Global Deletion of Tetraspanin CD82 Attenuates Bone Growth and Enhances Bone Marrow
Adipogenesis

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ABSTRACT

CD82 is a widely expressed member of the tetraspanin family of transmembrane proteins known to control cell signaling, adhesion, and migration. Tetraspanin CD82 is induced over 9-fold during osteoclast differentiation *in vitro*; however, its role in bone homeostasis is unknown. A globally deleted CD82 mouse model was used to assess the bone phenotype. Based on microCT and 4-point bending tests, CD82-deficient bones are smaller in diameter and weaker, but display no changes in bone density. Histomorphometry shows a decrease in size, erosion perimeter, and number of osteoclasts *in situ*, with a corresponding increase in trabecular surface area, specifically in male mice. Male-specific alterations are observed in trabecular structure by microCT and *in vitro* differentiated osteoclasts are morphologically abnormal. Histomorphometry did not reveal a significant reduction in osteoblast number; however, dynamic labeling reveals a significant decrease in bone growth. Consistent with defects in OB function, OB differentiation and mineralization are defective *in vitro*, whereas adipogenesis is enhanced. There is a corresponding increase in bone marrow adipocytes *in situ*. Thus, combined defects in both osteoclasts and osteoblasts can account for the observed bone phenotypes, and suggests a role for CD82 in both bone mesenchyme and myeloid cells.

1. INTRODUCTION

Tetraspanins (*Tspans*) are a family of 33 evolutionarily conserved transmembrane scaffolding proteins that behave as membrane organizing centers to facilitate a variety of cell functions including membrane signaling, cell-cell and matrix adhesion, membrane fusion, actin dynamics, immune surveillance, and motility [1, 2]. Structurally they are composed of four transmembrane spanning domains with palmitoylation sites at the intracellular juxtamembrane regions, two short intracellular tails and a small intracellular loop, a small extracellular domain, and a large extracellular domain composed of 2-4 cysteine-mediated disulfide bonds and a conserved CCG motif [3]. Tspans contain no catalytic activity and function by controlling the lateral organization of membrane proteins through direct interactions with other transmembrane proteins and other tetraspanin family members to form lipid-rich membrane microdomains [4]. The recent crystallization of a Tspan family member, CD81, revealed a hydrophobic pocket capable of sequestering a molecule of cholesterol between the four transmembrane domains, which could explain, in part, how tetraspanins are able to assemble lipid-enriched membrane complexes [5].

Some tetraspanins, such as CD37, CD81, and CD53, have been extensively studied in humoral and cellular immunity (reviewed in [6, 7]), while others, such as CD151, CD82, and CD9, have been extensively studied in tumor biology [8, 9]. However, the role of tetraspanin family members in bone homeostasis is essentially unknown. Assessment of tetraspanins in matrix-depositing osteoblasts, chondrocytes, and osteocytes has been limited to expression analysis; cellular function has not been studied [10-12] [13, 14] [15]. *In vitro* studies using RNAi in RAW264.7 cells demonstrated Tspan5 and Tspan10 are required for osteoclast fusion, whereas Tspan13 limits fusion [16, 17]. Similar approaches revealed the importance of Tspan7 for sealing zone function and actin polymerization [18]. One group reported that CD9 or CD81 null mice each displayed enhanced myeloid cell fusion *in vitro* and CD9/CD81 double-null mice display enhanced osteoclastogenesis *in situ* [19]. In contrast, an *in vitro* siRNA study found the

opposite, that CD9 is required for cell fusion during osteoclastogenesis [14]. However, none of these studies addressed the effect of blocking these tetraspanins on bone homeostasis *in vivo*.

A recent report indicated that CD82 is upregulated during murine osteoclast differentiation *in vitro*; however, the function of CD82 in osteoclasts or in the bone has yet to be delineated [20]. Studies on global CD82 KO mice have identified its crucial activity in several different biological processes including dendritic cell migration, angiogenesis, and platelet function [21] [22] [23]. Several studies indicate CD82 is crucial for hematopoietic stem cell (HSC) homing and bone marrow engraftment [24] [25]. CD82 null mice have reduced numbers of long-lived HSCs *in vivo* and reduced binding to the endosteal [26]. Given the potential role for CD82 in osteoclast differentiation, we hypothesized that loss of CD82 would result in elevated bone density due to defects in osteoclasts. Herein we assessed the bone phenotypes of our global deletion of mouse CD82.

2. Materials and Methods

2.1. Animals

CD82 null mice were generated as previously described [23]. Briefly, floxed mice were crossed to CMV-Cre mice to delete exons IV and V of the mouse CD82 sequence, resulting in global ablation of functional CD82. Mice were maintained in a pathogen-free and ALAAC certified facility. Littermates from heterozygous matings were used in all experiments and mice were all on a pure FVB/n background. Male and female mice were independently assessed to determine if any phenotypes were sex-dependent. At time of sacrifice all mice were weighed. CD82 KO female mice displayed a 20% reduction in body weight compared to wild type, while there was no significant difference in weight between the WT and KO males. Unless otherwise noted, animals were sacrificed by CO₂ asphyxiation and all studies were conducted in accordance with an IACUC approved protocol at the Van Andel Research Institute. All animal

experiments comply with the ARRIVE guidelines and were carried out in accordance with the National Institutes of Health guide for the care and use of Laboratory animals.

2.2. microCT

Femurs were harvested from 24- or 36-week old mice sacrificed via CO₂ asphyxiation. Bones were cleaned of excess tissue, fixed in a 10% neutral buffered formalin solution for 72 hours, and stored in 70% ethanol until use. Femurs were rehydrated in PBS for 12 hours prior to being scanned into a SkyScan1172 microCT scanner at 60kV (Bruker). NRecon, Dataviewer, and CTAn software were used for image reconstruction and data analysis. Cortical analysis began at 45 percent of the length of the bone, calculated up from the growth plate.

2.3. Mechanical Testing

Femurs from 36-week old mice were defleshed, wrapped in phosphate-buffered saline (PBS) pH 7.2-soaked gauze, and stored at -20°C, a rate of freezing that is not predicted to cause any cracking [27]. Femurs were thawed and allowed to equilibrate to room temperature for 2 h in PBS pH 7.2. Following equilibration, a standard four-point bend testing procedure was performed [28] using a TestResources 570L axial-torsional screw-driven testing system (TestResources, Shakopee, MN) with a displacement rate of 0.005 mm·s⁻¹. The distances between the lower and upper supports were 7.3 and 3.5 mm, respectively. The supports had radii of curvature of 0.5 mm at each point of contact with the femur. Displacement was applied by the upper supports in the anterior-posterior direction such that the anterior of the femur was in compression and the posterior was in tension. Force and displacement were directly measured from the load cell and crosshead, respectively.

2.4. Histomorphometry

Longitudinal undemineralized sections (5 μ m thick) were cut from Methyl Methacrylate (MMA) plastic embedded blocks of frontal sections of Femurs from mice aged 12 weeks, using a Leica 2265 microtome. These were stained with Goldner's Trichrome stain for the static measurements, and additional sections were cut at 10 μ m, and left unstained for dynamic (fluorescent) measurements. A region of interest was selected that was exactly 250 microns distal to the growth plate, and extending 1000 microns downward (thereby avoiding the primary spongiosa) through the metaphysis of the tibia. The cortical edges were avoided by placing the circle to be measured exactly 250 microns away from the endocortical edge. Standard bone histomorphometry was performed by the methods of Parfitt et al [29] using Bioquant Image Analysis software (R & M Biometrics, Nashville, TN) [30]. A two dimensional histologic section displays profiles of three-dimensional structures. Four types of primary measurements were made - area, length (or perimeter), distance between points or lines, and number. These referents, such as tissue volume, bone volume, bone surface, and osteoid surface are used to derive other indices, such as trabecular number and trabecular separation.

2.5. Tartrate Resistant Acid Phosphatase (TRAP) and Adipocyte Staining In Situ

Femurs and tibias were collected from 24-week old mice post-mortem, fixed in 10% neutral buffered formalin solution for 72 hours, and decalcified in 10% EDTA for 8 days prior to being embedded in paraffin. Osteoclasts were identified by TRAP staining. Briefly, slides were deparaffinized using a decreasing alcohol gradient and incubated in substrate solution (45mM Naphthol AS-BI Phosphate (Sigma) solubilized in 2-Ethoxyethanol (Sigma)), then diluted to 450nM into basic incubation medium (0.1M Sodium acetate trihydrate (Fisher Scientific), 75mM L-(+) tartaric acid (Fisher Scientific), and 0.3% glacial acetic acid, then warmed to 42°C) for 30 minutes at 42°C. Immediately following substrate solution incubation, slides were immersed in the color reaction solution (0.5M NaNO₂ (Fisher) in dH₂O and 150mM Pararosaniline HCl (Fisher Scientific) in 2N HCl, then diluted 1:50 in basic incubation medium) for 6 minutes. Slides

were rinsed, counterstained with Harris' Hematoxylin (Sigma) for 2 minutes, rinsed, placed in 0.1% ammonia water for 10 seconds, and rinsed again. Slides were dehydrated using an increasing alcohol gradient and coverslipped using Permount (Fisher Scientific).

Adipocytes were identified as "ghosts" resulting from the deparaffinization process during Pentachrome staining. Adipocytes were counted using Nikon NIS Elements Software and graphed as the number of adipocytes per unit bone length.

2.6. Calcein Labeling

Ten week-old mice were injected intraperitoneally with 20 mg/kg of calcein in a 2% sodium bicarbonate solution and injections repeated 10 days later. Mice were sacrificed at 12 weeks and femurs were harvested and fixed in 10% neutral buffered formalin for 3 days. Bones were placed in 70% ethanol until embedment into methyl methacrylate. Calcein labeling was visualized using fluorescent microscopy and mineral apposition rate was determined by histomorphometric analysis (using BioQuant Image Analysis). Sample information was blinded from viewer during analysis.

2.7. Osteoclast Differentiation In Vitro

Femurs and tibias were harvested from mice aged 4-6 weeks. One end from each bone was severed with scissors and placed in a 0.5mL microcentrifuge tube that was punctured in the bottom with a sterile needle. The 0.5mL tube was placed into a 1.5mL centrifuge tube and marrow was spun out of the bone by ramping up the microcentrifuge for 10 seconds. The 0.5mL tubes were removed from the 1.5mL tubes, and 0.5mL ACK buffer was used to reconstitute the red marrow that remained in the 1.5mL tube. Cells were incubated in ACK buffer (150mM NH₄Cl, 10mM KHCO₃, 0.1mM Na₂EDTA, pH 7.2-7.4) for 10 minutes at room temperature to allow red blood cells to lyse. Lysed red blood cells were separated from remaining cells by centrifugation at 3000RPM at room temperature for 5 minutes. White pellet

was resuspended in 10 mL α MEM1 (α MEM (ThermoFisher) supplemented with 10% heat-inactivated FBS, 1% Pen/Strep, and 1% L-glutamine) and allowed to grow overnight at 37°C. After 24 hours, cells in suspension were collected, counted, and plated at 400,000 cells/mL in 0.5mL α MEM2 (α MEM1 supplemented with 20ng/mL mCSF (R&D Systems)) in a 24-well plate for 48 hours to expand the osteoclast precursor population. After 48 hours, non-adherent cells were collected and plated in α MEM3 (α MEM2 supplemented with 20ng/mL RANKL (R&D Systems) and mCSF (R&D Systems)) and differentiated for 4 days. Differentiated osteoclasts were identified using a tartrate-resistant acid phosphatase staining kit (Sigma). Staining procedure was performed as instructed in the kit instructions with the exception of using Fast Garnet. We used Fast Red Violet Base Solution (Sigma) in place of Fast Garnet as it gave us more consistent results. For polarization analysis, only cells with ≥ 10 nuclei were considered. Scoring was based on the level of polarization from 1 to 5 and a blinded lab mate scored 20 images per genotype. Scores 1-2 were considered to be “normal” and scores of 3-5 were considered abnormal, which we termed “unpolarized”. Data is expressed as number of unpolarized cells with ≥ 10 nuclei per cm^2 .

2.8. Adipocyte Differentiation

Bone marrow was collected from femurs and tibias of 6-8 week old littermates. Bone marrow was resuspended in ACK lysis buffer (150mM NH_4Cl , 10mM KHCO_3 , 0.1mM Na_2EDTA , pH 7.2-7.4) for 5 min at room temperature to lyse red blood cells. After centrifugation for 5 min at 3000rpm, supernatant containing lysis buffer and red blood cells was removed. 1×10^8 cells were plated per 10cm dish in 10mL α MEM (ThermoFisher) supplemented with 10% FBS and 1% Pen/Strep. Medium was replaced every other day until cells gained confluency. Upon confluency, cells were trypsinized with 0.25% EDTA, counted, and seeded at 2×10^5 cells per well in a 12-well plate. After 24 hours, medium was replaced with adipogenic induction medium (α MEM supplemented with 10% FBS, 1% Pen/Strep, 1 μ M dexamethasone (Sigma), 5 μ g/mL

troglitazone (Sigma), 50uM 3-isobutyl-1-methylxanthine (Sigma), and 1ug/mL insulin (Sigma)). After 2 days in adipogenic induction medium, cells were treated with adipogenic maintenance medium (α MEM supplemented with 10% FBS, 1% Pen/Strep, and 1ug/mL insulin) for an additional 3 days. To assess adipogenic differentiation, cells were fixed with 10% formalin for 1 hour, washed with 60% isopropanol to dehydrate, and stained with 0.2% Oil-red O (Sigma) for 10 minutes at room temperature. Adipocytes were visualized using Nikon TE300 microscope and quantified using the NIS Elements Software counting tool. Sample information was blinded from viewer during quantification.

2.9. Osteoblast Differentiation

Mouse bone marrow cells were extracted from the long bones of 5 to 6 week old mice, plated in duplicate in α -MEM medium supplemented with 10%FBS, left to adhere for 3 d, and then differentiated to osteoblasts using 50 μ g/ml Vitamin C for another 7 d or 14 d. The medium was changed every 3 d until the end of the experiment. Alkaline phosphatase (ALP) staining were performed by fixing one set of cells in 10% neutral buffered formalin and then staining cells with NBT/BCIP (Thermo Scientific) at 37^oC. Osteoblast proliferation was monitored by 0.5% crystal violet (CV) staining of the second set of cells. After 14 days of differentiation, on set of cells was stained with 0.5% CV and one set stained with Alizarin Red (40mM, pH adjusted to 4.1 with 10% (v/v) ammonium hydroxide) (Sigma). Quantification was performed through ImageJ software where integrated density was calculated for Alizarin Red to calculate the color intensity of background staining versus the bright red mineralization deposits. The percent of each well covered in stain was calculated for ALP, CV, and Alizarin Red (mineralized area) as well.

2.10. qRT-PCR

RNA was collected at day 7 of osteogenic media treatment, at day 4 of osteoclast differentiation, and at day 5 of adipocyte differentiation using Qiagen RNeasy Mini Kit. cDNA was generated

using MuLV Reverse Transcriptase PCR Kit (NEB) with 0.5ug RNA. Subsequent cDNA was diluted 1:10 and subject to qPCR using FastStart Universal SYBR Green Master (Sigma) using the forward and reverse primers described in Table 1.

2.11. Statistical Analysis

Statistical analysis was performed using multiple unpaired t-tests with a 95% confidence interval. Statistical analysis and graphical representations of data were generated using GraphPad Prism.

3. RESULTS

3.1. Global deletion of CD82 in mice does not alter bone density, but KO bones are smaller in diameter and weaker.

To determine if CD82 loss significantly affects bone morphometry, femurs were harvested from 24- and 36- week-old mice and analyzed by micro-computed tomography (microCT). These end points were chosen to represent the mature skeleton before bone growth ceases and the aging skeleton to determine if there were any age-dependent phenotypes [31]. Because CD82 is upregulated during osteoclast differentiation [20], we initially anticipated that loss of CD82 would result in thicker, denser, and weaker bones due to osteoclast dysfunction. However, repeated analyses demonstrate that bone mineral density remained consistent between wild-type and CD82-null littermates (Fig. 1a). Similarly, cortical crosssectional thickness [Cs.Th] remained unchanged between wild-type and CD82-null mice (Fig. 1b). There was a decrease in total cortical cross sectional tissue area [T.Ar], which was limited to male mice at 24 weeks (Fig. 1c). However, there was an unexpected decrease in cortical perimeter [T.Pm] in both male and female mice, indicating narrower bones (Fig. 1d,e). Within the trabecular area, there was no change in the percent bone volume [BV/TV], despite a decrease in total trabecular volume [TV] (Fig. 1f,g), and an increase in bone surface/volume ratio [BS/BV] (Fig. 1h) that was

limited to the male mice. There was no change in bone length between wild-type and CD82-null mice (Fig. 1i). With respect to aging, there was the expected drop in BMD and BV between 24 and 36 weeks, but this was unaffected by CD82 loss. Overall, CD82 loss had no significant effect on any changes related to age.

To better assess the trabecular phenotype, bones from 12-week old mice were analyzed by static histomorphometry (Table 2). The most notable changes in these mice were in the CD82-null males consistent with microCT data. The same increase in BS/BV was also noted here, due mostly to the decrease in BV that did not quite reach statistical significance, but is consistent with narrower bones as assessed by microCT. However, here we observed an increase in total trabecular area (TV) seemingly in contrast to the microCT data. This difference is most likely due to limits of a 2D image, whereas the 3D analysis would account for the full bone shape. However, most striking was the large decrease in osteoclast parameters in male CD82-null mice, i.e. eroded surface (ES and ES/BS), osteoclast perimeter (OcS and OcS/BS), and number of osteoclasts (N.Oc/BS) (Table 2). Despite the bone area being smaller, there is increased trabecular surface area relative to bone volume most likely due to defects in osteoclasts. No statistical significant changes in these osteoclast parameters were seen in the female mice.

Despite bone density being largely unchanged between wild-type and CD82-null mice, femurs from male and female CD82-null mice have a significantly lower mean polar moment of inertia [MMI] and male knockout bones have decreased stiffness [Young's Modulus] (Fig 2). We did not notice any significant change in stiffness in bones from female knockout mice. This latter difference may be due to the altered osteoclasts in the male mice.

3.2. Osteoclasts are defective in CD82-null mice.

To better assess potential osteoclast defects, we looked more closely at osteoclasts *in situ* and carried out an *in vitro* differentiation analysis. CD82-null osteoclasts in 24-week old

male and female mice stain less intensely for TRAP *in situ* (Fig. 3a,b). *In vitro* differentiated CD82-null multi-nucleated (≥ 10 nuclei/cell) osteoclasts failed to properly polarize their nuclei and TRAP stain to the outer perimeter of the cell (Fig. 3c). Three times more cells from the CD82-null mice failed to properly polarize than cells from the wild-type mice (Fig. 3d). The overall size between CD82-null and wild-type multi-nucleated osteoclasts was not significantly different (not shown). Measurement of osteoclast differentiation markers indicated that wild-type and CD82-null differentiated osteoclasts express similar levels of differentiation markers (Fig. 3e). CD82 mRNA loss was also validated in the differentiated osteoclasts (Fig. 3e). Thus, the effect of CD82 loss on osteoclasts *in vitro* appears to be confined to functional aspects of relatively mature osteoclasts.

3.3. Reduced osteoblast function in CD82 null mice.

Bone formation rate (BFR) and bone mineralization are commonly reported parameters associated with new bone deposition [30]. Calcein labeling in CD82-deficient male mice (Fig. 4a) reveals a lower bone formation rate than wild-type littermates (BFR.WT.m = 0.373 ± 0.018 $\mu\text{m}/\text{day}$ vs. BFR.KO.m = 0.27 ± 0.030 $\mu\text{m}/\text{day}$), indicating that CD82-null bones have decreased bone deposition (Fig. 4b). There was no significant difference in the BFR in bones from female mice (BFR.WT.f = 0.444 ± 0.024 $\mu\text{m}/\text{day}$ vs BFR.KO.f = 0.505 ± 0.009 $\mu\text{m}/\text{day}$). Mineralized surface ratio to bone surface area was also significantly reduced in male mice (Fig. 4c).

Decreased bone deposition is most commonly the result of defective osteoblasts. Static histomorphometry staining (Fig. 4d) and quantification (Table 2) indicated no significant difference in osteoblast size or number. To further determine if loss of CD82 decreases osteoblastogenesis *in vitro*, we isolated osteoblast precursors from bone marrow of 5-week old mice and differentiated them into osteoblasts for 7 or 14 days (Fig. 4e). Consistent with the dynamic histomorphometry results, CD82-null-derived osteoblasts produce 5-times less alkaline phosphatase (ALP) (Fig. 4f). Proliferation, as monitored by crystal violet staining, is slightly

attenuated, 2-fold, though this did not reach significance (Fig. 4g). Alizarin Red staining intensity and mineralized area is dramatically reduced, about 5-fold, in CD82-null osteoblasts differentiated for 14 days (Fig. 4h,i) further validating the dynamic histomorphologic data that bone formation is decreased (Fig. 4a). Osteoblast differentiation is negatively influenced by CD82 loss as transcript levels of several differentiation markers, namely Osteocalcin, Collagen1 α 1, Runx2, Bone sialoprotein, and Osterix, are significantly decreased 2- to 3-fold in CD82-null populations (Fig. 4j). We further validated that CD82 mRNA is lost in the CD82-null cultured osteoblasts (Fig. 4k). While loss of CD82 does not significantly reduce osteoblast numbers, it severely affects their bone forming capacity.

3.4. Bone marrow adipogenic potential is enhanced upon CD82 loss.

Osteoblasts share a common progenitor with adipocytes, both cell types stemming from the mesenchymal lineage [32, 33]. Finding that CD82 loss attenuated osteoblast mineralization, we wanted to determine if adipocytes were affected by CD82 loss. Trabecular regions of CD82-deficient bones displayed an accumulation of more adipocyte ghosts *in situ*, the result of deparaffinization of tissue slides (Fig. 5a). Quantification revealed as much as a 6-fold increase in adipocyte ghosts in 24-week old CD82-null mice (Fig. 5b). While the adipocyte phenotype was most severe at 24- to 36- weeks of age, differences were easily detectable as early as 12 weeks. The increase in adipocytes was limited to the bone and not due to a global increase in fat cell production, as both liver and spleen displayed a reduced number of adipocytes (Fig. 5c,d) and body mass was not increased (not shown).

The drastic accrual specifically of bone marrow adipocytes spurred us to assess the effect of CD82 loss on adipogenic potential of bone marrow stromal cells. Primary bone marrow stromal cells from CD82-deficient mice that were subjected to adipogenic induction medium *in vitro* displayed enhanced differentiation into adipocytes, as determined by Oil Red O staining

(Fig. 5e) and increase expression of adipocyte differentiation markers PPAR γ and C/EBP α (Fig. 5f). Furthermore, adipocyte size was augmented nearly 2-fold in the absence of CD82 (Fig. 5g).

4. DISCUSSION

Using mice in which CD82 expression is globally deleted, we assessed bone morphology and function. *In vivo*, global CD82 loss did not affect bone length, density, cortical bone volume, or cross sectional thickness. However, in older mice it did result in reduced cortical perimeter leading to narrower bones. There was also a marked decrease in osteoclast function, size, and number in males leading to expansion of the trabecular surface area. Consequently, CD82-null femurs were less resistant to mechanical force by four-point bending and bones from male mice were less stiff than wild-type littermates. This latter phenotype, restricted to males, is consistent with the loss of osteoclast function seen only in males. Reduced osteoclast size and/or function may alter the resorption cavities. Prior studies indicate that increased trench-like cavities, rather than fully absorbed pits can lead to loss of stiffness [34].

Since microarray expression data indicated that CD82 is up-regulated during osteoclast differentiation [20], we expected to see an increase in bone density if loss of CD82 negatively affects osteoclast function. Instead, we found a decrease in cortical perimeter, but an increase in relative trabecular surface area. These data support a hypothesis that CD82 loss affects both osteoclasts and osteoblasts. The net effect at the cortical region would be balanced osteoclast and osteoblast function at the endosteal surface, but decreased osteoblast function at the periosteal surface leading to both a narrower inner and outer bone perimeter, with no net change in cortical cross sectional thickness. Consistent with this, not only did we detect intrinsic defects in osteoclast morphology *in vitro* and *in situ*, but also defects in osteoblast differentiation and mineralization *in vitro*, but minimal effect on osteoblast proliferation. The static histomorphometric data did not reveal major changes in number or size of osteoblasts, but

dynamic histomorphometry indicates significant reduction in bone growth and mineralization *in vivo*. The combined *in vivo* and *in vitro* data suggest that osteoblast numbers may be normal, but they are not functioning normally. Similarly, the osteoclasts produce polykaryons and express differentiation markers, but their morphology is abnormal making them appear smaller and covering less bone surface.

We found an unexpected sexual dimorphism in that the *in vivo/in situ* osteoclast phenotype was largely limited to the males. However, *in vitro* the abnormal polarization was observed in cells derived from both male and females, suggesting that hormones likely play an important role in this phenotype. Similarly, the *in vivo* defect in bone formation rate was limited to males, while the *in vitro* effect on osteoblast differentiation was independent of sex.

We initially thought that the increase in adipocytes in the bone marrow of CD82-null mice might reflect a lineage switch away from osteoblasts towards adipocytes [32, 33, 35]. However, the static histomorphometric data and the *in vitro* differentiation data indicate that this might not be the case. Rather that there might be an upstream precursor whose numbers are significantly increased by loss of CD82. It is interesting to note that osteoblast differentiation is dependent on proper adhesion via integrins and mechanical force [36-39]. Given the previous observations that CD82 loss alters integrin expression and actin dynamics in other cell types [21, 23], it is plausible that CD82 is controlling adhesion mechanics to maintain their commitment to the osteoblast lineage. In addition, we cannot rule out the possibility that some of the *in vivo* defects in osteoclast function are due to feed-back from defects in the osteoblasts and their inability to properly elicit osteoclasts. Furthermore, given the known defects in hematopoietic stem cell homing in CD82 null mice [26], there may be other unknown factors contributing to the observed phenotypes. Conditional deletion of CD82 in each of these lineages will be required to unequivocally resolve all these possibilities.

This is the first study to demonstrate that loss of CD82 expression has a negative impact on both mesenchymal and myeloid lineages in the bone. The myeloid lineage defect at this

point seems to be confined to the relatively mature osteoclast and is similar to that observed for the iRNA-mediated loss of Tspan 7 in osteoclast precursors [18]. This is in contrast to other tetraspanins that regulate the polykaryon fusion steps [17, 19]. Further analysis of the osteoclast phenotype in a myeloid-conditional deletion is currently underway. The mesenchymal phenotype is characterized by a reduction in bone formation rate and more adipocytes in the bone marrow *in vivo*, as well as reduced osteoblast differentiation and mineralization and increased adipogenesis *in vitro*. The loss of CD82 expression in osteoblasts may explain the puzzling reduction in the number of long-lived hematopoietic stem cells (HSCs) recently reported in CD82 KO mice [26]. Despite the loss in long-lived HSCs, the immune cell make up of these mice is essentially unaltered, indicating there is not a general disruption in immune cell ontology [23, 26]. This same report suggested there is a defect in adhesion of CD82 null HSCs to macrophages at the endosteal/marrow interface [26]. However, it is well-established that HSCs can bind the niche via osteoblasts [24, 40, 41]. Thus, a more likely alternative possibility is that the reduction in functional osteoblasts due to CD82 loss reduces the sites for and efficiency of HSC binding to osteoblast-associated niches.

5. CONCLUSIONS

This is the first study to report on the bone phenotypes of mice with homozygous global deletion of a tetraspanin protein. Loss of CD82 leads to weaker and smaller bones with no change in bone density. This is accompanied by a decrease in size and number of osteoclasts, a dramatic increase in bone marrow adipocytes, and reduced bone formation rate *in vivo*. The *in vitro* phenotypes are defective osteoclast polarization, increased adipogenesis, and reduced osteoblast differentiation and mineralization. These results are consistent with a defects in osteoclast, osteoblast, and adipocyte lineages.

Table 1. Primer sequences

Target	Forward Primer (5'→3')	Reverse Primer (5'→3')
TRAP	CACTCAGCTGTCCTGGCTCAA	CTGCAGGTTGTGGTCATGTCC
DCSTAMP	GCGGAACTTAGACACAGGG	CAAAGCAACAGACTCCCAAATG
CALCR	GACAACTGCTGGCTGAGTG	GAAGCAGTAGATAGTCGCCA
PPARγ	GGAAAGACAACGGACAAATCAC	TACGGATCGAAACTGGCAC
C/EBPα	TGGACAAGAACAGCAACGAG	TCACTGGTCAACTCCAGCAC
CD82	TACAAACCTCATCCAGCTCG	TCTTCACAATGAGCTGGTTG
ALP	ATCTTTGGTCTGGCTCCCATG	TTTCCCGTTCACCGTCCAC
Osteocalcin	GCAATAAGGTAGTGAACAGACTCC	GTTTGTAGGCGGTCTTCAAGC
Col1a1	GCAACAGTCGCTTCACCTACA	CAATGTCCAAGGGAGCCACAT
Runx2	CGGCCCTCCCTGAACTCT	TGCCTGCCTGGGATCTGTA
Bone Sialoprotein	AAGCAGCACCGTTGAGTATGG	CCTTGTAGTAGCTGTATTCGTCCT C
Osterix	AGCGACCACTTGAGCAAACAT	GCGGCTGATTGGCTTCTTCT

Table 2. Histomorphometry analysis of WT and CD82 KO bones

Measurement (n)	Female WT n=3	Female KO n=3	Male WT n=3	Male KO n=3
Bone Area (BV) mm ²	0.12±0.01	0.14±0.03	0.13±0.02	0.10±0.01
Tissue Area (TV) mm ²	1.54±0.02	1.43±0.11	1.75±0.036	1.87±0.02*
BV/TV	7.79±0.68	8.13±1.56	7.74±1.46	5.34±0.66
Bone Perimeter (BS) mm	8.03±0.24	7.65±1.34	9.19±1.15	8.80±0.88
BS/BV (mm ⁻¹)	69.64±4.08	70.13±3.32	72.38±4.17	89.02±2.64*
BS/TV (mm ⁻¹)	5.23±0.20	5.45±0.83	5.30±0.75	4.69±0.44
Trabecular Thickness (μm)	29.43±1.60	28.84±1.32	28.10±1.58	22.56±0.66*
Trabecular Number (mm ⁻¹)	2.61±0.10	2.72±0.42	2.65±0.37	2.34±0.22
Trabecular Space (μm)	358.23±16.29	386.58±66.33	394.22±65.64	416.88±41.41
Erosion Perim (ES) mm	0.12±0.01	0.10±0.04	0.18±0.02	0.09±0.03*
Quiescent Perim (QS) mm	7.91±0.24	7.55±1.31	9.01±1.14	8.71±0.85
Osteoblast Perim (ObS) mm	3.27±0.24	2.80±0.58	3.41±0.31	3.05±0.13
Osteoclast Perim (OcS)	0.12±0.01	0.10±0.04	0.18±0.02	0.09±0.03*
ES/BS	1.45±0.17	1.14±0.30	2.04±0.30	0.91±0.24*
QS/BS	98.56±0.17	98.87±0.15	97.97±0.29	99.09±0.24*
ObS/BS	40.09±1.92	35.72±1.85	38.09±2.25	35.39±3.69
OcS/BS	1.45±0.17	1.14±0.30	2.04±0.29	0.91±0.24*
N.Ob/BS	58.74±3.92	53.15±1.99	54.48±4.09	50.47±5.86
N.Oc/BS	0.67±0.15	0.67±0.10	1.03±0.14	0.59±0.11*

(*p<0.5 **p<0.01 ***p<0.001; significance determined by unpaired t-tests; mean ±SE is shown.)

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CONFLICT OF INTEREST

The authors have no conflicts of interest to report.

FIGURE LEGENDS

Figure 1. Global deletion of CD82 results in narrower bones. Trabecular and cortical parameters of formalin-fixed femurs from wild type (WT) and CD82 null (KO) littermates as analyzed by microCT. **(a)** trabecular bone mineral density [BMD], **(b)** cortical cross sectional thickness [Cs.Th], **(c)** total cortical cross sectional tissue area [T.Ar], **(d)** cortical perimeter [T.Pm], **(e)** cross sectional and cortical perimeter representative image of WT and CD82 KO bones, **(f)** percent bone volume [BV/TV], **(g)** total trabecular volume [TV], **(h)** trabecular bone surface volume ratio [BS/BV]. WT $n=12$, KO $n=14$ (24 weeks); WT $n=8$, KO $n=9$ (36 weeks). $*p<0.05$; error bars are SEM. **(i)** Bone length at age 24 weeks; WT $n=20$, KO $n=12$. Error bars are SEM. Statistical significance determined using multiple unpaired two-tailed t-tests with the Holk-Sidak method.

Figure 2. Global deletion of CD82 results in weaker bones. **(a)** Mean polar moment of inertia [MMI] and **(b)** Young's Modulus were calculated from mechanical testing using a four-point bending technique on fresh-frozen 36-week old femurs from male and female littermates. WT $n=8$, KO $n=9$. $*p<0.05$, $**p<0.01$; error bars are SEM. Statistical significance determined using multiple unpaired two-tailed t-tests with the Holk-Sidak method.

Figure 3. Osteoclast staining and polarization is altered upon CD82 loss. **(a)** Tartrate-resistant acid phosphatase (TRAP) staining on formalin-fixed, decalcified, paraffin-embedded femurs from wild type (WT) and CD82 null (KO) mice. **(b)** TRAP intensity staining quantified using ImageJ software. WT $n=12$, KO $n=14$. $**p<0.01$; error bars are SEM. Statistical significance determined using an unpaired two-tailed t-test. **(c)** TRAP staining on osteoclasts differentiated from female mice *in vitro* for 4 days with mCSF and RANKL. **(d)** TRAP-positive cells with ≥ 10 nuclei/cell were blindly scored based on level of nuclei within the center of the cell

(unpolarized) versus at the periphery of each cell. $n=13-15$ images per genotype, or approximately 70 cells per genotype. $*p<0.05$; error bars are SEM. Statistical significance determined using an unpaired two-tailed t-test. **(e)** Log₂ fold change in mRNA levels of osteoclast differentiation markers TRAP, DCSTAMP, CALCR, and CD82 from cells differentiated 4 days with mCSF and RANKL was assessed by qRT-PCR. $***p<0.001$; error bars are SEM. Statistical significance determined using multiple unpaired two-tailed t-tests with the Holk-Sidak method.

Figure 4. Osteogenesis is reduced upon CD82 loss. **(a)** Representative fluorescent images of calcein double-labeled femoral cross sections from male wild-type (WT) and CD82-null (KO) mice sacrificed at 12 weeks. Second injection was delivered 10 days after initial injection. **(b,c)** Dynamic histomorphometry analysis was performed as described in methods to determine bone formation rate [BFR] and mineralized surface/bone surface [MS/BS]. WT $n=3$, KO $n=3$; $*p<0.05$, ns = not significant; error bars are SEM. Statistical significance determined using multiple unpaired two-tailed t-tests with the Holk-Sidak method. **(d)** Representative images of osteoblasts lining trabecular bone (white arrows) from tissue stained with Goldner's Trichrome from static histomorphometry. **(e)** Osteoblasts from male mice were differentiated for 7 days (top;ALP) and 14 days (bottom;AR). Cells at day 7 were stained for Alkaline phosphatase (ALP) and total cell number using crystal violet (CV). Cells at day 14 were stained with Alizarin Red (AR) and crystal violet. **(f,g,h,i)** Quantifications of ALP, CV, and Alizarin Red (AR). Statistical significance determined using unpaired two-tailed t-tests. $n=3$, $*p<0.05$; ns = not significant; error bars are SEM. **(j)** Log₂ fold change in mRNA from osteoblast populations differentiated for 7 days was assessed by qRT-PCR. Collagen1 α 1 (Col1a1), Runx2, Osterix (OSX), Osteocalcin (Ocn), bone sialoprotein (BSP). WT $n=3$, KO $n=3$. $*p<0.05$, $***p<0.001$; error bars are SEM. Statistical significance determined using multiple unpaired two-tailed t-tests with the Holk-Sidak method. **(k)** CD82 mRNA from osteoblast populations differentiated for 7 days was assessed

by qRT-PCR. WT $n=3$, KO $n=3$; **** $p<0.0001$; error bars are SEM. Statistical significance determined using unpaired two-tailed t-test.

Figure 5. Bone marrow adipogenesis increases upon CD82 loss. (a) Femurs from wild type (WT) and CD82-null (KO) 24-week old mice were formalin-fixed, decalcified, paraffin-embedded, pentachrome-stained, and visualized by light microscopy at 4x magnification. 20x images are randomly identified close-ups of adipocyte ghosts from tissues stained with Goldner's Trichrome. (b) Number of adipocyte ghosts were quantified using ImageJ software as adipocytes per unit bone length/area. WT $n=12$, KO $n=14$; p values indicated; error bars are SEM. Statistical significance determined using unpaired two-tailed t-test. (c) Livers (LIV) and spleens (SPL) were isolated from wild type (WT) and CD82-null (KO) mice, embedded in paraffin, and then stained by H&E. (d) Number of adipocytes in tissues quantified. WT $n=4$, KO $n=3$; * $p<0.05$, ** $p<0.01$, *** $p<0.001$; error bars are SEM. Statistical significance determined using multiple unpaired two-tailed t-tests with the Holk-Sidak method. (e) Primary murine bone marrow cells were isolated from wild-type (WT) and CD82-null (KO) female and male 8-week old mice and differentiated *in vitro*. Adipocytes were visualized by Oil Red O-staining. WT $n=2$, KO $n=2$; p values indicated; error bars are SEM. Statistical significance determined using multiple unpaired two-tailed t-tests with the Holk-Sidak method. (f) Relative mRNA expression of adipocyte differentiation markers PPAR γ and C-EBP α in WT and KO adipocyte populations determined by aRT-PCR. p values indicated; error bars are SEM. (g) Size of the adipocytes were quantified using Nikon NIS Elements software's automatic shape area tool. Statistical significance determined using an unpaired two-tailed t-test.

REFERENCES

- [1] S.C. Sridhar, C.K. Miranti, Tetraspanin KAI1/CD82 suppresses invasion by inhibiting integrin-dependent crosstalk with c-Met receptor and Src kinases, *Oncogene* 25(16) (2006) 2367-2378.
- [2] C. Miranti, A. Bergsma, A. Van Sriel, Tetraspanins as master organizers of the plasma membrane, in: A. Cambi, D. Lidke (Eds.), *Cell membrane nanodomains: from biochemistry to nanoscopy*, CRC Press, Boca Raton, 2014, pp. 59-86.
- [3] C.S. Stipp, T.V. Kolesnikova, M.E. Hemler, Functional domains in tetraspanin proteins, *Trends Biochem Sci* 28(2) (2003) 106-12.
- [4] M.E. Hemler, Tetraspanin proteins mediate cellular penetration, invasion, and fusion events and define a novel type of membrane microdomain, *Annu Rev Cell Dev Biol* 19 (2003) 397-422.
- [5] B. Zimmerman, B. Kelly, B.J. McMillan, T.C. Seegar, R.O. Dror, A.C. Kruse, S.C. Blacklow, Crystal structure of a full-length human tetraspanin reveals a cholesterol-binding pocket, *Cell* 167(4) (2016) 1041-1051.e11.
- [6] E.L. Jones, M.C. Demaria, M.D. Wright, Tetraspanins in cellular immunity, *Biochem Soc Trans* 39(2) (2011) 506-11.
- [7] A.B. van Sriel, Tetraspanins in the humoral immune response, *Biochem Soc Trans* 39(2) (2011) 512-7.
- [8] C.K. Miranti, Controlling cell surface dynamics and signaling: How CD82/KAI1 suppresses metastasis, *Cell Signal* 21(2) (2009) 196-211.
- [9] M.E. Hemler, Tetraspanin proteins promote multiple cancer stages, *Nat Rev Cancer* 14(1) (2014) 49-60.
- [10] C. Niehage, C. Steenblock, T. Pursche, M. Bornhauser, D. Corbeil, B. Hoflack, The cell surface proteome of human mesenchymal stromal cells, *PLoS One* 6(5) (2011) e20399.
- [11] I. Kalajzic, A. Staal, W.P. Yang, Y. Wu, S.E. Johnson, J.H. Feyen, W. Krueger, P. Maye, F. Yu, Y. Zhao, L. Kuo, R.R. Gupta, L.E. Achenie, H.W. Wang, D.G. Shin, D.W. Rowe, Expression

549 profile of osteoblast lineage at defined stages of differentiation, *J Biol Chem* 280(26) (2005)
 550 24618-26.

551 [12] N. Hanagata, X. Li, Osteoblast-enriched membrane protein IFITM5 regulates the
 552 association of CD9 with an FKBP11-CD81-FPRP complex and stimulates expression of
 553 interferon-induced genes, *Biochem Biophys Res Commun* 409(3) (2011) 378-84.

554 [13] S. Hayashi, K. Miyake, P.W. Kincade, The CD9 molecule on stromal cells, *Leuk Lymphoma*
 555 38(3-4) (2000) 265-70.

556 [14] M. Ishii, K. Iwai, M. Koike, S. Ohshima, E. Kudo-Tanaka, T. Ishii, T. Mima, Y. Katada, K.
 557 Miyatake, Y. Uchiyama, Y. Saeki, RANKL-induced expression of tetraspanin CD9 in lipid raft
 558 membrane microdomain is essential for cell fusion during osteoclastogenesis, *J Bone Miner Res*
 559 21(6) (2006) 965-76.

560 [15] C.G. James, C.T. Appleton, V. Ulici, T.M. Underhill, F. Beier, Microarray analyses of gene
 561 expression during chondrocyte differentiation identifies novel regulators of hypertrophy, *Mol Biol*
 562 *Cell* 16(11) (2005) 5316-33.

563 [16] K. Iwai, M. Ishii, S. Ohshima, K. Miyatake, Y. Saeki, Expression and function of
 564 transmembrane-4 superfamily (tetraspanin) proteins in osteoclasts: reciprocal roles of Tspan-5
 565 and NET-6 during osteoclastogenesis, *Allergol Int* 56(4) (2007) 457-63.

566 [17] J. Zhou, T. Fujiwara, S. Ye, X. Li, H. Zhao, Downregulation of Notch modulators,
 567 tetraspanin 5 and 10, inhibits osteoclastogenesis in vitro, *Calcif Tissue Int* 95(3) (2014) 209-217.

568 [18] J.O. Kwon, Y.D. Lee, H. Kim, M.K. Kim, M.K. Song, Z.H. Lee, H.H. Kim, Tetraspanin 7
 569 regulates sealing zone formation and the bone-resorbing activity of osteoclasts, *Biochem*
 570 *Biophys Res Commun* 477(4) (2016) 1078-1084.

571 [19] Y. Takeda, I. Tachibana, K. Miyado, M. Kobayashi, T. Miyazaki, T. Funakoshi, H. Kimura,
 572 H. Yamane, Y. Saito, H. Goto, T. Yoneda, M. Yoshida, T. Kumagai, T. Osaki, S. Hayashi, I.
 573 Kawase, E. Mekada, Tetraspanins CD9 and CD81 function to prevent the fusion of
 574 mononuclear phagocytes, *J Cell Biol* 161(5) (2003) 945-56.

575 [20] T.N. Crotti, R.P. O'Sullivan, Z. Shen, M.R. Flannery, R.J. Fajardo, F.P. Ross, S.R. Goldring,
576 K.P. McHugh, Bone matrix regulates osteoclast differentiation and annexin A8 gene expression,
577 J Cell Physiol 226(12) (2011) 3413-21.

578 [21] E.L. Jones, J.L. Wee, M.C. Demaria, J. Blakeley, P.K. Ho, J. Vega-Ramos, J.A.
579 Villadangos, A.B. van Spriel, M.J. Hickey, G.J. Hammerling, M.D. Wright, Dendritic cell
580 migration and antigen presentation are coordinated by the opposing functions of the
581 tetraspanins CD82 and CD37, J Immunol 196(3) (2016) 978-87.

582 [22] Q. Wei, F. Zhang, M.M. Richardson, N.H. Roy, W. Rodgers, Y. Liu, W. Zhao, C. Fu, Y.
583 Ding, C. Huang, Y. Chen, Y. Sun, L. Ding, Y. Hu, J.X. Ma, M.E. Boulton, S. Pasula, J.D. Wren,
584 S. Tanaka, X. Huang, M. Thali, G.J. Hammerling, X.A. Zhang, CD82 restrains pathological
585 angiogenesis by altering lipid raft clustering and CD44 trafficking in endothelial cells, Circulation
586 130(17) (2014) 1493-504.

587 [23] K. Uchtmann, E.R. Park, A. Bergsma, J. Segula, M.J. Edick, C.K. Miranti, Homozygous loss
588 of mouse tetraspanin CD82 enhances integrin $\alpha\text{IIb}\beta 3$ expression and clot retraction in platelets,
589 Exp Cell Res 339(2) (2015) 261-9.

590 [24] J.M. Gillette, A. Larochelle, C.E. Dunbar, J. Lippincott-Schwartz, Intercellular transfer to
591 signalling endosomes regulates an ex vivo bone marrow niche, Nat Cell Biol 11(3) (2009) 303-
592 11.

593 [25] A. Larochelle, J.M. Gillette, R. Desmond, B. Ichwan, A. Cantilena, A. Cerf, A.J. Barrett, A.S.
594 Wayne, J. Lippincott-Schwartz, C.E. Dunbar, Bone marrow homing and engraftment of human
595 hematopoietic stem and progenitor cells is mediated by a polarized membrane domain, Blood
596 119(8) (2012) 1848-55.

597 [26] J. Hur, J.I. Choi, H. Lee, P. Nham, T.W. Kim, C.W. Chae, J.Y. Yun, J.A. Kang, J. Kang, S.E.
598 Lee, C.H. Yoon, K. Boo, S. Ham, T.Y. Roh, J.K. Jun, H. Lee, S.H. Baek, H.S. Kim, CD82/KAI1
599 maintains the dormancy of long-term hematopoietic stem cells through interaction with DARC-
600 expressing macrophages, Cell Stem Cell 18(4) (2016) 508-21.

601 [27] S. Cowin, van Rietbergen, B., Myers, E., Feinberg, S., Currey, J., Guo, X., Winet, H.,
602 Burger, E., Jee, W., Lakes, R., Martin, R., Young, M., Boskey, A., Brown, T., Davy, D., Fritton,
603 S., Goodship, A., Hart, R., Fritton, J., Keaveny, T., Knothe-Tate, M., Majeska, R., Odgaard, A.,
604 Turner, C., Kaufman, J., Arramon, Y., Jepsen, K., Lucchinetti, E., Pollack, S., Rueegsegger, P.,
605 Hollister, S., Prendergast, P., Villarraga, M., Doty, S., Schaffler, M., Siffert, R., Nauman, E.,
606 Sharkey, N., Burr, D., Dieudonne, S., Akkus, O., Chu, T., Ford, C., Halloran, J., Moss, M., Otter,
607 M., Rubin, C., Weinbaum, S., Xu, T., van der Meulen, M., Cunningham, J., Bostrom, M.,
608 Huiskes, R., Bone Mechanics Handbook, Second Edition ed., CRC Press, Boca Raon, 2001.

609 [28] C.H. Turner, D.B. Burr, Basic biomechanical measurements of bone: a tutorial, Bone 14(4)
610 (1993) 595-608.

611 [29] A.M. Parfitt, L.S. Simon, A.R. Villanueva, S.M. Krane, Procollagen type I carboxy-terminal
612 extension peptide in serum as a marker of collagen biosynthesis in bone. Correlation with Iliac
613 bone formation rates and comparison with total alkaline phosphatase, J Bone Miner Res 2(5)
614 (1987) 427-36.

615 [30] D.W. Dempster, J.E. Compston, M.K. Drezner, F.H. Glorieux, J.A. Kanis, H. Malluche, P.J.
616 Meunier, S.M. Ott, R.R. Recker, A.M. Parfitt, Standardized nomenclature, symbols, and units for
617 bone histomorphometry: a 2012 update of the report of the ASBMR Histomorphometry
618 Nomenclature Committee, J Bone Miner Res 28(1) (2013) 2-17.

619 [31] M. Piemontese, M. Almeida, A.G. Robling, H.N. Kim, J. Xiong, J.D. Thostenson, R.S.
620 Weinstein, S.C. Manolagas, C.A. O'Brien, R.L. Jilka, Old age causes de novo intracortical bone
621 remodeling and porosity in mice, JCI Insight 2(17) (2017).

622 [32] A.I. Caplan, Mesenchymal stem cells, J Orthop Res 9(5) (1991) 641-50.

623 [33] E.M. Horwitz, K. Le Blanc, M. Dominici, I. Mueller, I. Slaper-Cortenbach, F.C. Marini, R.J.
624 Deans, D.S. Krause, A. Keating, T. International Society for Cellular, Clarification of the
625 nomenclature for MSC: The International Society for Cellular Therapy position statement,
626 Cytotherapy 7(5) (2005) 393-5.

- [34] J. Vanderoost, K. Soe, D.M. Merrild, J.M. Delaisse, G.H. van Lenthe, Glucocorticoid-induced changes in the geometry of osteoclast resorption cavities affect trabecular bone stiffness, *Calcif Tissue Int* 92(3) (2013) 240-50.
- [35] Q. Chen, P. Shou, C. Zheng, M. Jiang, G. Cao, Q. Yang, J. Cao, N. Xie, T. Velletri, X. Zhang, C. Xu, L. Zhang, H. Yang, J. Hou, Y. Wang, Y. Shi, Fate decision of mesenchymal stem cells: adipocytes or osteoblasts?, *Cell Death Differ* 23(7) (2016) 1128-39.
- [36] M. Dejaeger, A.M. Bohm, N. Dirckx, J. Devriese, E. Nefyodova, R. Cardoen, R. St-Arnaud, J. Tournoy, F.P. Luyten, C. Maes, Integrin-linked kinase regulates bone formation by controlling cytoskeletal organization and modulating BMP and wnt signaling in osteoprogenitors, *J Bone Miner Res* 32(10) (2017) 2087-2102.
- [37] J. Li, Z. Wan, H. Liu, H. Li, L. Liu, R. Li, Y. Guo, W. Chen, X. Zhang, X. Zhang, Osteoblasts subjected to mechanical strain inhibit osteoclastic differentiation and bone resorption in a co-culture system, *Ann Biomed Eng* 41(10) (2013) 2056-66.
- [38] R. Li, L. Liang, Y. Dou, Z. Huang, H. Mo, Y. Wang, B. Yu, Mechanical strain regulates osteogenic and adipogenic differentiation of bone marrow mesenchymal stem cells, *Biomed Res Int* 2015 (2015) 873251.
- [39] P. Muller, A. Langenbach, A. Kaminski, J. Rychly, Modulating the actin cytoskeleton affects mechanically induced signal transduction and differentiation in mesenchymal stem cells, *PLoS One* 8(7) (2013) e71283.
- [40] L.M. Calvi, G.B. Adams, K.W. Weibrecht, J.M. Weber, D.P. Olson, M.C. Knight, R.P. Martin, E. Schipani, P. Divieti, F.R. Bringhurst, L.A. Milner, H.M. Kronenberg, D.T. Scadden, Osteoblastic cells regulate the haematopoietic stem cell niche, *Nature* 425(6960) (2003) 841-6.
- [41] J. Zhang, C. Niu, L. Ye, H. Huang, X. He, W.G. Tong, J. Ross, J. Haug, T. Johnson, J.Q. Feng, S. Harris, L.M. Wiedemann, Y. Mishina, L. Li, Identification of the haematopoietic stem cell niche and control of the niche size, *Nature* 425(6960) (2003) 836-41.

FIGURE 1

FIGURE 2

FIGURE 3

FIGURE 4

FIGURE 5

HIGHLIGHTS

- CD82-deficient bones are smaller in diameter due to defects in osteoblast-mediated bone growth and mineralization.
- Osteoclasts are smaller, erode less, there is increased trabecular bone surface area in situ; osteoclasts display morphological alterations in vitro.
- Bone, but not peripheral, adipocyte numbers and adipocyte differentiation are highly elevated in CD82-deficient mice.
- In vitro bone cell characteristics were independent of sex, but in vivo phenotypes were more pronounced in male mice.
- The combined defects in adipocytes, osteoclasts, and osteoblasts can account for the observed bone phenotypes, and suggests a role for CD82 in both bone mesenchyme and myeloid cells.