Mechanical stimulation of soft tissue cells regulates osteoblast differentiation and activity through soluble factors

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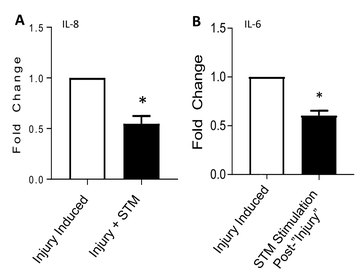
Abstract

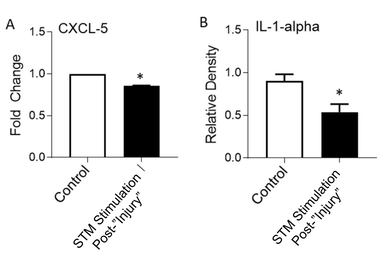
Osteoporosis is a disease of low bone mass that places individuals at enhanced risk for fracture, disability, and death. Hospitalizations for osteoporotic fractures exceeds those for heart attack, stroke, and breast cancer combined, and osteoporosis rates are expected to rise significantly in the coming decades. Despite this, there are limited pharmacological treatment options for osteoporosis, particularly for long-term management of this chronic condition, and the drug development pipeline is relatively bereft of new strategies and drug candidates. Consequently, there is an urgent need for new therapeutic strategies for treating osteoporosis. Here, we present a novel line of investigation examining the ability of non-invasive soft tissue manipulation (STM) to exert anabolic effects on the skeleton that may provide therapeutic benefit for individuals with low bone mass. Our rationale is premised on work showing that STM leads to decreased levels of chemokines and pro-inflammatory cytokines (such as Interleukin (IL)-1-alpha, IL-6, IL-8 and CXCL5) known to restrict the differentiation and/or activity of bone-forming osteoblasts. Additionally, STM is associated with increased serum levels of the bone formation marker N-terminal propeptide of type 1 procollagen and decreased serum levels of the bone resorption marker collagen type 1 C-telopeptide in young, healthy women and increased serum P1NP levels in some women with osteoporosis. To advance this work, we hypothesized that STM promotes the differentiation and/or activity of bone-forming osteoblasts and increases bone mass. Consistent with this, we show that conditioned media from primary dermal fibroblasts subjected to STM-like stimulation is bioactive and promotes a) increased osteoprogenitor cell proliferation and differentiation *in vitro* and b) increased bone formation in an *ex vivo* bone explant model using neonatal tibiae. Consistent with this, conditioned media from primary skeletal muscle myocyte and satellite cell cultures after STM-like stimulation promotes increased osteoprogenitor cell proliferation *in vitro*. Collectively, these data support the idea that STM stimulation of soft tissue cells may influence skeletal homeostasis. The experimental application of STM to improving bone mass is novel in its focus, which is significant given the relationship between low bone mass and high fracture risk in patients with osteoporosis and the need for new treatment strategies for this disease.

Primary Dermal Fibroblasts Preliminary Data



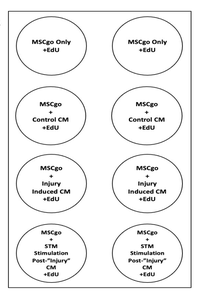
A&B: Quantification of these analytes are from the cytometric bead array assay. They are represented as means ±SEM normalized to Injury Induced Strain; n=3 per condition. *indicates p<0.05 against Injury Induced Strain by paired T-Test

HSkMCs & C2C12 Myoblasts Preliminary Data



A represents pilot data of HSkMC. **B** represents pilot data of C2C12 myoblasts. Quantification is by multi-analyte membrane array and is represented by means ±SEM normalized to control; n=2 per condition. *indicates p<0.05 against control by paired T-Test.

Osteoblast Differentiation Profile

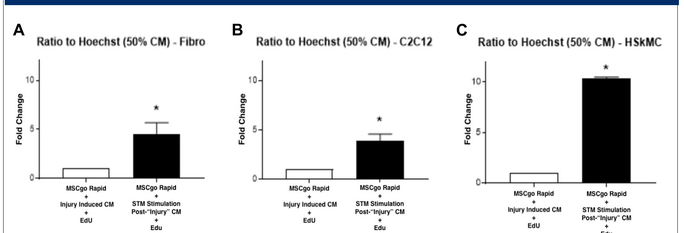


B

Sample Osteoblast Differentiation Schedule	
48 Hour EdU Proliferation	
Day -1	Plate 60,000 cells per well of W-20-17 cells (osteoblast precursors) w/ regular growth media
Day 0	Replace growth media with 500 ul 50/50 CM & MSCgo mixture for each condition + 0.5 ul EdU
Day 2	Collect media & use plate for EdU Proliferation Assay

A: Represents the plate layout of the conditions used for the osteoblast differentiation. These tests were done in duplicate using a 50:50 mix of MSCgo Rapid growth media and conditioned media obtained from our preliminary experiments seen to the left. **B:** Represents a normal schedule to collect differentiated media tagged with EdU for proliferation studies to be completed.

Osteoblast Differentiation



A-C represents data obtained using Click-iT™ Plus EdU Cell Proliferation Kit to quantify proliferation of various cell lines

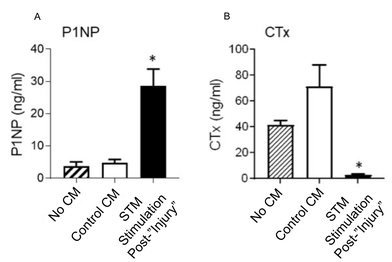
A: Primary Dermal Fibroblasts – represented as means ± SEM normalized to "MSCgo Rapid + Injury Induced CM + EdU"; n=3 per condition. *indicates p<0.05 against "MSCgo Rapid + Injury Induced CM + EdU" by paired T-Test.

B: C2C12 Myoblasts – represented as means ± SEM normalized to "MSCgo Rapid + Injury Induced CM + EdU"; n=5 per condition. *indicates p<0.05 against "MSCgo Rapid + Injury Induced CM + EdU" by paired T-Test.

C: Human Skeletal Myocytes – represented as means ± SEM normalized to "MSCgo Rapid + Injury Induced CM + EdU"; n=2 per condition. *indicates p<0.05 against "MSCgo Rapid + Injury Induced CM + EdU" by paired T-Test.

Tibiae Explant Differentiation

Enzyme-Linked Immunosorbent Assay (ELISA) results for bone formation marker P1NP (**A**) and bone resorption marker CTx (**B**) from neonatal tibiae explants exposed to osteogenic media +/- conditioned media (CM) from dermal fibroblasts subjected to cyclic short duration strain (CSDS) followed by acyclic long duration strain (ALDS) or control. * indicates p<0.05 compared to control conditioned media



Conclusions

Observing the osteoblast differentiation of W-20-17 cells after being exposed to conditioned media, we see statistically significant increases in proliferation in all three cell lines when comparing our "injury induced" conditioned media exposed cells to our "STM treatment" conditioned media exposed cells, with the largest proliferation increase seen in human skeletal myocytes with a 10-fold change. Combining these results with the original data from tibiae explants, which shows a statistically significant increase in bone formation marker P1NP and a statistically significant decrease in bone resorption marker CTx, we are provided key insight into how STM might promote the proliferation of osteoblast cells and eventually leading to the rebuilding of bone.

Continuing with this experimental design, we plan to further progress in the work of osteoblast differentiation by obtaining conditioned media through STM stimulation of tissue biopsies using a FlexCell machine. We plan to continue to support the idea that soft tissue manual therapy stimulation of soft tissue cells may influence skeletal homeostasis. If we can continue to prove this hypothesis, it provides hope that STM can one day be used as an alternative treatment of osteoporosis for patients with low bone mass or are high in risk for fractures.

References & Acknowledgements

Anloague, A., et al., Mechanical stimulation of human dermal fibroblasts regulates pro- inflammatory cytokines: potential insight into soft tissue manual therapies. BMC Res Notes, 2020. 13(1): p. 400.
Raisz, L.G., Pathogenesis of osteoporosis: concepts, conflicts, and prospects. J Clin Invest, 2005. 115(12): p. 3318-25.
Lebiome, A., et al., Osteoporosis and mortality. Joint Bone Spine, 2010. 77 Suppl 2: p. S107-12.
Wade, S.W., et al., Estimating prevalence of osteoporosis: examples from industrialized countries. Arch Osteoporosis, 2014. 9: p. 182.
Singer, A., et al., Burden of illness for osteoporotic fractures compared with other serious diseases among postmenopausal women in the United States. Mayo Clin Proc, 2015. 90(1): p. 53-62.
Bonjour, J.-P., et al., The importance and relevance of peak bone mass in the prevalence of osteoporosis. Salud publica de Mexico, 2009. 51: p. s5-s17.
Watts, N.B., et al., American Association of Clinical Endocrinologists Medical Guidelines for Clinical Practice for the diagnosis and treatment of postmenopausal osteoporosis. Endocr Pract, 2010. 16 Suppl 3: p. 1-37.
Suresh, E., M. Pazianas, and B. Abrahamsen, Safety issues with bisphosphonate therapy for osteoporosis. Rheumatology (Oxford), 2014. 53(1): p. 19-31.
Kerschan-Schindl, K., Romosozumab: a novel bone anabolic treatment option for osteoporosis? Wien Med Wochenschr, 2019.
Gullberg, B., O. Johnell, and J.A. Kanis, World-wide projections for hip fracture. Osteoporos Int, 1997. 7(5): p. 407-13.
Kaneshiro, S., et al., IL-6 negatively regulates osteoblast differentiation through the SHP2/MEK2 and SHP2/Akt2 pathways in vitro. J Bone Miner Metab, 2014. 32(4): p. 378-92.
Murakami, M. and N. Nishimoto, [IL-6 inhibitors prevent bone loss and cartilage degeneration in rheumatoid arthritis]. Clin Calcium, 2015. 25(12): p. 1851-7.
Loghmani, M.T., and M. Whitted, Soft Tissue Manipulation: A Powerful Form of Mechanotherapy. Journal of Physiotherapy & Physical Rehabilitation, 2016. 1: p. 122.
Thompson, W.R., et al., Understanding Mechanobiology: Physical Therapists as a Force in Mechanotherapy and Musculoskeletal Regenerative Rehabilitation. Phys Ther, 2016. 96(4): p. 560-9.
Anloague, A., et al., In vitro mimicking of therapeutic soft tissue stimulation regulates pro-inflammatory cytokines. Research Square Preprint, 2019.
Saetung, S., L.O. Chalurlut, and B. Ongphiphadhanakul, Thai traditional massage increases biochemical markers of bone formation in postmenopausal women: a randomized crossover trial. BMC Complement Altern Med, 2013. 13: p. 69.
Saetung, S., L.O. Chalurlut, and B. Ongphiphadhanakul, Acute changes in biochemical markers of bone resorption and formation after Thai traditional massage. J Med Assoc Thai, 2010. 93(7): p. 771-5.
Meltzer, K.R. and P.R. Standley, Modeled repetitive motion strain and indirect osteopathic manipulative techniques in regulation of human fibroblast proliferation and interleukin secretion. J Am Osteopath Assoc, 2007. 107(12): p. 527-36.
Eagan, T.S., K.R. Meltzer, and P.R. Standley, Importance of strain direction in regulating human fibroblast proliferation and cytokine secretion: a useful in vitro model for soft tissue injury and manual medicine treatments. J Manipulative Physiol Ther, 2007. 30(8): p. 584-92.
Lowery, J.W. and V. Rosen, Bone Morphogenetic Protein-Based Therapeutic Approaches. Cold Spring Harb Perspect Biol, 2017.

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