The CPPDD-Associated *ANKH M48T* Mutation Interrupts the Interaction of ANKH with the Sodium/Phosphate Cotransporter PiT-1

JOHN WANG, HING WO TSUI, FRANK BEIER, and FLORENCE W.L. TSUI

ABSTRACT. ***Objective.*** Numerous dominant human homolog of progressive ankylosis (*ANKH*) mutations have been identified in familial calcium pyrophosphate dihydrate crystal deposition disease (CPPDD). Due to the dominant nature of these mutations, we investigated whether ANKH interacts with other proteins; and if so, whether any CPPDD-associated *ANKH* mutation might disrupt such protein interactions.

Methods. Stable ATDC5 *ANKH wt*- and *ANKH M48T*-transfectants were generated. Lysates from these transfectants were used to identify candidate protein interaction with ANKH by coimmunoprecipitation followed by Western blot analysis. The effect of high phosphate on the expression of genes involved in modulating Pi (inorganic phosphate)/PPi (inorganic pyrophosphate) homeostasis in these transfectants was assessed.

Results. We showed that ANKH protein associates with the sodium/phosphate cotransporter PiT-1, and that ANKH M48T mutant protein failed to interact with PiT-1. We also showed that upon high phosphate treatment, the normally coordinated upregulation of endogenous *Ank* and *PiT1* transcript expression was disrupted in *ANKH M48T* transfectants.

Conclusion. Our results suggested that there is a coordinated interrelationship between 2 key participants of Pi and PPi metabolism, ANKH and PiT-1. (First Release April 15 2009; J Rheumatol 2009;36:1265–72; doi:10.3899/jrheum.081118)

Key Indexing Terms:
CALCIUM PYROPHOSPHATE DIHYDRATE CRYSTAL DEPOSITION DISEASE ANKH
PiT-1 INORGANIC PHOSPHATE INORGANIC PYROPHOSPHATE

Elegant molecular studies on the recessive *ank* (progressive ankylosis) mouse led to cloning of the *Ank* gene that codes for a regulator of inorganic pyrophosphate (PPi) transport, and identification of the molecular basis of the *ank* mutation in this mutant mouse[1]. Since then, in humans, various dominant human homolog of *Ank* (*ANKH*) mutations were detected in patients with 2 rare diseases [craniometaphyseal dysplasia (CMD)[2,3] and familial calcium pyrophosphate dihydrate crystal deposition disease (CPPDD)][4-8]. The precise mechanisms whereby these *ANKH* mutations lead to disease development are not entirely clear. Studies using transgenic mice bearing a couple of these mutations showed that it is likely that patients with CMD have dominant negative *ANKH* mutations, while patients with familial CPPDD have "gain-of-function" *ANKH* mutations[9]. The dominant nature of *ANKH* mutations in these 2 diseases suggests that the ANKH protein might interact with a membrane or signaling component(s). To date, there is no published report on proteins that interact with ANKH. Examinations of the functional consequences of CPPDD-associated *ANKH* mutations have thus far been focused on the effect of these mutations on extracellular PPi levels, and the results remain controversial[4,7,10]. In situations where gain-of-function mutations are subjected to feedback transcriptional down-regulation, the outcome measured from cell culture systems will depend on the delicate balance between the gain-of-function conferred by the mutation and the intrinsic compensatory dampening of the response, leading to inconsistent and contradictory results from different laboratories. Similar to an *Ank* wild-type transgene, a BAC transgene with the *M48T* mutation rescued the joint phenotype in *Ank* null mice. However, unlike patients with the *ANKH M48T* mutation, no pathological CPPDD was found in *Ank* null mice with the *Ank M48T* transgene[9]. It remains unclear whether this was due to the lack of an appropriate environment for CPPD crystal formation in mice.

From Toronto Western Research Institute, University Health Network; University of Toronto, Toronto; and University of Western Ontario, London, Ontario, Canada.

Supported by Canadian Institutes of Health Research (awarded to Dr. F.W.L. Tsui).

J. Wang, MSc, MBA, Research Assistant; H.W. Tsui, PhD, Research Associate, Toronto Western Research Institute; F. Beier, PhD, Associate Professor, University of Western Ontario; F.W.L. Tsui, PhD, Senior Scientist, Genetics and Development Division, Toronto Western Research Institute, Associate Professor, Department of Immunology, University of Toronto.

Address reprint requests to Dr. F.W.L. Tsui, Toronto Western Hospital, Mc14-419, 399 Bathurst Street, Toronto, Ontario M5T 2S8, Canada. E-mail: ftsui@uhnres.utoronto.ca

Accepted for publication January 8, 2009.

The control of systemic inorganic phosphate (Pi) homeostasis is known to be important for mineralization and bone formation. *ank/ank* mice and *Enpp1*$^{-/-}$ mice on high Pi diet showed increased mineralization of bones, and ectopic mineralization in the extracellular matrix (ECM) of arteries and skin[11], implying that these mutant mice cannot maintain a balance in Pi/PPi homeostasis. Numerous studies in chondrocytes[12], cementoblasts[13], and osteoblasts[14] showed that the expression profiles of genes that regulate [Pi] and [PPi] (such as *Ank*, *Enpp1*, *TNAP*, and *PiT-1*) are in turn changed by the Pi and PPi levels, indicating that the balance of Pi/PPi is strictly controlled by a complex interplay of these genes. Based on work with embryonic chick hypertrophic chondrocytes, Wang and colleagues proposed the following model for how Ank expression might affect Pi and PPi homeostasis[12]. Enhanced Ank PPi transport activity results in an increased e[PPi] and hydrolysis of e[PPi] by TNAP. Pi resulting from PPi hydrolysis then enters the cell through Na^+/Pi cotransporters (such as PiT-1). Transport of Pi into the cell results in a further stimulation of TNAP expression. The regulation of TNAP expression and activity by Ank ensure that sufficient TNAP activity is available at the outer membrane surface of growth plate chondrocytes and matrix vesicles to remove PPi, which is a potent inhibitor of mineralization. It is not entirely clear whether this model of regulation of TNAP expression by Ank in chicken hypertrophic growth plate chondrocytes is applicable to mammalian chondrocytes.

We recently showed that the *ANKH ΔE490* mutation in CPPDD affects TNAP activities in stable ATDC5 transfectants, suggesting an interplay of ANKH and TNAP activities[15]. In our study, we investigated whether another CPPDD associated mutation, *ANKH M48T*, might affect Pi homeostasis.

MATERIALS AND METHODS

DNA transfection. ANKH cDNA (*wt* and *M48T*) was subcloned into plasmid cytomegalovirus (pCMV) Tag 5A (Stratagene, La Jolla, CA, USA) in frame with the *c-myc* tag at the 3' end. All constructs were sequenced to ensure that no mutations occurred during the cloning process. The *ANKH* constructs or the empty vector (*neo*) were transfected into ATDC5 cells[16], using Fugene 6 (Roche). The transfectants were selected and maintained in G418. To avoid clonal bias[17], populations of stable transfectants (from 2 separate transfections) were used in all experiments. Differentiation of ATDC5 transfectants was induced in culture medium with 1% ITS (insulin, transferrin and selenium; Sigma). The cultures were fed with fresh ITS-containing medium every 2 days.

Immunoprecipitation and immunoblotting. ATDC5 transfectants were lysed with 50 mM Tris-HCl, pH 7.5, with 150 mM NaCl, 1% NP40, and a mixture of protease inhibitors (leupeptins, pepstatin A, and antipain). An aliquot of the lysates was first immunoprecipitated with an anti-myc antibody (Invitrogen). The immunoprecipitates were run on SDS-PAGE, and transferred to Immobilon-P. After blocking with 3% bovine serum albumin (Sigma) in TBST (10 mM Tris, pH 8, 500 mM NaCl, 0.1% Tween 20) for 1 h, the blots were incubated with a goat anti-ANK antibody (kindly provided by Dr. J.A. Winkles, University of Maryland School of Medicine, Baltimore, MD, USA)[18] or an anti-PiT-1 antibody (Alpha Diagnostic) for 45 min and then with horseradish peroxidase-conjugated anti-goat antibody (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) for 30 min. Specific signals were detected by chemiluminescence using Supersignal® West Femto maximum sensitivity substrates (Pierce Biotech) and imaging (BioRad).

Real-time reverse transcription-polymerase chain reaction (RT-PCR). RNA from the ATDC5 transfectants was prepared using the Trizol method. Oligo-dT primers were used for RT. Multiple dilutions of the RT mix were used for amplification to ensure linearity. The same primer pairs were used as reported[15]. *β-actin* expression was used for normalization. PCR reactions in triplicates were carried out for each sample using iQ SYBR mix (BioRad). Melt curves were done at the end of each PCR. Relative quantification of gene expression was carried out using the $2^{-\Delta\Delta Ct}$ method[19]. Each sample was compared to the expression of *neo* controls at Day 0. At least 2 different RNA preparations and 4 separate experiments were carried out for relative quantification of expression of each gene transcript. For each gene, statistical significance (p value < 0.05) among the transfectants was calculated using 2-way analysis of variance (ANOVA) and pairwise multiple comparison procedures (Holm-Sidak method).

RESULTS

Effect of ANKH M48T mutation on the interaction of ANKH with PiT-1. We recently reported that ATDC5 transfectants stably expressing the *ANKH ΔE490* mutation had low alkaline phosphatase activities throughout ITS treatment due to lower TNAP protein expression and the presence of intracellular low molecular weight inhibitors[15]. In view of recent reports that Pi/PPi homeostasis is strictly controlled by a complex interplay of genes that regulate Pi and PPi concentrations[12-14], we investigated whether ANKH physically associates with PiT-1 due to the dominant nature of the CPPDD-associated *ANKH* mutations. Using various transfectant lysates, we immunoprecipitated the tagged ANKH proteins using an anti-myc antibody. The Western blot of the immunoprecipitates was probed with an anti-PiT-1 antibody. The same Western blot was also reprobed with an anti-ANK antibody. As the *neo* control transfectants expressed no myc-tagged ANKH proteins, we detected neither the ANKH-myc nor the PiT-1 bands (Figure 1A, lane *neo*). From the *ANKH wt*-transfectants, PiT-1 was detected in the anti-myc immunoprecipitates, suggesting that PiT-1 associated with ANKH wt proteins (Figure 1A, lane wt). PiT-1 was also coimmunoprecipitated with the myc-tagged ANKH ΔE490 mutant proteins. Interestingly, PiT-1 failed to coimmunoprecipitate with the myc-tagged ANKH M48T proteins. Similar amounts of ANKH-myc proteins were detected in the immunoprecipitates from all the transfectants except the *neo* control (Figure 1A, bottom panel). Further, similar amounts of PiT-1 protein were present in the lysates of *ANKH-wt*- versus *ANKH-M48T*-transfectants, indicating that the failure to coimmunoprecipitate PiT-1 with ANKH M48T proteins was not due to the lack of PiT-1 proteins in the lysates (Figure 1B).

Coordinated upregulation of Ank and PiT1 gene expression was disrupted in ANKH M48T-transfectants upon treatment with high Pi. We next assessed the functional outcome of the disrupted ANKH-PiT-1 interaction consequent to the *ANKH*

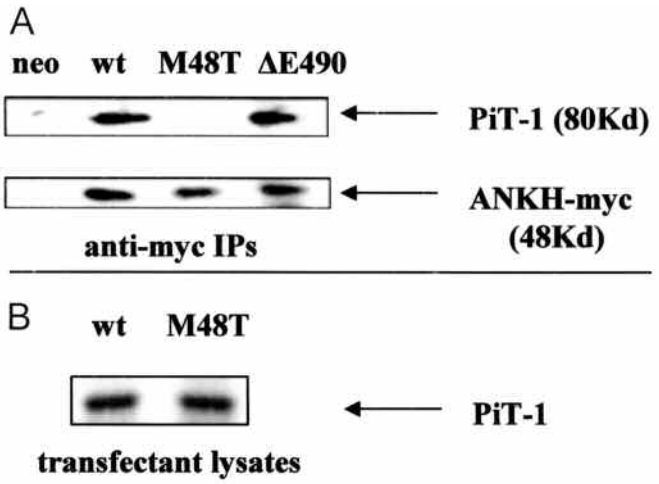

Figure 1. ANKH wt- but not ANKH M48T- protein interacts with PiT-1. A. Coimmunoprecipitation of ANKH and PiT-1 in the various transfectants. Western blots of anti-myc immunoprecipitates (IP) from different transfectants were probed with an anti-PiT-1 antibody (top panel) and with an anti-ANK antibody (bottom panel). B. A Western blot of lysates from *ANKH wt*- versus *ANKH M48T*-transfectant was probed with an anti-PiT-1 antibody.

M48T mutation. Our hypothesis is that ANKH-PiT-1 interaction ensures a balance in PPi export and Pi import. We anticipated that expression of genes involving Pi/PPi homeostasis would be dysregulated by treating *ANKH M48T* transfectants with high Pi. Thus, we treated ITS D17 *ANKH* transfectants (*wt* and *M48T*) and the *neo* controls with 0, 2, and 5 mM of Pi for 48 h and determined whether *PiT-1*, *Ank*, and *TNAP* transcript expressions were differentially affected. Real-time RT-PCR was carried out to assess the relative expression of *PiT-1*, *Ank*, *TNAP*, and *PiT-1*, and normalizations were carried out with *ß-actin* expression. The primer-pair for *Ank* amplified both endogenous *Ank* and transfected *ANKH* transcripts. We do not expect that the transfected *ANKH* levels would be modulated by Pi treatment, as the transfected cDNA is driven by a CMV promoter. Treatment of *neo* controls with Pi in the culture medium resulted in a 1.6 to 2.7-fold increase in *PiT-1* transcript expression (Figure 2A). Compared to *neo* controls, *ANKH wt*-transfectants had 1.6-fold higher, while *ANKH M48T*-transfectants had ~1.7-fold less *PiT-1* expression in the absence of exogenous Pi. There were no significant changes in the levels of *PiT-1* expression when either *wt*- or *M48T*-transfectants were treated with 2 or 5 mM Pi in the culture medium (Figure 2A). *Neo* controls cultured in 5 mM Pi showed a 1.8-fold increase in *Ank* transcripts (Figure 2B). No significant changes of total *Ank/ANKH* expression were detected in untreated or Pi treated *ANKH wt*-transfectants. In contrast, *ANKH M48T*-transfectants treated with 2 or 5 mM Pi showed a 2.4 and 1.9-fold increase in *Ank* transcript, respectively (Figure 2B). There was no change in *TNAP* expression in untreated or Pi treated *neo* controls (Figure 2C). Untreated *ANKH wt*-transfectants had 6-fold more *TNAP* transcripts than the *neo* controls. The *TNAP* transcript expression was downregulated (~4-fold) when the *ANKH wt*-transfectants were treated with 5 mM Pi (Figure 2C). Interestingly, untreated *ANKH M48T*-transfectants had 60-fold higher *TNAP* levels than the *neo* controls and Pi treatment led to a downregulation of *TNAP* expression in a dose-response manner. However, even at 5 mM Pi, *ANKH M48T* transfectants had ~9 to 14-fold more *TNAP* transcript than *neo* controls and *ANKH wt*-transfectants, respectively.

In summary, in *neo* controls, high [Pi] induced upregulation of *Ank* and *PiT-1* expression coordinately. However, in *ANKH M48T*-transfectants, high [Pi] induced upregulation of *Ank*, but not *PiT-1* expression. In addition, *TNAP* expression was significantly higher in untreated and Pi treated *ANKH M48T*-transfectants. We also investigated whether the expression of *ENPP-1* (which codes for a PPi-generating ectonucleotidase) is affected by the overexpression of wt or mutant *ANKH* in the presence or absence of exogenous Pi. The expression of *ENPP-1* was similar in *ANKH wt*-transfectants and *neo* controls (Figure 2D) in the absence of exogenous Pi. High [Pi] induced a 2-fold increase in *ENPP-1* expression in *ANKH wt*-transfectants, but resulted in a ~2-fold decrease in *ENPP-1* expression in *neo* controls. In contrast, in the absence of exogenous Pi, *ANKH M48T*-transfectants had a 2-fold increase in *ENPP-1* expression compared to that of *neo* controls, and this level of *ENPP-1* expression was maintained upon high [Pi] treatments (Figure 2D). As mentioned, these experiments were carried out in ATDC5 transfectants treated with ITS for 17 days (Day 17 ITS). The same set of experiments was also carried out in transfectants without ITS treatments, and similar results were obtained (data not shown).

Does overexpression of ANKH M48T change the kinetics of chondrocyte differentiation in ATDC5 transfectants? Neo controls and *ANKH*-transfectants (*wt* or *M48T*) were induced with ITS for 10, 17, and 23 days (ITS Day 10, Day 17, Day 23). At each timepoint, real-time RT-PCR was carried out to assess the relative expression of chondrocyte markers [3 Sox transcription factors: *Sox 9, Sox 5,* and *Sox 6*; 1 early chondrocyte marker: *collagen IIa1* (*Col 2α1*); and 3 hypertrophic chondrocyte markers: *bone sialoprotein* (*BSP*), *collagen X* (*ColX*), and matrix metalloproteinase (*MMP-13*). After normalization with *ß-actin* expression, the results were expressed as fold-changes relative to the expression in Day 0 *neo* controls. For each timepoint, we also determined the ratio of expression (listed below each plot at each timepoint in Figure 3). The stable *ANKH*-transfectants (*wt* or *M48T*) had similar *ANKH* transcript and ANKH protein expression (data not shown). Overexpression of ANKH wt-proteins in ATDC5 cells at the prechondrocyte stage (Day 0 cells with no ITS treatment) appears to disturb the normal induction of chondrogenesis by ITS. Similar to *ANKH wt*-transfectants, the *ANKH M48T* transfectants also appeared to be more differentiated than the *neo* controls at

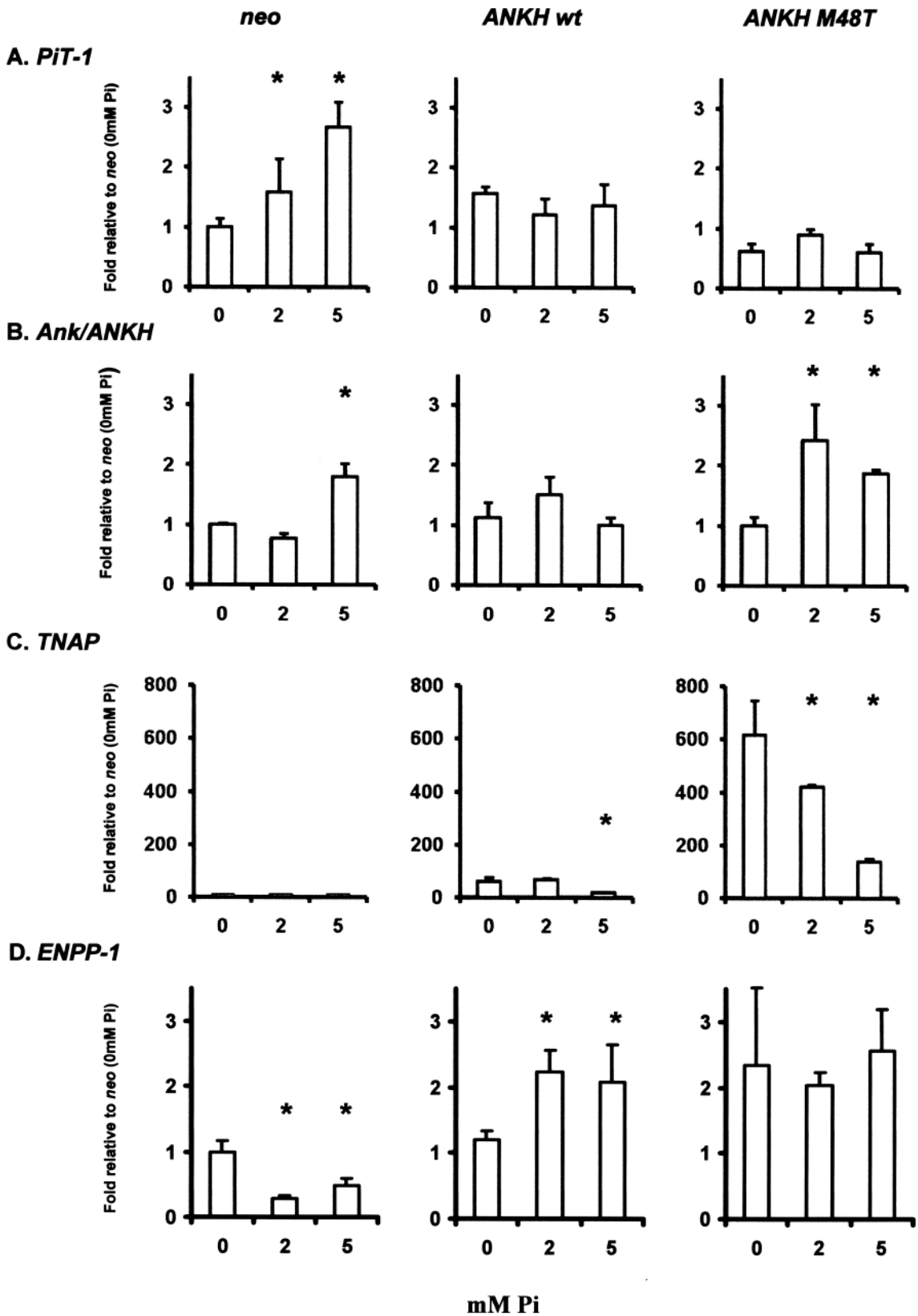

Figure 2. Expression of *PiT-1* (A), *Ank/ANKH* (B), *TNAP* (C), and *ENPP-1* (D) transcripts in different transfectants untreated (0) or treated with Pi (2 and 5 mM). Relative expression of different transcript was expressed as fold relative to that of *neo* controls (0 mM Pi). *Statistically significant difference compared to that of the corresponding transfectant without Pi treatment (0 mM Pi), using Holm-Sidak method of pairwise multiple comparison. Arrow bars denote standard deviation (n = 4).

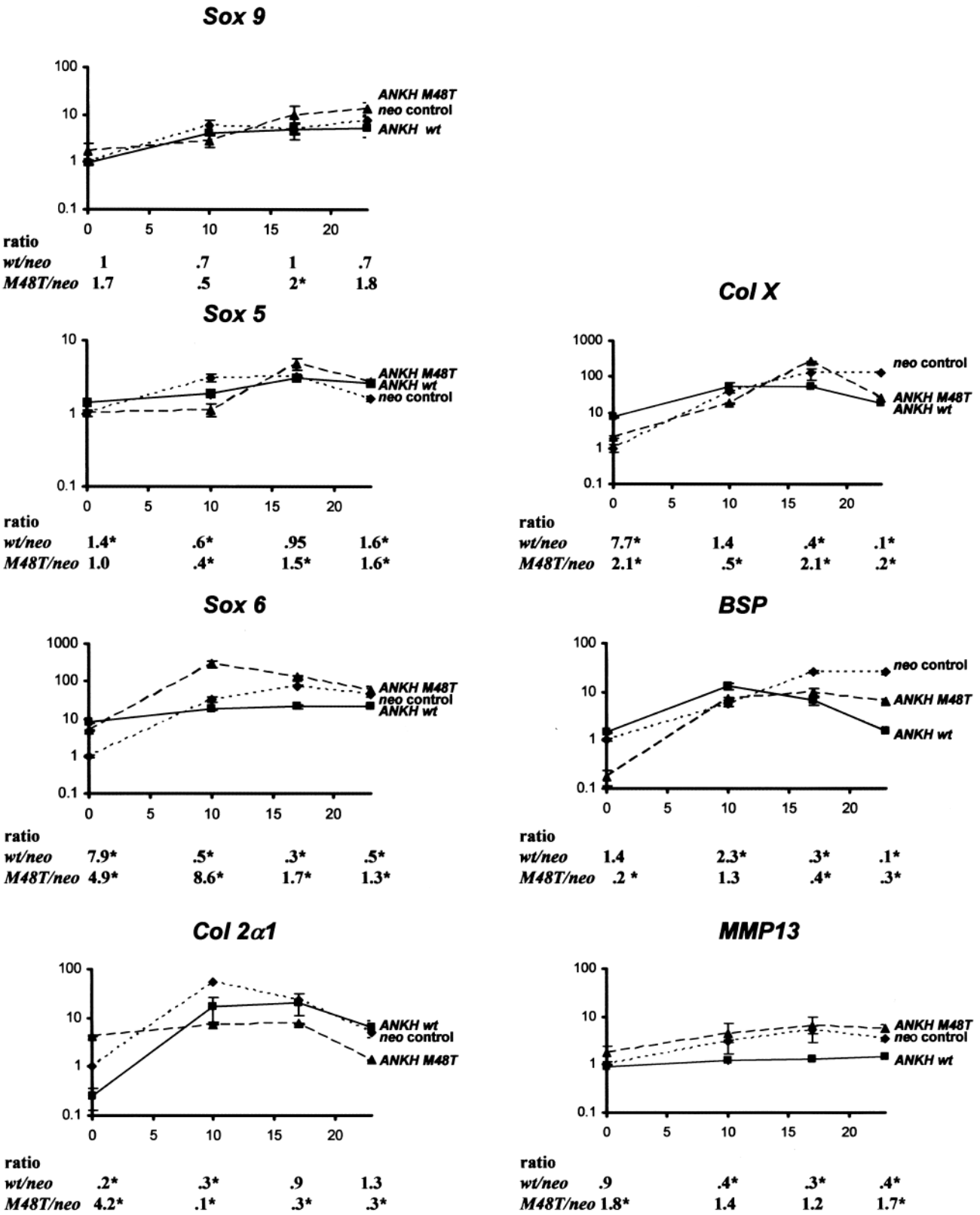

Figure 3. Comparison of the kinetics of expression of *Sox9*, *Sox5*, *Sox6*, *Col2a1*, *ColX*, *BSP*, and *MMP13* in *ANKH wt*-transfectants (solid lines) versus *neo* controls (dotted lines) versus *ANKH* M48T-transfectants (dashed lines) at Day 0, Day 10, Day 17, and Day 23 of ITS treatment. Expression of *β-actin* was used for normalization. "ratio" denotes expression in *ANKH* transfectants (either *wt*- or *M48T*-) over that of *neo*-controls. Arrow bars denote standard deviation (n = 4). *$p < 0.001$ (Holm-Sidak method of pairwise multiple comparison).

the prechondrocyte stage (Day 0), while lower levels of hypertrophic chondrocyte markers (such as *ColX* and *BSP*) were expressed at ITS Day 23 treatment (Figure 3 and Table 1). Thus, overexpression of ANKH M48T proteins affected the kinetics of normal hypertrophic chondrocyte differentiation in ATDC5 cells.

DISCUSSION

To mimic the dominant *ANKH* mutations found in familial CPPDD cells, we transfected ATDC5 cells that express Ank proteins with wt or mutant *ANKH-myc* constructs. Clonal ATDC5 *ANKH* transfectants have been used to study the effects of overexpressing either wild-type or various CPPDD-associated ANKH mutant proteins, and the results differed from our study. For example, Zaka, *et al* showed that overexpression of wt Ank protein had a 4.5-fold increase in *Col 10α* expression at ITS Day 28[10], while we showed that overexpression of wt ANKH protein in a mixed population of ATDC5 stable transfectants had a 10-fold decrease in *Col X* expression at ITS Day 23. The reason for this discrepancy is not clear, but could be due to clonal bias in the study by Zaka, *et al*[10]. One of the differences between clonal versus mixed populations relates to the ratio of transfected ANKH (wt or mutant) and the endogenous Ank present in the *ANKH* transfectants. Further, we found that ITS stimulated the upregulation of endogenous Ank, but not the transfected ANKH[15]. Thus during the course of induced hypertrophic differentiation of the ATDC5 transfectants, the ratio of the endogenous versus transfected forms also changed. In addition, Pi/PPi homeostasis is a dynamic process, likely subjected to feedback mechanisms. Taken together, *in vitro* culture systems investigated in different laboratories might have different [Pi]/[PPi] conditions and different ratio of transfected ANKH and endogenous Ank, leading to the generation of inconsistent and even contradictory results.

The physiological ionic extracellular [Pi] level is around 1 mM (varies from 0.8 to 1.2 mM). Feeding wild-type as well as *ank/ank* mice with a high phosphorus diet resulted in a 2-fold increase in serum [Pi], but enhanced joint mineralization was detected only in the *ank/ank* mice[11]. For this reason, we chose to treat the ATDC5 transfectants with 2 mM and 5 mM Pi, which would allow us to detect dose-response effects under conditions mimicking pathological [Pi] levels. We chose to transfect ATDC5 cells for the ease of generating mixed populations of stable transfectants and for the ability to follow the kinetics of endochondral chondrocyte differentiation. Although articular chondrocytes normally do not undergo hypertrophy in the uncalcified cartilage, chondrogenic events prior to chondrocyte hypertrophy are generally similar to those in endochondral chondrocytes. For example, similar levels of the major ECM proteins such as aggrecan and collagen II were found in both surface articular chondrocytes and the growth-plate (resting zone) chondrocytes[20]. Thus, we chose to examine the effects of high Pi treatment on various ATDC5 transfectants at ITS Day 17, just prior to chondrocyte hypertrophy.

Treatment of the *neo* transfectants (ITS Day 17) with 5 mM Pi resulted in a coordinated upregulation of *PiT-1* and endogenous *Ank* transcript, while there was no change in *TNAP* transcript expression. Treatment of the *ANKH wt*-transfectants (ITS Day 17) with 5 mM Pi did not lead to any significant changes in *PiT-1* and *Ank-ANKH* expression, but there was a significant drop in *TNAP* expression (about 4-fold less than that of the *ANKH wt* transfectants with no Pi treatment). It is possible that the combination of both *PiT-1* and *Ank/ANKH* expression was adequate to maintain Pi/PPi homeostasis in the presence of extracellular high Pi. However, an uncoordinated upregulation of *Ank* transcript, but not that of *PiT-1*, was detected when *ANKH M48T*-transfectants were treated with 5 mM Pi, possibly due to the inability of the mutant ANKH M48T protein to interact with PiT-1. Similar to the Pi-treated *ANKH wt*-transfectants, there was also a significant drop in *TNAP* expression when the *ANKH M48T*-transfectants were treated with 5 mM Pi (about 4-fold less than that of the *ANKH M48T* transfectants

Table 1. Significant changes in markers expressed in various *ANKH*-transfectants relative to that of the *neo* controls at each time point.

Transfectant (ITS)	*Sox9*	*Sox5*	*Sox6*	*Col-2αI*	*Col X*	*BSP*	*MMP-13*	*ENPP-1*	ALP Activity	TNAP Protein
ANKH-wt										
Day 0		↑1.4×	↑7.9×	↓5×	↑7.7×			ND		
Day 10		↓1.7×	↓2×	↓3×		↑2×	↓2.5×	ND		
Day 17			↓3×		↓2.5×	↓3×	↓3×			
Day 23		↑1.6×	↓2×		↓10×	↓5×	↓2.5×	ND	↓2×	↓2×
ANKH-M48T										
Day 0					↑2×	↓3.3×	↑2×	ND	↑2×	
Day 10			↑8.6×	↓10×				ND	↑2×	↑2×
Day 17	↑2×	↑1.5×	↑1.7×	↓3×	↑2×	↓2.5×		↑2×	↑2×	↑2×
Day 23		↑1.6×	↑1.3×	↓3×	↓5×	↓10×	↑2×	ND		

ANKH: human homolog of progressive ankylosis; Col: collagen; BSP: bone sialoprotein; MMP: matrix metalloproteinase; ND: not done.

with no Pi treatment). Possibly, these changes reflect an attempt to compensate for the Pi excess and restore an optimal Pi/PPi ratio. This notion is supported by the fact that a 2-fold increase in *ENPP-1* transcript expression was detected in both untreated and Pi-treated *ANKH M48T*-transfectants (Figure 2D). As shown in Table 1, *ANKH M48T*-transfectants had 2-fold higher alkaline phosphatase activities at Day 0, ITS Day 10, and Day 17. Taken together, our results suggest that the *ANKH M48T* mutation leads to dysregulation of participants (such as PiT-1, ENPP-1, and TNAP) involved in the Pi and PPi homeostasis.

Very little is known regarding the structure-function of the ANK/ANKH proteins. It is not clear how many transmembrane domains are present. Structural prediction of the ANKH protein using 11 different programs showed 7 to 12 predicted transmembrane domains[5]. We showed that the ANKH M48T proteins failed to interact with PiT-1. However, it is not clear whether ANKH binds to PiT-1 directly, and M48 might be a crucial contact point of the interaction. If the latter were true, M48 would be located intracellularly and thus would favor the 7-transmembrane model as predicted by the PRED-TMR program[5]. It is also unclear whether the ANKH M48T protein has the same conformation as the ANKH wt protein. Whether this disruption of ANKH-PiT-1 interaction is sufficient for the development of CPPDD is not known. The French kindred with the ANKH M48T mutation had earlier disease onset (before the age of 35 yrs) and acute articular attacks[21]. Due to the severity of the disease in patients from this kindred, it is possible that there are other yet-to-be-identified functional consequences of the *ANKH M48T* mutation. Similar to the finding in ANK-deficient mice bearing an *ANKH M48T* transgene[9], results from our studies using the ATDC5 transfectants also did not show any evidence that the *ANKH M48T* mutation is an activating mutation. However, based on our results, avoiding high-phosphorus diet might be beneficial to patients with the *ANKH M48T* mutation.

Previously, we showed that the CPPDD-associated *ANKH ΔE490* mutation affects TNAP activities[15]. In this study, we showed that the CPPDD-associated *ANKH M48T* mutation affects the interactions between ANKH and PiT-1, leading to a dysregulated expression of genes relating to Pi and PPi homeostasis. Taking these results together, we hypothesize that CPPDD-associated ANKH proteins lead to a dysregulation of modulators of Pi/PPi homeostasis such as PiT-1 and TNAP. It is likely that in familial CPPDD patients, the dynamic interplay of ANKH, PiT-1, and TNAP, consequent to the various *ANKH* mutations (*ANKH ΔE490* and *ANKH M48T*), led to the fluctuating local [Pi]/[PPi] ratio, resulting in the formation of CPPD/HA crystals in the joints.

ACKNOWLEDGMENT

We thank Dr. J.A. Winkles, University of Maryland School of Medicine, Baltimore, MD, USA, for his gift of anti-ANK antibody, and Dr. K.P.H. Pritzker, Mount Sinai Hospital, Toronto, Canada, for helpful discussions.

REFERENCES

1. Ho AM, Johnson MD, Kingsley DM. Role of the mouse ank gene in control of tissue calcification and arthritis. Science 2000;289:165-70.
2. Nurnberg P, Thiele H, Chandler D, et al. Heterozygous mutations in ANKH, the human ortholog of the mouse progressive ankylosis gene, result in craniometaphyseal dysplasia. Nature Genet 2001;28:37-41.
3. Reichenberger E, Tiziani V, Watanabe S, et al. Autosomal dominant craniometaphyseal dysplasia is caused by mutations in the transmembrane protein ANK. Am J Hum Genet 2001;68:1321-6.
4. Pendleton A, Johnson MD, Hughes A, et al. Mutations in ANKH caused chondrocalcinosis. Am J Hum Genet 2002;71:933-40.
5. Williams CJ, Zhang Y, Timms A, et al. Autosomal dominant familial calcium pyrophosphate dihydrate deposition disease is caused by mutation in the transmembrane protein ANKH. Am J Hum Genet 2002;71:985-91.
6. Williams CJ, Pendleton A, Bonavita G, et al. Mutations in the amino terminus of ANKH in two US families with calcium pyrophosphate dihydrate crystal deposition disease. Arthritis Rheum 2003;48:2627-31.
7. Zhang Y, Johnson K, Russell GG, et al. Association of sporadic chondrocalcinosis with a -4-basepair G-to-A transition in the 5'-untranslated region of ANKH that promotes enhanced expression of ANKH protein and excess generation of extracellular inorganic pyrophosphate. Arthritis Rheum 2005;52:1110-7.
8. McKee S, Pendleton A, Dixey J, Doherty M, Hughes A. Autosomal dominant early childhood seizures associated with chondrocalcinosis and a mutation in the ANKH gene. Epilepsia 2004;45:1258-60.
9. Gurley KA, Reimer RJ, Kingsley DM. Biochemical and genetic analysis of ANK in arthritis and bone disease. Am J Hum Genet 2006;79:1017-29.
10. Zaka R, Stokes D, Dion AS, Kusnierz A, Han F, Williams CJ. P5L mutation in Ank results in an increase in extracellular inorganic pyrophosphate during proliferation and nonmineralization hypertrophy in stably transduced ATDC5 cells. Arthritis Res Ther 2006;8:R164.
11. Murshed M, Harmey D, Millan JL, McKee MD, Karsenty G. Unique coexpression in osteoblasts of broadly expressed genes accounts for the spatial restriction of ECM mineralization to bone. Genes Dev 2005;19:1093-104.
12. Wang W, Xu J, Du B, Kirsch T. Role of the progressive ankylosis gene (ank) in cartilage mineralization. Mol Cell Biol 2005;25:312-23.
13. Foster BL, Hociti FH Jr, Swanson EC, et al. Regulation of cementoblast gene expression by inorganic phosphate in vitro. Calcif Tissue Int 2006;78:103-12.
14. Addison WN, Azari F, Sørensen ES, Kaartinen MT, McKee MD. Pyrophosphate inhibits mineralization of osteoblast cultures by binding to mineral, up-regulating osteopontin, and inhibiting alkaline phosphatase activity. J Biol Chem 2007;282:15872-83.
15. Wang J, Tsui HW, Beier F, Pritzker KPH, Inman RD, Tsui FWL. The *ANKH ΔE490* mutation in calcium pyrophosphate dihydrate crystal deposition disease (CPPDD) affects tissue non-specific alkaline phosphatase (TNAP) activities. Open Rheum J 2008;2:25-32.
16. Shukunami C, Ishizeki K, Atsumi T, Ohta Y, Suzuki F, Hiraki Y. Cellular hypertrophy and calcification of embryonal carcinoma-derived chondrogenic cell line ATDC5 in vitro. J Bone Miner Res 1997;12:1174-88.
17. Wang G, Woods A, Sabari S, Pagnotta L, Stanton L-A, Beier F.

Rho/ROCK signaling suppresses hypertrophic chondrocyte differentiation. J Biol Chem 2004;279:13205-14.
18. Yepes M, Moore E, Brown SA, et al. Progressive ankylosis (Ank) protein is expressed by neurons and Ank immunohistochemical reactivity is increase by limbic seizures. Lab Invest 2003;83:1025-32.
19. Schmittgen TD, Zakrajsek BA, Mills AG, Gorn V, Singer MJ, Reed MW. Quantitative reverse transcription-polymerase chain reaction to study mRNA decay: comparison of endpoint and real-time methods. Anal Biochem 2000;285:194-204.
20. Yamane S, Cheng E, You Z, Reddi AH. Gene expression profiling of mouse articular and growth plate cartilage. Tissue Engineering 2007;13:2163-73.
21. Gaucher A, Faure G, Netter P, et al. Hereditary diffuse articular chondrocalcinosis. Dominant manifestation without close linkage with the HLA system in a large pedigree. Scand J Rheumatol 1977;6:217-21.