The Paracrine Feedback Loop Between Vitamin D3 (1,25(OH)2D3) and PTHrP in Prehypertrophic Chondrocytes

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The endocrine feedback loop between vitamin D3 (1,25(OH)2D3) and parathyroid hormone (PTH) plays a central role in skeletal development. PTH-related protein (PTHrP) shares homology and its receptor (PTHR1) with PTH. The aim of this study was to investigate whether there is a functional paracrine feedback loop between 1,25(OH)2D3 and PTHrP in the growth plate, in parallel with the endocrine feedback loop between 1,25(OH)2D3 and PTH. This was investigated in ATDC5 cells treated with 10−8 M 1,25(OH)2D3 or PTHrP, Col2-pd2EGFP transgenic mice, and primary Col2-pd2EGFP growth plate chondrocytes isolated by FACS, using RT-qPCR, Western blot, PTHrP ELISA, chromatin immunoprecipitation (ChIP) assay, silencing of the 1,25(OH)2D3 receptor (VDR), immunofluorescent staining, immunohistochemistry, and histomorphometric analysis of the growth plate. The ChIP assay confirmed functional binding of the VDR to the PTHrP promoter, but not to the PTHR1 promoter. Treatment with 1,25(OH)2D3 decreased PTHrP protein production, an effect which was prevented by silencing of the VDR. Treatment with PTHrP significantly induced VDR production, but did not affect 1α- and 24-hydroxylase expression. Hypertrophic differentiation was inhibited by PTHrP and 1,25(OH)2D3 treatment. Taken together, these findings indicate that there is a functional paracrine feedback loop between 1,25(OH)2D3 and PTHrP in the growth plate. 1,25(OH)2D3 decreases PTHrP production, while PTHrP increases chondrocyte sensitivity to 1,25(OH)2D3 by increasing VDR production. In light of the role of 1,25(OH)2D3 and PTHrP in modulating chondrocyte differentiation, 1,25(OH)2D3 in addition to PTHrP could potentially be used to prevent undesirable hypertrophic chondrocyte differentiation during cartilage repair or regeneration.

J. Cell. Physiol. 229: 1999–2014, 2014. © 2014 The Authors. Journal of Cellular Physiology published by Wiley Periodicals, Inc.

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Abbreviations: 1α-OHase, 1α-hydroxylase; 24-OHase, 24-hydroxylase; AsAP, ascorbic acid 2-phosphate; bp, base pairs; BMSC, bone marrow-derived stem cells; BW, body weight; Ca, calcium; ChIP, chromatin immunoprecipitation; Co2Da1, collagen type 2; Co9, collagen type 9; CoX, collagen type X; FACS, fluorescence-activated cell sorting; FBS, fetal bovine serum; GPl.Th, mean growth plate height; GPl.Th.Pr,m, mean height of the proliferative growth plate zone; GPl.Th.Hp, mean height of the hypertrophic growth plate zone; HR, horseradish peroxidase; Mab, monoclonal antibody; MSC, mesenchymal stem cell; n.s., not significant; OA, osteoarthritis; P, inorganic phosphate; Pab, polyclonal antibody; PTH, parathyroid hormone; PTHrP, PTH-related protein; PTHR1, PTH/PTHrP receptor; RT, room temperature; SD, standard deviation; siRNA, silencing RNA; Tm, melting temperature; TSS, transcription start site; VDR, nuclear vitamin D3 receptor; VDRE, vitamin D3 response element.

The authors declare that they have no competing interests.

Contract grant sponsor: Dutch Arthritis Foundation;
Contract grant number: LLP22.
Contract grant sponsor: NWO (MEERVOUD grant);
Contract grant number: 836.07.003.
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Manuscript Received: 14 February 2014
Manuscript Accepted: 25 April 2014
Accepted manuscript online in Wiley Online Library (wileyonlineibrary.com): 29 April 2014.
DOI: 10.1002/jcp.24658
Longitudinal bone growth occurs at the growth plate, a highly organized cartilage structure that contains proliferating chondrocytes. These cells undergo a maturation process involving hypertrophy followed by apoptosis, thereby facilitating bone formation (Nilsson et al., 2005; Brochhausen et al., 2009). Some changes that occur in cartilage after injury or osteoarthritis (OA) resemble the processes that occur during the differentiation of growth plate chondrocytes (Driend, 2010; Zhang et al., 2012). In healthy articular cartilage, chondrocytes resist proliferation and terminal differentiation. In contrast, cartilage damage caused by injuries or OA reactivates chondrocyte hypertrophy as part of a repair mechanism, accompanied by acquisition of an autolytic phenotype and cartilage degradation (Driend, 2010; van der Kraan and van den Berg, 2012; Zhang et al., 2012). Ultimately, the hypertrophic chondrocytes undergo apoptosis to enable bone deposition (van der Kraan and van den Berg, 2012). The inferior quality of the repaired cartilage suggests that the inhibition of chondrocyte hypertrophy could be a target of treatment to improve cartilage repair (Zhang et al., 2012).

Chondrocyte proliferation and differentiation at the growth plate is regulated through the interaction of systemic hormones (endocrine level) and locally produced growth factors (autocrine and/or paracrine level). The endocrine feedback loop involves the active metabolite of vitamin D3 (1,25(OH)2D3) and parathyroid hormone (PTH) plays a central role in calcium and phosphate homeostasis during skeletal growth (Nilsson et al., 2005). Vitamin D3 is hydroxylated in the liver to 25-hydroxycholecalciferol (25(OH)D3), which is thereafter hydroxylated in various target cells into 1,25(OH)2D3 by the enzyme 1α-hydroxylase (St-Arnaud and Naja, 2011). 1,25(OH)2D3 in turn can be deactivated and catabolized by the enzyme 24-hydroxylase (Akeno et al., 1997; Tryfonidou et al., 2003). It has been shown that 1,25(OH)2D3 exerts its genomic effects by binding to its nuclear receptor (VDR), and that this complex then binds to vitamin D3 response elements (VDREs) in the promoter region of various target genes (Healy et al., 2003,2005b; St-Arnaud and Naja, 2011).

Both 1,25(OH)2D3 and PTH are also active at the growth plate and play an important autocrine and/or paracrine role during chondrocyte proliferation and/or differentiation (Kato et al., 1990; Klaus et al., 1991; Drissi et al., 2002). Growth plate chondrocytes express the VDR and the enzymes 1α- and 24-hydroxylase in vitro as well as in vivo (Boyan et al., 2002; Hugel et al., 2004; Nilsson et al., 2005; Naja et al., 2009; St-Arnaud and Naja, 2011). PTH-related protein (PTHRP) resembles PTH in genetic sequence and structure and both PTH and PTHR P share the same receptor: PTHR1 (Schipani and Provot, 2003; Zhang et al., 2012). PTHRI is expressed in low levels by proliferating chondrocytes and in high levels by pre/early hypertrophic chondrocytes (Kronenberg, 2003; Mak et al., 2008; Zhang et al., 2012). PTHrP is produced by proliferative growth plate chondrocytes and prevents proliferative cells from leaving the proliferating pool. In this way, hypertrophic chondrocyte differentiation is delayed (Kronenberg, 2003; Mak et al., 2008; Hirai et al., 2011; Zhang et al., 2012).

Understanding the processes behind chondrocyte differentiation is crucial, not only from a developmental point of view. Regenerative strategies for bone and cartilage make use of mesenchymal (stem) cells undergoing chondrogenic differentiation. The growth plate can be used as a model to study these processes, mainly because it has a highly organized structure, with chondrocytes undergoing differentiation in an orderly fashion (Nilsson et al., 2005; Brochhausen et al., 2009; Denison et al., 2009). Therefore, the main aim of this study was to investigate whether there is a functional paracrine feedback loop between 1,25(OH)2D3 and PTHrP in prehypertrophic growth plate chondrocytes, in parallel to the well-known endocrine 1,25(OH)2D3–PTH feedback loop.

We hypothesized that PTHrP increases the sensitivity of growth plate chondrocytes to 1,25(OH)2D3 either by increasing 1,25(OH)2D3 production by upregulating 1α-hydroxylase, and/or decreasing the catabolism of 1,25(OH)2D3 by downregulating 24-hydroxylase, and/or by upregulating VDR expression (Supplementary File 1). The feedback loop is closed by the inhibition of PTHrP and/or PTHRII transcription by the binding of 1,25(OH)2D3 to a VDRE located in the promoter region of (one of) these target genes.

Materials and Methods

In vitro studies with the ATDC5 cell line

Cell culture and experimental design. The mouse chondrogenic ATDC5 cell line was kindly provided by Dr. T. Welting (UMC Maastricht, The Netherlands). Cell culture was performed as described previously (Caron et al., 2012). Standard differentiation culture medium was supplemented with 0.2 mM L-aspartic acid 2-phosphate (AsAP, A8960, Sigma-Aldrich, Saint Louis) to reduce the time in culture until prehypertrophic differentiation (Altaf et al., 2006). Cells grown in a standard differentiation medium were compared with cells grown in a standard differentiation medium supplemented with 10–8 M PTHrP (pTH-Related Protein (1–34) amide, H-9095, Bachem, Bubendorf, Switzerland) or 10–8 M 1,25(OH)2D3 (kindly provided by Dr. C. Veldhuizen, Dishman, The Netherlands). Stripped fetal bovine serum (FBS) was used, which is devoid of vitamin D3 metabolites and growth factors. ATDC5 cells were plated on 24-well plates (Greiner Cellstar®) at a density of 6,400 cells/cm². Six hours later, cell differentiation was induced (day 0). The three different culture groups were studied from differentiation day 7 until day 10 (prehypertrophic phase) at the following time points: T0, T1, T2, T4, T8, T12, T16, T24, T32, and T48,10–8 M PTHrP or 1,25(OH)2D3 was first added to the cell culture medium. A T0–T4, and T48–10–8 M PTHrP or 1,25(OH)2D3 was added to the experimental plates. The experiment was repeated at least six times for each time point and culture condition.

RNA isolation and cDNA synthesis. Total RNA was extracted with the aid of the RNeasy® Mini kit (74104, Qiagen, Valencia, CA), according to the manufacturer’s protocol. An additional DNA digestion step with DNase (RNase-Free DNase Set, 79254, Qiagen) was included to ensure DNA removal. Total RNA was quantified with a Nanodrop® ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE). cDNA was synthesized using the Script™ cDNA Synthesis Kit (170-8891, Bio-Rad, Veenendaal, The Netherlands), according to the manufacturer’s instructions.

Quantitative determination of the expression of target genes. Primer nucleotide sequences for all reference genes were obtained from the Eccles Lab Reference Gene List (http://openwetware.org/wiki/Eccles:QPCR_reference_genes). Most primer nucleotide sequences for the target genes were obtained from PrimerBank (http://pga.mgh.harvard.edu/primerbank/index.html). For the other target genes, primer sequences were designed using PerlPrimer (http://perlprimer.sourceforge.net). Subsequently, M-fold version 3.2 was used to check for secondary structures in the primers. Thermal profiles were obtained with the aid of the RNeasy®® Mini kit (74104, Qiagen, Valencia, CA), according to the manufacturer’s protocol. An additional RNA isolation and cDNA synthesis. Total RNA was extracted with the aid of the RNeasy® Mini kit (74104, Qiagen, Valencia, CA), according to the manufacturer’s protocol. An additional DNA digestion step with DNase (RNase-Free DNase Set, 79254, Qiagen) was included to ensure DNA removal. Total RNA was quantified with a Nanodrop® ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE). cDNA was synthesized using the Script™ cDNA Synthesis Kit (170-8891, Bio-Rad, Veenendaal, The Netherlands), according to the manufacturer’s instructions.

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Hspca, Rpl32, Rps19, Ywhaz, B2m, Gapdh, Hbms, Hpfr-I, Sdha, and Tbp. The geNorm program was used to evaluate the stability of the housekeeping genes (Vandesompele et al., 2002). The three most stably expressed reference genes in the ATDC5 cell line, Rpl32, Rps19, and Sdha, were chosen to normalize gene expression. However, Sdha was not used as a reference gene in the experiment in which the VDR was silenced, because Sdha gene expression was not sufficiently stable under the experimental conditions used. RT-qPCR was performed using the iQ SYBR Green SuperMix Kit (Bio-Rad, Veenendaal, The Netherlands) and the MyiQ™ single color Real-Time PCR Detection System (Bio-Rad).

**Protein isolation and Western blot.** Semi-quantitative protein expression of the VDR and collagen type X was determined using Western blot. Protein was extracted from the ATDC5 cells with 50 μl RIPA buffer per well, and protein concentration was determined with a Lowry assay (500-0116, Bio-Rad). Thereafter, 30 μg protein was subjected to 12% SDS–PAGE, and electrophoretically onto a Hybond-C nitrocellulose membrane (90RPN203C, GE Healthcare Life Sciences, Diegem, Belgium). Only one SDS–PAGE gel and membrane was used per target protein. The membrane was blocked for 60 min, followed by overnight incubation at 4°C with the first antibody (Supplementary File 3). Thereafter, the membrane was washed and incubated for 60 min with a horseradish peroxidase (HRP)-conjugated second antibody. Protein was detected by enhanced chemiluminescence (Molecular Imager ChemiDoc XRS System, Bio-Rad). Control experiments were included in which the first antibody was omitted. After completion of Western blotting of the target proteins, the membranes were stripped using Stripping Buffer (Molecular Probes, Eugene, OR) at a density of 6,400 cells/cm² and 37°C. The ChIP assay was performed as described previously (Pandit et al., 2012), with minor modifications. Briefly, ATDC5 cells were seeded in Falcon Primaria petri dishes (353803, BD Biosciences, Breda, The Netherlands) at a density of 6,400 cells/cm² and cultured for 7 days (approximately 2 × 10⁶ cells per dish), as described earlier in this section. Cells were treated with 10⁻⁸ M 1,25(OH)₂D₃ for 24 h. For immunoprecipitation, rat anti-VDR (MAI-710, Affinity Bioreagents, Golden, CO) was used, whereas an equal amount of rat IgG (IgG2b, CBL606, Chemicon, Billerica, MA) was used as a normalization control. For more detailed information on the ChIP assay, see Supplementary File 5.

**Statistical analysis.** For determination of relative quantitative gene expression, the corrected ΔCt method was used (Pfaffl, 2001). ΔCt-values were determined for each time point by subtracting the mean reference gene Ct-value at the time point of interest from the target gene Ct-value at the same time point: 

$$\Delta\text{Ct} = \text{Ct}_{\text{Target}} - \text{Ct}_{\text{mean ref}}$$

Subsequently, all values were related to the T₀ time point by subtracting the ΔCt-value for T₀ from the ΔCt-value for Tₓ, the time point of interest: 

$$\Delta\text{Ct}_{\text{T₀}} - \Delta\text{Ct}_{\text{Tₓ}}$$

before the siRNA transfection medium was replaced by normal differentiation medium. On day 7 of differentiation (which was 72 h after siRNA initiation), 10⁻⁸ M PTHrP or 1.25(OH)₂D₃ was added to determine whether the effects of PTHrP and 1.25(OH)₂D₃ on the ATDC5 cells could be prevented by VDR silencing. From differentiation day 7 until day 10 (i.e., 72–144 h after siRNA initiation), the different culture groups were studied at the time points T₀, Tₕ, Tₐ, and T₄ₕ (the digits indicate the number of hours after siRNA or 1.25(OH)₂D₃ addition). 10⁻⁸ M PTHrP or 1.25(OH)₂D₃ was added at T₀, Tₕ, Tₐ, and T₄ₕ. Nine different cell culture conditions (control, control + scrambled mock, control + VDR-oligo, PTHrP, PTHrP + scrambled mock, PTHrP + VDR-oligo, 1.25(OH)₂D₃, 1.25 (OH)₂D₃ + scrambled mock, and 1.25(OH)₂D₃ + VDR-oligo) were studied and compared.

**Chromatin immunoprecipitation (ChIP) assay.** The sequence and location of the VDREs in the PTHrP promoter region have only been determined in the rat (Falzon, 1996; Kremer et al., 1996a). The Mus musculus and Rattus norvegicus PTHrP promoter regions were BLASTed (Altschul et al., 1997) to determine their homology (Supplementary File 4). Primers were designed and validated for the two VDRE regions of the mouse PTHrP promoter (Supplementary File 4, Fig. 3A). As a negative control, primers were designed at 1,000 bp upstream of VDRER. To date, there is no VDR reported for the PTHr1 gene. The PTHR1 gene has two VDRE regions, P1 and P2 (Fig. 3C). In bone and cartilage, the P2 promoter controls Pthr1 gene expression, and therefore we searched for possible VDREs in this P2 promoter with the aid of the core binding motif consensus sequence RGKTS {R=A or G, K=C or G} (Toell et al., 2000). This motif was included in two six-nucleotide sequences in the region upstream of U3: AGGTGA and GGTTGA, which are 2,092 and 2,027 bp upstream of the transcription start site (TSS), respectively. The distance between these sequences was 104 bp. The consensus is that a VDRE has normally three to six nucleotides between the two motif sequences (Carlberg, 1995). We cannot exclude that the area between these two sequences loops back to bring the sequences close together, enabling the VDR to bind. For this reason, primers were designed for each six-nucleotide sequence (VDRE1 and VDRE2), but also for the region containing these two core binding motif consensus sequences (VDRE combination). As negative and positive controls, primers were designed at 1,000 bp upstream of the expected VDREs and for one of the VDREs of the 24-hydroxylase promoter, respectively (Supplementary File 4). VDREs for 24-hydroxylase have been identified in the rat (Zierold et al., 1995), and therefore the rat promoter region was BLASTed against the mouse 24-hydroxylase promoter region. Both VDREs revealed 100% alignment. Primers were designed for the VDRE 5′-CGCACCCCGCTAGACC-3′. The ChIP assay was performed as described previously (Pandit et al., 2012), with minor modifications. Briefly, ATDC5 cells were seeded in Falcon Primaria petri dishes (353803, BD Biosciences, Breda, The Netherlands) at a density of 6,400 cells/cm² and cultured for 7 days (approximately 2 × 10⁶ cells per dish), as described earlier in this section. Cells were treated with 10⁻⁸ M 1,25(OH)₂D₃ for 24 h. For immunoprecipitation, rat anti-VDR (MAI-710, Affinity Bioreagents, Golden, CO) was used, whereas an equal amount of rat IgG (IgG2b, CBL606, Chemicon, Billerica, MA) was used as a normalization control. For more detailed information on the ChIP assay, see Supplementary File 5.
each individual experiment, target gene expression per time point of interest (n-fold change) was determined separately. Afterwards, for each target gene, the mean n-fold changes and standard deviations in gene expression per time point of interest were calculated. In the silencing study, VDR knockdown percentages in the siRNA VDR-oligo cultures were determined by subtracting the VDR Ct-value from the mean reference gene Ct-value for each time point: CtCt−CtVDR. Subsequently, this value was subtracted from the value for the siRNA scrambled mock cultures or the value for the control cultures, to obtain ΔCt-values. VDR knockdown percentages were calculated using the formula: 100 × 1−(1/ EΔCt).

Statistical analysis was performed using R Studio (version 0.96, http://www.rstudio.com) and R (version 2.15.2) (R Core Team, 2012). To determine whether the enrichment in the ChIP experiments was statistically significant, the data were examined for normal distribution, and a one-way ANOVA with Benjamini-Hochberg correction was used. For the rest of the data (target gene and PTHrP protein production), a mixed model for dependent data was used. This mixed model was optimized per target gene/protein and comparison. After it was determined whether the data were normally distributed, the random part of the model was determined (e.g., with random slopes and/or random intercepts). Thereafter, the fixed part of the model was optimized. Interaction of time and treatment (culture condition) appeared necessary in all cases. In the above mentioned tests, a P-value < 0.05 was considered significant.

In vivo studies

Animals. The animal studies were approved by the institutional animal care committee (DEK 2008.11.03.024), as required by Dutch law. The colony of the transgenic Col2-pd2EGFP reporter mice was maintained at the SPF facilities with approval from the Dutch Ministry of Infrastructure and Environment (DEM/SAS IG 99-057/03). The Col2-pd2EGFP transgenic mouse is appropriate for visualizing Col2a1 expression, for monitoring chondrocyte differentiation, and for isolating murine growth plate chondrocytes by fluorescence activated cell sorting (FACS) (Tryfonidou et al., 2011).

Diets. Vitamin D3 sufficient (control, TD 07370, 0.47% Ca, 0.3% P, 2,200 IU/kg vitamin D3) and deficient (TD 89123, 0.47% Ca, 0.3% P, 0 IU/kg vitamin D3) diets were purchased from Teklad and Food Research, Zeist, The Netherlands); the vitamin D3-sufficient diet contained 1,900 IU/kg and the vitamin D3-deficient diet less than 20 IU/kg.

Experimental design. The mice were kept under standard conditions in the experimental animal facility of the University of Utrecht. Control offspring were obtained from dams maintained on the vitamin D3-sufficient diet. Vitamin D3-deficient pups were obtained by feeding the dams a vitamin D3-deficient diet, from 2 weeks prior to mating until weaning. The weaned offspring were given the vitamin D3-deficient diet until euthanasia at 6 weeks of age. In order to ensure vitamin D3 deficiency, the pregnant females and their offspring were housed in filter-top cages, in which all fluorescent light was shielded, thereby preventing the endogenous production of vitamin D3. Only those pups that had a weight within 2 SD of the mean at 3 weeks of age were included. The pups were weaned at approximately 3 weeks of age, depending on whether they could feed independently. Weaned pups were housed in groups according to diet and gender (in order to prevent mating): vitamin D3 sufficient (VitD+), vitamin D3 deficient (VitD−), and vitamin D3 deficient supplemented with 1.25(OH)2D3 (VitD− + 1,25D). The animals were weighed every week at fixed times. At 3 weeks of age, the vitamin D3-deficient pups were given either 50 ng 1.25(OH)2D3 (VitD− + 1,25D), intraperitoneal (IP), 1 ng/μl in sterilized peanut oil; mean dose 5 ng/g body weight, BW) or placebo (VitD−, sterilized peanut oil). Thereafter, the dose of vitamin D3 metabolite was adjusted weekly, based on the mean BW of the respective group. The vitamin D3-sufficient mice (VitD+) received placebo (sterilized peanut oil) IP. The IP administration was performed five times a week, at 7 days postpartum, and followed by cervical dislocation.

Serum biochemistry. Blood samples for the measurement of calcium (Ca), inorganic phosphate (P), and vitamin D3 metabolites were collected in heparin-coated mini-collection-tubes (REF 450479, Greiner Bio-One, Monrovia, NC). For the measurement of PTH, blood was collected in EDTA-coated mini-collection tubes (REF 450475, Greiner Bio-One). Samples were immediately placed on ice until centrifugation and plasma was stored at −20°C until further analysis. Ca and P levels were measured according to standard procedures. Plasma 25(OH)D3 levels were measured to verify vitamin D3 deficiency in the respective groups. 25(OH)D3 was extracted from 25 μl plasma with the Bligh and Dyer method (Bligh and Dyer, 1959) and quantified with the aid of a competitive binding assay. Thereafter, plasma samples for pairs of mice were pooled within the ascribed group (due to sample volume limitations) and vitamin D3 metabolites were extracted from the pooled plasma using acetonitrile followed by a two-step phase extraction (C18 and Silicagel cartridge) and separated by straight phase HPLC. 25(OH)D3 was quantitatively determined using a competitive protein binding assay with a sensitivity of 2 nmol/L and 1,25(OH)2D3 was quantitatively determined with a radioreceptor assay (Reinhardt et al., 1984), with a sensitivity of 40 pmol/L. Levels were corrected for procedural losses (recovery) with the aid of the specific 3H-labeled vitamin D3 metabolite. PTH was determined according to the manufacturer’s instructions (intact PTH mouse EIA, Alpco Diagnostics, Salem, NH).

Growth plate histology. After euthanasia, the right tibia was removed and fixed in 4% formaldehyde (pH = 7.4, 4°C) for 24 h and decalcified in 0.5 M EDTA in Ca–Mg free Hanks solution (pH = 7.8, 4°C) for 7 days. After demineralization, the bones were washed and bisected in the sagittal plane. One half was embedded in Tissue Tek (O.C.T. compound, Sakura Fine Technical Co. Ltd., Tokyo, Japan) and stored at −70°C until further processing. The other half of the tibia was embedded in paraffin and stored at 4°C until further processing.

Quantitative determination of the expression of target genes. From each mouse, growth plate chondrocytes were isolated as described previously (Tryfonidou et al., 2011). Briefly, after euthanasia, the long bones and rib cartilage (except for the right tibia) were removed and submerged in cold (4°C) Hanks + 2% pen/strep. The growth plate and adjacent tissue from each animal was dissected with the aid of a stereoscope and scalpel blade, rinsed, and digested overnight at 37°C 5% CO2 with collagenase II (4176, Worthington, Lakewood, NJ). After digestion, cells were suspended in DMEM/F12 ± glutamine without phenol red (21041, Gibco, Bleswijk, The Netherlands) with 10% FCS (PAA) and 2% pen/strep. The cell solution was individually run through a FACS (BD Influx cell sorter; BD Biosciences) with a nozzle of 100 μm. After collection, the cells were lysed in 350 μL RLT buffer (Qiagen) containing 1% β-mercaptoethanol and stored at −70°C until further processing. RNA isolation, cDNA production, and RT-qPCR were performed as described for the in vitro studies. The relative level of gene expression of Pthrp, Pthr1, Vdr, and ColX was determined (Supplementary File 2).

Immunoﬂuorescent staining. Immunoﬂuorescent staining of bone for collagen type X was performed as described previously (Tryfonidou et al., 2011) on 10-μm cryosections, in order to deﬁne the pattern of GFP expression in relation to the phase of differentiation of growth plate chondrocytes. Briefly, after sections were rinsed with PBS, antigen retrieval with 4 mg/ml bovine hyaluronidase (450 IU/ml, Sigma-Aldrich, St. Louis, MO) was
performed. After blockade of aspecific binding sites with 10% goat serum, sections were incubated with rabbit polyclonal anti-mouse collagen type X (PXC2, 1:100) overnight at 4°C (Lunstrum et al., 1999). Goat anti-rabbit ALEXA 568 (1:100, Invitrogen) was used as second antibody. Nuclear counterstaining was performed with TO-PRO®-3, and the slides were mounted in Prolong Gold anti-fade reagent (Invitrogen). Confocal microscopy was performed using a Leica TCS SP confocal laser scanning microscope. Immunofluorescent staining images were prepared using Adobe Photoshop CSS. Linear adjustment of brightness, contrast, and color balance was applied to every pixel in the image.

**Immunohistochemistry.** Paraffin sections of 4 μm were cut and mounted on Microscope KP plus slides (Klinipath B.V., Duiven, The Netherlands). Each slide contained three sections: one section of each group in an ad random order. Slides were deparaffinized through xylene (two times 5 min) and graded ethanol (96%, 80%, 70%, 60%, 30%, 5 min each), followed by two rinses with PBS. Mid-sagittal sections of the three samples representing each group were included in the same P-pen circle and were thus incubated under identical conditions. Thereafter, antigen retrieval was performed (Supplementary File 3). After inhibition of endogenous peroxidase for 5 min, sections were preincubated with blocking for 30 min at room temperature (RT). The sections were incubated overnight with first antibody at 4°C. For further processing, depending on the first antibody, either the EnVision™-HRP detection system (Dako), the goat ImmunoCruz™ system (sc-2053, Santa Cruz Biotechnology, Inc., Heidelberg, Germany), or specific second antibody was applied for 30 min at RT, followed by incubation with streptavidin-conjugated with HRP for 30 min at RT. All antibodies were visualized with the liquid DAB+ substrate chromogen system (K3468, Dako, Glostrup, Denmark). In control experiments, the first antibody was omitted and, depending on the antibody, either substitution of the first antibody with its respective serum, and/or competition of the first antibody with corresponding peptides available for PTHrP (sc-12777 P) was performed. Raw images were made using a Colorview IIIU digital camera (Olympus, Zoeterwoude, The Netherlands) mounted to a BX-40 microscope (Olympus). Histomorphometry was performed on the raw images with ImageJ software package (Rasband, 2003). The isolation of primary growth plate chondrocytes from the limbs of three 9-day-old Col2-pd2EGFP mice was performed as described previously (Tryfonidou et al., 2011). Approximately 26% of the selected population was positive for GFP. On differentiation day 0 (T0), droplets of 10 μl (containing approximately 20,000 cells) were placed on a Falcon Primaria petri dish (353803, BD Biosciences), not touching each other to create multiple high-density microcultures. After 2 h, 10 ml differentiation medium was added as describe previously, and after 2 days (T2), 10⁻⁸ M 1,25(OH)₂D₃ (Dishman BV, Veenendaal, The Netherlands) was daily added to the 1,25(OH)₂D₃-treated cultures. In a similar manner, high-density microcultures on chamber slides (Lab-Tek®) were studied from differentiation day 0 until day 7. At the time points T0, T3, T5, and T7 (the digits indicate the number of days after differentiation culture medium was first added to the wells), medium for PTHrP ELISA and cells for RNA isolation were obtained, and formalin-fixed slices were stained for collagen type X (as described previously). Confocal microscopy was performed using a Leica SPEI confocal laser-scanning microscope (Leica Microsystems, Wetzlar, Germany). The concentration of PTHrP in culture media was measured with an ELISA (ELISA kit for mouse PLP, USCN E90819M). After it was determined that the data had a normal distribution, a one-way ANOVA with Benjamini-Hochberg correction was used to analyze the significance of differences in the mean (±SD) width of the growth plate (including the proliferative and hypertrophic zone), plasma parameters, target gene expression, and ChIP assay results between the different groups. For body weight and PTHrP protein levels, a mixed model for dependent data was used as described at the in vitro studies. In the above-mentioned tests, a P-value <0.05 was considered significant.

**Results**

**In vitro studies with the ATDC5 cell line**

**The effect of PTHrP on the vitamin D₃ pathway.** Protein and mRNA expression of 1α- and 24-hydroxylase was stable during culture and appeared not affected by treatment with 10⁻⁸ M 1,25(OH)₂D₃ (data not shown). At all time points, Vdr gene expression was significantly higher in the PTHrP-treated cultures than in the control cultures (P < 0.01, Fig. 1A). After the first addition of PTHrP at T₀, Vdr gene expression increased significantly to reach a maximum at T₄ and declined thereafter. At T₂₈, 4 h after the second addition of PTHrP, Vdr gene expression was significantly higher than that at T₂₄ (P < 0.01), but significantly lower than that at T₄ (P < 0.001). Vdr gene expression did not change substantially after T₂₈. VDR protein levels were also significantly higher at time points T₂₄ and T₄₈ in the PTHrP-treated cultures than in the control cultures (P < 0.01, Fig. 1B). Altogether, these data indicate that PTHrP treatment increased ATDC5 chondrocyte sensitivity for 1,25(OH)₂D₃ by upregulating VDR expression and not by influencing 1α- and/or 24-hydroxylase expression.

**The effect of vitamin D₃ on the PTHrP pathway.** Treatment of ATDC5 chondrocytes with 10⁻⁸ M 1,25(OH)₂D₃ increased the activity of the vitamin D₃ pathway (Armbricht and Bolzt, 1991), as evidenced by more than 1,000 times increased expression of the 24-OHase gene compared with control cultures (data not shown). 10⁻⁸ M 1,25(OH)₂D₃ treatment also significantly increased VDR gene and protein expression in ATDC5 chondrocytes in the prehypertrophic phase of differentiation (P < 0.05, Fig. 1AB) (Davoodi et al., 1995; Klaus et al., 1998; Healy et al., 2005a). Pthrp gene expression was significantly higher in the 10⁻⁸ M 1,25(OH)₂D₃-treated cultures than in the control cultures at all time points (P < 0.001, Fig. 1C). In contrast, the PTHrP protein content of
**Fig. 1.** VDR and PTHrP expression in 1,25(OH)2D3- and PTHrP-treated ATDC5 chondrocytes in the prehypertrophic phase of differentiation. ATDC5 cells were treated starting from day 7 of differentiation (T0) with 10^-8 M PTHrP or 1,25(OH)2D3 at T0, T24, and T48. A. VDRC relative gene expression (mean ± SD; n = 8). T0 in the control culture was set at 1. B: VDR protein expression expressed as VDR/β-actin ratio (mean ± SD; n = 6). T0 in the control culture was set at 1. C: PTHRp relative gene expression (mean ± SD; n = 8). T0 in the control culture was set at 1. D: PTHrP protein levels corrected for DNA content (pg PTHrP/ng DNA) in ATDC5 culture media of control cultures and 10^-8 M 1,25(OH)2D3-treated cultures, determined using a PTHrP ELISA (mean ± SD; n = 4). *P < 0.05, **P < 0.01, ***P < 0.001.
the medium from 1.25(OH)_{2}D_{3}-treated cultures was significantly lower than that of control cultures at T_{48} and T_{72} (P < 0.001, Fig. 1D). This seemingly contradictory result could be attributed to a phenomenon called “mRNA superinduction.” It has been reported that the inhibition of PTHR1 protein synthesis leads to the induction of Pthrp mRNA expression (“mRNA superinduction”) in a number of cell lines (Ikeda et al., 1990). We explored this possibility in the ATDC5 cell line by measuring PTHrP mRNA and protein levels (in culture medium and cell lysate, corrected for DNA content) in the first 24 h after treatment with 1.25(OH)_{2}D_{3}. PTHR1 protein production decreased by 10^{-8} M 1.25(OH)_{2}D_{3} treatment from T_{4} forward (P < 0.01, Fig. 2B,C) and remained lower in the 10^{-8} M 1.25(OH)_{2}D_{3}-treated cultures than in the control cultures until T_{72} (Fig. 1D). In contrast, Pthrp mRNA expression significantly increased from T_{24} onward by 10^{-8} M 1.25(OH)_{2}D_{3} treatment (Figs. 1A and 2C). Given the time line of these events, where the increase in Pthrp mRNA expression (T_{24}) is preceded by an initial significant decrease in PTHrP protein production (T_{4}/T_{8}), it seems reasonable to assume that the increased Pthrp mRNA levels were due to the “mRNA superinduction” phenomenon. To determine whether this observed effect was physiological or pharmacological, we conducted an additional experiment investigating the time line of PTHrP mRNA and protein expression in the presence of 10^{-8}, 10^{-10}, and 10^{-12} M 1.25(OH)_{2}D_{3}. PTHrP protein levels were dose-dependently influenced by 1.25(OH)_{2}D_{3} treatment (Fig. 2B,C). Moreover, the increased Pthrp mRNA expression observed in the 10^{-8} M 1.25(OH)_{2}D_{3}-treated ATDC5 chondrocytes (at T_{24}), was not observed in the 10^{-10} and 10^{-12} M 1.25(OH)_{2}D_{3}-treated cells. This indicates that the “Pthrp mRNA superinduction” phenomenon, only observed in the 10^{-8} M 1.25(OH)_{2}D_{3}-treated ATDC5 chondrocytes, is pharmacological and can be avoided with lower physiological 1.25(OH)_{2}D_{3} regimes.

**Functional binding of 1.25(OH)_{2}D_{3} to the promoter region of the PTHrP gene.** Computational analysis of the PTHrP promoter revealed the presence of two VDR binding elements (Fig. 3A). ChIP experiments showed a significant 2-fold enrichment of VDRE1 (P < 0.01) and a 1.6-fold enrichment of VDRE2 (n.s.) in the PTHrP promoter (Fig. 3B). No enrichment was observed in the PTHrP negative control (sequence 1,000 bp upstream of VDRE2) and a significant 2.5-fold enrichment was observed in the positive control (24-hydroxylase promoter, P < 0.05, Fig. 3B). Altogether, this indicates that in ATDC5 chondrocytes in the prehypertrophic phase of differentiation, 1.25(OH)_{2}D_{3} binds to its nuclear receptor, the VDR, and together they bind to a 1.25(OH)_{2}D_{3}-responsive region (VDRE1) in the PTHrP promoter. In this way, 1.25(OH)_{2}D_{3} directly regulates Pthrp expression via genomic effects.

Computational analysis of the PTHr1 promoter revealed the presence of two VDREs (Fig. 3C). ChIP experiments showed a 1.4-fold enrichment of both VDRE1 and VDRE2, and a 2.1-fold enrichment of the VDRE combination (region containing both the expected VDRE1 and VDRE2) in the PTHr1 promoter (Fig. 3D). However, these results were not significant and the PTHr1 negative control (sequence 1,000 bp upstream of the expected VDREs) also revealed a 1.5-fold enrichment of the VDR antibody. Altogether, this indicates that there is no functional binding of 1.25(OH)_{2}D_{3} and the VDR to a VDRE in the PTHr1 promoter of ATDC5 chondrocytes in the prehypertrophic phase of differentiation.

**Determination of the role of the VDR in the paracrine feedback loop between PTHrP and 1.25(OH)_{2}D_{3}.** To clarify the role of the VDR in the paracrine feedback loop between PTHrP and 1.25(OH)_{2}D_{3}, we successfully silenced the VDR in 100% confluent ATDC5 chondrocytes in the prehypertrophic phase of differentiation; significant VDR knockdown was seen at all time points in the control + VDR-oligo cultures and the 1.25(OH)_{2}D_{3} + VDR-oligo cultures (P < 0.05, Fig. 4A,C).

Up- and downregulated PTHPr protein production in the scrambled mock cultures compared with the control cultures was observed at several time points (both with and without 1.25(OH)_{2}D_{3} supplementation, Fig. 4D), which can be explained by off-target effects of the scrambled mock siRNA sequence (Persengiev et al., 2004). The culture medium PTHrP protein content increased over time in the control cultures, whereas it decreased over time in the 1.25(OH)_{2}D_{3}-treated cultures, with the difference being significant at T_{48}–T_{72} (P < 0.05, Fig. 4D). At T_{48} and T_{72}, PTHrP protein levels in the 1.25(OH)_{2}D_{3} + VDR-oligo cultures were significantly higher than in the 1.25(OH)_{2}D_{3} cultures (P < 0.05), indicating that VDR silencing counteracted the 1.25(OH)_{2}D_{3}-mediated inhibitory effect on PTHrP protein production in ATDC5 chondrocytes in the prehypertrophic phase of differentiation.

**The role of the paracrine PTHrP-1.25(OH)_{2}D_{3} feedback loop in hypertrophic chondrocyte differentiation.** 1.25(OH)_{2}D_{3} treatment of the prehypertrophic ATDC5 cultures decreased the DNA content by about 40% whereas...
the addition of PTHrP increased the DNA content by about 30% compared with the control cultures (data not shown).

Gene expression of the (pre) hypertrophic differentiation markers Col9, Pthr1, and ColX increased significantly over time in the control cultures ($P < 0.05$), but not in the PTHrP- and 1,25(OH)$_2$D$_3$-treated cultures (Fig. 5A). Generally, Pthr1, Col9, and ColX gene expression was higher in the control cultures than in the PTHrP- or 1,25(OH)$_2$D$_3$-treated cultures at all time points. However, Western blot analysis indicated that only on differentiation day 14 ($T_{168}$), collagen type X protein expression was significantly lower in the PTHrP- and 1,25(OH)$_2$D$_3$-treated cultures than in the control cultures ($P < 0.001$, Fig. 5B), but not on differentiation day 10 ($T_{72}$). This indicates that both 1,25(OH)$_2$D$_3$ and PTHrP treatment inhibited hypertrophic differentiation of the ATDC5 chondrocytes, which was noticed earlier at the mRNA than at the protein level.

To clarify the role of the paracrine feedback loop between PTHrP and 1,25(OH)$_2$D$_3$ in growth plate chondrocyte differentiation, we silenced the VDR in ATDC5 chondrocytes in the prehypertrophic phase of differentiation. In the control + scrambled mock and the control + VDR-oligo
cultures, ColX gene expression did not increase over time, in contrast to what was observed in the control cultures (Fig. 6A). For the PTHrP- and 1,25(OH)2D3-treated ATDC5 cultures, no significant differences in collagen type X expression between the non-silenced (PTHrP/1,25(OH)2D3) and the VDR silenced (PTHrP/1,25(OH)2D3 + VDR-oligo) cultures were observed (Fig. 6A,B), indicating that VDR silencing did not counteract the PTHrP/1,25(OH)2D3-mediated inhibitory effect on collagen type X gene and protein expression in ATDC5 chondrocytes in the prehypertrophic phase of differentiation. In contrast, Pthr1 expression was significantly upregulated in the 1,25(OH)2D3 + VDR-oligo cultures at T48 and T72 (P < 0.01, Fig. 6), indicating that VDR silencing counteracted the 1,25(OH)2D3-mediated inhibitory effect on Pthr1 expression in ATDC5 chondrocytes in the prehypertrophic phase of differentiation.

Generally, ColX and Vdr gene expression was significantly higher in the scrambled mock cultures than in the non-silenced control cultures, whereas the scrambled mock treatment did not affect Pthr1 and Pthrp expression (Figs. 4A,B and 6A).

The difference in ColX and Vdr gene expression was, however, not
accompanied with differences in VDR and collagen type X protein expression between the scrambled mock and the non-silenced control cultures (Figs. 4C and 6B), indicating that the scrambled mock-induced upregulated mRNA expression was not translated into increased protein expression. Most probably, the upregulated \textit{ColX} and \textit{Vdr} mRNA expression can be attributed to off-target effects of the scrambled mock sequence (Persengiev et al., 2004).

In vivo studies

Animals and biochemistry. From 4 weeks of age onward, the control vitamin D$_3$-sufficient (VitD$^+$) mice weighed significantly more than the VitD$^-$ mice ($P < 0.001$, Supplementary file 6A). Plasma 25(OH)D$_3$ levels confirmed vitamin D$_3$ deficiency in VitD$^-$ mice ($P < 0.001$, Supplementary file 6A). As expected, the plasma concentration of 1,25(OH)$_2$D$_3$ was significantly higher in VitD$^+$; $+1,25D$ mice than in VitD$^+$ and VitD$^-$ mice ($P < 0.01$). Plasma calcium (Ca) levels were significantly lower in VitD$^-$ mice than in VitD$^+$ mice ($P < 0.01$), but were significantly higher in VitD$^-$; $+1,25D$ mice than in VitD$^-$ and VitD$^+$ mice ($P < 0.01$). Inorganic phosphate (P) plasma levels were not significantly different between groups, but PTH levels were significantly higher in VitD$^-$ mice than in VitD$^+$ and VitD$^-; +1,25D$ mice ($P < 0.001$).
Growth plate histomorphometry. The mean growth plate height (GPl.Th) was significantly higher in VitD− mice than in VitD+ and VitD−; +1.25D mice (P < 0.001, Fig. 7A). The mean GPl.Th.Hyp and the ratio of GPl.Th.Hyp/GPl.Th were significantly higher in VitD− mice than in VitD+ or VitD−; +1.25D mice (P < 0.05). The standard deviation of GPl.Th.Hyp, a measure of the irregularity of the hypertrophic zone, was also significantly higher in VitD− mice than in VitD+ and VitD−; +1.25D mice (P < 0.01, Fig. 7A). The Col2-pd2EGFP vitamin D3-deficient mice thus showed the classical signs of low body weight, hypocalcemia, hyperparathyroidism, and rickets with an enlarged growth plate. The latter was mainly due to an increased and irregular hypertrophic zone (Donohue and
Fig. 7. Results of the in vivo study (A + B) and the primary cell culture (C + D) of Col2-pd2EGFP transgenic mice growth plate chondrocytes. A: Histomorphometry (H&E) and immunofluorescent staining for collagen type X (red) of tibial growth plates from Col2-Col2-pd2EGFP transgenic mice (6 weeks of age) indicating that vitamin D3 deficient mice (VitD−/−) developed rickets. Supplementation with 1,25(OH)2D3 (VitD−/+1,25D) reversed the rachitic phenotype as compared with controls (VitD+/+). Note that Col2-pd2EGFP fluorescence (green) is native (mean ± SD; n = 7). B: Relative ColX, Vdr, Pthrp, and Pthr1 gene expression corrected for reference genes in sorted Col2-pd2EGFP positive growth plate chondrocytes after termination of the study, at 6 weeks of age (mean ± SD; n = 6). Relative target gene expression in the growth plate of control (VitD+/+) mice was set at 1. C: Relative ColX, Vdr, Pthrp, and Pthr1 gene expression corrected for reference genes in control or 1,25(OH)2D3-treated cultured primary Col2-pd2EGFP growth plate chondrocytes. Time is shown in days after the start of differentiation (T0, T3, T5, and T7). From T2 onward, 10−8 M 1,25(OH)2D3 was daily added to the culture medium. Relative target gene expression at T0 in the control culture was set at 1. These data were not subjected to statistical analysis due to the small sample size (n = 1 experimental replicate). D: From left to right: Immunofluorescent staining for collagen type X and native expression of Col2-pd2EGFP in control and 10−8 M 1,25(OH)2D3-treated cultured primary Col2-pd2EGFP growth plate chondrocytes. Transcription of collagen type 2 (green) and hypertrophic differentiation of chondrocytes (red) were detected in control cultures, whereas the 10−8 M 1,25(OH)2D3-treated cells expressed no Col2-pd2EGFP and only occasionally collagen type X immunofluorescence in their pericellular matrix. A ChIP assay revealed enrichment of PTHrP VDRE1, but not the VDRE2. A PTHrP ELISA performed on culture media revealed decreased PTHrP production in the 10−8 M 1,25(OH)2D3-treated primary growth plate chondrocytes compared with control cultures (mean ± SD; n = 4). *P < 0.05, **P < 0.01, ***P < 0.001.
expression was higher in 1,25(OH)2D3-treated compared with control primary growth plate chondrocytes. However, in accordance with gene expression levels, PTHR1 staining was present in all growth plate zones and seemed to be in greater amounts in VitD+ mice than in mice from the other two groups (Supplementary file 6B).

In vitro studies with primary growth plate chondrocytes
As a further step, we aimed to better understand the role of 1,25(OH)2D3 and PTHrP in the regulation of gene expression in primary growth plate chondrocytes, which undergo differentiation in an orderly manner. The effect of PTHrP on the vitamin D3 pathway was investigated in vitro (ATDC5 and primary growth plate chondrocytes) and in vivo (Col2-pd2EGFP mice) models and demonstrated that there is a functional paracrine feedback loop between 1,25(OH)2D3 and PTHrP in prehypertrophic growth plate chondrocytes (Fig. 8).

Discussion
The regulation of chondrocyte differentiation is a key event in skeletal development. Regenerative strategies for cartilage engineering use mesenchymal stem cells (MSCs), but are hampered by the inherent capacity of chondrogenically differentiating MSCs to undergo hypertrophic differentiation (Hellingman et al., 2012). Hence, understanding the processes that regulate chondrocyte differentiation is crucial to further fine-tune regenerative strategies for cartilage and bone engineering. In order to do so, we used growth plate chondrocytes, which undergo differentiation in an orderly fashion. We used complementary in vitro (ATDC5 and primary growth plate chondrocytes) and in vivo (Col2-pd2EGFP mice) models and demonstrated that there is a functional paracrine feedback loop between 1,25(OH)2D3 and PTHrP in prehypertrophic growth plate chondrocytes (Fig. 8).
endocrine level—PTHrP stimulates 1α-hydroxylase in the kidney in the same way that PTH does (Henry et al., 1997). However, in the current study, PTHrP did not significantly affect the expression of 1α- and 24-hydroxylase in ATDC5 chondrocytes in the prehypertrophic phase of differentiation. Although we did not determine the actual activity of the respective enzymes, it is tempting to speculate that PTH and PTHrP influence 1α- and 24-hydroxylase expression in a tissue/cell-specific manner.

We found that PTHrP significantly increased VDR expression (Fig. 1A,B). Vdr gene expression was upregulated 4 h after initiation of PTHrP treatment (T0), whereas repeated treatment resulted in a lower increase in Vdr gene expression at T28 (Fig. 1A). Desensitization of the PTH-induced cAMP response (Jongen et al., 1996) might explain the decreased response of the ATDC5 cells to PTHrP after the second addition of PTHrP (at T24) compared with the first addition (at T0). Previous studies found a PTH/PTHrP-mediated decreased VDR expression in renal, intestinal, and osteoblast-like cells (Reinhardt and Horst, 1990; Sruissadaporn et al., 1995; Healy et al., 2005b), but increased VDR expression in growth plate chondrocytes and osteoblast-like cells (Pols et al., 1988; Klaus et al., 1997). A possible explanation for these contradictory results is again a cell-line/tissue specific effect.

The effect of vitamin D3 on the PTHrP pathway

We hypothesized that treatment with 1,25(OH)2D3 would decrease PTHrP production, because Pthrp expression is reported to be suppressed by 1,25(OH)2D3 in several other cell types (Kremer et al., 1991; Falzon, 1996; Sepulveda et al., 2006).

In contrast to our hypothesis, in the in vivo studies with the Col2-pd2EGFP mouse model, Pthrp gene expression was increased in Col2-pd2EGFP growth plate chondrocytes from mice treated with 1,25(OH)2D3 compared with VitD- or VitD+ mice (Fig. 7B). However, these in vivo studies are limited by several factors. First, growth plate chondrocytes were isolated on the basis of FACs of Col2-pd2EGFP-positive chondrocytes and, hence, may not be representative of the total population of growth plate chondrocytes. Second, the altered calcium homeostasis in the vitamin D3-deficient mice treated with 1,25(OH)2D3 resulted in hypercalcemia (Supplementary file 6A), which could have contributed to the increased Pthrp and Vdr expression in VitD--; +1,25D mice compared with VitD- or VitD+ mice (Fig. 7B). Calcium has been shown to regulate PTHrP secretion (Kremer et al., 1996b; Chattopadhyay et al., 2000) and to have an additive effect on the homologous upregulation of VDR expression during treatment with 1,25(OH)2D3 (Davoodi et al., 1995; Klaus et al., 1998; Healy et al., 2005a). To exclude the effects of interfering factors, we also performed in vitro studies with ATDC5 and primary Col2-pd2EGFP growth plate chondrocytes and studied the pattern of expression of target genes and proteins in the presence and absence of 1,25(OH)2D3. In line with our hypothesis, PTHrP gene and protein expression was lowered in the 1,25(OH)2D3-treated primary Col2-pd2EGFP growth plate chondrocytes compared with control cultures (Fig. 7C,D). Also in prehypertrophic ATDC5 chondrocytes, PTHrP protein production was decreased by 1,25(OH)2D3-treatment (Figs. 1D and 2B,C). The counterintuitive increase of Pthrp gene expression in 10−8 M 1,25(OH)2D3-treated ATDC5 cultures compared with control cultures from 24 h of treatment onward (Figs. 1C and 2A) could be explained by "Pthrp mRNA superinduction" (Ikeda et al., 1990), which is only provoked by the preceding decreased PTHrP protein expression in 10−8 M 1,25(OH)2D3-treated prehypertrophic ATDC5 chondrocytes (Fig. 2B,C), and not at lower dosages.

We furthermore silenced the VDR to clarify its role in the regulation of PTHrP expression by 1,25(OH)2D3. VDR silencing counteracted the inhibitory effect of 1,25(OH)2D3 on PTHrP protein production (Fig. 4D). In addition, we observed significant enrichment of the PTHrP VDRE1 in 1,25(OH)2D3-treated ATDC5 and primary growth plate chondrocytes in the prehypertrophic phase of differentiation (Figs. 3B and 7D). This is the first study to report functional binding of 1,25(OH)2D3 through its receptor to the promoter region of the PTHrP gene in growth plate chondrocytes. The 1,25(OH)2D3-responsive region (VDRE) in the PTHrP promoter has already been characterized in other tissues (Kremer et al., 1991, 1996a; Falzon, 1996). Taken together, these findings prove that in the growth plate, the functional paracrine feedback loop between PTHrP and 1,25(OH)2D3 is closed by the inhibition of PTHrP transcription by the binding of 1,25(OH)2D3 to a VDRE located in the PTHrP (and not the PTHR1) promoter region (Fig. 8).

The role of the paracrine PTHrP-1,25(OH)2D3 feedback loop in hypertrophic chondrocyte differentiation

Having established that there is a functional paracrine feedback loop between PTHrP and 1,25(OH)2D3 in growth plate chondrocytes (Fig. 8), we wanted to define the role of this feedback loop in hypertrophic chondrocyte differentiation. Both 1,25(OH)2D3 and PTHrP affected the proliferation and differentiation of growth plate chondrocytes. 1,25(OH)2D3 treatment resulted in a decreased DNA content of the hypertrophic ATDC5 chondrocytes, indirectly indicating that 1,25(OH)2D3 had an anti-proliferative effect, which is in line with previous reports (Klaus et al., 1991). Moreover, 1,25(OH)2D3 had an inhibitory effect on chondrocyte hypertrophy, based on the reduced Col9, ColX, and Pthr1 gene expression and collagen type X protein expression in 1,25(OH)2D3-treated ATDC5 and primary Col2-pd2EGFP positive growth plate chondrocytes (Figs. 5 and 7C,D). This is by no means a new finding, since 1,25(OH)2D3 has been shown to inhibit terminal chondrocyte differentiation both in vitro and in vivo (Kato et al., 1990; Drissi et al., 2002; Idelevich et al., 2011; Castillo et al., 2012). In addition, VDR silencing in 1,25(OH)2D3-treated ATDC5 cells only partially reversed the anti-hypertrophic effects of 1,25(OH)2D3; it prevented the inhibitory effect of 1,25(OH)2D3 on Pthrp gene expression, but it did not affect collagen type X expression (Fig. 6). In unpublished studies, we found that treatment with 10−6 and 10−12 M 1,25(OH)2D3 decreased the expression of (pre) hypertrophic differentiation markers, and hence VDR silencing may have been ineffective in counteracting the strong inhibitory effect of 10−12 M 1,25(OH)2D3 on hypertrophic differentiation. An alternative explanation is that 1,25(OH)2D3 also exerts effects by binding to a membrane-associated receptor PDIA3 (Boyan et al., 2006; St-Arnaud and Naja, 2011).

PTHrP is a well-known suppressor of hypertrophic chondrocyte differentiation (Ballock and O’Keefe, 2003; van der Eerden et al., 2003; Hoogendam et al., 2007; Brochhausen et al., 2009). Accordingly, in the control ATDC5 cultures, Col9 gene and collagen type X gene and protein expression increased with time, but this increase was prevented by the addition of PTHrP (Fig. 5). Furthermore, the addition of PTHrP to ATDC5 cells in a prehypertrophic differentiation phase significantly downregulated the expression of the gene for the receptor of PTHrP, Pthr1 (Fig. 5A), which is also in line with previous reports (Weisser et al., 2002; Wealthall, 2009). This homologous downregulation of Pthr1 is possibly a measure to prevent overstimulation, but another explanation lies in the physiological role of PTHrP, namely, to prevent proliferative cells leaving the proliferating pool. In this way, hypertrophic chondrocyte differentiation—and thus PTHR1 production—is delayed (Kronenberg, 2003; Mak et al., 2008; Hirai et al., 2011; Zhang et al., 2012). Taken together, the results of our study indicate that PTHrP could be used clinically to inhibit...
undesirable hypertrophic chondrocyte differentiation. Accordingly, in vitro work has already demonstrated that PTHrP successfully inhibited hypertrophic differentiation of articular chondrocytes (Wang et al., 2011; Zhang et al., 2013) and cartilage constructs engineered from bone marrow-derived mesenchymal stem cells (BMSCs), without losing cartilage-specific matrix proteins (US patent 20080318859, Kafifah et al., 2007). Moreover, in vivo, intra-articular PTHrP injection together with collagen-silk scaffold implantation (4–6 weeks post-injury) inhibited terminal differentiation and enhanced chondrogenesis in induced osteochondral defects in rabbits (Zhang et al., 2013). In contrast, in chondrogenically differentiated BMSC pellets, PTHrP could not diminish the T3-induced enhancement of hypertrophy (Mueller et al., 2013). Despite significant reduction of some hypertrophic markers, the absolute level of expression was still high compared with articular chondrocyte-based cartilage constructs (Hellingman et al., 2012). Furthermore, PTHrP has even been reported to suppress chondrogenic differentiation of BMSC pellets (Weiss et al., 2010). Noteworthy is the fact that above-mentioned studies started the PTHrP treatment at different time points, that is, before or after manifestation of hypertrophy. Thus, the use of PTHrP needs to be further investigated with regard to the inhibition of hypertrophic chondrocyte differentiation, articular cartilage repair, and the generation of state-engineered cartilage from MSCs (Zhang et al., 2012).

In order to further study how the interaction between 1,25(OH)2D3 and PTHrP influences hypertrophic chondrocyte differentiation, we evaluated whether PTHrP prevents the differentiation of chondrocytes through a VDR-dependent mechanism. For this purpose, the VDR was successfully silenced in PTHrP-supplemented cultures, but the expression of Pthri gene and collagen type X protein was hardly influenced (Fig. 6). As it is not possible to discuss these results in the light of earlier reports, we can only postulate that the anti-hypertrophic effect of PTHrP is independent of the VDR. This indicates that 1,25(OH)2D3 and PTHrP individually influence hypertrophic chondrocyte differentiation and thus may have a synergistic effect in suppressing hypertrophic differentiation.

Conclusions

Taken together, the data obtained using an integrative approach involving in vitro studies with ATDC5 and primary growth plate chondrocytes and in vivo studies with Col2α2EGFP transgenic mice led us to conclude that there is a functional paracrine feedback loop between PTHrP and 1,25(OH)2D3 in prehypertrophic growth plate chondrocytes. 1,25(OH)2D3 inhibits PTHrP production through a functional binding place (VDRE) in the PTHrP promoter, and PTHrP increases chondrocyte sensitivity to 1,25(OH)2D3 by increasing VDR production (Fig. 8). The results of this study furthermore indicate that 1,25(OH)2D3 and PTHrP individually influence hypertrophic chondrocyte hypertrophy and, hence, may have the potential to inhibit undesirable hypertrophic chondrocyte differentiation during cartilage repair or engineering. To our knowledge, the effect of 1,25(OH)2D3 and the synergistic effect of a combination of PTHrP and 1,25(OH)2D3 on MSC-based cartilage regeneration has not yet been evaluated and might provide leads for new strategies to improve the quality of engineered cartilage.

Acknowledgments

We are very grateful to Rob Bleumink (Center for Cell Imaging) for his assistance with microscopy, Jan Jaap Stevenhagen (TNO Nutrition and Food Research, Zeist, The Netherlands) for the vitamin D3 content analysis, Ms. Jane Sykes for language corrections, and Mr. Hans Vermoij

(Utrecht University, The Netherlands) for assistance in statistical analysis. Furthermore, we thank Dr. G. P. Lunstrum (Oregon Health Sciences University), Dr. T. Welting (UMC Maastricht) and Dr. C. Veldhuizen (Dishman, The Netherlands) for their generous gifts.

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