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14. ABSTRACT

Our application seeks to develop myostatin inhibitors as novel therapeutic agents for improving muscle and bone strength, and preventing falls and fractures for aging veterans. Our goals for year 1 of the project were to determine how the expression of myostatin, its receptor, and the myostatin antagonist follistatin change with age in musculoskeletal tissues from aged mice and bone marrow samples obtained from young (18-30) and older (50-70) patients in the orthopaedic clinic. Our findings so far demonstrate that, in skeletal muscles from mice, myostatin expression does not change with age but expression of the myostatin antagonist follistatin decreases significantly with age. Data from bone marrow samples show that while myostatin shows a moderate increase with age, and follistatin levels show no change, levels of activin A increase significantly with age. Activin binds the same receptor as myostatin, and inhibits bone mineralization. These data suggest that a decoy receptor that binds both myostatin and activin A may prevent loss of muscle and bone mass with age

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Introduction

Loss of muscle mass with age is implicated in age-related bone loss, and muscle frailty contributes to an increased incidence of falls and fractures. Yet, the molecular mechanisms underlying age-related muscle wasting, and the ability of muscle to promote bone formation and fracture healing, are unknown. We have focused our research on the role of myostatin (GDF-8) in muscle-bone interactions in order to develop more effective treatment and prevention strategies for muscle injury, frailty, and bone fracture. We have previously shown that myostatin deficiency increases bone strength and biomineralization throughout the skeleton, and that a new myostatin inhibitor (propeptide) increases both muscle mass and bone formation (Hamrick et al., 2007, 2010; Elkasrawy and Hamrick, 2010). Our research therefore suggests that myostatin is a key factor regulating both myogenesis and osteogenesis.

The goal of our CDMRP-sponsored research is to better characterize myostatin's role in age-related bone loss, so that targeted therapies to prevent bone fractures by enhancing muscle and bone strength can be developed. We hypothesize that the expression of myostatin and its receptor are elevated with aging in bone and muscle, which antagonizes the osteogenic and myogenic capacity of stem cells in these tissues, but that myostatin inhibitors will reverse this age-related decline in musculoskeletal function. Year 1 of the project is to determine how the expression of myostatin, its receptor, and the myostatin antagonist follistatin change with age in musculoskeletal tissues. We investigated age-related alterations in the expression of myostatin in muscle and bone marrow from mice. We also examined changes in the expression of myostatin, follistatin, and the myostatin receptor in human bone marrow-derived stromal cells obtained from young (18-30) and older (50-70) patients in the orthopaedic clinic. Our findings so far demonstrate that, in skeletal muscles from mice, expression of the myostatin antagonist follistatin decreases significantly with age. Data from patient bone marrow samples show that while myostatin shows a moderate increase with age, and follistatin levels show no change, levels of activin A increase significantly with age. Activin binds the same receptor as myostatin, and inhibits bone mineralization. These data suggest that a decoy receptor that binds both myostatin and activin A may prevent loss of muscle and bone mass with age.

Body

<u>Changes in myostatin signaling with age in mouse skeletal muscle</u>:

We used real-time PCR (RT-PCR) to analyze gene expression and ELISA to measure protein levels of myostatin, the myostatin receptor (ActRIIB), activin A, and follistatin in slow-twitch (soleus) and fast-twitch (EDL) muscles from young (12 months) and aged (24 months) mice. The data show that while the expression of myostatin, activin, and their receptor are not altered significantly with age in muscle, follistatin expression increases both in terms of gene expression and in protein levels in the fast-twitch extensor digitorum longus muscle (Fig.1). These data are particularly relevant for aging because it is most often fast-twitch fibers that are reduced in size and strength with age, contributing directly to a greater risk for falls.

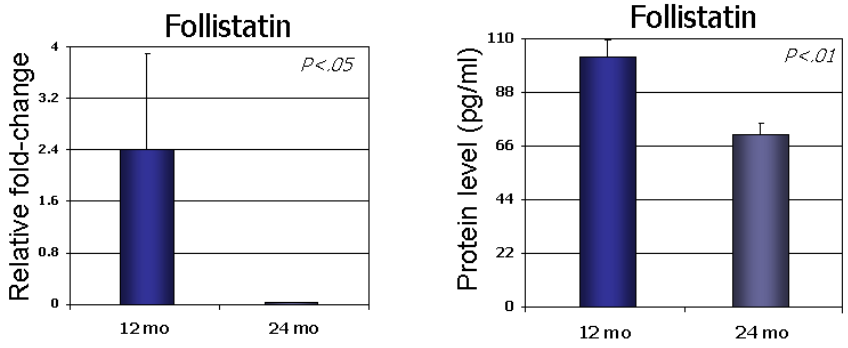

Figure 1. *Relative gene expression (left) and protein levels (right) of the myostatin antagonist follistatin decline significantly with age in the mouse extensor digitorum longus muscle, a muscle that is composed primarily of fast-twitch (type II) fibers*.

Changes in myostatin signaling with age in the bone marrow microenvironment of mice and humans:

In vitro and in vivo studies have demonstrated that both activin A and its antagonist follistatin play important roles in bone formation. Activin A treatment can inhibit mineralization in cultured osteoblasts, whereas follistatin increases mineralization (Eijken et al., 2007). Furthermore, inhibiting activin A in vivo using a decoy soluble activin A receptor (ActRIIA) increases bone formation in mice and monkeys (Pearsall et al., 2008; Lotinun et al., 2010). Follistatin also antagonizes myostatin (GDF-8), and mice lacking myostatin show increased bone density. Thus, interactions among these factors are likely to be involved in the regulation of bone mass throughout growth, development and aging. Yet, changes in the relative levels of these factors within the bone marrow microenvironment with increasing age are not well understood. We investigated age-related changes in the activin A-myostatin-follistatin system using femoral bone marrow aspirates from young (12 months) and aged (24 months) mice, as well as bone marrow samples from young (<50 years, n=7) and older (>70 years, n=10) knee arthroplasty patients.

Samples were processed using centrifugation to obtain bone marrow supernatant fluid from both mice and humans, since the soluble factors within the marrow supernatant in part define the extracellular milieu to which bone cells are regularly exposed. Supernatant samples were analyzed using ELISA. Results indicate that follistatin levels are not significantly altered with age in bone marrow supernatants from either mice or humans (Figs. 2, 3). Myostatin levels were significantly (+75%) increased in mouse bone marrow with age, but did not increase with age in human bone marrow (Figs. 2, 3). Activin A levels increased with age (+17%) in mouse bone marrow, and increased by more than 120% in human bone marrow (Figs. 2, 3). The marked increase in activin A levels with age in the patient samples was associated with a similar increase in the activin A: follistatin ratio. These findings suggest that activin A bioavailability is altered with age in the bone marrow microenvironment, and may therefore play a role in age-associated changes in bone metabolism.

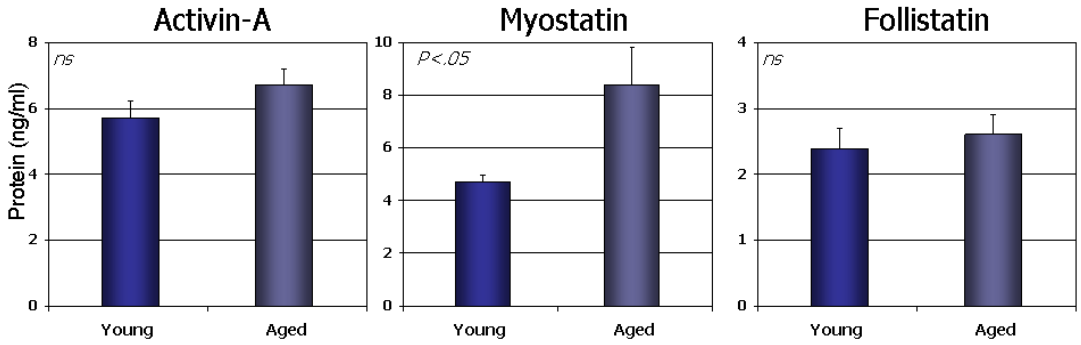

***Figure 2**. Protein levels (right) of activin A, myostatin, and follistatin in bone marrow supernatants from young (12 mo) and aged (24 mo) mice determined using ELISA assays.*

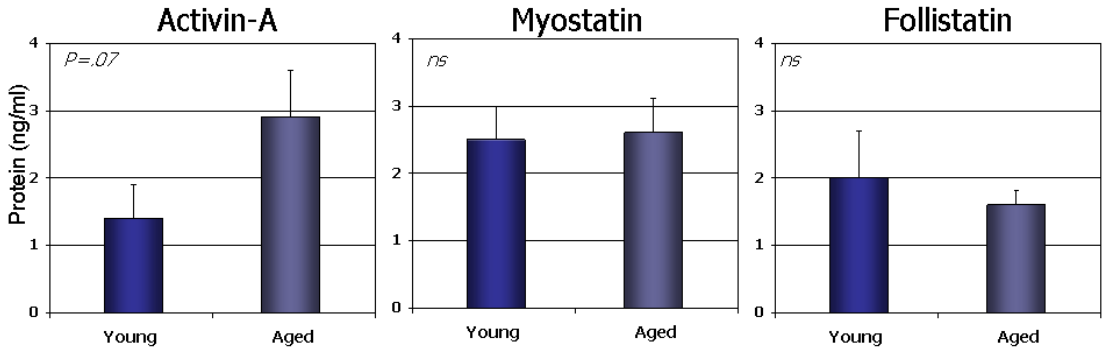

***Figure 3**. Protein levels (right) of activin A, myostatin, and follistatin in bone marrow supernatants from young (12 mo) and aged (24 mo) mice determined using ELISA assays.*

Completion of year 1 objectives:
We collected approximately 50 bone marrow samples from knee arthroplasty patients, and of these n=7 fell into our young age group (<50 years) and n=10 fell into our older (>70 years) age group. In order to increase our sample size we have continued to collect marrow samples. We have obtained an additional four samples from young patients and one additional sample from an 83 y.o. patient since the data presented above were analyzed. We will run the assays for these samples and add them to the data set shown above in August, 2011. In addition, we are collecting mRNA from bone marrow stromal cells isolated from the patient marrow samples using a magnetic bead sorting technique. These samples will also be analyzed for expression of myostatin, its receptor, activin A and follistatin in August, 2011.

Key Research Accomplishments:

- First-ever documentation of myostatin levels in human and mouse bone marrow.
- Discovery of significantly elevated levels of activin A in the bone marrow microenvironment with aging, revealing a novel therapeutic target for the prevention of osteoporosis.
- Discovery that the myostatin antagonist follistatin declines with age in fast-twitch skeletal muscle, revealing a novel mechanism underlying age-related muscle loss.

Reportable Outcomes:

Manuscripts:

in prep — Hamrick MW, Bowser M, Chutkan N, Martell J, Corpe S, Park MA, Hillman D, Ahsan S, Arounleut P, Isales CM, Shi XM. Age-related changes in the activin A-myostatin-follistatin system within the bone marrow microenvironment. *Bone*.

in press — Elkasrawy M, Fulzele S, Bowser M, Wenger K, **Hamrick MW**. Myostatin (GDF-8) inhibits chondrogenesis and chondrocyte proliferation in vitro by suppressing Sox-9 expression. *Growth Factors*.

2011 — **Hamrick, MW**. A role for myokines in muscle-bone interactions. *Exercise & Sports Science Reviews* 39: 43-47.

Published Abstracts from Professional Presentations:

2011 — Bowser M, Chutkan N, Martell J, Corpe R, Isales CM, Park MA, Hillman D, **Hamrick MW**. Age-related changes in the activin A-myostatin-follistatin system within the bone marrow microenvironment. Journal of Bone & Mineral Research SA0001.

2011 — Zhang W, **Hamrick MW**, Ding K, Wenger K, Hill W, Isales CM, Shi XM. Bone marrow mesenchymal stem cell and bone loss with aging. ASBMR Forum on Aging & Skeletal Health 29: P8.

2011 — Isales CM, **Hamrick MW**, Ding K, Zhong Q, Bollag W, Shi XM, Hill W, Rowse J, Elsalanty M, Chutkan N, Insogna K. The impact of dietary protein on bone mass and strength in the aging animal. ASBMR Forum on Aging & Skeletal Health 34: P17.

2011 Elkasrawy M, Immel D, Wen X, Liu L, Lian L, **Hamrick MW**. Effects of myostatin on muscle and bone healing following deep penetrant musculoskeletal injury. British Journal of Bone & Joint Science: P053.

Invited Seminars:

2011 Program in Musculoskeletal Research, Eli Lilly & Co., Indianapolis, Indiana.

2011 Department of Pathology & Anatomical Sciences, University of Missouri, Columbia MO.

Conclusions:
Falls and debilitating bone fractures are a major problem for veterans, and more than 40,000 veterans suffered hip fractures from 2000-2002. Men have a higher fracture-related mortality than women, and one out of every three male veterans that sustains a hip fracture dies within one year. Falls are the main etiological factor in more than 90% of fractures, and so treatments that can improve muscle strength while at the same time increasing bone mass will significantly reduce fracture-related morbidity and mortality. Myostatin is a factor that induces muscle wasting and suppresses bone formation. Our data collected thus far demonstrate i) the myostatin antagonist follistatin declines significantly with age in mice, and ii) activin A, which inhibits bone mineralization, is elevated with age in patient bone marrow supernatants. These findings suggest that blocking both myostatin and activin A with a decoy receptor that can bind both (ActRIIB) may preserve both muscle strength and bone mass with aging, decreasing fracture risk in veterans. We will further investigate this prediction in years 2 and 3 of the project.

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Hamrick MW, Arounleut P, Kellum E, Cain M, Immel D, Liang L. 2010. Recombinant myostatin (GDF-8) propeptide enhances the repair and regeneration of both muscle and bone in a model of deep penetrant musculoskeletal injury. *Journal of Trauma* 69: 579-83.

Hamrick MW, Shi X, Zhang W, Pennington C, Kang B, Thakore H, Haque M, Isales CM, S. Fulzele, K. Wenger. 2007. Loss of myostatin function increases osteogenic differentiation of bone marrow-derived mesenchymal stem cells but the osteogenic effect is ablated with unloading. *Bone* 40: 1544-1553.

Lotinun S, Pearsall RS, Davies MV, Marvell TH, Monnell TE, Ucran J, Fajardo RJ, Kumar R, Underwood KW, Seehra J, Bouxsein ML, Baron R. 2010. A soluble activin receptor type IIA fusion protein (ACE-011) increases bone mass via a dual anabolic-antiresorptive effect in Cynomolgus monkeys. *Bone* 46(4):1082-8.

Pearsall RS, Canalis E, Cornwall-Brady M, Underwood KW, Haigis B, Ucran J, Kumar R, Pobre E, Grinberg A, Werner ED, Glatt V, Stadmeyer L, Smith D, Seehra J, Bouxsein ML. 2008. A soluble activin type IIA receptor induces bone formation and improves skeletal integrity. *Proc Natl Acad Sci U S A* 105(19):7082-7.

Appendices: None attached.