The role of PTHrP in chondrocyte differentiation.

Hoogendam, J.

Novel early target genes of PTHrP in chondrocytes

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Abstract

We have performed microarray analysis to identify PTHrP target genes in chondrocytes. ATDC5 cells were cultured as micromasses to induce chondrocyte differentiation. At day 8 of culture the cells had a pre-hypertrophic appearance. This time point was chosen for isolation of RNA at 0h, 1h, 2h, and 4h after a challenge with 10^{-7} M PTHrP. Samples were subjected to a cDNA microarray using competition hybridization.

A list of 12 genes ($p< 10^{-3}$), of which the expression regulation by PTHrP was confirmed by qPCR analysis, was generated. This included 7 upregulated and 5 downregulated genes. Three genes were known to be involved in PTHrP regulation and 6 were previously found in growth plate chondrocytes. Most of the genes (10 out of 12) were implicated in signal transduction and regulation. PTHrP also induced the expression of the upregulated genes in KS483 osteoblasts, suggesting involvement in a more generalized response to PTHrP. The vast majority of the upregulated genes (6 out of 7) contained CREB and/or AP-1 transcription factor binding sites in their promoter regions. Remarkably, a number of PTHrP regulated genes contained Stat transcription factor binding sites in their promoters. In transient transfection assays we show that PTHrP is able to positively regulate the activity of Stat3- and negatively regulate the activity of Stat5-specific promoter-reporter constructs in ATDC5 and UMR106 cells. In combination with the expression regulation of genes involved in Jak/Stat signalling, this data is suggestive for a previously not recognized interaction between PTHrP and Jak/Stat signalling.

Introduction

Longitudinal growth results from chondrocyte proliferation and subsequent differentiation in the epiphyseal growth plate, by a process called endochondral ossification. During this process resting chondrocytes in the stem cell zone enter the proliferative zone, start dividing and arrange in typical columns. These cells are characterized by secreting high amounts of collagen II. Subsequently, cells stop proliferating and start to differentiate into pre-hypertrophic chondrocytes, secreting collagen IX. Pre-hypertrophic cells further increase in size and become hypertrophic chondrocytes, which secrete high amounts of collagen X. The extracellular matrix becomes calcified and finally, mature chondrocytes undergo apoptosis, leaving a scaffold for bone formation.

Systemic hormones regulate longitudinal growth, partly via direct actions on growth plate chondrocytes through their receptors[1-3]. The direct and indirect actions of systemic hormones on growth plate chondrocytes were recently reviewed by Van der Eerden et al.[4]. The mechanism of action of the various factors involved in regulating the proliferation and differentiation of chondrocytes within the growth plate is still largely unknown. Systemic hormones probably interact with locally acting growth factors present in the growth plate, such as the Fibroblast Growth Factors (FGFs) or the members of the Indian Hedgehog (IHh)/ Parathyroid Hormone related Peptide (PTHrP) negative feedback loop, which is described to control the pace of chondrocyte differentiation[5;6].

In this study we have focused on the actions of PTHrP on growth plate chondrocytes. PTHrP plays a crucial role in controlling the pace of chondrocyte proliferation and differentiation in the growth plate, which is recognized by a number of studies. Knockout mice for PTHrP

show accelerated chondrocyte differentiation leading to dwarfism[7], while ectopic expression of PTHrP in chondrocytes inhibited their differentiation leading to a smaller cartilaginous skeleton[8]. In addition, PTHrP, the expression of which is tightly controlled by IHh[5], regulates both the rate and extent of chondrocyte proliferation by directly regulating the cell cycle machinery, partly by down regulation of the cyclin-dependent kinase inhibitor $p57^{Kip2}$[9].
PTHrP signals through the PTH/PTHrP Receptor 1 (PTHR1), which is predominantly expressed in pre-hypertrophic chondrocytes in the transition zone of the growth plate[10]. Binding of PTHrP to its receptor activates various signal transduction pathways. The dominant pathways result in activation of adenylate cyclase/protein kinase A (AC/PKA) and phospholipase C/protein kinase C (PLC/PKC)[11]. Downstream targets of PTH-signalling include the transcription factors cAMP response element-binding protein (CREB) and members of the AP-1 family, which are responsible, at least partly, for the genomic response. Indeed various PTH response genes have binding sites for these transcription factors in their promoters[12;13]. Recently, early response genes of Parathyroid Hormone (PTH) in osteoblasts were identified by microarray analysis[14].
In this study the chondrogenic ATDC5 cell line was used to identify PTHrP target genes in pre hypertrophic-like chondrocytes[15]. ATDC5 cells reproducibly differentiate into chondrocytes in four weeks in a monolayer culture[16]. This cell line is a representative model for studying the actions of PTHrP on chondrogenesis. During this process cells become responsive to PTHrP and in agreement with in vivo studies PTHrP inhibits hypertrophic chondrocyte differentiation[17;18].
We selected this cell line for the identification of early response genes of PTHrP. Using cDNA microarray analysis we identified 12 early response genes and confirmed their regulation by PTHrP using quantitative PCR in different cell culture models. Bio-informatic and functional analysis of a subset of these response genes, using transient transfection assays, revealed a previously not recognized level of interaction between PTHrP and Jak/Stat signalling.

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. KS483 mesenchymal progenitor cells were differentiated into osteoblasts as described previously[20]. UMR106 cells were cultured in DMEM (Invitrogen) containing 100 U/ml penicillin (Invitrogen), 100 U/ml streptomycin (Invitrogen), 10% FCS (Integro BV).

cAMP enzymimmunoassay

To establish the responsiveness of ATDC5 cells to PTHrP, intracellular cAMP accumulation was measured as previously described[21], using an enzymimmunoassay (Amersham, Freiburg, Germany), according to the manufacturers protocol. For this purpose, ATDC5 micromasses were challenged with a dose range of PTHrP(1-34) at day 7 and 14 of culture.

RNA isolation and amplification

Medium was refreshed after 7 days and the ATDC5 micromasses were challenged with 10^{-7} M PTHrP(1-34) at day 8 of culture (time point 0h) and total RNA was extracted at different time points in triplicate by 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[22], 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 dNTP's, 10U DNA Ligase (Invitrogen), 40U DNA polymerase I (Invitrogen), and 2U Rnase H (Invitrogen). This was followed by addition 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 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, 2U 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.[23]. 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.[24], 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 13.000 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[25] 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 experiments, 0h vs 0h, were hybridized in duplicate, 1h vs 0h and 2h vs 0h in triplicate, and 4h vs 0h in quadruplicate. 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). 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 if the spot settles the selection criteria in all time points. Spots showing an absolute fold change of 2 or spots showing significantly regulation ($p<0.01$) in the reference array, and flagged spots in all arrays were excluded from analysis. For further analysis, spots were selected from the remaining list if the signal intensity of Cy3 or Cy5 was above a cut off level (0.05), and the absolute fold change was under 50.

Analysis of variance (ANOVA) was performed between the remaining spots of the 4 different hybridizations. Spots showing significant ($p<0.001$) differential expression regulation, with intensity value above background value, and with a fold change >1.8, for upregulated genes, and <0.55, for downregulated genes, were selected for further analysis. 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.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

RNA was isolated from ATDC5 micromasses, cultured for 7 and 14 days, using Trizol LS Reagent (Invitrogen), and reverse transcribed into cDNA, using random hexamer primers (Amersham). Semiquantitative PCR was performed for beta-2 microglobulin (β2m), collagen II, collagen IX, collagen X, and PTHR1 under the following conditions: cDNA was denatured at 94°C for 5 minutes, followed by cycles of 30 s at 94°C, 30 s at 56°C, and 30 s at 72°C, and final extension at 72°C for 10 minutes (table 1).

Name	F/R	Primer	cycli
β2µ	F	TGACCGGCTTGTATGCTATC	25
	R	CAGTGTGAGCCAGGATATAG	
PTHR1	F	TGCTTGCCACTAAGCTTCG	30
	R	TCCTAATCTCTGCCTGCACC	
Collagen II	F	GCCAAGACCTGAAACTCTGG	27
	R	GCGATGCTGTTCTTACAGTGG	
Collagen IX	F	CTGTGTGTGCAGTTGTCTGG	27
	R	CCAGTGCTTTTCAAGTGTGC	
Collagen X	F	GCCTCGAGCCCAAAAGGCGTCTCATATTT	35
	R	GGCTCGAGCCCAGGAGCAGTGGATAATG	

Table 1 : RT-PCR primer sets
F: forward primer, R: reverse primer

RNA isolation from KS483 osteoblasts

After 11 day of culture, KS483 cells have formed bone nodules and the cells within the nodules start to mineralize the extracellular matrix. This time point was chosen to stimulate the cells with 10^{-7} M PTHrP(1-34). RNA was isolated after 1, 3, and 6 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 target genes, quantitative PCR was performed using the BioRad iCycler (Biorad, Veenendaal, The Netherlands). For each gene a set of primers was designed (table 2), which spanned at least 1 intron-exon boundary and had an optimal annealing temperature of 60°C, using the Applied Biosystems software program Primer Express. 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 30 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 2), 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[26].

Name	F/R	Forward primer	MgCl
β2µ	F	CACTGACCGGCCTGTATGC	3 mM
	R	GAATTCAGTGTGAGCCAGGATATAGA	
RGS2	F	GGAAGCCCAAAACTGGCAA	3 mM
	R	CTGCCCAGAGCTGCGCT	
SGK	F	CTCCTCCGCCAAGTCCCT	3 mM
	R	TGCCTAGCCAGAAGAACCTTTC	
Ptp4a1	F	CCTTGGCAGAGCTCCGGT	3 mM
	R	GGAGCCGCATTTTCGGAC	
Upar	F	CAATGGTGGCCCAGTTCTG	3 mM
	R	TCCCAGCACATCTAAGCCTGT	
IER3	F	CTCTACCCTCGAGTGGTCCG	4 mM
	R	CTCAGGTGTCACGGCGC	
Stat3	F	AGCCCCGGAGACAGTCGA	3 mM
	R	AAACACCAACGTGGCATGTG	
Csrp2	F	CCACTCGGAATGCCTGTCTG	3 mM
	R	GCCACTGTTGTGCTGTCTAAATTTT	
Sf3a2	F	CATCAGACTAACTTGGCCCGG	3 mM
	R	TCTCAGGGTAGTCAATCTGGAACAG	
Acvr2b	F	GACGGGCCTGTCGATGAGTA	3 mM
	R	TCATGGTCCCAGCACTCCTC	
Gab1	F	GACAGAAAAGTCAAGCCGGC	3 mM
	R	GGACATGGGAAACCTAGAGGAGT	
LamRI	F	CAACAACAAGGGAGCTCACTCA	4 mM
	R	TCTCAATCTCCTCTGGGTCTCTG	
Dym	F	AAAGTCCCCCGGCAGCTA	3 mM
	R	TGGGCCTTGCATCAGGTACT	
Arpc4	F	CGCGATGACTGCCACTCTC	3 mM
	R	CAATTTCATCGGCCTGCTTC	
Aurkb	F	GGACTGCCACGATCATGGA	4 mM
	R	CGCACATGGTCTTCCTCCTC	
Uchl5	F	TTTTTGCCAAGCAGGTAATTAATAATG	4 mM
	R	GCCCCTTCATAGCTGCATCA	
Akr1b8	F	CACCTGGAAGTCTCCCCCA	3 mM
	R	AATGAAGAGGTCCTCCCGCT	

Table 2 : qPCR primer sets.
F: forward primer, R: reverse primer

Identification of transcription factor binding sites

To find evolutionary conserved transcription factor binding sites in promoters of target genes, sequences surrounding the transcription start site, from –1000 bp to +100 bp, were extracted from the NCBI database and were aligned to either the human or rat genome using the evolutionary conserved regions (ECR) browser[27]. rVista 2.0 was used to predict conserved regulatory elements and define transcription factor binding sites, with similarity predefined as 0.85 (complete match has a value of 1)[28]. Consensus binding sequences, which were predicted in 4 or more promoters, were selected.

Transient transfection

ATDC5 cells were seeded at a density of 10000 cells/cm^2 and UMR106 cells were seeded at a density of 30000 cells/cm^2 in a 24-wells plate. The cells were kept in a humidified atmosphere of 5% CO_2 and 95% O_2 at 37°C. The second day the cells were transiently co-transfected with 1 μg of the reporter construct and with either 100 ng HA-Stat3 expression vector (kindly provided by T. Hirano, Department of Molecular Oncology, Osaka university, Japan[29]), 100 ng Stat5a, 100 ng Stat5b expression vector (kindly provided by Warren J. Leonard, National Institutes of Health, Bethesda, Maryland, USA[30]) or 100 ng pcDNA3.1 expression construct using Fugene 6 transfection reagent (Roche, Basel, Switzerland), according to the manufacturers protocol. The following reporter constructs were used: a Stat3 specific reporter promoter construct (kindly provided by Dr. I. Touw, Erasmus Medical Center, Rotterdam, The Netherlands[31]), and a Stat5 specific reporter promoter construct (kindly provided by Peter Storz, University of Stuttgart, Stuttgart, Germany[32]). To correct for transfection efficiency, 25 ng CMV renilla was included in all transfection experiments. The next morning the medium was changed and at the end of the day cells were treated with a dose range of PTHrP(1-34) (10^{-9}, 10^{-8} and 10^{-7} M). After 20 hours luciferase assays were performed using the Dual-Luciferase Reporter assay system (Promega) according to the manufacturers protocol. Luciferase activity was measured using the Wallac 1450 Microbeta Trilux luminescence counter (Perkin-Elmer, Boston, MA, USA). Firefly luciferase activity was corrected for renilla luciferase activity.

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

Characterization of ATDC5 micromass cultures

To induce chondrocyte differentiation, ATDC5 cells were cultured as micromasses. Already after 7 days a homogeneous cell pellet was formed, containing an alcian blue positive cartilage matrix. Both the size of the micromasses and the intensity of the alcian blue staining increased further after 14 days of culture (fig. 1A). Histological analysis at 7 days showed rounded chondrocytes embedded in an alcian blue positive extracellular matrix (fig.1B). RNA was isolated and used for PCR analysis to study the expression of typical cartilage markers (fig. 1C). Collagen II was expressed at 7 days and tended to decline at 14 days. Markers for pre-

hypertrophic (collagen IX) and for hypertrophic chondrocytes (collagen X) were present at 7 days and were increased at 14 days. PTHR1 mRNA did not change in the differentiation process (fig. 1C), but the responsiveness of the ATDC5 cells to PTHrP increased with differentiation (fig. 2).
Taken together, culturing the ATDC5 cells as micromasses efficiently induced chondrocyte differentiation. After 1 week of culture, the cells responded to PTHrP and expressed markers of pre-hypertrophic chondrocytes, which are the main target cells for PTHrP in the growth plate. For this reason, this time point was chosen for identification of PTHrP target genes.

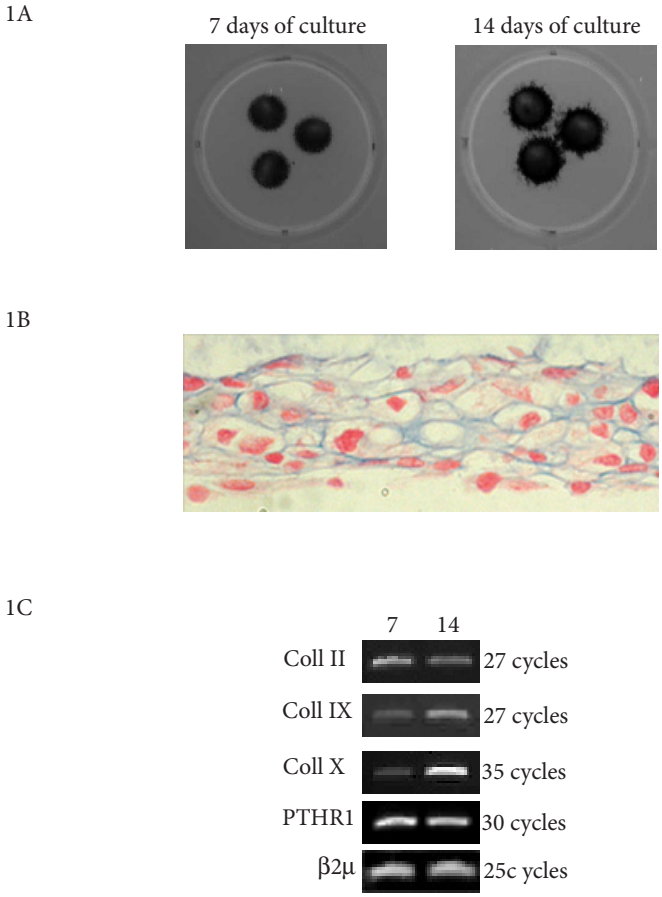

Figure 1: Phenotypic characterization of ATDC5 micromass cultures.
(A) ATDC5 micromass cultures were cultured for 7 and 14 days and stained for alcian blue. (B) Section of an ATDC5 micromass, cultured for 9 days, and stained for alcian blue (C) Expression pattern of coll II, coll IX, coll X, PTHR1, and β2μ mRNAs at day 7 and 14 of ATDC5 micromass differentiation.

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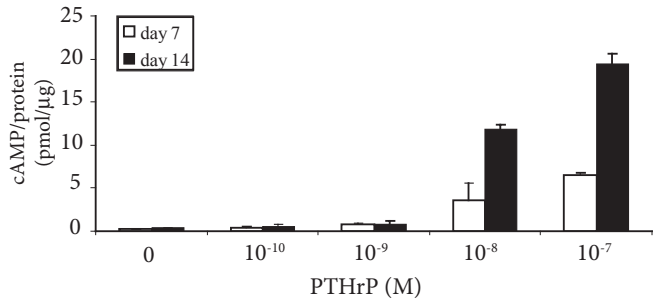


Figure 2: Responsiveness of ATDC5 cells to PTHrP during differentiation.
Intracellular cAMP concentration corrected for protein was measured at day 7 and 14 of culture, following stimulation with a dose range of PTHrP.

Identification and selection of PTHrP target genes

ATDC5 cells were cultured as micromasses and stimulated at day 8 with 10^{-7} M PTHrP for 1, 2, and 4 hours and cDNA microarray analysis was performed. After applying stringent selection criteria, 7843 out of the 15442 spots were taken in the analysis. A list of 31 spots, which exhibited significant ($p<0.001$) differential expression after 1, 2 or 4 hours following PTHrP stimulation, was generated. Sequence analysis revealed that 3 genes were present in two different spots and 1 gene in 3 spots. The expression patterns for the duplicate and triplicate spots were identical. This reduces the number of response genes to 26 (table 3). From this list 16 genes were chosen for validation experiments in ATDC5 cells. Exclusion criteria were insufficient information on gene identity, for example ESTs, and insufficient information for the design of qPCR primer sets, which span intron-exon boundaries.

The expression patterns of the selected genes were first validated by studying expression profiles using qPCR on the same RNA samples subjected to microarray analysis (fig. 3B, D, and F). As internal standard β2µ, which was not regulated by PTHrP in microarray analysis (data not shown), was used. Four out of 16 genes could not be validated and were discarded from further study, leaving 12 genes for more detailed analysis (table 4). In qPCR experiments RGS2, Stat3, Csrp2, Upar, and IER3 showed the same expression profile compared to the data from microarray analysis (fig. 3B and D). The expression pattern of early upregulated target genes SGK and Ptp4a1 were slightly different. SGK was only upregulated after 1 hour and Ptp4a1 after 2 and 4 hours, instead of continuously up regulation of both target genes revealed by microarray analysis. The downregulated target genes, Sf3a2, Acvr2b, Gab1, LamRI, and DYM showed in the qPCR data down regulation at all time points, instead of transient down-regulation at 1 hour after PTHrP stimulation found by microarray analysis (fig. 3F). The expression patterns were confirmed in RNA samples from ATDC5 cells isolated in a new independent experiment (data not shown).

Name	Acc. Nr.	up/down	Ratio 0h[2]	Ratio 1h[2]	Ratio 2h[2]	Ratio 4h[2]	selected
Regulator of G-protein signaling 2 (RGS2)	NM_053453	up	1,00	8,53	22,18	2,85	X
Serum glucocortioid regulated kinase (SGK)	NM_011361	up	0,90	2,63	1,88	2,82	X
Protein tyrosine phosphatase 4a1 (Ptp4a1)	NM_011200	up	1,01	1,78	2,63	4,13	X
Urokinase plasminogen activator receptor (Upar)	NM_011113	up	0,90	1,84	3,74	1,16	X
Immediate early response 3 (Ier3)	NM_13366	up	0,86	2,75	3,54	2,93	X
Signal transducer and activator of transcription 3 (Stat3)	NM_011486	up	0,86	0,74	1,17	2,72	X
Cysteine and glycine-rich protein 2 (Csrp2)	NM_007792	up	1,12	1,15	1,78	2,26	X
Splicing factor 3a, subunit 2 (Sf3a2)	NM_013651	down	1,04	0,34	0,74	0,85	X
Activin receptor IIb (Acvr2b)	NM_007397	down	1,13	0,47	0,93	0,955	X
Growth factor receptor bound protein 2–associated protein 1 (Gab1)	NM_021356	down	1,38	0,42	1,01	0,92	X
Laminin receptor I (LamRI)	NM_011029	down	1,62	0,22	0,96	1,34	X
Dymeclin (Dym)	NM_027727	down	0,87	0,17	0,59	0,71	X
GDP-mannose pyrophosphorylase B (gmppb)	NM_177910	down	1,06	0,12	0,58	0,99	
actin related protein 2/3 complex, subunit 4 (Arpc4)[1]	NM_026552	down	0,85	0,55	1,01	0,83	X
RNA binding motif protein 16 (Rbm16)	BC075621	down	1,02	0,27	0,88	0,89	
aldolase 1 (Aldoa)	NM_007438	down	0,88	0,53	0,89	0,94	
Aurkb: aurora kinase B[1]	NM_011496	down	0,78	0,38	0,60	0,67	X
heparan sulfate (glucosamine) 3-O-sulfotransferase 1 (Hs3st1)	NM_010474	down	0,79	0,30	0,60	0,71	
ubiquitin carboxyl-terminal esterase L5 (Uchl5)[1]	NM_019562	down	1,03	0,04	0,65	0,86	X
aldo-keto reductase family 1, member B8/7 (Akr1b8)[1]	NM_008012	down	0,94	0,49	0,85	0,89	X
phosphatidylinositol glycan, class T (Pigt)	NM_133779	down	1,20	1,89	0,75	0,84	
KCNQ1 overlapping transcript 1 (Kcnq1ot1)	AF119385	down	1,03	0,07	0,57	0,64	
betaine-homocysteine methyltransferase (Bhmt)	NM_016668	down	1,18	0,24	0,79	0,84	
eukaryotic translation elongation factor 1 alpha 1 (Eef1a1)	NM_010106	down	0,76	0,48	0,75	0,81	
EST	NM_133349	down	0,83	0,84	0,53	1,09	
EST	AC155250	down	1,11	0,21	0,71	0,87	

Table 3: 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.

Reg.[1]	Abbr.[2]	Name	Function	rp[3]	gp[4]
up	RGS2	Regulator of G-protein signaling 2	Inhibitor of G-protein coupled receptor protein activation.	yes	no
up	SGK	Serum glucocortioid regulated kinase	involved in signal transduction pathways.	yes	no
up	Ptp4a1	Protein tyrosine phosphatase 4a1	Plays a regulatory role in a variety of cellular processes.	no	yes
up	Upar	Urokinase plasminogen activator receptor	Regulator of cell-surface plasminogen activation.	yes	no
up	IER3	Immediate early response 3	Anti apoptotic characteristics.	no	no
up	Stat3	Signal transducer and activator of transcription 3	Transcription factor involved in GH signalling.	no	yes
up	Csrp2	Cysteine and glycine-rich protein 2	partner of protein inhibitor of activated STAT1 (PIAS1).	no	yes
down	Sf3a2	Splicing factor 3a, subunit 2	Binds microtubules and can bundle microtubules in vitro.	no	no
down	Acvr2b	Activin receptor IIb	Member of TGFb-R superfamily, expressed during chondrogenesis.	no	yes
down	Gab1	Growth factor receptor bound protein 2–associated protein 1	Involved in signalling from various receptors (GH/IL6).	no	no
down	LamRI	Laminin receptor I	Involved in interactions between cells and ECM.	no	yes
down	Dym	Dymeclin	Participates in proteoglycan metabolism.	no	yes

Table 4: List of validated PTHrP target genes.

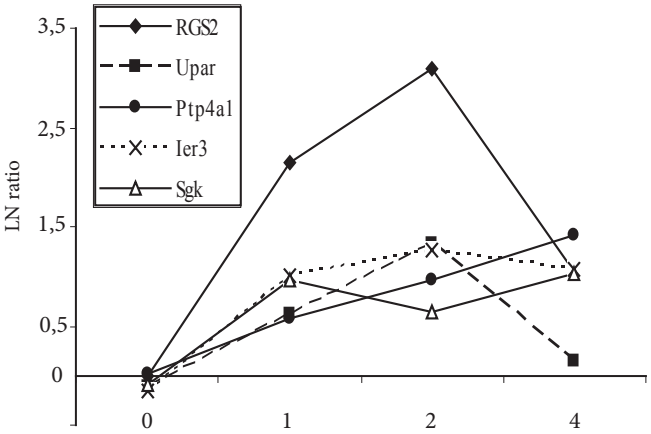
[1]) Reg. = regulation

[2]) Abbr. = abbreviation

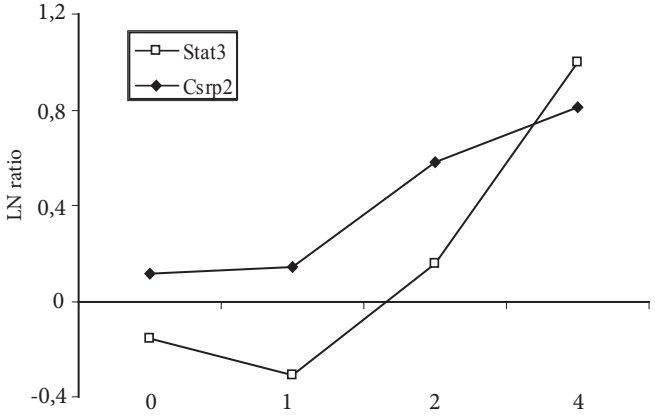
[3]) rp = previously been implicated in PTHrP signaling

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

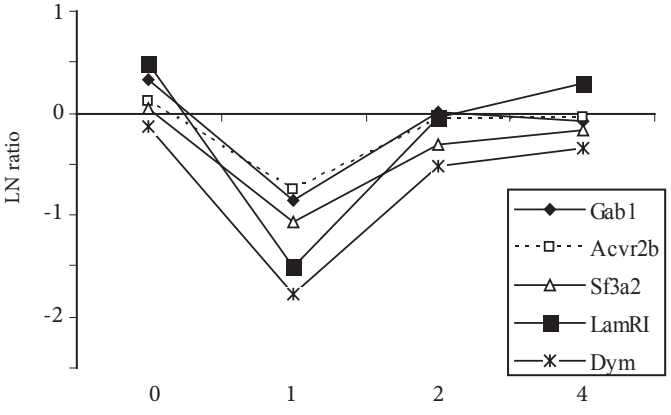
3A


RGS2
Upar
Ptp4a1
Ier3
Sgk
LN ratio
3,5
2,5
1,5
0,5
0
0
1
2
4

3C


Stat3
Csrp2
LN ratio
1,2
0,8
0,4
0
-0,4
0
1
2
4

3E


Gab1
Acvr2b
Sf3a2
LamRI
Dym
LN ratio
1
0
-1
-2
0
1
2
4

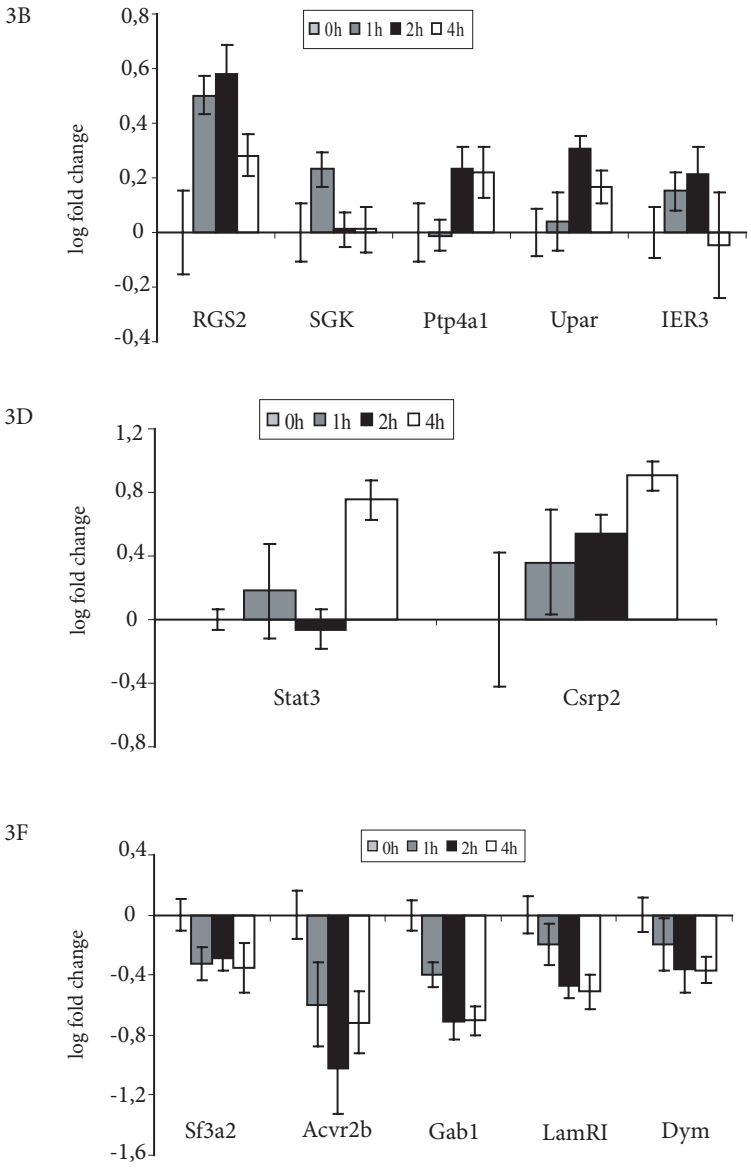


Figure 3: expression profiles of PTHrP target genes in ATDC5 chondrocytes revealed by microarray analysis and qPCR analysis.

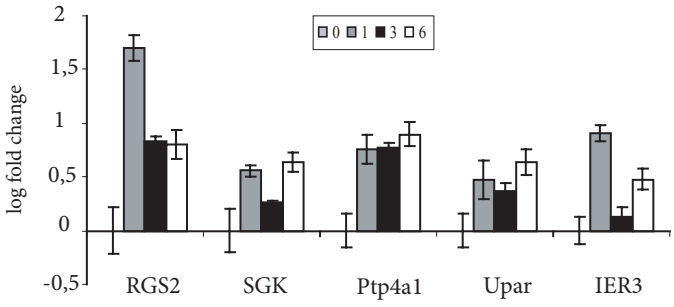
RNA was isolated from ATDC5 micromass cultures at 0h, 1h, 2h and 4h after PTHrP stimulation, amplified and labelled as described. Samples were hybridized against 0h. Based on the expression profiles revealed by microarray analysis, the genes were divided over 3 clusters. (A) Cluster 1 contained 5 immediate early upregulated genes, i.e. RGS2, SGK, Ptp4a1, UPAR, and IER3. The expression pattern revealed by qPCR is shown in (B). (C) Cluster 2 contained 2 immediate early upregulated genes, i.e. STAT3 and Csrp2. The expression pattern revealed by qPCR is shown in (D). (E) Cluster 3 contained 5 immediate downregulated genes, i.e. Sf3a2, Acvr2b, Gab1, LamRI, and DYM. The expression pattern revealed by qPCR is shown in (F).

Validation of PTHrP target genes by qPCR

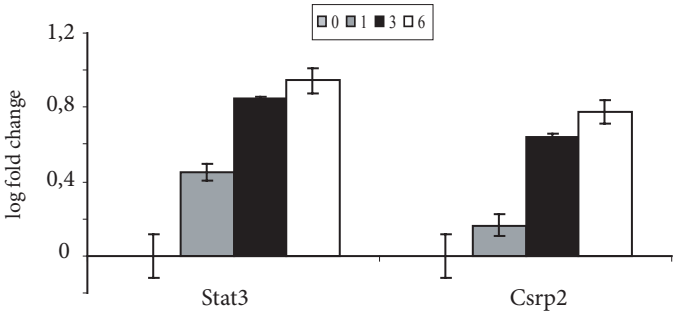
Among these 12 identified PTHrP target genes (table 3) 7 genes were upregulated and 5 genes were downregulated. Most of the genes were implicated in signal transduction and regulation and were intracellular mediators or receptors. Three genes were already known target genes of PTHrP in osteoblasts and 6 genes have previously been found in growth plate chondrocytes. Based on the expression patterns, the genes were divided into 3 groups, 2 groups for upregulated genes and 1 group for downregulated genes (fig. 3A, C, and E). Genes in expression pattern 1 were upregulated after 1 hour and genes in expression pattern 2 were upregulated after 2 and 4 hours. The genes in expression pattern 3 were downregulated after 1 hour. Expression pattern 1 contained RGS2, SGK, Upar, IER3, and Ptp4a1; Stat3 and Csrp2 formed expression pattern 2; and expression pattern 3 contained Sf3a2, Gab1, DYM, LamRI and Acvr2b.

To test whether the response of these target genes was restricted to chondrocytes or whether it was 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 0, 1, 3, and 6 hours after a challenge with PTHrP. The expression patterns of all upregulated genes were comparable with the expression patterns found in chondrocytes. The expression patterns of the downregulated genes in KS483 osteoblasts were less clear. All genes responded to PTHrP. Only Gab1 was downregulated, although this regulation was transiently instead of continuously as in ATDC5 cells. Remarkably, Sf3a2 and Dym were upregulated instead of downregulated. No consistent pattern of regulation was found for Acvr2b and LamRI.

4A



4B



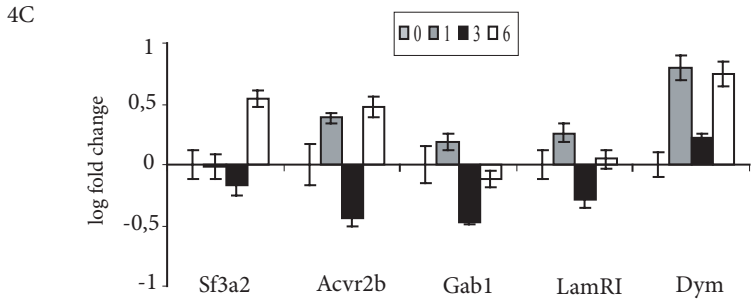


Figure 4: Expression patterns of PTHrP target genes in KS483 osteoblasts.
The genes were grouped according to expression profiles revealed by microarray analysis in chondrocytes as described in fig. 3. RNA was isolated from KS483 osteoblasts, stimulated with PTHrP at day 11, after 0h, 1h, 3h and 6h and qPCR was performed. The expression of the genes in expression pattern 1 in chondrocytes (A), in expression pattern 2 in chondrocytes (B), and in expression pattern 3 in chondrocytes (C) were determined by qPCR.

Transcription factor binding sites

We subsequently analyzed promoter regions (sequences from -1000 bp to + 100 bp from the transcription start site) of target genes to identify common regulatory elements involved in their expression regulation. To decrease the number of false positive results, we searched for transcription factor binding sites that were conserved in human and rat genomes. The promoter regions of RGS2, SGK, Ptp4a1, Stat3, Acvr2b, Gab1, and LamRI were aligned to the human genome and the promoter regions of IER3, Upar and Csrp2 to the rat genome. Insufficient information was available in the database to align the promoter regions of Sf3a2 and Dym with the human and the rat genomes, preventing the selection of evolutionary conserved binding elements in these genes. Table 5 shows transcription factors of which binding sequences have been found in 4 or more promoters of the identified PTHrP target genes.

ATF/CREB binding sites were predicted in the promoter regions of 6 out of 7 upregulated genes, 4 times in combination with an AP-1 binding site. ATF/CREB binding sites were also predicted in 2 downregulated genes, but never in combination with an AP-1 binding site. Remarkably, 1 of these genes was Dym, which was upregulated in KS483 osteoblasts. Another interesting finding was the identification of Stat transcription factor binding sites, predominantly in the promoter regions of upregulated PTHrP target genes. Binding sites for the transcription factor ELK1, which is, like ATF/CREB, AP-1, and Stat, involved in immediate early responses, were predicted in 7 promoter regions. Binding sites for transcription factor E2F and SP1, both of which are facilitating factors involved in gene transcription, were distributed over up and downregulated target genes and were predicted in 10 out of 12 and 6 out of 12 promoter regions, respectively.

Reg.[1]	Name	ATF/ CREB[2]	AP1	E2F[2]	SP1[2]	ELK1[2]	Stat1[2]	Stat3[2]	Stat6[2]
up	RGS2	X	X				X		X
up	SGK	X	X	X		X	X	X	X
up	Ptp4a1	X(3)	X	X(2)					
up	Upar	X	X		X		X		
up	IER3			X(2)	X(2)	X(2)	X	X	X(2)
up	Stat3	X(2)		X	X(2)	X(2)	X	X	
up	Csrp2	X		X(4)	X	X(2)		X(3)	X(2)
down	Sf3a2			X(3)	X	X(2)	X(2)	X	
down	Acvr2b			X(2)	X(4)				
down	Gab1			X					
down	LamRI	X		X		X			
down	Dym	X(2)		X(4)		X(2)	X(2)	X	

Table 5: Transcription factor binding sites.
Conserved transcription factor binding sites predicted 4 times or more in the promoter regions of the PTHrP target genes. X: transcription factor binding site is present in promoter region. [1]) Reg.: Regulation [2]) In case more than 1 site is present in the promoter regions the number of hits is indicated between brackets.

Stat regulation by PTHrP

In order to investigate the biological significance of the interactions between PTHrP and Stat signalling, transient transfection experiments were performed. In contrast to undifferentiated cells, differentiated ATDC5 (and KS483) cells could not be transfected. Since undifferentiated ATDC5 cells express very low amounts of the PTHR1, experiments were also performed in UMR106 cells, which can easily be tranfected and have higher PTHR1 expression levels.
In line with the upregulation of Stat3 mRNA revealed by microarray analysis, PTHrP induced activity of a Stat3 reporter in both cell types dose dependently with a maximal fold induction of 1.4 in ATDC5 cells (fig. 5A) and 2.5 in UMR106 cells (fig. 5B). In the presence of Stat3 expression vector, luciferase activity of the Stat3 reporter was enhanced in ATDC5 cell, but not in UMR106 cells (fig. 5A and B). PTHrP also further increased Stat3 reporter activity in the presence of Stat3 expression vector in both cell lines.
To investigate whether the regulation of Stat proteins by PTHrP is part of a more generalized interaction between PTHrP and Jak/Stat signalling, we subsequently studied the regulation of Stat5a and Stat5b by PTHrP. Comparable to Stat3, the mRNA expression of Stat5a (fig. 6A) and Stat5b (fig. 6B) was upregulated after 4 hours of PTHrP treatment in ATDC5 cells by qPCR. Transient transfection experiments were performed in ATDC5 cells and in UMR106 cells, using a Stat5 promoter-reporter construct in the absence of Stat5 expression vector. PTHrP inhibited luciferase activity of the Stat5 reporter, with a maximal fold change of 0.5, in UMR106 cells (fig. 6D), but had no effect in ATDC5 cells (fig. 6C). This observation is in contrast to the increase in mRNA expression of Stat5a and Stat5b. Addition of Stat5a or Stat5b enhanced the reporter activity in ATDC5 and in UMR106 cells. In both cell lines, PTHrP inhibited Stat5 reporter activity in the presence of Stat5b expression vector, whereas in the presence of Stat5a expression vector reporter activity was inhibited in UMR106 cells only.

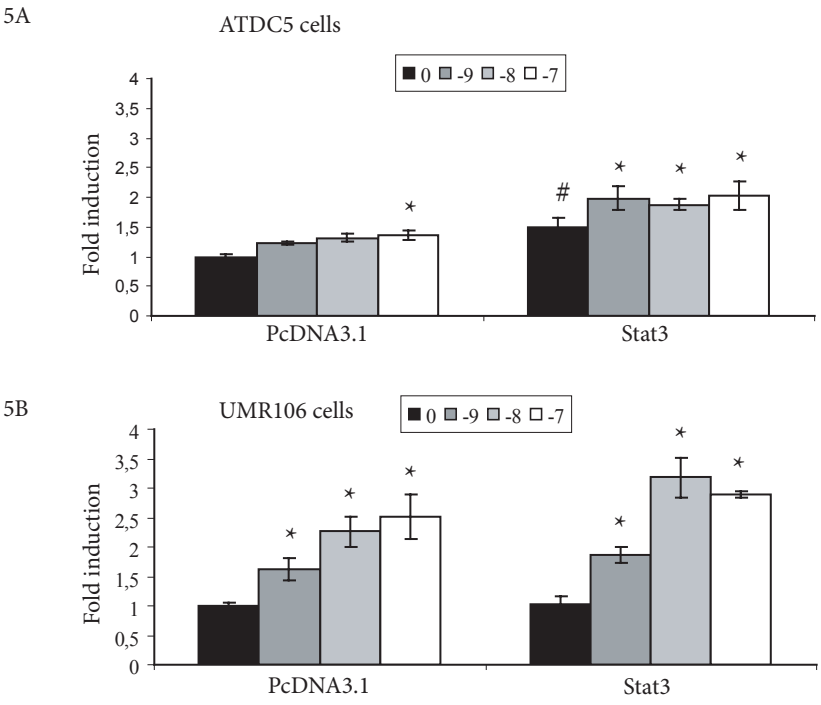
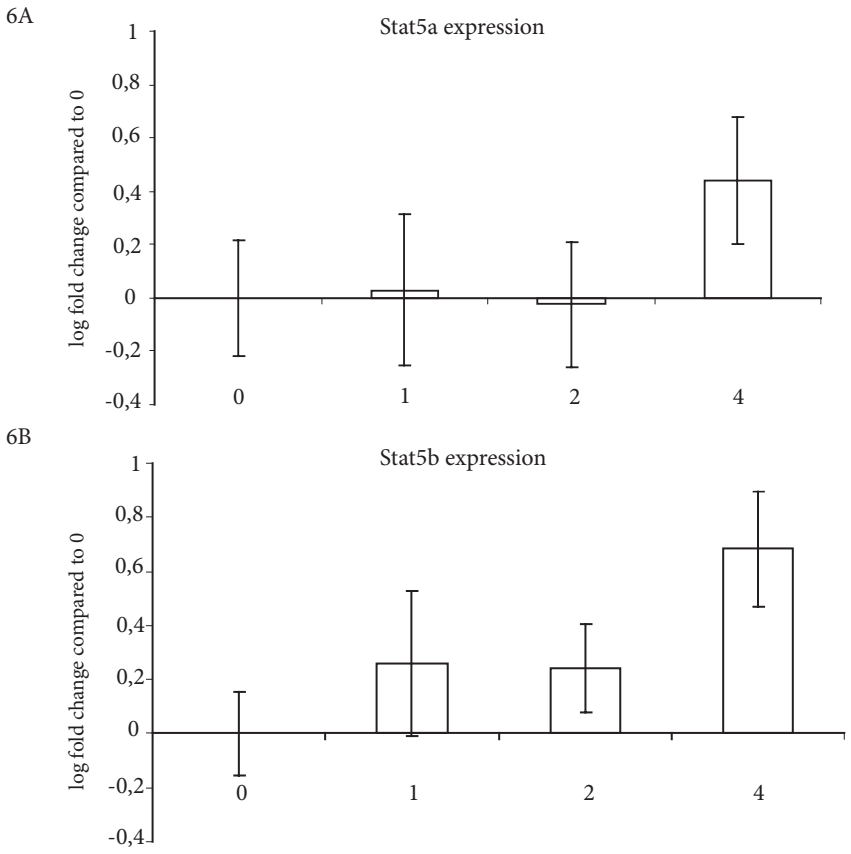


Figure 5: Regulation of the activity of Stat3 protein by PTHrP.
A) ATDC5 cells and B) UMR106 cells were transiently transfected with the stat3 specific promoter-reporter construct and co-transfected with 100 ng pcDNA3.1 or 100 ng stat3 expression vectors and treated with a dose range of PTHrP (0, 10-9, 10-8, 10-7 M). Data are expressed as fold induction compared to control after correction for transfection efficiency. Experiments were performed in quadruplicate and repeated at least twice. *Significant $p<0.05$ compared to vehicle stimulation. #Significant $p<0.05$ compared to vehicle stimulation in pcDNA3.1 co-transfections.

Discussion

In the present study, we have identified early response genes of PTHrP in chondrocytes using the chondrogenic ATDC5 cell line. Disadvantages of the ATDC5 cell line differentiated under standard conditions in monolayer are the formation of a heterogeneous culture and the necessity for prolonged culture periods of at least 28 days[15]. In monolayer cultures chondrogenesis is initiated when multi cell layers appear, suggesting that cell-cell contacts are crucial for chondrocyte differentiation. These cell-cell contacts are the initial trigger for mesenchymal stem cells (MSCs) to form chondrocytes in embryogenesis. To accelerate the formation of cell-cell contacts, we used the micromass culture technique, resembling the condensation of MSCs *in vivo* by formation of a three-dimensional spheroid structure[19;33]. Aggregation of ATDC5 cells resulted in more homogeneous cultures. Already after 7 days type II collagen was abundantly expressed compared to expression after 14 days in monolayer culture[16]. Type IX and type X collagen were expressed at low levels after 7 days compared to expression after 14 and 21 days, respectively, in monolayer culture[16]. In both cultures the responsiveness of the ATDC5 cells to PTHrP increased during differentiation[18]. We decided to stimulate the



micromasses at day 8 with PTHrP. At this time point, the chondrocytes respond to PTHrP and have pre-hypertrophic characteristics as demonstrated by the expression of collagen type IX. Late-proliferating and pre-hypertrophic cells are the main target cells of PTHrP in the growth plate[34]. The higher levels of collagen X expression at day 14 of culture suggested that more cells have already undergone chondrocyte hypertrophy at this time point.

To identify early response genes of PTHrP the NIA 15k mouse cDNA bank was used. This bank was amplified and spotted by the Leiden Genome Technology Center. The quality control of custom-made cDNA microarray, compared to commercially available microarrays, 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[35]. 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[36]. Because of the uncertainty of the identity of the spots, our results could not be used for pathway screening or genome wide analysis. By applying very stringent selection criteria, the microarray could still be used to identify a subset of PTHrP target genes. The

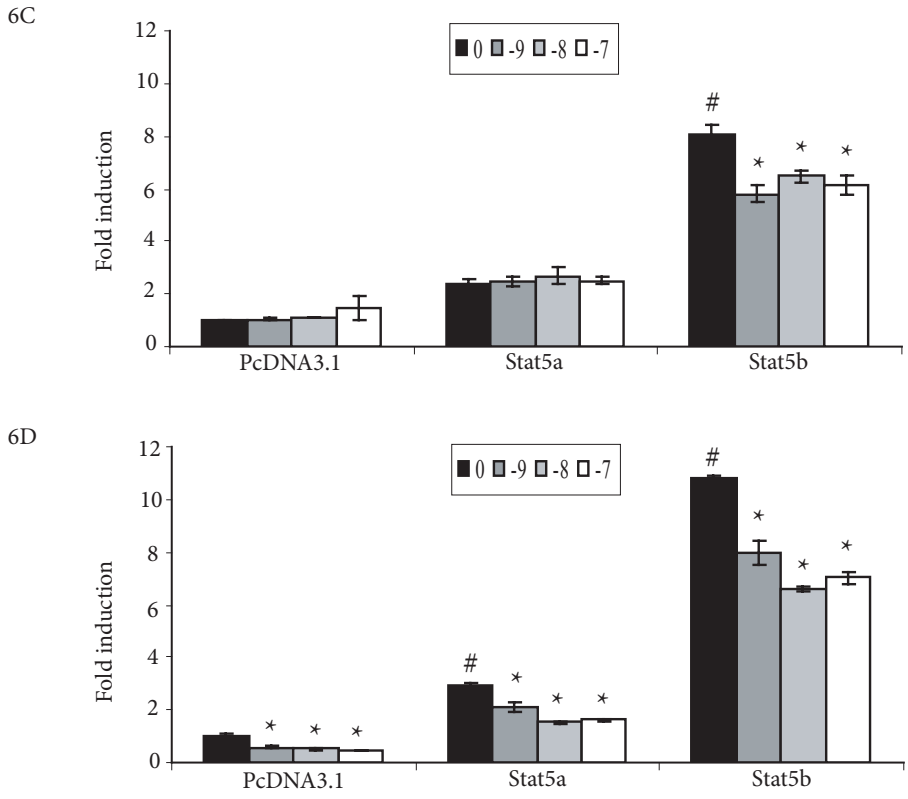


Figure 6: Regulation of Stat5 mRNA expression and Stat5 protein activity by PTHrP.
RNA was isolated at day 11 from ATDC5 micromass cultures at 0h, 1h, 2h and 4h after PTHrP stimulation and qPCR was performed for A) Stat5a and B) Stat5b. C) ATDC5 cells and D) UMR106 cells were transiently transfected with the stat5 specific promoter-reporter construct and co-transfected with 100 ng pcDNA3.1, 100 ng Stat5a or 100 ng Stat5b expression vectors and treated with a dose range of PTHrP (0, 10-9, 10-8, 10-7 M). Data are expressed as fold induction compared to control after correction for transfection efficiency. Experiments were performed in quadruplicate and repeated at least twice. *Significant $p<0.05$ compared to vehicle stimulation. #Significant $p<0.05$ compared to vehicle stimulation in pcDNA3.1 co-transfections.

validity of this approach was subsequently shown by qPCR in different cell models and by bio-informatics analysis. We were able to classify 12 out of 16 genes as bonafide target genes of PTHrP. qPCR analysis is an established method for validation of microarray data. 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[37-39].

Bio-informatic analysis revealed that RGS2, Upar, and SGK were already identified as PTH target genes in osteoblasts[14;40;41]. Indeed, in this study RGS2, Upar and SGK were also induced in osteoblasts as well as in chondrocytes by PTHrP. Six out of 12 target genes have not been demonstrated in growth plate chondrocytes before. The majority of the identified PTHrP target genes (10 out of 12) were involved in signal transduction pathways and modulation

of these pathways. These include RGS2, SGK, Ptp4a1, Ier3, Stat3, Csrp2, and Gab1. These factors are involved in various signal transduction pathways, like AC/PKA, ERK, Jak/Stat and PI3-kinase/AKT pathways, suggesting that PTHrP signalling could influence these signalling cascades[41-46]. In addition, PTHrP could also influence other pathways via regulation of expression of receptors, like Upar, Acvr2b, and LamRI.

Quantitative PCR resulted in validation of the upregulated PTHrP target genes identified by microarray analysis, in chondrocytes as well in osteoblasts. These data suggest that the upregulated genes are part of a more generalized response to PTHrP, which is not restricted to chondrocytes. Verification of the downregulated target genes revealed a more cell type dependent picture. For instance, two genes, Dym and Sf3a2, were downregulated in chondrocytes, but upregulated in osteoblasts.

The dominant pathway activated by PTHrP is the AC/PKA pathway, which results in activation of the transcription factors CREB and/or AP-1[47]. Indeed, in various early response genes of PTH or PTHrP functional CREB and AP-1 response elements have been identified[48]. In line with this, promoter analysis revealed CREB transcription factor binding sites predominantly in the upregulated genes (6 out of 7), 4 times in combination with an AP-1 site. A less consistent picture was found for the downregulated genes. In the promoter regions of 2 downregulated genes (LamRI and Dym) CREB transcription factor binding sites were predicted. Remarkably, Dym was induced by PTHrP in KS483 osteoblasts. In addition, the presence of SP1, E2F and ELK1 were predicted in both up- and downregulated genes. Recently, Qin et al used a statistical approach to identify transcription factor binding sites used by PTH-signalling in osteoblasts[14] instead of enrichment for evolutionary conserved binding sites applied in this paper. Comparable to our study were the predictions of CREB and AP-1 transcription factor binding sites predominantly in upregulated genes. Also the presence of Sp1 sites in up- and downregulated genes were predicted by both methods. The validity of our approach was furthermore underscored by previous data showing the presence of a CREB transcription factor binding site in the Stat3 promoter[49]. Furthermore, RGS2 is induced by cAMP, suggesting a CREB transcription factor binding site in its promoter[50]. In addition, SP1 binding sites have been described before in the promoter regions of IER3 and Csrp2[51;52].

A remarkable finding in our study was the expression regulation of proteins involved in the Jak/Stat signalling cascade, like Stat3 and Csrp2. Csrp2 is a binding partner of Pias1, which is an inhibitor of Stat1[42]. In addition, a novel observation was the prediction of several Stat transcription factor binding sites in the promoter regions of genes induced by PTHrP (6 out of 7), often in combination with CREB and AP1 sites. These observations were of biological significance, since we also observed that PTHrP induced the activity of a Stat3 reporter construct, either in the presence or absence of extra Stat3. This result is comparable with another study, in which Stat3 reporter activity was induced by activation of the AC/PKA pathway, by increasing the posttranslational activation of Stat3 proteins in rat thyroid cells[53]. In addition, we showed that the regulation of Stat proteins by PTHrP was not only restricted to Stat3, but also included other members of the Jak/Stat family, like Stat5a and Stat5b. Despite the induction of Stat5a and Stat5b mRNA, PTHrP inhibited Stat5 reporter activity in the absence and presence of exogenous Stat5a and Stat5b. This observation is most likely explained by an effect of PTHrP on the posttranslational actions of Stat5a and Stat5b, mediated by the

AC/PKA pathway. Indeed, the inhibiting effect on Stat5 activity after activation of the AC/PKA pathway has been described before in T lymphocytes[54]. It was shown that AC/PKA signalling inhibited tyrosine phosphorylation of Stat5a and Stat5b, thereby preventing their activation. Our results suggest that this mechanism may also be operation in chondrocytes and osteoblasts after activation of PTHR1 signalling.

The effects of PTHrP on Stat3 and Stat5 reporters were observed in ATDC5 and UMR106 cells with only slight differences. Generally, the responses in ATDC5 cells were lower than observed in UMR cells. This is most likely explained by low PTHR1 responses of the undifferentiated ATDC5 cells. Due to the excessive formation of cartilage matrix, which prevented efficient transfections, the transfection experiment could not be repeated in differentiated ATDC5 cells and KS483 cells, which express higher levels of PTHR1. The data suggest, however, that a crosstalk between PTHR1 and Jak/Stat signalling is a more generalized mechanism. Taken together, we provide evidence for interactions between PTHrP and Jak/Stat signalling, not only at the level of mRNA expression regulation, but also at the level of posttranslational modification, resulting in either activation of Stat3 or repression of Stat5a and Stat5b mediated gene transcription.

The involvement of Jak/Stat proteins in signalling cascades of other growth factors in chondrocyte differentiation has been described before. Stat1 and Stat3 are involved in the effects of fibroblast growth factor (FGF) on chondrocyte proliferation within the growth plate[55]. In addition, the Jak/Stat cascade is involved in growth hormone (GH) signalling. Stat5b is the most important Stat protein with respect to the actions of GH on growth and is responsible for the induction of insulin-like growth factor-1 (IGF-1)[56]. Since Jak/Stat signalling plays an important role in chondrocyte proliferation, modulation of this pathway by PTHrP might be an essential mechanism involved in the actions of PTHrP in keeping the growth plate chondrocytes in a proliferation competent state.

In summary, we have applied new culture conditions to induce chondrogenic differentiation of ATDC5 cells. In addition, we have identified 12 PTHrP target genes. Among them were several genes involved in distinct signalling pathways operational within the growth plate, suggesting the presence of a crosstalk with PTHrP signalling. In addition, we report for the first time the presence of a previously not recognized interaction between PTHrP and Jak/Stat signalling.

Acknowledgements

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