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Vanin-1 Pantetheinase Drives Increased Chondrogenic Potential of Mesenchymal Precursors in *ank/ank* Mice

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Widespread endochondral and intramembranous ectopic bone formation is mediated by extracellular PP_i deficiency that develops in *ank/ank* mice. Herein we report on the rapid condensation into chondrogenic nodules of cultured *ank/ank* bone marrow stromal cells (BMSCs). We compared the roles of increased chondrogenic potential versus altered osteoblast function in the *ank/ank* phenotype. To do so, we crossbred *ank/ank* mice with mice lacking Vanin-1 pantetheinase, which inhibits synthesis of the chondrogenesis regulator glutathione, since we observed increased Vanin-1 expression and pantetheinase activity and decreased glutathione in *ank/ank* BMSCs. $Vnn1^{-/-}$ BMSCs demonstrated delayed chondrogenesis mediated by increased glutathione. Moreover, increased chondrogenesis of *ank/ank* BMSCs and increased chondrogenic transdifferentiation and calcification by *ank/ank* aortic smooth muscle cells and explants were corrected by Vanin-1 knockout. Osteoblastogenesis was accelerated in *ank/ank* mesenchymal stem cells. However, in cultured *ank/ank* osteoblasts, Vanin-1 knockout actually increased specific alkaline phosphatase activity and lowered extracellular PP_i, and did not correct increased calcification. Moreover, Vanin-1 knockout failed to correct the *ank/ank* skeletal soft tissue phenotype. Therefore, *ank/ank* periskeletal soft tissue calcification appears more dependent on altered osteoblastic function than enhanced chondrogenic potential and is not dependent on Vanin-1; however, Vanin-1 regulates chondrogenesis via glutathione metabolism and is critical for accelerated chondrogenesis of *ank/ank* mesenchymal precursors and P_i donor-driven chondrogenic transdifferentiation and calcification of aortic smooth muscle cells. ***(Am J Pathol 2008, 172:440–453; DOI: 10.2353/ajpath.2008.070753)***

Calcification is induced and controlled by factors including chondrocyte, osteoblast, and osteoclast precursor recruitment and differentiation and modification of the extracellular matrix to regulate hydroxyapatite crystal growth.[1–4] Heritable deficiencies in mineralization regulators have been particularly informative about hierarchical, co-operative, and antagonistic relationships among such factors.[2,5,6] Indeed, the particularly potent capacity of PP_i to inhibit hydroxyapatite crystal growth[7] has been underscored by the phenotypic effects of alteration of PP_i transport and generation.[8,9] Homozygosity for the murine *ank* mutant of the multiple-pass transmembrane protein ANK, whose PP_i transport function is disabled by a spontaneous ANK C-terminal truncation, is a striking example.[8] The *ank/ank* mice, which are markedly depleted in extracellular PP_i,[5,8] do not exhibit any developmental or gross skeletal abnormality at time of birth, but go on to spontaneously develop pathological soft tissue calcification by 2 months of age.[10] The process culminates in spinal and peripheral joint bony ankylosis by 3 to 4 months of age,[11] events suppressed by sustained administration of the PP_i analogue phosphocitrate.[12]

A remarkably similar phenotype to that of the *ank/ank* mouse, including ankylosing bone formation within perispinal ligaments and peripheral joint synovium and calcification of large arteries,[13] occurs in mice deficient in

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the PP_i-generating ecto-enzyme nucleoside pyrophosphatase phosphodiesterase 1 (NPP1; previously termed PC-1, *npps*, and *ttw*).[5,9,14] Furthermore, a primary role of PP_i depletion in the mineralization disorder of NPP1 null and *ank/ank* mice has been suggested by the partial correction of *in vitro* and *in vivo* mineralization abnormalities in both these mouse models via crossbreeding with mice deficient in the PP_i-hydrolyzing ecto-enzyme tissue-nonspecific alkaline phosphatase[6,15,16] and essentially total correction by systemic P_i deficiency.[2]

Robust endochondral and intramembranous bone formation at peripheral joint and spinal entheses and within intervertebral ligaments is a pathological hallmark of murine ANK and NPP1 deficiency.[10,17] The development of organized and tissue-restricted soft tissue calcification rather than dystrophic global calcification in extracellular PP_i-deficient animals[8,9,11–14,17] is consistent with *in vitro* evidence for direct regulatory effects of PP_i on gene expression and cellular function in the postnatal skeleton. For example, extracellular PP_i induces the hydroxyapatite crystal growth inhibitor and skeletal remodeling regulator osteopontin in osteoblasts.[5,6] Osteopontin depletion in *ank/ank* and NPP1 null primary calvarial osteoblasts critically mediates increased calcification *in vitro*.[5] Functionally significant effects of ANK and extracellular PP_i on differentiation in chondrocytes also include promotion of chondrocyte maturation and terminal differentiation and regulation of expression of matrix metalloproteinase-13, tissue-nonspecific alkaline phosphatase, and osteocalcin.[18,19] Moreover, increased chondrogenic transdifferentiation of cultured aortic smooth muscle cells (SMCs) and intra-arterial chondroid metaplasia occur in association with aortic calcification in both *ank/ank* and NPP1$^{-/-}$ mice.[13]

Chondrogenesis is modulated by the metabolism of glutathione (GSH),[20,21] a redox stress regulator that is the major reduced intracellular thiol.[22,23] Conversely, deficiency of γ-glutamyltranspeptidase, an ecto-enzyme that catalyzes GSH cleavage as a critical recycling event in cysteine metabolism, is associated with reduced tissue stores of GSH as well as dwarfism mediated not only by effects on osteoclast development[24] but also by a proliferative defect of chondrocytes rescued *in vivo* by supplementation with *N*-acetyl cysteine.[21] Vanin-1 pantetheinase is a glycosylphosphatidylinositol-anchored plasma membrane ecto-enzyme involved in cysteine and GSH metabolism.[9,22,23,25] Pantetheinases specifically hydrolyze pantetheine to pantothenic acid (vitamin B_5) and the cell-permeant sulfhydryl cysteamine (NH_2-CH_2-CH_2-SH).[26,27] Cysteamine directly inhibits γ-glutamylcysteine synthetase, the rate-limiting enzyme in synthesis of GSH.[22,23] Vnn1$^{-/-}$ mice, which have a grossly normal phenotype, lack free cysteamine in tissues and demonstrate elevated stores of GSH in multiple tissues.[22,23,25]

In this study, we defined a central role of Vanin-1 in chondrogenesis of undifferentiated *ank/ank* mesenchymal precursor cells. Our results also indicate that Vanin-1 is critical for P_i-driven chondrogenic transdifferentiation of *ank/ank* aortic SMCs and calcification by *ank/ank* artery explants. However, we observed that pathological periskeletal soft tissue calcification in *ank/ank* mice is more dependent on osteoblastic function than on the increased chondrogenic potential of *ank/ank* mesenchymal precursor cells.

Materials and Methods

Reagents

All chemical reagents were obtained from Sigma (St. Louis, MO), unless otherwise indicated. Human recombinant bone morphogenetic protein (BMP)-2, human transforming growth factor (TGF) β1, human TGFβ3, and enzyme-linked immunosorbent assay kits for assay of murine BMP-2 and active TGFβ1 were obtained from R&D Systems (Minneapolis, MN).

Mice Studied

All animal procedures were performed humanely and following institutionally approved protocols. The *ank/ank* breeding colony used was originally on a hybrid background (derived originally from crossing a C3H and C57BL/6 hybrid male with BALB/c female).[12] Heterozygote breeders were used to generate and study *ank/ank* mice and wild-type littermate progeny, with genotypes analyzed by polymerase chain reaction (PCR), as described.[5] Vnn1$^{+/-}$ mice[26] were backcrossed for more than nine generations on a C57BL/6 background and then interbred to generate and study Vnn1$^{-/-}$ mice and wild-type littermate progeny on the same background. Vanin-1 genotyping was done by PCR.[26] The *ank/ank*/Vnn1$^{-/-}$ mice were generated by crossing Ank/*ank* and Vnn1$^{-/-}$ mice to generate double heterozygotes which were bred to generate Ank/Ank/Vnn1$^{+/+}$, Ank/Ank/Vnn1$^{-/-}$, *ank/ank*/Vnn1$^{+/+}$, and *ank/ank*/Vnn1$^{-/-}$ littermates.

Isolation of Plastic-Adherent Bone Marrow Stromal Cells (BMSCs) and Mesenchymal Pluripotential Cell Enrichment

Femurs of euthanized mice were flushed with 1% fetal calf serum (FCS) containing Dulbecco's modified Eagle's medium low glucose. Washed cells removed from the femurs were subsequently depleted of hematopoietic cells via 1.44 g/L Ficoll density gradient centrifugation for 20 minutes at 800 × *g*. Remaining cells were cultured for 14 days in basal mesenchymal stem cell medium (Lonza, Walkersville, MD) supplemented with 1% glutamine (w/v), 100 U/ml penicillin, 50 μg/ml streptomycin, and 10% FCS. For chondrogenic differentiation studies of BMSCs, the adherent high-density culture system was used to study aliquots of 3×10^5 cells in a 10 μl volume placed in a 9-mm dish and allowed to adhere at 37°C for 1 hour, followed by the addition of 0.5 ml of basal medium for 24 hours, after which the medium was replaced with 0.5 ml of complete serum-free medium (CSFM) (Mediatech, Herndon, VA), supplemented with BMP-2 and TGFβ1 where indicated and replaced every 3 days. For isolation

of enriched mesenchyme-derived cell lines with chondrogenic potential, murine BMSCs were grown as above until the cells reached confluency. Cells were split 1:2 every 5 to 7 days over a period of 12 weeks as described.[27] Two successive enrichments via magnetic cell separation were performed on aliquots of 1×10^7 cells, first applying negative selection, using the Lineage Depletion Kit (Miltenyi Biotec, Auburn, CA), to reduce cells expressing hematopoietic lineage markers. Second, we applied positive selection for CD117,[28,29] using a kit from Miltenyi Biotec. Cell lines thus obtained were confirmed to maintain growth and chondrogenic potential for more than 50 passages and to retain adipogenic and osteoblastogenic potential and were therefore termed mesenchymal stem cells (MSCs). Isolated MSC lines were carried in the basal mesenchymal stem cell medium described above. In high-density nonadherent pellet culture chondrogenic differentiation studies of MSCs, aliquots of 1×10^6 cells were plated in round-bottomed 96-well plates and centrifuged for 10 minutes at $400 \times g$. After 24 hours, chondrogenesis was stimulated by change of medium to CSFM supplemented each with 10 ng/ml BMP-2 and TGFβ3, and the medium was replaced every 3 days.

Assay for Multipotential Bone Marrow Stromal Precursor Cells (Colony-Forming Unit Fibroblastoid Cells (CFU-F))

BMSC preparations were washed once and centrifuged for 10 minutes at $400 \times g$, and viable cells (assessed by trypan blue staining) were resuspended at 2.25×10^5/ml, with aliquots of 0.4 ml plated in 2-cm^2 dishes in Dulbecco's modified Eagle's medium supplemented with 10% FCS and 3.7 g/L HEPES, pH 7.3, as described.[13] Medium was replaced on days 3 and 8. CFU-F and colonies were counted on day 13 after fixation and Giemsa staining, with a colony defined as constituting a minimum of five cells per group.

Immunocytochemical Analyses of Protein Expression

For immunocytochemical analysis of Vanin-1 expression, a rabbit polyclonal antibody was generated using the keyhole limpet hemocyanin-tagged Vanin-1-specific peptide NH_3-EQTKTPTSEVSSAYSTWN-COOH as the immunogen. Cells were plated on glass coverslips coated with poly-L-lysine. After 24 hours, the cells were fixed for 15 minutes with 4% paraformaldeyhyde. Cells were then stained with 1:500 dilution of rabbit anti-Vanin-1 or 1:100 dilution of rabbit α-ANK[8] and counterstained with hematoxylin.

$^{35}S/^{3}H$ Incorporation Assay for Sulfated Proteoglycan Synthesis

To quantify the amount of ^{35}S incorporation into sulfated proteoglycans, we adapted previously described methods for study of proteoglycans.[30] In brief, cells undergoing chondrogenic differentiation were labeled with 1 μCi/ml [^{35}S]sulfur and [^{3}H]proline[31] for 24 hours before collection. Medium was removed, and cells were washed three times with phosphate-buffered saline and sulfated proteoglycans were then extracted in 8 mol/L guanidine HCl, 0.01 mol/L sodium acetate, 0.02 mol/L EDTA, 0.2 mol/L 6-aminocaproic acid, 5 mmol/L benzamidine HCl, 10 mmol/L *N*-ethylmaleimide, and 0.5 mmol/L phenylmethylsulfonyl fluoride for 24 hours at 4°C under constant rotation. Extracted samples were centrifuged for 15 minutes at $14{,}000 \times g$, with supernatants analyzed by liquid scintillation counting.

Pantetheinase Activity, GSH, and PP_i Assays

For studies of bone marrow pantetheinase activity *in situ*, 2-week-old mice were euthanized and the bone marrow was flushed from each femur with 0.5 mol/L potassium phosphate buffer, pH 8.0, containing 1% (v/v) Nonidet P-40. The samples were incubated with 30 μmol/L β-mercaptoethanol for 10 minutes at 30°C followed by the addition of 500 nmol/L S-pantetheine-3-pyruvate. The aminoethylcysteine production was recorded at 296 nm (at 30°C) at 0 and 10 minutes. Using the same approach, we determined pantetheinase activity from aliquots of 1×10^6 cells carried in high-density culture and extracted in 0.5 mol/L potassium phosphate, 1% Nonidet P-40, pH 8.0. To determine GSH and oxidized glutathione levels, we used an enzymatic recycling assay (glutathione assay kit, Cayman Chemicals, Ann Arbor, MI) in the presence of glutathione reductase and spectophotometrically determined 5-thio-2-nitrobenzoic acid generation in deproteinated cells.[32] To do so, we used 30 μg of total cell lysate protein for each sample, an amount determined by bicinchoninic acid protein assay before deproteinization.[32] Conditioned media PP_i was determined radiometrically following centrifugation at $20{,}000 \times g$ for 10 minutes to remove cellular debris, and samples were normalized per DNA concentration.[18] Alkaline phosphatase specific activity was determined as described.[13]

RT-PCR Analyses

For RT-PCR, total RNA was isolated using TriZOL (Invitrogen, San Diego, CA) and reverse-transcribed as described.[16] To perform quantitative PCR, 1 μl of a 25-fold dilution of the cDNA from specific reverse transcription reactions was amplified using the LightCycler FastStart DNA MasterPlus SYBR Green I kit (Roche Diagnostics, Indianapolis, IN) with addition of 0.5 μmol/L of each primer in the LightCycler 2.0 (Roche Diagnostics). Following amplification, a monocolor relative quantification of the target gene and reference (glyceraldehyde-3-phosphate dehydrogenase; GAPDH) analysis determined the normalized target gene to GAPDH mRNA copy ratios by the manufacturer's LightCycler software (version 4.0). All primers were designed using the LightCycler Probe Design software 2.0, and the sequences are listed in Table 1.

Arterial SMC and Aortic Ring Organ Culture Studies

Aortas from groups of three animals were pooled for digestion with 1 mg/ml collagenase I (Worthington Biochemical,

Table 1. Primers Designed for qPCR Analyses

Name	Sequence	Accession number (designed/BLAST result)
Aggrecan F	5′-TTCCATCTGGAGGAGAGGG-3′	NM_007424
Aggrecan R	5′-ATCTACTCCTGAAGCAGATGTC-3′	
ANK F	5′-ATGAGTCAGCCACCGAG-3′	AF274752/NM_020332
ANK R	5′-GGAGGAAAGAGACGACAGTT-3′	
GAPDH F	5′-CATCCCAGAGCTGAACG-3′	DQ403054/NM_199472
GAPDH R	5′-CTGGTCCTCAGTGTAGCC-3′	
MSX2 F	5′-GAGCCCGGCAGATACTC-3′	NM_013601
MSX2 R	5′-CCCGCTCTGCTATGGAC-3′	
SOX9 F	5′-CGACGTGGACATCGGTGAA-3′	NM_011448
SOX9 R	5′-GCTGCTGATGCCGTAAC-3′	
Type II collagen F	5′-CCCTGGTATGACTGGCTT-3′	NM_007743
Type II collagen R	5′-GACCACGAATCCCTTCCT-3′	
Vnn1 F	5′-TGGTAGTTCAGTGGACACG-3′	NM_011704
Vnn1 R	5′-AGGGAAGACATACCGGG-3′	
Vnn3 F	5′-CCGTTTGGGAAGTTTGGC-3′	NM_011979
Vnn3 R	5′-CGAATGGAATGGAACTGCTGA-3′	

All primers were designed from murine sequences.
F, forward primer; R, reverse primer.

Lakewood, NJ) for 10 minutes to remove remaining adventitia and endothelium, followed by placement in medium containing 2 mg/ml collagenase I, 25% elastase, and 20% FCS for 1.5 hours. Washed cells were plated in M231 medium (Cascade Biologics, Portland, OR) containing SMC growth supplement (basic fibroblast growth factor, epidermal growth factor, insulin, 5% FCS). Staining for smooth muscle actin (>95% positive) and von Willebrand factor (<1% positive) verified specificity of each SMC isolate. SMCs initially on tissue culture plates coated with 1 $\mu g/cm^2$ murine laminin to promote maintenance of contractile differentiation state were expanded for two passages before experimentation. Calcification was induced by adding 2.5 mmol/L β-glycerolphosphate and 50 μg/ml ascorbic acid, and deposited Ca^{2+} quantified by release of bound Alizarin Red S by 10% cetylpyridinium chloride.[13] Cultures of 2- to 3-mm aortic rings were performed in the aforementioned SMC growth medium supplemented with 2.5 mmol/L sodium phosphate and 7 U/ml alkaline phosphatase for 7 to 9 days.[33] To measure calcification, the aortic ring cultures were decalcified in 0.6 N HCl for 24 hours, and free calcium determined colorimetrically by stable interaction with phenolsulfonephthalein (Bioassay Systems, Hayward, CA),[34] corrected for total protein concentration (SMCs) or dry weight (aortic rings). Alternatively, aortic ring explants were treated with 0.3 μCi/ml ^{45}Ca for 24 hours before collection and incorporated ^{45}Ca was quantified by liquid scintillation counting.[33]

Studies of Primary Calvarial Osteoblasts

Mice were euthanized at 3 days of age for calvarial osteoblast isolation by sequential collagenase digestion.[15] Confluent osteoblasts were grown in α minimal essential medium containing 10% FCS, 1% glutamine, penicillin, and streptomycin, 50 μg/ml ascorbate, and 2.5 mmol/L β-glycerophosphate to induce calcification.[5]

Micro-Computed Tomography (Micro-CT) Analysis

Paws and T11–T12 thoracic vertebrae were scanned and measured by micro-CT (using vivaCT 40 scanner, SCANCO Medical, Bassersdorf, Switzerland), with an isotropic resolution of 10 μm in all three spatial dimensions operated at an energy level of 55 kV and the current of 145 μA using a 300-ms integration with 2X averaging. The number of slices varied according to the sizes of the paw and the thoracic vertebral bodies, ranging from 200 to 650 per specimen. For the trabecular compartment of the thoracic vertebral bodies, mineralized bone was separated from bone marrow with a matching cube three-dimensional segmentation algorithm. Bone volume was calculated using tetrahedrons corresponding to the enclosed volume of the triangulated surface, with total volume representing the volume of sample examined. A normalized index, bone volume/total volume, was used to compare samples of varying size. Methods used for calculating connectivity density (Conn.D.), trabecular number (Tb.N), trabecular thickness (Tb. Th), and trabecular separation (Tb.Sp) were described previously.[35] The three-dimensional images generated for each animal included the front and back view of whole paws.

Statistics

Where indicated, all error bars represent standard deviation. Statistical analyses were performed using the Student's *t*-test (paired two-sample testing for means).

Results

Increased Chondrogenic Potential of Cultured ank/ank BMSCs

The *ank/ank* BMSC preparations contained a ~70% larger pool of multipotential cells (CFU-F cells) than did

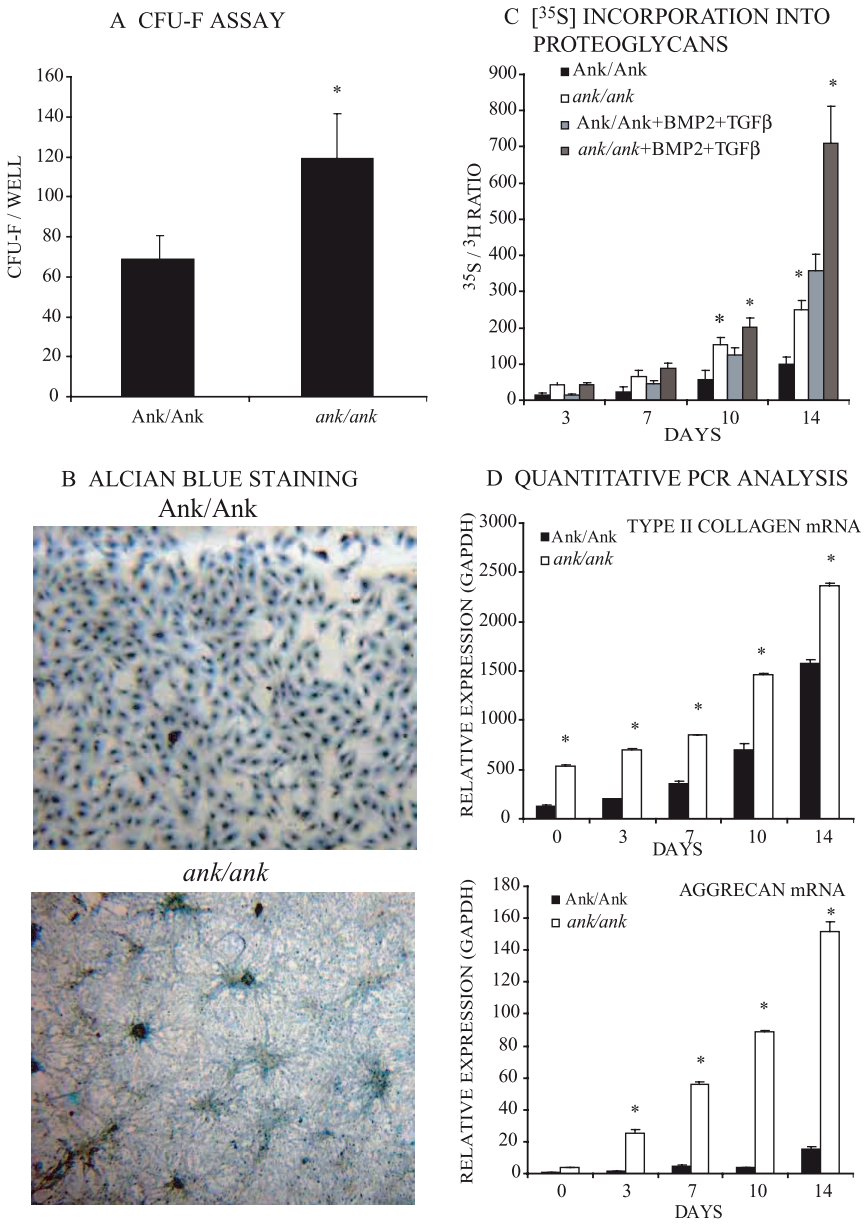

Figure 1. Increased chondrogenic differentiation of *ank/ank* BMSCs. **A:** To determine CFU-F formation in the *ank/ank* BMSCs, harvested BMSC preparations from 4-month-old wild-type and littermate *ank/ank* mice were resuspended at 2.25×10^5 cells/ml. Cell aliquots (0.4 ml) were plated in 2-cm^2 dishes in Dulbecco's modified Eagle's medium supplemented with 10% FCS and 3.7 g/L HEPES, pH 7.3. On day 13 the cells were fixed, Giemsa stained and the numbers of colonies (CFU-F) were counted. ($n = 6$). **B:** Spontaneous formation of condensed chondrogenic nodules in *ank/ank* BMSCs were determined after growth of the BMSC in basal medium for mesenchymal stem cells supplemented with 10% FCS for 14 days, at which time the cells were stained with Alcian blue/nuclear fast red. Images are representative of results from separate experiments on 10 individual mice of each genotype. Magnification, ×63. **C:** Sulfated proteoglycan synthesis in *ank/ank* BMSCs was determined with or without the addition of 10 ng/ml BMP-2 and 10 ng/ml TGFβ1 versus buffer to high-density cultures in CFSM. All cells were labeled with 1 μCi/ml [^{3}H] and [^{35}S] for 24 hours before extraction in guanidine-containing buffer on the days indicated. Data pooled from five mice per genotype. **D:** Development of cartilage-specific gene expression in BMSCs in high density culture was determined by qPCR. RNA was extracted from BMSCs pooled from four mice of each indicated genotype and cells were grown as in **C** but with no exogenous BMP-2 and TGFβ1. The RNA was reverse-transcribed, and the cDNA was amplified using the LightCycler FastStart DNA MasterPlus SYBR Green I kit for type II collagen, aggrecan, and GAPDH mRNA quantification. Data are expressed as relative expression of type II collagen/GAPDH and aggrecan/GAPDH mRNA copies (as determined by the LightCycler software) at each time point. $^*P < 0.05$.

congenic wild-type controls (Figure 1A). In addition, within 14 days in monolayer culture in a complete medium supplemented with serum and designed simply to support mesenchymal precursor cells, the *ank/ank* BMSCs, but not those from congenic wild-type controls, developed condensation into Alcian blue-staining nodules consistent with accelerated chondrogenesis (Figure 1B). To evaluate further the chondrogenic differentiation of *ank/ank* BMSCs, the cells were transferred into high-density culture in serum-free conditions after 14 days of monolayer culture, performed as above, in the basal medium to support mesenchymal stem cells. We observed heightened sulfated proteoglycan synthesis and expression of aggrecan and type II collagen mRNA, consistent with active chondrogenesis in *ank/ank* BMSC preparations (Figure 1, C and D). The enhanced sulfated proteoglycan synthesis seen in *ank/ank* BMSCs occurred in cells cultured in serum-free conditions with or without addition of recombinant BMP-2 and TGFβ1 to promote chondrogenesis (Figure 1C). Conditioned media levels of active TGFβ1 and of BMP-2, measured by enzyme-linked immunosorbent assay, did not significantly differ in *ank/ank* cells relative to controls under serum-free and exogenous cytokine-free culture conditions (data not shown).

We observed that *ank/ank* plasma had a more than fivefold elevation of pantetheinase activity relative to wild-type controls (Figure 2). Mice express two pantetheinase isoenzymes, Vanin-1 and Vanin-3.[25,26] Because active forms of both murine pantetheinase isoenzymes are released from cells,[26] we evaluated unfractionated bone marrow extracts. We focused on mice 2 weeks of age, a time point that precedes development of a gross phenotype in the *ank/ank* mouse.[11,12] RT-PCR analysis of cells in whole marrow extracts and of isolated BMSCs detected Vanin-1 expression but only trace Vanin-3 expression under these conditions (data not shown), indicating

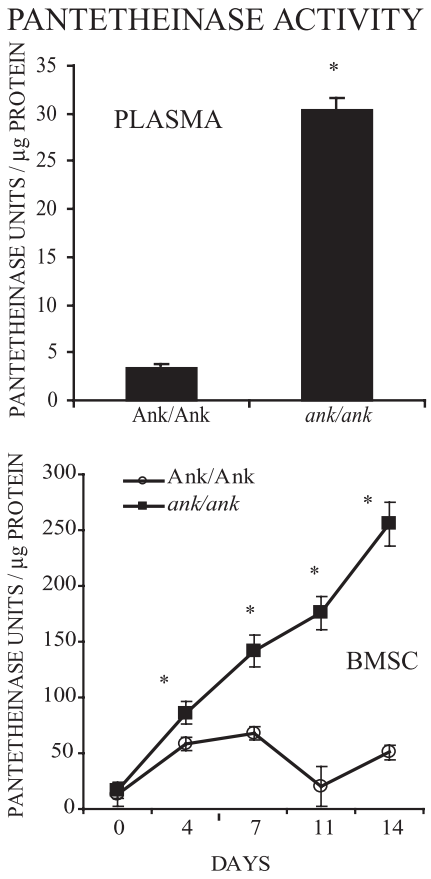

Figure 2. Up-regulated pantetheinase activity in *ank/ank* mouse plasma and BMSCs. We determined plasma pantetheinase activity levels in 2-week-old *ank/ank* and Ank/Ank mice, with data pooled from individual samples of 20 mice of each genotype (left panel). Additionally, BMSCs were grown in high density culture conditions in CSFM, and pantetheinase activity measured in cell lysates (right panel). $*P < 0.05$.

Vanin-1 to be the predominant pantetheinase in the *ank/ank* bone marrow. Studying BMSCs in high density culture conditions under which cells were undergoing chondrogenesis, we observed that *ank/ank* BMSCs had a more than twofold increase in pantetheinase activity by day 7 and more than fivefold increase by day 14 relative to wild-type littermate BMSCs (Figure 2).

Increased Vanin-1 and ANK Expression in Both BMSCs and MSC Lines Established from ank/ank Mice

RT-PCR and immunocytochemical analyses revealed constitutive, low-level expression of both Ank and Vnn1 mRNA (Figure 3A), but ANK and Vanin-1 protein expression were below limits of detection by Western blotting (data not shown) in resting wild-type BMSCs After induction of chondrogenesis by placement in high-density culture, wild-type BMSCs developed up-regulated expression of mRNA for Vanin-1 (but not Vanin-3) (Figure 3A). After induction of chondrogenesis in *ank/ank* BMSCs by placement in high-density culture, the up-regulated Vanin-1 mRNA expression gradually subsided (Figure 3B). Vanin-1 protein expression was below detection limits in wild-type Ank/Ank BMSCs but was dramatically increased by the 14th day after isolation of the *ank/ank* BMSCs. Next, MSC lines enriched from BMSCs of congenic wild-type and *ank/ank* mice were studied, and incidental note was made of accelerated osteoblastogenesis of the *ank/ank* MSCs relative to wild-type MSCs (see Supplementary Figure 1 on *http://ajp.amjpathol.org*). Significantly, the *ank/ank* MSCs provided comparable results to those with BSMCs for regulated changes in Ank and Vanin-1 expression during chondrogenic differentiation (see Supplementary Figure 2 on *http://ajp.amjpathol.org*). Notably, Vanin-1 (but not Vanin-3) mRNA expression demonstrated several hundredfold up-regulation as *ank/ank* MSCs underwent chondrogenesis (see Supplementary Figure 2 on *http://ajp.amjpathol.org*).

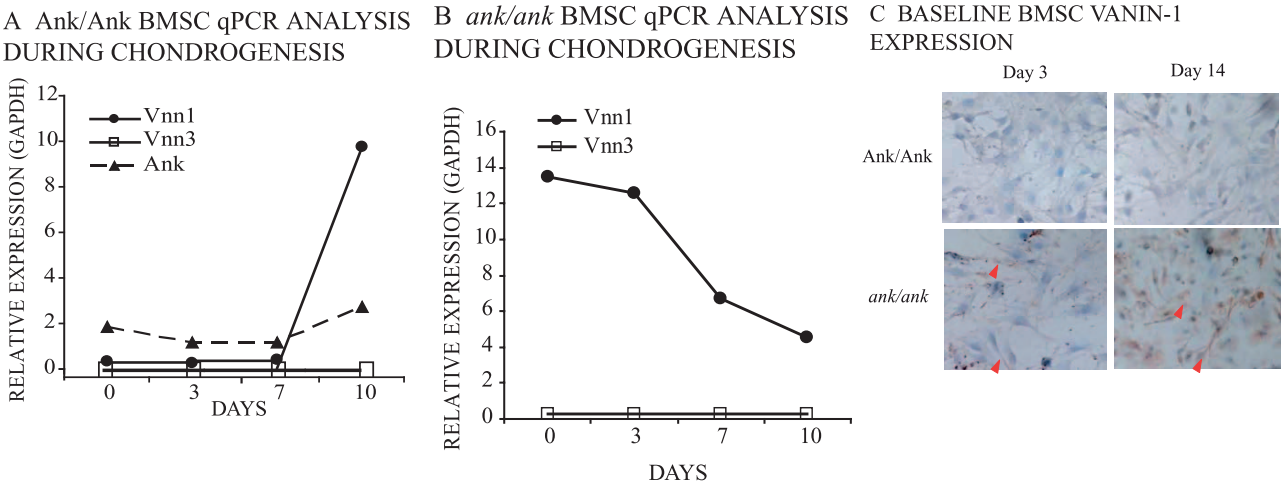

Figure 3. Expression analyses of Ank, Vanin-1, and Vanin-3 in BMSCs. **A** and **B:** We used qPCR to quantify Vnn1, Vnn3, and Ank mRNAs in BMSCs in high-density culture for 10 days in CSFM. Data reflect mRNA copies of each gene relative to GAPDH. Cells were pooled from five animals of each genotype. **C:** To examine Vanin-1 expression, aliquots of 1×10^5 BMSCs from wild-type and *ank/ank* mice, 3 and 14 days after initial isolation, were plated on coverslips coated with poly-L-lysine. After 24 hours, cells were fixed with 4% paraformaldehyde and stained with polyclonal antibodies to Ank protein (ANK), Vanin-1, and a rabbit IgG control (not shown). Proteins were visualized with AEC and counterstained with hematoxylin. Cultures pooled from five animals and images representative of five slides. Magnification, ×100.

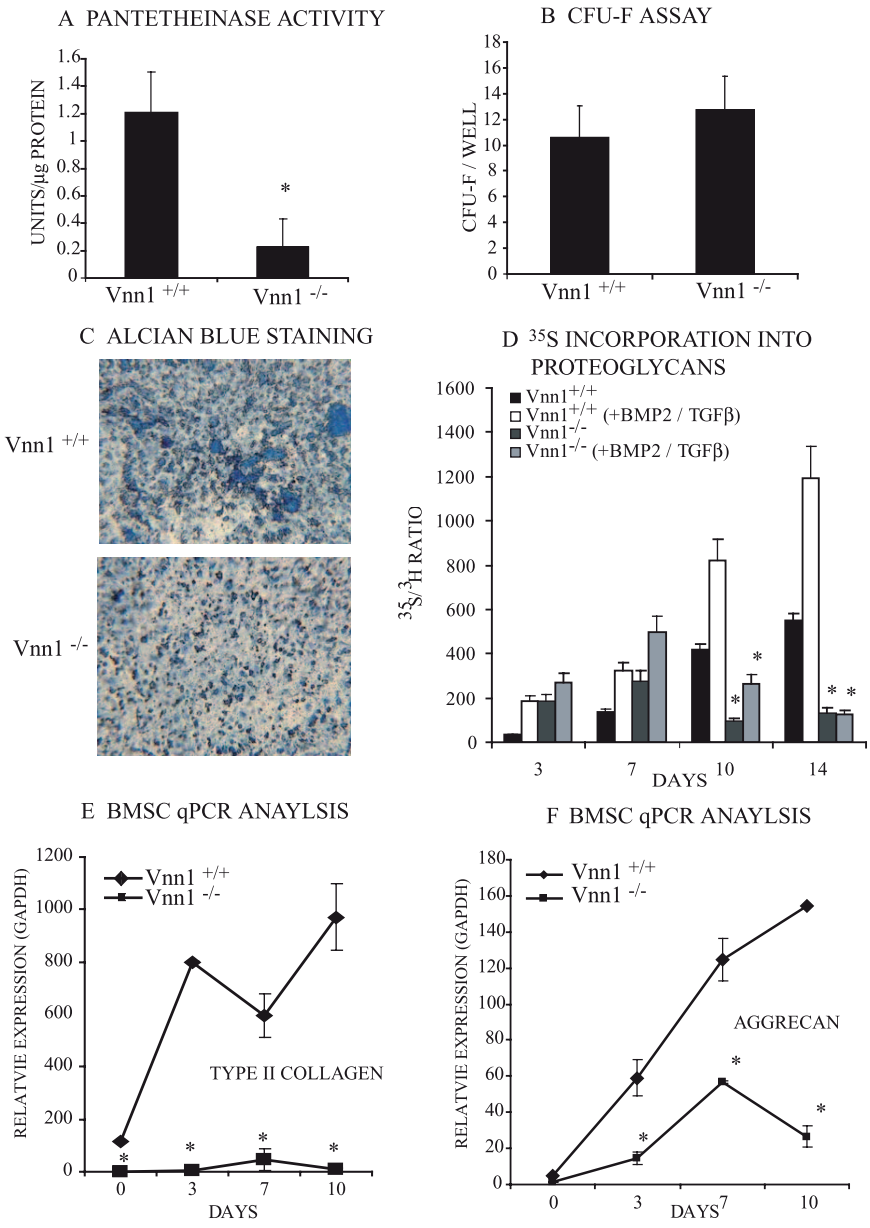

Figure 4. Delayed chondrogenesis of Vnn1$^{-/-}$ BMSCs in high-density culture. **A:** Aliquots of 3×10^5 Vnn1$^{+/+}$ and Vnn1$^{-/-}$ BMSCs from 3-month-old littermates were studied in high-density culture in CSFM. After 3 days in culture, BMSCs from each animal were extracted and assessed for pantetheinase activity in triplicate ($n = 20$). **B:** Vnn1$^{+/+}$ and Vnn1$^{-/-}$ BMSCs on day 13 were fixed and Giemsa-stained, and numbers of CFU-F were counted as in Figure 1 above ($n = 5$). **C:** Delayed formation of Alcian blue-positive nodules was found for Vnn1$^{-/-}$ BMSCs grown in high-density culture and stimulated for 10 days with 10 ng/ml each of BMP-2 and TGFβ1. Data shown are from cells pooled from five animals of each genotype. Magnification, ×63. **D:** Vnn1$^{+/+}$ and Vnn1$^{-/-}$ BMSCs were grown in high-density culture in CSFM with or without the addition of 10 ng/ml each of BMP-2 and TGFβ1, and sulfated proteoglycans synthesis assessed as above. Data pooled from three experiments done in triplicate. **E** and **F:** Vnn1$^{+/+}$ and Vnn1$^{-/-}$ BMSCs were grown in high-density culture in CSFM with 10 ng/ml each of BMP-2 and TGFβ1 and qPCR performed for type II collagen (**E**) and aggrecan mRNA (**F**) copies relative to GAPDH mRNA. Data pooled from three experiments. *$P < 0.05$.

Delayed Chondrogenic Potential of Cultured Vnn1$^{-/-}$ BMSCs

Cultured Vnn1$^{-/-}$ BMSCs were more than 80% deficient in pantetheinase activity relative to wild-type littermate controls (Figure 4A). There was no significant difference in numbers of CFU-F in these Vnn1$^{-/-}$ mouse BMSC preparations (Figure 4B), but delayed chondrogenic potential was revealed via depression of BMP-2/TGFβ1-induced condensation of cells into Alcian blue-staining chondrogenic nodules (Figure 4C). To examine further the chondrogenesis in Vnn1$^{-/-}$ BMSCs, the cells were grown in serum-free conditions with and without BMP-2 and TGFβ1. Over 14 days in culture, BMP-2 and TGFβ1 stimulated greater sulfated proteoglycan synthesis and type II collagen mRNA expression in Vnn1$^{+/+}$ cells than Vnn1$^{-/-}$ BMSCs, whose chondrogenic response to BMP-2 and TGFβ1 was suppressed (Figure 4, D and E). Unlike *ank/ank* BMSCs, Vnn1$^{-/-}$ BMSCs required BMP-2 and TGFβ1 for optimum induction of type II collagen and aggrecan mRNA (Figure 4, E and F) and sulfated proteoglycans synthesis (Figure 4D).

Modulation of Chondrogenesis by Cysteamine and GSH Stores with Vanin-1 and ANK Deficiencies

Vnn1$^{-/-}$ BMSCs had elevated GSH stores relative to wild-type cells, an abnormality reversed by treatment with the GSH synthesis inhibitor buthionine sulfoximine (BSO) at micromolar concentrations[20] (Figure 5A). In Vnn1$^{-/-}$ BMSCs, 10 to 100 μmol/L BSO treatment corrected the depression of BMP-2- and TGFβ1-stimulated sulfated

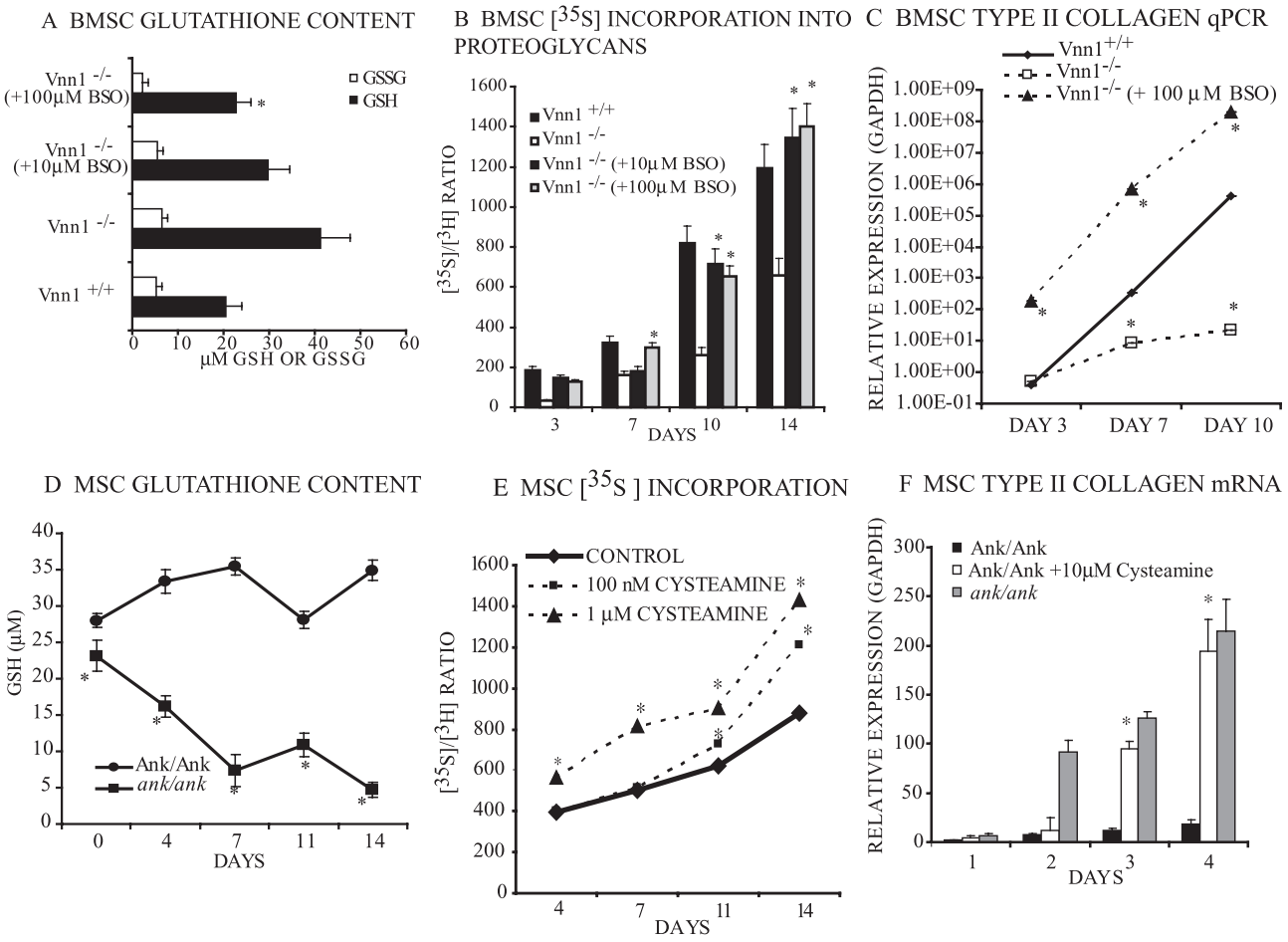

Figure 5. GSH content associated with alterations of chondrogenesis in Vnn1$^{-/-}$ BMSCs and *ank/ank* MSCs *in vitro*. Vnn1$^{+/+}$ and Vnn1$^{-/-}$ BMSCs from 3-month-old littermate animals in high-density culture in CSFM were stimulated with 10 ng/ml each of BMP-2 and TGFβ1 and treated with the GSH synthesis inhibitor BSO where indicated. **A:** GSH and oxidized glutathione (GSSG) content were determined after deproteinization of BSMCs isolated on day 7 in culture. Data pooled from three experiments in triplicate. **B:** Reversal of decreased sulfated proteoglycans synthesis in Vnn1$^{-/-}$ BMSCs by treatment with BSO. BMSCs were cultured, as described above, under conditions to promote chondrogenesis, which here included addition of 10 ng/ml each of both TGFβ and BMP-2. Data pooled from three experiments in triplicate. **C:** Effects of BSO treatment on the decreased type II collagen expression in Vnn1$^{-/-}$ BMSCs, evaluated by qPCR. **D–F:** MSCs isolated from wild-type and *ank/ank* BMSCs were carried in pellet culture in CSFM. **D:** Decreased total GSH content in *ank/ank* MSCs. Data pooled from two experiments in triplicate. **E:** Cysteamine-induced increase in sulfated proteoglycans synthesis in wild-type MSCs. Data pooled from three experiments done in triplicate. **F:** Cysteamine (10 μmol/L)-induced elevation of type II collagen mRNA in wild-type MSCs to levels comparable to those in *ank/ank* MSCs. Cells were grown in pellet culture and stimulated for 4 days with cysteamine where indicated. Data expressed as relative expression of type II collagen to GAPDH mRNA copies. Data pooled from three experiments. *$P < 0.05$.

proteoglycan synthesis in high-density culture (Figure 5B) under conditions where BSO did not increase sulfated proteoglycans synthesis in Vnn1$^{+/+}$ control cells (not shown). BSO treatment (100 μmol/L) also reversed the delay in BMP-2- and TGFβ1-stimulated type II collagen expression in Vnn1$^{-/-}$ BMSCs in high-density culture (Figure 5C).

The *ank/ank* MSCs demonstrated progressive GSH depletion in pellet culture relative to wild-type MSCs (Figure 5D). Thus, we directly tested for a role in enhancing MSC chondrogenic potential of the Vanin-1 enzymatic product cysteamine, which suppresses GSH synthesis. Cysteamine (100 nmol/L and, more potently, 1 μmol/L) increased [^{35}S]sulfur incorporation into proteoglycans in wild-type MSCs in pellet culture (Figure 5E). Furthermore, cysteamine (1 μmol/L) increased type II collagen expression in wild-type MSCs to levels comparable to those seen in untreated *ank/ank* MSCs in pellet culture (Figure 5F).

Deficiency of Vanin-1 Corrects Accelerated Chondrogenesis of ank/ank BMSCs as Well as Artery SMC and Explant Chondrogenic Transdifferentiation and Calcification

We observed correction of increased chondrogenesis of *ank/ank* BMSCs by Vanin-1 deficiency (Figure 6). Specifically, we first confirmed increased Alcian Blue-staining chondrogenic nodule formation in *ank/ank*/Vnn1$^{+/+}$ BMSCs, whereas a decrease was seen in the Ank/Ank/Vnn1$^{-/-}$ cultures (Figure 6A). In the *ank/ank*/Vnn1$^{-/-}$BMSCs in high-density culture, early formation of chondrogenic nodules was seen at day 3 but was not sustained by day 14 (Figure 6A). Furthermore, the increase in sulfated proteoglycans synthesis (assessed by [^{35}S] incorporation) was corrected in *ank/ank*/Vnn1$^{-/-}$BMSCs, as was the increase of type II collagen and aggrecan mRNA (Figure 6, B and C). Under these conditions, Va-

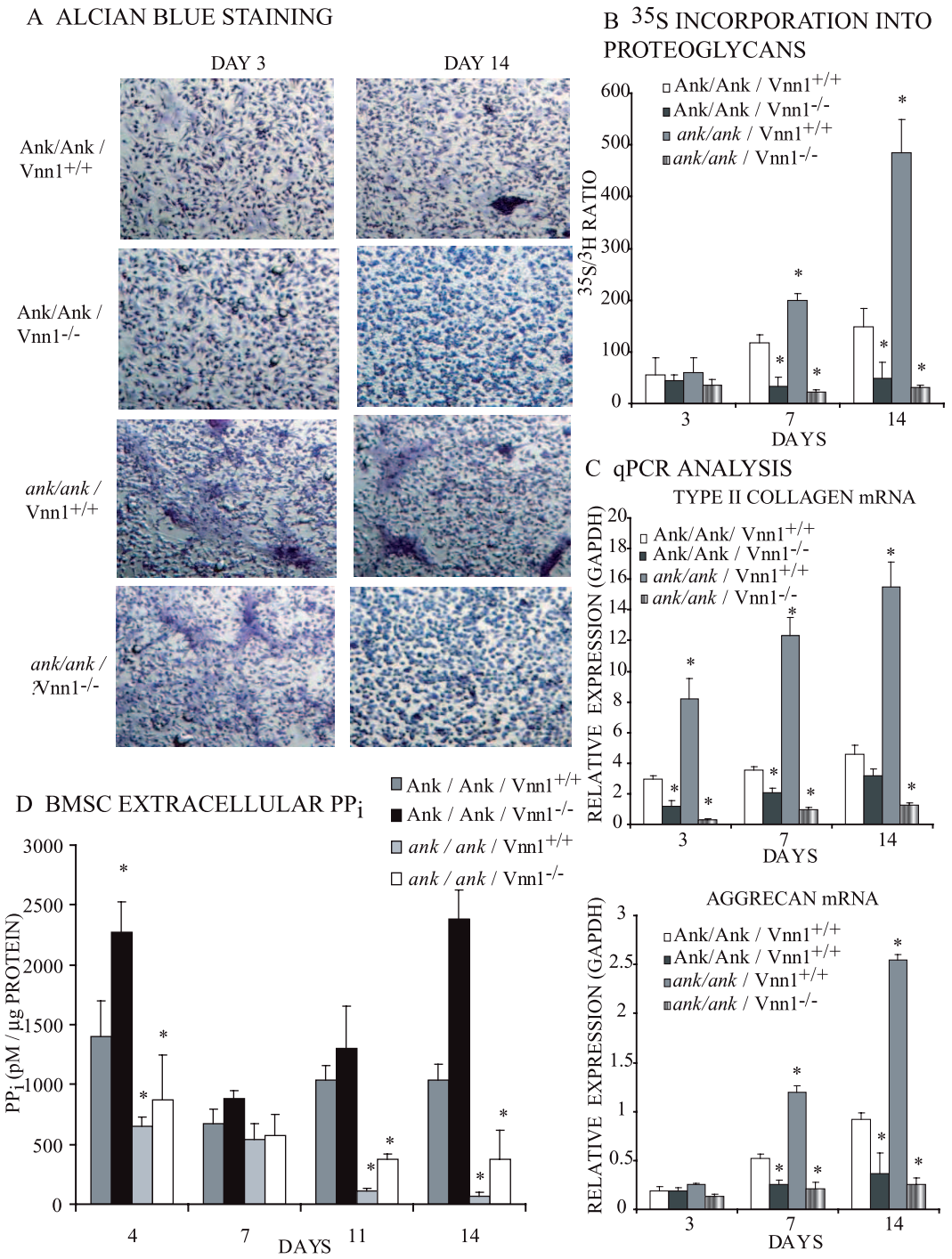

Figure 6. Alteration of the intense chondrogenic differentiation displayed by *ank/ank* BMSCs via Vanin-1 gene knockout. **A:** BMSCs of the indicated Ank and Vanin-1 genotypes from littermate animals derived by crossbreeding were studied in high-density culture with addition of BMP-2 and TGFβ1 for 3 to 14 days. Cells were fixed with 4% paraformaldehyde and stained with Alcian blue to assess chondrogenic nodule formation. Data shown are from cells pooled from four animals of each genotype. Magnification, ×40. **B:** BMSCs of *ank/ank*/Vnn1$^{-/-}$ mice had decreased extracellular PP_i during chondrogenesis. **C:** Assessment of sulfated proteoglycans synthesis in BMSCs. Data pooled from five mice each genotype. **D:** The mRNA levels of type II collagen and aggrecan relative to GAPDH were determined by qPCR. $^*P < 0.05$.

nin-1 deficiency did not correct the depressed extracellular PP_i levels of *ank/ank* BMSCs (Figure 6D). Last, we observed marked Alcian Blue staining *in situ* consistent with ectopic chondrogenesis that developed at the xiphoid process in *ank/ank* mice, a finding corrected by Vanin-1 gene knockout (see Supplementary Figure 3 on *http://ajp.amjpathol.org*).

Next, we tested if Vanin-1 deficiency corrected the known heightened propensity for cultured *ank/ank* aortic SMCs to calcify, an event associated with chondro-

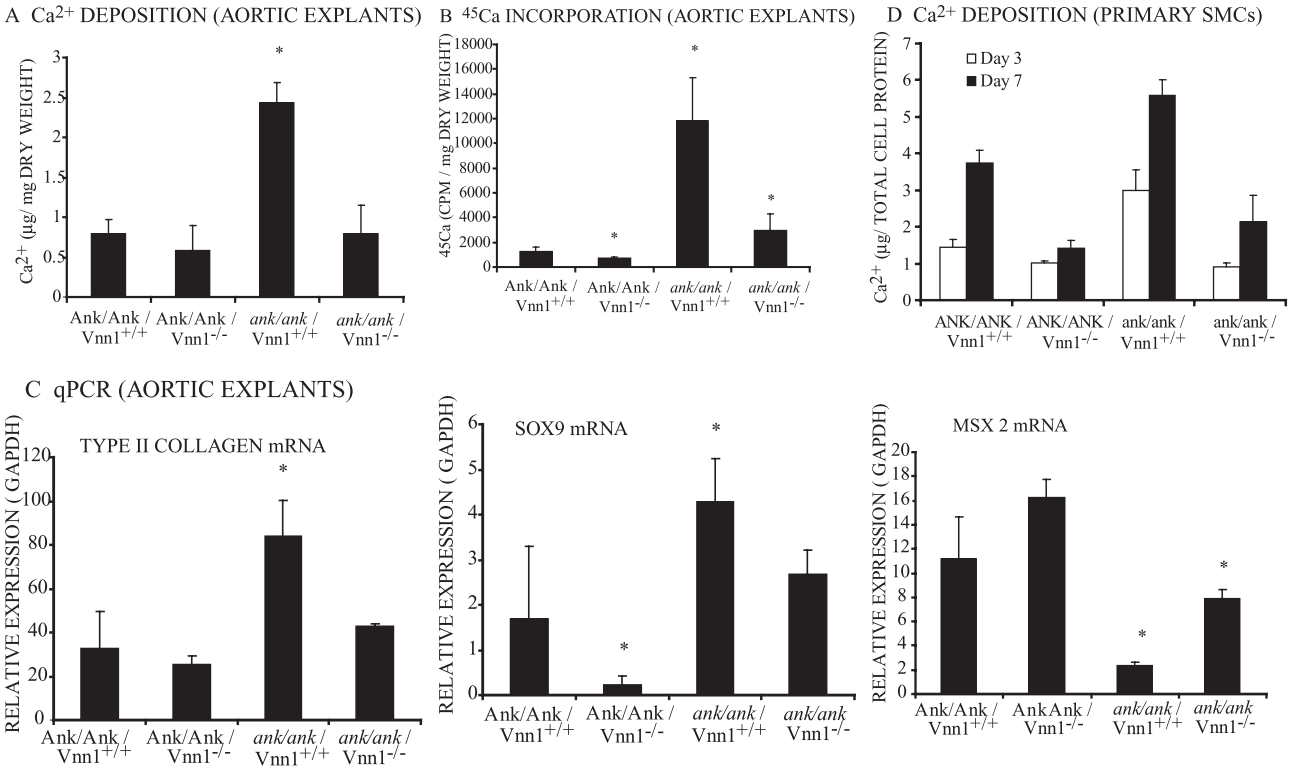

Figure 7. Correction of increased calcification of *ank/ank* aortic ring explants and cultured SMCs by Vanin-1 gene knockout. Slices of 2 to 3 mm from isolated whole aortas from 3-month-old littermate mice of the indicated genotypes were cultured in SMC growth media supplemented with 2.5 mmol/L NaP_i and 7U/ml alkaline phosphatase for 7 days. **A:** Total RNA was isolated from the aortic cultures for quantification of type II collagen, Sox9, and MSX 2 relative to GAPDH mRNA copies by qPCR. **B:** Free Ca^{2+} deposition/mg dry weight in aortic ring explants was determined by phenolsulfonephthalein binding after decalcification in 0.6 N HCl for 16 hours. Data pooled from 10 animals. **C:** Aortic explant cultures were incubated as above for 7 days prior addition of 0.3 μCi/ml ^{45}Ca for 24 hours. Aortas were collected on days 5 and 7, washed three times with phosphate-buffered saline, dried, weighed, and incorporated ^{45}Ca cpm quantified. **D:** Aliquots of 1×10^5 primary SMCs/well from 2-month-old littermate mice of the indicated genotypes were grown in 12-well dishes for 3 to 7 days in SMC growth medium supplemented with 2.5 mmol/L β-glycerophosphate and 50 μg/ml ascorbic acid. Note that the heightened Ca^{2+} deposition by *ank/ank* SMCs was corrected by Vanin-1 gene knockout, similar to the results for calcification and chondrogenic gene expression in *ank/ank* aortic explants. Data pooled from eight animals, replicates of three.

genic transdifferentiation of SMCs.[13] In addition, we examined calcification of aortic rings in organ culture, adapting a rat model[33] in which 2- to 3-mm sections of the abdominal aorta were treated with sodium phosphate and alkaline phosphatase. After 7 days, a significant increase in calcification was found in the *ank/ank*/Vnn1$^{+/+}$ aortic explants, which was corrected by Vanin-1 knockout (Figure 7, A and B). Aortic explants from *ank/ank*/Vnn1$^{+/+}$ mice demonstrated increased chondrogenic differentiation, as evidenced by increased cartilage-specific mRNA for type II collagen and the cartilage master transcription factor SOX9 (Figure 7C). In contrast, there was decreased expression in the *ank/ank*/Vnn1$^{+/+}$ aortic rings of MSX2, a transcription factor that promotes maintenance of multipotentiality but suppresses chondrogenesis[36] (Figure 7C). Each of these changes in gene expression was significantly reversed by Vanin-1 knockout in *ank/ank* aortic explants. Primary SMCs isolated from each genotype examined for calcification yielded similar results as for aortic explants, since increased calcification by *ank/ank*/Vnn1$^{+/+}$ SMCs at days 3 and 7 was corrected by Vanin-1 knockout (Figure 7D).

Vanin-1 Knockout Does Not Suppress Calcification by ank/ank Differentiated Osteoblasts and Fails to Correct the ank/ank Skeletal Phenotype

Primary calvarial osteoblasts from the Ank/Ank/Vnn1$^{-/-}$ mice demonstrated decreased matrix calcification (Figure 8A). However, Vanin-1 knockout failed to correct increased calcification by *ank/ank* primary osteoblasts (Figure 8A). In this context, Ank/Ank/Vnn1$^{-/-}$ osteoblasts demonstrated decreased extracellular PP_i, and there was unexpected further reduction in the decreased extracellular PP_i of *ank/ank* osteoblasts via Vanin-1 knockout. These findings were associated with more than doubling of specific activity of alkaline phosphatase (Figure 8, B and C), an enzyme that not only degrades PP_i but also is critically up-regulated with osteoblast maturation that drives bone mineral formation.

Initial gross observations on 3-month-old animals revealed no alteration of the joint stiffness or loss of mobility of *ank/ank* mice by Vanin-1 deficiency. The *ank/ank* mice failed to thrive.[3,8,11,12] However, there was no significant difference in body weight of 10 male mice of each geno-

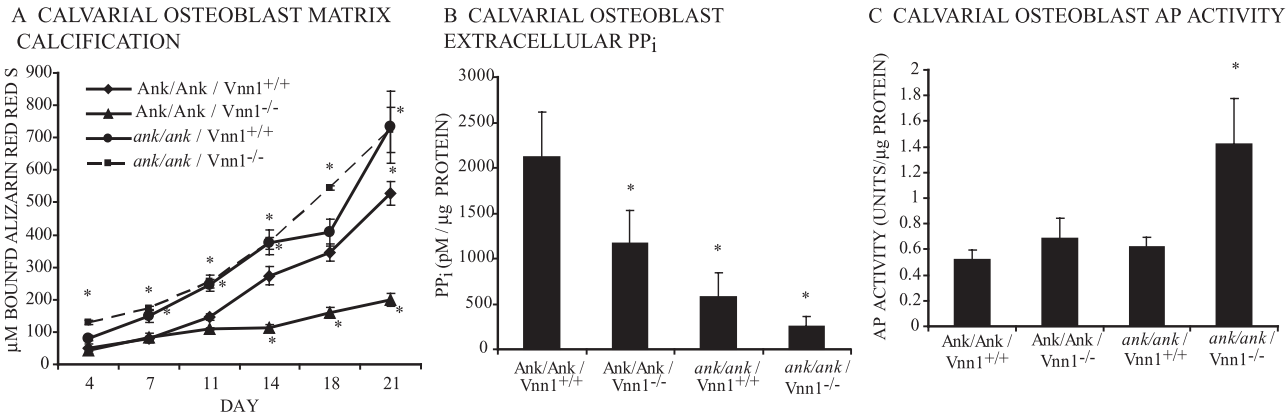

Figure 8. Vanin-1 gene knockout does not correct the increased matrix calcification of *ank/ank* calvarial osteoblasts. **A:** Calvarial osteoblasts were isolated from 3-day-old mice of the indicated genotypes and aliquots of 1×10^5 osteoblasts from each genotype plated in 12-well dishes. After adherence, the medium was replaced with α minimal essential medium containing 10% FCS, 50 μg/ml ascorbic acid, and 2.5 mmol/L β-glycerophosphate. On the days indicated, the cells were washed with phosphate-buffered saline, fixed with 4% PFA, and stained for 15 minutes with Alizarin Red S. The dye was released with cetylpryridinium chloride and quantified at OD_{570}. **B–D:** After 24 hours, the conditioned media were collected for determination of extracellular PP_i levels (**B**) and cell lysates were collected for determination of alkaline phosphatase (AP) (**C**) specific activity. Data pooled from three animals each genotype studied in triplicate. $^*P < 0.05$.

type at 2 months of age (data not shown). Vanin-1 knockout did not grossly alter thoracic vertebral mineralization in *ank/ank* mice. Specifically, in a direct comparison of T11 thoracic vertebrae analyzed by micro-CT, there were no significant differences between when comparing *ank/ank* mice to *ank/ank* mice also bearing the Vanin-1 knockout genotype (see Supplementary Table 1 and Figure 4 on *http://ajp.amjpathol.org*). Last, comparing front paws of animals of all genotypes by micro-CT analysis, we observed that the extensive ectopic calcification at the peripheral joint margins of *ank/ank* mice was not corrected by knockout of Vanin-1 (Figure 9). Our results and their significance are summarized in the schematic of Figure 10.

Discussion

Marked extracellular PP_i deficiency states are associated with tissue-restricted ectopic endochondral and intramembranous bone formation rather than being a product of simple dystrophic calcification. The first question tackled by this study was the possibility of increased chondrogenic potential of *ank/ank* mesenchymal precursor cells including MSCs. We observed that *ank/ank* BMSC preparations contained an expanded pool of multipotential CFU-F cells. Moreover, there was increased chondrogenic differentiation in not only *ank/ank* BMSC preparations enriched in multipotential cells from the bone marrow stroma but also in *ank/ank* MSC lines enriched from BMSCs. Importantly, these cells did not calcify under the experimental conditions and timeframe used[13] (K. Johnson et al, unpublished observations). Hence, our findings were not attributable to a secondary cellular reaction to matrix calcification and buttress the role of extracellular PP_i as a regulator of chondrogenesis.[13] The current study determined that ANK not only is constitutively expressed by BMSCs and MSCs but also undergoes robust down-regulation during chondrogenic differentiation.

The second question addressed was the potential role of GSH metabolism due to increased Vanin-1 in increased chondrogenic differentiation of mesenchymal

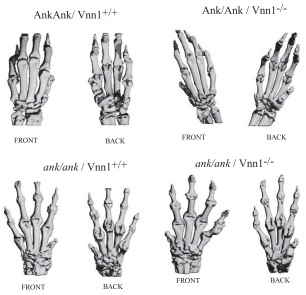

Figure 9. Lack of alteration by Vanin-1 knockout of the phenotype of ectopic calcification at the front paws of *ank/ank* mice, as evidenced by micro-CT. The front paws from five animals of each genotype were studied by micro-CT with an isotropic resolution of 10 μm in all three spatial dimensions, operated at an energy level of 55 kV and the current of 145 μA using a 300-ms integration with 2X averaging. Displayed here are images representative of the findings with each genotype indicated.

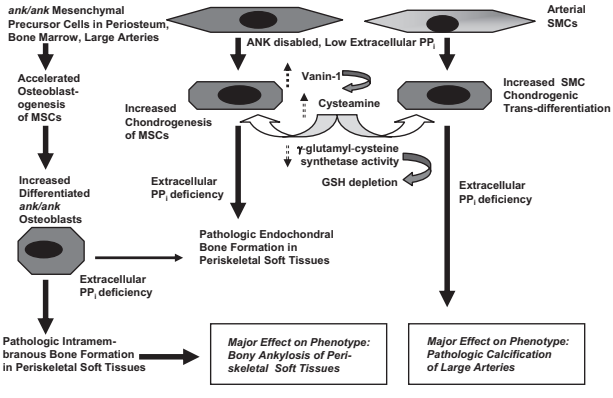

Figure 10. Schematic summarizing the results of this study. This model is based on the combined results of analyses of chondrogenesis of *ank/ank* MSCs, osteoblastogenesis of MSCs, and calcification of differentiated osteoblasts from *ank/ank* mice and the effects of Vanin-1 knockout on MSCs and tissues of *ank/ank* mice and on specific phenotypic features. In brief, the data revealed that *ank/ank* periskeletal soft tissue calcification appears more dependent on altered osteoblast development and function than accelerated chondrogenesis and is not dependent on Vanin-1. These results are reviewed in detail in the Discussion.

precursor cells in *ank/ank* mice. We implicated up-regulated expression of Vanin-1, associated with the primary defect in ANK function, as an enhancing mechanism for chondrogenic differentiation in *ank/ank* mesenchymal precursors. Increased pantetheinase activity was seen in *ank/ank* plasma and in cultured *ank/ank* BMSCs. Furthermore, cultured *ank/ank* BMSCs and MSCs constitutively expressed Vanin-1, and there was robust, Vanin isoenzyme-selective up-regulation of Vanin-1 as chondrogenic differentiation progressed in response to BMP-2 and TGFβ3. Under these conditions, marked GSH depletion also developed in *ank/ank* MSCs. Modulation of GSH stores is tissue-selective in Vnn1$^{-/-}$ mice, consistent with differential Vanin-1 expression at the tissue level.[22,23,26] Our findings suggest that modulation of both ANK function and Vanin-1 pantetheinase in MSCs are associated with autocrine regulation of GSH stores and chondrogenic commitment. This synergistic restraining mechanism for chondrogenic differentiation appears compromised in *ank/ank* mesenchymal precursors.

Our findings of Vanin-1 up-regulation, cysteamine generation, and GSH depletion as mediators of increased chondrogenic potential in *ank/ank* mesenchymal precursor cells were reinforced by the observation of decreased chondrogenic potential in cultured Vnn1$^{-/-}$ BMSCs, which was reversed by suppression of GSH generation using BSO. Vnn1$^{-/-}$ mice exhibit no gross skeletal developmental abnormalities.[26] The suppressed chondrogenic potential of cultured Vnn1$^{-/-}$ BMSCs might reflect the potential for stress-inducible skeletal development and growth abnormalities in Vnn1$^{-/-}$ mice. Vanin-1 accounted for most but not all of wild-type cultured BMSC pantetheinase activity. Though mRNA levels of the only other mouse pantetheinase isoenzyme, Vanin-3, did not significantly change during chondrogenic commitment *in vitro*, the normal skeletal development of Vnn1$^{-/-}$ mice might reflect compensatory effects of skeletal tissue pantetheinase activity attributable to Vanin-3.

The effects of Vanin-1 on chondrogenesis further uncover the networked actions of redox stress, GSH stores, and cysteine metabolism on chondrogenesis. For example, direct measures to augment cellular GSH promote chondrogenic differentiation in limb bud micromass cultures *in vitro*.[20,21] Oxidative stress and tissue GSH stores can modulate activation of the redox-sensitive transcription factors AP-1 and nuclear factor κB, which regulate chondrogenesis.[2,37–39] In addition, degradation of GSH stores by γ-glutamyl transpeptidase critically supports intracellular levels of cysteine, a requisite mechanism to maintain endochondral chondrocyte proliferation.[21] In this study, free cysteamine alone induced sulfated proteoglycans synthesis and collagen II expression in wild-type MSCs, elevating collagen II expression in pellet culture in wild-type MSCs to levels comparable to those in cultured *ank/ank* MSCs without cysteamine treatment. Though generation by Vanin-1 of cysteamine at physiological concentrations inhibits GSH generation by suppressing γ-glutamylcysteine synthetase activity,[22,26] cysteamine acts as an antioxidant at relatively high concentrations (ie, $\geq$0.1 mmol/L), at which provision of cell-permeant free SH groups by cysteamine directly promotes intracellular GSH formation.[40] Direct examination of the role of cysteamine in *ank/ank* skeletal pathology *in vivo* will be of interest. We speculate that regulation of GSH stores by Vanin-1 and regional accumulation of cysteamine could have effects on chondrogenic differentiation and on chondrocyte growth and mineralization in *ank/ank* mice that change over time. Physiological cysteamine and cystamine interconversion[26] also could indirectly regulate osteoblast differentiation and chondrocyte maturation in *ank/ank* mice, since cystamine inhibits caspase-3[41–43] and transglutaminase activities,[41,44,45] respectively.

Our results revealed that Vanin-1 was critical for increased chondrogenic transdifferentiation and P_i donor-induced calcification of cultured *ank/ank* artery SMCs and of aortic ring sections in organ culture. Chondrogenesis is a multistep transcriptionally regulated process[46] that requires recruitment and commitment of undifferentiated mesenchymal cells into chondroprogenitors, which condense in an N-cadherin-mediated manner and differentiate into chondrocytes.[39,47,48] Sox9 promotes multiple steps in this process, subject to effects of direct interaction with β-catenin.[1] Sox9-mediated expression of Sox5 and Sox6 further promotes condensation and chondrocyte differentiation.[49] Sox9 also can cooperatively promote Vanin-1 transcription.[50] Thus, accelerated chondrogenesis by itself could potentially promote increased Vanin-1 expression in *ank/ank* mice. However, in this study, up-regulation of bone marrow pantetheinase activity was observed in 2-week-old *ank/ank* mice, a point before development of a gross skeletal phenotype. Additionally, robust up-regulation in Vnn1 mRNA expression was seen early (day 5) during chondrogenic differentiation in cultured *ank/ank* MSCs in this study. Hence, Vanin-1 might play an early amplifying role in pathological chondrogenesis in soft tissues in *ank/ank* mice.

The third question addressed was the possible propensity for pathological soft tissue calcification of *ank/ank* mice requiring increased chondrogenic potential mediated by Vanin-1, and whether such a requirement might differ for soft tissue calcification at skeletal sites versus the artery wall. The knockout of Vanin-1, despite correcting increased chondrogenic potential of *ank/ank* mesenchymal precursor cells, failed to correct either the enhanced calcification by *ank/ank* differentiated calvarial osteoblasts in culture or the ectopic mineral formation around the *ank/ank* skeleton that causes the lethal immobility of the *ank/ank* phenotype.[2] Clearly, any effects of Vanin-1 on pathological soft tissue calcification *in vivo* in *ank/ank* mice are exceeded by effects of P_i,[2] possibly mediated by effects of P_i on cell differentiation in addition to matrix calcification.

Limitations of this study included analyses confined to mixed cell populations of BMSCs enriched in multipotential cells[51] and of BMSC-derived MSCs with chondrogenic potential. Phenotypic abnormalities in *ank/ank* mice may not be primarily mediated by bone marrow function, as transplantation of normal bone marrow into lethally irradiated *ank/ank* mice has failed to inhibit the characteristic phenotype from developing.[11] Conversely, transplantation of *ank/ank* bone marrow into lethally irradiated

normal mice failed to induce characteristic *ank/ank* phenotypic changes.[11] Our findings in BMSCs and bone marrow-derived MSCs appear in line with the long-suspected increase in chondrogenic potential of multipotential cells in arteries, periosteum, perispinal ligaments, and synovium in *ank/ank* mice.[5,9,12–14] This study did not define how increased Vanin-1 expression arose in association with deficient ANK function. A role of extracellular PP_i depletion is suspected as NPP1$^{-/-}$ mice also demonstrate increased pantetheinase activity (K. Johnson et al, unpublished observations), and the capacity of ANK to promote movement of both PP_i into and out of cells[52] may play a role by tuning how cells sense extracellular PP_i. The observed changes in alkaline phosphatase activity, and PP_i levels in Vnn1$^{-/-}$ primary calvarial osteoblasts likely contributed to lack of phenotype correction for *ank/ank* mice. However, we have not mechanistically addressed if altered PP_i metabolism specific to *ank/ank* osteoblasts accounted for their altered calcification and alkaline phosphatase activity associated with Vanin-1 deficiency. Last, preliminary evaluation of thoracic vertebral mineralization by micro-CT did not reveal significant differences between the *ank/ank* mice and the *ank/ank* mice additionally bearing the Vnn1$^{-/-}$ genotype. Extensive study, including assessment of multiple regions, will be needed to test for changes in bone mineralization in due to Vanin-1 deficiency in mice with normal ANK function and PP_i metabolism.

Significantly, this study revealed accelerated osteoblastogenesis of *ank/ank* MSCs relative to wild-type controls in this study. However, we have not yet assessed the potential role of Vanin-1 in this finding, and it will be of interest to ascertain if Vanin-1 plays a functional role in an early branch point where chondrogenesis and osteoblastogenesis are separated from adipogenesis. For example, we have not yet tested if Vanin-1, as in other cells,[53] suppresses the expression and function in BMSCs and MSCs, of peroxisome proliferator-activated receptor γ, a promoter of adipogenesis but suppressor of osteochondral differentiation in mesenchymal precursor cells.[54] We have not directly examined long-term effects of Vanin-1 gene knockout on phenotype and *ank/ank* mortality.[2] Last, because artery calcification is mild and is detected later than skeletal abnormalities in *ank/ank* mice,[13] we did not evaluate the effects of Vanin-1 deficiency on artery calcification *in situ* in *ank/ank* mice.

In conclusion, we have demonstrated that defective ANK function promotes chondrogenic differentiation in BMSCs and MSCs. Furthermore, up-regulation of Vanin-1 that develops in cells with defective ANK function has the potential to amplify chondrogenic differentiation, mediated in part by cysteamine generation and effects on GSH stores in MSCs. Our results add to growing evidence[5,6,13,18,19] that local ANK expression and regulated PP_i generation and transport function to modulate cell differentiation in not only physiological suppression of soft tissue calcification but also in postnatal skeletal remodeling. Further *in vivo* analyses of *ank/ank* mice will be pertinent to fully dissect the temporal, mechanistic, and spatial relationships between extracellular PP_i depletion, Sox9 expression, Vanin-1 expression, osteochondral development, and pathological calcification. Nevertheless, our study indicates that *ank/ank* periskeletal soft tissue calcification appears more dependent on altered osteoblast development and function than accelerated chondrogenesis and is not dependent on Vanin-1.

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