The Use of Coacervate Sustained Release System to Compare the Chondrogenic Potential of 5 BMPs In Vitro and Microfracture Mediated Cartilage Repair In Vivo

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Introduction. There are 14 Bone Morphogenetic Proteins (BMPs) identified to date, each with distinct and versatile functional roles [1, 2]. Our previous study compared 5 BMPs for bone regeneration and revealed that BMP2 is the most potent BMP to promote bone regeneration using the coacervate sustain release system [3]. Further, BMPs are required for chondrogenic differentiation of cells. Various BMPs have been used to promote cartilage repair in vivo using either gene therapy or biomaterials [4-11]. We previously showed that the coacervate sustained released BMP2/sFLT1 can promote cartilage repair to the same extent as lenti-BMP2 transduced human muscle-derived stem cells using a monoiodoacetate (MIA) induced osteoarthritis rat model [12]. Coacervate is a polymer of poly (ethylene argininylaspartate diglyceride) (PEAD) and heparin that can sustain-release growth factors and has been used to repair a varieties of tissues including skin wound, heart, bone, cartilage and so on [13-17]. The aim of this study is to use the coacervate sustained release platform to compare the potency of 5 BMPs for promoting chondrogenic differentiation in vitro and microfracture-mediated cartilage repair.

Materials and Methods. **1. Synthesis of PEAD** was performed as previously described [18]. **2. Comparison of the potency of 5 BMPs to promote chondrogenic differentiation of human bone marrow mesenchymal stem cells (hBMMSCs)**. Chondrogenic pellet cultures were performed for 3 populations of human BMMSCs isolated as previously described [19], using Stem-Pro complete chondrogenic medium supplement with 50ng/ml BMP2,4,6,7,9 and compared to chondrogenic medium control (N=4 pellets each treatment). The pellets were harvested at 24 days after differentiation and fixed in neutral buffered formalin (NBF) and pellet diameters were measured. The pellets were then embedded in NEG-50 freezing medium and cryosections were cut. Safranin O staining and immunohistochemistry staining of Collagen 2A (Col2A) was performed and matrix percentages were measured. **3**. **In vivo cartilage repair using osteochondral defect (1.5mm diameter and 1.5mm depth) plus microfracture (one 0.7mm drill hole) model in rat trochlear groove.** Sprague Dawley (SD) rats were divided into 6 groups (N=8 with 4 males and 4 females): (1)PBS+coacervate group; (2)500ng BMP2+coacervate; (3)500ng BMP4+coacervate; (4) 500ng BMP6+coacervate; (5) 500ng BMP7+coacervate;(6) 500ng BMP9+coacervate. BMPs in 1.25µl were added to 2ul heparin (10mg/ml) and allowed to bind for at least 10 minutes, and then 7.2 µl PEAD (10mg/ml) was added to form coacervate. Before adding to the osteochondral defect, 7.5 µl thrombin was mixed with coacervate and added to the defect, and then 7.5 µl fibrinogen was added and the coacervate complex was allowed to gel in the defect area. 8 weeks after surgery, rats were sacrificed and the injured entire distal femurs were dissected, and gross images were taken for ICRS scoring, and then fixed in NBF for 4 days for micro-CT scanning and histology. **4. MicroCT and histology:** The entire distal femur was scanned with Vivo-CT 80 using 30 µm voxel size. The tissues were then decalcified with 5% formic acid for 2 weeks, and paraffin embedded and sectioned. Alcian blue and Safranin O staining was performed to reveal the cartilage matrix. Seller's histology scoring was performed. Statistical analysis was performed using Graphpad Prism 9.

Results. 1. In vitro chondrogenesis: Gross images of pellets and diameter quantification showed all BMPs formed significant larger pellets than control group. BMP2,4 9 also formed significant larger pellets than BMP 6 and 7 **(Fig.1A.D).** Safranin O staining showed all BMPs groups have significantly higher Safranin O positive matrix than the control group. BMP4 group also has significant higher Safranin O positive matrix than BMP7 group (**Fig.1B,E**). Immunohistochemistry staining of Col2A1 and Col2A1 positive matrix percentage showed all BMP groups have significant higher percentage Col2A matrix than the control group and BMP4,7 9 showed higher percentage than BMP2 and 6 **(Fig. 1C,F).** 2. **In vivo cartilage repair**: MicroCT results showed BMP groups to have more osteochondral defect healing but none of the groups showed complete healing **(Fig.2A).** Gross images and ICRS macro score indicated all BMP groups have higher scores (better repair) than control while BMP4 and BMP9 showed slightly higher score **(Fig.2.B and C)**. Alcian blue staining showed the best and worst cartilage repair. All BMP groups showed better repair than the control group (**Fig.3A-D**). Safranin O staining and Seller's histology score showed all BMP groups have improved cartilage repair, with BMP2, 6, 9 showing significant lower scores (better repair) compared to the control group, but no significant difference compared to BMP4 and 7 groups (**Fig. 3.E-H**). However, Safranin O staining was faint in most samples.

Discussion. The in vitro study indicates that BMP2,4,9 are the most potent to promote chondrogenesis. In vivo, Macro score revealed all BMPs enhanced microfracture-mediated cartilage repair. Histology scores were significantly improved in BMP2,6,9 groups compared to the other groups. The overall lower Safranin O intensity might be caused by the coacervate complex not being entirely maintained in the defect area due to the small defect and short observation time (8 weeks). **Significance/clinical relevance**. Coacervate sustained-release BMP 2,6,9 could be an efficient way to promote microfracture-mediated cartilage repair. **Acknowledgement:** This project is supported by a Philanthropy gift from the Musculoskeletal Regeneration Partnership Fund by Mary Sue and Michael Shannon and NIH RO1(R01NR016436) to Dr. Yadong Wang.

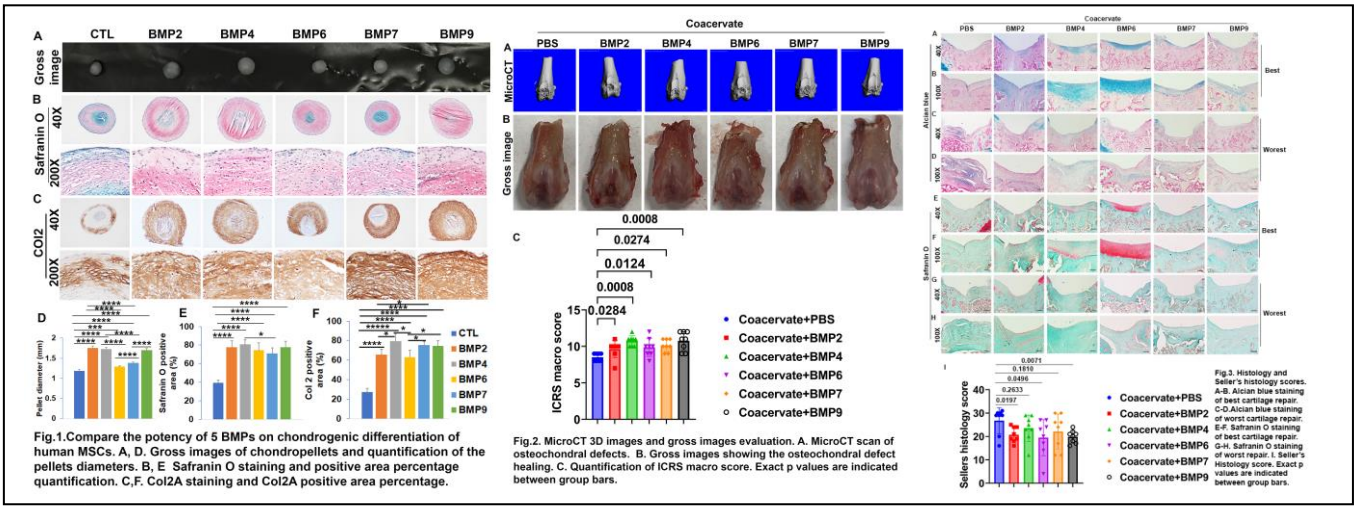

Fig.1.Compare the potency of 5 BMPs on chondrogenic differentiation of human MSCs. A, D. Gross images of chondropellets and quantification of the pellets diameters. B, E Safranin O staining and positive area percentage quantification. C,F. Col2A staining and Col2A positive area percentage.

Fig.2. MicroCT 3D images and gross images evaluation. A. MicroCT scan of osteochondral defects. B. Gross images showing the osteochondral defect healing. C. Quantification of ICRS macro score. Exact p values are indicated between group bars.

Fig.3. Histology and Seller's histology scores. A-B. Alcian blue staining of best cartilage repair. C-D.Alcian blue staining of worst cartilage repair. E-F. Safranin O staining of best cartilage repair. G-H. Safranin O staining of worst repair. I. Seller's histology score. Exact p values are indicated between group bars.