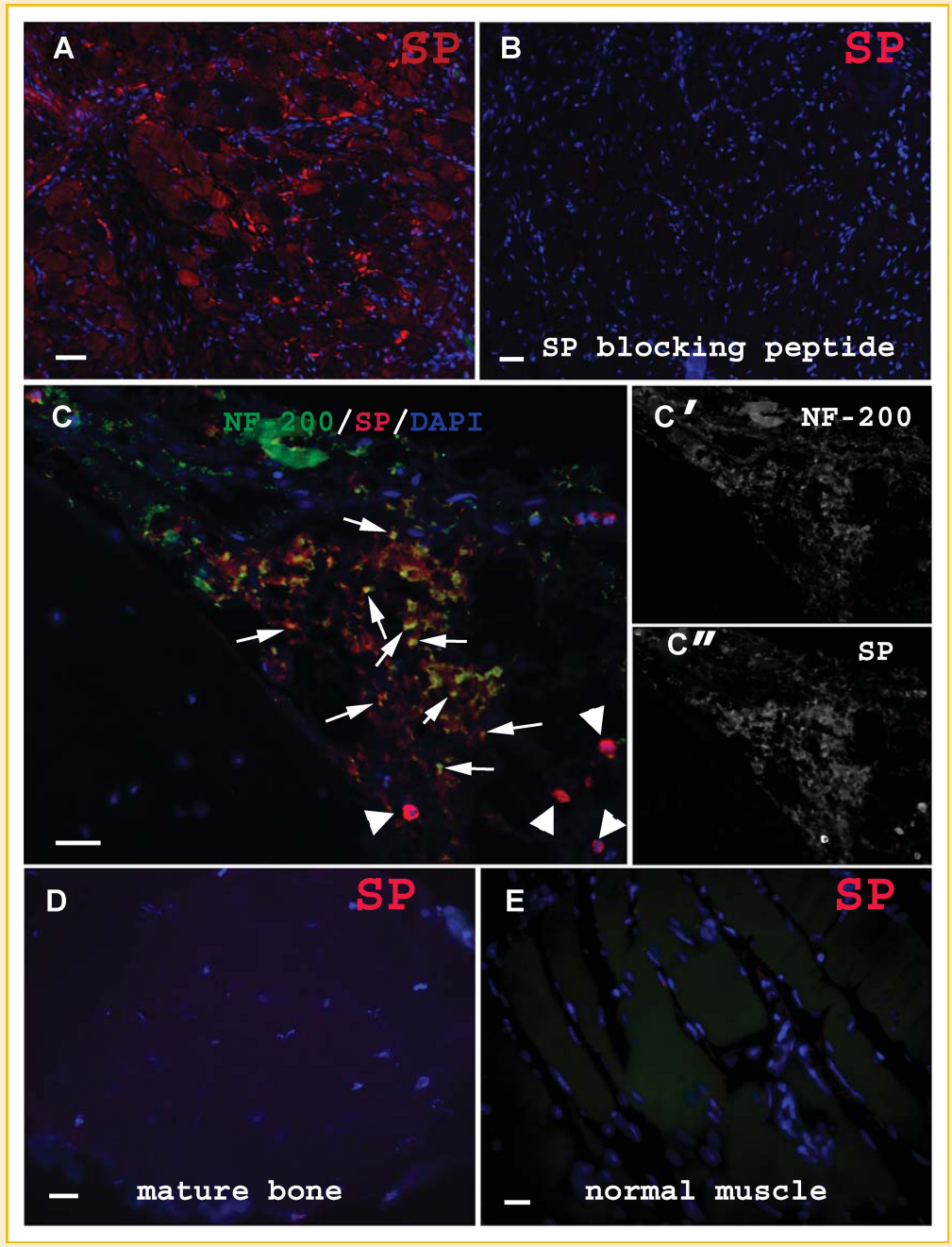

Fig. 2. SP staining is specific, and co-localized with a neuronal marker. A,B: Peptide blocking experiment indicated that both punctate and the semi-diffuse staining observed in early lesions are specific. A: Representative image of SP staining in an early lesion from a FOP patient. B: Both punctate and the semi-diffused staining in a adjacent section is blocked by SP peptide. C: Representative image of SP/NF-200 double staining in an early lesion from a traumatic brain injury (TBI) patient. Note that there is extensive co-localization of SP and NF-200 (white arrows in C). Also note that there are some NF-200$^-$ cells also express high level of SP (white arrow heads in C). C′,C″: Split channel of NF-200 and SP, respectively. D: Representative image of SP stained in mature HO from a sample of a TBI patient. E: Typical image of SP staining from a sample of normal control muscle. Bar = 40 μm.

early FOP lesions (Supplementary Fig. 2A) and in early lesions of acquired HO (Fig. 2C). However, some NF-200$^-$ cells that express high levels of SP were also found in the early lesions of acquired HO (Fig. 2C), interestingly, these NF-200$^-$ and SP$^+$ cells were not closely associated with or surrounded by diffused SP staining as NF-200$^+$ cells are, suggesting that they do not contribute significantly to the SP up-regulation in the lesion. In contrast, SP signals rarely co-localized with NF-200 in mature stages of FOP HO (Supplementary Fig. 2C). These data suggest that the predominant source of SP in the early pre-osseous lesions is neuronal (NF-200$^+$). Overall, our data support that SP dysregulation may play key roles in the pathophysiology of common human HO and in response to dysregulated BMP signaling in patients with FOP.

SP UP-REGULATION IN TARGET TISSUES OF ANIMAL MODELS IS BMP DEPENDENT AND INJURY INDUCED

In order to better understand the functional implications of SP up-regulation in HO, we studied the Nse-BMP4 transgenic mouse model that closely recapitulates FOP and also displays the histological hallmarks of acquired HO [Kan et al., 2009]. We first examined the expression of SP in subcutaneous connective tissue, and muscles of the hind limbs, target tissues where HO formation occurs in response to injury [Kan et al., 2009]. As a control, we also compared SP expression in other neuronal and non-neuronal tissues that could potentially be indirectly involved in the HO process, including the secondary immune system (spleen and lymph nodes), primary immune system (thymus), DRG, and central nervous system (CNS) (Supplementary Fig. 5, and data not shown). There were no obvious transgene dependent changes in SP expression in any tissues from postnatal or young uninjured Nse-BMP4 mice.

To determine whether the SP up-regulation in target tissues is triggered by injury, we performed superficial and deep muscle injury in young (1-month old) Nse-BMP4 mice and examined SP expression in the injured and uninjured limbs of the same mice. No transgene-dependent SP up-regulation is detected in naïve animals (data not shown). However, in response to injury, the limbs of Nse-BMP4 mice showed substantially increased SP expression compared to the uninjured limbs as early as 1.5 h after injury (Fig. 3A–C). In contrast, the increase in SP was minimal in WT mice under the same conditions (data not shown). At 1 day after injury,

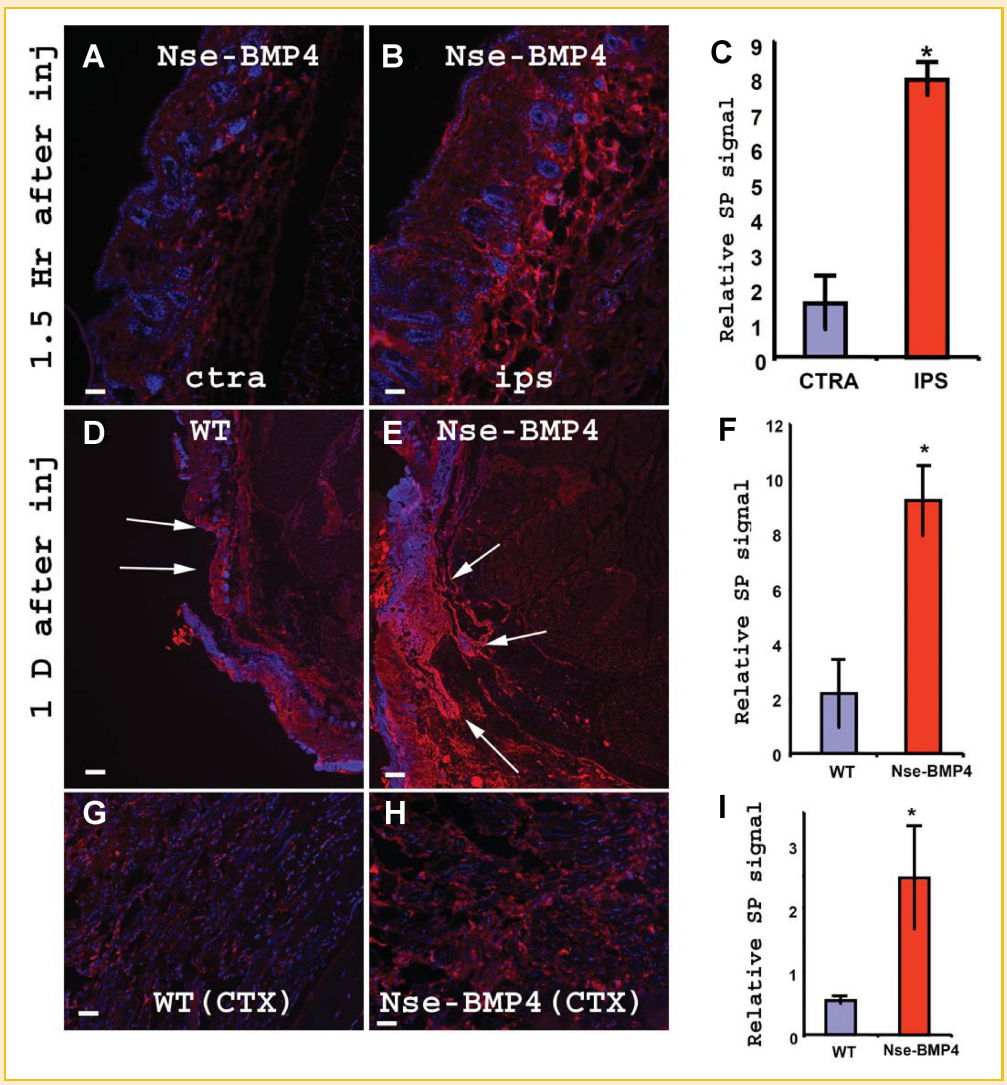

Fig. 3. Injury induced transgene dependent SP up-regulation in connective tissue and muscles. Both superficial muscle injury (A–F) and CTX-induced deep muscle injury (G–I) induced SP up-regulation. A,B: Representative images of SP immunostained cross sections of contralateral uninjured (A: control) and injured (B) limbs 1.5 h after superficial muscle injury. Note the dramatic SP up-regulation and edema in the injured site (B). C: Quantification of the relative SP signal in (A,B) ($^*P < 0.01$ vs. control by ANOVA). Error bars represent SD. D,E: Representative images of SP-immunostained cross sections of injured WT (D) and Nse-BMP4 (E) limbs 1 day after superficial muscle injury. Note the dramatic SP up-regulation and edema in Nse-BMP4 mice (E) arrows in (D) and (F) point to injury site. Note the dramatic SP up-regulation and edema in (E). F: Quantification of the relative SP signal in (D,E) ($^*P < 0.01$ vs. control by ANOVA). Error bars represent SD. G,H: Representative images of SP-immunostained cross sections of injured WT (G) and Nse-BMP4 (H) limbs after CTX-induced injury. I: Quantification of the relative SP signal in (G,H). ($^*P < 0.01$ vs. control by ANOVA). Error bars represent SD. Bar = 40 μm in A, B, G and H, Bar = 200 μm in D and E.

dramatically increased SP expression was observed in injured Nse-BMP4 mice compared to WT mice (Fig. 3D–F). More importantly, Similar SP up-regulation was also observed in CTX-induced deep muscle injury model (Fig. 3G–I), which further strengthened our conclusion.

The observed SP up-regulation in the Nse-BMP4 mouse model could arise from neuronal tissue, non-neuronal tissue, or a combination of both. However, data from double staining of human samples suggested that neuronal SP is the predominant source, at least in early lesions. Double staining of the mouse sections also supports this conclusion (Supplementary Fig. 6).

To explore the underling mechanism and further confirm the injury induced and BMP signaling-dependent SP up-regulation in other in vivo systems, we took the advantage of two other well-established mouse models: the floxed caALK2 mouse model (used as a mouse model of FOP) [Yu et al., 2008], and BMP4 matrigel injection model. Adenovirus-Cre was mixed with CTX and injected into hindlimb muscles of caALK2 transgenic mice to induce muscle injury and local caALK2 expressing cells. We repeated the injury-induced, caALK2-dependent, SP up-regulation in this model (Supplementary Fig. 7). Co-localization study with NF200 further suggested the neuronal contribution to SP up-regulation (Supplementary Fig. 8). Detailed study suggested a paracrine, rather than an autocrine mediated mechanism of action, because robust SP did not co-localize with GFP (caALK2+) cells (Supplementary Fig. 8). To further test whether injury and exogenous BMP4-signaling function synergistically in SP up-regulation and HO induction, we mixed BMP4 with matrigel, with or without CTX, followed by intramuscular injection to induce HO. Indeed, we also confirmed the BMP4-dependent SP up-regulation in this model (Supplementary Fig. 9). Interestingly, even though CTX alone does not appreciably up-regulate SP, local SP up-regulation was more dramatic in the mice that were treated with CTX + BMP4 matrigel than that of BMP4 matrigel alone (Supplementary Fig. 9).

Since hind limb musculature is richly innervated by SP^+ sensory nerve fibers, we examined lumbar dorsal root ganglion (DRG) in Nse-BMP4 mice to determine the possible contribution of SP^+ sensory neurons to the injury induced increase in SP expression. In young (3–4 weeks old), uninjured Nse-BMP4 mice, the number and pattern of SP expression by DRG neurons did not differ from WT mice (Supplementary Fig. 10). However, in injured, or adult Nse-BMP4 mice with HO, we observed an unusual mesh-like pattern of SP expression in which cellular staining of SP was not prominent, whereas staining in the tissue surrounding neurons was dramatically increased (Fig. 4C). The p75 low-affinity neurotrophin receptor (p75) is expressed by almost all sensory neurons in the adult DRG [Lee et al., 1992]. Quantitative analysis found that the number of SP^+ $p75^+$ cells in the DRG of old Nse-BMP4 mice that had HO was lower, even though the number of total $p75^+$ neurons was similar (Fig. 4E). We reasoned that over-release of SP from peripheral sensory neurons could lead to this staining pattern by depleting the cytoplasmic SP in the cell bodies of the DRG. To directly test this hypothesis, we pretreated Nse-BMP4 old mice that had HO with colchicine, which disrupts and blocks the axoplasmic transport and release of SP [Paulson and McClure, 1975]. We then compared the SP expression pattern of treated and untreated lumbar DRG, and found that the normal expression pattern of SP was largely restored in colchicine-treated Nse-BMP4 mice (Fig. 4D). Further, the number of SP^+ neurons was dramatically increased after colchicine treatment in Nse-BMP4 mice but not in age-matched WT mice (Fig. 4E). These observations exclude the possibility that the low number of SP^+ neurons in DRG of older Nse-BMP4 mice was due to reduced survival and strongly support the hypothesis of injury-induced over-release of the peptide.

Since the observed peripheral SP up-regulation was induced by injury, we speculated that SP over-release/depletion in DRG neurons could also be induced by injury. To directly test the hypothesis, superficial muscles were injured in young (1-month old) Nse-BMP4 mice which do not display the mesh-like pattern of SP immunostaining. The mesh-like pattern was reproduced as early as 1.5 h after injury (Fig. 4F,G), coincident with the up-regulation of SP in the injured skin (Fig. 3). These observations suggest that SP^+ DRG neurons contribute to the injury-induced increase in SP levels.

Since the specific SP receptor, NK1r is expressed by DRG neurons [Andoh et al., 1996], it is possible that the SP release acts through paracrine and/or autocrine mechanisms to regulate SP expression. To test this hypothesis, SP levels were examined in DRG of NK1r antagonist-treated old Nse-BMP4 mice. Unlike colchicine, RP-67580 treatment of old Nse-BMP4 mice restored only a small and variable degree of normal immunostaining (Fig. 4H,I) suggesting that paracrine/autocrine SP signaling is not the predominate mechanism underlying the increased release of SP. However the mesh-like pattern of SP immunostaining was less prominent after RP-67580 treatment suggesting at least some role for paracrine/autocrine signaling.

To further test the role of neuronal SP, we selectively ablated SP^+ neurons in DRG of neonatal (1–2 days old) Nse-BMP4 mice by capsaicin treatment [Sugimoto et al., 1998] and waited until the treated animals were 1–2 months old to test injury-induced short-term and long-term effects. Neonatal capsaicin treatment is known to selectively and irreversibly destroy small diameter DRG sensory neurons without obvious effects on non-neuronal cells [Nagy et al., 1981]. The effectiveness of ablation was further confirmed by comparing SP stained lumbar DRG sections from the capsaicin and vehicle pretreated young WT and Nse-BMP4 mice (1-month old). We found that the small diameter SP^+ neurons were almost completely absent in lumbar DRG of capsaicin-treated mice (Supplementary Fig. 11). Once the effectiveness was confirmed, capsaicin and vehicle-pretreated adult WT and Nse-BMP4 mice were subjected to standard injury. Injured and control hind limbs and DRG were harvested at 1 h and 1 day after injury and examined for SP expression. We found that in DRG, the injury-induced mesh-like pattern was absent in capsaicin-pretreated Nse-BMP4 mice (Fig. 5B). In hind limbs, injury-induced SP up-regulation in target tissues of the capsaicin treatment group was also minimal, compared to vehicle-treated mice (Fig. 5E, F, and I). Consistently, H&E staining demonstrated the lack of massive inflammatory response in capsaicin-pretreated Nse-BMP4 mice, compared to vehicle-pretreated ones (Fig. 5G, H, and J). These data further support the hypothesis that the mesh-like pattern is caused by over release of SP, that neuronal SP is necessary for the injury-induced SP up-regulation in target tissues, and that SP is necessary to amplify the

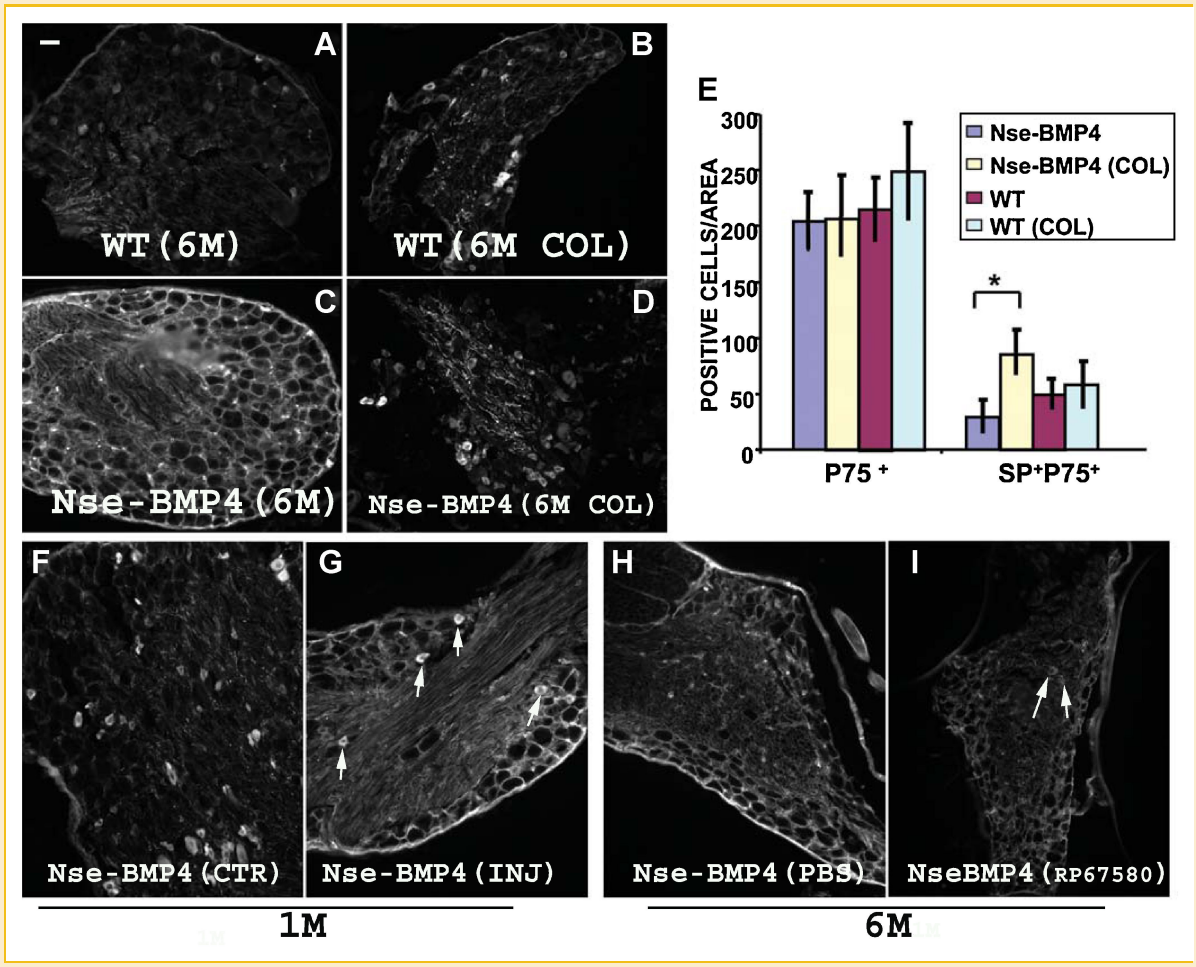

Fig. 4. Injury induced a unique immunohistochemical phenotype (mesh-like SP staining pattern) of lumbar dorsal root ganglion (DRGs) of Nse-BMP4 mice. Sections of lumbar DRGs from age-matched 6-month-old WT (A,B) and Nse-BMP4 with HO (C,D) mice were immunostained for SP. A,C untreated, and B,D, pretreated with colchcine (COL). Note that the unique mesh-like SP-staining pattern was found only in Nse-BMP4 (C), and that the normal pattern was restored by colchcine treatment in Nse-BMP4 mice (D). E: Depicts the SP^+ cell densities of different subpopulations of neurons in 6-month-old Nse-BMP4 and age-matched WT lumbar DRGs. Note that colchcine treatment increased the density of SP^+ neurons in Nse-BMP4 DRGs without significantly changing the total number of $P75^+$ neurons. $^*P < 0.01$ vs control by ANOVA. F,G: The mesh-like staining pattern is induced by superficial muscles injury (G, 1.5 h after injury) in younger (1-month old) Nse-BMP4 mice. F: SP-immunostained sections of lumbar DRGs from 1-month-old naïve Nse-BMP4. Note that cytoplasmic staining of SP is still observed at this time point. White arrows point to the SP^+ cell bodies in (G). NK1r antagonist (RP-67580) treatment had variable efficiency in restoring the normal staining pattern of SP in old Nse-BMP4 mice (H,I). SP stained sections of lumbar DRGs from 6-month-old Nse-BMP4 treated with PBS (H), and 6 month old Nse-BMP4 pretreated with NK1r antagonist for 2 weeks (I). Note that the normal staining pattern of SP in old Nse-BMP4 mice was only partially restored with NK1r antagonist treatment. White arrows point to the SP^+ cells. Bar = 40 μm. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

inflammatory response. Importantly, the efficiency of HO formation (4 weeks after injury) was greatly reduced in capsaicin-pretreated adult Nse-BMP4 mice (Fig. 6A), a finding that further supports that the abnormal inflammatory response induced by neuronal SP secretion mediates HO formation.

Taken together, these in vivo studies suggest that SP up-regulation is dependent on increased BMP signaling, and that the neuronal source is the major contributor. To directly test whether BMP4 could influence the expression and release of SP in a dissociated neuronal population, lumbar DRGs were harvested, dissociated and cultured with or without BMP4 treatment. We found that SP release and expression was up-regulated by BMP4 treatment in WT sensory neurons. Interestingly, both the expression and release of SP from cultured Nse-BMP4 neurons were up-regulated, and this effect was blocked by Noggin treatment indicating a ligand-dependent paracrine effect (Supplementary Fig. 12 and data not shown). The caveat of this in vitro study is that behavior of dissociated and cultured sensory neurons likely reflect the in vivo function of injured instead of naïve sensory neurons, and the observed plateaued response of Nse-BMP4 neurons to BMP4 treatment likely reflects the saturation of BMP signaling in this condition.

NK1r ANTAGONIST INHIBITS INJURY-INDUCED INFLAMMATION AND HO

These observations suggested that inhibition of the SP receptor, NK1r, might therefore be a novel treatment for preventing the early events that lead to HO. To test this hypothesis, we treated Nse-BMP4

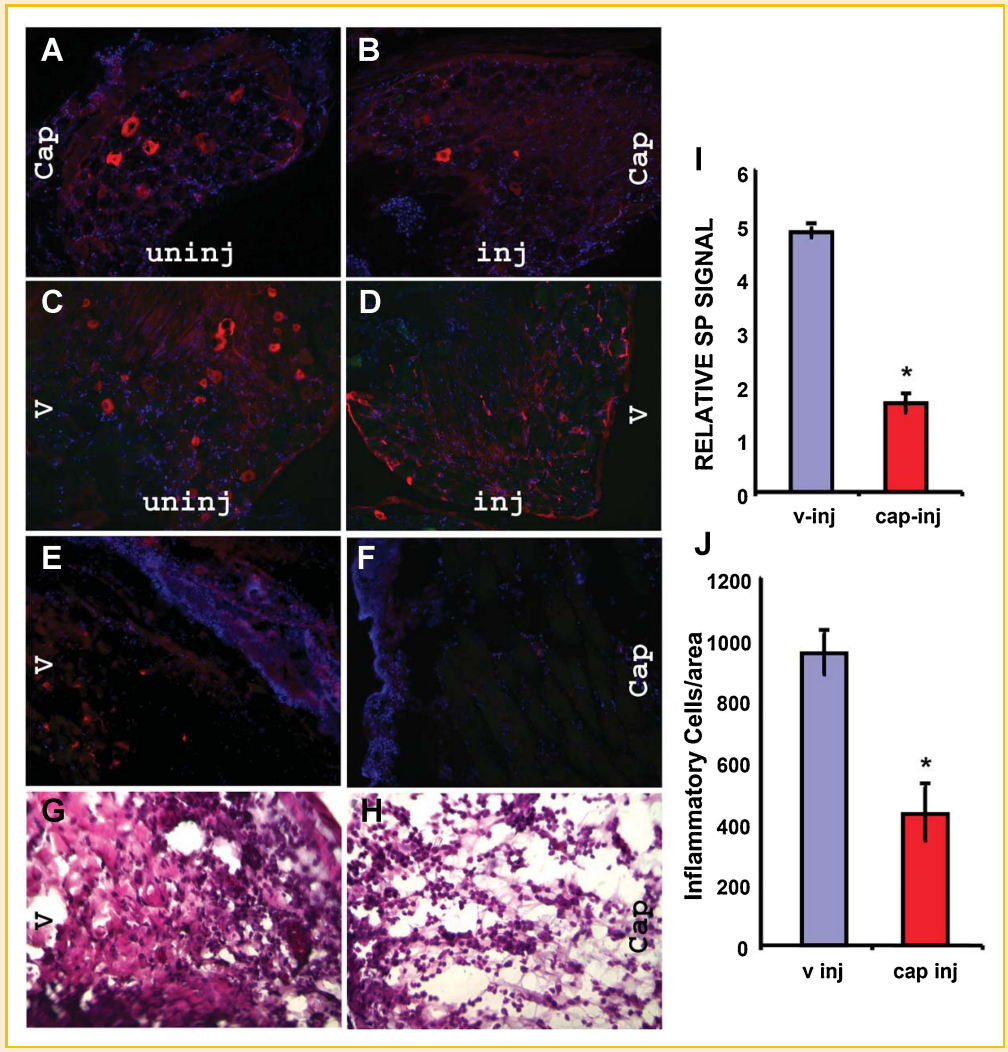

Fig. 5. Ablation of SP^+ neurons inhibits injury-induced SP up-regulation and inflammation. Small diameter SP^+ sensory neurons were virtually absent in capsaicin-pretreated Nse-BMP4 DRG (A,B), compared to vehicle-treated ones (C,D). DRG depicted in (A,C) are from uninjured side, while (B,D) DRG are from injured side. Note that injury did not lead to injury-induced mesh-like pattern in capsaicin-pretreated Nse-BMP4 mice (B), compared to vehicle-treated one (D). Ablation of SP^+ neurons inhibits injury-induced SP up-regulation in target tissues of Nse-BMP4 mice (E,F). Cross sections of hind limbs from the injury site are shown here. Vehicle (E) and capsaicin-pretreated (F) mice were subjected to similar superficial muscles injury, hind limbs were harvested 1 h after injury (shown) or 1 day after injury (not shown). Reduced SP up-regulation was observed in lesions from capsaicin-pretreated Nse-BMP4 mice (F), compared to vehicle-pretreated mice (E). The strong SP staining was found closely associated with muscle fibers. Infiltrating cells are indicated by dense $DAPI^+$ regions. G,H: H&E staining clearly indicated that ablation of SP^+ neurons inhibited injury-induced inflammatory response. G: Image from injured site (1 h after injury) of vehicle-pretreated Nse-BMP4 mice demonstrated massive infiltration of inflammatory cells. In contrast, much less inflammatory cells infiltration was observed in injured site of capsaicin-pretreated Nse-BMP4 mice (H). I: Quantification of the relative SP levels observed in (E,F). $^*P < 0.01$ versus vehicle control by ANOVA. J: Quantification of the numbers of inflammatory cells observed in (G,H). $^*P < 0.01$ versus vehicle control by ANOVA.

mice (2 months old, without pre-existing HO) with the specific NK1r antagonist RP-67580 [Moussaoui et al., 1993; Shepheard et al., 1993; Kennedy et al., 1997] or PBS after superficial or deep muscle injury. The percentage of mice that developed HO after superficial muscle injury in RP-67580-treated groups (40%) was significantly lower than in the control group (80%) 4 weeks after injury (Fig. 6), indicating that RP-67580 inhibited HO formation. Similar results were also observed by micro-CT analysis from CTX-induced deep muscle injury model (Fig. 6). Overall these findings suggest that an NK1r antagonist can effectively prevent HO.

To directly test if NK1r antagonists prevent HO by inhibiting the early inflammatory response, two additional experiments were preformed. We found the edema and inflammation were markedly reduced in RP-67580-treated Nse-BMP4 mice both grossly and histologically (Fig. 6). Conversely, administering the same treatment to older Nse-BMP4 mice that had already developed HO provided no observable beneficial effect in RP-67580-treated group (data not shown). Both experiments suggested that the Nk1r antagonist (RP-67580) worked mainly through blocking the initial inflammatory response.

ENDOGENOUS SP SIGNALING IS ESSENTIAL FOR HO

To directly test the requirement for SP signaling for HO, we mated Nse-BMP4 mice with SP precursor null mutant (PPT-A−/−) mice to

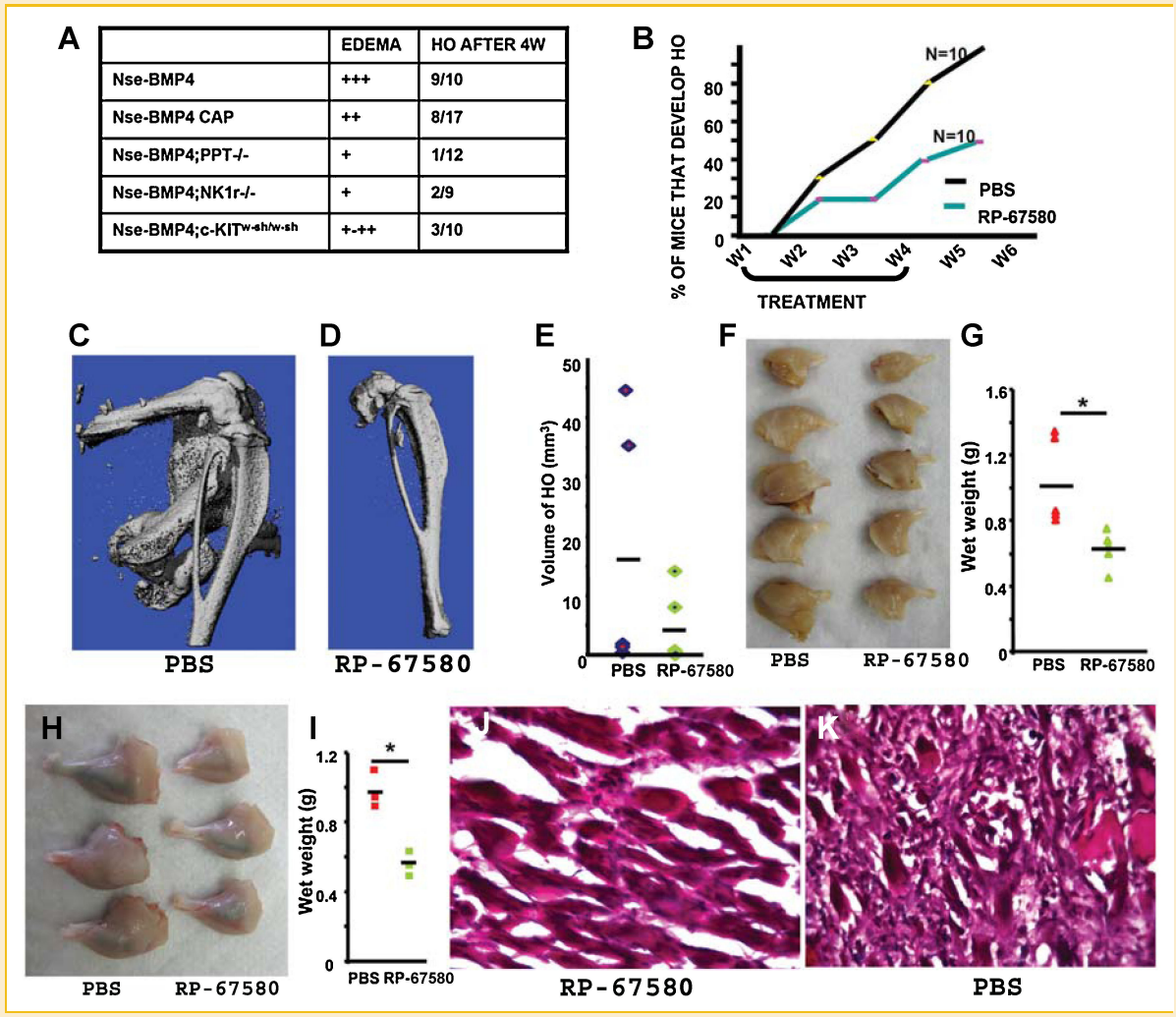

Fig. 6. Inhibition of SP-signaling blocks injury-induced inflammation and HO. A: summarizes superficial muscles injury-induced edema and HO in different conditions. Nse-BMP4 mice without endogenous SP signaling (PPT-A−/−, Nk1r−/−, or ablation of SP+ neurons), or mast cells (c-kit$^{w-sh/w-sh}$) did not develop injury-induced HO efficiently. B: Effect of SP receptor (NK1r) inhibition (RP-67580) in response to superficial muscles injury. Frequency (percent of mice) of HO formation over time (1–6 weeks) with RP-67580 or PBS treatment is shown. (C-G) RP-67580 inhibited HO in CTX-induced muscle injury model. C: CTX-induced HO (3D reconstruction) from a PBS-treated Nse-BMP4 mouse. D: CTX-induced HO was inhibited by RP-67580 treatment. E: Total HO volumes 1 month after muscles injury in RP-67580 and PBS-treated groups. F: Gross images from the same groups. G: Total wet weights of affected hind limbs in the same groups. $^{*}P < 0.01$ versus control by ANOVA. Note that the total HO volumes, total wet weights, and gross images consistently indicated the inhibition effect of RP-67580. H–K: RP-67580 specifically inhibited injury-induced edema and inflammation. H: Gross images 7 days after injury from RP-67580 and PBS-treated limbs. Note that PBS-treated limbs show increased edema (reflected by sample circumference) compared to RP-67580-treated limbs. I: Total wet weights of affected hindlimbs from the same groups. $^{*}P < 0.01$ versus control by ANOVA. J,K: Typical images of H&E staining from RP-67580 and PBS-treated group, respectively. Note that the inflammatory response is dramatically reduced in RP-67580-treated lesion (J), comparing to PBS (K)-treated lesion.

generate Nse-BMP4; PPT-A−/− double mutant mice. These mice survive without any gross phenotype. However, unlike the Nse-BMP4 mice, the double mutant mice failed to form HO in response to injury (Fig. 6) indicating that endogenous SP signaling is essential for HO formation. To further determine if the observed phenotype is SP signaling specific, we mated Nse-BMP4 mice with SP receptor NK1r−/− mice [Bozic et al., 1996] and subjected them to similar injury. We found that Nse-BMP4;NK1r−/− mice form HO with reduced efficacy (Fig. 6), which essentially mimicked the phenotype of Nse-BMP4; PPT-A−/− double mutant mice, and further supporting the conclusion that SP signaling plays a central role in HO formation.

MAST CELLS ARE REQUIRED FOR THE SP-MEDIATED INDUCTION OF HO

SP induces release of other inflammatory mediators through Nk1r mast cell-dependent pathways [Yano et al., 1989; Saban et al., 2002], which stimulates further leukocyte recruitment, thereby amplifying the inflammatory response. We reasoned that this amplification might be crucial for the transgene-dependent abnormal injury responses. More importantly, recent work further suggested that degranulation of mast cells required direct interaction between mast cells and sensory nerve terminals [Suzuki et al., 2004; Folgueras et al., 2009] and specific NK1r expression was observed on cell surface of mast cells [Okada et al., 1999].

Not surprisingly, we observed massive mast cells infiltration after muscle injury in Nse-BMP4 mice, compared to that of WT controls. More interestingly, mast cells were highly enriched in areas surrounding early inflammation, often immediately adjacent to, or contacted directly with fibers of neurons ($NF200^{+}$) (data not shown), and also in proximity to small-medium-sized blood vessels. This pattern is consistent with the proposed early proinflammatory function of mast cells (Supplementary Figs. 13 and 14).

To directly test whether mast cells are necessary for HO, we utilized the mast cell deficient mouse line, $c\text{-}kit^{w\text{-}sh/w\text{-}sh}$ [Duttlinger et al., 1993]. We first generated Nse-BMP4; $c\text{-}kit^{w\text{-}sh/w\text{-}sh}$ double transgenic mice and confirmed that mast cells are indeed deficient in these double transgenic mice by counting the toluidine blue-positive cells (mast cells) in the skin of Nse-BMP4;$c\text{-}kit^{w\text{-}sh/w\text{-}sh}$ double mutant and $c\text{-}kit^{w\text{-}sh/w\text{-}sh}$ mice. We found that mast cells were virtually absent in these double mutants (Supplementary Fig. 15). Previous reports have indicated that mast cells mediate downstream effects of SP [Saban et al., 2002]. Consistently, we found that both Nse-BMP4 single transgenic mice and Nse-BMP4; $c\text{-}kit^{w\text{-}sh/w\text{-}sh}$ double mutant mice had similar levels of injury-induced SP up-regulation (data not shown). However, the efficiency of HO formation was dramatically reduced in Nse-BMP4; $c\text{-}kit^{w\text{-}sh/w\text{-}sh}$ double mutant mice in response to injury, compared to Nse-BMP4 single transgenic mice (Fig. 6), indicating that mast cells (or factors from mast cells) are required to mediate the downstream injury response to produce HO. Since mast cells are not known to produce any chondrogenic or osteogenic factors directly, it is likely that mast cells contribute to HO indirectly by amplifying the injury response, at least in part through the SP receptor (NK1r).

DISCUSSION

An elusive neuro-inflammatory connection to HO has long been suspected. We show here that neuro-inflammatory signaling through Substance P (SP) induces and mediates BMP-dependent HO. Our study shows that a critical inductive event in HO is the neural release of BMP-dependent SP. Dramatic up-regulation of SP was observed not only in patients with FOP and acquired HO, but also in three independent animal models of HO. Importantly, blocking neuron-specific SP signaling through the NK1r receptor abrogates HO formation. Since the Nk1r inhibitor RP-67580 crosses the blood-brain barrier poorly, this drug likely acted on peripheral tissues rather than the CNS [Barr and Watson, 1993; Holzer-Petsche and Rordorf-Nikolic, 1995] This conclusion is supported by the observations that ablation of SP^{+} neurons in DRG or ablation of the mast cell-dependent local amplifying circuitry dramatically reduced injury-induced HO. Null mutation of the PPT gene completely blocked HO formation, further implicating SP signaling in the pathogenesis of the disorder. Taken together, these data implicate neuron-specific SP up-regulation as a common neuro-inflammatory inductive factor for hereditary and sporadic HO and provide a molecular target for therapeutic intervention.

Our data also show that anterograde stimulation of DRG likely induces the retrograde transport of SP not only to the skin, but also to skeletal muscle and other connective tissues where it acts on NK1r receptors on tissue mast cells to amplify the inflammatory response and stimulate HO. Taken together, our studies in three independent animal models suggest that elevated BMP signaling indirectly influences the expression and release of SP in injured site through a paracrine-mediated mechanism.

Although it is generally recognized that SP expression is increased in many inflammatory conditions, there is currently no consensus on how SP is regulated at the mRNA and protein levels in DRG in response to injury [Reinshagen et al., 1994; Castagliuolo et al., 1998]. Measurement of total levels of SP or its transcripts did not reveal the critical pattern changes that were found in this study suggesting that these measures are not sufficient to gauge changes in the regulation of SP in DRG. Rather, our data suggest that the unique staining pattern in DRG reflect increased release of SP in response to injury. DRG neurons are pseudo-unipolar with axons that bifurcate into two branches, a distal process that innervates peripheral tissues (e.g., skin and muscles) and a central process that innervates spinal cord. The velocity or efficiency of axoplasmic transportation along the two branches is asymmetrical, even though the diameters of two branches are similar [Ochs et al., 1978], and 80% of SP is transported peripherally and only 20% centrally [Harmar and Keen, 1982]. This may explain why the dramatic SP up-regulation was observed in the periphery, but not in the spinal cord. SP is also known to be expressed by some non-neuronal cells, and release of the peptide from these cells could be up-regulated in some pathological conditions [Castagliuolo et al., 1997]. However, the role of the non-neuronal SP in HO is still unclear.

Our demonstration that mast cells are a downstream target of SP in this neuro-inflammatory process has important therapeutic implications and is consistent with prior studies [Gannon et al., 2001; Kulka et al., 2008; Freeman et al., 2010]. Mast cells are present in all HO lesions and are particularly abundant at all stages of FOP lesions and in BMP4-induced HO [Gannon et al., 2001]. Our findings are also consistent with previous findings showing that sprouting peripheral nerve fibers is a common observation in new bone formation [Hedberg et al., 1995] and fracture healing [Madsen et al., 1998]. Interestingly, it was also reported that, in context of other lesions, SP-positive nerves had more contacts with mast cells compared to VIP- or CGRP-containing fibers, and tryptase, a mast cell-specific proteinase, could degrade VIP and CGRP, but not SP [Naukkarinen et al., 1994]. These, together with our data, may explain why only SP is specifically up-regulated.

Our current working hypothesis is that following injury, BMP signaling up-regulates SP release in DRG neurons which, in turn, activates mast cells that produce factors that amplify local inflammation. Little is known about the post-inflammatory pre-osseous events in HO, but SP may also recruit local progenitors/stem cells to form a fibroproiferative lesion that is common to all forms of sporadic and hereditary heterotopic endochondral ossification [Hong et al., 2009]. A recent study indicates that vascular endothelial cells can be transformed into multipotent stem-like cells in an ACVR1 or BMP4-dependent manner through an endothelial-to-mesenchymal transition, consistent with both the molecular genetics and pathology of FOP [Medici et al., 2010]. Based on the study, it is reasonable to postulate that dysregulated BMP signaling plays dual roles in the chronicle phase of HO induction:

first, in the induction of stem-like cells, and then in their endochondral transformation to heterotopic bone. Our data show that SP acts downstream of BMP signaling to orchestrate this complex process.

Overall, the current study has profound therapeutic implications. Our study identifies SP as a potent inductive neuro-inflammatory factor for HO and places it in a neuro-inflammatory amplification circuit that provides novel cellular and molecular targets for therapeutic intervention. Our findings specifically suggest that NK1r antagonists or mast cell inhibitors might provide specific chronic targets for preventing bone formation, in both hereditary and acquired forms of HO.

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