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Inhibin A is an Endocrine Stimulator of Bone Mass and Strength

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Non-standard Abbreviations: InhA, Inhibin A; ORCH, orchidectomized, μCT, micro computed tomography; BMD, bone mineral density; BM, bone marrow; FEM, finite element modeling; OB, osteoblast; PABM, peak adult bone mass

Abstract – Gonadal function plays a major role in bone homeostasis. It is widely held that the skeletal consequences of hypogonadism are solely due to a loss of sex steroids; however, increases in bone turnover begin during perimenopause prior to decreases in serum estradiol levels. These data and our demonstration that Inhibins acutely regulate bone cell differentiation *in vitro* led us to test whether Inhibin A (InhA) regulates bone mass *in vivo*. Using a transgenic model of inducible human InhA expression, InhA increased total body BMD, increased bone volume, and improved biomechanical properties at the proximal tibia in intact mice, and also prevented the loss of BMD, and bone volume and strength associated with gonadectomy at both the spine and proximal tibia. In addition, InhA increased mineral apposition rate, double labeled surface, and serum osteocalcin levels *in vivo* and osteoblastogenesis *ex vivo*, without affecting osteoclast number or activity. Together these results demonstrate novel stimulatory effects of InhA on the skeleton *in vivo*. These studies provide *in vivo* evidence demonstrating that gonadal factors other than sex steroids play an important role in regulating bone mass and strength, and, combined with our previous clinical data, suggest that gonadal InhA may be a component of the normal endocrine repertoire that regulates bone quality in both the axial and appendicular skeleton.

Introduction

Bone mass and strength in adult mammals is controlled by a delicate balance between formation and resorption known collectively as bone turnover (1) Bone turnover is tightly regulated in order to maintain sufficient bone mass and strength to prevent fracture during normal physical activity (1-3). In diseases of bone loss, such as osteoporosis, decreased bone mass and strength leading to non-traumatic fractures of the spine, hip, and other bones is common (4) and the result of an imbalance in bone turnover. The availability of effective anti-catabolic agents has had a significant impact on slowing osteoporosis progression. However, there remains a significant need for anabolic agents capable of increasing bone mass and strength in patients with extensive bone loss.

In both genders, gonadal function is critical for the maintenance of bone quality. Consequently, hypogonadism is one of the most common causes of osteoporosis (5, 6). In women, this is widely attributed primarily to the loss of gonadal steroids (5, 6) that occurs during the menopausal transition. However, many other gonadally derived factors, including the Inhibins, contribute to the regulation of bone turnover and bone quality (7-12).

Inhibin B (InhB) and Inhibin A (InhA) are heterodimeric proteins in the TGFß superfamily composed of αßB or αßA subunits, respectively (13). Inhibins were originally identified based on their ability to suppress pituitary FSH secretion (13). Although Inhibin α-subunit expression (required for Inhibin dimer formation) is very low in human and rat bone marrow, (14, 15) Inhibin accumulates in the bone marrow of 25 day-old rats within 10 min of i.v. injection of [^{125}I]-InhA and is retained for at least an hour (16). These results are consistent with the idea that the effects of Inhibin on marrow cell hematopoiesis (17-19) are attributable to Inhibin derived from gonadal sources (20). Collectively, these data led to our hypothesis that changes in the gonadal Inhibins may have direct effects on osteoblast and osteoclast development, thereby regulating increases in bone turnover and bone mass. In support of this hypothesis, we previously demonstrated *in vitro* effects of Inhibins on osteoblast and osteoclast differentiation in both mice (7) and humans (8).

In the study described here, the effect of InhA on bone volume, architecture and strength was tested using an inducible murine model of human InhA expression (21). Our findings demonstrate that continuous InhA exposure *in vivo* increases bone volume and strength in intact adult mice, and prevents the bone loss associated with gonadectomy. Interestingly, InhA effects are mediated by a mechanism that increases bone formation, with little or no discernable effect on osteoclasts or bone resorption. These data reveal a new role for InhA as a non-steroidal, gonadally derived endocrine regulator of bone mass.

Materials and Methods

Experimental Animals. Transgenic mice engineered to inducibly express InhA, originally developed by Pierson et al. (21, 22), were obtained from Dr. Teresa Woodruff (Northwestern University, IL). These mice carry two unique transgenes. One gene encodes a designer chimeric nuclear receptor that binds mifepristone (MFP), termed GLVP, that is under the control of a liver specific promoter (21, 22) . When activated by binding MFP, the GLVP receptor activates transcription of the second transgene (*inh*) that encodes linked sequences of the human Inhibin α and Inhibin βA subunits. Subsequently, InhA is selectively and exclusively expressed from the liver of MFP-treated bigenic animals. An internal ribosome entry site (IRES) sequence inserted between the Inhibin α and Inhibin βA reading frames dramatically decreases the production of βA-subunit relative to α-subunit, thereby preventing the formation of

Activin A (21, 22) . MFP induced expression of InhA in bigenic (*inh/glvp*) mice produces the expected suppression of serum FSH, confirming that the liver-derived InhA is secreted into the circulation and down-regulates pituitary FSH levels (21, 22). The genetic control for the inducible *Inh/Glvp* mice were monogenic animals expressing only the MFP-activated receptor and not the human InhA transgene (*Glvp/-*).

All animals, identified by unique ear tags, were housed (four per cage) with free access to water and maintained at a constant temperature, on a 12 h light-dark cycle. The animal treatment and care protocols conformed to National Institute of Health guidelines and all studies were performed using a University of Arkansas for Medical Sciences (UAMS) IACUC approved protocol.

Controlled release pellets (Innovative Research of America, Toledo, OH) designed to deliver vehicle or 6 micrograms per day of MFP to induce continuous InhA expression were subcutaneously implanted in 5.5-month-old male and female mice (10 per group). We previously determined 5.5 months to be the time of peak adult bone mass; PABM (data not shown) in these mice. The mice were followed for 4 weeks before sacrifice and detailed skeletal analysis.

Similarly, in additional experiments orchidectomy (ORCH) or sham surgery was performed as described previously (21), and controlled releases pellets inserted at the time of surgery. The mice (12 per group) were followed for 4 weeks post-surgery before sacrifice and skeletal analysis.

The skeletal analyses performed were the same for all animals. At sacrifice, blood was collected by cardiac puncture and seminal vesicle or uterine weight determined to verify gonadectomy if performed (data not shown). In all cases, one tibia was excised for decalcified paraffin histology, the other tibia was harvested for microcomputed tomography (microCT) analysis and subsequent non-decalcified, methyl methacrylate (MMA) embedded histology. The fifth lumbar vertebrae (L5) were also harvested and used for non-decalcified MMA histology, while the 6th lumbar (L6) vertebrae were used for microCT and biomechanical testing. Bone marrow was harvested from both femurs for *ex vivo* bone marrow culture.

Bone Mineral Density (BMD). Total body BMD and total body bone mineral content (BMC) were determined *in vivo* using a PIXImus2 bone densitometer (GE Medical Systems, Madison, WI), as described (23-25). The precision of this technique in our laboratory is 1.7% (23-25). BMD and BMC were measured in mice from each genotype at monthly intervals from 10 weeks of age to determine the age at which these mice attained PABM, which was 5.5 months of age in both genders and both genotypes (data not shown). Subsequently, total body BMD measurements were obtained at sacrifice, excluding the head as previously described (24, 25). In addition, sub-region analysis of the mid-shaft of the tibia and femur of all mice was performed, as we have described previously (23-25), to determine the effect of InhA treatment on cortical BMD.

Human-Specific Serum Inhibin A assay. Blood was collected at sacrifice by cardiac puncture, allowed to clot and serum obtained. Human-specific Inhibin A was measured by 2-site ELISA according to manufacturer's protocol (Diagnostics Systems Laboratories, Webster, TX) and as previously described (8).

MicroCT Analysis of Bone. Ethanol fixed tibiae and formalin fixed L6 vertebrae were imaged using a microCT-40 (Scanco Medical AG, Bassersdorf, Switzerland) using a voxel size of 12 μm in all dimensions. The region of interest (ROI) selected for tibial analysis comprised 240 transverse CT slices representing the entire medullary volume extending 1.24 mm distal to the end of the primary spongiosa with a border lying approximately 100 μm from the cortex. Vertebrae were evaluated using approximately 250 transverse CT slices encompassing the central trabecular bone in the anterior compartment of the vertebral body between the cranial and caudal end plates, excluding 100 μm near each endplate. Three dimensional reconstructions were created by stacking the ROIs from each 2-dimensional slice then applying a grey-scale threshold and Gaussian noise filter (26) specifically optimized for murine trabecular bone. Morphometric variables were computed from the binarized images using direct, three-dimensional techniques that do not rely on any prior assumptions about the underlying structure. Fractional bone volume (BV/TV, %) and architectural properties of trabecular reconstructions (apparent trabecular thickness (TbTh, μm), trabecular number (TbN, mm^{-1}), and connectivity density (ConnD, mm^{-3})) were calculated using published methods. (26)

Finite Element Modeling (FEM) of Tibial Cancellous Bone. Finite element modeling (FEM) was used to determine the contribution of bone volume and architecture to bone biomechanical properties. Using FEM version 1.0 (Scanco Medical), the 3D reconstructions of trabecular bone in the proximal tibia were converted to brick element mesh and assigned material properties with Poisson's ratio = 0.2 and Young's modulus = 1.8 GPa. A 1% apparent axial compression was simulated, and the reaction force (or force, in Newtons, required to create the compression) was calculated (27, 28).

Compression Testing of L6 Vertebrae. The compressive strength of L6 vertebrae were determined in a single load-to-failure compression test as we have previously described

(29), using a MTS 858 Bionex Test Systems load frame (MTS, Eden Prairie, MN) with computer control, data logging, and calculations of load to failure using TestWorks version 4.0 (MTS). The load frame was operated at a constant rate of 0.1 mm/sec with load and displacement recorded at 100 Hz. Load to failure was recorded as the load after a 2% drop from peak load.

Histomorphometric Analysis of Bone. Tibiae were harvested at sacrifice and the muscle dissected away before fixation in Mallonig's as previously described (24, 30). For static histomorphometric analyses, 4-5 μm-thick central saggital sections of undecalcified MMA embedded tibiae were stained for tartrate-resistant acid phosphatase (TRAP) and counterstained with hematoxylin to determine osteoclast numbers and eroded surface per cancellous bone surface within the region of interest, or with Masson's Trichrome for all other measurements, as defined by Parfitt (31) using Osteomeasure software (Osteometrics, Atlanta, GA), and as we have previously described (24). Dynamic *in vivo* measurement of bone formation was performed by injecting each mouse with 30 mg/kg of calcein 8-days before sacrifice and 30 mg/kg of either tetracycline or Alizarin Red S 2-days before sacrifice. The proportion of single and double labeled surfaces and the interlabel distance between the fluorochrome labels were measured in the cancellous bone of unstained sections adjacent to those used for static measurements. Appropriate measurements were normalized to total cancellous bone perimeter or tissue volume (24, 30; 66). All cancellous bone measurements were made within the area defined by 700-1400 μm distal to the growth plate and 150 μm away from either endocortical surface.

C-terminal Telopeptide Measurement in Serum. Serum levels of the C-terminal telopeptide of Collagen I (CTx), a specific marker of bone resorption, were determined using a RatLaps ELISA assay (Nordic, Herlev, Denmark) according to protocols from the manufacturer.

Osteocalcin Measurement in Serum. Serum levels of osteocalcin, a specific marker of bone formation, were determined using the mouse osteocalcin single-plex kit assay (Linco Research Inc, St. Charles, MO) according to protocols from the manufacturer.

***Ex vivo* Bone Marrow Culture.** Bone marrow cells were harvested from femurs for osteoblastogenic culture as previously described (7) at specific time points following Inhibin A induction or placebo treatment *in vivo*. Briefly, cells were flushed from femurs, washed, and cultured in 12-well plates at a density of $2x10^6$ cells per well in αMEM, supplemented with 15% FCS, 50 μg/ml ascorbic acid and 10mM betaglycerolphosphate, in the presence or absence of 50 ng/ml Inhibin A (R&D Systems) or follicle stimulating hormone (FSH, National Hormone and Peptide Program) in triplicate wells per treatment. Cells were fed every 3 days with half-volumes of medium, until day 28, when cells were fixed and mineral stained with Alizarin Red to facilitate determination of the number of bone nodules (CFU-OB) formed per well (7).

Statistical Analysis. All experimental data that passed standard normalization tests were analyzed by one-way ANOVA and Student-Neuman-Keuls post-hoc test. Data that were not normally distributed were analyzed by Kruskall-Wallis ANOVA on ranks and Dunn's post-hoc tests. Parametric data are presented at mean +/- SEM, and non-parametric data are presented as median (25th percentile, 75th percentile). P values <0.05 were considered statistically significant and are reported as such.

Results

InhA Increases Bone Mass and Strength.

Human InhA Expression.

To determine if InhA is able to increase bone mass, mice at peak adult bone mass (5.5 months of age) were anesthetized and implanted with a time-release pellet containing Vehicle (Veh) or MFP (to induce InhA expression). In bigenic *Glvp/InhA* mice treated with MFP, the mean serum concentration of human InhA at the time of sacrifice 4 weeks post-induction (400-880pg/ml) was within the normal range of previous reports of total murine Inhibin measured by RIA (400-600 pg/ml) (32-34). However, in monogenic animals expressing only the MFP-activated receptor and not the human InhA transgene (*Glvp/-*), human InhA levels were never detectable, regardless of MFP treatment.

Human InhA Increases Bone Mass and Strength in Intact Mice.

To control for any potential effects of MFP treatment alone on skeletal physiology, *Glvp/-* mice were analyzed as controls for *Glvp/InhA* mice. Three-dimensional analysis of trabecular microarchitecture by μCT was performed on the proximal tibial metaphysis (Figure 1A) and L6 vertebrae (data not shown), and 2-dimensional analysis of total body bone mineral density (BMD) was performed using Piximus DEXA (Figure 2A) to exclude the possibility that any observed skeletal effects were site-specific (35).

MFP treatment had no effect on BV/TV of the proximal tibia of *Glvp/-* mice (male and female) (Figure 1A) or on the total body BMD (Figure 2A). Similarly, there was no demonstrable effect of MFP on L6 vertebrae or any other skeletal parameter measured (data not shown). In contrast, in intact MFP-treated *Glvp/InhA* mice, the expression of human InhA significantly increased bone volume fraction (BV/TV) in both male and female mice (Figure 1A), and also increased total body BMD (Figure 2A).

During the course of our day-to-day management of the mouse colony, we noted variable levels of human InhA levels in female mice, independent of MFP pellet insertion (Figure 1B). In particular, we repeatedly measured elevated human InhA expression in female *Glvp/InhA* mice that that received no MFP pellet or had never been housed with mice harboring an MFP pellet, strongly suggesting that the InhA transgene had become non-MFP-inducible in female mice. No male mice were observed to have detectable human InhA levels (assay detection limit is 20 pg/ml) in the absence of MFP induction (Figure 1B). The uncontrolled expression of human InhA (42 – 367 pg/ml), independent of MFP pellet insertion selectively in female *Glvp/InhA* mice made any subsequent use of female mice unreliable. Females were therefore excluded from further investigation. Nonetheless, measurement of bone volume in the original MFP-inducible intact *Glvp/InhA* female mice (Figure 1A), demonstrated that human InhA expression stimulated increases in BV/TV in intact female mice.

InhA Prevents ORCH-Induced Loss of Mass and Strength.

Vehicle treated and MFP treated, *Glvp/InhA* (bigenic) mice were of similar body weight, and did not demonstrate a significant difference in body weight gain over the 4 week experimental period, regardless of gonadal status.

ORCH initiated at peak adult bone mass induced the expected loss of total body BMD by DEXA (Figure 2A) and trabecular bone volume fraction (BV/TV) in both the tibiae and vertebrae of both *Glvp/-* and *Glvp/InhA* mouse strains, as determined by microCT (Figure 2B, 3A). These findings demonstrate that the skeletons of both the *Glvp/-* and *Glvp/InhA* mice are sensitive to ORCH. In preliminary studies in females, we noted that the skeletons of both the *Glvp/-* (and *Glvp/InhA*) mouse strains were insensitive to ovariectomy (OVX), as has been reported in a number of mouse strains and transgenic mouse lines (36, 37).

MFP alone in male *Glvp/-* mice had no effect on BMD (Figure 2A), BV/TV (Figure 2B) or trabecular architecture (data not shown), regardless of gonadal status. These data clearly demonstrate that any effects on the skeleton of *Glvp/InhA* mice are not the result of effects of MFP on bone, but are associated with the induced expression of human InhA.

In contrast to the lack of MFP effect on *Glvp/-* mice, the effect of human InhA expression in Sham (intact) *Glvp/InhA* mice was to increase bone mass. Total body BMD was increased (Figure 2A), and trabecular bone volume in the proximal tibia (Figures 2B, 3A; Table 1) and the lumbar vertebrae (Figure 3A; Table 1) were also increased. MicroCT analysis of the trabecular microarchitecture revealed that the stimulatory effect of human InhA expression in Sham (intact) *Glvp/InhA* mice was mediated by an increase in trabecular number (TbN) and connectivity density (ConnD), leading to a decrease in the structure model index (SMI) (Table 1).

As in the *Glvp/-* mice, ORCH reduced total body BMD (Figure 2A) and BV/TV in both the proximal tibiae (Figures 2B, 3A; Table 1) and L6 vertebrae (Figure 3A, Table 1) of *Glvp/InhA* mice as expected. However, human InhA expression in ORCH *Glvp/InhA* mice prevented the gonadectomy-induced loss of total body BMD (Figure 2A) as well as the loss of BV/TV at both tibial and vertebral sites (Figures 2B, 3A; Table 1).

Similar to the observations in the intact *Glvp/InhA* mice, InhA preserved BV/TV in ORCH mice by maintaining TbN and ConnD (Table 1).

To determine if InhA maintenance of tibial bone volume and architecture in ORCH mice correlated with an improvement in biomechanical properties, finite element modeling (FEM) was used to calculate the compressive stiffness of trabecular bone in the proximal tibia (27, 28). Human InhA expression prevented the ORCH-induced loss of bone stiffness in the tibia (Figure 3B), and remarkably, even increased stiffness in intact (Sham) animals (Figure 3B). In addition, direct biomechanical compression testing of L6 vertebrae (29) demonstrated that InhA expression also prevented the ORCH-induced loss of bone stiffness that occurs in the axial skeleton (Figure 3C).

In addition to examining the effects of human InhA expression on trabecular bone, we also examined the effect of InhA on cortical bone. Sub-region analysis of the mid-shaft of both the tibia and femur revealed no significant effect of InhA on cortical BMD in intact or ORCH male mice (Figure 3D, E), in agreement with the cortical analyses performed using microCT (data not shown). Thus, the protective and stimulatory effects of InhA on cancellous bone mass, architecture and strength that occur in both the vertebrae and the proximal tibiae are not associated with any demonstrable changes in cortical BMD or architecture.

Inhibin Stimulates Osteoblast Activity

Lack of InhA Effects on Osteoclastogenesis or Bone Resorption

Several quantitative analyses were performed to determine whether the stimulatory effects of InhA on bone mass and strength in *Glvp/InhA* mice were mediated by decreased bone resorption, increases in bone formation, or a combination of both. As has been previously described, ORCH-stimulated bone turnover was associated with increases in serum CTx (38). However, human InhA expression did not affect bone resorption in either ORCH or Sham operated mice, as measured by serum CTx (Figure 4A). Similarly, static histomorphometric analysis of the secondary spongiosa of the proximal tibia (30) showed that InhA expression had no effect on osteoclast number, or on the proportion of osteoclast eroded surfaces, or any measured osteoclast parameter, regardless of gonadal status (Table 2).

InhA Stimulates Osteoblast Activity

In contrast to the lack of InhA effect on bone resorption, the measurement of a serum marker of bone formation, osteocalcin, showed that InhA expression significantly increased serum osteocalcin in intact animals, and also increased osteocalcin levels in ORCH animals (Figure 4B). This effect on a systemic measure of osteoblast activity suggests that InhA stimulates bone formation via changes in osteoblast activity.

To support the notion that the bone forming effect of InhA is mediated by effects on osteoblastic activity, histomorphometric parameters of osteoblastic activity were also measured in the secondary spongiosa of the proximal tibia as described (24, 30). Surprisingly, InhA did not significantly affect the number of osteoblasts on the trabecular bone surface (NOb/BS), or the number of osteoblasts per total area (NOb/TA) regardless of gonadal status (Table 2). Similarly, InhA expression did not affect osteoblast surface per BS (ObS/BS), osteoid surface per BS (OS/BS), or osteoid thickness (OsTh) (Table 2) nor any other static osteoblastic parameter measured (data not shown). Interestingly, dynamic histomorphometry demonstrated that human InhA expression in intact Sham mice significantly increased (at least 2-fold) osteoblast activity, as measured by an increased mineral apposition rate (MAR) (Figure 4C; Table 2), increased double labeled surface per bone surface (dLS/BS; Table 2), and the inter-label distance (Figure 4D-E), in the absence of an increase in bone formation rate (BFR). The additional increases in MAR observed in response to InhA expression in ORCH mice did not reach significance, presumably due to the increased bone formation and bone turnover rates known to be associated with ORCH alone (38). Thus in agreement with microCT measurements of BV/TV and microarchitecture, serum osteocalcin levels and dynamic bone histomorphometry confirmed that InhA expression stimulated bone formation in *Glvp/InhA* mice and demonstrated that the enhanced bone formation occurred predominantly as the result of an increase in osteoblast activity, as measured by MAR and dLS/BS.

InhA *in vivo* Stimulates Osteoblast Recruitment in *ex vivo* Bone Marrow Cultures.

To better elucidate the effect(s) of InhA on osteoblast progenitors, *ex vivo* femoral bone marrow cultures were established using bone marrow harvested from vehicle-treated or InhA overexpressing *Glvp/InhA* animals. In direct support of the observed stimulatory effects of InhA on osteoblastic cells *in vivo*, osteogenic differentiation of marrow cells derived from animals exposed to MFP-stimulated InhA for 4 weeks *in vivo* was significantly increased compared to cells from mice not exposed to InhA *in vivo* (Figure 5). In addition and entirely consistent with our previously published results (7, 8), direct *in vitro* treatment of bone marrow cells with exogenous human InhA significantly reduced osteoblast differentiation, (Figure 5) regardless of the *in vivo* exposure to InhA.

To gain insight into the time course of InhA action, we evaluated the trabecular

BV/TV in the proximal tibia of InhA expressing MFP treated *Glvp/InhA* mice. In addition, osteoblast differentiation in *ex vivo* bone marrow cultures was also evaluated in marrow harvested from these same animals (Figure 6). Interestingly, the stimulatory effect of InhA expression on trabecular BV/TV was observed only after 4 weeks of InhA treatment *in vivo* (Figure 6A). Furthermore, the osteogenic differentiation of marrow cells derived from these same animals exposed to InhA *in vivo* was significantly increased compared to cells from mice not exposed to InhA *in vivo,* only at week 3 (Figure 6B). Most notably, short-time *in vivo* exposure to InhA (1 week) resulted in suppression of *ex vivo* osteogenic differentiation that was comparable to that observed in cells from vehicle treated mice following exogenous administration of InhA *in vitro* (Figure 6B). In addition, as we have shown previously (Figure 5) (7, 8), the administration of exogenous InhA *in vitro* significantly inhibited osteoblast differentiation, independent of InhA exposure *in vivo* (Figure 6B). These data provide compelling mechanistic information suggesting that the osteoblasts (and/or their precursors) are the target(s) of InhA action, and that the effect of InhA is dependent upon the duration of *in vivo* exposure.

An important endocrine marker of Inhibin action *in vivo* is the suppression of circulating FSH (7-12). Recently, much interest has arisen in the role of FSH in the regulation of bone mass (7-12) (39). In the presence of InhA expression in MFP treated *Glvp/InhA* mice, serum FSH is dramatically reduced (7-12). In order to confirm that the bone stimulatory effects of InhA were not associated with changes in FSH, we evaluated the effects of exogenous FSH on murine *ex vivo* marrow cultures obtained from animals that had been exposed to InhA *in vivo* for 1-3 weeks. As shown in Figure 7, FSH addition had no effect on the osteogenic differentiation of marrow cells derived from either control animals or animals exposed to InhA *in vivo.* In addition, FSH had no influence on the osteogenic suppression due to *in vitro* InhA exposure. These data confirm the effects of InhA on osteoblast differentiation and demonstrate that they are not associated with FSH.

Discussion

Osteoporosis is widely acknowledged in women (5, 40, 41) and affects more than 8 million females in the United States alone (40). However, osteoporosis is also a rapidly increasing problem in men, affecting up to 2.5 million men in the United States and many more worldwide (40, 42, 43). Thus, the prevalence of this disease and the associated morbidity accentuate the need for new preventive and anabolic therapies. Loss of gonadal function is a major determinant of bone loss leading to disease, which has been previously attributed primarily to the loss of sex steroids. A variety of clinical studies in perimenopausal women have suggested that other gonadal factors may contribute to the increasing bone turnover that occurs in the absence of changes in serum estradiol levels (8-10, 44-47).

The early rise in FSH levels that occurs in perimenopausal women is attributable to a selective decrease in InhB secretion that occurs in the presence of normal levels of estradiol, InhA, GnRH, and LH (45-47). Similarly, testicular InhB is a primary regulator of FSH secretion in men (45, 48), although FSH has been demonstrated to be similarly suppressed by exogenous administration of hInhA (49, 50). Because both InhA and InhB isoforms selectively inhibit pituitary FSH secretion, these data suggest that the increased

FSH in perimenopausal women is attributable to a loss in feed-back inhibition by gonadal InhB (reviewed in (51)). Our recent clinical (8) and *in vitro* (7, 8) findings suggest that decreases in circulating Inhibin levels, due to reduced ovarian function, contribute to perimenopausal bone loss (44). As the loss of gonadal function progresses in postmenopausal women, the well-established decreases in estradiol accompany declining levels of both InhB and InhA, further increasing serum FSH (45-47) and markedly increasing bone loss. Recent evidence has implicated elevated FSH as an independent stimulator of the postmenopausal increase in osteoclastic bone resorption (52) (39). However, given that the target of InhA action in our study appears to be cells in the osteoblastic lineage, and the lack of effect of FSH in *ex vivo* bone marrow cultures, the bone forming and/or protective effects of InhA on bone appear independent of the reported FSH mediated changes in osteoclast function.

Few animal models exist in which to study the action of Inhibins on bone. The alpha-Inhibin gene has been deleted (53), but these mice develop gonadal tumors at 6 weeks of age, during the phase of longitudinal bone growth. After gonadectomy, the resulting lack of sex steroids prevents the mice from reaching peak adult bone mass (53), and precludes the analysis of any selective effects of Inhibin on the adult skeleton. The inducible transgenic mouse model utilized in the study reported here permits the inducible expression of human InhA at any stage of growth and/or adulthood (21). This model is uniquely suited to study the effects of InhA on the adult skeleton. The data demonstrate that InhA increases BV/TV by 36% and 54% in intact male and female mice, respectively, and also prevents the 43% loss of BV/TV that occurs due to ORCH (Figure 2B). These observations provide additional evidence to support the emerging idea that non-steroidal gonadal hormones contribute to the regulation of bone homeostasis (8, 12, 44).

The ability of InhA to increase bone mass is somewhat unexpected given Inhibin's well-documented ability to antagonize the effects of Activin (54), which has been shown to stimulate bone formation *in vivo* (55, 56). However, unlike Activin, Inhibins are not normally produced within the bone microenvironment (57) and are gonadally-derived, circulating endocrine hormones (13). Thus, the stimulatory action of InhA on the skeleton significantly differs from those of other TGFβ superfamily members such as Activin, TGFβ and BMP. The normal physiological effects on the skeleton of these TGFβ superfamily members are entirely due to their local production in bone (58).

In addition to the stimulatory effects on bone volume, the effects of InhA on trabecular bone microarchitecture demonstrate that InhA is also able to prevent the perforations that occur after ORCH. Such perforations typically raise the SMI and decrease ConnD (26, 59). InhA expression was able to lower the SMI and increase the ConnD in Sham operated mice, suggesting that InhA increased trabecular connections and closed preexisting perforations in the bones of these animals. The stimulatory effect of InhA expression on bone microarchitecture was also associated with similar beneficial effects on both tibial and vertebral biomechanical properties. Furthermore, InhA expression also prevented ORCH-induced losses of bone density, volume, microarchitecture, and strength in both the axial and appendicular skeleton. Thus, the stimulatory effects of InhA in intact Sham mice resulted in increased bone quality and strength.

Four weeks exposure to InhA *in vivo* is required to induce increases in tibial BV/TV, whereas increases in *ex vivo* osteoblastogenesis are observed after 3 weeks of InhA *in vivo*. In addition, *in vivo* exposure to InhA for only 1 week resulted in significant suppression of osteogenesis in *ex vivo* marrow cultures. This short-term suppressive effect of osteogenesis is entirely consistent with our clinical observations that serum Inhibin levels are inversely correlated with markers of bone formation and bone resorption (8). Moreover, our collective, *in vivo* and *in vitro* data suggest that Inhibins exert a temporal bimodal effect on the regulation of bone metabolism. We propose that the normal physiological role of pulsatile (short duration) exposure to Inhibins is to suppress bone turnover (8), whereas longer-term continuous exposure to Inhibins (*Glvp/InhA* mice) is anabolic. The stimulatory effect of InhA on the skeleton is mediated by increases in mature osteoblast activity and osteoblast differentiation. The significant increases in bone mass and architecture induced by InhA are independent of changes in osteoclast number or function.

Direct bone histomorphometry (Table 2) suggested that InhA expression was anabolic in *Glvp/InhA* mice and revealed that the increase in bone mass was caused by enhanced bone formation, predominantly as the result of an increase in MAR and dLS/BS, with no effect on bone formation rate (BFR). This observation is not new, as others have reported anabolic effects on the skeleton mediated by increased MAR, with or without effects on BFR (60-64). The InhA-induced increase in MAR and dLS/BS reported here suggests that InhA increases bone formation primarily by stimulating osteoblast activity, an idea supported by the observation that InhA expression also induced significant increases in serum osteocalcin levels. However, these data do not preclude the possibility that InhA also increased BFR, which may have occurred earlier or be more easily detected prior to the 4 week time point analyzed here.

As has been observed for other endocrine regulators of bone turnover (1), the effects of InhA *in vitro* and short continuous duration *in vivo* are distinct from those of long-term continuous exposure to InhA *in vivo*. Our data suggest that the endocrine effects of continuous exposure *in vivo* overrides the direct suppressive effects of exogenous InhA on osteoblastogenesis *in vitro*. These differential effects of InhA are similar to those demonstrated for estrogens and glucocorticoids.

It is well documented that physiological estrogens suppress bone turnover, but that high-dose estrogen treatment increases bone formation and is strongly anabolic (65-67). Similarly, it is also well-accepted that glucocorticoids increase osteoblastogenesis *in vitro* (68-73) whereas continuous *in vivo* exposure decreases osteoblastogenesis, increases osteoblast apoptosis, and increases osteoclast activity leading to bone loss (38, 74-78). Furthermore, the distinct InhA actions *in vitro* and *in vivo* are reminiscent of the well-documented paradigm of continuous versus pulsatile PTH that have driven mechanistic studies by numerous investigators for decades (79-85). Thus, we hypothesize that the *in vivo* effects of InhA, like a host of other endocrine hormones, are pleitropic. Further experiments are ongoing to elucidate the differential mechanisms by which Inhibins exert their profound effects on the skeleton.

Continuous exposure to physiological levels of InhA *in vivo* increases MAR, dLS/BS, BV/TV and increases bone strength. *Ex vivo* culture of bone marrow harvested from animals exposed to InhA *in vivo* demonstrate an increased recruitment of bone marrow cells into the osteoblastic lineage (increasing CFU-OB), which is suppressed by exogenous *in vitro* treatment with InhA, as we have shown previously (7) (8). These data suggest strongly that the endocrine effects of continuous InhA exposure *in vivo* overrides the direct suppressive effects of InhA on osteoblastogenesis *in vitro.* Presumably, the effects of InhA *in vivo* are mediated by cell types either absent or underrepresented in primary *ex vivo* murine bone marrow cultures. The identity of the cellular mediators and mechanisms of InhA action on bone mass remains the focus of intensive investigation.

Given that the primary endocrine role of the Inhibins is to suppress FSH (86, 87) and FSH has been recently implicated as a pro-osteoclastogenic agent (52),(39) it is unlikely that decreased FSH is a mediator of the stimulatory effects of InhA on the skeleton. As shown here, FSH treatment did not effect osteoblast differentiation *in vitro*. *In vivo*, InhA action increases osteoblast activity, whereas FSH has been shown to exert effects on osteoclasts and their precursors, without effects on osteoblasts (52, 88, 89). The bone stimulatory effect of InhA demonstrated here resulted from continuous exposure; whether InhA increases bone mass when delivered by pulsatile (cyclic) administration remains to be determined. Unlike other endocrine regulators of bone mass, the lack of InhA effects on bone resorption and osteoclast number suggest the likelihood of an InhA induced imbalance between bone resorption and bone formation.

Collectively, the data presented here demonstrate that InhA acts as a potent endocrine stimulator of bone mass and strength *in vivo* that can also prevent the bone loss associated with ORCH, implicating InhA as a bone anabolic agent. Given the complicated nature of the dimeric Inhibin peptides, further studies elucidating the mechanisms of InhA action are warranted to determine the possible utility of targeting InhA signaling as a therapeutic modality to stimulate bone formation. Together, these data provide direct evidence supporting the emerging idea that gonadal factors other than sex steroids play an important role in regulating both bone mass and bone strength (8, 12). Furthermore, it appears that InhA may be a component of the normal endocrine repertoire that regulates bone volume and strength in both the axial and appendicular skeleton.

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Figure Legends

Figure 1 –InhA increases BV/TV in intact mice.

(A) InhA increases bone volume in intact female and male mice. Female and male *Glvp/- and Glvp/InhA* mice were continuously treated for 4 weeks with either vehicle (Veh) or MFP, which in *Glvp/InhA* mice will induce expression of human InhA (MFP/InhA). Trabecular bone volume in the proximal tibia was examined by microCT 4 weeks later. Bone volume per tissue volume (BV/TV) was significantly increased by InhA only in *Glvp/InhA* mice. MFP alone had no effect on BV/TV in *Glvp/-* mice. * =$p < 0.05$. **(B) Human InhA levels are detected in uninduced female mice, but not in uninduced male mice**. Serum human InhA levels were measured in male and female *Glvp/InhA* mice during routine management of the colony. Some of the 9 sham female mice that were uninduced (no MFP pellet) had variable levels of human InhA, as did some of the 10 uninduced ovariectomized (OVX) females. Two females that had never been implanted with a pellet also showed substantial human InhA levels. Dotted line shows detection limit of the human InhA ELISA. Uninduced sham males (n=12) and orchidectomized (ORCH) males (n=11) had undetectable human InhA levels.

Figure 2 – Human InhA expression prevents loss of Bone Mineral Density and Bone Volume in ORCH *Glvp/InhA* male mice. Male *Glvp/- and Glvp/InhA* mice were either sham-operated (Sham) or orchidectomized (ORCH). ORCH induced the expected loss in total body BMD measured at sacrifice **(A)** and in tibial cancellous BV/TV **(B)** in both genotypes. **(Left panels)** *Glvp/-* mice (Intact and ORCH) were continuously treated with either vehicle (Veh) or MFP. As expected ORCH significantly reduced BV/TV;

however, no effect of MFP was observed on BMD or BV/TV in Sham or ORCH *Glvp*/- mice. **(Right panels)** *Glvp/InhA* mice (Intact or ORCH) were continuously treated with either vehicle (Veh) or MFP to induce expression of human InhA (MFP/InhA) for 4 weeks. As expected, ORCH significantly reduced both total body BMD and tibial cancellous BV/TV. InhA expression increased total body BMD and tibial cancellous BV/TV in Sham *Glvp*/*InhA* mice and prevented the ORCH induced loss of BMD and BV/TV (MFP/InhA). Within each genotype, different letters are significantly different at $p<0.05$

Figure 3 - InhA effects on the proximal tibia and L5/L6 vertebrae of *Glvp/InhA* mice. **(A)** *Glvp/InhA* mice were either orchidectomized (ORCH) or sham-operated (Sham) and continuously treated with either vehicle (Veh) or MFP to induce expression of human InhA (InhA) for 4 weeks. Trabecular bone volume in the proximal tibia and L5/L6 vertebrae was examined by microCT and in Masson's trichrome stained histological sections. The images illustrate the expected loss of bone volume in ORCH-Veh mice at both skeletal sites and the prevention of the ORCH-induced loss in ORCH-InhA mice. In addition, InhA increased trabecular bone volume in intact, Sham operated mice (Sham-InhA). **(B)** The stiffness of cancellous bone in the proximal tibia was examined by finite element modeling of the μCT reconstructions of tibial cancellous bone. Human InhA expression in *Glvp/InhA* mice significantly increased the calculated stiffness (reaction force to a 1% strain) of tibial cancellous bone in Sham mice and significantly prevented the ORCH-induce loss of strength in the tibia. (**C**) Load-to-failure (N) of the isolated intact L6 vertebra was determined using a standard

biomechanical compression test. InhA expression in *Glvp/InhA* mice significantly prevented the ORCH-induced loss of strength in ORCH mice (ORCH InhA) compared to ORCH-Veh treated mice. Different letters are significantly different at $p<0.05$. Percent change in cortical BMD in the mid-shaft of the femur **(D)** and tibia **(E)** were determined as described in Methods and previously (23-25) from longitudinal BMD measurements obtained at the beginning and end of the 4 week experiment. Percent difference was calculated as previously described (23-25). Neither ORCH nor MFP/InhA had any effect on cortical BMD.

Figure 4 – Inhibin A stimulates osteoblast activity *in vivo* without associated increases in osteoclast activity. **(A)** Serum C-terminal crosslinks of Collagen I were measured at sacrifice as a systemic indicator of osteoclast activity. ORCH caused the expected increase in Collagen Crosslinks (ORCH-Veh), which was unaffected by InhA expression, regardless of surgery. **(B)** Serum osteocalcin was measured as a systemic indicator of osteoblast activity. In intact animals, InhA expression significantly increased serum osteocalcin (Sham MFP/InhA). ORCH did not increase serum osteocalcin at the time point measured here (4 weeks) while InhA expression (ORCH MFP/InhA) increased osteocalcin levels. Different letters are significantly different at $p<0.05$. Unmarked bars are not significantly different from any other treatment. **(C)** The mineral apposition rate (MAR), a direct measure of osteoblast activity *in vivo*, was measured in central histological sections of the proximal tibia. InhA expression significantly increased MAR in Sham mice. The MAR of ORCH-InhA mice was equivalent to that in SHAM-InhA mice, but neither was significantly different from ORCH-Veh. Different letters are

significantly different at $p<0.05$. Unmarked bars are not significantly different from any other treatment. **(D, E)** Representative double fluorochrome labeled regions of trabecular bone in the proximal tibia of Vehicle treated (D) or Inhibin A overexpressing (E) intact Sham mice. Arrows indicate the distance between the two labels used to calculate MAR.

Figure 5 – Inhibin A has distinct effects on osteoblastogenesis *in vivo* and *in vitro*. Marrow harvested from the femora of Sham mice treated with Vehicle (black bars) or from mice implanted for 4 weeks with MFP pellets to stimulate expression of Inhibin A (white bars) was cultured in osteogenic media in the absence (-) or presence (+) of exogenously added 30 ng/ml human Inhibin A *in vitro*. *Ex vivo* osteoblast differentiation was assessed at culture day 28 by staining with Alizarin Red and the number of mineralized nodules per well enumerated. Expression of InhA *in vivo* significantly increased *ex vivo* osteoblast differentiation, regardless of *in vitro* treatment. In contrast, and as expected, direct treatment with InhA *in vitro* suppressed *ex vivo* osteoblast differentiation, regardless of the *in vivo* exposure to InhA. Different letters are significantly different from each other at $p<0.05$.

Figure 6 – Time Course of Inhibin A increases in BV/TV and osteoblastogenesis. (A) Male *Glvp/InhA* mice were continuously treated for 1-4 weeks with either vehicle (Veh; solid black bars) or MFP (white bars) to induce expression of human InhA (InhA). Trabecular bone volume in the proximal tibia was examined by microCT in animals sacrificed at weekly intervals (n = 6-8 animals per group per week). Bone volume per tissue volume (BV/TV) was significantly increased by InhA only after 4 weeks of

exposure. * = $p < 0.05$. (**B**) Marrow was harvested from the femora of the same *Glvp/InhA* mice treated *in vivo* with a Vehicle pellet (Veh) or MFP pellet to overexpress Inhibin A (InhA). Marrow cells were cultured in osteogenic media in the absence (Con) or presence (InhA) of an exogenously added 30 ng/ml human Inhibin A *in vitro*. *Ex vivo* osteoblast differentiation was assessed at culture day 28 by staining with Alizarin Red and the number of mineralized nodules per well enumerated. Expression of InhA *in vivo* significantly suppressed *ex vivo* osteoblast differentiation at week 1, but significantly increased differentiation at week 3. As expected, exogenous InhA treatment significantly inhibited all cultures, regardless of *in vivo* exposure. Different letters are significantly different from each other at $p<0.05$.

Figure 7 – FSH is not responsible for InhA effects on osteoblastogenesis. Marrow was harvested from the femora of *Glvp/InhA* mice treated *in vivo* with a Vehicle pellet (Veh) or MFP pellet to overexpress Inhibin A (InhA) for 1-3 weeks. The harvested marrow cells were cultured in osteogenic media in the absence (Con) or presence (FSH) of an exogenously added 30 ng/ml human FSH *in vitro*. *Ex vivo* osteoblast differentiation was assessed at culture day 28 by staining with Alizarin Red and the number of mineralized bone nodules (CFU-OB) per well enumerated. FSH had no effect on osteoblastogenesis, regardless of *in vivo* exposure to InhA. Nor did FSH have any effect on the stimulatory or suppressive effects of InhA. Different letters are significantly different from each other at $p<0.05$.

Table 1 – InhA Improves the Microarchitectural Properties of Trabecular Bone in the Proximal Tibia and Lumbar Vertebrae

Location	Treatment	Conn D (mm^{-1})	SMI	TbN (mm^{-1})	TbTh (mm)	TbSp (mm)	DA
Prox Tibia	Sham-Placebo	114.1 +/-46.1[a]	1.042 +/-0.717[a]	4.93 +/-1.00[a]	0.061 (0.060, 0.064)	0.180 (0.163, 0.226)	2.06 +/-0.16
	Sham-InhA	160.3 +/-26.6[b]	0.350 +/-0.408[b]	5.82 +/-0.53[b]	0.064 (0.057, 0.072)	0.157 (0.138, 0.169)[a]	2.01 +/-0.20
	ORCH-Placebo	50.4 +/-27.9[c]	2.016 +/-0.397[c]	3.36 +/-0.71[c]	0.061 (0.057, 0.064)	0.296 (0.258, 0.364)[b]	1.98 +/-0.23
	ORCH-InhA	98.7 +/-46.7[ac]	1.165 +/-0.525[a]	4.37 +/-1.02[ac]	0.064 (0.060, 0.070)	0.228 (0.181, 0.268)	1.93 +/- 0.10
L6 Vertebra	Sham-Placebo	139.4 +/-21.0	-0.629 +/-0.623[a]	5.67 +/-0.68[a]	0.066 +/-0.004	0.167 +/-0.027[a]	1.76 +/-0.09
	Sham-InhA	137.9 +/-18.0	-1.276 +/-0.979[a]	5.99 +/-0.73[a]	0.071 +/-0.005	0.154 +/-0.022[a]	1.71 +/-0.07
	ORCH-Placebo	131.3 +/-21.3	0.274 +/-0.792[b]	4.91 +/-0.65[b]	0.065 +/-0.006	0.198 +/-0.032[b]	1.72 +/-0.16
	ORCH-InhA	144.5 +/-18.3	-0.628 +/-0.916[a]	5.41 +/-0.69	0.070 +/-0.004	0.178 +/-0.028	1.68 +/-0.08

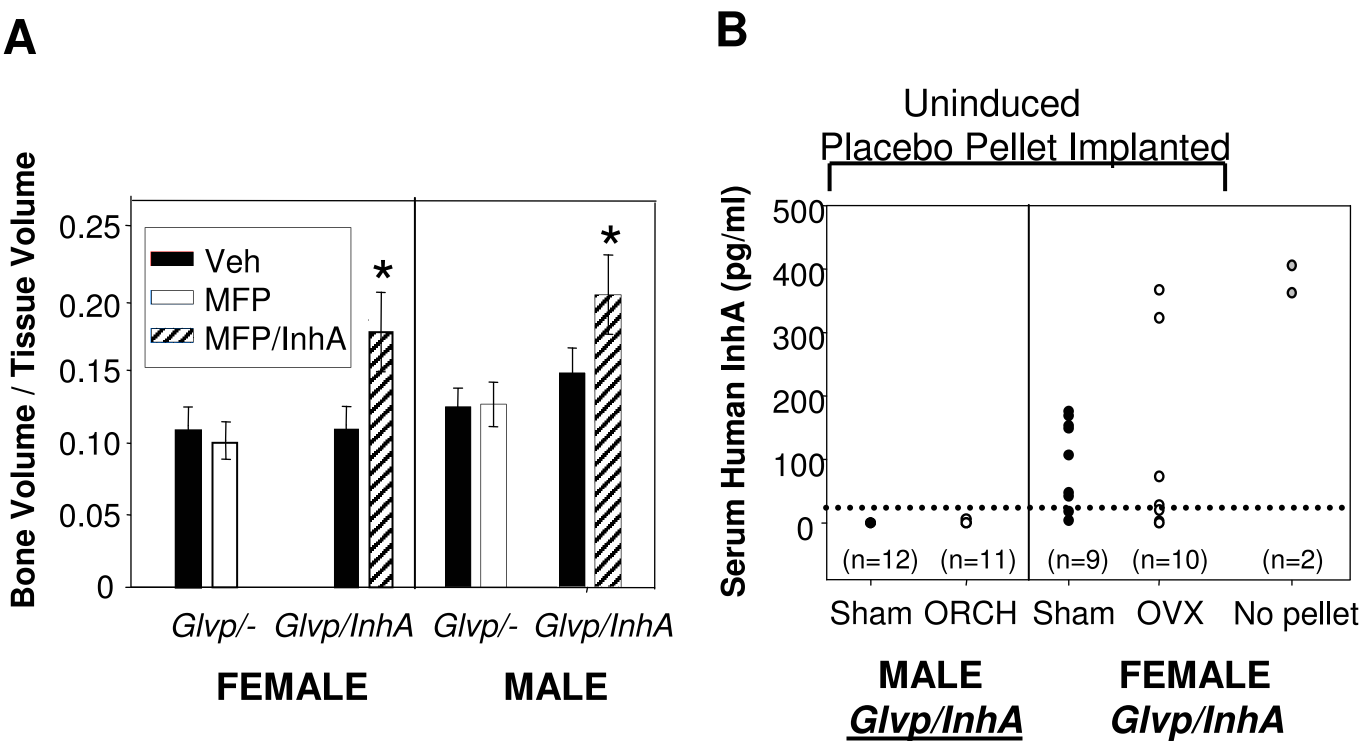
Different superscripts are significantly different with $p<0.05$; unscripted data are not significantly different from any other treatment. For the proximal tibia, connectivity density (Conn D), structure model index (SMI), trabecular number (TbN), and degree of anisotropy (DA) were analyzed by one-way ANOVA and SNK post-hoc test. Data for trabecular thickness (TbTh) and separation (TbSp) were not normally distributed and were analyzed by Kruskall-Wallis ANOVA on ranks and Dunn's post-hoc tests. For L6, all data were analyzed by one-way ANOVA and SNK post-hoc test. Parametric data are presented at mean +/-standard deviation and non-parametric data are presented as median (25th percentile, 75th percentile).

Table 2 – Inhibin A Stimulates Mineral Apposition Rate But Does Not Affect Static Indices of Bone Turnover *in vivo*.

	Sham-Veh	Sham-InhA	ORCH-Veh	ORCH-InhA
Oc.N/BS	5.06 +/-2.21	6.14 +/-1.96	5.32 +/-2.38	7.21 +/-3.05
ES/BS	0.318 +/-0.160	0.275 +/-0.138	0.319 +/-0.109	0.360 +/-0.123
Ocl.N/TA	44.13+/-8.14	61.70+/-8.22	33.18+/-6.91	64.99+/-19.70
Ob.N./BS	8.06 +/-6.13[a]	10.2 +/-3.42[a]	22.6 +/-10.4[b]	20.8 +/-6.7[b]
Ob.S/BS	0.085 +/-0.070	0.103 +/-0.035	0.242 +/-0.118	0.209 +/-0.049
Ob.N/TA	67.85+/-18.19[a]	103.33+/-21.32	145.12+/-31.02[b]	175.63+/-45.80
OS/BS	0.103 +/-0.098	0.111 +/-0.045	0.239 +/-0.146	0.304 +/-0.100
O.Th.	4.46 +/-0.97	3.47 +/-0.66	3.62 +/-1.23	4.32 +/-0.96
BFR	0.183+/-0.152	0.295+/-0.269	0.271+/-0.390	0.116/-0.127
BFR/TV	0.266+/-0.077	0.313+/-0.165	0.366+/-0.161	0.199/-0.082
MAR	0.283+/-0.112[a]	0.750+/-0.376[b]	0.496+/-0.320	0.807+/-0.621
dLS/BS	0.022+/-0.005[a]	0.047+/-0.009[b]	0.010+/-0.003[c]	0.027+/-0.010

Different superscripts are significantly different with $p<0.05$. For static histomorphometric analyses, 4-5 µm-thick central saggital sections of undecalcified methyl methacrylate embedded tibiae were stained for TRAP and counterstained with hematoxylin to measure osteoclasts and eroded surface or with Masson's Trichrome for all other measurements. Oc.N/BS – Osteoclast Number per Bone Surface; Oc.N/TA – Osteoclast Number/Total Area; ES/BS – Eroded Surface/BS; Ob.N/BS – Osteoblast Number/BS; Ob.S/BS – Osteoblast Surface/BS; Ob.N/TA – Osteoblast Number/Tissue Area OS/BS – Osteoid Surface/BS; O.Th. – Osteoid thickness; BFR – Bone Formation Rate; BFR/TV – BFR/Tissue Volume; MAR – Mineral Apposition Rate; dLS/BS – double Labeled Surface/BS.

Figure 1



A
Bone Volume / Tissue Volume
0.25
0.20
0.15
0.10
0.05
0
Veh
MFP
MFP/InhA
*
*
Glvp/-
Glvp/InhA
Glvp/-
Glvp/InhA
FEMALE
MALE
B
Uninduced
Placebo Pellet Implanted
Serum Human InhA (pg/ml)
500
400
300
200
100
0
(n=12)
(n=11)
(n=9)
(n=10)
(n=2)
Sham
ORCH
Sham
OVX
No pellet
MALE
Glvp/InhA
FEMALE
Glvp/InhA

Figure 2

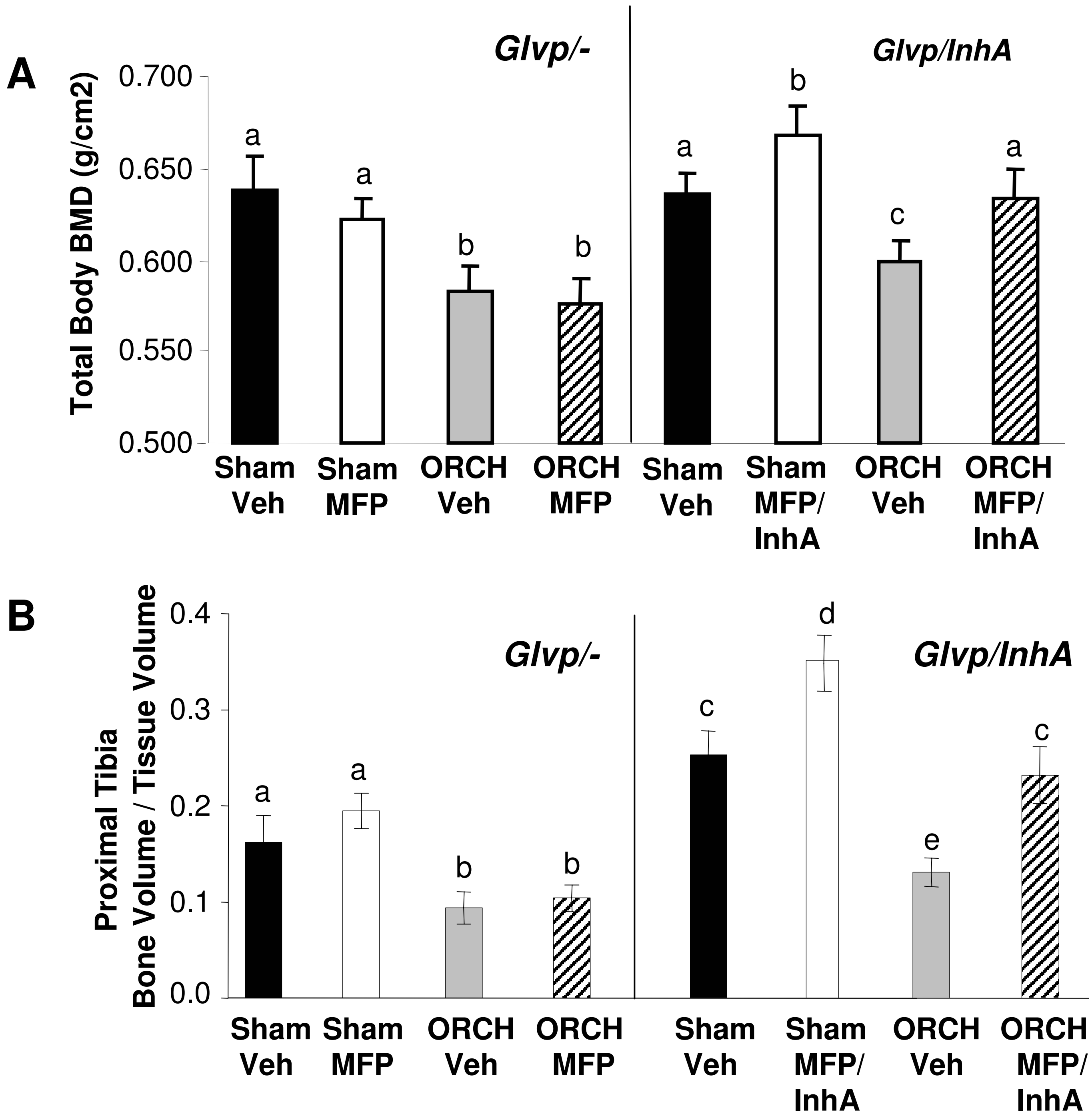
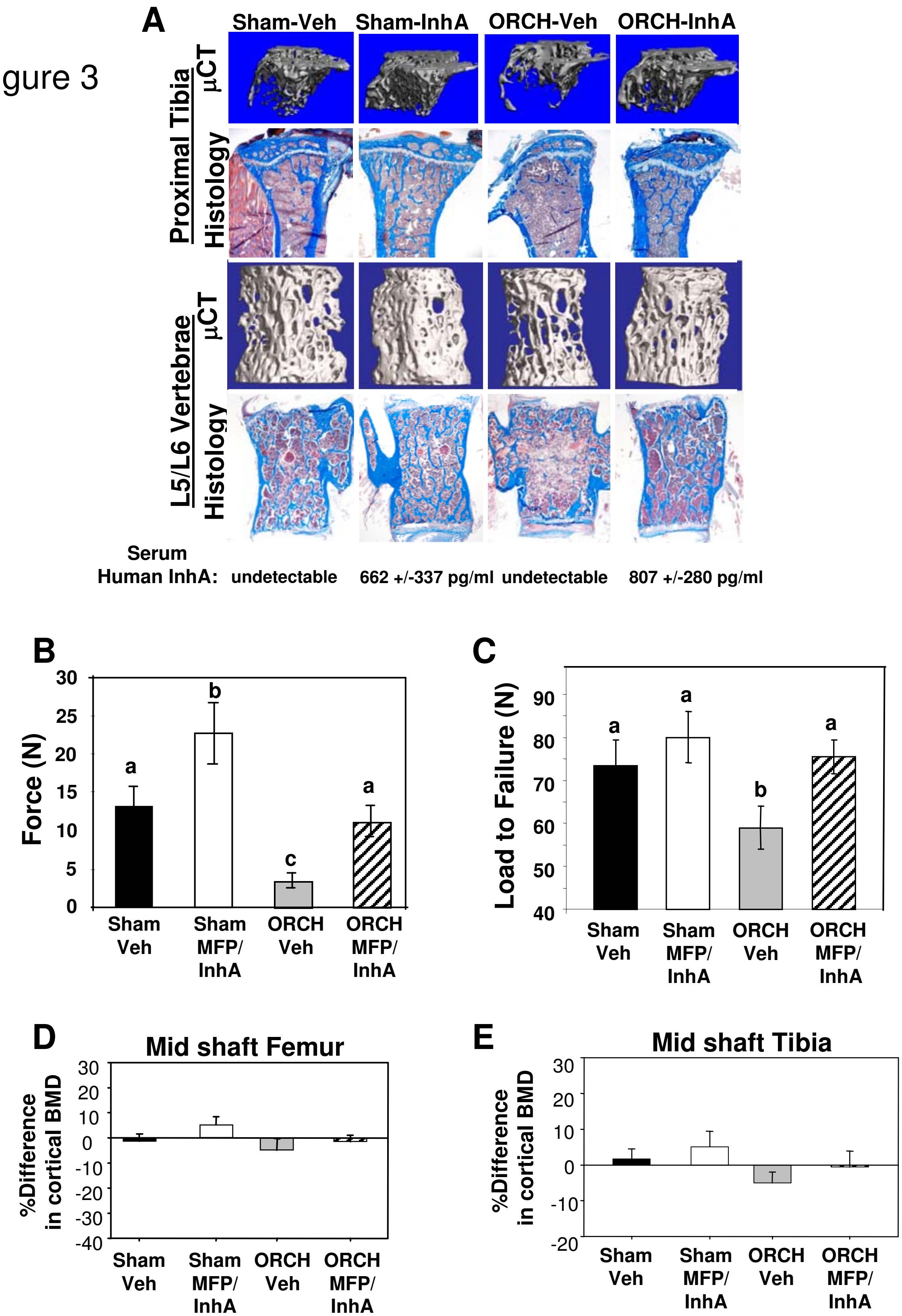

A
Total Body BMD (g/cm2)
0.700
0.650
0.600
0.550
0.500
Glvp/-
Glvp/InhA
a
a
b
b
a
b
c
a
Sham Veh
Sham MFP
ORCH Veh
ORCH MFP
Sham Veh
Sham MFP/ InhA
ORCH Veh
ORCH MFP/ InhA
B
Proximal Tibia
Bone Volume / Tissue Volume
0.4
0.3
0.2
0.1
0.0
Glvp/-
Glvp/InhA
a
a
b
b
c
d
e
c
Sham Veh
Sham MFP
ORCH Veh
ORCH MFP
Sham Veh
Sham MFP/ InhA
ORCH Veh
ORCH MFP/ InhA

Figure 3



A
Sham-Veh
Sham-InhA
ORCH-Veh
ORCH-InhA
Proximal Tibia
µCT
Histology
L5/L6 Vertebrae
µCT
Histology
Serum
Human InhA:
undetectable
662 +/-337 pg/ml
undetectable
807 +/-280 pg/ml
B
Force (N)
30
25
20
15
10
5
0
a
b
c
a
Sham Veh
Sham MFP/ InhA
ORCH Veh
ORCH MFP/ InhA
C
Load to Failure (N)
90
80
70
60
50
40
a
a
b
a
Sham Veh
Sham MFP/ InhA
ORCH Veh
ORCH MFP/ InhA
D
Mid shaft Femur
%Difference in cortical BMD
30
20
10
0
-10
-20
-30
-40
Sham Veh
Sham MFP/ InhA
ORCH Veh
ORCH MFP/ InhA
E
Mid shaft Tibia
%Difference in cortical BMD
30
20
10
0
-10
-20
Sham Veh
Sham MFP/ InhA
ORCH Veh
ORCH MFP/ InhA

Figure 4

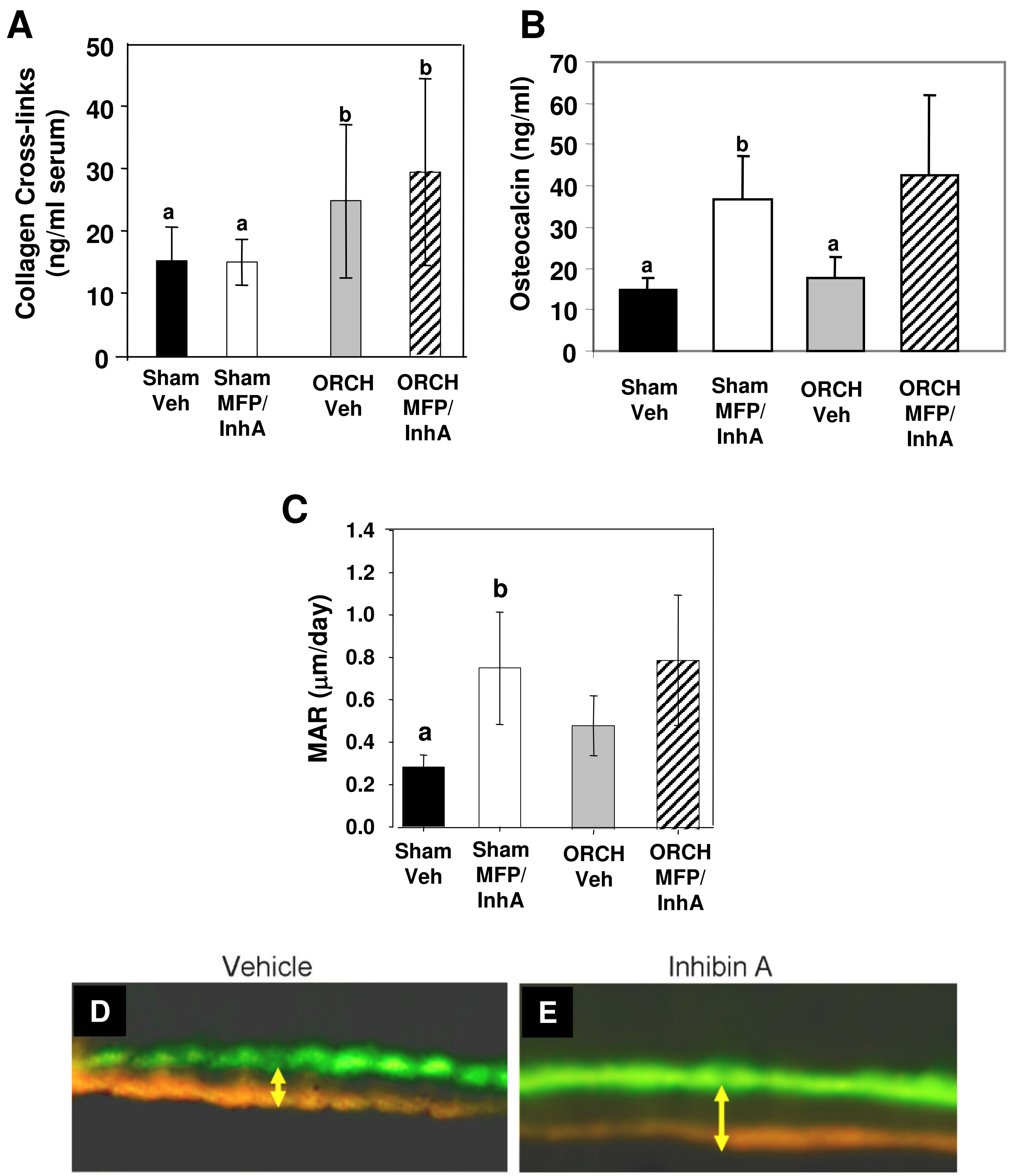
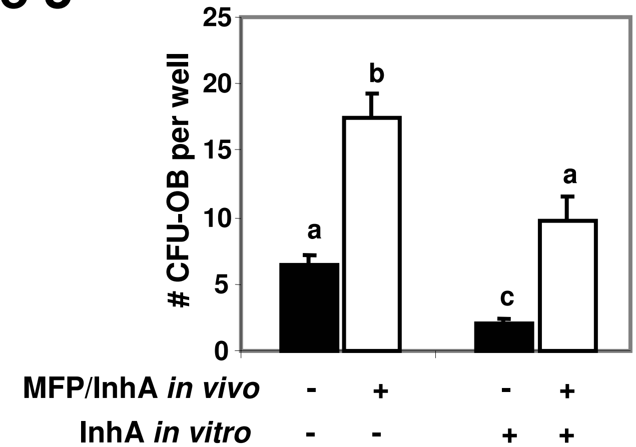

A
Collagen Cross-links (ng/ml serum)
50
40
30
20
10
0
a
a
b
b
Sham Veh
Sham MFP/ InhA
ORCH Veh
ORCH MFP/ InhA
B
Osteocalcin (ng/ml)
70
60
50
40
30
20
10
0
a
b
a
Sham Veh
Sham MFP/ InhA
ORCH Veh
ORCH MFP/ InhA
C
MAR (µm/day)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
a
b
Sham Veh
Sham MFP/ InhA
ORCH Veh
ORCH MFP/ InhA
Vehicle
Inhibin A
D
E

Figure 5



CFU-OB per well
0
5
10
15
20
25
a
b
c
a
MFP/InhA *in vivo*
InhA *in vitro*
-
+
-
+
-
-
+
+

Figure 6

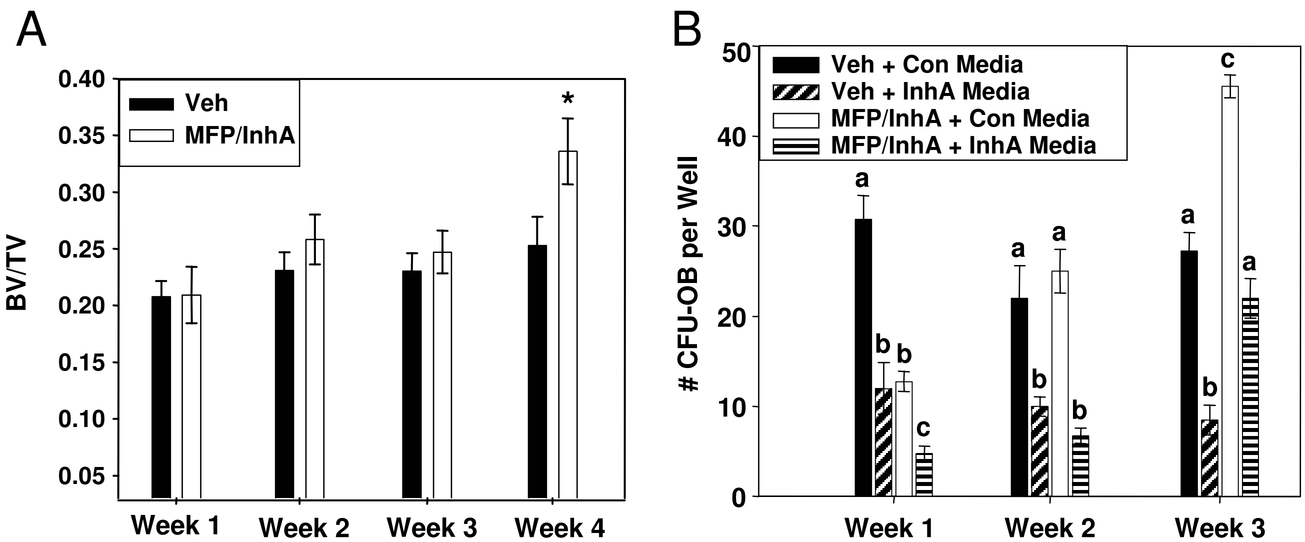
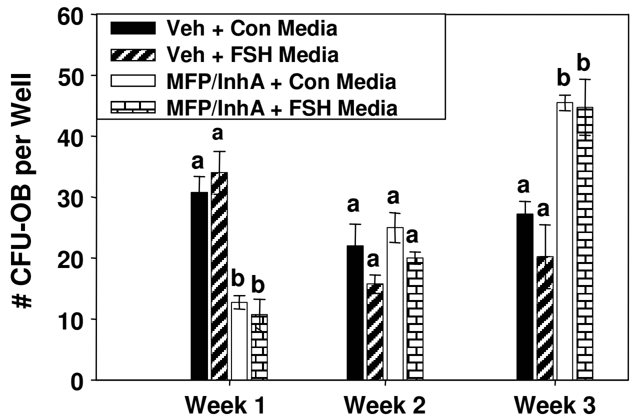

A
BV/TV
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
Veh
MFP/InhA
*
Week 1
Week 2
Week 3
Week 4
B
CFU-OB per Well
50
40
30
20
10
0
Veh + Con Media
Veh + InhA Media
MFP/InhA + Con Media
MFP/InhA + InhA Media
a
b
b
c
a
b
a
b
a
b
c
a
Week 1
Week 2
Week 3

Figure 7



Veh + Con Media
Veh + FSH Media
MFP/InhA + Con Media
MFP/InhA + FSH Media
CFU-OB per Well
60
50
40
30
20
10
0
a
a
b
b
a
a
a
a
a
a
b
b
Week 1
Week 2
Week 3