Early Cellular Responses in Male and Female Rats to BMP-2 cmRNA Delivered into Critical Size Femoral Defects

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INTRODUCTION: Growth factors such as BMP-2 hold promise as agents of osteogenesis in clinical settings where bone healing is defective. Their clinical application is held back by problems of delivery that lead to serious side effects[1]. Delivery of mRNA encoding human BMP-2 provides an attractive alternative to circumvent these clinical translation hurdles. Our group has shown that chemically modified mRNA (cmRNA) is an efficient and reliable vector of BMP-2 delivery for regeneration of long bone critical size defects in male[2] but not female rats[3]. The objective of the present study was to investigate the early cellular mechanisms triggered by cmRNA or recombinant human (rh) BMP-2 delivery in bone critical size defects and how these differ between sexes.

METHODS: BMP-2 cmRNA was produced from cDNA by in vitro transcription, purified and complexed with a liponanoparticle; 50 µg cmRNA were loaded onto clinically approved collagen sponges (6 x 3 x 3 mm) 15 min prior to implantation, as before[2, 3]. This study was approved by IACUC (#A4410-19). Male and female Fischer rats at 16 weeks of age were randomly divided into 3 groups and used for surgery (n=6/sex/group). A 5 mm critical sized defect was created in the right femur of each rat and received a collagen sponge containing either BMP-2 cmRNA, non-coding (NC) cmRNA as a control, or 11 µg rhBMP-2. Animals were euthanized at day 3, the femora harvested for histology (Masson's trichrome) and RNAscope analysis (n=3/sex/group) or single cell RNA sequencing (scRNAseq) analysis according to 10x protocols (n=3/sex/group). The *R* package *Seurat* was used for all scRNAseq analysis.

RESULTS: ScRNAseq analysis uncovered 13 unique cell types at the femoral defect site (Fig. 1A) identified by marker gene expression, for a total of 168,768 cells, with PMNs being the largest cluster by far (n=71,132). PMN clusters of cmRNA treated groups more than doubled the cell number of the rhBMP-2 group (n=27,887 & n=29,402 vs. n=13,843). Bmp2 gene expression, both endogenous and cmRNA encoded, across all cell types was mainly seen in PMNs (Fig. 1B) and was notably highest in the BMP-2 cmRNA group (Fig. 1C). Interestingly, male rats within the BMP-2 cmRNA group displayed a large upregulation in overall Bmp2 expression compared to similarly treated females (Fig. 1D). Module scores showed upregulated expression of genes linked to Interleukin (IL) -1 and IL-6 signaling within cmRNA-treated groups, with the Bmp2 construct having no notable differences from the non-coding controls (Fig. 1E). Bone defects treated with rhBMP-2 resulted in enhanced expression of genes linked to osteogenesis while those treated with BMP-2 cmRNA resulted in a more chondrogenic transcriptional profile (Fig. 1E), predominantly driven by MSCs. Histological analysis showed high cellular infiltration at the defect borders and surrounding the collagen sponge (Fig. 2A). Fluorescent RNAscope analysis validated scRNAseq findings by demonstrating strong binding of the BMP-2 cmRNA on the collagen sponge surface with marked infiltration of S100a8^{+} neutrophils (PMN) in the BMP-2 cmRNA group (Fig. 2B), and a strong proliferative response by Postn^{+} skeletal stem cell progenitors (MSC) (Fig. 2C) in male rats.

DISCUSSION: By incorporating scRNaseq and RNAscope technologies, we are the first group to detect the major cell types present in a rat long bone critical size defect in the context of regenerative, transcript therapy, identifying the cells uptaking (PMNs) and responding (MSCs) to BMP-2 cmRNA. Such an early timepoint is bound to capture a large number of immune cells that may obscure the underlying mechanistic processes but by using a total of 18 animals (n=9/sex), we were able to obtain a clear picture of the cellular mechanisms within the bone defect. The scRNAseq results confirmed our prior observation that BMP-2 cmRNA treated defects heal via endochondral ossification, while rhBMP-2 treated defects do so via intramembranous ossification[2]. Moreover, the significant difference in BMP-2 cmRNA uptake and Bmp2 expression by PMNs between male and female rats provides the foundation for future studies to investigate the complex interactions of RNA-liponanoparticles with intra-lesional cells of males and females.

SIGNIFICANCE/CLINICAL RELEVANCE: Defective bone healing remains a pressing clinical problem and cmRNA therapy is an emerging and appealing solution, with high potential for accelerated clinical translation. This study describes important cellular mechanisms and populations within bone defects that can be targeted by future therapeutics.

REFERENCES: 1. James AW, et al. Tissue Eng Part B Rev. 2016. PMC4964756. **2.** De La Vega RE, et al. Sci Adv. 2022. PMC8849297. **3.** De La Vega RE, et al. ORS Annual Meeting; PS-1-LB176, #2377; Long Beach, CA; 2024.

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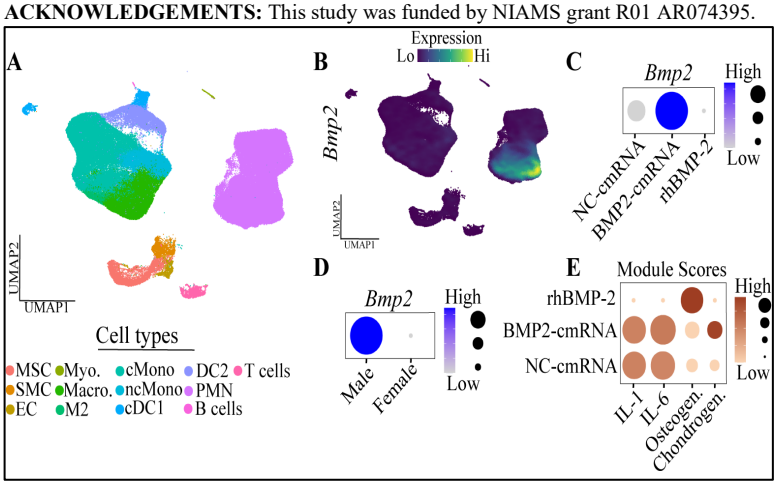

Figure 1. ScRNAseq analysis of bone defect tissue at day 3. (A) UMAP of bone defect cells colored by cell types. **(B)** Feature plot depicting Bmp2 expression in all clusters. **(C)** Dot plot of Bmp2 expression by group. **(D)** Dot plot of Bmp2 expression by sex. **(E)** Module score depicting the strength of signaling pathways between groups.

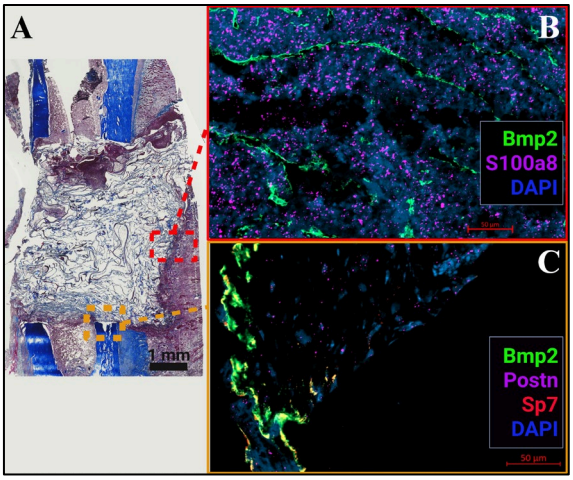

Figure 2. Histology and RNAscope of bone defects at day 3. (A) Trichrome stained histology slide of the femoral bone defect filled with a collagen sponge. **(B)** RNAscope staining for Bmp2 and S100a8 at the sponge border. **(C)** RNAscope staining for Bmp2, Postn and Sp7 at the bone-defect interface.