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The Role of the Progressive Ankylosis Protein (ANK) in Adipogenic/Osteogenic Fate Decision of Precursor Cells

Takeshi Minashima^{a,1}, Martin Quirno^a, You Jin Lee^a, and Thorsten Kirsch^{a,*}

^aMusculoskeletal Research Center, Department of Orthopaedic Surgery, New York University School of Medicine, New York, New York, United States of America

Abstract

The progressive ankylosis protein (ANK) is a transmembrane protein that transports intracellular pyrophosphate (PPi) to the extracellular milieu. In this study we show increased fatty degeneration of the bone marrow of adult *ank/ank* mice, which lack a functional ANK protein. In addition, isolated bone marrow stromal cells (BMSCs) isolated from *ank/ank* mice showed a decreased proliferation rate and osteogenic differentiation potential, and an increased adipogenic differentiation potential compared to BMSCs isolated from wild type (WT) littermates. Wnt signaling pathway PCR array analysis revealed that Wnt ligands, Wnt receptors and Wnt signaling proteins that stimulate osteoblast differentiation were expressed at markedly lower levels in *ank/ank* BMSCs than in WT BMSCs. Lack of ANK function also resulted in impaired bone fracture healing, as indicated by a smaller callus formed and delayed bone formation in the callus site. Whereas 5 weeks after fracture, the fractured bone in WT mice was further remodeled and restored to original shape, the fractured bone in *ank/ank* mice was not fully restored and remodeled to original shape. In conclusion, our study provides evidence that ANK plays a critical role in the adipogenic/osteogenic fate decision of adult mesenchymal precursor cells. ANK functions in precursor cells are required for osteogenic differentiation of these cells during adult bone homeostasis and repair, whereas lack of ANK functions favors adipogenic differentiation.

Keywords

adipogenesis; bone fracture healing; osteogenesis; progressive ankylosis protein (ANK); Wnt/ β -catenin signaling

*Corresponding author: Musculoskeletal Research Center, Department of Orthopaedic Surgery, NYU School of Medicine, 301 East 17th Street, Suite 1500, Phone: 212-598-6589, thorsten.kirsch@nyumc.org (TK).

¹Present Address: Department of Physiology and Regenerative Medicine, Kinki University School of Medicine, Osaka, Japan

Conflict of Interest

None of the authors have a conflict of interest.

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1. INTRODUCTION

ANK is a transmembrane protein that transports intracellular pyrophosphate (PPi) to the extracellular milieu [1]. The progressive ankylosis allele (*ank*) is a spontaneous recessive mutation that causes the lack of a functional progressive ankylosis protein (ANK) in *ank/ank* mice [1]. We have previously shown that *ank/ank* mice show an osteopenic phenotype [2]. Several studies have now established that ANK is an important regulator of extracellular PPi homeostasis in skeletal tissues [1–5]. Previous findings from our laboratory have suggested that extracellular PPi directly affects osteogenic differentiation [2]. Other studies have suggested that ANK may play a role of mediating ATP efflux in musculoskeletal cells [6]. ATP and its hydrolysis products have been shown to affect osteoblast differentiation and mineralization [7–12]. Therefore, it is possible that ANK is an important regulator of osteoblast differentiation. However, it is not clear how ANK affects osteogenic differentiation of BMSCs.

Wnt/ β -catenin signaling has been shown to induce osteogenic differentiation in osteoprogenitor cells [13]. Consequently, activating mutations in Wnt/ β -catenin signaling pathway in humans causes high bone-mass phenotype such as van Buchem disease, whereas inactivating mutations cause osteopenic diseases such as osteoporosis-pseudoglioma syndrome [14]. In addition, loss of Wnt/ β -catenin signaling is mainly responsible for the loss of osteogenic potential of aging bone marrow stromal cells (BMSCs), the osteogenic precursor cells in the bone marrow [15, 16]. These cells have the potential to differentiate into adipocytes, chondrocytes and osteoblasts [17]. As a consequence of loss of Wnt/ β -catenin signaling, aging BMSCs decrease their ability to differentiate into osteoblasts and increase their ability to differentiate into adipocytes [18–20]. Little, however, is known about upstream factors that regulate Wnt/ β -catenin signaling in aging BMSCs.

Since Wnt/ β -catenin signaling in osteoprogenitor cells induces their differentiation, Wnt/ β -catenin signaling is also essential during bone healing [21]. The declining Wnt/ β -catenin signaling in aging osteogenic precursor cells has been suggested as a main reason for delayed fracture healing in elderly. In fact, a recent study has shown that treatment of a bone fracture with liposomal Wnt3a resulted in markedly faster and more robust bone healing with earlier onset of mineralization and new osteoid deposition than happens normally in young animals [15].

ank/ank mice have a limited life span of 4 to 6 months [22]. Previously we have shown that adult *ank/ank* BMSCs show reduced osteogenic differentiation [2], suggesting that these cells rapidly lose their osteogenic differentiation potential. The goal of this study was to determine how lack of ANK function affects the osteogenic differentiation potential of adult BMSCs. Specifically, we asked the question of whether ANK directly affects Wnt/ β -catenin signaling and ultimately osteogenic differentiation of adult BMSCs. To accomplish this goal we analyzed the trabecular bone and bone marrow structure in adult *ank/ank* mice and WT littermates and analyzed the adipogenic and osteogenic differentiation potentials of BMSCs isolated from *ank/ank* mice and WT littermates. In addition, we analyzed how lack of ANK function affects bone fracture healing in mice.

2. MATERIALS AND METHODS

2.1. Animals

The *ank/ank* breeding colony used was originally on a hybrid background derived from crossing a C3H and C57BL/6 hybrid male with BALB/c female. Heterozygote breeders (C3FeB6 A/A^{w-J}-Ank^{ank/J}) were obtained from The Jackson Laboratory (Bar Harbor, ME). Heterozygote breeders were used to generate and study *ank/ank* and wild-type (WT) littermates, with genotypes analyzed by polymerase chain reaction (PCR) as previously described [1].

To analyze bone fracture healing, we utilized a well-established femur fracture model in 6-week-old *ank/ank* mice and WT littermates [23]. Briefly, a medial parapatellar incision was used to expose the femoral condyles. A hole was drilled into the femoral intramedullary canal at the intracondylar notch using a 30-gauge needle. A 0.01-inch diameter stainless steel pin was inserted into the intramedullary canal to stabilize the impending fracture. A closed diaphyseal femoral fracture was created using a blunt impact force in a three-point bending technique. Pain was managed postoperatively with subcutaneous doses of buprenorphine (0.05mg/kg). Mice were euthanized at 2 weeks and 5 weeks post fracture. Surgery was performed on approximately equivalent numbers of male and female mice. Each group consisted on 5 mice. Protocols were approved by the Institutional Animal Care and Use Committee at New York University School of Medicine in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

2.2. Histology

Hindlimbs from 2 month-old and 4-month-old female and male *ank/ank* mice and WT littermates were used. We analyzed the hindlimbs of 4 animals in each group (*ank/ank* and WT). Dissected femurs and tibias from *ank/ank* mice and WT littermates were fixed in 4% paraformaldehyde, decalcified in 0.2M EDTA pH 7.4 for 14 days, and embedded in paraffin. Five-micrometer sections were cut and stained with hematoxylin and eosin. For immunohistochemical staining, sections were pretreated with bovine testicular hyaluronidase (2mg/ml; Sigma) for 30 min at 37°C, blocked with goat serum for 20 min at room temperature, and incubated over night with a primary antibody specific for perilipin A (ab3526, Abcam, Cambridge, MA) followed by biotinylated secondary antibodies for 30 min at room temperature. After washing, sections were incubated with a streptavidin-peroxidase conjugate for 10 min at room temperature followed by a solution containing diaminobenzidine (chromogen) and 0.03% hydrogen peroxide for 5 min at room temperature. Control sections were incubated with nonimmune rabbit serum. Specimens were viewed under Evos FL Auto Cell Imaging System (Thermo Fisher Scientific). Quantitative analysis of the total area of the fat vacuoles in the bone marrow of the tibia was performed on sections from 2-month old and 4-month old tibiae from *ank/ank* mice (n = 3) and wild type littermates (n = 3). The analysis was performed in an area 100µm to 700µm distal to the growth plate using the Evos FL Auto Cell Imaging System software. In addition, the number of perilipin immune-positive cells in this area was counted using the Evos FL Auto Cell Imaging System software.

For histological evaluation of bone fracture healing, femurs were excised, the needle was removed, and the samples were fixed and decalcified as described above. Five-micrometer sections were cut and stained with hematoxylin and eosin or alcian blue.

2.3. MicroCT Evaluation

Specimens were fixed in 4% paraformaldehyde and analyzed by high-resolution microCT using a Skyscan 1172 (Bruker, Billerica, MA). To analyze the degree of bone fracture healing, the following parameters were determined: total callus volume (TCV), bone tissue volume in the callus (BV), bone tissue volume fraction of the total callus volume (BV/TCV).

2.4. Plain Radiography

Serial radiographs were taken directly after the creation of the fracture and 2 weeks after fracture surgery using the digital radiographic function of the IVIS Lumina III In Vivo Imaging System (PerkinElmer, Shelton, CA).

2.5. Cell Cultures

Bone marrow stromal cells (BMSCs) were isolated from femurs of 8-week-old female and male *ank/ank* mice or WT littermates and cultured at 25×10^6 cells per 10-cm in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 15% fetal calf serum as described previously [24–26]. To obtain 25×10^6 cells for one experiment, we used 4 hindlimbs from 2 *ank/ank* mice and 4 hindlimbs from 2 WT littermates. For osteogenic differentiation, BMSCs were cultured in osteogenic differentiation medium (MEM supplemented with 10% fetal calf serum, 5mM β -glycerophosphate, 50 μ g/ml ascorbic acid, 10^{-7} M dexamethasone) [2, 26, 27]. After 7, 14, and 21 days of culture, cells were stained for alkaline phosphatase (ALP) activity using Alkaline Phosphatase Magenta Immunohistochemical Substrate Solution (Sigma). To determine mineralization in these cultures, alizarin red S staining was performed on day 14 and day 21 as described previously [28]. For adipogenic differentiation, BMSCs were cultured in adipogenic differentiation medium (DMEM supplemented with 10% fetal calf serum, 0.5 mM 3-isobutyl-1-methylxanthine, 5 μ g/ml insulin, and 1 μ M dexamethasone) as described previously [26]. Oil red staining was performed after 7, 14, and 21 days of culture.

For Wnt PCR array using the RT2 Profiler PCR array for the Wnt signaling pathway (Qiagen) we cultured *ank/ank* and WT BMSCs after these cells have reached confluency for 2 days in osteogenic differentiation medium.

2.6. Luciferase Reporter Assays

For luciferase assays to determine β -catenin activity, cells were co-transfected with a firefly TCF/LEF-specific luciferase reporter vector (TOPFlash Reporter; EMD Millipore, Billerica, MA) and a constitutively expressed Renilla luciferase reporter, which served as an internal control for normalizing transfection efficiencies and monitoring cell viability. Luciferase activities from both reporters were measured using a dual luciferase assay kit (Promega Corp., Madison, WI). Luciferase activities were measured using the Tristar LB 941 luminometer (Berthold Technologies, Oak Ridge, TN). All experiments were performed in triplicate and repeated three times.

2.7. Real-time PCR analysis

Total RNA was isolated from cell cultures using the RNeasy Minikit (Qiagen). Gene expression was quantified by real-time PCR analysis as described previously [28]. Briefly, 1 µg of total RNA was reverse-transcribed using the Omniscript RT kit (Qiagen). A 1:100 dilution of the resulting cDNA was used as a template to quantify the relative content of mRNA by real-time PCR using SYBR Green. PCRs were performed with a SYBR Green PCR Master Mix kit (Applied Biosystems), with 95°C for 10 min followed by 40 cycles at 95°C for 15 s and 60°C for 1 min, and 1 cycle at 95°C for 15 s and 60°C for 1 min. The 18S rRNA was amplified at the same time and used as an internal control. The cycle threshold values for 18S rRNA and the samples were measured and calculated by computer software. Relative transcription levels were calculated as $x = 2^{-CT}$, in which $CT = E - C$, $E = Ct_{exp} - Ct_{18S}$, and $C = Ct_{ctrl} - Ct_{18S}$. The gene-specific primers used are as follows: *ank*-forward, 5'-GCC CAT TGT CAA CCT CTT CGT-3', and *ank*-reverse, 5'-GAA TGG CCA CTG CCT CTG TAG-3'; ALP-forward, 5'-AAC ACC AAT GTA GCC AAG-3', and ALP-reverse, 5'-TCG GGC AGC GGT TAC TGT-3'; adipocyte protein 2 (AP2)-forward, 5'-CTT CAA ACT GGG CGT GGA A-3', and AP2-reverse, 5'-CCA TCT AGG GTT ATG ATG CTC TTC A-3'; osteocalcin (OC)-forward, 5'-CCA GCG ACT CTG AGT CTG ACA A-3', and OC-reverse, 5'-CCG GAG TCT ATT CAC CAC CTT ACT-3'; osterix-forward, 5'-TTC TGT CCC CTG CTC CTT CTA G-3', and osterix-reverse, 5'-CGT CAA CGA CGT TAT GCT CTT C-3'; peroxisome proliferator-activated receptor gamma (PPAR γ)-forward, 5'-CCC AAT GGT TGC TGA TTA CAA A-3', and PPAR γ -reverse, 5'-AAT AAT AAG GTG GAG ATG CAG GTT CT-3'; runx2-forward, 5'-AGT AGC CAG GTT CAA CGA TCT GA-3', and runx2-reverse, 5'-GAC TGT TAT GGT CAA GGT GAA ACT CTT-3'.

To profile gene expression of Wnt proteins, Wnt receptors and Wnt target genes in *ank/ank* and WT BMSCs, we carried out a PCR array using the RT2 Profiler PCR array for the Wnt signaling pathway (Qiagen) following the manufacturer's instructions. The average threshold cycle value (Ct value) was calculated and normalized to that of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase. These experiments were repeated three times independently.

2.8. Cell Proliferation

For analysis of cell proliferation, 5000 *ank/ank* or WT BMSCs were dispensed in a 96-well plate. The plate was incubated for 12 h, 24 h or 48 h in a humidified incubator at 37°C followed by the addition of 10 µl Cell counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Inc., Rockville, MD) and incubation for 4 h at 37°C. After the incubation, the absorbance of the collected medium was measured at 450 nm. The absorbance of the collected medium after 12 h incubation of WT cells was set as 100%.

2.9. Statistical analysis

All measurements were performed at least in triplicates. Numerical data are presented as mean $\pm$ S.D. ($n = 3$). Statistical analysis was performed by Student's *t*-test to evaluate differences between the two groups. Analysis of variance was performed when the examined experimental groups exceeded three. Tukey's multiple comparison test was applied as post

hoc test. Statistical significance was defined as $p < 0.05$ (p -values are reported in the figure legends).

3. RESULTS

3.1. Lack of ANK function accelerates age-related fatty bone marrow

We have previously shown that *ank/ank* mice have an age-related osteoporotic phenotype, decreased osteoblast number and decreased bone formation compared to WT littermates [2]. Histological analysis of the femur and the tibia of 2-month old ($n=4$) and 4-month old ($n=4$) *ank/ank* mice revealed besides the osteoporotic phenotype increased fatty degeneration in the bone marrow compared to WT littermates ($n=4$ for 2-month old mice, and $n=4$ for 4-month old animals) as indicated by the increased number of fat vacuoles in the bone marrow (Fig. 1A). Increased fatty degeneration of the bone marrow became evident in 2-month-old *ank/ank* mice (Fig. 1A), and markedly increased in 4-month old *ank/ank* mice (Fig. 1A). In addition, immunostaining with an antibody specific for perilipin, a protein that coats lipid droplets in adipocytes, revealed markedly increased immunostaining for this protein in the bone marrow of 2-month old *ank/ank* mice compared to WT littermates (Fig. 1B). Quantitative analysis revealed that the total area of fat vacuoles in the bone marrow of the tibia was increased in 2-month and 4-month-old *ank/ank* mice compared to wild type littermates (Fig. 1C). In addition, the total area of fat vacuoles in the bone marrow of the tibia increased in 4-month old *ank/ank* mice and wild type littermates compared to 2-month old *ank/ank* mice and wild type littermates, respectively (Fig. 1C). This increase in 4-month old mice, however, was more pronounced in *ank/ank* mice than wild type littermates (Fig. 1C). Furthermore, the number of perilipin immuno-positive cells in the bone marrow of 2-month old *ank/ank* tibiae was markedly higher than the number of immunopositive cells in the bone marrow of 2-month old wild type tibiae (Fig. 1D). These findings together with our previous findings suggest enhanced adipogenesis and decreased osteogenesis of precursor cells in vivo in adult *ank/ank* mice compared to WT littermates.

3.2. Lack of ANK function inhibits proliferation and osteogenic differentiation and accelerates adipogenic differentiation of BMSCs

We analyzed proliferation of BMSCs isolated from *ank/ank* mice and WT littermates using the Cell Counting Kit-8 (CKK8). BMSCs isolated from *ank/ank* mice showed a markedly decreased rate of proliferation in culture at 1, 3 and 7 days compared to BMSCs isolated from WT littermates (Fig. 2A).

Next, we analyzed the osteogenic and adipogenic differentiation potential of BMSCs isolated from the bone marrow of 8-week old *ank/ank* mice and WT littermates in in vitro cell cultures. When BMSCs were cultured in osteogenic differentiation medium decreased ALP activity and bone nodule formation was evident in BMSCs obtained from *ank/ank* mice after 7, 14 and 21 days of culture in osteogenic differentiation medium compared to BMSCs isolated from WT littermates (Fig. 2B). In addition, markedly reduced mineralization as indicated by reduced alizarin red S staining was evident in *ank/ank* BMSCs cultured for 14 or 21 days in osteogenic differentiation medium compared to WT BMSCs cultured in osteogenic differentiation medium (Fig. 2B). The mRNA level of ALP, an early bone marker

gene was markedly decreased in *ank/ank* BMSCs cultured in osteogenic differentiation medium during the 14-day culture period compared to the ALP mRNA levels of WT BMSCs cultured in osteogenic differentiation medium (Fig. 2C). The mRNA levels of osteocalcin (OC), a late osteogenic marker were only detectable in WT BMSCs cultured in osteogenic differentiation medium at day 7 and day 14, but were not detectable in *ank/ank* BMSCs cultured in osteogenic differentiation medium during the 14-day culture period (Fig. 2C). In addition, the mRNA levels for osterix and runx2, two major osteogenic transcription factors were markedly decreased in *ank/ank* BMSCs cultured in osteogenic differentiation medium over the 14-day culture period compared to WT BMSCs (Fig. 2C). The mRNA levels of ALP, osteocalcin, osterix and runx2 were low or not detectable in *ank/ank* and WT BMSCs cultured in growth medium and the mRNA levels of these bone markers did not change over the 14-day culture period in growth medium (Fig. 2C).

ANK mRNA levels in WT BMSCs cultured in growth medium did not change during the 14-day culture period (Fig. 3). In contrast, ANK mRNA levels increased during the first seven days of WT BMSCs cultured in osteogenic differentiation medium and reached the highest mRNA level on day 7 (Fig. 3). At day 14 the ANK mRNA levels in WT BMSCs cultured in osteogenic differentiation medium decreased to levels similar to the levels in WT BMSCs cultured in growth medium. ANK mRNA levels in WT BMSCs cultured in adipogenic differentiation medium decreased below the levels in WT BMSCs cultured in growth medium (Fig. 3).

ank/ank BMSCs differentiated into oil-red O-positive adipocytes more efficiently than WT BMSCs. When cultured in growth medium for 21 days, the number of oil-red O-positive adipocytes in *ank/ank* BMSC cultures were more compared to oil-red O-positive WT cells (Fig. 4A). The number of oil-red O-positive adipocytes markedly increased when *ank/ank* BMSCs were culture in adipogenic differentiation medium compared to *ank/ank* BMSCs cultured in growth medium for 21 days (Fig. 4A). More importantly, notably more oil red O-positive adipocytes were evident in *ank/ank* BMSCs cultured for 21 days in adipogenic differentiation medium compared to WT BMSCs cultured for 21 days in adipogenic differentiation medium (Fig. 4A). Real time PCR analysis confirmed increased mRNA levels of PPAR γ , a key transcription factor for adipogenesis, as well as acid binding protein AP2, a marker for the adipocyte phenotype, in *ank/ank* BMSCs cultured for 21 days in growth medium compared to the levels of these adipogenic markers in WT BMSCs cultured in growth medium (Fig. 4B). In addition, at day 21 the mRNA levels of PPAR γ and AP2 were markedly higher in *ank/ank* BMSCs cultured in adipogenic differentiation medium compared to the levels in WT BMSCs cultured in adipogenic differentiation medium (Fig. 4B).

Next, we performed rescue experiments in which we transfected *ank/ank* BMSCs with empty pcDNA expression vector or pcDNA expression vector containing full-length *ank*. When cultured for 5 days in osteogenic differentiation medium, the mRNA levels of the early osteogenic marker ALP were lower in *ank/ank* BMSCs transfected with empty expression vector and cultured in osteogenic differentiation medium compared to the levels in WT cells (Fig. 5, ALP). Transfection of *ank/ank* BMSCs with expression vector containing *ank* increased mRNA levels of ALP to levels similar to WT cells cultured for 5

days in osteogenic differentiation medium (Fig. 5, ALP). In addition, transfection of *ank/ank* BMSCs with expression vector containing *ank* resulted in increased Wnt/ β -catenin signaling as indicated by increased luciferase activity of the TOPFlash reporter compared to the levels in *ank/ank* BMSCs transfected with empty expression vector and cultured for 3 days in osteogenic differentiation medium (Fig. 5, TOPFlash). The luciferase activity from the TOPFlash reporter in *ank/ank* BMSCs transfected with expression vector containing *ank* was similar to the luciferase activity in WT BMSCs cultured for 3 days in osteogenic differentiation medium (Fig. 5, TOPFlash).

When *ank/ank* BMSCs transfected with empty expression vector were cultured in adipogenic differentiation medium for 5 days, C/EBP β , a transcription factor involved in early adipogenic differentiation [29], and PPAR γ were increased compared to the levels in WT BMSCs cultured in adipogenic differentiation medium (Fig. 5 C/EBP β , PPAR γ). The mRNA levels of C/EBP β and PPAR γ were reduced to levels similar to the levels of WT BMSCs when *ank/ank* BMSCs were transfected with expression vector containing *ank* and cultured for 5 days in adipogenic differentiation medium (Fig. 5 C/EBP β , PPAR γ). These findings show that the transfection of *ank/ank* BMSCs with an expression vector that encodes functional ANK protein increases their osteogenic differentiation potential and decreases their adipogenic differentiation potential to levels similar to the osteogenic and adipogenic differentiation potential of WT BMSCs.

3.3. Lack of ANK Function Decreases the Expression of Wnt Ligands, Wnt Receptors and Wnt Signaling Proteins

Wnt/ β -catenin signaling plays a crucial role in osteogenic differentiation of precursor cells [30]. We performed a Wnt signaling pathway PCR array analysis to obtain insights of whether ANK affects the expression of Wnt ligands, Wnt receptors and Wnt signaling proteins. We used RNA isolated from *ank/ank* and WT BMSCs and cultured for 2 days in differentiation medium for the Wnt signaling pathway PCR array. PCR array analysis of the expression levels of the Wnt ligands revealed that Wnt2b, 4, 5a, 6, 7b, 9a, 10a, 11, and 16 were expressed in WT BMSCs cultured for two days in osteogenic differentiation medium with Wnt2b being expressed at the highest level (Fig. 6). Wnt2b stimulates Wnt/ β -catenin signaling [31]. The expression levels of Wnt2b, 4, 5a, 6, 7b, 10a, 11 and 16 were markedly reduced in *ank/ank* BMSCs (Fig. 6). Wnt9a was the only Wnt protein that was expressed at markedly higher levels in *ank/ank* BMSCs compared to WT BMSCs (Fig. 6). Interestingly, Wnt9a expression was shown to be downregulated during the differentiation of precursor cells into mature osteoblasts, and knockdown of Wnt9a in osteoblasts increased expression of ALP, runx2, a key transcription factor for osteoblast differentiation, and osteocalcin, a late marker of osteoblast differentiation [32, 33]. In addition, knockdown of Wnt9a in osteoblasts increased their degree of mineralization [33], indicating that Wnt9a is an inhibitor of osteoblast differentiation. PCR array analysis revealed that the Wnt receptors Fzd1, 2, 3, 4, 5, 6, 7, and Lrp5 and Lrp6 were expressed in WT BMSCs cultured for 2 days in osteogenic differentiation medium. Fzd3, Fzd4, Fzd5 and Lrp5 were expressed at high levels in WT cells (Fig. 6). Fzd5 mediates canonical (β -catenin) and non-canonical Wnt signaling, and Lrp5 and Lrp6 are essential co-receptors for Wnt/ β -catenin signaling in osteoblasts [30]. All these Wnt Fzd receptors and co-receptors were expressed at markedly lower levels in

ank/ank BMSCs cultured for two days in osteogenic differentiation medium compared to WT cells (Fig. 6). Finally, the mRNA levels of Wnt signaling proteins, including members of the destruction complex (adenomatous polyposis coli (APC), axin 2, casein kinase I isoform alpha (CSNK1A1)), β -catenin (CTNNB1), disheveled segment polarity protein 1 (Dvl1), lymphoid enhancer-binding factor 1 (LEF1), and transcription factor 7 (TCF 7) were decreased in *ank/ank* BMSCs cultured for two days in osteogenic differentiation medium compared to WT BMSCs (Fig. 6). In summary, our findings revealed that most of the Wnt ligands, Wnt receptors, and Wnt signaling proteins that stimulate osteoblast differentiation were expressed at lower levels in *ank/ank* BMSCs cultured for two days in osteogenic differentiation medium than in WT BMSCs, whereas Wnt9a, an inhibitor of osteoblast differentiation, was expressed at higher levels in *ank/ank* BMSCs compared to WT BMSCs.

3.4. Lack of ANK Function Delays Bone Fracture Healing

In the final set of experiments, we determined how lack of ANK function affects bone fracture healing. We utilized a well-established femur fracture model in 6-week-old *ank/ank* mice and WT littermates [23]. X-ray analysis of the fractured hindlimb of *ank/ank* mice and WT littermates 2 weeks after the fracture revealed a markedly reduced callus size in *ank/ank* mice compared to WT littermates (Fig. 7A). Three-dimensional reconstructed microCT images of *ank/ank* mice and WT littermates 2 weeks after fracture confirmed the X-ray findings and quantitatively showed a markedly reduced size of the fracture callus in *ank/ank* mice compared to WT littermates (Fig. 7B). In addition, lower proportion of mineralized bone in the callus was observed in *ank/ank* mice at week 2 compared to WT littermates (Fig. 7B). At 5 weeks the fractured bone in WT mice was further remodeled and restored to original shape, whereas the fractured bone in *ank/ank* mice was not fully restored and remodeled to original shape (Fig. 7B). Average BV/TV in the callus of fractured bones from WT mice reached 20% on week 2 after fracture and 80% on week 5. In contrast, average BV/TV in the callus of fractured bones from *ank/ank* mice only reached 4% on week 2 and 30% on week 5 (Fig. 7C).

Histological analysis of sections from the callus formed 2 weeks after fracture surgery confirmed microCT analysis and revealed a smaller size callus (C) in the fracture site of *ank/ank* mice compared to WT littermates (Fig. 8A, B). However, alcian blue staining revealed that the *ank/ank* callus contained more cartilaginous tissue (CT) and less bony tissue than the WT callus (Fig. 8A, B). In summary, these experiments reveal delayed fracture healing in *ank/ank* mice compared to WT littermates.

4. DISCUSSION

In this study we demonstrate increased fatty degeneration of the bone marrow in femur and tibia of 2-month and 4-month old *ank/ank* mice similar to the age-related fatty degeneration of the bone marrow in human patients [15, 18, 20, 34]. Fatty degeneration of the bone marrow in the *ank/ank* mice was observed in 2-month old mice and severely increased in 4-month old mice. In addition, we show that lack of ANK function in BMSCs delays their osteogenic differentiation and promotes adipogenic differentiation of these cells. Expression of functional ANK in *ank/ank* BMSCs was able to rescue the phenotype resulting in

increased osteogenesis and decreased adipogenesis. Finally, we demonstrate delayed bone fracture healing with delayed formation of bony tissue in *ank/ank* mice. Previous findings have shown that adipogenic-osteogenic fate decision plays a key role in bone remodeling and bone healing and that this fate decision alters during aging with a decline in the osteogenic differentiation potential of aging BMSCs [34–36]. Interestingly, the current study reveals that ANK expression is upregulated during the initial stages of osteogenic differentiation of precursor cells, whereas ANK expression is downregulated in these cells when they undergo adipogenic differentiation. Therefore, our data suggest that ANK plays a key role in the osteogenic fate decision of adult precursor cells.

Recent studies have revealed that Wnt/ β -catenin signaling is a key signaling pathway that regulates osteogenic fate decision. Abrogation of Wnt/ β -catenin signaling leads to adipogenic differentiation, whereas potentiation of Wnt/ β -catenin signaling leads to osteogenic differentiation of mesenchymal stem cells [15, 16]. In addition, other studies have shown that Wnt ligands, Wnt receptors, Wnt signaling proteins and ultimately Wnt/ β -catenin signaling is increased during bone fracture healing and plays a major role in the repair process [37]. Specifically, Wnt/ β -catenin signaling promotes osteoblast differentiation and thereby stimulates bone fracture healing [37]. Our findings show that lack of ANK function results in decreased expression of several Wnt ligands, Wnt receptors and Wnt signaling proteins in BMSCs when cultured in osteogenic differentiation medium that have been shown to stimulate Wnt/ β -catenin signaling in these cells and ultimately osteogenesis [14]. In contrast, Wnt9a, a Wnt ligand that has been shown to inhibit osteogenesis was increased in *ank/ank* BMSCs [33]. These findings suggest that the increased expression of ANK during early osteogenesis is a key mediator of stimulating the expression of Wnt ligands and receptors, including Lrp5 and Lrp6, key co-receptors for the activation of Wnt/ β -catenin signaling, and Wnt signaling proteins that are required for osteogenic fate decision of adult mesenchymal stem cells [15]. In addition, our findings suggest that the reduced expression of Wnt ligands, receptors and signaling proteins in *ank/ank* osteogenic precursor cells may be a reason for the delayed bone fracture healing in *ank/ank* mice.

At this time we can only speculate how ANK mediates the stimulation of the expression of Wnt ligands, receptors and signaling proteins, and ultimately is required for osteogenic differentiation of precursor cells. ANK was shown to be a transmembrane protein that transports intracellular PPi to the extracellular milieu [1, 3]. Extracellular PPi has been shown to act as a mineralization inhibitor by preventing the growth of hydroxyapatite crystals [38]. Extracellular PPi, however, can easily being hydrolyzed by enzymes, such as ALP, into inorganic phosphate (Pi) [39]. Previous studies from our and other laboratories have shown that extracellular Pi stimulates osteogenic differentiation [2, 40, 41]. Interestingly, a recent study has shown that high extracellular Pi induced phenotypic transformation of vascular smooth muscle cells into osteogenic-like cells via the activation of Wnt/ β -catenin signaling in these cells [42]. Our previous study, however, also suggested that extracellular PPi directly stimulates osteogenesis [2, 43]. Therefore, it is possible that extracellular PPi resulting from ANK transport directly or via its hydrolysis to extracellular Pi may affect Wnt/ β -catenin signaling in osteogenic precursor cells. This possibility is supported by previous findings showing that extracellular PPi affects Wnt ligand expression and Wnt/ β -catenin signaling in articular chondrocytes [44].

We cannot, however, rule out the possibility that ANK uses other mechanisms than the regulation of PPI homeostasis to control osteogenic differentiation of precursor cells. For example a recent study has shown that ANK regulates ATP efflux in articular chondrocytes [6]. Extracellular ATP has been shown to stimulate osteogenic differentiation of mesenchymal stem cells via purinergic receptors [7]. In addition, we and others have shown that ANK interacts with other intracellular proteins [45, 46]. For example, we have shown that ANK interacts with sphingosine kinase 1 (SPHK1) in chondrocytes [46]. This interaction resulted in increased SPHK1 activity and as a consequence increased sphingosine-1 phosphate (S1P) as the actual modulator of SPHK1 activity affecting cellular function levels in chondrocytes [46]. Recent studies have suggested that S1P may play an important role in the regulation of osteogenesis. Specifically, these studies have shown that S1P promotes osteogenic differentiation of precursor cells and inhibits their adipogenic differentiation [47–49]. Future studies have to determine the exact mechanisms of how ANK affects osteogenic and adipogenic differentiation of precursor cells.

5. CONCLUSION

Our study provides evidence that ANK plays a critical role in the adipogenic/osteogenic fate decision of adult mesenchymal precursor cells. ANK functions in precursor cells are required for osteogenic differentiation of precursor cells, whereas lack of ANK function favors adipogenic differentiation of mesenchymal precursor cells. In addition, lack of ANK function resulted in delayed bone fracture healing in mice as a consequence of delayed osteogenesis. With age, mesenchymal stem cells in the bone marrow become inclined to undergo adipogenic differentiation rather than osteogenic differentiation, resulting in increased adipocytes and fat deposition in the bone marrow and a decreased number of osteoblasts causing osteoporosis. Our findings suggest that ANK may play a key role in the regulation of bone formation during bone homeostasis and bone fracture healing during adulthood and that a detailed understanding of the mechanisms of how ANK regulates osteogenic fate decision in precursor cells may provide novel anti-aging therapeutic targets in the skeletal system.

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Abbreviations

ADM	adipogenic differentiation medium
ALP	alkaline phosphatase
ANK	progressive ankylosis protein
<i>ank</i>	progressive ankylosis gene
AP2	adipocyte protein 2
APC	adenomatous polyposis coli

beta-catenin	CTNNB1
BMSCs	bone marrow stromal cells
BV	bone tissue volume in callus
BV/TCV	bone tissue volume fraction of the total callus volume
C	callus
CAT	cartilaginous tissue
C/EBPβ	CCAAT-enhancer-binding protein beta
CKK-8	cell counting kit-8
CSNK1A1	casein kinase I isoform alpha
CT	cycle value
DMEM	Dulbecco's Modified Eagle Medium
Dvl1	disheveled segment polarity protein 1
GM	growth medium
LEF1	lymphoid enhancer-binding factor 1
n.d	not detectable
OC	osteocalcin
ODM	osteogenic differentiation medium
PCR	polymerase chain reaction
Pi	inorganic phosphate
PPARγ	peroxisome proliferator-activated receptor gamma
PPi	pyrophosphate
S1P	sphingosine-1 phosphate
SPHK1	sphingosine kinase 1
TCF 7	transcription factor 7
TCV	total callus volume
WT	wild type

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Highlights

- Lack of functional ANK results in decreased proliferation and osteogenic differentiation of precursor cells.
- Lack of functional ANK results in increased adipogenic differentiation and increased fatty degeneration in the bone marrow of aging mice.
- Lack of functional ANK results in decreased expression of Wnt ligands, receptors and signaling proteins in osteogenic precursor cells.
- Lack of functional ANK results in impaired bone fracture healing.

Figure 1.

A: Histological analysis of the bone marrow of 2-month and 4-month old WT and *ank/ank* mice (tibia, safranin O staining). Note the increased fat fraction in the bone marrow of 2-month and 4-month old *ank/ank* mice compared to WT littermates. Bar, 200 μm . **B:** Immunohistological staining with antibodies specific for perilipin, a protein that coats lipid droplets in adipocytes, of sections from the bone marrow of 2 month-old WT and *ank/ank* mice. Bar, 200 μm . **C:** Quantitative analysis of the total area (μm^2) of the fat vacuoles in the bone marrow of 2-month and 4-month old *ank/ank* and wild type tibiae **D:** Number of cells immunopositive for perilipin in an area 100 μm to 700 μm distal to the growth plate. Sections

from three different *ank/ank* mice and three different wild type littermates were analyzed. Data are expressed as mean $\pm$ SD. (^a $p < 0.01$ vs. 2-month old WT tibia; ^b $p < 0.01$ vs. 2-month old *ank/ank* tibia).

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Figure 2.

A: Proliferation rate of WT and *ank/ank* BMSCs cultured for 12 h, 24 h and 48 h using the Cell counting Kit-8 (CCK-8). After the incubation with CCK-8 solution, the absorbance of the collected medium was measured at 450 nm at various time points. All the absorbance rates are expressed as percent of the absorbance rate of WT cells after 12 h culture period, which was set as 100%. Data were obtained from 3 different cultures and are expressed as mean $\pm$ S.D. (^a $p < 0.01$ vs. WT cells cultured for 24 h; ^b $p < 0.01$ vs. WT cells cultured for 48 h). **B:** ALP activity and alizarin red S staining of WT and *ank/ank* BMSCs cultured for 7, 14 and 21 days in growth medium (GM) or osteogenic differentiation medium (ODM). **C:** mRNA levels of ALP (early osteogenic marker), osteocalcin (OC, late osteogenic marker) and the two osteogenic transcription factors osterix and runx2 in WT and *ank/ank* BMSCs cultured for 1d, 3d, 7d and 14d in GM or ODM. mRNA levels were determined by real time PCR using SYBR Green and normalized to the level of 18S RNA. The mRNA levels of ALP, osterix and runx2 are expressed as relative to the levels of WT BMSCs cultured in growth medium for 1 day, which were set as 1. mRNA levels of OC are expressed as relative to the levels of WT BMSCs cultured in ODM for 7 days, which was set as 1. mRNA levels of OC were not detectable (n.d.) in WT BMSCs cultured in GM, WT BMSCs cultured in

ODM for 1 and 3 days, and *ank/ank* BMSCs cultured in GM or ODM. Data were obtained from triplicate PCRs using RNA from 3 different cultures. Data are expressed as mean $\pm$ S.D. (^a $p < 0.01$ vs. WT cells cultured in GM; ^b $p < 0.01$ vs. WT cells cultured in ODM).

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Figure 3.

mRNA levels of *ank* in WT BMSCs cultured for 1, 3, 7 and 14 days in growth medium (GM), osteogenic differentiation medium (ODM), or adipogenic differentiation medium (ADM). mRNA levels were determined by real time PCR using SYBR Green and normalized to the level of 18S RNA. The mRNA levels are expressed as relative to the levels of WT BMSCs cultured in growth medium for 1 day, which were set as 1. Data were obtained from triplicate PCRs using RNA from 3 different cultures. Data are expressed as mean $\pm$ S.D. ($^a p < 0.01$ vs. WT cells cultured in GM for 1 day).

Figure 4.

A: Adipocyte formation in WT and *ank/ank* BMSCs cultured for 21 days in growth medium (GM) or adipogenic differentiation medium (ADM) (oil red O staining). Bar, 200 μ m. **B:** mRNA levels of PPAR γ and AP2 in WT and *ank/ank* BMSCs cultured for 21d in growth medium (GM) or adipogenic differentiation medium (ADM). mRNA levels were determined by real time PCR using SYBR Green and normalized to the level of 18S RNA. The mRNA levels of PPAR γ and AP2 are expressed as relative to the levels of WT BMSCs cultured in growth medium for 1 day, which were set as 1. Data were obtained from triplicate PCRs using RNA from 3 different cultures. Data are expressed as mean $\pm$ S.D. (^ap < 0.01 vs. WT cells cultured in GM for 1 day, ^bp < 0.01 vs. WT cells cultured in ADM for 21 days).

Figure 5.

Restoration of osteogenic and adipogenic differentiation potential of *ank/ank* BMSCs after transfection with expression vector containing *ank*. *ank/ank* BMSCs were transfected with empty expression vector (pcDNA) or expression vector containing *ank* (pcDNA-*ank*). In addition, *ank/ank* BMSCs and WT BMSCs were co-transfected with the TOPFlash luciferase reporter. **A:** Twenty-four hours after transfection cells were cultured in osteogenic differentiation medium for 5 days. mRNA levels of ALP were determined by real time PCR using SYBR Green and normalized to the level of 18S RNA. In addition, cell extracts were analyzed for luciferase activity from the TOPFlash reporter (TOPFlash). Transfection efficiency was monitored by co-transfection with Renilla luciferase vector, which provides constitutive expression of the Renilla luciferase reporter. **B:** Twenty-four hours after transfection cells were cultured in adipogenic differentiation medium for 5 days. mRNA levels of C/EBPβ and PPARγ were determined by real time PCR using SYBR Green and normalized to the level of 18S RNA. The mRNA levels of ALP, C/EBPβ and PPARγ are expressed as relative to the levels of WT BMSCs cultured in osteogenic or adipogenic differentiation medium for 5 days, which were set as 1. Data in **A** and **B** were obtained from three different experiments and are expressed as mean ± S.D. (^a*p* < 0.01 vs. WT cells, ^b*p* < 0.01 vs. *ank/ank* transfected with empty pcDNA vector).

Figure 6.

PCR array analysis of Wnt proteins, Wnt receptors/co-receptors and Wnt signaling proteins in *ank/ank* and WT BMSCs cultured for two days in osteogenic differentiation medium. Total RNA was isolated from *ank/ank* and WT BMSCs cultured in osteogenic differentiation medium for 2 days. RNA was subjected to PCR array analysis of the expression of Wnt ligands, Wnt receptors and Wnt signaling proteins. Values represent expression levels of Wnt ligands, Wnt receptors/co-receptors and Wnt signaling proteins relative to glyceraldehyde-3-phosphate dehydrogenase. Values represent mean $\pm$ S.D. obtained from three independent samples.

Figure 7. Reduced callus size and less mineralized bone in the callus of fractured femurs from *ank/ank* mice compared to WT littermates. **A:** Representative radiographs of the fracture site from *ank/ank* mice and WT littermates two weeks after the fracture. **B:** Representative 3D images of fractured femur from *ank/ank* mice and WT littermates at week 2 and week 5 after fracture. **C:** Bone volume within the total callus volume (BV/TCV) in WT and *ank/ank* mice 2 weeks and 5 weeks after fracture (mean $\pm$ S.D., n=4 in each group, ^ap < 0.01).

Figure 8.

A: Representative histological sections of the fracture callus from *ank/ank* mice and WT littermates 2 weeks after fracture stained with alcian blue (cartilage) and H & E. Bar, 200 μ m. **B:** Area of the total callus (C), cartilage tissue (CAT, blue), and bone tissue (BT) within the callus. Data are expressed as mean $\pm$ S.D. (n = 4 in each group; ^ap < 0.01)