Double Knockout of R-spondin-2 and R-spondin-3 in Osteoblasts Alters Bone Mechanical Properties

Nicholas Chiaramonti[1,2], Kyle Maas[1], Noah Sinishtaj[1], Conor S. Locke[1], Archana Sanjay[3], Kurt D. Hankenson[1]
[1]University of Michigan, Ann Arbor, MI, [2]Central Michigan University, Mt. Pleasant, MI, [3]UConn Health, Farmington CT
chiar1ni@cmich.edu

DISCLOSURES: The authors do not have anything to disclose.

INTRODUCTION: With 178 million cases reported globally in 2019, long bone fractures comprise a significant portion of injuries to the musculoskeletal system.[1] By 2030, 20% of the US population will be over the age of 65 with an increased risk for long bone fractures.[1] Attainment of peak bone mass is essential to prevent adverse fracture in this population. Wnt signaling is a well-recognized pathway that modulates the formation of bone, and it has been shown that activation of Wnt signaling can increase bone mass both in animal models and in humans. Wnt signaling can be stimulated by proteins in the R-spondin family, including R-spondin-2 (Rspo2) and R-spondin-3 (Rspo3), which show the greatest sequence homology and overlapping expression patterns in bone. Our prior work has shown that, in mice, disruption of Rspo2 reduces peak bone mass and strength[2], while Rspo3 disruption has been shown to decrease trabecular bone mass and vertebral bone strength[3]. Herein, we advance our understanding of Rspo2 and Rspo3 in bone by concurrently disrupting both genes in osteoblasts constitutively. We hypothesized that genetic knockout of Rspo2 and Rspo3 together would impair bone mechanical properties.

METHODS: With animal care and use committee approval, to assess the roles of Rspo2 and Rspo3 in bone growth and development, Osteocalcin Cre positive (+) mice were crossed with Rspo2 floxed and Rspo3 floxed mice to obtain Cre- (wildtype) and Cre+ Rspo2 Rspo3 double floxed knockouts (DfKO). Male and female mice were double labeled with calcein and harvested when 1-, 3-, 6-, and 9-month-old. Femora were wrapped in saline soaked gauze and kept frozen at -20°C. MicroCT scans of each femur were obtained with a Bruker/Skyscan 1176 High Resolution micro-CT imaging system prior to mechanical testing. Femora underwent 4-point bend mechanical testing to interrogate mechanical function.

RESULTS: Constitutive knockout of Rspo2 and Rspo3 in Osteocalcin Cre+ cells during mouse development (DfKO) leads to significant differences in several metrics of the mechanical properties of female femurs at 3 and 6 months (Figure 1A-D), as well as male femurs at 6 months (Figure 1E and 1F). At 3 months, female DfKO femurs required significantly lower forces to reach the yield point than wild type femurs (15.32 N vs. 17.95 N, $p < 0.05$). At 6 months, female DfKO femurs displayed significantly lower femur stiffness than wildtype femurs (158.2 N/mm vs. 262.7 N/mm, $p < 0.05$). Also at 6 months, female DfKO femurs exhibited a greater displacement of the femur at the yield point than wildtype femurs (0.134 mm vs. 0.0965 mm, $p < 0.05$). For 6-month-old male femurs lacking Rspo2/3 expression, there was a significantly lower yield load, ultimate load, and failure load as compared to wildtype male femurs at this time point (yield load = 17.6 N vs. 25.0 N, $p < 0.05$; ultimate load = 26.3 N vs. 31.7 N, $p < 0.05$; failure load = 23.3 N vs. 29.9 N, $p < 0.05$). 6-month DfKO males also had significantly decreased femur stiffness compared to wildtype males (199.02 N/mm vs. 245.89 N/mm, $p < 0.05$).

DSICUSSION: From these data, we conclude that constitutive dual knockout of both Rspo2 and Rspo3 during osteoblast differentiation results in significant differences in the mechanical properties of mouse femurs. These findings are congruent with previous work in our laboratory which established the capacity of Rspo2 to activate Wnt signaling and regulate osteoblast activity. Specifically, Rspo2 disruption in mature osteoblasts was shown to reduce peak bone mass, weight, and crown-rump length in mice, and to decrease mechanical properties of bone.[2] Rspo2 is thought to be required for Wnt signaling, osteoblast activity, and bone formation. Rspo3 also has a known role as a Wnt signaling agonist[4], and previous studies have identified Rspo3 as a regulator of trabecular bone development and bone strength. Rspo3 appears to have variable roles depending on the bone compartment (long bone trabecular vs cortical bone vs vertebral bone).[3] While generally our work supports the concept that R-spondins positively regulate cortical bone mechanical strength, contextualized relative to the work of Knight et al 2018 when Rspo2 was disrupted in osteoblasts, it is surprising that strength deficits were not more pronounced when both genes were disrupted. Despite structure and functional homology, Rspo2 and Rspo3 may have both overlapping and unique roles in regulating bone formation, structure, and function. On-going microCT analysis is exploring femoral structural and mineral density parameters of DfKO femurs, and dynamic histomorphometry will be used to assess bone formation.

CLINICAL RELEVANCE: These findings support a role for R-spondin proteins in adult bone function. Additional studies are needed to test R-spondin proteins as therapeutic agents in diseases with deficient bone development, helping to translate these results into improved patient outcomes.

REFERENCES:

(1) GBD 2019 Fracture Collaborators, *Lancet Healthy Longev* 2021, *2* (9), e580-e592. DOI: 10.1016/S2666-7568(21)00172-0.; **(2)** Knight, et al. *Bone Res* 2018, *6*, 24. DOI: 10.1038/s41413-018-0026-7.; **(3)** Nilsson, et al. *Nat Commun* 2021, *12* (1), 4923. DOI: 10.1038/s41467-021-25124-2. **(4)** Kim, et al. *Mol Biol Cell* 2008, *19* (6), 2588-2596. DOI: 10.1091/mbc.e08-02-0187.

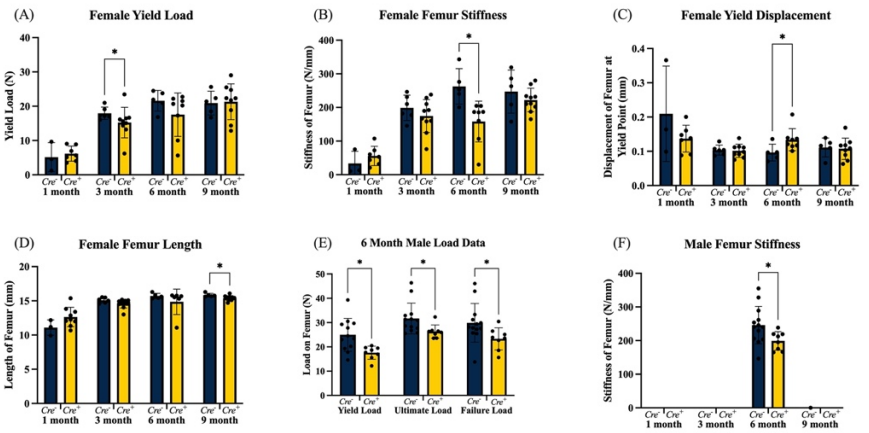

Fig. 1 Results from 4-point bend mechanical testing of 1-, 3-, 6-, and 9-month female and 6-month male femurs. (A) Load required to reach the yield point. (B) Stiffness of femur in response to increasing load. (C) Displacement of femur at the yield point. (D) Length of femur measured from greater trochanter to lateral condyle. (E) All load data from 6-month male femurs: yield load measured at yield point, ultimate load measured at maximum load applied, failure load measured at time of fracture. (F) Stiffness of femur in response to increasing load. * = P<0.05.