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Advance Publication

ORIGINAL

Osteocalcin Attenuates T3- and Increases Vitamin D3-Induced Expression of MMP-13 in Mouse Osteoblasts

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Abstract. Osteocalcin (OCN), the most abundant non-collagenous protein of the bone matrix, whose function is not fully understood, was recently suggested to act as endocrine factor regulating energy metabolism. Besides OCN, osteoblasts also express MMP-13, a matrix metallo-proteinase important for bone development and remodeling. Although differentially, both genes are regulated by 1,25-dihydroxy vitamin D3 (1,25D3) and T3, important hormones for bone metabolism. In mouse osteoblasts with a distinct differentiation status, T3 increases the expression of both proteins. By contrast, 1,25D3 stimulates the expression of MMP-13 but inhibits the expression of OCN in these cells. In humans, however, 1,25D3 up-regulates both genes while T3 inhibits the OCN expression. Using northern blot hybridization we studied gene expression in the mouse osteoblastic cell line MC3T3-E1. We show that MMP-13 expression was strongly increased by T3 when the stimulation of OCN was low and, inversely, that the MMP-13 increase was low when T3 strongly stimulated the OCN expression. These findings suggest an interrelationship between OCN and MMP-13 expression. In fact, we observed that externally added OCN attenuated the T3 induced MMP-13 expression dose dependently and, furthermore, increased the 1,25D3 stimulated MMP-13 expression. Using a protein kinase A inhibitor we were able to show that this inhibitor mimics the effect of OCN suggesting a PKA dependent pathway to be involved in this regulatory process. We therefore hypothesize that OCN is a modulator of the hormonally regulated MMP-13 expression.

Key words: Thyroid hormones, Osteoblasts, Osteocalcin, MMP-13, Gene expression

RECENTLY, it was recognized that osteocalcin (OCN) plays a key role in the cross talk between energy and bone metabolism [1] which both are tightly regulated by hormones such as thyroid hormones (T3, T4) and calcitriol (1,25D3) [2]. Moreover, these hormones, members of the thyroid/steroid nuclear receptor family [3], regulate OCN expression. However, this regulation depends strictly on species as well as differentiation status of the osteoblasts. OCN expression is up regulated by 1,25D3 in human [4] and rat [5] but not in mouse osteoblasts [6,7]. By contrast, T3 - induced OCN-expression is specific for murine osteoblasts [7,8]. 1,25D3, however, attenuates the T3 stimulated expression dose dependently in mouse osteoblasts but shows a synergistic effect on the T3 regulation in rat osteosarcoma cells [9]. In human osteoblasts, T3 attenuates the 1,25D3-induced transcription of OCN [10,11]. Clinically, OCN, a marker protein of the mature osteoblasts, is routinely used as a serum marker for bone formation [12], as well as for bone turnover

[13] and gains an increasing importance as a marker for malignancies such as prostate carcinoma [14,15] and leukemia [16].

Recently, it was clearly demonstrated that the uncarboxylated form of osteocalcin functions as a hormone regulating glucose metabolism and fat mass in mice [1]. It affects pancreatic ß-cell proliferation by regulating Cyclin D1 and Cdk4 expression. Moreover, OCN increases the expression of insulin as well as genes regulating adipocyte development and differentiation [17].

Like OCN, MMP-13 (collagenase-3) has a double role in cancer [18] and bone metabolism. MMP-13 efficiently degrades collagen type II most as well as collagens type I, III, and X, which are the major components of cartilage and bone [19]. Although not only expressed in bone tissue [20], MMP-13 has a critical role during growth plate development and maturation and mice lacking MMP-13 have profound deficiencies in the morphological transformations preceding endochondral bone formation [21,22].

MMP13-expression depends on the exposure to a variety of systemic and local factors including hormones like 1,25D3 [23,24] and triiodothyronine (T3) [23,25]. We recently compared the T3 and 1,25D3 regulated expression of MMP-13 in cell lines with different differentiation status at various conditions by northern hybridization, and found that MMP-13 mRNA expression is inversely proportional to OCN mRNA expression [23]. Those observations suggest a regulatory interaction between hormones, OCN and MMP-13.

In this study, the regulatory interaction of OCN, T3 and 1,25D3 on MMP-13 expression was investigated. We show that OCN stimulates the 1,25D3-induced and attenuates the T3-stimulated MMP-13 expression in MC3T3-E1 mouse osteoblastic cells probably by a cAMP mediated pathway. From our data we suggest that OCN in combination with steroid hormones influences bone and growth plate development.

Materials and Methods

Cell culture

Murine MC3T3-E1 cells (referred as original MC3T3-E1 cells, kindly donated by Dr. Kumegawa, Meikai University, Dept. of Oral Anatomy, Sakado, Japan) were cultured in αMEM and MC3T3-E1 clone 4 or clone 30 (referred as clone 4 or clone 30, kindly donated by Dr. Franceschi, University of Michigan, Dept. of Periodontics, USA) were cultured in DMEM (Biochrom AG, Berlin, Germany) supplemented with 4.5 g/l glucose, 5% FCS (Sigma) and 30 µg/ml Gentamycin (Sigma) at 37°C under 5% CO2 in humidified air. They were sub-cultured twice a week using 0.001% pronase E (Roche) and 0.02% EDTA in Ca^{2+} and Mg^{2+} free phosphate-buffered saline (PBS). To prevent a potential phenotypic drift during repeated subcultures the cells were not used for more than four weeks after thawing.

Treatment with antisense oligonucelotides

Original MC3T3-E1 cells were cultured for 6 days in αMEM. They were thereafter treated with either 0.1 µg/ml or 0.2 µg/ml phosphorothioate (PTO) modified oligonucleotides (5´-GCAGAGAGAGGGTCCTCAT-3´) or 100 nM T3, or both, for 48 hours. For these experiments we used the original MC3T3-E1 cell line because these cells more efficiently take up foreign DNA.

RNA-isolation and northernblot hybridization

Because of the strong MMP-13 and weak OCN regulation by T3 (Fig. 1), for northernblot analysis, MC3T3-E1 cells clone 30 were seeded at a density of about 20,000 cells/cm^2 and cultured in the medium described above. After 4 days of culture a medium

change was performed and 48 hours later cultures were continued for 48 hours either untreated or treated with 10^{-7} M T3 (Sigma) or 1,25D3 (kindly provided by Roche) at the indicated concentrations or in combination of both. Treatment with bovine OCN (Calbiochem) was performed for the same culture time as the hormonal treatment at the indicated concentrations. RNA was isolated using either a mini-prep method [26] or Trizol reagent (Sigma) according to the supplier's suggestions. The total amount of RNA was determined by measuring the absorption at 260 nm with a spectrophotometer (Hitachi). Northern hybridization was performed by fractionating 10 µg total RNA on a 1% agarose gel containing 2.2 M formaldehyde. After electrophoresis the gel was transferred to a nylon filter (NEN, Brussels) with 20xSSC (1xSSC is 0.15 M NaCl and 0.015 M sodium citrate) and baked for 2 hours at 80°C. Prehybridization was performed one hour in 10% sodium-dodecylsulfate, 1 M sodium-phosphate buffer pH 7.4. For hybridization the buffer was changed to a new one with the same compounds and the filters were incubated over night with the labeled probe. The amount of hybridized RNA was determined by exposing and evaluating the filters in an InstantImager (Packard Instruments Company, Meriden, CT, USA). As hybridization probe we used the mouse MMP-13 cDNA ([27], kindly provided by Yves Eeckhout, Université Catholique de Louvain, Faculté de médecine, Unité de biologie cellulaire, B-1200 Bruxelles, Belgium) and the mouse OCN cDNA ([28], kindly provided by Dr. Busslinger, IMP, Vienna, Austria). For control we hybridized the same northern blots using the EcoR I fragment of the human ribosomal 28S cDNA. The probes were labeled by multi-prime labeling according to the supplier's suggestions (Roche).

Expression analysis by quantitative reverse transcription polymerase chain reaction (QRT-PCR)

mRNA was extracted using a mRNA Isolation Kit (Roche) and cDNA was synthesized from the mRNA using the 1st Strand cDNA Synthesis Kit (Roche). The obtained cDNA was subjected to PCR amplification with a real time cycler using SYBR-Green reaction mix (Roche). The MMP-13 forward primer was 5'-CAT TCA GCT ATC CTG GCC ACC TTC-3' and the reverse primer was 5'-CAA GTT TGC CAG TCA CCT CTA AG C-3'. Glyceraldehydephosphate-dehydrogenase (GAPDH) with the forward primer 5'- CTG CAC CAC CAA CTG CTT AGC C -3' and the reverse primer 5'- GTC CAC CAC CCT GTT GC GTA G -3' was used as a housekeeping gene for normalization. All PCR´s were performed as triplicate.

After 10 minutes of initial denaturation at 95°C the PCR was performed with 60 cycles: 10 seconds denaturation at 95°C; 30 seconds annealing and extension at 60°C. Quantification was achieved using the 2^ [-δδC (T)] method [29].

Statistical analyses

Statistical analyses were performed by ANOVA (Scheffe's post hoc test) or student's t-test using StatView 4.5 (Abacus Concepts Inc., Berkley, CA, USA). A $P \leq 0.05$ was considered to be significant and the data are presented as mean ± standard deviation (mean ± S.D.). All experiments were repeated at least twice.

Results

Vitamin D3 selectively stimulates MMP-13 but not OCN in osteoblast-like cells with different stages of differentiation

Based on preliminary data [23] we studied regulation of MMP-13 by 1,25D3 as well as T3. We compared the regulation in osteoblast-like cells with different differentiation status, namely MC3T3-E1 clone 4 and 30 [30]. This well characterized cell lines when

cultured with ascorbic acid show either a pre-osteoblastic phenotype (clone 30), with low expression of OCN, PTH-receptor and no mineralization, or a phenotype of a mature osteoblast (clone 4) with high OCN and PTH-receptor expression and the potential to mineralize in vitro as well as in vivo after transplantation into animals [30]. In these cell lines 1,25D3 strongly increased the mRNA levels of MMP-13 while it completely inhibited OCN expression (Fig. 1). A different behavior was observed for the T3 regulation. In the case of clone 30 there was a significant increase in the mRNA expression of MMP-13 unlike in clone 4, where T3 increased it weakly. Comparing these expression patterns in both cell lines, it could be observed that MMP-13 mRNA levels were inversely proportional to those of OCN (Fig. 1). Being aware that the expression pattern could depend on the differentiation status of the cells, an alternative explanation for this result could be that secreted OCN influences the expression of MMP-13.

Inhibition of OCN-expression by antisense oligonucleotides stimulates T3-induced expression of MMP-13

Antisense oligonucleotides against OCN mRNA, which are commonly used to inhibit protein expression, should eradicate any influence of this protein on MMP-13 expression. Figure 2 shows that antisense oligonucleotides against OCN mRNA dose dependently increased the T3 regulated expression of MMP-13, supporting the assumption that OCN expression attenuates MMP-13 transcription.

Addition of OCN attenuates MMP-13 gene expression

MC3T3-E1 cells of clone 30 in which T3 stimulated MMP-13 expression was found to be strong, were cultured in αMEM with 5% BSA, and treated with either 100 nM T3 or bovine OCN, or both. The OCN concentrations used were chosen in the concentration range which we had previously determined when MC3T3-E1 cells have been treated with T3 [8]. Comparative analysis of MMP-13 expression to untreated cultures is shown in figure 3: T3 increased the MMP-13 expression about five-fold. At a concentration of 30 nM, bovine OCN significantly ($P \leq 0.05$) attenuated the mRNA level of MMP-13 to about 2/3 of the T3 induced level. This attenuation depended strictly on the dose of OCN as shown in figure 4.

Supplementation of OCN increases 1,25D3-stimulated MMP13-stimulation

Based on the known interaction of both hormones on bone metabolism, we examined whether OCN has an influence on 1,25D3 regulated MMP-13 expression as well. Adding OCN to 1,25D3 treated cultures, the opposite behavior to T3 was observed: 1,25D3 significantly increased MMP-13 expression (Fig. 5, 2-fold, $P \leq 0.05$). 30 nM OCN, having no influence on basal MMP-13 mRNA expression, significantly enhanced the 1,25D3 stimulated MMP-13 expression (Fig. 5, 1.5-fold, $P \leq 0.01$).

PKA-inhibitor HA1004 attenuates T-stimulated and enhances 1,25D3-stimulated MMP13-expression

These experiments were based on data indicating that a possible OCN receptor might act through changing the intracellular cAMP concentration that antagonizes the activity of PTH, which is known to, at least partially, increase intra cellular cAMP levels [31]. HA1004, a substance that preferable inhibits protein kinase A and therefore mimics the effect of low cAMP, had no influence on basal expression (Figs. 6 and 7). T3 and 1,25D3 both stimulated MMP-13 expression, HA1004 however, attenuated T3- and increased 1,25D3-stimulated MMP-13 expression as expected when protein kinase A is involved in the modulating activity of OCN (Figs. 6 and 7).

Discussion

Our experiments show that OCN modulates MMP-13 expression induced by the calciotropic hormones T3 and 1,25D3. The present data are in agreement with recently published findings that OCN acts, possibly via receptor, on pancreatic ß-cells in regulating Insulin, Adiponectin, and CyclinD1 [1] and on osteoblasts, increasing proton secretion after treatment with bovine OCN [31]. Our results support on the one hand the function of OCN as an endocrine factor and extend on the other hand the general effect on proton secretion to a more functional level, namely to a modulatory effect on the hormonal regulation of MMP-13 expression, which acts as a central proteinase in bone metabolism. Initial evidence for OCN's involvement in the regulation of MMP-13 emerged when the regulation pattern of both proteins by T3 and 1,25D3 was compared. In osteoblastic cells with a preosteoblastic phenotype like MC3T3-E1 clone 30 [30], T3 regulated expression of MMP-13 was high, while that of OCN was low. In the MC3T3-E1 cell line clone 4, a cell line with a more mature osteoblastic phenotype, which is able to mineralize in vitro and in vivo, T3 could induce OCN expression very strongly while MMP-13 was virtually undetectable. Recently, it was shown that in osteoblasts, the expression of MMP-13 and of OCN are mutually exclusive [32-34] suggesting that there exist osteoblasts with different gene expression patterns and functions [23]. The finding that these proteins are expressed successively during the developmental process suggests that OCN could advance the differentiation of osteoblasts to the next differentiation status by down regulating genes of the previous phenotype. This process could be limited to certain tissue areas rather than throughout the whole skeletal system.

T3 has multiple effects on bone metabolism [2,35]. Lack of this hormone or of its receptor results in severe developmental disturbances [36] but hyperthyroidism is known to increase bone cell functions and cause osteoporosis. At the cellular level, this hormone regulates many genes and their functions during the osteoblastic differentiation process like alkaline phosphatase and its activity [8,37] and OCN [7,9], and it additionally enhances the functional activity of differentiated osteoblasts [38]. The accelerated remodeling process encountered in hyperthyroidism could easily be explained by the fact that increased MMP-13 is synthesized thus enhancing the initiation of resorption. Increased OCN, however, could accelerate the differentiation process of osteoblasts and both could be responsible for an increased bone turnover.

The recent finding that OCN acts as an endocrine factor and regulates metabolic activity of the ß-cells [1,17] does not exclude that this protein besides its systemic function also acts locally. In addition to the recently reported endocrine function of OCN, our results indicate the possibility that it operates both autocrine and paracrine. Furthermore, the involvement of OCN in energy metabolism provides some evidence for a recent exciting finding: different to T3 in mouse, in human and rat 1,25D3 upregulates OCN expression in osteoblasts [7]. There are two important physiological processes, which are different in humans and rats on the one side and mice on the other side, namely a prolonged life span and the ability to develop symptoms of ageing and age-associated obesity in the elderly. It is common knowledge that thyroid hormones known as "juvenile hormones", especially with their teammate prolactin, are important for the early development of an organism. A similar regulatory system had already been developed earlier in evolution as known from organisms like D. melanogaster [39]. 1,25D3, however, is important for the maintenance of many functions within the elderly organism, including skeletal, muscle, neuronal systems and fat metabolism. This was clearly demonstrated by thyroid hormone and vitamin-D receptor depleted mice: while the thyroid hormone receptor knock out mouse had severe developmental distortions [36], the phenotype of the vitamin-D receptor knock-mouse could be rescued by a high calcium diet [40]. The

relation between obesity and low serum levels of vitamin-D is well documented [41,42] and the new finding of OCN as a regulator of this processes seems promising.

In this study, addition of antisense oligonucleotides against OCN increased both basal as well as T3 regulated MMP-13 expression. Although, we are aware that treatment with antisense_oligonucleotides does not necessarily down regulate protein synthesis, this experiment supports the findings with externally added OCN. The concentration of OCN used for the experiments was comparable to the concentrations found in previously published experiments of T3 treated MC3T3-E1 cells [8] and 1,25D3 completely down regulated transcription and synthesis of OCN [7,43]. Therefore, experiments with 1,25D3 and antisense oligonucleotides to OCN were not promising. The stimulatory effect of OCN on the 1,25D3-regulated MMP-13 expression was about two-fold and the inhibitory effect on T3 regulated expression about one third. The regulatory potential of OCN on ß-cells was in the same order of magnitude [1], especially when we take into consideration that the used OCN in our experiments was bovine OCN that is partially carboxylated. At the tissue level in vivo, OCN is integrated into the extracellular matrix, possibly adsorbed to hydroxylapatite, where OCN also acts as a regulator of the size of the mineral crystals [44]. One might speculate that OCN signals to osteoblasts binding to this bone matrix to differentiate and this results in down regulation of MMP-13 expression preventing initiation of bone resorption. 1,25D3 or other hormones like PTH could activate the expression of MMP-13 locally to start a new resorption cycle. Especially, PTH is an excellent candidate to antagonize the action of OCN, because this hormone is known to increase intracellular cAMP [45] and regulates MMP-13 [46]. Using the microphysiometer to measure the proton transport out of osteoblasts after OCN stimulation, a significant decrease of intracellular cAMP was found and from that an effect of the putative OCN-receptor on the adenylate cyclase was postulated [31]. This is in line with our results using protein kinase A inhibitors, which increased 1,25D3 stimulated MMP-13 expression as OCN did, although, we are aware that this is only an indirect evidence for the involvement of that pathway. Differences in non-genomic actions of both hormones could also be responsible for their different modulatory activiy on MMP-13 expression [47].

Thus, our results provide an additional confirmation to previous findings that OCN influences the differentiation status of osteoblasts. Furthermore, our results attribute paracrine or autocrine functions of OCN to recent findings where this protein was suggested to act as an endocrine factor in regulating the metabolism of ß-cells. In conclusion, our study clearly demonstrates that OCN is a modulator of hormone regulated osteoblastic gene expression.

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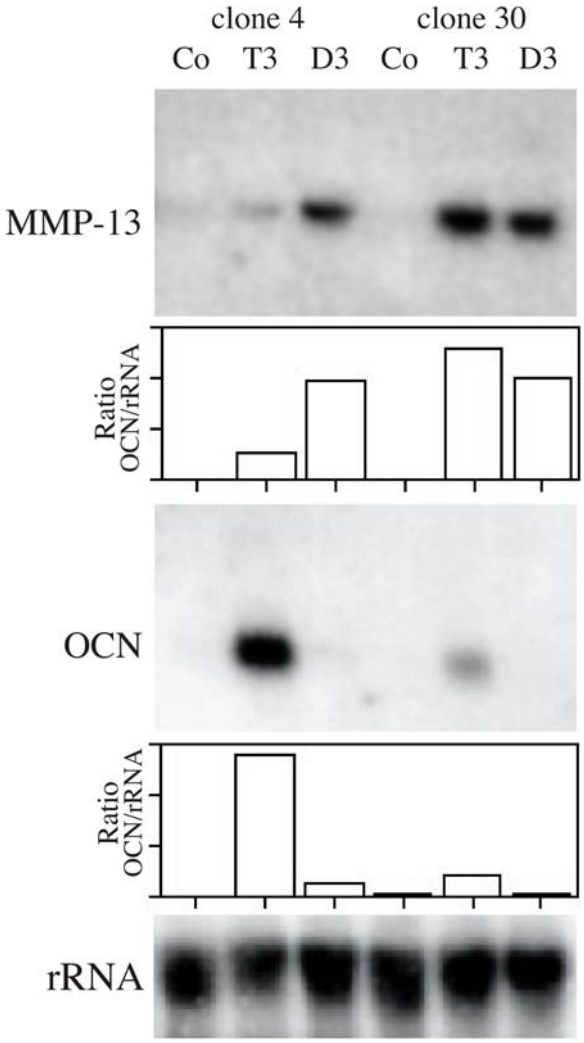

Fig. 1. The T3 stimulated expression of MMP-13 and OCN was inversely related in osteoblast-like cells with different differentiation status. MC3T3-E1 cells clone 4 and clone 30 were cultured for 6 days and treated without or with 100 nM T3 or 10 nM 1,25D3. The MC3T3-E1 clone 30 consists mainly of preosteoblastic cells. In these cells T3 only marginally induced OCN expression. T3 and 1,25D3, however, equally induced MMP-13 mRNA expression. The MC3T3-E1 clone 4 consists of differentiated osteoblasts. In these cells T3 strongly stimulated the OCN expression. In these cultures T3 only marginally induced MMP-13 while 1,25D3 induced MMP-13 expression as strong as in clone 30. Shown are northern blots hybridized with the mouse MMP-13 cDNA probe. For normalization of loading a 28S rDNA probe was used. The pictures represent the printouts of the counted radioactivity measured with an InstantImager. The bar charts below the northern blot printouts illustrate the amount of OCN or MMP-13 mRNA normalized to 18S rRNA, respectively.

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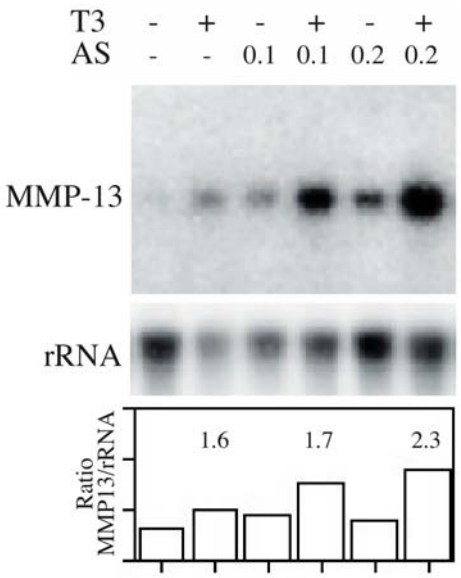

Fig. 2. Antisense oligonucleotides against OCN mRNA increased T3 stimulated MMP-13 expression in MC3T3-E1 cells. Original MC3T3-E1 cells were cultured for 6 days in αMEM and treated either 0.1 μg/ml or 0.2 μg/ml PTO modified antisense oligonucleotides against OCN mRNA or 100 nM T3, or both, for 48 hours. The antisense oligonucleotides itself only marginally increased MMP-13 expression but they strongly increased T3 stimulated MMP-13 expression. This indicates that blocking OCN translation by antisense oligonucleotides increases T3 stimulated MMP-13 expression. Shown are northern blots hybridized with the mouse MMP-13 cDNA probe. For normalization a 28S rDNA probe was used. The pictures represent the printouts of the counted radioactivity measured with an InstantImager. The bar chart below the northern blot printouts illustrates the amount of MMP-13 mRNA normalized to 18S rRNA, respectively. The numbers above the bars indicate the increase by the T3 treatments.

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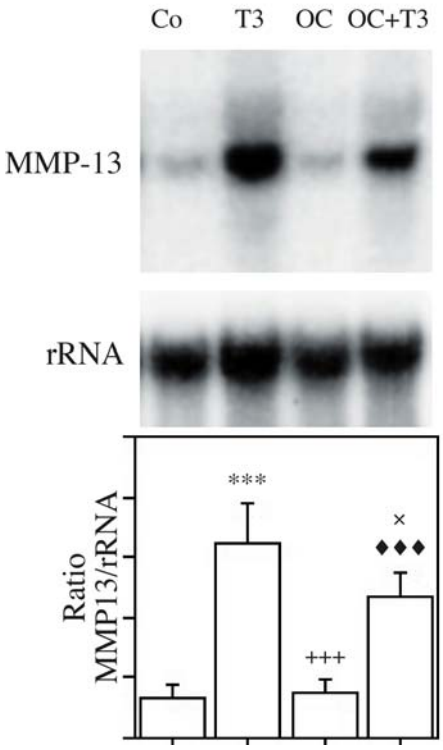

Fig. 3. Bovine OCN attenuated T3 stimulated MMP-13 expression in MC3T3-E1 cells. MC3T3-E1 cells clone 30 were cultured for 4 days in αMEM and treated without (Co) or with 100 nM T3 or 30 nM bovine OCN (OC) or with both T3 and OCN (OC+T3) for 48 hours. T3 increased MMP-13 expression that was attenuated by OCN. This protein alone had no influence on MMP-13 expression. Shown are northern blots hybridized with the mouse MMP-13 cDNA probe. For normalization a 28S rDNA probe was used. The pictures represent the printouts of the counted radioactivity measured with an InstantImager. The bars represent the mean ±SD of the MMP-13 mRNA signals normalized to the 28S rRNA signals. (n = 3; ANOVA: x, $P \leq 0.05$ T3 vs. OC+T3; ***, +++, ♦♦♦, $P \leq 0.001$, Co vs. T3, T3 vs. OC, OC vs. OC+T3).

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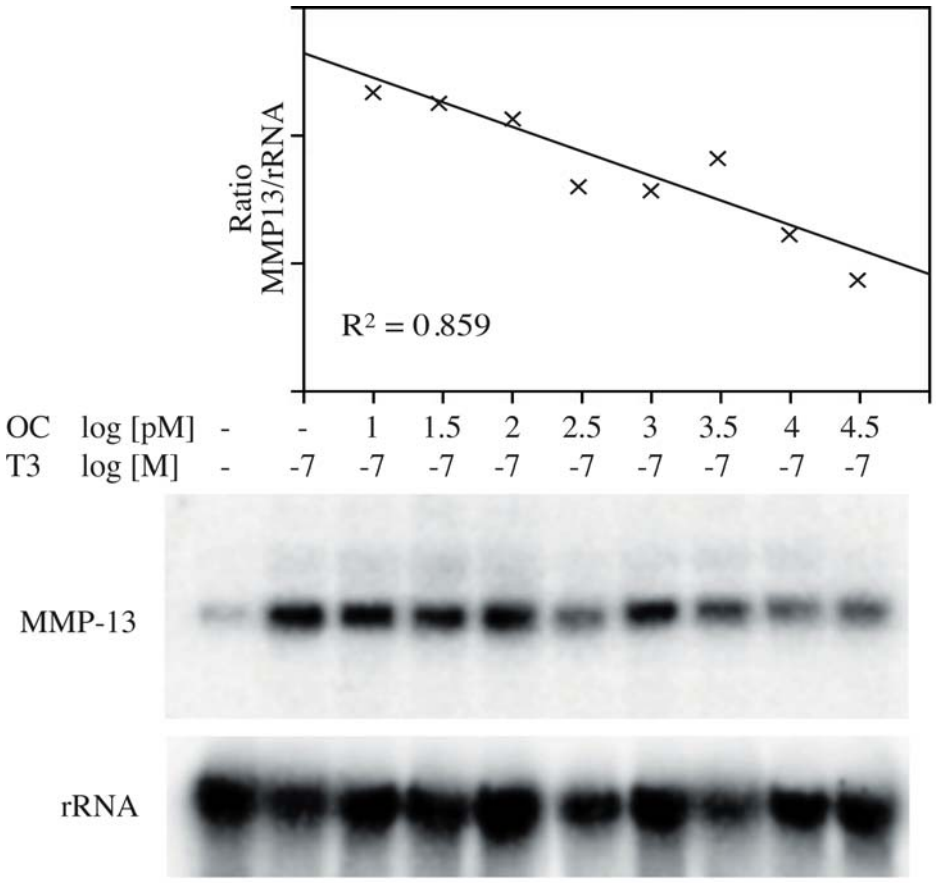

Fig. 4. OCN attenuated T3 stimulated MMP-13 expression in MC3T3-E1 cells dose dependently. MC3T3-E1 cells clone 30 were cultured for 4 days αMEM and treated without or with 100 nM T3 and increasing concentrations of bovine OCN for 48 hours. The concentrations of OCN are given in log [pM] corresponding to a range from 0.01 to 30 nM. T3 significantly stimulated the MMP-13 mRNA expression, which effect was attenuated by increasing amounts of OCN. The dose dependent inhibition suggests that OCN acts through a specific receptor. Shown are northern blots hybridized with a mouse MMP-13 cDNA probe. For normalization a 28S rDNA probe was used. The pictures represent printouts of the counted radioactivity measured with an InstantImager. The graph shows the linear regression of the logarithms of the OCN concentrations vs. the radioactivity reflecting the normalized MMP-13 cDNA expression.

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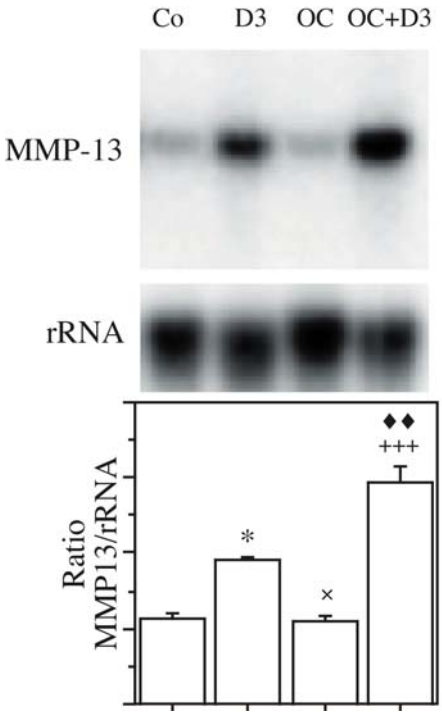

Fig. 5. Bovine OCN increased 1,25D3 stimulated MMP-13 expression in MC3T3-E1 cells. MC3T3-E1 cells clone 30 were cultured for 4 days in αMEM and treated without (Co) or with 10 nM 1,25D3 (D3) or 30 nM bovine OCN (OC) or with both 1,25D3 + OCN (OC+D3) for 48 hours. 1,25D3 significantly increased MMP-13 expression. 30 nM OCN, having no influence on basal MMP-13 mRNA expression, significantly increased the 1,25D3 stimulated MMP-13 expression. Shown is one of three northern blots hybridized with the mouse MMP-13 cDNA probe. For normalization a 28S rDNA probe was used. The pictures represent the printouts of the counted radioactivity measured with an InstantImager. The bars represent the mean ±SD of the MMP-13 mRNA signals normalized to the 28S rRNA signals. (n = 3; ANOVA: *, x, $P \leq 0.05$ Co vs. D3, D3 vs OC; +++, $P \leq 0.001$ Co vs. OC+D3; ♦♦, $P \leq 0.01$ D3 vs. OC+D3. Co vs. OC was not significant different.

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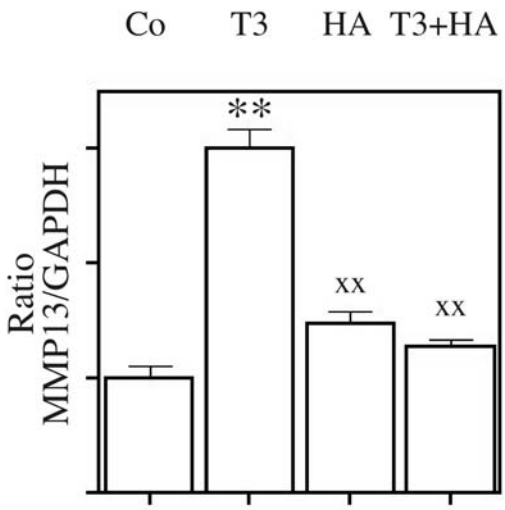

Fig. 6. The PKA inhibitor HA1004 resembles a possible PKA dependent action of OCN on the T3 regulated MMP-13 expression. MC3T3-E1 cells clone 30 were cultured for 4 days in αMEM and treated without (Co) or with 100 nM T3 or HA1004 (HA) or with both T3 + HA for 48 hours. T3 significantly increased MMP-13 expression. HA, having no influence on basal MMP-13 mRNA expression, significantly decreased the T3 stimulated MMP-13 expression. mRNA was isolated and gene expression was analyzed by QRT-PCR as triplicates. Expression of MMP-13 was normalized to GAPDH. Bars represent means ± SD; **, xx, $P \leq 0.01$; n = 3.

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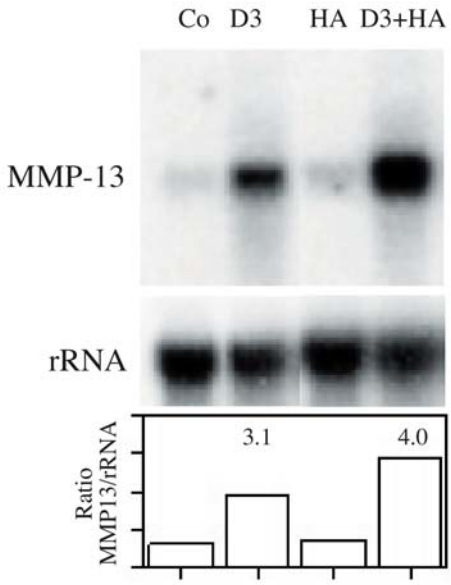

Fig. 7. The PKA inhibitor HA1004 resembles a possible PKA dependent action of OCN on the 1,25D3 regulated MMP-13 expression. MC3T3-E1 cells clone 30 were cultured for 4 days in αMEM and treated without (Co) or with 10 nM 1,25D3 (D3) or HA1004 (HA) or with both 1,25D3 + HA for 48 hours. 1,25D3 significantly increased MMP-13 expression. HA, having no influence on basal MMP-13 mRNA expression, significantly increased the 1,25D3 stimulated MMP-13 expression. Shown are northern blots hybridized with the mouse MMP-13 cDNA probe. For normalization a 28S rDNA probe was used. The pictures represent the printouts of the counted radioactivity measured with an InstantImager. The bar chart below the northern blot printouts illustrates the amount of MMP-13 mRNA normalized to 18S rRNA, respectively. The numbers above the bars indicate the increase by the 1,25D3 treatments.