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Fractone-heparan sulfates mediate BMP-7 inhibition of cell proliferation in the adult subventricular zone

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HIGHLIGHTS

- ► Bone morphogenetic protein-7 inhibits cell proliferation in the subventricular zone.
- ► Heparan sulfate proteoglycans mediate BMP-7 inhibition of cell proliferation.
- ► Extracellular matrix fractones bind BMP-7 via heparan sulfates proteoglycans.
- ► Fractones and vascular basement membranes promote BMP-7 in the adult brain.

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ABSTRACT

Bone morphogenetic protein-7 (BMP-7) is a heparin-binding growth factor that inhibits cell proliferation in the subventricular zone (SVZ) of the lateral ventricle, the primary neurogenic niche in the adult brain. However, the physiological mechanisms regulating the activity of BMP-7 in the SVZ are unknown. Here, we report the inhibitory effect of BMP-7 on cell proliferation through the anterior SVZ after intracerebroventricular injection in the adult mouse. To determine whether the inhibition of cell proliferation induced by BMP-7 is dependant on heparin-binding, heparitinase-1 was intracerebroventricularly injected to N-desulfate heparan sulfate proteoglycans before BMP-7 was injected. Heparatinase-1 drastically reduced the inhibitory effect of BMP-7 on cell proliferation in the SVZ. To determine where BMP-7 binds within the niche, we visualized biotinylated-BMP-7 after intracerebroventricular injection, using streptavidin Texas red on frozen brain sections. BMP-7 binding was seen as puncta in the SVZ at the location of fractones, the particulate specialized extracellular matrix of the SVZ, which have been identified primarily by N-sulfated heparan sulfate immunoreactivity (NS-HS+). BMP binding was also seen in NS-HS+ blood vessels of the SVZ. Injection of heparitinase-1 prior to biotinylated BMP-7 resulted in the absence of signal for biotinylated-BMP-7 in the fractones and blood vessels, indicating that the binding is heparan sulfate dependant. These results indicate that BMP-7 requires heparan sulfates to bind and inhibit cell proliferation in the SVZ neurogenic niche. Heparan sulfates concentrated in fractones and SVZ blood vessels emerge as a functional stem cell niche component involved in growth factor activity.
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1. Introduction

Bone morphogenetic protein-7 (BMP-7) is a member of the 15-member BMP family and of the transforming growth factor-beta (TGF-β) super-family. In the brain, members of the BMP family are involved in maintaining the pool of stem cells and the balance of neural stem cell quiescence/cell proliferation [4,24]. BMP-7, which is produced by the meninges and choroid plexus [31], intervenes in brain development [5] and inhibits neural stem cell proliferation in adulthood [14,17]. In the body, BMP-7 strongly influences cell proliferation and differentiation and plays crucial role in embryogenesis and osteogenesis. BMP-7 is a heparin-binding protein, i.e. a signaling molecule that requires heparan sulfate proteoglycans (HSPG) at the cell surface to exert its biological activity [14,17,30]. To our knowledge, it has not been determined whether BMP-7 requires HSPG to inhibit cell proliferation in the brain. The means by which BMP-7 achieves locally restricted activity in the anterior portion of the lateral ventricle subventricular zone (SVZa), the primary neurogenic niche in adulthood [6,8,18], remains to be clarified.

Pioneering studies have shown that hematopoetic growth factors require HSPG to promote blood cell proliferation [11,29]. More recent studies have shown that HSPG are required for the

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recognition and activation of FGF-2 by FGFR (receptors) at the surface of target cells during development [25] and adulthood [2,12]. HSPG, which consist of a core protein and covalently attached disaccharide chains, are found at the cell surface interstitial matrix or are concentrated in basement membranes [32] at the connective tissue/parenchyma interface [15]. Although not yet characterized, the high-diversity of heparan-sulfated motifs attached to the HSPG core protein may reflect the need for numerous binding sites specific for individual growth factors. Growth factor binding to HSPG protects growth factors from enzymatic degradation and provides a mechanism for growth factor storage in the extracellular matrix (ECM). Even more essential, the binding to HSPG facilitates growth factor recognition by their cognate receptors at the cell surface, initiating a cascade of intracellular signals that will ultimately promote growth factor biological activity. Therefore, HSPG serve as extracellular modulators that store and promote growth factor function in specific locations.

The question arises as to whether HSPG promote growth factors in the adult neurogenic zones. We recently demonstrated that N-sulfated HSPG are expressed along the neurogenic axis of the adult brain [19]. This study demonstrated that cell proliferation occurs in a N-sulfated HSPG immunoreactive (N-sulfated HSPG+) niche that consists of particulate subventricular ECM structures that we previously named fractones [21,22], and of ECM in blood vessels, as a continuum throughout the neurogenic zones. Fractones contain HSPG, collagen-IV and laminin [15,21,22], therefore they chemically resemble basement membranes [32] but display a punctate morphology in light microscopy after immunolabeling for laminin or HSPG, and a fractal morphology in transmission electron microscopy [21,22]. In the adult brain, extensive cell processes from neural stem/progenitor cells converge toward fractones, with more than 100 cell contacts per individual fractone being apparent [21]. Fractones and SVZ vascular basement membranes bind FGF-2 via their heparan sulfates [15]. However, the role of this binding has not yet been identified.

Here, we examine the effect of BMP-7 inhibition of cell proliferation along the rostro-caudal axis of the SVZa after intracerebroventricular (ICV) injection. To determine whether the BMP-7 effect on cell proliferation is mediated by HSPG, we examine the loss of BMP-7 inhibition of cell proliferation and of BMP-7 binding to SVZ niche structures after desulfation by heparitinase-1 [25].

2. Materials and methods

2.1. Animals and injection schedules

8–10 week old male and female Balb/c mice ($n = 24$) were anesthetized with ketamine/xylazine mix (ratio 80/12) at 50 mg/kg of body weight. Two successive ICV injections (lasting 15 min each) were performed unilaterally at bregma +0.5 mm, +0.6 mm lateral to the midline and 2 mm depth with 1 μl of heparitinase-1 (32 mU/injection, from Flavobacterium heparinum, Seikagaku, Japan) and/or 1 μl of BMP-7 (0.5 μg/injection, 10-783-79729, GenWay Biotech, Inc., San Diego, CA) diluted in artificial cerebrospinal fluid (CSF, Harvard Apparatus, Holliston, MA) using a micro-4 microsyringe controller (WPI, Saratosa, FL) connected to a stereotaxic apparatus (Stoelting, Wood Dale, IL). ICV injections were performed sequentially with either CSF + CSF (termed CSF condition) or CSF + BMP-7 (BMP-7 condition) or heparatinase-1 + BMP-7 (Hep + BMP-7 condition) or heparitinase-1 + CSF (Hep condition) were performed at day 1 and day 3. At day 5, a single intraperitoneal injection of bromodeoxyuridine (BrdU, 50 mg/kg of body weight; Sigma; St. Louis, MO) was performed 6 h prior to animal termination to assess cell proliferation (pre-mitotic cells). The brain was dissected, frozen in isopentane at −80 °C and stored at −20 °C without fixation. The animal experimental protocol followed NIH guidelines and was approved by the University of Hawaii IACUC.

2.2. In vivo binding of BMP-7

BMP-7 was biotinylated using EZ-link Micro-sulfo-NHS Biotinylation kit (21425, Pierce, Rockford, IL).

The binding of BMP-7 was detected on brain frozen sections with streptavidin-Texas red as previously described [15]. When ICV injections of biotin only or artificial CSF only were carried out, no streptavidin Texas red signal was detected in the brain (data not shown).

2.3. Immunohistochemistry

Immunohistochemistry (IHC) was performed as previously described on serial frozen sections fixed with acetone at −20 °C just prior to the IHC procedure [21]. Fractones, blood vessels and choroid plexus were imunolabeled with anti-laminin antibodies (1/1000, L9393, Sigma, St. Louis, MO) [21] or anti-N-sulfate glycosamine (1/500, 10E4, Seikagaku, Japan) [7,15], then visualized with AlexaFluor-647 goat anti-rabbit (1/400), AlexaFluor-488 goat anti-mouse IgM (1/400, A21042, InVitrogen, Carlsbad, CA) or Cy-3 donkey anti-mouse IgM (1/400, 715-165-140, Jackson Laboratories, Bar Harbor, ME). Pre-mitotic cells (S-phase) were labeled with anti-BrdU antibodies (1/500, OBT0030, Oxford Biotechnology, UK) as previously described [15] and visualized with AlexaFluor-488 goat anti-rat (1/400, A11-006, InVitrogen). The results were recorded with a Zeiss confocal laser scanning microscope. Images were processed with Adobe Photoshop CS3 (Adobe Systems, Mountain view, CA). Adjustments for brightness/contrast were minimal. Control experiments for imunolabeling specificity were performed by omitting primary antibodies and by comparing dual immunolabelings with two antibodies against the same molecules (described in previous publications [15,21]).

2.4. Cell counts and statistical methods

BrdU+ cells were counted per SVZa of the lateral ventricle in series of whole-brain 25-μm thick coronal sections ranging from 1.2 mm to 0.5 mm anterior to bregma, using a 20× Plan Apo objective and a DMIL Leica immunofluorescence microscope. To compensate for over-counting, the observed numbers of BrdU+ cells were subjected to corrections given by Abercrombie's formula [1]. The corrected numbers were submitted to statistical analysis with XLStat. The means and respective standard deviations were reported per SVZa for each condition. A one-way ANOVA ($a = 0.01$), Tukey's and two-sided Dunnett's tests to compare each condition to the control (CSF condition) and a REQWQ's procedure for multiple comparison tests were performed to evaluate the significance of the differences observed. A confidence interval of 99% was applied and a p-value <0.001 was obtained for all comparisons except when comparing CSF and Hep ($p = 0.006$). All statistical models applied gave a significant difference between 2 conditions.

3. Results

3.1. Intracerebroventricular injection of BMP-7 inhibits cell proliferation in the SVZa: quantitative inhibition of mitogenesis along the rostro-caudal axis

BMP-7 is an inhibitor of neural stem cell proliferation *in vitro* [30] and its over-expression by ependymal cells results in the inhibition of neurogenesis [17]. Therefore, BMP-7 is a good candidate to inhibit cell proliferation *in vivo* after ICV injection. We examined the effect of BMP-7 ICV injection on cell proliferation in the

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