The role of PTHrP in chondrocyte differentiation.

Hoogendam, J.

Novel late target genes of PTHrP in chondrocytes

5

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Abstract

To gain more insight into the downstream effectors of PTHrP signalling in chondrocytes, we performed microarray analysis to identify late PTHrP response genes using the chondrogenic ATDC5 cell line. At day 8 of micromass culture ATDC5 cells have pre-hypertrophic-like characteristics and at this time point the cells were stimulated with 10^{-7} M PTHrP for 24h and 72h and RNA was isolated. PTHrP treatment inhibited the outgrowth of cartilage matrix and decreased the expression of Col10a1 mRNA, which is in line with the inhibitory effects of PTHrP on chondrocyte differentiation.

Using cDNA microarray analysis, a list of 9 genes ($p< 10^{-3}$) was generated, including 3 upregulated (IGFBP4, Csrp2, and Ecm1) and 6 downregulated (Col9a1, Col2a1, Agc, Hmgn2, Calm1, and Mxd4) response genes. Four out of 9 genes (Ecm1, Calm1, Hmgn2, and Mxd4) are novel PTHrP response genes and 2 out of 9 (Calm1 and Hmgn2) have not yet been identified in cartilage. Four out of 9 (Ecm1, Col2a1, Col9a1, and Agc) genes are components of the extra cellular matrix and the remaining genes are involved in signal transduction and transcription regulation.

The response to PTHrP was validated by quantitative PCR, using the same RNA samples as labelled in the microarray experiments and RNA samples isolated from a new experiment. In addition, we examined whether these genes also reacted to PTHrP in other PTHrP responsive models, like KS483 osteoblasts and explanted metatarsals. The expression of late PTHrP response genes varied between ATDC5 chondrocytes, KS483 osteoblasts and metatarsals, suggesting that the expression of late response genes is dependent on the cellular context of the PTHrP responsive cells.

Introduction

Parathyroid hormone (PTH) related peptide (PTHrP) regulates the pace of chondrocyte differentiation during endochondral bone formation[1;2]. In the growth plate the main target cells for PTHrP are late-proliferating and pre-hypertrophic chondrocytes, which express the PTH/PTHrP receptor (PTHR1)[1;3]. Since both decreased and increased PTHrP signalling lead to severe growth plate abnormalities, the expression of PTHrP must be tightly controlled[4-7]. The protein controlling PTHrP expression in the growth plate is the growth factor Indian Hegdehog (IHh), which is expressed by pre-hypertrophic chondrocytes. Vice versa, PTHrP regulates the expression of IHh by inhibiting the transition from proliferating chondrocytes into hypertrophic chondrocytes, thereby delaying IHh production, which completes the negative feedback loop.

PTHrP not only controls the pace of chondrocyte differentiation in the growth plate, but is required for keeping the chondrocytes in the proliferative competent stage as well[8]. The effect of PTHrP on proliferation is mediated by controlling the expression of cell cycle regulators, like cyclin dependent kinase inhibitor p57kip2, as described recently[9].

Two major pathways are involved in PTHR1 transduction, namely the adenylate cyclase (AC)/ protein kinase A (PKA) and the phopholipase C (PLC)/ protein kinase C (PKC) pathway. Recently it was shown that the AC/PKA pathway is sufficient for the effects of PTHrP on chondrocyte differentiation, suggesting only a minor role for the PLC/PKC pathway[10]. Signalling via AC/PKA leads to the activation of the transcription factors, cAMP

response-element binding protein (CREB) and members of the AP-1 family. These two classes of transcription factors are largely responsible for the biological response to PTHrP. In concordance with this, a majority of the early target genes of PTHrP contain binding sites for CREB and/or AP-1 in their promoters[11-13]. Furthermore, phosphorylation by PKA regulates the activity of one of the master transcription factors in cartilage formation, Sox9[14]. This is another illustration that PTHrP can influence chondrocyte differentiation via the AC/PKA pathway.

Despite the critical role of PTHrP in endochondral bone formation, downstream targets of PTHrP, which transduce its effect on chondrocyte differentiation, are largely unknown. For this reason, we have started microarray experiments with the chondrogenic ATDC5 cell line[15]. This cell line is a representative model for studying the actions of PTHrP on chondrogenesis. The cells reproducibly differentiate into chondrocytes in 7 days in micromass culture. In addition, during this process cells become responsive to PTHrP and in agreement with in vivo studies PTHrP inhibits hypertrophic chondrocyte differentiation[16;17]. Previously, we have identified novel early response genes of PTHrP[18]. In this study we focus on the identification of late PTHrP response genes, regulated 24 or 72 hours after PTHrP treatment. Comparison of the expression patterns of these response genes in osteoblasts and explanted metatarsals with ATDC5 cells, demonstrates that the regulation of these genes by PTHrP is cell type specific and dependent on the cellular context of the PTHrP responsive cells.

Materials and Methods

Cell culture

ATDC5 cells were grown in Dulbecco's modified Eagle's medium /F-12 (DMEM/F12) (Invitrogen, Breda, The Netherlands) containing 100 U/ml penicillin (Invitrogen), 100 U/ml streptomycin (Invitrogen), 10% charcoal stripped foetal calf serum (FCS; Integro BV, Zaandam, The Netherlands), 10 µg/ml insulin (Sigma Chemical Co., St Louis, MO, USA), 10 µg/ml bovine transferrin (Roche, Almere, The Netherlands), and 3 x 10^{-8} M sodium selenite (Roche), in a humidified atmosphere of 5% CO_2 and 95% O_2 at 37°C. The micromass culture technique was modified from Ahrens et al.[19]. Trypsinized cells were resuspended in medium at a concentration of 2 x 10^7 cells/ml, and 3 drops of 10 µl of this cell suspension were placed in a well of a standard 12 wells culture plate. The cells were allowed to adhere for 2 hours at 37°C and 5% CO_2, and then 1 ml medium was added to each well. The medium was replaced every other day.

RNA isolation and amplification

Medium was refreshed after 7 days and the ATDC5 micromasses were challenged with 10^{-7} M PTHrP or vehicle at day 8 of culture (time point 0h) and total RNA was extracted at different time points in triplicate using Trizol LS Reagent (Invitrogen), followed by RNA cleanup with RNeasy mini kit (Qiagen, Maryland, USA). RNA concentrations were determined by measuring the absorbance at 260 nm. Next, RNA samples were pooled and time point 0h was chosen as reference sample. Total RNA (3 µg/reaction) was amplified as described before[20], with slight modifications. In short, first strand cDNA was synthesized by adding 500 ng T7-oligodT primer (5'-TCTAGTCGACGGCCAGTGAATTGTAATACGACTCACTATAGGGC

$G(T)_{21}$-3') to 10 µl RNA sample. Samples were incubated for 10 minutes at 70°C, followed by 60 minutes at 42°C in a total volume of 20 µl, containing 5x first strand buffer, 10 mM DTT, 0.5 mM dNTPs, 2U Rnasin (Promega, Leiden, The Netherlands), and 200U Superscript II RT (Invitrogen). Next, second strand cDNA was synthesized for 2 hours at 16°C in a total volume of 150 µl, containing 5x second strand buffer, 0.2 mM dNTPs, 10U DNA Ligase (Invitrogen), 40U DNA polymerase I (Invitrogen), and 2U Rnase H (Invitrogen). This was followed by addition of 2 µl T4 polymerase (5 U/µl) (Invitrogen) and incubation for 5 minutes at 16°C. The double-stranded cDNA reaction was stopped and the remaining RNA in the mixture was degraded by addition of 7.5 µl of 1 M NaOH, 2 mM EDTA followed by incubation at 65°C for 10 minutes. Samples were purified by phenol/chloroform/isoamylalcohol (25:24:1) extraction. The volume of the aqueous phase was increased to 450 µl using H_2O. For further purification, samples were transferred to a Centricon-100 microconcentrator column (Millipore, Amsterdam, The Netherlands) (pre-spinned with 450 µl H_2O) and centrifuged for 12 minutes at 2500 rpm. After 3 wash steps with 450 µl H_2O, cDNA was collected in a total volume of 7 µl by inverting the column and by centrifuging for 30 s at 13000 rpm. Subsequently, cDNA was transcribed into cRNA using the T7 high yield transcription kit (Epicentre, Madison, USA). The cDNA solution was incubated at 42°C for 3 hours in a total volume of 20 µl, containing 10x T7 reaction buffer, 7.5 mM ATP, CTP, GTP, and UTP, 10 mM DTT, 2 U Rnasin, and 2 µl Ampliscribe T7 enzyme solution, followed by sample concentration using centricon-100 microconcentrator columns. This method was based on the original protocol of Van Gelder et al.[21]. Finally, cRNA concentration was determined by measuring the absorbance at 260 nm.

Probe labelling

cRNA (1.2 µg) was reverse transcribed with random hexamer primers, and labelled by incorporation of cyanine 5-dUTP (Cy5) or cyanine 3-dUTP (Cy3) (NEN, Boston, USA) according to the protocols of Ross et al.[22] with slight modifications. In short, cRNA and 8 µg random primers (Roche) in a total volume of 15 µl was incubated for 10 minutes at 70°C. Subsequently, 6 µl 5x first strand buffer, 3 µl 0.1 M DTT, 0.6 µl low-T dNTPs, 3 µl Cy3 dUTP (time point 0h) or Cy5-dUTP (other time points), and 1 µl Superscript II RT (200 U/µl) were added, incubated for 10 minutes at room temperature, followed by incubation at 42°C for 90 minutes. After 60 minutes fresh Superscript II RT (1 µl) was added. Next, RNA was degraded, by addition of 15 µl 0.1 M NaOH and incubation for 10 minutes at 70°C, after which the solution was neutralized by addition of 15 µl 0.1 M HCl. The labelled samples, supplemented with 180 µl 10 mM Tris, 1mM EDTA, pH 8 (TE) and 10 µl mouse Cot-1 DNA (10 mg/ml) (Invitrogen), were pooled and purified using a centricon-30 microconcentrator column (Millipore) (pre-spinned with 450 µl TE for 8 minutes at 13000 rpm). PolyA RNA (20 µg) (Amersham) and yeast tRNA (20 µg) (Invitrogen) were added to 450 µl TE during the second wash step. The purified product was collected, by inverting the column and by centrifuging for 1 minutes at 13000 rpm, and finally, resuspended in a total volume of 45 µl hybridization solution, containing 7.65 µl 20x SSC and 1.35 µl 10% SDS.

(Pre-) hybridization

For the hybridization experiments microarrays, on which the NIA 15k mouse cDNA clone

set[23] was spotted, were purchased from the Leiden Genome Technology Center (LGTC). DNA was crosslinked by UV irradiation at 65 mJ/cm^2 (Stratalinker mode 1800 UV Illuminator, Stratagene). To prevent non-specific hybridization, the slides were incubated in 45 µl hybridization solution (400 ng/µl yeast tRNA, 400 ng/µl poly(A) RNA, 400 ng/µl herring sperm DNA (Invitrogen), 100 ng/µl mouse Cot1 DNA, 5x Denhardt's solution, 3.2x SSC and 0.4% SDS) at 65°C for 30 minutes. Prior to hybridization, the slides containing the pre-hybridization mixture were incubated for 2 minutes at 80°C to denature the spotted DNA. After pre-hybridization, the slides were washed twice in 2x SSC for 5 minutes at room temperature and dehydrated with subsequent steps of 5 x 5 minutes 70%, 5 minutes 90% and 5 minutes 100% ethanol. For hybridization, the probes were denatured by heating for 2 minutes at 100°C, left at room temperature for 15 minutes, centrifuged for 10 minutes, and placed under a 24 mm x 60 mm glass coverslip. The slides were incubated overnight at 65°C in a hybridization chamber (Corning, Amsterdam, The Netherlands) and washed the next day in 2x SSC for 5 minutes at room temperature and dehydrated using graded ethanols.

Microarray design and statistical analysis

The reference array experiment, 0h vs 0h, was hybridized in duplicate, 24h vs 0h and 72h vs 0h for PTHrP and vehicle treated samples were hybridized in triplicate. Following hybridization, slides were scanned in the Agilent DNA Microarray scanner (Agilent Technologies, Amstelveen, The Netherlands). Genepix 3.0 software (Axon Instruments Inc.) was used to quantify the resulting images. Subsequently, normalization and gene expression analysis were performed with Rosetta Resolver (Rosetta Biosoftware, seattle, USA). Due to the overall poor quality of the cDNA spots on the microarray, stringent selection criteria were used for inclusion of spots, to minimize the risk of false positive signals. A spot was only included in the analysis if it passed all of the following selection criteria. 1) Spots should have an absolute fold change of less than 2 in the reference array. 2) Spots should show no significantly regulation ($p<0.01$) in the reference array. 3) Only non-flagged spots in any of the arrays were included. 4) The signal intensity of Cy3 or Cy5 of the spots should be above a cut off level (0.05). 5) Spots should show an absolute fold change less than 50. Next, analysis of variance (ANOVA) was performed between the remaining spots of the reference and PTHrP treated hybridizations. 6) Spots should show significant ($p<0.001$) differential expression regulation, with intensity value above background value. 7) Spots should not be differentially expressed ($p<0.01$) during time in the vehicle treated cultures. To identify the selected spots, the PCR-amplified cDNA of each spot (500 ng) was sequenced by the LGTC using 12 pmol M13 primers in a total volume of 24 µl.

Histology

Micromasses were fixed for 10 minutes in 10% formalin, subsequently dehydrated using graded ethanols, and embedded in paraffin. Sections of 5 micron were deparaffined with Paraclear (EarthSafe Technologies Inc, Belle Mead, NJ, USA) and hydrated using graded ethanols. Subsequently, the sections were treated for 3 minutes with 3% acetic acid, stained with 1% Alcian Blue for 30 minutes, rinsed first with 3% acetic acid and then with bidest. Hereafter, the sections were immersed in Nuclear Fast Red for 5 minutes and then rinsed with

bidest. Next, the sections were dehydrated using graded ethanols. Finally, the sections were embedded in histomount-diluted 1:1 xylene.

RNA isolation from mouse bone explants

Metatarsalsia were isolated from 15-days old Swiss Albino mouse embryos. The mice were kept in a light and temperature controlled room (12 hours light, 20-22°C) with food and water available *ad libitum*. Experiments were approved by the local ethical committee for animal experiments. The bone explants were cultured in αMEM with 10% FCS and the next day metatarsals were challenged with 10^{-7} M PTHrP or vehicle in αMEM containing 1% BSA. RNA was isolated after 24 and 72 hours from metatarsals, using Trizol LS Reagent (Invitrogen) and reverse transcribed into cDNA using random hexamer primers (Amersham).

RNA isolation from KS483 osteoblasts

KS483 mesenchymal progenitor cells were differentiated into osteoblasts as described previously[24]. At day 11, KS483 cells have formed bone nodules and the cells within the nodules start to mineralize the extracellular matrix. This time point was chosen to treat the cells with 10^{-7} M PTHrP. RNA was isolated after 24 and 72 hours using Trizol LS Reagent (Invitrogen) and reverse transcribed into cDNA using random hexamer primers (Amersham).

Quantitative PCR (qPCR)

To validate the expression patterns of PTHrP response genes, quantitative PCR 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[25]. Significant changes were calculated using the Double Delta Model (DDM) (Chapter 6).

Name	F/R	Forward primer	MgCl
β2μ	F	CACTGACCGGCCTGTATGC	3 mM
	R	GAATTCAGTGTGAGCCAGGATATAGA	
IGFBP4	F	AACTGTGACCGCAACGGC	4 mM
	R	ACGCTGTCCGTCCAGGG	
Csrp2	F	CCACTCGGAATGCCTGTCTG	3 mM
	R	GCCACTGTTGTGCTGTCTAAATTTT	
Ecm1	F	TCGCCCACCTAGCTCCCTATC	4 mM
	R	AGCAGCGGACGGTCATATTCTG	
Col2a1	F	CTTCGGAGAGTGCTGTCCCATC	4 mM
	R	CCCTTGTCACCACGATCACCTC	
Col9a1	F	CACTGGGCTGCCTGGTATCC	4 mM
	R	GGCTCCTGTGTCTGGTCTCTTG	
Agc	F	CGAATGGAACGACGTCCC	3 mM
	R	CTGATCTCGTAGCGATCTTTCTTCT	
Calm1	F	GTGTCCGTGGTGCCGTTACTC	3 mM
	R	TGCGAGCGAAGGAAGGAAGAAC	
Hmgn2	F	TCCAAAGCCAGAGCCCAAACC	4 mM
	R	GGCATCTCCAGCACCTTCAGC	
Mxd4	F	CCCGAACAACAGGTCTTCACAC	4 mM
	R	GCACGCTTCAGAAGGCTCAG	
Ap3d	F	AATGCGGAGGCGGTGAAGTC	4 mM
	R	GGGTGGTGGACAGCCAGAAGC	
Brd4	F	GGCTCGAAAACTCCAGGATGTG	4 mM
	R	TGTCGCTGCTGCTGTCACTAG	
Nme1	F	CAGGCAGGGGACCAGCAAC	4 mM
	R	GCCTGAAGGAGGTAAGGAATGC	
Nsd1	F	CACACAGAACAAGGGCTTCAGG	3 mM
	R	CCACAGGACAGCGGACACAG	
Tgfβ1i4	F	CTCCCTCCCGCCCTCTCTC	4 mM
	R	GGTTCCCAGCAAAGACGACAAG	

Table 1 : qPCR primer sets

Results

Effect of PTHrP treatment on ATDC5 micromass cultures

ATDC5 cells were cultured as micromasses for 8 days to stimulate chondrocyte differentiation. Previously we have shown that the cells have a pre-hypertrophic phenotype at this stage of culture[18]. PTHrP treatment at day 8 resulted in inhibition of the outgrowth of cartilage matrix 72 hours later (fig. 1A). Histological analysis of the micromasses showed rounded chondrocytes embedded in an alcian blue positive extracellular matrix (fig.1B). Treatment with PTHrP for 24 and 72 hours did not change the cell structure or the intensity of the alcian blue staining in the micromasses. RNA was isolated 24 and 72 hours after PTHrP treatment and used for qPCR analysis to study the expression of collagen 10 (Col10a1), a marker for hypertrophic chondrocytes. PTHrP decreased Col10a1 mRNA expression after 72 hours of treatment (fig. 1C).

Taken together PTHrP treatment at day 8 of culture inhibited new formation of cartilage and inhibited the differentiation into hypertrophic Col10a1 expressing chondrocytes. For this reason, this time point was chosen for identification of PTHrP response genes.

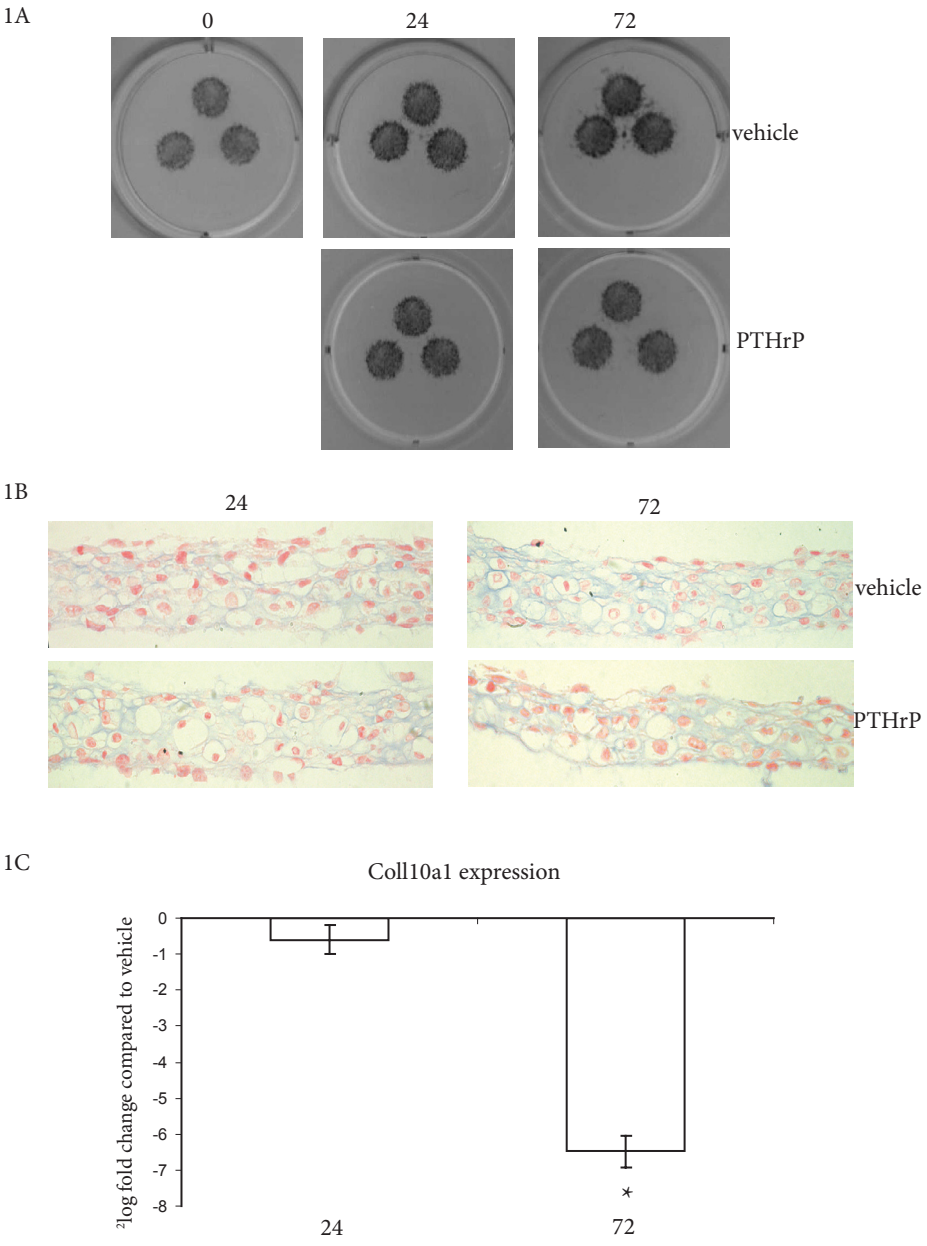


Figure 1: characterization of ATDC5 micromass cultures.
(A) ATDC5 micromass cultures were cultured for 8 days and then treated with vehicle or 10-7 M PTHrP for 0, 24 and 72 hours. Cultures were stained with alcian blue. (B) Alcian blue stained sections of ATDC5 micromasses, cultured for 8 days and then treated with vehicle or 10-7 M PTHrP for 24 and 72 hours. (C) qPCR analysis of col10a1 mRNA in micromasses cultured for 8 days and then treated with 10-7 M PTHrP for 24 and 72 hours. Expression in PTHrP treated cultures was compared to vehicle treated cultures and expressed as log fold change. * Significant vs vehicle treated samples ($p<0.05$).

Name	Accession number	Up/down	Ratio 0h[2]	Ratio 24h[2]	Ratio 72h[2]	selected
Extracellular matrix protein 1 (Ecm1)	NM_007899	up	0.75	1.56	1.82	X
Transforming growth factor beta 1 induced transcript 4 (TGFβ1i4)1	NM_009366	up	1.01	1.71	2.36	X
Cysteine and glycine-rich protein 2 (Csrp2)	NM_007792	up	1.12	2.71	1.92	X
CD2 antigen (cytoplasmic tail) binding protein 2 (Cd2bp2)	NM_027353	up	1.11	4.94	3.26	
Insulin-like growth factor binding protein 4 (IGFBP4)	NM_010517	up	0.81	1.84	1.42	X
Interferon induced transmembrane protein 3 (Ifitm3)	NM_025378	up	0.85	1.85	2.14	
Carbonyl reductase 2 (Cbr2)	NM_07621	up	0.86	2.42	2.12	
Nuclear receptor-binding SET-domain protein 1 (NSD1)1	AF419220	up	1.12	1.71	2.07	X
NADH dehydrogenase (ubiquinone) Fe-S protein 5 (Ndufs5)	NM_134104	up	0.76	1.60	3.17	
Guanosine monophosphate reductase (Gmpr)	NM_025508	up	0.91	1.25	2.56	
EST	AC122296	up	1.11	3.82	2.60	
Phosphatidylinositol glycan, class T (Pigt)	NM_133799	down	1.31	0.38	0.99	
Adaptor-related protein complex 3, delta 1 subunit (Ap3d)1	NM_007460	down	0.86	0.45	0.52	X
Aggrecan (Agc)	NM_007424	down	0.86	0.25	0.14	X
Procollagen, type IX, alpha 1 (Col9a1)	NM_007740	down	0.91	1.03	0.20	X
Stearoyl-Coenzyme A desaturase 2 (Scd2)	NM_009128	down	1.00	0.69	0.40	
Max dimerization protein 4 (Mxd4)	NM_010753	down	0.79	0.55	0.40	X
Expressed in non-metastatic cells 1 (Nme1)1	NM_008704	down	1.26	0.61	0.51	X
Splicing factor, arginine/serine-rich 1 (ASF/SF2)	NM_173374	down	0.92	0.60	0.39	
Hypoxanthine guanine phosphoribosyl transferase 1 (Hprt1)	NM_013556	down	0.96	0.64	0.49	
Procollagen, type II, alpha 1 (Col2a1)	NM_031163	down	1.08	0.81	0.28	X
SID1 transmembrane family, member 2	NM_172257	down	0.90	0.71	0.56	
Ubiquitin specific protease 34 (usp34)	AK033182	down	0.96	0.66	0.44	
Selenoprotein (Sep15)	NM_053102	down	0.96	0.79	0.52	
Calmodulin 1 (Calm1)	NM_009790	down	0.87	0.63	0.40	X
Bromodomain-containing 4 (Brd4)1	NM_020508	down	0.84	0.53	0.42	X
High mobility group nucleosomal binding protein domain 2 (Hmgn2)	NM_016957	down	0.97	0.64	0.42	X
EST	AC127579	down	0.84	0.71	0.52	
EST	NM_026561	down	0.87	0.38	0.35	
EST	NM_028043	down	0.91	0.66	0.40	
EST	NM_134007	down	1.04	0.72	0.57	

Table 2: Genes regulated by PTHrP in chondrocytes revealed by statistical analysis of the microarray data.

[1]) These genes could not be validated in qPCR analysis

[2]) Significant ($p<0.001$) differential expression regulation.

Selection of PTHrP response genes

ATDC5 cells were cultured as micromasses and treated at day 8 with 10^{-7} M PTHrP for 24 and 72 hours and cDNA microarray analysis was performed. After applying stringent selection criteria, 8319 out of the 15442 spots were taken in the analysis. A list of 31 spots, which exhibited significant ($p<0.001$) differential expression after 24 or 72 hours following PTHrP treatment and did not change in the vehicle treated cultures, was generated (table 2). From this list 14 genes were chosen for validation experiments in ATDC5 cells. Exclusion criteria were insufficient information on gene identity, for example ESTs, or insufficient information for the design of qPCR primer sets, which span intron-exon boundaries.

Validation of PTHrP response genes in ATDC5 cells by qPCR

The expression patterns of the selected genes were first validated by studying expression profiles using qPCR in the same RNA samples subjected to microarray analysis (fig. 2B, D, F and H) and in RNA samples from ATDC5 cells isolated in a new independent experiment (data not shown). Beta-2-microglobulin (β2μ) was used as internal standard, as it was not regulated by PTHrP in microarray analysis (data not shown). Five out of 14 genes could not be validated in the second RNA panel and were discarded from further study, leaving 9 genes for more detailed analysis (table 3). Among these 9 identified PTHrP response genes, 3 genes were upregulated and 6 genes were downregulated. Five genes were already known response genes of PTHrP and 7 genes have previously been found in growth plate chondrocytes (table 3). Four out of 9 response genes were extracellular matrix proteins, namely Ecm1, Col2a1, Col9a1 and Agc. The other response genes were implicated in protein binding and were involved in the regulation of signalling and transcription.

Based on the expression patterns, the genes were divided into 4 groups, 2 groups for upregulated genes and 2 groups for downregulated genes (fig. 2A, C, E and G). The expression of the genes in expression pattern 1 peaked at 24 hours while the gene in expression pattern 2 was continuously upregulated. The gene in expression pattern 3 was only downregulated after 72 hours. In expression pattern 4 the genes were continuously downregulated. Expression

Reg.[1]	Abbr.[2]	Name	Function	rp[3]	gp[4]
up	IGFBP4	Insulin-like growth factor binding protein 4	Negative modulator of IGF-1 activity	yes	yes
up	Csrp2	Cystein and glycine-rich protein 2	Binding partner of protein inhibitor of activated STAT1 (PIAS1)	yes	yes
up	Ecm1	Extracellular matrix protein 1	Negative regulator of endochondral bone formation	no	yes
down	Col9a1	Collagen, type IX, alpha 1	Extracellular matrix protein	yes	yes
down	Col2a1	Collagen, type II, alpha 1	Extracellular matrix protein	yes	yes
down	Agc	Aggrecan	Extracellular matrix protein	yes	yes
down	Calm1	Calmodulin 1	Principal mediator of the calcium signal	no	no
down	Hmgn2	High mobility group nucleosome binding protein 2	Nucleosome binding protein	no	no
down	Mxd4	Max dimerization protein 4	Member of Myc/Max/Mad network	no	yes

Table 3 : List of validated PTHrP target genes.

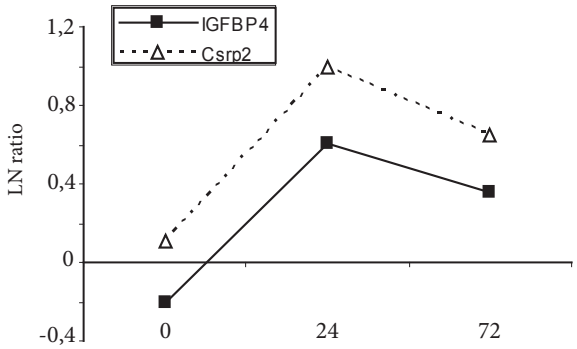
[1]) Reg. = regulation

[2]) Abbr. = abbreviation

[3]) rp = previously been implicated in PTHrP signaling

[4]) gp = previously been found in growth plate chondrocytes

2A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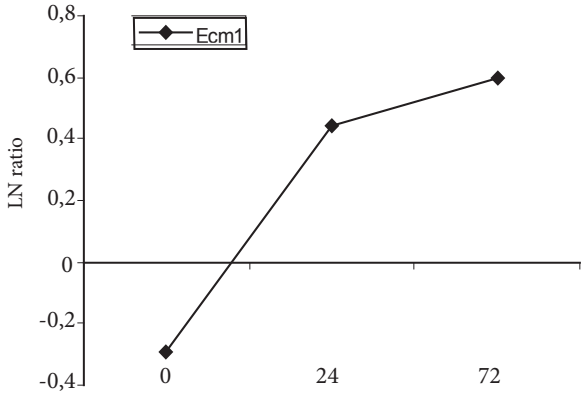
72

2C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-0,4
0
24
72

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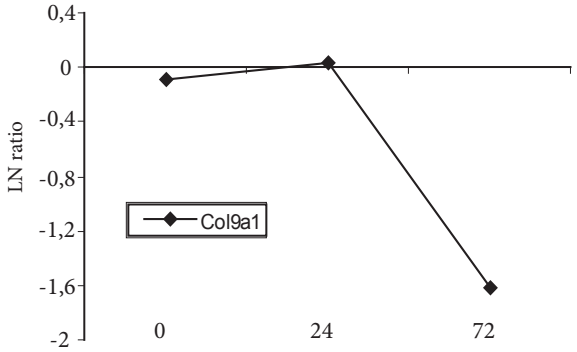
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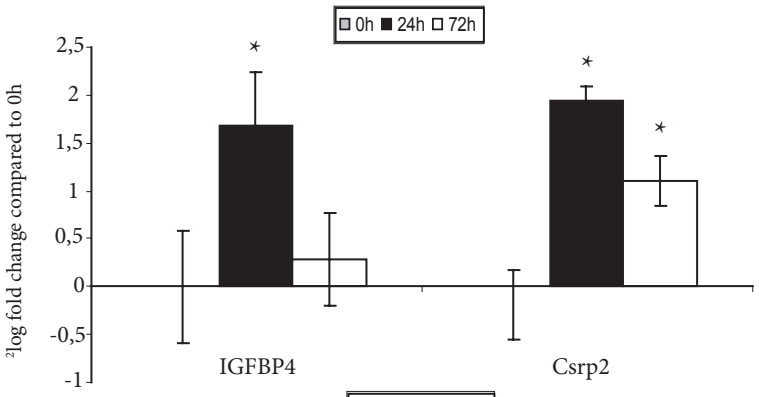
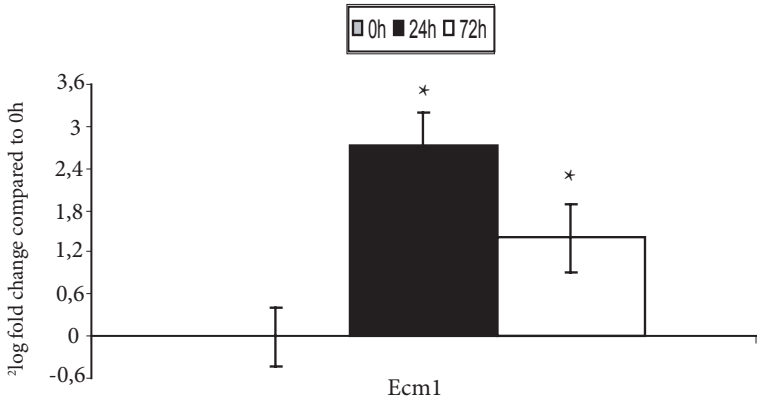
-0,8
-1,2
-1,6
-2
0
24
72

2B


0h 24h 72h
2,5
2
1,5
1
0,5
0
-0,5
-1
2log fold change compared to 0h
IGFBP4
Csrp2

2D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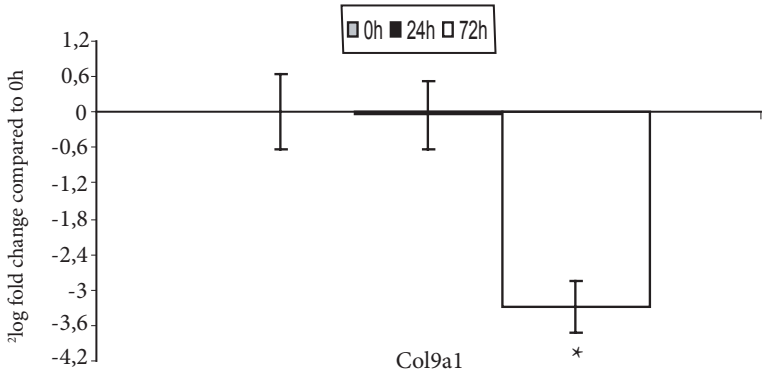
Ecm1

2F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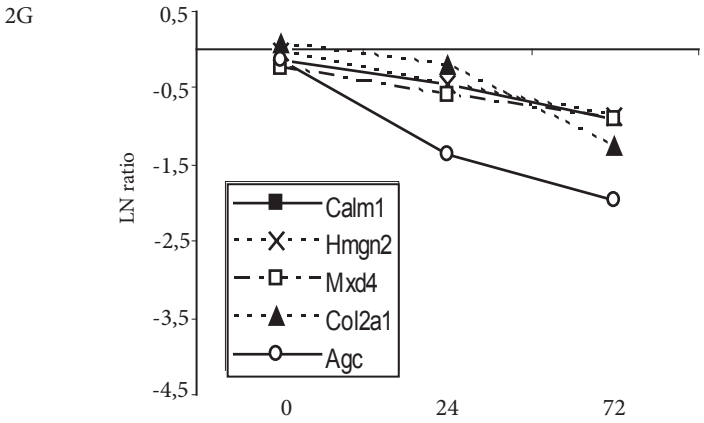
Col9a1



pattern 1 contained Csrp2 and IGFBP4 and expression pattern 2 contained Ecm1. Col9a1 formed expression pattern 3. In expression pattern 4 were Col2a1, Agc, Calm1, Hmgn2, and Mxd4.
In qPCR experiments all genes, except for Ecm1, showed the same expression profile compared to the data from microarray analysis (fig. 2). Ecm1 showed the same expression pattern as the genes in expression profile 1 (fig. 2B and D), upregulation at 24 hours followed by a slight decrease of mRNA expression after 72 hours, instead of continuously upregulation revealed by microarray analysis.

Validation of PTHrP response genes in metatarsals by qPCR

To test whether the identified response genes also respond to PTHrP in chondrocytes *ex vivo*, metatarsals from 15-day old mouse embryos were treated with 10^{-7} M PTHrP for 24 and 72 hours and qPCR analysis was performed. Metatarsals from 15-day old mouse embryos consist of undifferentiated cartilage surrounded by a perichondrium in which osteoblast and osteoclast precursors are present. They differentiate normally during a 1 week culture period into a mineralized bone explant[26]. In response to PTHrP the expression of Col10a1 decreased after 24 hours, indicating a biological response to PTHrP (fig. 3A). All genes except 1, Calm1, responded to PTHrP in the metatarsal model. However, there were remarkable differences, both in the temporal regulation and in the direction of regulation, in the response to PTHrP when compared to the response in ATDC5 cells (compare fig. 3 with fig. 2). For instance, all downregulated genes in the ATDC5 cultures were upregulated in the metatarsal model. In addition, the peak in expression of the upregulated genes, IGFBP4, Csrp2, and Ecm1 differed between the two models. In metatarsals the peak expression of IGFBP4, Csrp2, and Ecm1 was after 72 hours compared to the peak expression in ATDC5 cells after 24 hours.

Validation of PTHrP response genes in KS483 osteoblasts by qPCR

To test whether the response of the response genes were restricted to chondrocytes or whether

2H

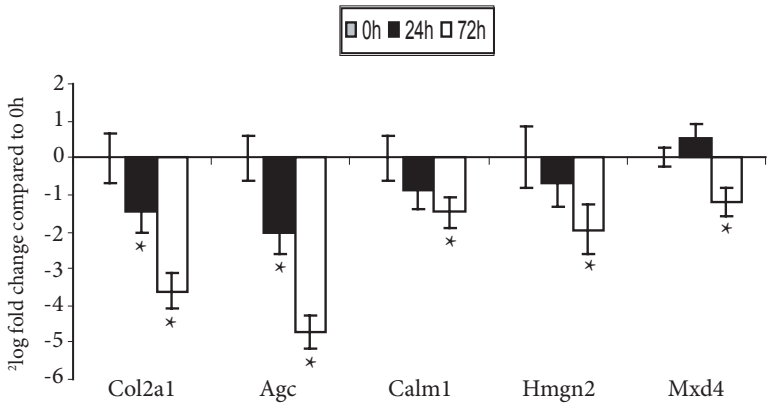


Figure2: expression profiles of PTHrP response genes in ATDC5 chondrocytes revealed by microarray analysis and qPCR analysis.
RNA was isolated from ATDC5 micromass cultures, at 0, 24 and 72 hours after PTHrP (10-7 M) stimulation, amplified and labelled as described. Samples were hybridized against 0 hours in duplicate or triplicate (for details see materials and methods). In the left panel the expression profiles revealed by microarray analysis are shown (A, C, E, and G). In the right panel the corresponding patterns revealed by qPCR are shown (B, D, F, and H). Every sample was run in triplicate.
(A and B) Expression profile 1 contained 2 upregulated genes, i.e. IGFBP4 and Csrp2. (C and D) Expression profile 2 contained 1 upregulated gene, i.e. Ecm1. (E and F) Expression profile 3 contained 1 downregulated gene, i.e. Col9a1. (G and H) Expression profile 4 contained 5 downregulated genes, i.e. Col2a1, Agc, Calm1, Hmgn2, and Mxd4. Expression in PTHrP treated cultures was compared to vehicle treated cultures and expressed as log fold change. *Significant vs 0h ($p<0.05$).

they were part of a more generalised response to PTHrP, the expression patterns were analysed in differentiated KS483 osteoblasts using qPCR (fig. 4). For this purpose, RNA was isolated after 24 and 72 hours after a challenge with 10^{-7} M PTHrP. The expression patterns of the typical chondrocyte markers Agc, Col2a1 and Col9a1 were not established in this osteoblast culture system. The expression of 3 out of 6 response genes changed after PTHrP treatment in the KS483 culture system, including IGFBP4, Ecm1, and Mxd4. The expression regulation of IGFBP4 and Ecm1 was comparable to the regulation in metatarsals and was different in peak expression compared to the ATDC5 cells. Mxd4 mRNA expression increased in response to PTHrP in osteoblasts, instead of a decrease in expression in ATDC5 chondrocytes, however the expression was comparable to the PTHrP response in metatarsals. Csrp2, Calm1, and Hmgn2 were not significantly regulated by PTHrP in KS483 osteoblasts.

Discussion

In the present study, we have identified late response genes of PTHrP in chondrocytes using the chondrogenic ATDC5 cell line. ATDC5 cells were cultured as micromasses to induce chondrocyte differentiation. After 1 week this resulted in a homogeneously differentiated cell culture, which contained chondrocytes with pre-hypertrophic-like characteristics. Previously, we have shown high expression of PTHR1 in these cells[18]. Because late-proliferating and pre-hypertrophic cells are the main target cells of PTHrP in the growth plate[2], we decided to stimulate the micromasses at day 8 with PTHrP for 24 and 72 hours, to identify late PTHrP

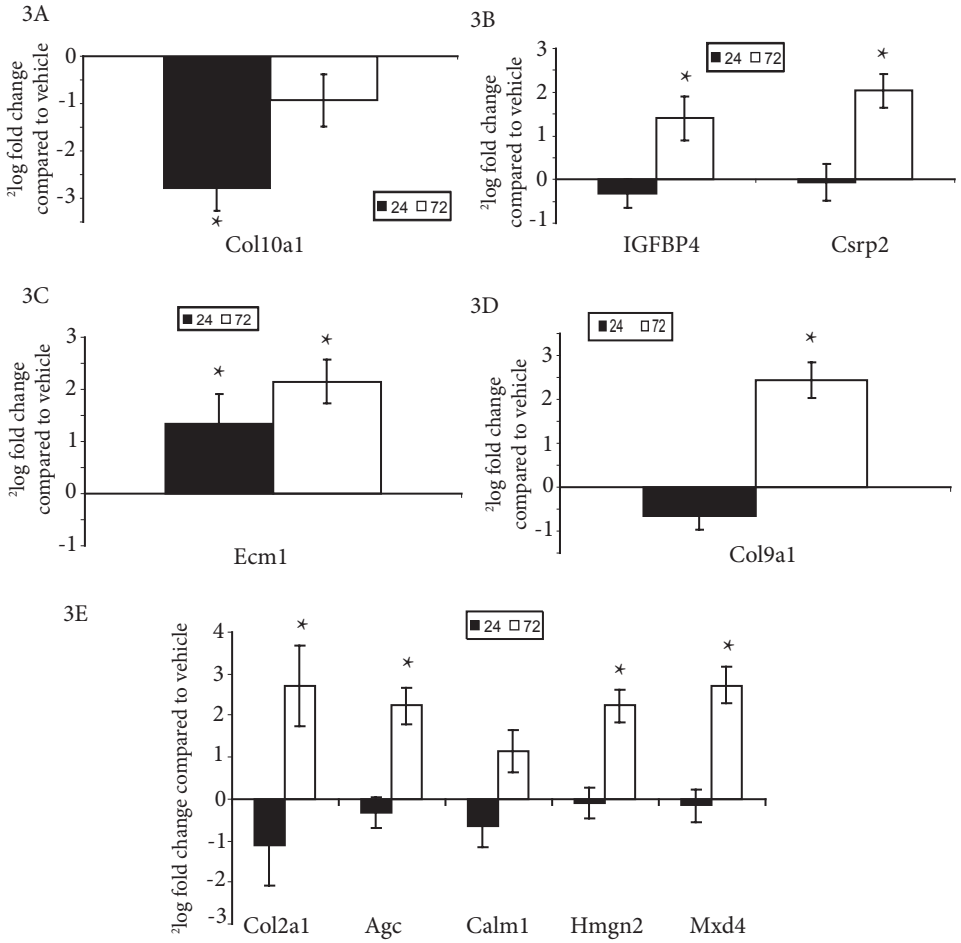


Figure 3: expression patterns of PTHrP response genes in 15-day old metatarsals.
RNA was isolated from 15-day old metatarsals and treated with 10-7 M PTHrP for 24 and 72 hours, and qPCR was performed. Every sample was run in triplicate. (A) PTHrP treatment decreased Col10a1 mRNA expression as expected. (B) Expression regulation of genes from expression pattern 1, as revealed by microarray analysis in ATDC5 cells, in explanted metatarsals. (C) Expression regulation of genes from expression pattern 2, as revealed by microarray analysis in ATDC5 cells, in explanted metatarsals. (D) Expression regulation of genes from expression pattern 3, as revealed by microarray analysis in ATDC5 cells, in explanted metatarsals. (E) Expression regulation of genes from expression pattern 4, as revealed by microarray analysis in ATDC5 cells, in explanted metatarsals. Expression in PTHrP treated cultures was compared to vehicle treated cultures and expressed as log fold change. *Significant vs vehicle treated samples ($p<0.05$).

response genes. A challenge with PTHrP resulted in a biological response at 72 hours, as shown by downregulation of the hypertrophic marker Col10a1 and inhibition of the outgrowth of the cartilage matrix, but did not result in a change of cell structure within the micromasses. This is in agreement with current evidence that PTHrP does not inhibit formation of chondrocytes per se, but inhibits their differentiation into hypertrophic cells.
To identify late response genes of PTHrP we used the NIA 15k mouse cDNA bank, which was

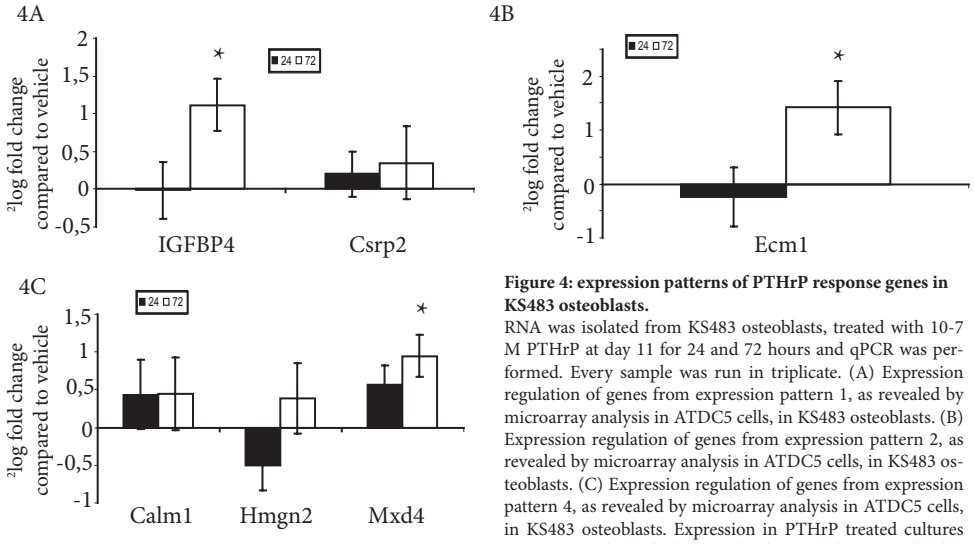


Figure 4: expression patterns of PTHrP response genes in KS483 osteoblasts.
RNA was isolated from KS483 osteoblasts, treated with 10-7 M PTHrP at day 11 for 24 and 72 hours and qPCR was performed. Every sample was run in triplicate. (A) Expression regulation of genes from expression pattern 1, as revealed by microarray analysis in ATDC5 cells, in KS483 osteoblasts. (B) Expression regulation of genes from expression pattern 2, as revealed by microarray analysis in ATDC5 cells, in KS483 osteoblasts. (C) Expression regulation of genes from expression pattern 4, as revealed by microarray analysis in ATDC5 cells, in KS483 osteoblasts. Expression in PTHrP treated cultures was compared to vehicle treated cultures and expressed as log fold change. Typical cartilage markers were not studied. *Significant vs vehicle treated samples ($p<0.05$).

amplified and spotted by the Leiden Genome Technology Center. Compared to commercially available microarrays, the quality control of custom made cDNA microarray is a well known problem. The quality of the spots of the microarray used in this study was low, due to heterogeneous spot morphologies ("doughnuts"), deposition inconsistencies, and oversized spots as described previously[18;27]. In addition, identification of the spots was only possible by direct sequencing the cDNAs used in the spotting process, because of contamination. This contamination is most likely introduced during multiple rounds of replication of the bank by PCR, as previously suggested[28]. Pathway screening or genome wide analysis could not be used in this study, because of the uncertainty of the identity of the spots. However, the microarray could still be used to identify PTHrP response genes by applying very stringent selection criteria. Using qPCR in different cell models and bio-informatics analysis we showed the validity of this approach. We were able to classify 9 out of 14 genes as bonafide response genes of PTHrP. However, due to the distinct methodologies and in our case also the relative poor quality of the custom made arrays, the overlap was not 100%. Others have also reported this, particularly with respect to the fold changes[29-31].

Bio-informatic analysis revealed that 4 genes, Ecm1, Calm1, Hmgn2 and Mxd4, have not yet been described as response genes of PTHrP. IGFBP4 was already identified as PTHrP response gene in osteoblasts[32] and the typical chondrocyte markers, Agc, Col2a1, and Col9a1, in chondrocytes[17]. In addition, Csrp2 has previously been identified as an early target gene of PTHrP in chondrocytes as well as in osteoblasts in our previous study[18]. Two out of 9 late response genes, Calm1 and Hmgn2, have not been demonstrated in growth plate chondrocytes before. Furthermore, 4 out 9 genes (Ecm1, Col2a1, Col9a1, and Agc) were matrix proteins compared to 0 out of 12 genes among the early response genes. In addition,

4 out of 9 late response genes (Csrp2, Hmgn2, Calm1 and Mxd4) were involved in signal transduction and regulation, compared to 10 out of 12 early response genes[18]. Although we had only a small list of response genes, this is in agreement with the notion that the majority of early target genes are implicated in signal transduction and regulation and that the majority of late response genes code for structural proteins belonging to the extracellular matrix[29;33]. The remaining late response gene, IGFBP4, is involved in protein binding and modulation of signal transduction.

Detailed analysis on the role of some of our targets in chondrocyte differentiation is currently lacking. For example, an interesting feature of the transcription factor Hmgn2 is the presence of a HMG domain. Remarkably, the three major transcription factors involved in chondrocyte differentiation, Sox9, Sox6, and L-Sox5, contain a HMG box. The HMG domain containing protein family can be divided in two subfamilies, the first one containing the subclass of Sox proteins and Hmgn2 belongs to the second subfamily[34]. Together with other as yet unidentified HMG-box containing proteins, the Sox transcription factors form a complex, which binds to several consensus HMG binding sites in the collagen 2 promoter[35;36]. Hmgn2 may be one of these unknown binding proteins. Thus besides regulation of Sox9 activity by phosphorylation, PTHrP may also influence the expression of putative binding partners of Sox9. Remarkably, Hmgn2 is downregulated in ATDC5 and upregulated in metatarsals, but not regulated in osteoblasts, suggesting chondrocyte specific regulation of Hmgn2 expression by PTHrP.

Another response gene of PTHrP involved in protein binding and transcription regulation was Mxd4, a member of the Mad gene family. Mxd4 heterodimerizes with Max forming a transcriptional repression complex. Mxd4 competes for Max binding with Myc, which heterodimerizes with Max forming a transcriptional activation complex. Through regulation of Mxd4 expression, PTHrP could influence the activity of c-Myc, which plays an important role in cell proliferation and prevents the differentiation of cultured chondrocytes into hypertrophic chondrocytes[37;38]. Regulation of c-Myc activity might be one of the mechanisms by which PTHrP keeps the chondrocytes in a proliferative competent stage.

The remaining proteins involved in protein binding and signalling regulation were IGFBP4, Csrp2, and Calm1. PTHrP may play a role in changing intracellular Ca2+ levels by regulating Calm1 expression, which appears to be limited to the ATDC5 cells[39]. The expression regulation of Csrp2, a binding partner of Pias1, which inhibits Stat1, could indicate a possible crosstalk between PTHrP signalling and FGF or Growth Hormone (GH) signalling cascades as mentioned before[18;40]. Both factors use Jak/Stat signalling to exert their effects on chondrocyte proliferation and differentiation. In addition, our data indicates a crosstalk between PTHrP and the IGF signalling cascade as well. IGFBP4 is shown to exert inhibitory effects on IGF1-promoted growth[41;42]. Via upregulation of IGFBP4, PTHrP might be able to counteract IGF1-activity.

A remarkable finding in this study was the decrease of Agc mRNA expression in ATDC5 chondrocytes and an increase of Agc mRNA expression in explanted metatarsals after PTHrP treatment. In contrast to the metatarsals, which contain chondrocytes at various stages of differentiation as well as other cell types like osteoblasts and –clasts, the cultures of the ATDC5 cells are more homogenous. It may well be that regulation of Agc by PTHrP is dependent on the developmental and differentiation stage of the chondrocyte. This is supported by

observations in rat costal chondrocytes in which PTHrP either induced, slightly repressed or had no effect on Agc mRNA expression depending on the gestational age of the cells[43].
Previously we have shown that the majority of early PTHrP effects were part of a more generalised response, not only restricted to chondrocytes[18]. To address whether this was also the case for late PTHrP responses, the expression regulation of these genes by PTHrP were also established in KS483 osteoblasts and in explanted metatarsals, which consist of different cell types, like chondrocytes and osteoblast and osteoclast precursors in the perichondrium. In both systems differences were found in gene expression compared to the expression in ATDC5 chondrocytes in response to PTHrP. Most of the upregulated genes showed a temporal change in expression in metatarsals and osteoblasts. In addition, the direction of the expression of downregulated genes was changed or the expression was not regulated in osteoblasts and metatarsals. This suggest that between early and late effects mechanisms must be operational that translate the PTHrP responses from a more generalised effect, as seen in the immediate early response, into a cell type and cellular context dependent effect, as seen in the late response. Results of other studies also indicate such a mechanism[29;33]. The factors involved in this process are not known.
In summary, we have identified 9 late PTHrP response genes, including 4 novel target genes, Ecm1, Calm1, Hmgn2, and Mxd4. Among them were structural proteins, binding proteins and regulators of signalling. The expression of the late PTHrP response genes varied between cell types, suggesting a functional mechanism, translating the PTHrP responses from a more generalised early response, into a cell type and cellular context dependent late response.

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