The role of PTHrP in chondrocyte differentiation.

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Nherf2, but not Nherf1 overexpression, stimulates osteoblast and chondrocyte matrix mineralization

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Abstract

The PDZ domain containing proteins Na+/H+ exchanger regulatory factor 1 (Nherf1) and Nherf2 redirect type 1 Parathyroid Hormone (PTH)/PTH related Peptide (PTHrP) receptor (PTHR1) signalling in renal tubuli. Whether Nherf1 and Nherf2 are also expressed during endochondral bone formation and whether they play a role in osteoblast and chondrocyte differentiation is not known. Furthermore, it is not known whether they modulate PTHR1 signalling in these cells.

We showed that Nherf1 and Nherf2 are expressed by growth plate chondrocytes and by osteoblasts in the bone collar using in situ hybridization. In addition, overexpression of Nherf1 and Nherf2 in KS483 mesenchymal progenitor cells resulted in opposite effects on terminal osteoblast differentiation. Nherf1 overexpression inhibited, while Nherf2 overexpression stimulated matrix mineralization. Cartilage matrix mineralization was increased in the Nherf2, but not in the Nherf1 overexpressing cell line. Overexpression of Nherf1 or Nherf2 had no effect on the production of the early chondrocyte marker, the glycosaminoglycans. PTHrP inhibited osteoblastic matrix mineralization and GAG production in chondrocytes. Overexpression of Nherf1 or Nherf2 did not change the effect of PTHrP treatment on osteoblast and chondrocyte differentiation.

The distinct actions of Nherf1 and Nherf2 are probably due to the recruitment of different target molecules, like transcription factors, receptors and signalling molecules.

Introduction

The Na+/H+ exchanger regulatory factor 1 (Nherf1) and Nherf2 form a family of adaptor proteins and are characterized by the presence of two tandem psd95, discs large protein, ZO1 (PDZ) protein interaction domains. They were first found in the apical membranes of renal epithelial cells, where they are involved in regulating the sodium-hydrogen exchange[1]. Other studies revealed that Nherf proteins also play a role in the regulation of phosphate and calcium transport in kidney cells[2;3]. Beside interaction with ion channels, Nherf1 and Nherf2 also cooperate with transcription factors, signalling proteins, structural proteins, and with G-protein coupled receptors, including the type 1 Parathyroid Hormone (PTH)/PTH related Peptide (PTHrP) receptor (PTHR1)[4;5].

The interactions of Nherf1 and Nherf2 with the PTHR1 are best studied in kidney cells. Immunohistochemical analysis suggests that the PTHR1 co-localizes with Nherf1 and Nherf2 in the tubuli. Binding of PTHR1 to Nherf1 occurs through the PDZ1 domain and binding to Nherf2 through the PDZ2 domain[4;6;7]. Both adaptor proteins bind, beside to the PTHR1, also to the intracellular signalling mediator phospholipase C beta (PLCβ), which results in a switch of PTHR1 signalling from the adenylate cyclase (AC)/protein kinase A (PKA) pathway to the PLCβ/protein kinase C (PKC) pathway[4;6]. It is unclear whether Nherf1 or Nherf2 are also expressed in osteoblasts and chondrocytes, the other main targets of PTH and PTHrP signalling, and whether they play a comparable role in redirecting PTHR1 signalling in these cells.

In chondrocytes and osteoblasts PTHR1 signalling results in the activation of two main pathways, the AC/PKA pathway via the Gαs-protein and PLCβ/ PKC pathway via the Gαq-protein. Activation of the Gαs-protein results in phosphorylation of the plasma membrane

bound enzyme AC, which synthesizes cyclic AMP (cAMP) from ATP. Subsequently, cAMP exerts its effects by activating the enzyme PKA, which finally leads to the activation of cAMP response element binding protein (CREB). In addition, Gαq-protein activates PLCβ, which in turn cleaves phosphatidylinositol 4,5-biphosphate (PIP2) to generate two products: diacylglycerol (DAG) and inositol 1,4,5-triphosphate 3 (IP3). At this step the signalling pathway splits in two branches. First, IP3 leaves the plasma membrane and diffuses rapidly through the cytosol, to release Ca(2+) from the endoplasmatic reticulum. Second, DAG activates PKC, which in turn activates downstream mediators, resulting in genomic responses.
Both pathways are essential in the regulation of chondrocyte proliferation and differentiation. This can be deduced from comparing the bone phenotypes of transgenic mice. PTHR1 knockout mice show a severe inhibition of chondrocyte proliferation and premature maturation of chondrocytes[8]. A similar, but less severe phenotype is observed in PTHrP knockout mice[9]. Chondrocyte specific knockout mice, carrying a mutation in the Gαs-protein, leading to disruption of the AC/PKA pathway, displayed a comparable phenotype to the growth plate phenotype observed in PTHrP knockout mice[9;10]. The opposite was found in mice carrying a mutant form of the PTHR1, which specifically interrupts signalling via the PLCβ/PKC pathway and signals normally via the AC/PKA pathway[11]. These mice showed an increase in chondrocyte proliferation and a delay in chondrocyte hypertrophy and vascular invasion. These data indicate that the PLCβ/PKC pathway regulates the transition from proliferating into hypertrophic chondrocytes. In addition, it suggest that the AC/PKA pathway is the dominant pathway after PTHR1 signalling in chondrogenesis and that PLCβ/PKC signalling opposes the effects of the AC/PKA pathway[11].
Whether Nherf1 and Nherf2 are involved in directing PTHR1 signalling towards the PLCβ/PKC pathway in chondrocytes and osteoblasts is unclear. Therefore, in this study we first addressed whether Nherf1 and Nherf2 are expressed during endochondral bone formation *in vivo*. In addition, the actions of Nherf1 and Nherf2 overexpression on chondrocyte and osteoblast differentiation *in vitro* were examined. Finally, the influence of Nherf1 and Nherf2 overexpression on the effect of PTHR1 signalling *in vitro* was investigated.

Materials and Methods

In situ hybridization

Nherf1 and Nherf2 full-length cDNA were obtained from Image Consortium and they were sequence verified. For in situ hybridizations, plasmids were linearized with the appropriate restriction enzymes and antisense and sense cRNA probes were generated using T7 or Sp6 RNA polymerase, respectively. The probes were labelled and in situ hybridization was performed as previously described[12].

Generation of stable cell lines

Stable cell lines were generated using the Flp-In system (Invitrogen) according to the manufacturer's protocol. In short, first a KS483 Flp-in host cell line was generated by stable introduction of a single copy of an Flp-Recombinase target (FRT) site as an integral part of an antibiotic resistancy gene in the genome of KS483. One clone, KSfrt 4D3, was selected that was comparable to the parental KS483 cells in differentiation characteristics and retained

full capacity to differentiate into osteoblasts and chondrocytes. This clone was used for the generation of isogenic stable cell lines by transient co-transfection of an FRT-targeting vector and an Flp-recombinase expression vector. FRT-targeting vectors were a derivation of the pEF5/FRT/V5-DEST vector (Invitrogen), containing either luciferase cDNA as a control or full length human Nherf1 or human Nherf2 cDNAs, under the control of the EF1α promoter. Due to Flp-mediated recombination at the genomic FRT-site, this targeting vector was incorporated in the genome. Simultaneously, a shift in antibiotic resistancy was introduced allowing positive selection for integrants in the genomic FRT-site only and negative selection for random integrants in one single step. This method allows the generation of isogenic stable cell lines after a single transfection by eliminating variability of multiple integrated copies and results in consistent long-term stable expression of the transgene[13].

Cell culture

For osteoblast differentiation KS483 cells were seeded at a density of 12000 cells/cm^2 as described previously[14]. Medium was changed every 3 to 4 days. At confluence (from day 4 of culture onward), ascorbic acid (50 μg/ml. Merck Inc., NY, USA) and when nodules appeared (from day 11 of culture onward) β-glycerol phosphate (5mM, Sigma Chemical Co., St Louis, MO, USA) were added. From day 11 the cells were cultured in the absence or presence of 10^{-7} M PTHrP (1-34) (Bachem, PA, USA), stained at day 18 for matrix mineralization with alizarin and alizarin was subsequently measured as described before[14].

For chondrocyte differentiation KS483 cells were cultured as pellets. For this, 300000 cells were seeded in a 96 wells plate (Greiner, Alphen a/d Rijn, The Netherlands) in high glucose DMEM (Invitrogen, Breda, The Netherlands), containing 40 μg/ml proline (Sigma, Zwijndrecht, The Netherlands), 100 μg/ml pyruvate (Sigma), 50 mg/ml ITS + premix (BD Biosciences, San Jose, USA), and 50 μg/ml ascorbic acid and centrifuged for 5 min at 1238 rpm. Medium was changed every 3 to 4 days. From day 1 to 14 medium was supplemented with 10^{-7} M dexamethasone (Sigma), and 10 ng/ml TGFβ3 (R&D systems, Minneapolis, USA). From day 14 to 28 medium was supplemented with 5mM β-glycerol phosphate and 500 ng/ml BMP6 (kindly provided by dr. S. Vukicevic, University of Zagreb, Croatia). At day 28 pellets were used for glycosaminoglycan (GAG) analysis or used for histology.

Glycosaminoglycan assay

After 28 days 3 to 4 pellets were washed with PBS and incubated overnight in 0.1 mM EDTA, containing 40 μg/ml proteinase K (Invitrogen) at 56°C. From each sample 50 μl was used for GAG analysis and 10 μl was used for DNA measurements. For GAG analysis, 50 μl sample was calibrated against a GAG standard (0,1,2,3, and 5 μg) using the Blyscan sulphated GAG assay (Biocolor, Krimpen a/d IJssel, The Netherlands) according to the manufacturers protocol, with small modifications. In short, each sample was diluted 1:1 in 0.1 mM EDTA/40 μg/ml proteinase K and 500 μl Blyscan dye reagent was added. Next, the samples were mixed for 30 minutes and centrifuged for 10 minutes at 13000 rpm. Then, pellets were dissolved in 200 μl dye dissociation reagent and mixed for 15 minutes. Absorbance was measured at 650 nm. For DNA measurements, 10 μl sample was calibrated against a DNA standard (0.5 – 10 μg) as described before[14].

Von Kossa staining

At day 28, two pellets were fixed in 10% formalin for 1 hour and embedded in paraffin. Section of 5 µm were deparaffined and hydrated through graded ethanol steps and rinsed with bidest. Subsequently, they were incubated in 1% $AgNO_3$ for 30 seconds and rinsed with bidest. Next, sections were treated for 3 minutes in 0.01% pyrogallol and rinsed with bidest. Then, they were incubated in 5% Nathiosulfate and rinsed with bidest. Sections were counterstained with light green and afterwards rinsed three times with bidest. Finally, the sections were air dried and embedded in histomount-diluted 1:1 in xylene.

RNA isolation

Cells were cultured under osteoblastic conditions as described before[14], for 4, 7, 11, 14, 18 days and RNA was isolated using Trizol LS reagent (Invitrogen) and reverse transcribed into cDNA using random hexamer primers (Amersham).

Quantitative PCR

Quantitative PCR (qPCR) was performed using the BioRad iCycler (Biorad, Veenendaal, The Netherlands). For each gene a set of primers was designed (table 1), which spanned at least 1 intron-exon boundary and had an optimal annealing temperature of 60˚C, using Beacon designer (United Bioinformatica Inc., Calgary, Canada). cDNA (5 ng) was amplified in triplicate using the qPCR core kit for SYBR green 1 (Eurogentec, Maastricht, The Netherlands), under the following conditions: cDNA was denatured for 10 minutes at 95˚C, followed by 40 cycles, consisting of 15 s at 95˚C, 20 s at 60˚C, and 40 s at 72˚C. From each sample a melting curve was generated to test for the absence of primer dimer formation and DNA contamination. Each reaction contained 5 µl cDNA (1 ng/µl), 10x reaction buffer, 3 or 4 mM $MgCl_2$ (table 1), 40 µM dNTP's, 300 nM primer, 0,75 µl SYBR green, and 0,1 µl HotGoldStar polymerase in a total volume of 25 µl. Fold changes, adjusted for the expression of β2m, were calculated and log transformed using the comparative method[15]. Significant changes were calculated using the Double Delta Model (DDM) (J. Hoogendam et al., Chapter 6).

Name	F/R	Primer	MgCl
β2µ	F	CACTGACCGGCCTGTATGC	3 mM
	R	GAATTCAGTGTGAGCCAGGATATAGA	
mouse nherf1	F	CCAGCGATACCAGTGAGGAGC	3 mM
	R	GAGGAGGAGGAGGAGGTAGATGAG	
mouse nherf2	F	GCTCTCCTGCTTCTCACTCTGG	3 mM
	R	TCGTCCTCCTGTGCCTTGATTC	
human nherf1	F	TACAGAAGGAGAACAGTCGTGAAGC	4 mM
	R	GCCAGGGAGATGTTGAAGTCTAGG	
human nherf2	F	CCGACAAGGACACTGAGGATGG	4 mM
	R	CGCTTGTTGACTCGCATGGC	

Table 1: quantitative PCR primer sets.
F: forward primer, R: reverse primer.

Statistics

Values represent mean ± SEM. Differences were examined by analysis of variance (ANOVA) followed by the post-hoc least significant difference test (LSD). Results were considered significant at $p<0.05$.

Results

Nherf1 and Nherf2 mRNA expression during endochondral bone formation in vivo

In order to establish whether Nherf1 and Nherf2 mRNAs are expressed by chondrocytes and osteoblasts during endochondral bone formation, in situ hybridizations were performed on 18-days old mouse embryos and on hind paws of 17-days old mouse embryos. Nherf1 (fig. 1 A-D) and Nherf2 (fig. 1 E-H) showed a similar expression pattern in kidney, vertebrae, as well as in metatarsals. The negative controls are shown in figure 1A (Nherf1) and 1E (Nherf2). In kidney Nherf1 and Nherf2 mRNAs were expressed in the tubuli, whereas no expression was found in the glomeruli (fig. 1B and 1F). Nherf1 and Nherf2 mRNAs were expressed by chondrocytes throughout the growth plate and by the osteoblasts in the bone collar (fig. 1C and 1G). In addition, the chondrocytes in vertebrae showed Nherf1 and Nherf2 mRNA expression as well (fig. 1D and 1H). Remarkably, the pre-chondrocytes in the intervertebrae region and the pre-osteoblasts in the perichondrium showed no Nherf1 and Nherf2 mRNA expression. Taken together, Nherf1 and Nherf2 mRNAs are co-expressed by chondrocytes and osteoblasts during endochondral bone formation.

The effect of Nherf1 and Nherf2 overexpression on chondrocyte and osteoblast differentiation in vitro

We subsequently studied Nherf1 and Nherf2 mRNA expression during osteoblast differentiation *in vitro* using KS483 cells. In agreement with the results of the in situ hybridization, we found stable Nherf1 (fig. 2A) and Nherf2 (fig. 2B) mRNA expression during KS483 osteoblast differentiation. In order to investigate the role of Nherf1 and Nherf2 in osteoblast and chondrocyte differentiation, we generated human Nherf1 and Nherf2 overexpressing KS483 cell lines using the Flp-In system. As a control cell line, cells were stably transfected with the luciferase gene.

The Nherf1 and Nherf2 overexpressing cell lines showed stable overexpression of about 10-fold of human Nherf1 and human Nherf2 mRNA compared to endogenous (mouse) Nherf1 and endogenous (mouse) Nherf2 mRNA (fig. 3). In addition, the expression of endogenous Nherf1 and Nherf2 mRNA at the end of culture (day 18) was not different from the expression at the beginning of culture (day 4) in both overexpressing cell lines, which is comparable to Nherf1 and Nherf2 mRNA expression in the control cell line.

To establish whether Nherf1 and Nherf2 overexpression influences osteoblast differentiation of KS483 cells, the cells were cultured in monolayer for 18 days under osteoblastic conditions. At the end of culture the mineralized osteoblastic matrix was stained with alizarin red (fig. 4A) and alizarin red was quantified (fig. 4B). Nherf2 overexpression stimulated the production of a mineralized osteoblastic matrix. This result was in contrast to the production

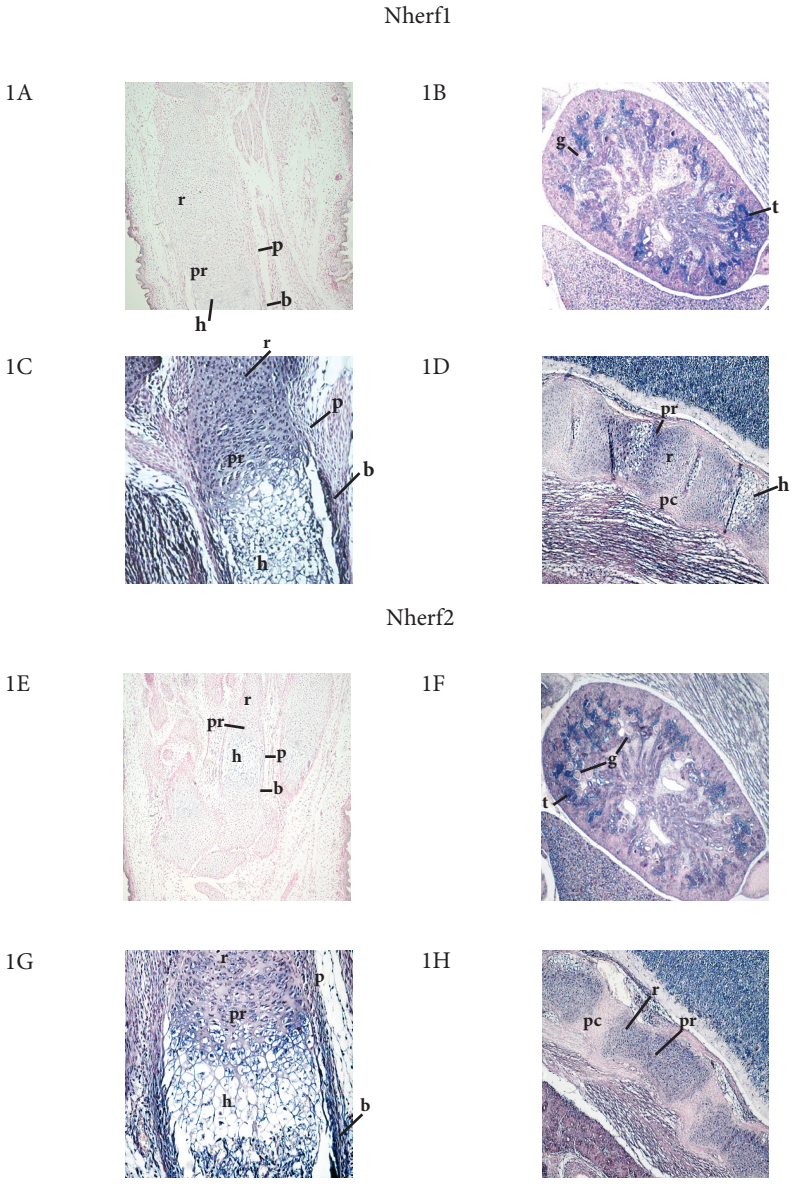

Figure 1: Nherf 1 and Nherf2 mRNA expression in mouse embryo
Nherf1 and Nherf2 mRNA expression were established by performing in situ hybridizations on parasagital sections of 18-days old mouse embryos and on hind paws of 17-days old mouse embryos. A) The negative control (sense probe) for Nherf1 and E) the control for Nherf2 were not stained. In the kidney B) Nherf1 and F) Nherf2 mRNA were expressed in tubuli (t), but not in the glomeruli (g). In the growth plate C) Nherf1 and G) Nherf2 mRNA was expressed throughout the growth plate by resting (r), proliferating (pr), and hypertrophic (h) chondrocytes. Both mRNAs were also expressed by osteoblasts in the bone collar (b) but not by pre-osteoblasts in the perichondrium (p). In vertebrae D) Nherf1 and H) Nherf2 were expressed throughout the growth plate as well. Pre-chondrocytes (pc) in the intervertebrae did not express both Nherf mRNAs. r = Resting, pr = proliferating, and h = hypertrophic chondrocytes.

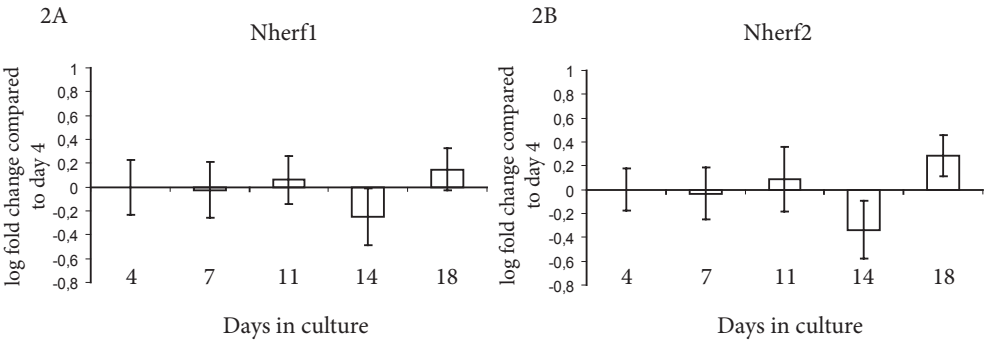

Figure 2: Expression Nherf1 and Nherf2 during KS483 osteoblast differentiation
The mRNA expression of Nherf1 and Nherf2 during KS483 osteoblast differentiation was established using qPCR analysis. Both mRNAs were highly and continuously expressed during osteoblast differentiation. Ct-value β2μ: 29.4; Ct-value Nherf1: 28.5; Ct-value Nherf2: 30.9.

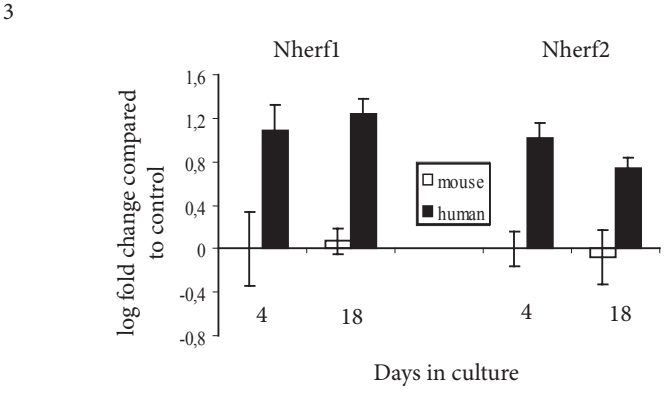

Figure 3: Overexpression of Nherf1 and Nherf2 in KS483 cells
Full length human Nherf1 and human Nherf2 cDNAs were stably transfected in the KS483 cell line. Using qPCR, the mRNA expressions of mouse and human were measured at day 4 and day 18 of osteoblast culture. Mouse mRNAs were continuously expressed during culture. Both human mRNAs showed a stable overexpression of about 10 fold compared to the mouse mRNA expression during differentiation.

of the mineralized matrix by Nherf1 overexpressing cell line, in which the mineralization was decreased, compared to the control cell line.
To examine whether Nherf1 or Nherf2 overexpression affected chondrocyte differentiation as well, cells were cultured as pellets for 4 weeks under chondrogenic conditions. At the end of culture the GAG production was measured (fig. 5) and the mineralized cartilage matrix was stained with von Kossa (fig. 6). While the production of GAGs in the Nherf1 and Nherf2 overexpressing cell lines was comparable to the GAG production in the control cell line (fig. 5),

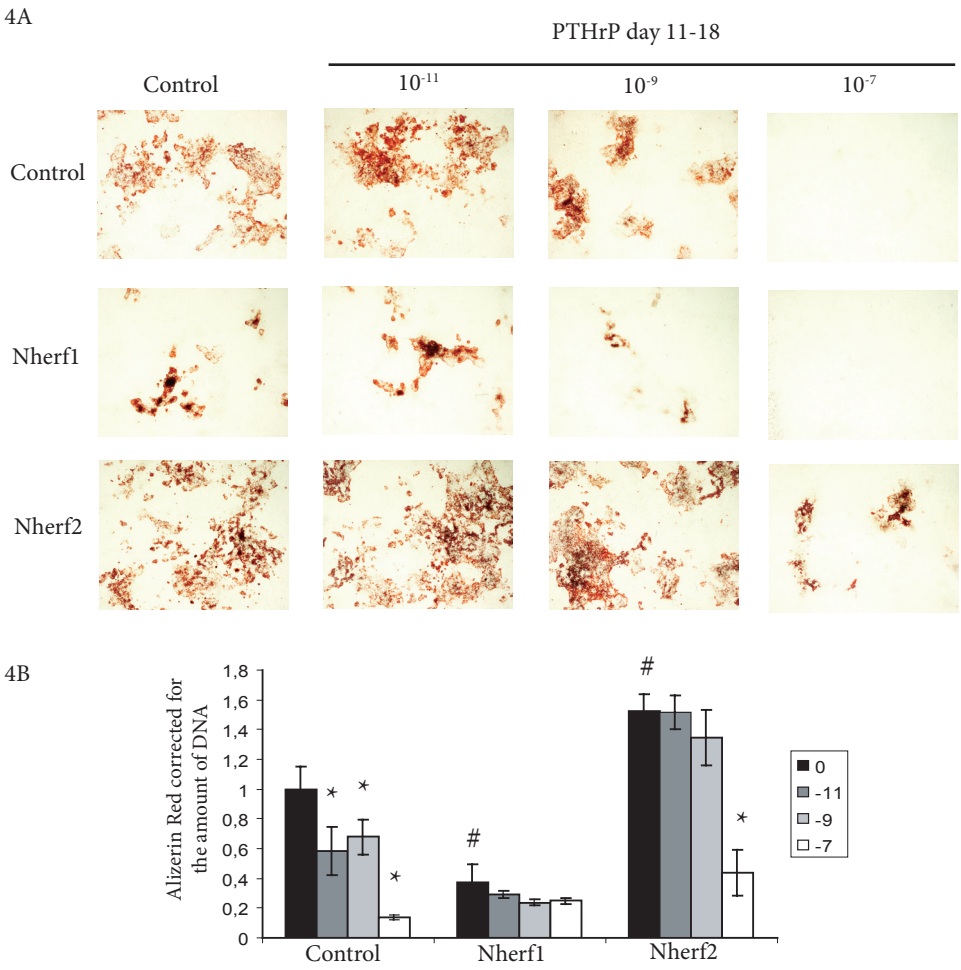

Figure 4: The effects of Nherf overexpression and PTHrP treatment on osteoblastic matrix mineralization
Cells were cultured under osteoblastic conditions for 18 days. From day 11-18, cells were treated with a dose range of PTHrP (10-11, 10-9, 10-7 M). After 18 days the mineralized osteoblastic matrix was stained with alizarin Red (A) and the amount of alizarin Red was measured (B). Nherf1 overexpression inhibited, while Nherf2 overexpression stimulated matrix mineralization. In the control cell line, PTHrP dose dependently inhibited matrix mineralization. The highest dose completely inhibited mineralization. In addition, the highest dose of PTHrP also completely inhibited mineralization of the Nherf1 overexpressing cell line, this was, however, not significant. Matrix mineralization of the Nherf2 cell line was inhibited, although not completely by 10-7 M PTHrP. *Significant $p<0.05$ compared to untreated cultures.

mineralization of the cartilage matrix was not similar (fig. 6). Nherf2 induced mineralization of the cartilage matrix, whereas the Nherf1 overexpressing cell line, like the control cell line, had not produced a mineralized cartilage matrix.
Thus, Nherf1 and Nherf2 overexpression have distinct effects on terminal chondrocyte and osteoblast differentiation of KS483 cells.

The effect of PTHrP treatment in Nherf1 and Nherf2 overexpressing cell lines

In order to investigate whether Nherf1 and Nherf2 overexpression changed the biological response to PTHrP treatment in osteoblast differentiation, monolayer cultures were treated with a dose range of PTHrP from day 11-18. The osteoblastic matrix was stained with alizarin red (fig. 4A) and afterwards alizarin red was measured (fig. 4B). PTHrP inhibited the production of a mineralized matrix in the control cell line dose dependently. Nherf2 overexpression partly reversed this inhibition. Only the highest concentration (10^{-7} M) inhibited matrix mineralization in the Nherf2 overexpressing cell line, whereas all doses of PTHrP inhibited in the control cell line. Treatment with the highest dose of PTHrP also inhibited mineralization of the Nherf1 cell line (fig. 4A), this was however not significant (fig. 4B).

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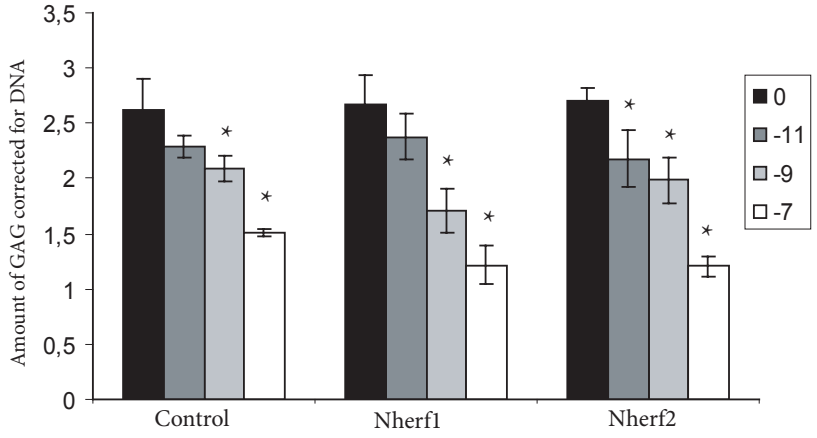

Figure 5: The effects of Nherf overexpression and PTHrP treatment on GAG production
Cells were cultured as pellets, to stimulate chondrocyte differentiation. Pellets were treated from week 2 to 4 with a dose range of PTHrP (10-11, 10-9, 10-7 M). After 4 weeks the GAG production corrected for DNA was determined. The GAG production was not affected by Nherf overexpression. In all cell lines PTHrP inhibited dose dependently the GAG production. *Significant $p<0.05$ compared to untreated pellets.

To examine whether Nherf1 and Nherf2 overexpression altered the biological response to PTHrP treatment in chondrocyte differentiation, pellets were treated with a dose range of PTHrP from week 2-4. After 4 weeks, the GAG production was measured and the mineralized cartilage matrix was stained with von Kossa. In all cell lines, PTHrP treatment resulted in a dose dependent decrease of GAG production (fig. 5). In the control and Nherf1 overexpressing cell lines, a dose of 10^{-9} M PTHrP inhibited GAG production, while in the Nherf2 overexpressing cell line a dose of 10^{-11} M PTHrP already inhibited GAG production. Only the Nherf2 overexpressing cell line had produced a mineralized cartilage matrix and the highest dose of PTHrP (10^{-7} M) inhibited the production of mineral (fig. 6).
Thus, overexpression of Nherf1 or Nherf2 had only minor consequences for the biological actions of PTHrP.

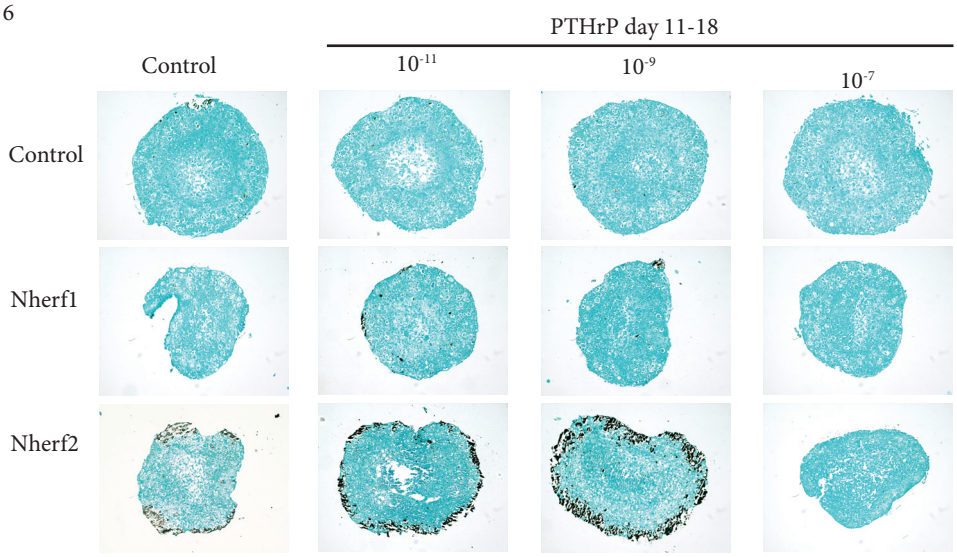

Figure 6: The effects of Nherf overexpression and PTHrP treatment on cartilage matrix mineralization
Cells were cultured as pellets, to stimulate chondrocyte differentiation. Pellets were treated from week 2 to 4 with a dose range of PTHrP (10-11, 10-9, 10-7 M). After 4 weeks the matrix was coloured with von Kossa to stain the mineralized matrix. The control and Nherf1 overexpressing cell line had not produced a mineralized cartilage matrix after 4 weeks. Nherf2 overexpression induced mineralization of the cartilage matrix. The highest dose of PTHrP inhibited the mineralization.

Discussion

Nherf has first been found in renal tubular cells as a regulator of the Na+/H+ exchanger[1]. The two isoforms of Nherf, Nherf1 and Nherf2, have not only been associated with the regulation of ion channels, but they also bind to other proteins, like transcription factors, signalling molecules and receptors, including the PTHR1[4;5]. The interactions of the Nherf proteins and PTHR1 are best studied in renal tubuli, where they redirect signalling from the AC/PKA pathway to the PLCβ/PKC pathway[4]. In this study we examined whether Nherf1 and Nherf2 mRNAs are also expressed by chondrocytes and osteoblasts *in vivo*. In addition, we investigated

the effects of Nherf1 and Nherf2 overexpression on chondrocyte and osteoblast differentiation *in vitro*. Furthermore, we examined whether Nherf1 and Nherf2 overexpression alters the biological response to PTHR1 signalling in chondrocyte and osteoblast differentiation.

To address the first question, whether Nherf1 and Nherf2 are expressed during endochondral bone formation, we performed in situ hybridizations for Nherf1 and Nherf2 on 18-days old mouse embryos and hind paws of 17-days old mouse embryos. We found a widespread expression of Nherf mRNAs, including expression in renal tubuli, which is line with other studies[16;17]. During endochondral bone formation, both Nherf1 and Nherf2 mRNAs were expressed by chondrocytes and by osteoblasts in the bone collar. However, no expression was found in pre-chondrocytes and pre-osteoblasts, suggesting that Nherf1 and Nherf2 exert their actions during later stages of chondrocyte and osteoblast differentiation and have no effect on progenitor cells. In line with the *in vivo* data, we detected Nherf1 and Nherf2 mRNA expression during osteoblast differentiation *in vitro* using KS483 progenitor cells.

The second question we addressed was whether Nherf1 and Nherf2 overexpression altered chondrocyte and osteoblast differentiation of KS483 progenitor cells. Generation of Nherf1 and Nherf2 overexpressing cell lines resulted in stable overexpression of Nherf1 and Nherf2 mRNA of about 10 fold during culture. No data about overexpression of Nherf1 and Nherf2 protein have been collected. Since overexpression of Nherf1 and Nherf2 resulted in biological responses, it is likely that Nherf1 and Nherf2 protein are overexpressed as well. This needs to be confirmed.

Overexpression of Nherf1 and Nherf2 had distinct effects on osteoblast differentiation. Overexpression of Nherf2 resulted in an increase in matrix mineralization. Probably, Nherf2 overexpression results in the recruitment of signalling mediators, as receptors and signalling molecules, involved in mineralization and thereby inducing the mineralization of the osteoblastic matrix. Since Nherf2 overexpression induced cartilage matrix mineralization as well, this mechanism is perhaps also operational in chondrocyte differentiation. In contrast to overexpression of Nherf2, overexpression of Nherf1 inhibited osteoblastic matrix mineralization. The negative effect of Nherf1 in bone formation has been demonstrated before in Nherf1 knockout mice[17]. Some, but not all female Nherf1 knockout mice, showed dwarfism and displayed severe osteoporosis and bone fractures. This is presumably a secondary effect of the mild hypophosphatemia due to ablation of Nherf1, since Nherf1 regulates the phosphate transport by binding to the sodium/phosphate co-transporter type IIa (Npt2a). However, our data suggests that the bone phenotype may also be explained partly by direct effects on osteoblastic matrix mineralization. The effect of Nherf1 overexpression on cartilage matrix mineralization was less clear. However, the control cell line had not yet produced a mineralized cartilage matrix after a culture period of 4 weeks as well.

Distinct functions for Nherf1 and Nherf2 have been reported in the kidney, in the regulation of ion channels[17-19]. Our results also suggest diverse functions in osteoblast and chondrocyte differentiation for Nherf1 and Nherf2. This might be explained by their distinct construction, which consists of several functional domains. Therefore, Nherf1 and Nherf2 may have a preference for binding to different sets of other PDZ proteins, like receptors, ion channels, signalling proteins, transcription factors and cellular structural proteins[2;3;16;20].

The third aim was whether Nherf overexpression alters the biological effect of PTHR1 signalling on osteoblast and chondrocyte differentiation. We have shown earlier that the PTHR1 is

expressed during KS483 osteoblastic differentiation and that PTHR1 signalling inhibits the mineralization of the osteoblastic matrix via various mechanisms[13]. Despite overexpression of Nherf1 and Nherf2, the highest dose of PTHrP inhibited matrix mineralization. Thus, Nherf1 and Nherf2 had only minor effects on the biological effects of PTHrP in this cell model.
Remarkably, while the lowest dose of PTHrP already inhibited osteoblastic matrix mineralization in the control cell line, only a high dose of PTHrP inhibited osteoblastic mineralization in Nherf2 overexpressing cell line. This discrepancy could be a direct effect of Nherf2, but we hypothesize that it is an indirect effect, through the presumably larger amount of mineralization at the time point of PTHrP treatment compared to the control cell line. For complete inhibition of the mineralization of the osteoblastic matrix, PTHrP treatment should start earlier during differentiation. Comparable to the control cell line, PTHrP treatment completely inhibited osteoblastic matrix mineralization in the Nherf1 overexpressing cell line. This inhibition was, however, not significant, because of the already low amount of mineralization in the untreated cells.
The effects of PTHrP on chondrocyte proliferation and differentiation in the growth plate have been studied extensively[8;21;22]. In agreement with these studies, we found an inhibition of PTHrP treatment on cartilage matrix mineralization in the Nherf2 overexpressing cell line. The effects of PTHrP treatment on cartilage matrix mineralization in the control and Nherf1 cell lines were less clear, due to the lack of mineralization in these pellets. Nherf1 and Nherf2 overexpression did not alter the effect of PTHrP treatment on GAG production. PTHrP dose dependently inhibited GAG production with only small differences, irrespective of Nherf overexpression.
Despite overexpression of Nherf1 or Nherf2 mRNA, overexpression did not result in major changes on biological actions of PTHrP. This may be explained by a lack of translation from Nherf mRNA into protein. However, it is likely that Nherf2 protein is overexpressed, since the Nherf2 overexpressing cell line induced biological responses in osteoblast and chondrocyte differentiation. In addition, the Nherf1 overexpressing cell line altered the biological response in osteoblast differentiation, indicating that Nherf1 protein is overexpressed as well. Another explanation could be the inefficient coupling of Nherf1 and Nherf2 to the PTHR1 in osteoblasts and chondrocytes, in contrast to renal kidney cells. In addition, levels of PLCβ could be too low to affect the dominant AC/PKA pathway. The measurements of the activation of the AC/PKA pathway and the PLCβ/PKC pathway are currently underway.
In summary, we showed that Nherf1 and Nherf2 are expressed during endochondral bone formation by chondrocytes and osteoblasts. In addition, our data suggest that Nherf predominantly alters the matrix mineralization of KS483 osteoblasts and chondrocytes. Finally, in our study Nherf overexpressing did not alter the biological response to PTHR1 signalling.

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Reference list

1. Yun CH, Oh S, Zizak M, Steplock D, Tsao S, Tse CM, Weinman EJ, Donowitz M 1997 cAMP-mediated inhibition of the epithelial brush border Na+/H+ exchanger, NHE3, requires an associated regulatory protein. Proc Natl Acad Sci U S A 94:3010-3015
2. Mahon MJ, Cole JA, Lederer ED, Segre GV 2003 Na+/H+ exchanger-regulatory factor 1 mediates inhibition of phosphate transport by parathyroid hormone and second messengers by acting at multiple sites in opossum kidney cells. Mol Endocrinol 17:2355-2364
3. Palmada M, Poppendieck S, Embark HM, van de Graaf SF, Boehmer C, Bindels RJ, Lang F 2005 Requirement of PDZ domains for the stimulation of the epithelial Ca2+ channel TRPV5 by the NHE regulating factor NHERF2 and the serum and glucocorticoid inducible kinase SGK1. Cell Physiol Biochem 15:175-182
4. Mahon MJ, Donowitz M, Yun CC, Segre GV 2002 Na(+)/H(+) exchanger regulatory factor 2 directs parathyroid hormone 1 receptor signalling. Nature %20;417:858-861
5. Shenolikar S, Weinman EJ 2001 NHERF: targeting and trafficking membrane proteins. Am J Physiol Renal Physiol 280:F389-F395
6. Mahon MJ, Segre GV 2004 Stimulation by parathyroid hormone of a NHERF-1-assembled complex consisting of the parathyroid hormone I receptor, phospholipase Cbeta, and actin increases intracellular calcium in opossum kidney cells. J Biol Chem 279:23550-23558
7. Wang S, Raab RW, Schatz PJ, Guggino WB, Li M 1998 Peptide binding consensus of the NHE-RF-PDZ1 domain matches the C-terminal sequence of cystic fibrosis transmembrane conductance regulator (CFTR). FEBS Lett 427:103-108
8. Lanske B, Karaplis AC, Lee K, Luz A, Vortkamp A, Pirro A, Karperien M, Defize LH, Ho C, Mulligan RC, Abou-Samra AB, Juppner H, Segre GV, Kronenberg HM 1996 PTH/PTHrP receptor in early development and Indian hedgehog-regulated bone growth. Science 273:663-666
9. Karaplis AC, Luz A, Glowacki J, Bronson RT, Tybulewicz VL, Kronenberg HM, Mulligan RC 1994 Lethal skeletal dysplasia from targeted disruption of the parathyroid hormone-related peptide gene. Genes Dev 8:277-289
10. Sakamoto A, Chen M, Kobayashi T, Kronenberg HM, Weinstein LS 2005 Chondrocyte-specific knockout of the G protein G(s)alpha leads to epiphyseal and growth plate abnormalities and ectopic chondrocyte formation. J Bone Miner Res 20:663-671
11. Guo J, Chung UI, Kondo H, Bringhurst FR, Kronenberg HM 2002 The PTH/PTHrP receptor can delay chondrocyte hypertrophy in vivo without activating phospholipase C. Dev Cell 3:183-194
12. Van der Eerden BC, Karperien M, Gevers EF, Lowik CW, Wit JM 2000 Expression of Indian hedgehog, parathyroid hormone-related protein, and their receptors in the postnatal growth plate of the rat: evidence for a locally acting growth restraining feedback loop after birth. J Bone Miner Res 15:1045-1055
13. Van der Horst G, Farih-Sips H, Lowik CW, Karperien M 2005 Multiple mechanisms are involved in inhibition of osteoblast differentiation by PTHrP and PTH in KS483 Cells. J Bone Miner Res 20:2233-2244
14. Van der Horst G, Van Bezooijen RL, Deckers MM, Hoogendam J, Visser A, Lowik CW, Karperien M 2002 Differentiation of murine preosteoblastic KS483 cells depends on autocrine bone morphogenetic protein signaling during all phases of osteoblast formation.

Bone 31:661-669
15. Livak KJ, Schmittgen TD 2001 Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25:402-408
16. Weinman EJ, Steplock D, Wang Y, Shenolikar S 1995 Characterization of a protein cofactor that mediates protein kinase A regulation of the renal brush border membrane Na(+)-H+ exchanger. J Clin Invest 95:2143-2149
17. Shenolikar S, Voltz JW, Minkoff CM, Wade JB, Weinman EJ 2002 Targeted disruption of the mouse NHERF-1 gene promotes internalization of proximal tubule sodium-phosphate cotransporter type IIa and renal phosphate wasting. Proc Natl Acad Sci U S A %20;99:11470-11475
18. Weinman EJ, Steplock D, Shenolikar S 2003 NHERF-1 uniquely transduces the cAMP signals that inhibit sodium-hydrogen exchange in mouse renal apical membranes. FEBS Lett 536:141-144
19. Wade JB, Welling PA, Donowitz M, Shenolikar S, Weinman EJ 2001 Differential renal distribution of NHERF isoforms and their colocalization with NHE3, ezrin, and ROMK. Am J Physiol Cell Physiol 280:C192-C198
20. Weinman EJ, Hall RA, Friedman PA, Liu-Chen LY, Shenolikar S 2006 THE ASSOCIATION OF NHERF ADAPTOR PROTEINS WITH G PROTEIN-COUPLED RECEPTORS AND RECEPTOR TYROSINE KINASES *. Annu Rev Physiol 68:491-505.:491-505
21. Karaplis AC, Goltzman D 2000 PTH and PTHrP effects on the skeleton. Rev Endocr Metab Disord 1:331-341
22. Weir EC, Philbrick WM, Amling M, Neff LA, Baron R, Broadus AE 1996 Targeted overexpression of parathyroid hormone-related peptide in chondrocytes causes chondrodysplasia and delayed endochondral bone formation. Proc Natl Acad Sci U S A 93:10240-10245