Comparative Effect of BMP-2 and BMP-9 for Bone Regeneration by Mesenchymal Stem Cells in a Biomimetic Hydrogel

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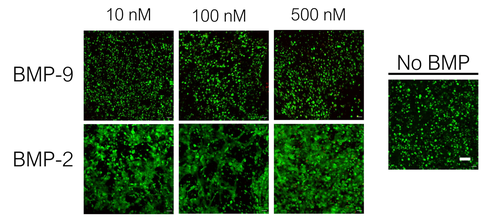
Background

The skeletal system.

Treatment approaches seeking to regenerate bone tissue often look to the native environment to select bone mimetic components for incorporation in a cell-laden scaffold to guide cellular differentiation. Literature indicates that extracellular matrix (ECM)-bound growth factors, specifically bone morphogenetic proteins (BMPs), are an important component of the ECM.[1,2] Previous work from our lab has shown that **BMP-2** can be covalently tethered into a poly(ethylene glycol) – based hydrogel network, maintain bioactivity, and have a measurable effect on the cellular response of encapsulated MC3T3-E1 cells.[2] Although BMP-2 is a well-established osteogenic cue, it has been shown to have confounding effects on cellular differentiation, as it is able to stimulate chondrogenesis, osteogenesis, and endochondral ossification all within the same culture system.[3] Additionally, investigation into the osteogenic potential of various members of the BMP family of growth factors (GFs), have found that a somewhat lesser investigated member, **BMP-9,** may be the most potent osteoinductive BMP.[4,5] Therefore, **in this work we sought to compare the osteogenic response elicited by BMP-2 and BMP-9 on encapsulated human MSCs (hMSCs),** when tethered into an enzyme degradable bone mimetic PEG-hydrogel, over a range of tethering concentrations. The effects of these growth factors, on known osteomarkers, was assessed.

Results and Discussion

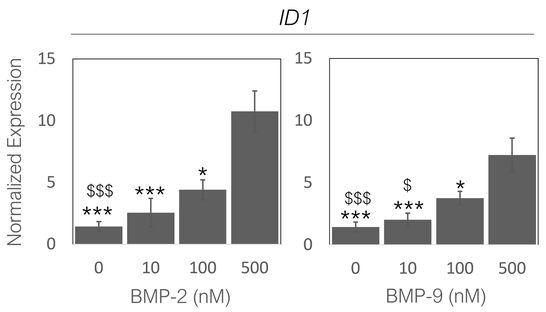
Tethered BMP-2 enhanced cell spreading at week one.



Live/Dead staining at week 1.
Scale bar = 200 µm

- Abundant green and minimal red staining indicates that the cells survived encapsulation and up to one week of culture across all conditions.
- Qualitative assessment of cell morphology indicates that tethered BMP-2 is more effective at inducing cell spreading than BMP-9 or the mimetic gel alone.

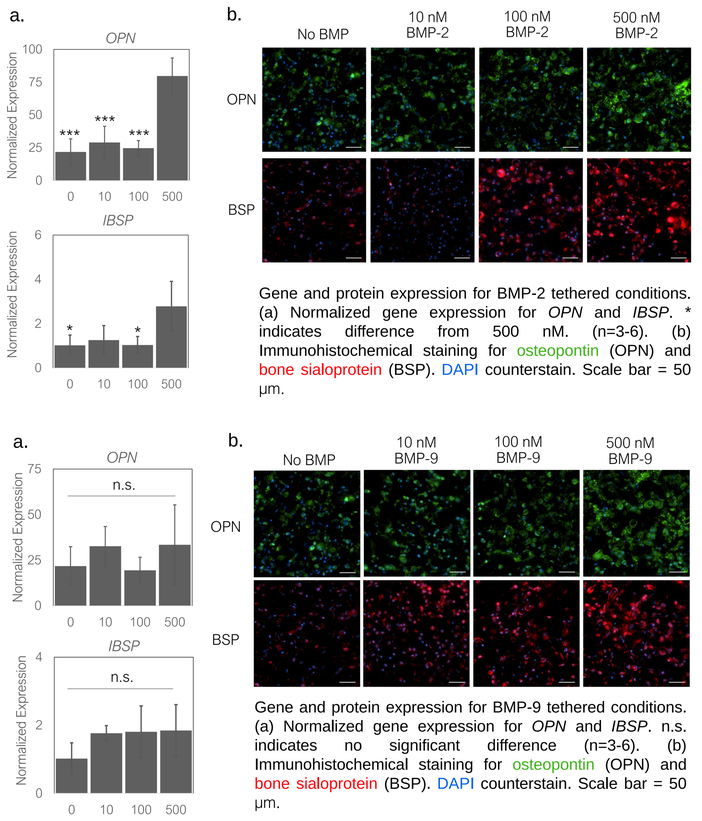
BMP-2 and BMP-9 induced expression of an early osteogenic marker, *ID1*.



Normalized gene expression of *ID1* at week one. * indicates difference from 500 nM and $ indicates difference from 100 nM conditions.(n=3-6)

Dose responsive increase in gene expression of *ID1*, a direct target of BMP-2 and BMP-9, at week one.

500 nM tethered BMP-2, has a significant effect on the gene and protein expression of osteopontin and bone sialoprotein at week eight.

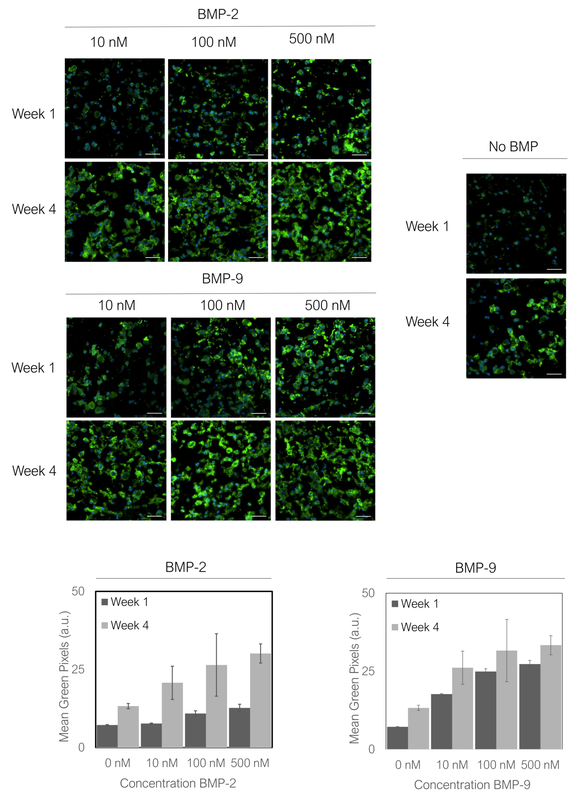


Gene and protein expression for BMP-2 tethered conditions. (a) Normalized gene expression for *OPN* and *IBSP*. * indicates difference from 500 nM. (n=3-6). (b) Immunohistochemical staining for osteopontin (OPN) and bone sialoprotein (BSP). DAPI counterstain. Scale bar = 50 µm.

Gene and protein expression for BMP-9 tethered conditions. (a) Normalized gene expression for *OPN* and *IBSP*. n.s. indicates no significant difference (n=3-6). (b) Immunohistochemical staining for osteopontin (OPN) and bone sialoprotein (BSP). DAPI counterstain. Scale bar = 50 µm.

- Normalized gene expression for *OPN* and *IBSP* were significantly upregulated in the 500 nM tethered BMP-2 condition. Immunohistochemical staining shows positive staining on the protein level for both OPN and BSP.
- Although there was no statistical increase on gene level expression for *OPN* or *IBSP* for tethered BMP-9 conditions, qualitative observation on the protein level, particularly for BSP, indicated deposition increased with increasing concentrations of tethered BMP-9.

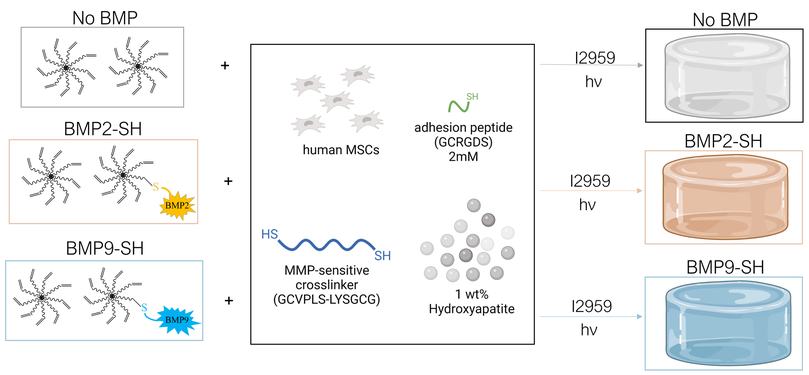
Tethered BMP-2 and BMP-9 enhance early expression of collagen I



Quantification of green pixels in RGB image as a proxy for relative amounts of collagen I staining. Immunohistochemical staining for collagen I with DAPI counterstain. Scale bar = 50 µm.

- Average collagen I deposition appears to increase from weeks one to four across all conditions.
- Tethered BMP-9 appears to more greatly enhance collagen I deposition, as compared to BMP-2, at week one across all tethering concentrations.
- Images indicate that the inclusion of BMP-2 and BMP-9 increased collagen I deposition at weeks one and four.

Materials and Methods



Human MSC Culture Human MSCs, derived from the bone-marrow of a male donor (age 22 years), were obtained from RoosterBio. MSCs were expanded to passage one and reached ~80% confluency. **Monomer Synthesis, Hydrogel Formation and Cell Encapsulation** PEGNB was prepared by reacting 8-arm PEG amine (10kDa) with excess 5-norbornene-2-carboxylc acid with N,N-diisopropylethylamine (DIEA) and 1-[Bis(dimethylamaino0methylene]-1H-1,2,3-triazolog[4,5-b]pyridinium 3-oxid hexafluorophosphate (HATU). BMP2-SH and BMP9-SH were synthesized by reacting the growth factor with four molar excess of Traut's reagent in buffer for 1 hour. Cellular gels were prepared by encapsulating human MSCs in the bone-mimetic hydrogel whose base formulation was 6% w/w PEG-NB with either no BMP, tethered BMP2-SH (10, 100, or 500 nM), or tethered BMP9-SH (10, 100, or 500 nM), an MMP sensitive crosslinker (GCVPLS-LYSGCG; 1:0.80 thiol:ene ratio), GCRGDS (2 mM), and hydroxyapatite (1% w/w). under 352 nm light at ~5 W/cm^2 for 8 minutes. Cell-laden gels were cultured up to 12 weeks in a defined medium that included; α-MEM, 1% ITS+premix, 100 nM dexamethasone, 50 µg mL^{-1} ascorbate-2-phosphate, 1X MEM non-essential amino acids, 10 mM β-glycerophosphate, penicillin/streptomycin, and fungizone. **Live/Dead Imaging** Hydrogels from each condition were taken a soaked in a solution of calcein AM (1 hour) and ethidium homodimer (10 minutes). Gels were rinsed and imaged on a confocal microscope. Images were processed using ImageJ (n=3 per condition). **Characterization of Cell-Laden Hydrogels.** MSC-laden hydrogels were cultured for up to 12 weeks. *qPCR* Samples were collected at weeks 1, 4, and 8 and stored at -80^{0}C. RNA was isolated and converted into cDNA. Quantitative real-time polymerase chain reaction (qPCR) was performed with a Fast SYBR Green Master Mix. Gene expression was normalized to cells at day zero prior to encapsulation. *Immunohistochemistry:* Cell-laden hydrogels were fixed and processed for immunohistochemistry at prescribed timepoints. Sections (5µm) were stained for osteopontin, bone sialoprotein or collagen I. For osteopontin: sections were treated with Retrievagen and then by anti-osteopotin (1:200, ab8448, Abcam) and a secondary antibody (n=3). Abcam**)** and a secondary antibody (n=3). For bone sialoprotein: sections were treated with Retrievagen and then by anti-bone sialoprotein (1:15, WVID1(9C5), DSHB) and a secondary antibody (n=3). For collagen I: sections were treated consecutively with pepsin and Retrievagen. Samples were then treated with anti-collagen I (1:200, ab34710, Abcam) and a secondary antibody (n=3). All samples were imaged on a Nikon widefield and processed using Nikon Elements and ImageJ. Color histograms were made using ImageJ to compare amount of green present in RGB images from collagen I stain. *Statistics* Statistical analysis was performed using the Real Statistics plug-in for Excel. One symbol indicates p<0.05 and three symbols indicates p<0.001, two symbols indicate p<0.01, and one symbol indicates p<0.05.

Conclusions

The system described is a **highly tunable hydrogel platform** where growth factors can be tethered into the network to control MSC response. Results from this study suggests the successful tethering of BMP2-SH and BMP9-SH to the hydrogel network and that the bone-mimetic hydrogel with BMP-2 and BMP-9, **enhanced osteogenic response of human MSCs**. Results also indicate that there may be differences in the response elicited by each growth factor. BMP-2 appears to have a greater effect on cell spreading by week one and on the gene expression of non-collagenase proteins *OPN* and *IBSP*. On the protein level, BMP-2 and BMP-9 appear to have comparable degrees of expression. Imaging from collagen I staining indicates that BMP-9 may elicit a more rapid deposition of collagen I compared to BMP-2 or the gel alone.

Future Work

Future work will compare the relative tethering efficacies of BMP-2 and BMP-9 by modified ELISA to better understand differences in cellular response. Additionally, we will investigate the expression of mature osteoblast and osteocytic markers, including; *DMP1* and *PHEX* on the gene and protein levels. Additionally, the extent of mineral deposition will be assessed by von Kossa staining.

References

1. Migliorini, E., et al., Bone (2020):115540, 2. Nistala, H., et al., J. Cell Biol. (2010):1107-1121, 3.Schoonraad, S.A., et al., Biomacromolecules (2021):1065-1079 4. Zhou, N., et al. Cell Tissue Res (2016): 101-111 3. Lamplot, J. D., et al., Am J Stem Cell, (2013):(1-21), 5. Zhao,X., et al., Am J Trans Res (2021):4233-4250

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