Crosstalk between Sensory Neuropeptides Regulating Heterotopic Ossification in Tendon

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Introduction: Heterotopic ossification (HO) is bone formation within soft tissues, including the tendons. It is a costly medical problem with no effective cure. Two main causes are neurogenic and musculoskeletal traumas, where in both cases inflammation leads to a release of certain factors, which can in turn trigger overexpression of bone morphogenetic proteins (BMPs). BMP-2, one of the most studied of the BMPs, has been shown to be involved in most common cases of HO by promoting osteogenic differentiation of primitive stem cells into osteocytes within the injury site. Despite advances in understanding the pathophysiology of HO, the associated cellular and molecular mechanisms are not well understood. The peripheral nervous system, through specific neuromediators, plays an active role in regulation of cellular and extracellular changes observed in ossified tendons. Sensory neuropeptides, including substance P (SP) and calcitonin gene-related peptide (CGRP), are maintained at low levels in asymptomatic tendons but upregulated in patients with injured or degenerative tendons, as well as in animal disease models[1-4]. Both SP and CGRP are known to be involved in BMP-2 induced osteogenic differentiation in vitro and HO in vivo, however, further investigation of the interaction between the two neuropeptides on BMP-2 signaling is warranted. Here, we assessed the effect of SP and/or CGRP on BMP-2 induced osteogenic differentiation and possible interactions between the three pathways in vitro and in vivo. Unique to this study is to apply biopatterning technology to deliver SP and/or CGRP at millimeter scale resolution and in physiologically relevant doses for spatial control of the tendon microenvironment in vivo[5].

Methods: For the first set of in vitro experiments, a representative pluripotent myoblastic progenitor cell line, C2C12, was cultured and stimulated with SP, CGRP, SP and CGRP (100ng/ml), with or without BMP-2 (100ng/ml), replacing treatments every after 48h. Osteogenic differentiation was measured 24h after the second treatment through alkaline phosphatase activity (n=9). The second set of experiments assessed osteogenic differentiation through a late osteogenic marker, mineralization, where a pre-osteoblastic cell line, MC3T3, was cultured in osteogenic media and stimulated with the same set of treatments for 18 days with media changed every 72 h. Mineralization was measured through Alizarin Red Staining (ARS) (n=6). For the in vivo experiments, DermaMatrixTM (DM)(Synthes Inc., West Chester, PA) constructs (2mm x 4mm patterns in the central portion 2mm x 8mm strips) were biopatterned with neuropeptides (SP, CGRP, SP and CGRP) using our custom inkjet-based bioprinter at a spatial resolution of 80 µm to yield a final concentration of 60ng/mm2 as described in detail previously [6]. Achilles tendons of 3-5 months old C57BL/6 male mice were surgically exposed and tunnels were drilled through the calcaneus as an anchor point for suturing (n=4-6 for each treatment). The unprinted regions of DM (2mm x 2mm on each end) were sutured to the calcaneus and to the gastrocnemius, while the biopatterned region was sutured over the entire tendon body. Control animals received DM with no treatment. At week 6, the animals were sacrificed, and high-resolution microcomputed tomography (µCT) was performed to measure HO around the calcaneus and mid-tendon area. All animal studies were done under approved IACUC protocol.

Results: Neither of treatments had a significant effect on BMP-2 -induced ALP activity in C1C12s (Fig. 1A,B), whereas SP by itself enhanced BMP-2 induced mineralization in MC3T3s compared to controls, and CGRP had no significant effect (Fig.2A,B). Remarkably, CGRP when added with SP reversed the effect of BMP-2 where mineralization was significantly reduced even compared to controls with BMP-2 treatment alone (Fig. 2A,B) ($p \leq 0.001$). Fig. 3 shows µCT images from all treatment groups and quantification of bone volume. These data indicate SP by itself can promote HO in our Achilles tendon model, while CGRP by itself has no effect. However, when CGRP is delivered with SP, CGRP reverses the effect of SP, inhibiting SP-induced HO formation within the Achilles tendon of mice and furthermore CGRP reduces bone formation significantly compared to control HO ($p \leq 0.01$).

Discussion: Our in vitro and in vivo data suggest a possible connection between SP and CGRP pathways and the BMP-2 pathway. In vitro, SP alone can enhance osteogenesis while CGRP has no significant effect on BMP-2 -induced osteogenic differentiation. In vivo, SP promotes HO but CGRP has no effect on HO. Interestingly, when SP and CGRP are delivered together, both in vitro and in vivo, CGRP can counteract the effect of SP on BMP-2 induced osteogenic differentiation and bone formation.

Significance: We were able to promote and further reverse HO in Achilles tendons of mice using biopatterned DM constructs. To our knowledge there is no other study reported that involves administration of SP and/or CGRP in tendon to elucidate the effect of these neuropeptides in tendon HO as well as the interaction between SP and CGRP related to tendon HO. Characterization of the interaction between the SP, CGRP and BMP-2 pathways might be of clinical interest.

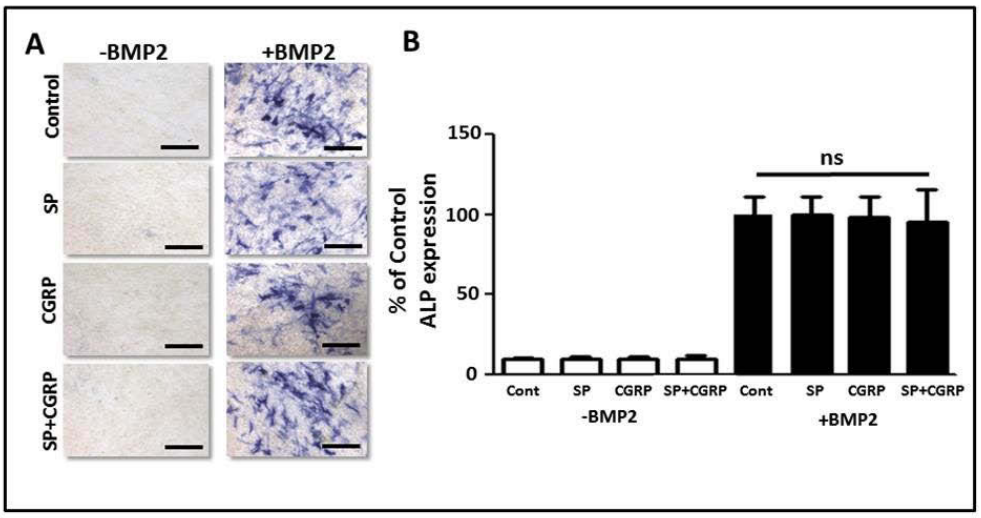

Figure 1 C2C12s cultured with neuropeptides ±BMP-2 **A.** Cells cultured in normal growth medium (Control); growth medium with SP, CGRP, SP and CGRP; with or without BMP-2. Blue stands for the ALP stain. All treatments have a final concentration of 100ng/ml. Scale bar: 200µm. **B.** Quantification of ALP staining for each experimental group are normalized to control. n=9.

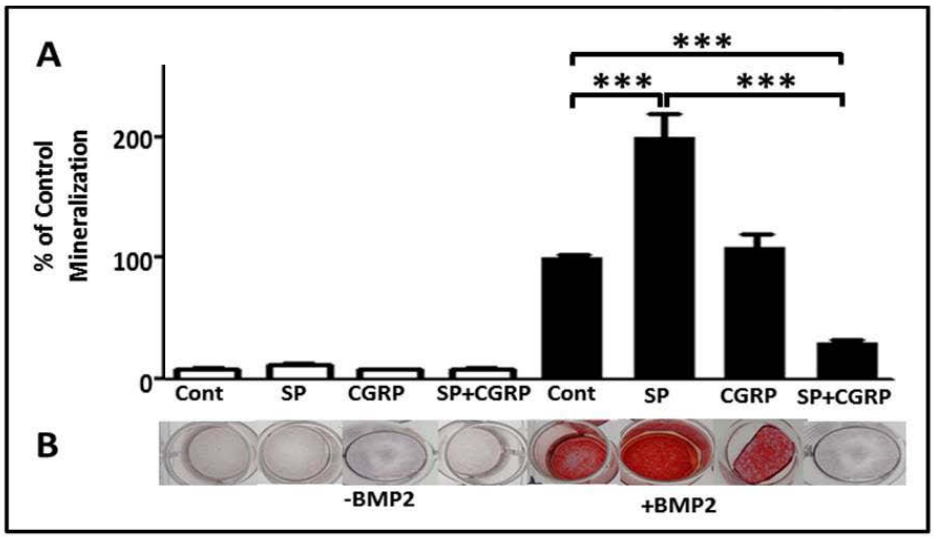

Figure 2 MC3T3s cultured in osteogenic media with neuropeptides ±BMP-2 **A.** Quantification of ARS for each experimental group are normalized to control. n=6-9, *** indicates p≤0.001. **B.** Whole plate images of pre-osteoblasts stimulated with SP, CGRP, SP and CGRP, with or without BMP-2. Red stands for ARS. All treatments have a final concentration of 100ng/ml. Controls did not receive any neuropeptide treatment.

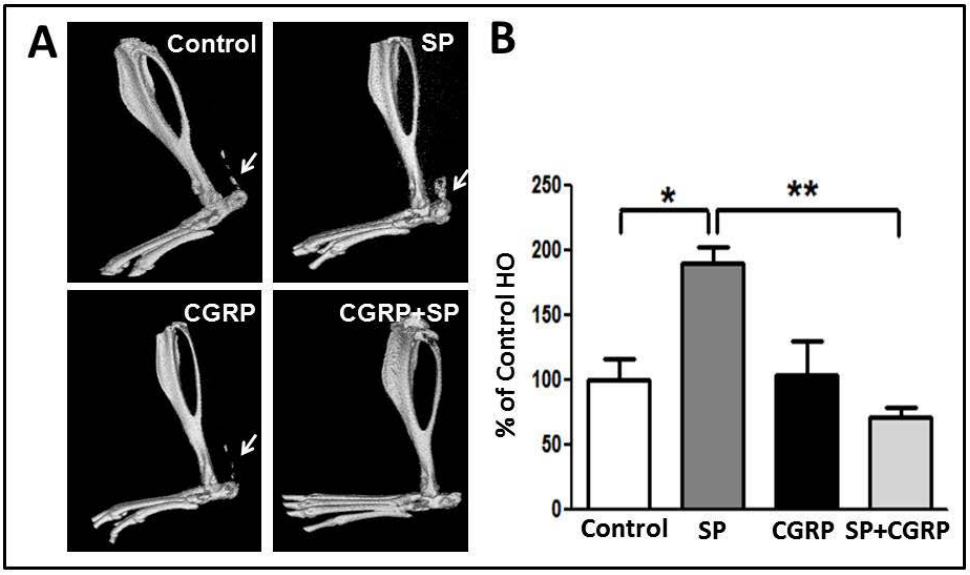

Figure 3. Biopatterned DM constructs implanted to Achilles tendon of mice **A.** µCT scans of the operated legs 6 weeks post-surgery, control legs receive DM with no treatment. **B.** Quantification of HO around calcaneus and mid-tendon, all measurements are normalized to control. n=4-6, * indicates p≤0.05, ** indicates p≤0.01.