**The Development And Characterization Of RNA Interference Therapeutics For Fibrodysplasia Ossificans Progressiva**

Arun R. Shrivats1, Mike McDermott1, Saadyah Averick1, Hong Cho1, Matthew Mackenzie1, Krzysztof Matyjaszewski1, Yuji Mishina2, Jeffrey O. Hollinger1.

1Carnegie Mellon University, Pittsburgh, PA, USA, 2University of Michigan, Ann Arbor, MI, USA.

**Disclosures:**

**Introduction:** Fibrodysplasia ossificans progressiva (FOP) is a severely disabling condition of extraskeletal ossification caused by gain-of-function mutations occurring in ACVR1/ALK2_a type I bone morphogenetic protein (BMP) receptor. One of the commonly found mutations in FOP patients is the R206H mutation, which renders signaling activity of ACVR1 hyperactive upon binding of BMP ligands. This dysregulation of BMP signaling leads to osteogenic differentiation and, in turn, extraskeletal bone formation. FOP patients experience highly painful soft-tissue swellings known as flare-ups that trigger these uncontrolled bone formation processes. There is no known cure for FOP and current treatment strategies for flare-ups are clinically unacceptable. It is hypothesized that the silencing of key agents in the BMP signaling pathway may arrest the FOP-induced morphogenesis of soft tissues into bone. The BMP signaling pathway and other bone-forming signaling pathways converge at RUNX2_a transcription factor that is a gatekeeper for osteogenic differentiation. We seek to silence this pivotal factor and, subsequently, prevent osteoblast lineage progression and heterotopic bone formation. To silence the Runx2 gene, we harness the endogenous silencing machinery of RNA interference (RNAi) by delivering short interfering ribonucleic acids (siRNAs) against Runx2 to target cells. Though RNAi is a powerful tool, the biggest hurdle preventing it from widespread clinical use is achieving safe and effective delivery of siRNAs to target cells. We employ cationic nanostructured polymers (NSPs) to overcome these obstacles; in this capacity, NSPs will bind, protect, and transport anti-Runx2 siRNAs to target cells.

The severity of FOP necessitates the use of robust in vitro testing platforms that mimic the BMP dysregulation that is the hallmark of FOP. Thus, we harvest calvarial osteoblasts from mice with the Q233D mutation, which renders BMPR1A_another type I BMP receptor_constitutively active (ca). These caBMPR1A osteoblasts provide an in vitro model comparable to the R206H mutation by priming cells for BMP-based osteogenic triggers.

The development of successful polymer-based RNAi therapeutics for FOP is a tremendous challenge; variables such as the optimal NSP:siRNA ratios and dosages required to achieve success have yet to be identified. Further, in order to successfully prevent osteoblast lineage progression, we must match the delivery of RNAi therapeutics with the temporal expression profile of RUNX2. These unanswered questions impede the development of successful RNAi therapeutics for FOP. Here, we report the successful calibration of NSP:siRNA ratios, as well as siRNA dosing and timing variables to silence BMP-induced RUNX2 expression in caBMPR1A osteoblasts.

**Methods:** NSPs with three distinct architectures_nanogels, stars and block-copolymers_were synthesized by ATRP(1, 2). Both star and nanogel NSPs contain the cationic monomer DMAEMA, while block co-polymers contained the novel t-MTEMA monomer. Biocompatibility of NSPs was assessed by MTS in MC3T3 E1.4 pre-osteoblasts and was normalized to untreated cells. Complexation ratios (wt:wt) of NSPs with siRNAs were determined by gel electrophoresis. To establish in vitro testing models for RUNX2 knockdown, calvarial osteoblasts were harvested from seven-day old mice with the Q233D mutation in BMPR1A. All knockdown studies were performed in cells with passage numbers below five. Selectively methylated siRNAs were synthesized against the Runx2 gene. The investigation into NSP:siRNA ratios, siRNA doses and the temporal delivery considerations required to knockdown Runx2 mRNA was conducted by quantitative reverse-transcriptase PCR (qRT-PCR).

**Results:** Biocompatibility of star, nanogel, and block NSPs was assessed by MTS assay in MC3T3 cells (Fig. 1A). Star and nanogel NSPs demonstrated 100% cell viability up to 800 μg/ml; block NSPs were biocompatible at 100 μg/ml. Based on the significant differences in polymer architecture, composition and cationic charge, a range of NSP:siRNA complexation ratios were expected (Fig. 1B-1D). Star NSPs complexed with siRNA at ratios > 200:1; nanogels and block NSPs complexed at ratios > 10:1. Runx2 mRNA knockdown was determined by delivery of anti-Runx2 siRNA by each NSP at specific ratios. Results demonstrated that peak knockdown occurred at NSP:siRNA ratios of ~500:1 for stars (Fig. 2A), 1 to 100:1 for nanogels (Fig. 2B) and 10 to 100:1 for blocks (Fig. 2C). An analysis of siRNA dosing with nanogel NSPs (Fig. 2D) indicated that a reduction from the 30 pmol (used in Figures 2A-C) to 5 pmol does not significantly reduce knockdown efficiency.

Specificity of RNA interference therapeutics was evaluated by delivery of control siRNA for star, nanogel and block NSPs at ratios of 500:1, 1:1 and 50:1, respectively (Fig. 3A). Control siRNA treatments demonstrated insignificant changes in Runx2 mRNA expression while the delivery of Runx2 siRNA via nanogel and block NSPs demonstrated significant reductions in Runx2 expression. Knockdown efficiency was compared to delivery of naked siRNAs, which demonstrated insignificant knockdown of Runx2 mRNA, validating the necessity of NSPs for siRNA delivery. Successful silencing of Runx2 requires that we match administration of therapeutics with the temporal expression profile of this key transcription factor. An investigation into the optimal time for siRNA delivery with respect to rhBMP-2 delivery was conducted (Fig. 3B). Temporal periods tested included 24,
12, 6 and 0 hours prior to rhBMP-2 administration. The optimal rhBMP-2 delivery time tested for nanogel and star NSPs was 24 and 12 hours prior to the differentiation trigger, respectively.

Discussion: This difference in the optimal delivery window suggests that star and nanogel NSPs have differing speeds of siRNA delivery and, potentially, different internalization mechanisms as well. This result also compels us to investigate the inclusion of additional targets to prevent osteogenic differentiation. Osterix (OSX), for instance, is another pivotal osteoblast transcription factor that operates downstream of RUNX2 and plays a pivotal role in regulation of bone formation. We are currently investigating a coordinated RNAi attack on Runx2 and Osx to prevent osteogenic differentiation.

Significance: FOP is a devastating pathology that currently lacks clinically efficacious treatments. The future of FOP therapies may be at the intersection of RNAi technology, polymer chemistry and bone biology; we have harnessed these tools and used a drug delivery approach to develop anti-BMP therapeutics targeted against RUNX2—a master regulator of bone formation. Here we demonstrate the ability to knockdown expression of Runx2 in caBMPR1A (Q233D) osteoblasts by delivery of siRNAs via star, nanogel and block NSPs. We report the calibration of NSP ratios, siRNA dosing and timing of RNAi treatments against RUNX2. The specificity achieved against RUNX2 here can translate to other targets; for FOP, we anticipate the coordinated silencing of Runx2 and Osx may be able to prevent osteogenic differentiation and the progression of the FOP pathology. This approach has great promise and may translate to the development of therapeutics for other pathologies, including trauma-induced heterotopic ossification and osteoarthritis.

Acknowledgments: We would like to acknowledge the Department of Defense via DMRDP W81XWH-11-2-0073 for funding this research.
