



Published in final edited form as:

J Mol Med (Berl). 2013 December ; 91(12): . doi:10.1007/s00109-013-1068-3.

R-Spondin 1 promotes vibration-induced bone formation in mouse models of osteoporosis

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Abstract

Bone tissue adapts to its functional environment by optimizing its morphology for mechanical demand. Among the mechanosensitive cells that recognize and respond to forces in the skeleton are osteocytes, osteoblasts, and mesenchymal progenitor cells (MPCs). Therefore, the ability to use mechanical signals to improve bone health through exercise and devices that deliver mechanical signals is an attractive approach to age-related bone loss; however, the extracellular or circulating mediators of such signals are largely unknown. Using SDS-PAGE separation of proteins secreted by MPCs in response to low magnitude mechanical signals and in-gel trypsin digestion followed by HPLC and mass spectroscopy, we identified secreted proteins up-regulated by vibratory stimulation. We exploited a cell senescence-associated secretory phenotype screen, and reasoned that a subset of vibration-induced proteins with diminished secretion by senescent MPCs will have the capacity to promote bone formation *in vivo*. We identified one such vibration-induced bone-enhancing (*vibe*) gene as R-Spondin 1, a Wnt pathway modulator, and demonstrated that it has the capacity to promote bone formation in three mouse models of age-related bone loss. By virtue of their secretory status, some *vibe* proteins may be candidates for pre-clinical development as anabolic agents for the treatment of osteoporosis.

Keywords

Mechanical signals; vibration; R-spondin 1; telomerase; telomere; aging; osteoporosis; mesenchymal stem cells

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Disclosure statement

The authors declare no conflicts of interest related to the work presented in this paper.

Introduction

Bone tissue adapts to its functional environment by optimizing its morphology for mechanical demand (e.g. work against gravity or resistance exercise). Mechanosensitive cells that recognize and respond to forces in the skeleton include mesenchymal progenitor cells (MPCs), osteoblasts, osteoclasts, osteocytes and cells of the vasculature [1]. Proximal mechanosensing mechanisms may involve ion channels, integrins, connexins, caveolar and noncaveolar lipid rafts, as well as cell shape alteration at the membrane or cytoskeleton [1]. G-proteins, MAPKs, and nitric oxide have been implicated in downstream intracellular signaling [1].

The skeleton's sensitivity to mechanical stimuli represents a critical determinant of bone mass and physical activity is an important strategy to reduce osteoporosis and fractures in the elderly. It is well-established that increases in bone mineral density (BMD) or reductions in bone loss occur with sufficient exercise or mechanical loading [2, 3]. Despite potentially missed opportunities to maintain a strong skeleton into adulthood and old age, and minimizing bone loss in peri-menopausal years and later life, physical activity and exercise at virtually any age can still increase BMD and potentially reduce fracture risk with minimal therapeutic harm [3, 4]. Extremely low level mechanical stimuli, such as that received through vibration, improve both the quantity and the quality of trabecular bone [5], are anabolic to trabecular bone in children [6], and increase bone and muscle mass in the weight-bearing skeleton of young adult females with low BMD [7]. Therefore, the ability to use mechanical signals to improve bone health through exercise and devices that deliver mechanical signals is an attractive approach to age-related bone loss. Although the extracellular or circulating mediators of such signals are largely unknown, that they exist is supported by site-specificity of adaptive responses to bone mechanical loading and unloading [8–10].

MPCs can differentiate into cells that form mesodermal tissues such as bone and fat. Low magnitude mechanical signals (LMMS) have been shown to increase the number of MPCs, as well as their potential to differentiate into osteoblasts versus adipocytes [11]. The number and activity of osteoblasts responsible for synthesizing new bone matrix are substantially reduced with aging, and limitations in the ability of osteogenic precursors to replace osteoblasts may potentially explain many aspects of age-related bone loss [12, 13]. Here we report the identification of secreted proteins up-regulated by vibratory stimulation of human MPCs and demonstrate that one such protein (R-spondin 1; *Rspo1*) has the capacity to promote bone formation in three mouse models of age-related bone loss. Thus, secreted vibration-induced bone-enhancing (*vibe*) genes may potentially serve as extracellular mediators of mechanical signals.

Materials and Methods

Animals

Fourth-generation 6 month old telomerase single mutant mice (*Terc*^{-/-}) and 3-month old Werner helicase/telomerase double mutant mice (*Wtn*^{-/-} *Terc*^{-/-}) [14–16], as well as 18.5 month old physiologically aged male mice (all on the C57Bl/6 background) were used for experiments. The University of Pennsylvania Institutional Animal Care and Use Committee (IACUC) approved the use of mice described in this paper.

MPC cell strains and culture

Femoral head samples were collected from three men, age range 40–50 years old, after elective hip arthroplasty for degenerative joint disease. Bone marrow was flushed from samples and suspended in α -Minimal Essential Medium (MEM) with nucleosides (Gibco/

Invitrogen, Grand Island, New York, USA) plus 10% fetal bovine serum (FBS) (Gibco/Invitrogen, Grand Island, New York, USA). Marrow was spun out of suspension at 300×g for 5 minutes and the pellet resuspended in α -MEM + 10% FBS before seeding cells into tissue culture flasks. Non-adherent cells were rinsed from the flasks after 24 hours, and MPC strains were grown and maintained in α -MEM + 10% FBS. Cells were seeded at a density of 1×10^4 cells/cm² at each passage and grown until confluent, usually by 10 days. Cells were used at the third confluent *in vitro* passage, after no more than ~6 population doublings after outgrowth from explant, and were considered “early passage. Cultures were defined as being at the end of their proliferative lifespan or “senescent” when they were unable to complete one population doubling during a 4-week period that included three consecutive weeks of refeeding with fresh medium containing 10% FBS. Population doublings were calculated as previously described [17]. Collection of femoral head samples was approved by the Institutional Review Board of the University of Pennsylvania.

Delivery of LMMS

The Juvent 1000 Dynamic Motion Platform was used to deliver LMMS [18]. Amplitude was delivered at a sinusoidal frequency of 32–37 Hz (displacement frequency), acceleration “g” force of 0.3 g (peak to peak) (+/- 20%), and vertical displacement of ~ 85 μ m with a continuous duty cycle. Cultures and additional mass 15.8 kg were placed on the platform to obtain a normal operating load. MPCs, serum-starved for 24 hr in α -MEM, were stimulated by LMMS for 10 min at room temperature, and then incubated for an additional 1 or 2 hrs at 37°C prior to collection of secreted proteins, whole cell lysate, and whole cell RNA. For vibratory stimulation in a 29 year-old male subject, the same device was used and LMMS were delivered for 20 minutes.

Blood collection and isolation of plasma

Blood was collected prior to and one hour after delivery of LMMS by a vibratory platform as described above. Whole blood was collected into Vacutainer® ethylenediaminetetraacetic acid (EDTA)-treated specimen tubes (BD, Franklin Lakes, New Jersey). Cells were removed from plasma by layering blood over Ficoll-Paque PLUS (GE Healthcare, Mickleton, NJ) and centrifugation per the manufacturer’s instructions. This protocol was approved by the University of Pennsylvania Institutional Review Board.

SDS-PAGE, in-gel trypsin digestion, and mass spectroscopy

Secreted proteins were precipitated from serum-free conditioned media with acetone at a 5:1 ratio, spun down for 10 min at 15,000×g at -20°C, and the pellet dried for 30 min. The protein was dissolved in RIPA buffer (150 mM NaCl, 1.0% IGEPAL® CA-630, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0.) with 1x proteinase inhibitor cocktail (Sigma, St. Louis, Missouri, USA) and phosphatase inhibitor cocktail (Pierce, Rockford, Illinois, USA) and quantified by the BCA assay (Pierce, Rockford, Illinois, USA). One-dimensional SDS-PAGE was performed using standard techniques. After Coomassie blue staining and washing of the gel, contiguous gel fragments were excised and each slice cut into 1×1 mm pieces. The in-gel tryptic digestion kit #89871; (Pierce Biotechnology, Rockford, Illinois, USA) was used according to the manufacturer’s instructions with both reduction and alkylation steps as described. Extracted proteins were dried and refrigerated until submission for mass spectroscopy analysis by the Proteomics Core at the University of Pennsylvania. Identification of genes and relative expression patterns (prior to Western blot confirmation) was performed using Scaffold 3.0 software (Proteome Software, Portland, Oregon, USA). Relative expression of secreted proteins was normalized to the average abundance of 4 secreted proteins that did not change significantly with LMMS after replicate analysis.

Western blot analysis

After removal of conditioned media, LMMS-stimulated MPCs and controls (no LMMS stimulation) were lysed in RIPA buffer with 1x proteinase inhibitor cocktail (Sigma, St Louis, Missouri, USA) and phosphatase inhibitor cocktail (Pierce, Rockford, Illinois, USA) to recover total protein. The lysate was incubated on ice for 15 min and centrifuged at 12,000 g for 10min. Secreted protein from conditioned media and plasma was prepared as described for SDS-PAGE. Protein was quantified using the BCA Assay kit (Pierce, Rockford, Illinois, USA). Fifty micrograms of total protein was separated on a 10% SDS-PAGE gel and then transferred to PVDF membranes (Bio-Rad, Hercules, California, USA) by electroblotting. Membranes were incubated overnight at 4°C with a 1:1000 dilution of antibody specific for Rspo1 (ABCAM, Cambridge, Massachusetts, USA). Membranes were washed with PBST (Phosphate buffered saline + 0.1% Tween-20) and incubated for 1 hr with a 1:5000 goat anti-rabbit IgG-HRP (sc-2004; Santa Cruz Biotechnology, Santa Cruz, California, USA). Antibody against albumin (Cell Signaling, Danvers, MA), against β -actin, (Santa Cruz Biotech, Dallas, Texas) and type 1 collagen (ABCAM, Cambridge, MA) were used at dilutions of 1:2000, 1:1000, and 1:1000, respectively. Appropriate anti-mouse or anti-rabbit HRP-conjugated secondary antibodies (Santa Cruz biotech, Dallas, Texas) were used at a dilution of 1:5000.

RNA extraction and Real-time PCR

Whole cell RNA was isolated at the same time as protein extraction from duplicate cultures using the RNeasy mini-kit (Qiagen, Valencia, California, USA). Real-time PCR was performed by standard methods.

Preparation and administration of purified Rspo1

Human recombinant Rspo1 was prepared by the Wistar Institute Protein Expression Laboratory as previously described [19]. Animals were administered Rspo1 at 3.3 μ g/g body weight as daily peritoneal injections for seven days.

Bone Histomorphometry

Bone histomorphometry was carried out essentially as previously described [20]. Briefly, to determine mineral apposition rate (MAR), each animal was injected intraperitoneally with 30 mg/kg calcein (Sigma, St Louis, MO, USA) at 9 days and 2 days before necropsy. For all measured parameters, mouse hind limbs were excised, cleaned of soft tissue, and fixed in 3.7% formaldehyde for 72 hours. Isolated bone tissue was dehydrated in graded alcohols (70 to 100%), cleared in xylene and embedded in methyl methacrylate. Plastic tissue blocks were cut into 5 μ m sections using a Polycut-S motorized microtome (Reichert-Jung, Nossloch, Germany). For TRAP staining, sections were incubated with substrate solution (112mM sodium acetate, 77 mM L-(+) tartaric acid, 0.3% glacial acetic acid) at 37°C for 5 hrs. This solution was then replaced with substrate solution plus 11.6mM sodium nitrite and 2.6mM pararosaniline dye, and incubated at room temperature for an additional 2 hrs before rising and dehydration by standard procedures. Goldner's Trichrome staining, was performed by standard methods.

Three consecutive sections per limb were visualized for fluorochrome labeling using a Nikon Eclipse 90i microscope and Nikon Plan Fluor 10X objective (Nikon Inc., Melville, New York, USA). For other measurements, consecutive sections were visualized using 4X and 20X objectives. Image capture was performed using NIS Elements Imaging Software 3.10 Sp2 and a Photometrics Coolsnap EZ camera. The Bioquant Osteo II digitizing system (R&M Biometrics, Nashville, TN) was used according to the manufacturer's instructions.

The terminology and calculations used are those recommended by the Histomorphometry Nomenclature Committee of the ASBMR [21].

Statistical Methods

The t test (Student's t test; two-sided and paired) was used to determine whether the average value for a bone histomorphometric parameter differed significantly between Rspo1 treated and untreated animals. One-way ANOVA was performed to determine if mRNA expression of Rspo1 varied significantly with time after LMMS. Statistical significance was set to $p = 0.05$. Statistical analysis was performed using GraphPad Prism 4.0 (San Diego, CA, USA). Error is expressed as standard error of the mean.

Results

Identification of secreted proteins from MPCs in response to low magnitude mechanical signals

MPCs are among the mechanosensitive cells that reside in bone tissue and we used human CD73⁺ CD90⁺ CD105⁺ CD45⁻ cells capable of differentiation into osteoblasts and adipocytes in the current study (Fig. 1). In order to identify secretory, vibration-induced bone-enhancing (*vibe*) genes in human MPCs, proteins were precipitated from conditioned media 2 hours after LMMS and separated by 1-dimensional SDS-PAGE. After in-gel trypsin digestion of LMMS-enhanced Coomassie blue stained protein bands (centered at 75 kDa, 70kDa, 45kDa, and 30kDa), HPLC, and mass spectroscopy analysis, 46 proteins were found to be up-regulated in response to LMMS compared to unstimulated controls. In response to LMMS, relative expression varied from about 2.5-to almost 30-fold. The most highly expressed LMMS-induced proteins secreted by human MPCs (relative to expression without stimulation) are shown in Table 1.

Vibration-enhanced expression of R-Spondin 1 in human MPCs *in vitro* and in a young healthy male *in vivo*

Of the known protein products the species most highly up-regulated by LMMS was R-Spondin 1 (Rspo1), a Wnt pathway modulator with few reported effects in bone [22]. We confirmed the vibration-enhanced expression of Rspo1 in human MPCs (Fig. 2 a-c) as well as in the circulation of a healthy 29 year-old male (Fig. 2 d). Success in finding that both known and unknown secretory proteins are responsive to LMMS and that circulating Rspo1 is increased after LMMS, as well as the identification of genes that play known or suspected roles in bone or matrix remodeling, strongly suggested that a subset of vibration-induced genes have the capacity to promote bone formation *in vivo*, and thus are true *vibe* genes.

R-Spondin 1 is a secretory, vibration-induced bone-enhancing (*vibe*) protein

We chose Rspo1 as a likely *vibe* gene based on its enhanced induction by LMMS (Table 1, Fig. 2), potential relevance to bone remodeling based on its characterization as a Wnt pathway modulator [22, 23], and its diminished secretion in senescent MPCs (Fig. 2e). We evaluated its ability to increase mineral apposition *in vivo* using three mouse models of age-related bone loss. Fig. 3a shows that mineral apposition rate (MAR) is significantly increased when recombinant Rspo1 is administered to physiologically aged mice, as well as in two telomere dysfunction-based models of accelerated aging (*Terc*^{-/-} and *Wrn*^{-/-} *Terc*^{-/-} mutants) where osteoporosis is a known phenotype. Significant increases in MAR were accompanied by increases in bone turnover, and importantly, significant increases in normalized osteoid surface and bone volume (Fig. 3b). These findings are consistent with a strong anabolic effect where bone resorption is coupled to enhanced deposition of mineralized matrix.

Discussion

We have developed a screening strategy to isolate a new class of genes, vibration-induced bone-enhancing (*vibe*) genes, whose protein products are secreted and have the capacity to promote bone formation. By virtue of their secretory status, some *vibe* proteins may be candidates for pre-clinical development as anabolic agents for the treatment of osteoporosis. We have identified R-Spondin 1, a Wnt pathway modulator, as one such *vibe* gene. This report represents an initial characterization of the “vibration secretome” induced by LMMS, with important implications for understanding the response of MPCs to mechanical signals which are transduced by molecular pathways that ultimately lead to new bone formation.

Adaptation to mechanical loading at cortical and cancellous sites is well described [24]. For example, adaptation to daily, cyclic, axial loading of a long bone results in the inhibition of bone loss, elevated bone mineral content, greater effects at cancellous versus cortical sites, and variation in response depending on the term and level of loading [25]. Disuse or paralysis of limbs shows extensive loss of trabecular bone [26].

Enhanced external load intensity (amplitude and frequency) and local elevations of strain at resorption cavities can induce bone formation [27]. However, with aging the skeleton becomes less responsive to loads. Historically, the osteocyte has been considered the bone cell predominately responsible for the transduction of mechanical signals [28]. Located within the bone matrix, osteocytes arise from MPCs through osteoblast differentiation. Osteocyte damage or apoptosis in the young skeleton leads to osteoclastic bone resorption followed by formation, but in the aged skeleton can lead to empty lacunae or micropetrosis where the lacuna fills in with mineral [28]. Changes in peri-lacunar mineral density, elastic modulus of the peri-lacunar matrix, and in the size of lacunae and canaliculi affect mechanosensation by the aging osteocyte [28]. Even if the osteocyte remained viable for decades, its mechanoresponsiveness would be compromised. However, other mechanosensitive cells that recognize and respond to forces in the skeleton include MPCs, which respond to LMMS by increasing proliferation and potential to differentiate into osteoblasts [11]. In the case of Rspo1, it is rapidly secreted and can be found in MPC supernatants as well as in circulation by one hour after stimulation by LMMS. Regulation of secretion is the most likely explanation for these observations, and is consistent with the differential expression of secreted (but not cellular) Rspo1 between vibration-induced and uninduced conditions in early-passage cells.

Noninvasive delivery of LMMS improves both quantity and quality of trabecular bone, are anabolic to trabecular bone in children, increase bone and muscle mass in the weight-bearing skeleton of young adult females with low BMD, and increase spinal trabecular bone while keeping visceral fat at baseline levels in young women with osteopenia [5–7]. However, 202 healthy postmenopausal women with osteopenia who received whole-body vibration therapy for 12 months did not alter BMD or bone structure [29]. Delivery of such therapy involves standing on an oscillating platform which produces vertical accelerations that are transmitted from the feet to the weight bearing skeleton. Since transmission of whole-body vibration depends on its intensity, knee-joint angle, distance from the vibratory source, and dampening by soft tissue [30] using circulating (systemic) mediators of mechanical signals for bone loss is appealing, and may overcome these limitations.

R-spondins are secreted Wnt signaling agonists that regulate embryonic patterning and stem cell proliferation in the intestinal crypt and hair follicle. R-spondins, including Rspo1, can bind to Lgr4, Lgr5 and Lgr6 in the Frizzled/Lrp Wnt receptor complex suggesting that their activity enhances pleiotropic functions in development and stem cell growth [31–34]. Little is known about the effects of RSp1 in the skeleton, but one report suggests that it is

protective against inflammatory bone damage in a mouse model of arthritis [22]. Given the roles of Wnt signaling in bone remodeling [35], the application of R-spondins for regenerative purposes holds future promise.

We previously showed that deficiencies in genome maintenance molecules, such as Werner helicase (Wrn) and telomerase (Terc), are related to a low bone mass phenotype due to impairment in osteogenic potential (decreased MPCs) and osteoblast differentiation (decreased expression of osteoblast markers) [15]. We also showed that MPCs derived from Wrn^{-/-} Terc^{-/-} double mutants or Terc^{-/-} single mutants have a reduced *in vitro* lifespan concomitant with impaired osteogenic potential and osteoblast differentiation, but that telomere dysfunction mediates decreased osteogenesis independent of proliferation [16]. We employed both accelerated aging models in our functional characterization of Rspo1 since they recapitulate many aspects of senile bone loss at early ages, with dysfunctional telomeres as the basis for defects in both proliferation and differentiation in MPCs.

We observed responsiveness to Rspo1, in terms of bone turnover and mineral acquisition, in both Terc^{-/-} and Wrn^{-/-} Terc^{-/-} mutants. Although speculative, perhaps the response suggests that telomere dysfunction, or other stresses (e.g. DNA damage) leading to the same cellular consequences as telomere dysfunction (e.g. cellular senescence), may be operational in normal skeletal aging. However, Rspo1 cannot be acting via upregulation of telomerase activity. Outside of its role in contributing to telomerase activity, mTert has been reported serve other functions (e.g. to physically occupy gene promoters of Wnt-dependent genes) and to thus serve as a transcriptional modulator of the Wnt signaling pathway [36]. Importantly, Wnt signaling leads to upregulation of mTert gene via cooperation between β -catenin and Klf4 [37]. The implications of this are that Wnt agonists (such as Rspo1) may promote bone formation in old wild-type mice by promoting the up-regulation of mTert in mTerc-deficient mice, thus contributing to mTert function(s) that do not depend on telomerase activity. It is also possible that Rspo1 is involved in multiple mechanisms that preserve bone structure, including telomerase-independent functions of Tert such as preservation of stemness in mesenchymal precursors, upregulation of growth factor receptor expression and cell proliferation, as well as regulation of Wnt target genes [38–41].

If functional deficits in osteoblasts that occur with aging play a major role in the uncoupling of bone formation and resorption, then recruitment of osteoblast precursors and osteoblast differentiation become critical components in maintaining skeletal homeostasis. Aging effects on human MPCs manifest as declines in measures associated with osteogenic potential, particularly after the age of forty [12, 13]. MPCs from aged donors also tend to have decreased proliferative potential [13].

Many changes in gene expression that occur with senescence appear to be unrelated to growth arrest, especially in fibroblasts [42]. For example, senescent cells overexpress genes that encode secreted proteins that can alter the tissue microenvironment, including proteins that remodel the extracellular matrix or mediate local inflammation [43, 44]. Interestingly, regulation of secretion may also account for the differential expression of secreted Rspo1 between vibration-induced and uninduced conditions in early-passage cells, as well as between early- and late-passage (senescent) cells after LMMS. These findings raise the possibility that as senescent cells increase in number with age, they might contribute to age-related decrements in tissue structure and function by impaired responsiveness to external cues. Thus, a senescence-associated secretory phenotype is evident in MPCs and our data indicates that it results in the diminished secretion of LMMS-induced proteins, a finding that can be exploited to identify other *vibe* genes.

Acknowledgments

This work was supported by National Institutes of Health/ National Institute on Aging grants R01AG028873 (R.J.P), and P01AG031862 (D.C.S., F.B.J.). The Juvent 1000 Dynamic Motion Platform was a generous gift from Mary Leonard, MD, MSCE (Children's Hospital of Philadelphia, Philadelphia, Pennsylvania).

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- Mesenchymal stem cells respond to low magnitude mechanical signals (vibration).
- R-Spondin 1 is upregulated by mechanical signals and secreted.
- R-Spondin 1 promotes bone formation in three mouse models of osteoporosis.

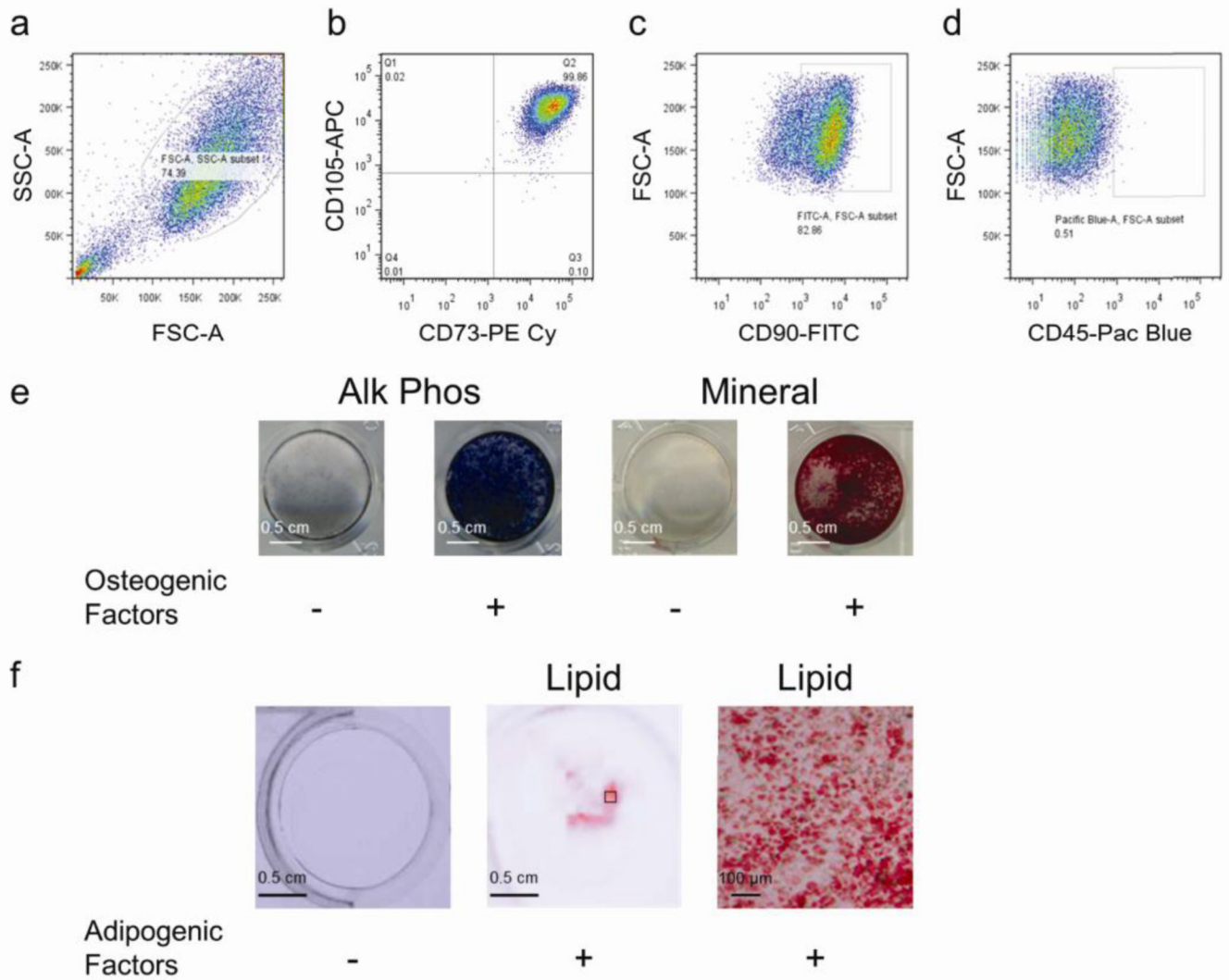


Fig 1. Phenotype of human mesenchymal progenitor cells (MPCs). MPCs are CD 73⁺ CD90⁺ CD105⁺ CD45⁻ cells by flow cytometric analysis (**a-d**). MPCs are capable of differentiating into osteoblasts and adipocytes in vitro (**e, f**).

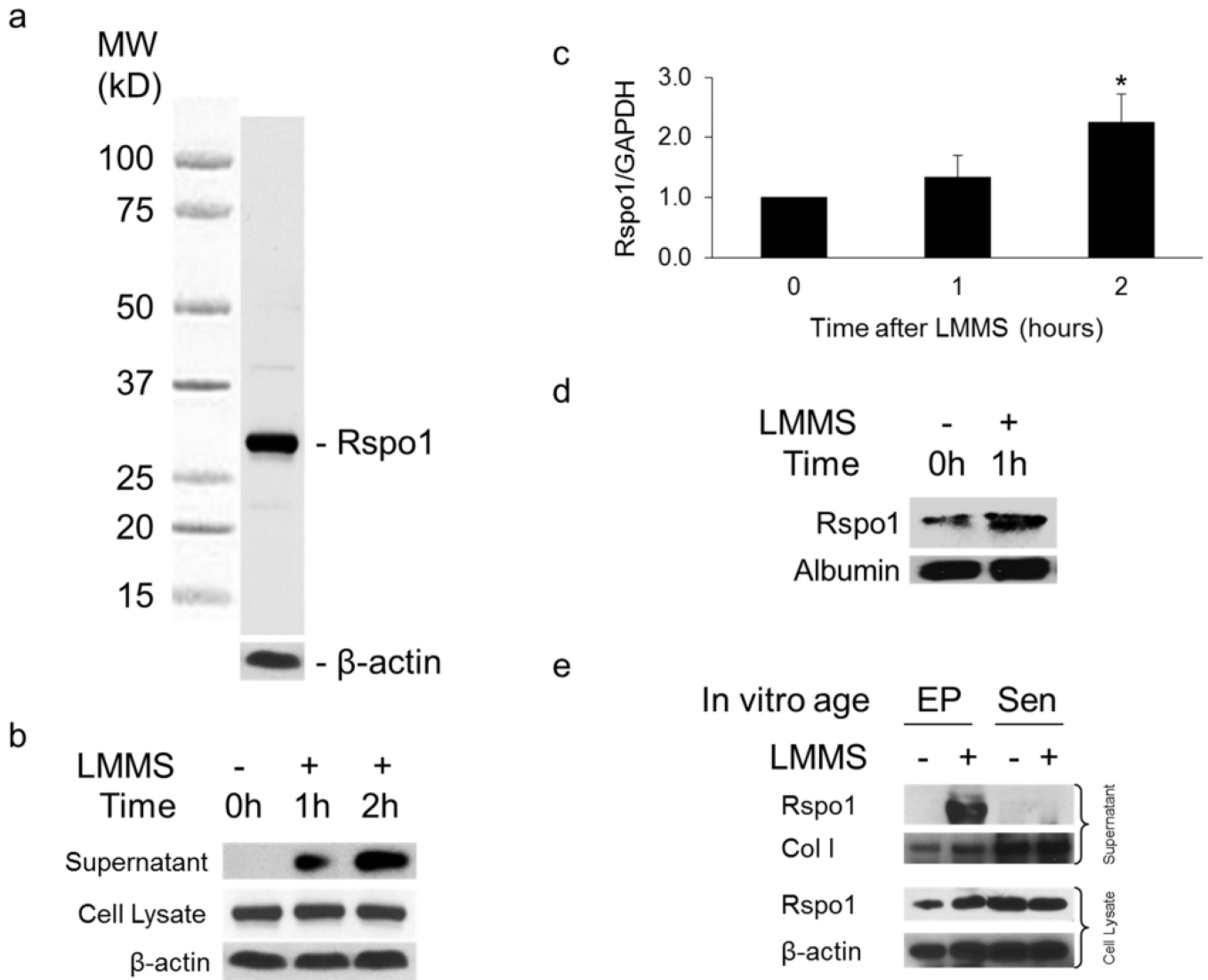
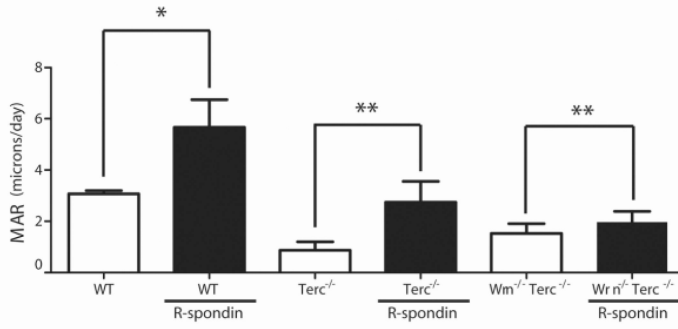


Fig. 2. Vibration (LMMS)-induced expression of Rspo1. **(a)** Rspo1 is recognized as a protein of ~30 kD by Western blot analysis of whole cell protein. **(b)** Rspo1 Western blot analysis of secreted protein (supernatant) and whole cell protein (cell lysate) produced in response to LMMS. **(c)** RT-PCR analysis of the Rspo1 transcript. *, $p < 0.05$. $n = 3$ for all conditions. **(d)** The circulating level of Rspo1 is increased in response to LMMS in a 29 year-old healthy male. **(e)** Senescent MPCs do not secrete Rspo1 in response to LMMS. Shown are Western blot analyses of secreted protein (supernatant) and whole cell protein (cell lysate) produced by senescent (Sen) and early passage (EP) MPCs using antibodies against Rspo1, type I collagen (Col I), and β -actin. $n = 3$ for all conditions.

a



b

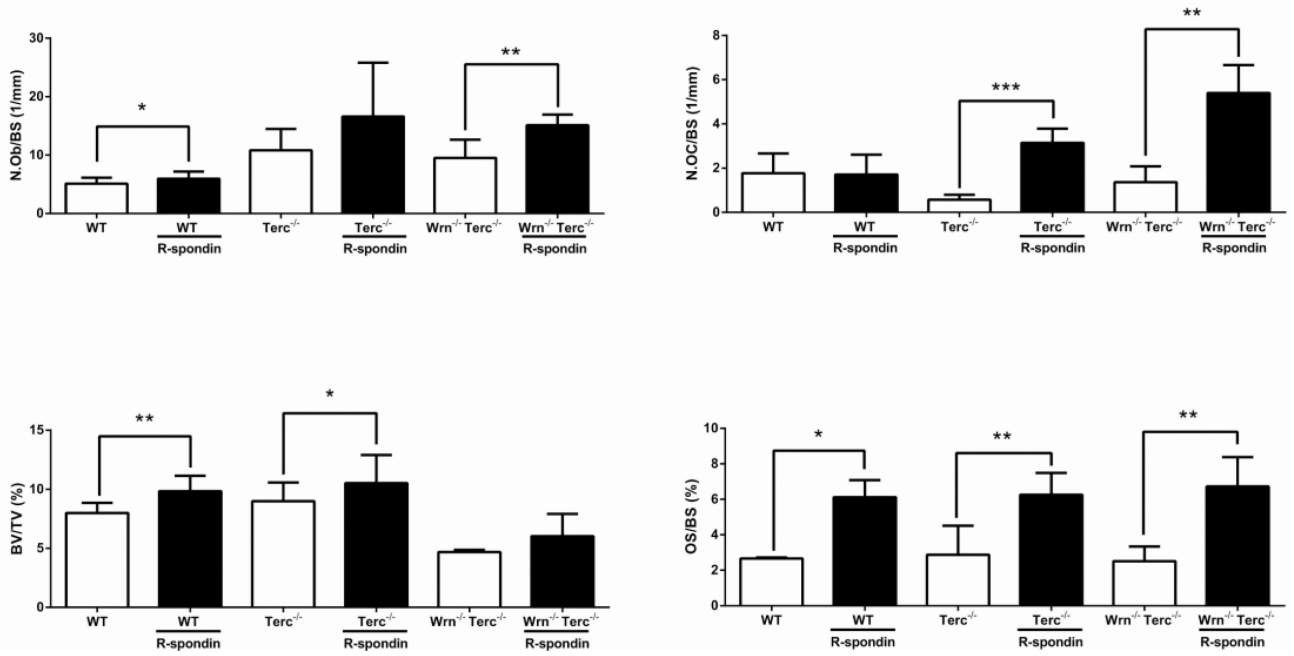


Fig. 3.

Rspo1 promotes bone formation. (a) Rspo1 enhances mineral apposition rate (MAR) in three mouse models of age-related bone loss. (b) Rspo1 promotes bone turnover and bone formation in mouse models of osteoporosis. Animals used were 18.5 month old wild-type mice (WT; n = 4 per group), 6 month old Terc^{-/-} single mutants (n = 3 per group) and 3-month old Wrn^{-/-} Terc^{-/-} double mutants (n = 3 per group). N.Ob/BS, number of osteoblasts/bone surface; N.OC/BS, number of osteoclasts/bone surface; BV/TV, bone volume/total volume; OS/BS, osteoid surface/bone surface. *, p<0.05; **, p<0.02, ***, p<0.005.

Table 1

Identification of LMMS-responsive secretory proteins in human MPCs.

Molecular weight* (kDa)	Gene	Unique Peptides	Highest relative expression (Fold)
19	tissue inhibitor of metalloproteinases	4	12.5
29	R-Spondin 1	4	28.0
36	unnamed protein product gi62822120	4	11.6
42	unnamed protein product gi189053417	10	28.2
45	unnamed protein product gi189055325	9	17.9
45	plasminogen activator inhibitor-1 precursor	14	9.9
61	unnamed protein product i158258302	6	7.2
129	unnamed protein product gi158256710	6	6.4
139	Collagen alpha 1 chain precursor variant	7	8.1
312	fibrillin-1 precursor	3	4.5

* Based on Scaffold 3.0 software analysis. Molecular weights are given for the unprocessed proteins. For unnamed protein products, the National Center for Biotechnology Information (NCBI; <http://www.ncbi.nlm.nih.gov/>) identifier is given.