Effects of sFRP1 inhibitor Way 316606 on the bone, muscle, and heart of Dystrophin$^{-/-}$/Utrophin$^{-/-}$Double Knockout Mice

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Introduction: Duchenne's Muscular Dystrophy (DMD) is a severe genetic muscle disease due to the mutation of the dystrophin gene [1]. DMD patients also have high risks of bone fracture. Dystrophin$^{-/-}$/Utrophin$^{-/-}$ (DKO-Hom) is a mouse model that recapitulates the clinical manifestations of DMD better than dystrophin$^{-/-}$ (Mdx*)* mice with severe muscle histopathology including muscle necrosis, fibrosis, fat infiltration, heterotopic bone formation (HO), kyphosis and short lifespan [1]. DKO-Hom mice exhibit bone osteopenia as early as 4 weeks and secondary to muscle pathology as well as delayed fracture healing [2, 3]. We hypothesize that the detrimental factors released from the diseased muscle of DKO-Hom cause bone osteopenia. The aim of this study is to investigate if secreted frizzled-related protein 1 (sFRP1) plays an important role in the development of osteopenia.

Methods: 1. Animal breeding and tissue collection. DKO-Hom mice were generated using dystrophin$^{-/-}$/Utrophin$^{+/-}$ mice bred at the Animal Facility of Colorado State University. All experiments were approved by IACUC animal protocol of CSU (#1234). 4 weeks old C57BL/10J (WT), Mdx and DKO-Hom mice (n=5 each) were sacrificed, thigh muscles were collected for RNA isolation. After extraction of RNA, cDNA synthesis, then quantitative polymerase chain reaction (Q-PCR) was performed to detect sFRP1. **2. Mice treatment.** DKO-Hom mice at 4 weeks-old were then divided into 2 groups (n=5/group including both males and females) and treated with vehicle (5%DMSO in PBS) or sFRP1 inhibitor Way 316606 at 1mg/kg using IP injection twice a week a previously used [4]. Mice were then sacrificed at 2 weeks after treatment. **3. MicroCT scanning and analysis for bone tissues.** Lumbar spine, tibia, and femur were harvested and fixed in formalin for microCT and histology analysis. MicroCT scanning was performed using 15µm voxel size using Viva-CT 80. **4. Muscle and heart histology analysis.** Gastrocnemius muscle tissues and heart were collected, and H&E staining was performed using ANATECH. Inc reagents. Data were analyzed using Graphpad Prism 10.1. P<0.05 was considered statistical significance.

Results: 1. SFRP1 is significantly increased in the thigh muscle of DKO-Hom mice. Q-PCR results indicated sFRP1 was significantly increased in the thigh muscle of DKO-Hom mice compared to WT or Mdx mice (P=0.0053 and 0.0059 respectively) (**Fig.1A**). **2. Effects of sFRP1 treatment on the spine and long bone microarchitecture.** Gross microCT images for the spine and femur showed similar HO formation between Way 316606 treated mice and non-treated control mice (**Fig.2A-B**). For the spine L5 bone microarchitecture, we found Way316606 treated mice showed a trend of increase in trabecular thickness (Tb.Th), trabecular separation (Tb.Sp) and a significant increase in the bone volume (BV) density. No significant differences for bone volume/total volume (BV/TV) and trabecular number (Tb.N) between Way316606 treated and control mice were observed (**Fig.2C-H**). For the proximal tibia trabecular bone, Way316606 treatment did not significantly change the BV/TV, Tb.N, Tb.Th, Tb.Sp and BV density compared to control mice (**Fig.2I-N**). For the femur cortical bone, Way316606 did not significantly affect the cortical thickness of midshaft femur, but significantly increased BV density compared to the control (**Fig.2.Q-N**). **3. Effects of Way316606 treatment on muscle and heart pathology.** H&E staining showed Way 316606-treated gastrocnemius muscle still showed severe inflammation and fibrosis compared to the control. No obvious improvement was observed (**Fig.3A-B**). Furthermore, H&E staining for the heart showed heart tissues have relative mild inflammation and fibrosis, there was no improvement observed in the Way316606 treated group compared to the control group (**Fig.3C-D**).

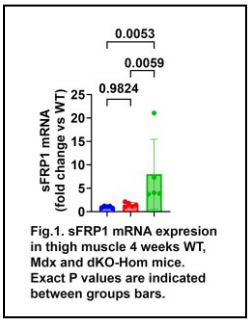

Fig.1. sFRP1 mRNA expresion in thigh muscle 4 weeks WT, Mdx and dKO-Hom mice. Exact P values are indicated between groups bars.

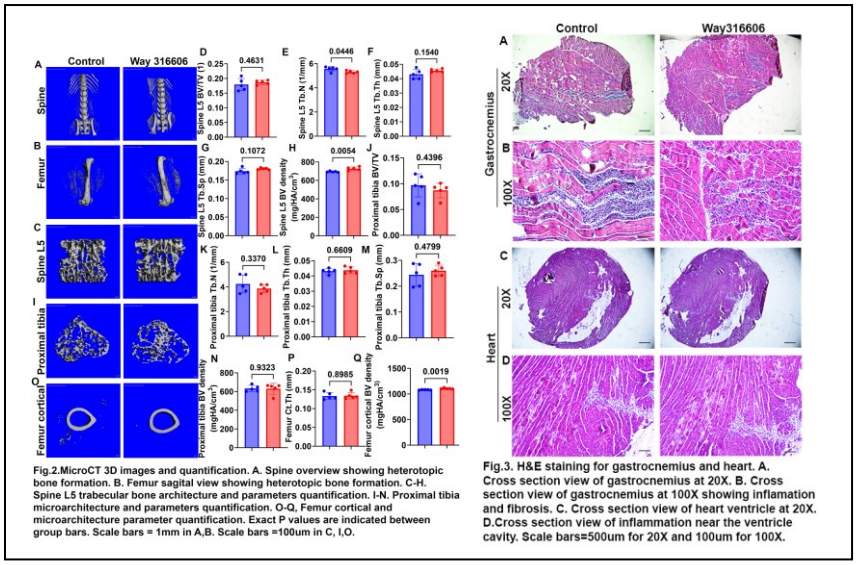

Fig.2.MicroCT 3D images and quantification. A. Spine overview showing heterotopic bone formation. B. Femur sagital view showing heterotopic bone formation. C-H. Spine L5 trabecular bone architecture and parameters quantification. I-N. Proximal tibia microarchitecture and parameters quantification. O-Q, Femur cortical and microarchitecture parameter quantification. Exact P values are indicated between group bars. Scale bars = 1mm in A,B. Scale bars =100um in C, I,O.

Fig.3. H&E staining for gastrocnemius and heart. A. Cross section view of gastrocnemius at 20X. B. Cross section view of gastrocnemius at 100X showing inflamation and fibrosis. C. Cross section view of heart ventricle at 20X. D.Cross section view of inflammation near the ventricle cavity. Scale bars=500um for 20X and 100um for 100X.

Discussion and conclusion: Our previous study showed sFRP1 was significantly increased in the 6-8 weeks old DKO-Hom mice compared to WT mice. It was also significantly increased in the non-myogenic mesenchymal stromal cells (platelet-derived growth factor receptor α (PDGFRα)$^+$ cells) in DKO-Hom mice compared to WT mice [5]. This study revealed sFRP1 was significantly increased at 4 weeks DKO-Hom mice compared to WT and Mdx mice which is the time when we found DKO-Hom mice already exhibited osteopenia. Further, we treated mice with sFRP1 inhibitor Way 316606, but we did not find significant improvement of BV/TV of both spine L5 and proximal tibia. We did find that Way316606 treatment increased BV density of spine L5 trabecular bone and femur cortical bone. Way316606 did not significantly improve the muscle HO bone formation (microCT) as well as inflammation of gastrocnemius muscle and heart tissues. A previous study showed Way 316606 treatment significantly increased the bone microarchitecture of ovariectomy-induced osteoporosis in mice after 6 weeks of treatment [4]. The insignificant improvement on bone, muscle and heart observed in this study may be due to the short time treatment. In conclusion, sFRP1 is significantly increased in the DKO-Hom mice but may not play a critical role in the bone and muscle pathology and bone osteopenia observed in DKO-Hom mice.

Significance/clinical relevance: Targeting sFRP1 to treat osteopenia and high fracture risk may be complicated by other abnormal signaling pathways in DMD patients. Further study with longer time treatment is required. **Acknowledgments:** This project was funded by NIH R01AR065445 to Dr. Huard.