SHP2 Depletion Promotes Cartilage Anabolic Gene Expression by Increasing SOX9 Protein Stability

Alexandria Martinez[1], Lijun Wang[1], Huiliang Yang[1], Jiahui Huang[1], Thedoe Nyunt[1], Maurizio Pacifici[2], Véronique Lefebvre[2], Shaomeng Wang[3], Douglas C. Moore[1], and Wentian Yang[1]*. 1. Department of Orthopaedic Surgery, Brown University Alpert Medical School and Rhode Island Hospital, Providence, RI 02903, USA; 2. the Department of Pediatrics, Children's Hospital of Philadelphia, Philadelphia, Pennsylvania 19104, USA. 3. Departments of Internal Medicine, Pharmacology, and Medicinal Chemistry, University of Michigan, Ann Arbor, Michigan 48109, USA.

Disclosures: A Martinez (N), L Wang (N), H Yang (N), J Huang (N), S Sun (N), M Pacifici (N), V Lefebvre (N), DC Moore (N), and S Wang (N).

INTRODUCTION Cartilage diseases remain among the costliest and most clinically burdensome musculoskeletal disorders. Therefore, understanding of articular cartilage (AC) and growth plate cartilage (GPC) development and homeostatic mechanism(s) could lead to novel therapeutics for sustaining AC homeostasis, preventing cartilage degeneration, and promoting the repair of injured cartilage. Reversible protein phosphorylation is one of many post-translational protein modifications (PTMs) that are crucial for regulating tissue development and homeostasis. To date, most of the work on PTMs in cartilage has focused on protein phosphorylation by tyrosine kinases (PTKs). However, dephosphorylation by protein tyrosine phosphatases (PTPs) has equally profound functional consequences but is understudied. SHP2, encoded by *PTPN11*, is a cytoplasmic PTP. Somatic SHP2 loss-of-function mutations in human cause a benign cartilage tumor syndrome, and conditional SHP2 deletion in chondrogenic cells in mice provoke the formation of osteochondromas and thicken GPC, though the cellular and molecular mechanisms remain incompletely understood. Here, we interrogate the impact of SHP2 depletion in *Prg4*+ cells on AC in mice. Investigating the impact of SHP2 depletion in AC should provide important insights into fundamental processes of cartilage biology and the potential translational application of SHP2 agonists and antagonists.

METHODS *Rosa26*^{ZSG}-bearing Control (*Ptpn11*$^{fx/+}$;*Prg4*^{CreER}) and SHP2 knockout mice (*Ptpn11*$^{fx/fx}$;*Prg4*^{CreER}) were generated, genotyped, and used per the IACUC approved protocols. To induce *Tg(Prg4*^{CreER}*)* activity *in vivo*, tamoxifen (TM) was injected intraperitoneally (50 mg/kg body weight) into 5 of 2 week-old mice, and the mice were euthanized at 6 weeks of age. Plain and 3D μCT radiographs of mice skeletons were carried out after euthanasia using high-resolution X-ray and desktop μ-CT40 systems. The knee joints were dissected from mice and fixed in 4% PFA at 4°C. For H&E and Safranin O staining, 14% EDTA solution-decalcified skeletal specimens were embedded in paraffin, and 5 μm of sections were cut. For RNAScope and immunostaining, undecalcified skeletal tissues were embedded in OCT, and 7 μm of frozen sections were collected using the Kawamoto tape method [1]. Antibodies and DNA probes were purchased from commercial sources and used per manufacturer's instructions. To generate Prg4+ AC cell lines, newborn *Tg(Prg4*^{CreER}*;Ptpn11*$^{fx/+}$*;R26*^{ZSG}*)* and *Tg(Prg4*^{CreER}*;Ptpn11*$^{fx/fx}$*;R26*^{ZSG}*)* mice received two doses of TM at P1 and P3, and were sacrificed at P5. The knee joints were dissected and incubated with collagenase/hyaluronidase digestion solution to release Prg4+ (R26^{ZSG+}) cells. After immortalization with SV40 large T antigen, R26^{ZSG+} AC cells were sorted and cell lines were established. All cells were cultured in DMEM/F12 culture medium supplemented with 10% FBS and 1% penicillin/streptomycin. Protein synthesis inhibitor cycloheximide (CHX) and proteosome inhibitor MG132 were purchased from commercial sources.

RESULTS SHP2CTR^{Prg4ER} and SHP2KO^{Prg4ER} mice were born at the expected Mendelian ratios and exhibited similar body weight and length, growth plate thickness and overall joint structure over postnatal life. However, AC thickness increased significantly in SHP2KO^{Prg4ER} mutants, compared to SHP2CTR^{Prg4ER} controls (Fig. 1a). The increase in AC thickness was also accompanied by an increase in the abundance of anabolic gene transcripts, such as *Col2a1* and *Acan*, and SOX9 protein, and the cellularity of *Sox9*+ AC cells (Figs. 1b). These observations were replicated in mice and in cultured chondrocytes following treatment with the SHP2 PROTAC inhibitor, SHP2D26, which efficiently and rapidly depleted intracellular SHP2[2]. Our mechanistic studies indicate that SHP2 negatively regulates SOX9 stability and attenuates its degradation by proteosomes, since MG132 treatment of Control Prg4+ AC cells markedly increased SOX9 in vitro (Fig. 1c). Additional evidence that SHP2 negatively regulates SOX9 stability include the significant increase in SOX9 expression in cultured cells (Fig. 1d) and SOX9+ cells in both AC and GPC *in vivo* (Fig. 1e) after SHP2D26 administration.

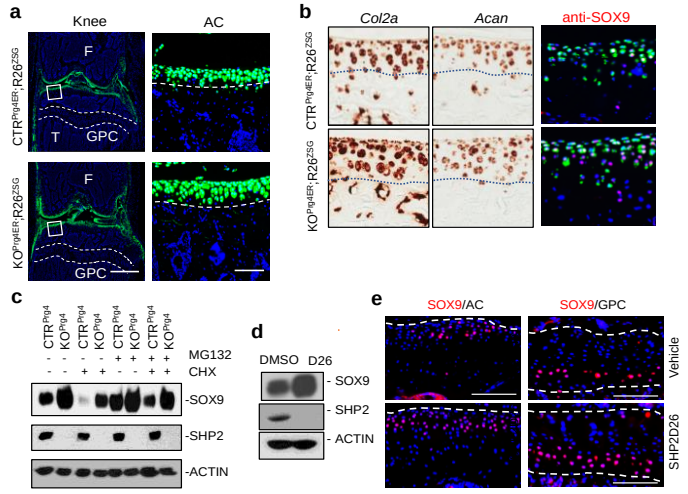

Fig. 1 a. Fluorescent microscopic images demonstrating the distribution of Prg4+ AC cells (Green) in the knee joints mice, and increased cartilage thickness in SHP2 KO mice. (TM injection at wk 2, euthanasia at wk 6). **b.** RNAScope and immunostaining demonstrating increased abundance of *Col2a1*, *Acan*, and SOX9 in AC of KO mice. **c,d.** Western blots of SOX9 expression in AC cells treated with MG132, CHX or SHP2D26. **e.** Fluorescent immunostaining demonstrating the distribution of SOX9+ cells (red) in AC (L) and GPC (R) of mice treated with vehicle or SHP2D26 for 3 weeks.

DISCUSSION Articular cartilage homeostasis is crucial for life-long joint function, but the underlying cellular and molecular mechanisms governing chondrocyte stability remain poorly understood. In this study, we have found the SHP2 supports AC development and homeostasis, in part, by influencing SOX9 stability and SOX9 responsive anabolic gene expression in the Prg4+ cartilage cells. SHP2 regulation of SOX9 stability is mainly mediated by PTM, since *Sox9* abundance is comparable in Prg4+ AC cells between SHP2CTR^{Prg4ER} and SHP2KO^{Prg4ER} mice. Importantly, MG132 but not CHX treatment stabilizes SOX9 in control cells, suggesting that SHP2 positively modifes lysosome-mediated SOX9 degradation. This finding is further supported by our in vivo findings.

SIGNIFICANCE/CLINICAL RELEVANCE: This study suggests that SHP2 is indispensable for joint development and homeostasis, and that targeting the SHP2 signaling pathway has a potential to mitigate AC degeneration by sustaining SOX9 expression and anabolic gene expression. Our findings could form the basis for novel therapeutics to a series of cartilage degenerative diseases.

REFERENCES:

1. Tadafumi Kawamoto and Komei Kawamoto. Methods in Molecular Biology, vol. 2230, https://doi.org/10.1007/978-1-0716-1028-2_15.
2. Mingliang Wang et al. J. Med. Chem. 2020, 63, 14, 7510–7528

ACKNOWLEDGEMENTS: This study was supported by NIH and NIAMS Grants R21 AR081642-01 and RO1AR066746, and the Rhode Island Hospital Orthopaedic Foundation (W.Y.).