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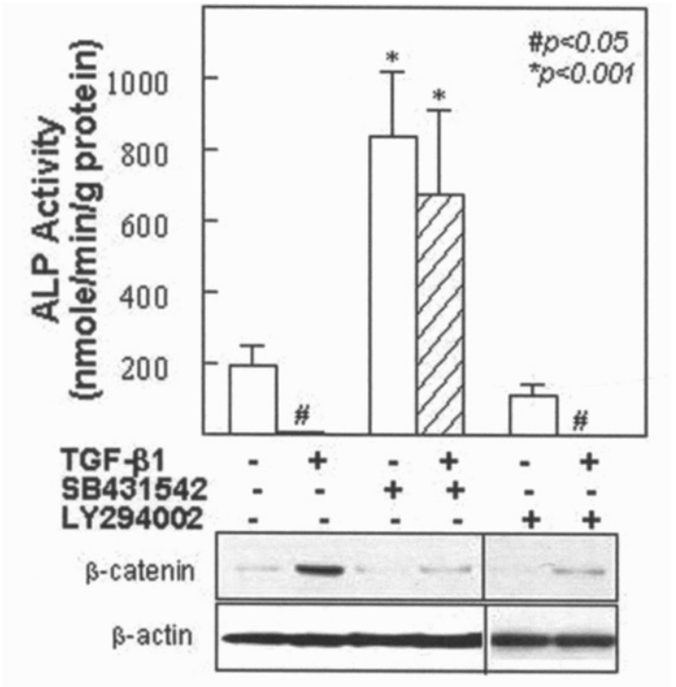
Blocking TGF-β and Wnt Signaling Stimulates Osteoblastogenesis of Human Bone Marrow Mesenchymal Stem Cells. S. Zhou, J. Glowacki. Orthopedic Surgery, Brigham and Women's Hospital, Harvard Medical School, Boston, MA, USA.

Adult human bone marrow stromal cells or mesenchymal stem cells (hMSCs) have the potential to differentiate to osteoblasts, chondrocytes or adipocytes. Our previous data showed that TGF-β and Wnt interact to promote chondrocyte differentiation and inhibit adipocyte differentiation [JBMR 19(3):463-470, 2004]. In other processes, TGF-β and Wnt/wingless signaling pathways control developmental events and activation of specific target genes. In this study, we tested the hypothesis that these pathways interact in osteoblast differentiation of hMSCs. Low-density mononuclear cells were isolated from fresh marrow by centrifugation on Ficoll/Histopaque 1077; adherent hMSCs were used at early passage. Effects of TGF-β1 on Wnt signaling molecule β-catenin were evaluated at 2 days with Western immunoblot. Osteoblast differentiation was assessed by biochemical assays for alkaline phosphatase (ALKP) activity at 2 weeks. First, we examined the mechanism by which TGF-β regulates Wnt signaling pathway. TGF-β1 (1 ng/mL) increased nuclear accumulation and stability of β-catenin in a time dependent manner. Use of small, specific chemical kinase inhibitors showed that TGF-β type I receptor (ALK-5) inhibitor, SB431542 (10 μM), and PI-3 kinase inhibitor, LY294002 (40 μM), but not p42/44MAPK, p38MAPK, JNK, PKA or PKC inhibitors, blocked TGF-β1 stimulation of β-catenin in hMSCs. Marrow cells from Smad3-/- mice showed that Smad3 is necessary for β-catenin accumulation. Thus, TGF-β stimulation of β-catenin requires ALK5/Smad3 and PI-3 kinase signaling. Second, we showed that TGF-β1 inhibited osteoblast differentiation in hMSCs, synergistically with Wnt signal (5 mM LiCl). The ALK5 inhibitor, SB431542 (10 μM), stimulated ALP activity 8.8-fold (Figure, $p<0.001$, ANOVA) and antagonized the inhibitory effects of TGF-β1 on ALP activity. Thus blocking endogenous and exogenous TGF-β1 stimulated osteoblast differentiation. PI-3 kinase inhibitor, LY294002 (10 μM), did not mitigate TGF-β1's inhibition of osteoblastogenesis although it too blocked TGF-β1 stimulation of β-catenin. In sum, TGF-β activates Wnt signal pathway via ALK5/Smad3 and PI-3 kinase, and blocking TGF-β and Wnt signaling with ALK5 inhibitor stimulated osteoblast differentiation of human bone marrow mesenchymal stromal/stem cells.



Disclosures: S. Zhou, None.
This study received funding from: NIH, MTF.

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Bone Mineralization and Inflammation Genes Increase in Muscles as a Consequence of Age and Performance of a High Repetition Task. M. F. Barbe[1], F. F. Safadi[2], S. N. Popoff[2], M. Amin*[1], M. Harris*[1], A. E. Barr[1]. [1]Physical Therapy, Temple University, Philadelphia, PA, USA, [2]Anatomy and Cell Biology, Temple University School of Medicine, Philadelphia, PA, USA.

We have shown that performance of highly repetitive tasks is associated with increased serum levels of IL-1alpha and increased activated macrophages in musculoskeletal tissues. Our purpose was to examine expression patterns of genes related to bone mineralization and inflammation in flexor forelimb muscles from young adult and aged (16 months) rats performing a repetitive reaching and grasping task. Twelve young adult and 12 aged female Sprague-Dawley rats were used. Six of each age group performed a high repetition low force (HRLF) task for 2 hrs/dy, 3 dys/wk for 3 weeks. The remaining were age-matched controls. Half of the rats were perfused with paraformaldehyde for immunohistochemistry while tissues from the remaining were collected unfixed and flash frozen for molecular analyses. mRNA was extracted using TRizol, Affymetrix oligonucleotide 230A genechip analysis performed and analyzed statistically (3-4/group). A significant upregulation of Osteoactivin, a gene related to bone mineralization, was found in aged rats (both control and task), while alpha-2-HS-glycoprotein was significantly down-regulated in young adult 3 week HRLF task rats only. Clear trends of upregulation of other genes related to bone mineralization were seen as a consequence of 1) age (matrix Gla protein and ameloblastin); 2) both age and task performance (osteopontin); and 3) task performance in aged rats only (soluble fibroblast growth factor receptor IIIb, osteoregulin, and osteocalcin). Significant upregulation of genes related to inflammation were also seen as a consequence of 1) age (interleukin 6 signal transducer, toll protein, complement component 5 receptor 1); 2) task performance in young rats only (attractin, CD59 antigen, complement component factor h); and 3) task performance in aged rats only (corticotropin releasing hormone, interleukin 4, interferon regulatory factor 1, inhibin alpha, and interleukin 6). Also, CD4 antigen, IL-4 and IL-10 mRNA decreased in young 3 week HRLF rats, but not in aged HRLF rats. In contrast, IL-4 was significantly increased in aged 3 week HRLF rats. Microarray results were verified using immunohistochemistry and ELISA for osteoactivin and several interleukins. These results show that a high repetition-low force reaching and grasping task induces genes related to both inflammation and bone mineralization in muscle tissues, suggesting that these molecules play a role in either inflammation or injury healing. Some of these genes are increased as a consequence of age.

Disclosures: M.F. Barbe, None.
This study received funding from: NIOSH and NIAMS.

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Caveolin-1 Scaffolds Are not Necessary for Lipid Raft Microdomains Involved in the Mechanical Strain Response. N. Case*[1], H. Jo*[2], J. Rahnert*[3], X. Fan[3], T. Gross[4], J. Rubin[1]. [1]University of North Carolina, Chapel Hill, NC, USA, [2]Georgia Tech/Emory, Atlanta, GA, USA, [3]VAMC/Emory, Decatur, GA, USA, [4]University of Washington, Seattle, WA, USA.

Mechanical loading regulates bone remodeling; recent work has provided insights into the molecules involved in mechanical signal transduction. We have shown that H-Ras GTPase activation of ERK1/2 is required for mechanical inhibition of RANKL expression. H-Ras is known to be localized to lipid raft microdomains, and disruption of raft domains blocks mechanical activation of H-Ras. An association between H-Ras and caveolin-1 (Cav-1), a scaffolding protein found in caveolar lipid rafts, has been reported. Indeed, Cav-1 was shown to be necessary for shear-dependent activation of ERK1/2 in endothelial cells and has been suggested to play a role in other mechanoresponsive pathways. In this study we investigated the role of Cav-1 in the mechanical regulation of bone remodeling effector genes. We first evaluated a potential interaction between Cav-1 and H-Ras in mechanically responsive CIMC-4 cells. Immunoprecipitation of culture lysates indicated that Cav-1 and H-Ras were not associated. Immunofluorescent double labeling and evaluation with confocal microscopy revealed that Cav-1 and H-Ras did not co-localize in CIMC-4 osteoblasts. Interestingly, we did observe co-localization between H-Ras and flotillin-1, a raft-associated protein absent in caveolar microdomains also known as reggie-2. These results suggest that H-Ras is localized to lipid raft microdomains that lack Cav-1 but do contain flotillin-1. To further probe a potential role for Cav-1 in the strain response, we utilized siRNA against Cav-1. siCav-1 (60 nM) inhibited Cav-1 mRNA at 48 hours and decreased Cav-1 protein at 72 hours. Cultures were incubated with siCav-1 or a negative control siRNA prior to application of strain overnight (2%, 0.17Hz) and were examined for strain responses dependent on mechanically activated H-Ras. Silencing Cav-1 did not alter the mechanoregulation of RANKL, osterix, or RUNX2 gene expression: all strained cultures showed a decrease in RANKL mRNA and increases in both osterix and RUNX2 mRNAs as compared to unstrained cultures ($p<0.05$). In confirmation, Cav-1 null stromal cells were shown to be similarly mechanoresponsive. Overall, our data indicate that Cav-1 is not required for strain regulation of those bone remodeling effector genes mediated via activation of H-Ras GTPase. Having confined H-Ras to a subset of lipid raft microdomains that contain flotillin-1 but not Cav-1 may allow us to narrow our search for mechanically responsive molecules to those that co-localize with flotillin-1.

Disclosures: N. Case, None.