Injectable Microparticles Deliver R-Spondin-2 mRNA and may Enhance Osteogenesis and Myogenesis

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INTRODUCTION: Bone defects with overlying muscle injury have limited therapeutic options and may result in amputation. Protein based therapies, like bone morphogenetic protein-2 (BMP-2), promote regeneration of large bone defects. However, composite muscle and bone injuries are recalcitrant to BMP-2 treatment[1]. New strategies are needed to address polytraumatic injuries addressing both muscle and bone regeneration. Gene delivery with biomaterials is an area of interest to repair tissue defects. Our lab has created calcium phosphate mineral-coated microparticles that improve delivery of therapeutic mRNA and sequester cell-secreted proteins[2,3]. This localizes and prolongs the activity of the transgene, which is important for regenerative medicine applications.

WNT/ β-catenin signaling is naturally involved in musculoskeletal healing and may be of interest for composite injuries. R-Spondins are WNT agonists implicated in improvement of osteogenesis in the presence of BMP-2[4] and also promote myogenic differentiation[5]. R-Spondin-2 is the most potent R-Spondin but also contains a TSP-1 domain that inhibits BMP receptors[6]. However, RSPO-1 does not impact BMP-R activity but has reduced potency[6]. Mutant RSPO-2 with the TSP-1 domain from RSPO-1 is functionally active[6] and may promote BMP signaling when combined with BMPs. The use of "myogenic RSPO-2" (native RSPO-2) and "osteogenic RSPO-2" (RSPO-2$^{dTSP\text{-}1\ (RSPO\text{-}1)}$) with BMP-2 could promote muscle and bone regeneration. Further, the anti-BMP-R activity of WT-RSPO-2 could inhibit heterotopic ossification from the underlying regenerating bone while actively inhibiting heterotopic ossification. Taken together, we hypothesized that a single dose mRNA delivery strategy using mineral-coated microparticles to deliver mRNA for R-Spondins could promote myogenesis and osteogenesis.

METHODS: Synthetic mRNA for RSPO-2, RSPO-1, RSPO-2$^{dTSP\text{-}1\ (RSPO\text{-}1)}$ and BMP-2 were created by cloning the coding sequences into a plasmid DNA expression vector flanked by 5' and 3' untranslated regions from the beta-globin gene. DNA templates were created using PCR with an appended 120 nt long polydT tail in the 3' primer. mRNA were synthesized with co-transcriptional capping using CleanCap-AG (Trilink Biotechnologies) and uridine were fully substituted with N1-methylpseudouridine. Lipofectamine™ Messenger Max (Thermofisher Scientific) was used as the transfection reagent. Mineral-coated microparticles (MCM) were synthesized by incubation in a modified simulated body fluid solution with the addition of 5mM citric acid and 1mM NaF as previously described[2]. mRNA complexes were bound to MCMs at a ratio of 125 µg MCM per µg of mRNA for 30 minutes under constant vertical rotation. MCMs were centrifuged and resuspended to deliver only the bound mRNA complexes. Myogenesis: Human H9 embryonic stem cells or murine C2C12 myoblasts were cultured in growth media (DMEM, 1% Pen/Strep, 10% FBS) until 75% confluence, then were cultured in low serum media: DMEM, 1% Pen/Strep, 2% B-27 supplement (Gibco). RSPO-2 mRNA +/- MCMs were delivered two days after myogenic induction and were fixed on d7 or d14 and stained for myosin heavy chain expression (MYHC). On d7 mRNA was extracted for gene expression for *myog*, *myf5*, *myostatin* or *axin2*. Osteogenesis: Human mesenchymal stromal cells (hMSC) were grown to 100% confluence prior to treatment with 1:1 mut/WT RSPO-2 + BMP-2 mRNA. Cells were fixed on d21 and stained with Alizarin red for calcification. N=3 biological replicates were used for each assay and were analyzed by one-way ANOVA when applicable.

RESULTS SECTION: Both WT and mut-RSPO-2 mRNA delivered via MCMs resulted in robust calcification, as assessed by alizarin red staining after 21 days of culture (Fig. 1C-D). Conversely, without MCMs only mut-RSPO-2 delivery resulted in calcification (Fig. 1C), while WT-RSPO-2 mRNA produced no calcification (Fig. 1A). To investigate myogenesis we delivered RSPO-2 mRNA to C2C12 cells. MYHC$^+$ myotubes formed in culture after 7 days of differentiation (Fig. 1 E). Treatment with MCMs increased MYHC$^+$ cells and myotube fusion (Fig. 1F), which were further enhanced with RSPO-2 mRNA with (Fig. 1G) or without MCMs (Fig 1H). RSPO-2 mRNA delivery increased *myf5* expression in differentiating C2C12 cells after 7 days (Fig 1I) but only RSPO-2 mRNA delivered with MCMs decreased expression *myostatin* (Fig. 1J).

DISCUSSION: There are conflicting reports on the role of R-Spondins in relation to BMP signaling but consensus is forming around RSPO-2 being inhibitory for BMP receptors[6]. Here we reaffirmed that WT-RSPO-2 inhibits mineralization in the presence of BMP-2, and that mutation of the BMP receptor region in RSPO-2 - replacing with the TSP-1 region from RSPO-1 - permits BMP-2 mediated mineralization *in vitro*. Interestingly, the presence of MCMs is sufficient to allow complete mineralization of the tissue culture even when combined with WT-RSPO-2. Calcium phosphates are known to be osteoconductive and sequester proteins. This may help to override any inhibitory effects of WT-RSPO-2. RSPO-2 has been shown to enhance myogenesis in C2C12 cells. Here we used a mRNA delivery strategy that promoted myogenesis in C2C12 cells as assessed by MYHC expression and gene expression of the pro-myogenic gene *myf5*. MCMs alone improved myotube formation without RSPO-2. Interestingly, RSPO-2 or MCM delivery seemed to promote alignment of the myotubes relative to the untreated control. Furthermore, RSPO-2 mRNA with MCMs reduced *myostatin*, an inhibitory protein for myogenesis, while MCMs alone increased *myostatin* expression. Future work will investigate the ability of RSPO-2 to regenerate musculoskeletal tissues *in vivo*.

SIGNIFICANCE/CLINICAL RELEVANCE (1-2 sentences): Polytraumatic injury to muscle and bone has few therapeutic options. This work may lead to the development of a method to promote healing of muscle and bone defects while inhibiting heterotopic ossification.

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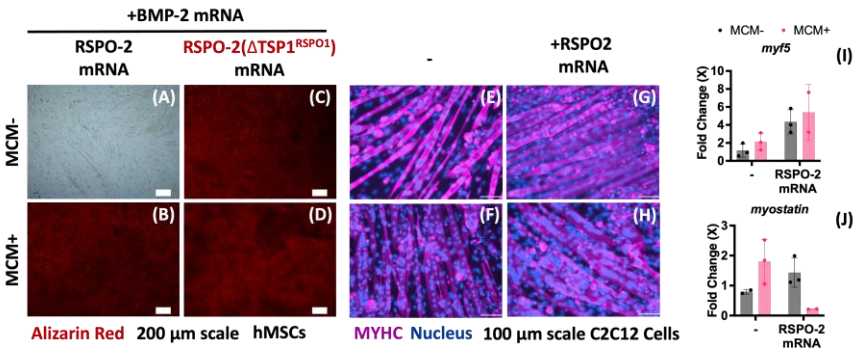

Figure 1. A-D. Alizarin Red Staining of hMSCs on d21 of differentiation after treatment with mRNA for **A/B.**WT-RSPO-2 or **C/D.** Mut-RSPO-2 without (top row) or with (bottom row) MCM treatment. **E-H**. Myosin Heavy Chain (MYHC) and nuclear (DAPI) staining of C2C12 cells on d7 of differentiation after treatment with **E.** negative control **F.** MCM only **G.** RSPO2 mRNA alone **H.** RSPO2 mRNA + MCM. **I/J**. Gene expression on d7 of myogenic differentiation from C2C12 cells for **I.** *myf5* and **J.** *myostatin*.