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## The essential role of glucocorticoids for proper human osteoblast differentiation and matrix mineralization

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### Abstract

Glucocorticoids (GCs) exert profound effects on bone and are essential for human osteoblast differentiation. However, GCs are still interpreted as negative regulators of bone formation, mainly caused by the detrimental effects on bone after clinical use of GCs. In this paper we emphasize the importance of GCs for proper human osteoblast differentiation and matrix mineralization. We show that human osteoblast differentiation needs to be triggered by GCs in a specific time-window during the early stages of development. Exposure to GCs in the beginning of osteoblast development induces a dose dependent increase in alkaline phosphatase activity and matrix mineralization. GC-induced differentiation stimulated expression of genes involved in bone formation and suppressed genes that negatively regulate bone formation and mineralization. Furthermore we highlight the importance of local cortisol activation in osteoblasts by expression of 11 $\beta$ -hydroxysteroid dehydrogenase 1 (11 $\beta$ -HSD1).

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### 1. Introduction

Osteoblast differentiation is a complex process regulated by many endocrine, paracrine and autocrine factors. In human and rat bone marrow stromal cells GCs are crucial for the induction of osteoblast differentiation and formation of a mineralized extracellular matrix (Bellows et al., 1987; Cheng et al., 1996; Herbertson and Aubin, 1995; Iba et al., 1995; Jorgensen et al., 2004; Maniopoulos et al., 1988). Although the exact role of GCs in regulating bone formation is unclear, it might be dependent on the duration and concentration of GC treatment and on the differentiation stage of both osteoblasts and osteoclasts (Hirayama et al., 2002; Ishida and Heersche, 1998; Pockwinse et al., 1995; Smith et al., 2000). At a molecular level GC signaling is mediated via the GC receptor (GR $\alpha$ ). GR $\alpha$  is expressed in almost all cell-types including osteoblasts (Liesegang et al., 1994) where it regulates gene expression by binding to GC responsive elements in the regulatory regions of several target genes, including osteocalcin, collagenI $\alpha$ 1 and transforming growth factor- $\beta$ 1 (Heinrichs et al., 1993; Parrelli et al., 1998; Peterkofsky et al., 1999). Despite clear stimulation of bone for-

mation by GCs in vitro, GCs are still interpreted as negative regulators of bone formation. This idea is mainly caused by the clinical use of GCs. GC therapy frequently results in bone loss and increased risk for fractures (Canalis, 1996; Clowes et al., 2001; Weinstein, 2001). This negative effect on bone is caused by high levels of GCs for longer periods and might not reflect the normal in vivo role of GCs in bone. Furthermore, most studies on osteoblast differentiation are performed using mouse osteoblasts that lack the need for GC treatment for the induction of differentiation (Ecarot-Charrier et al., 1983; Sudo et al., 1983), which is in contrast to the human situation. Therefore we emphasize in this paper the importance of GCs for proper human osteoblast differentiation and matrix mineralization. Moreover we want to highlight the significance of pre-receptor regulation of GCs in osteoblasts by expression of 11 $\beta$ -hydroxysteroid dehydrogenase 1 (11 $\beta$ -HSD1). 11 $\beta$ -HSD1 is found in almost all GC target tissues including osteoblasts (Bland et al., 1999; Cooper et al., 2000) and primarily displays reductase activity converting relative high levels of free cortisone into the biologically active cortisol. As a consequence 11 $\beta$ -HSD1 activity in osteoblasts provides an efficient mechanism for the local activation of GCs in bone and is an important autocrine determinant of osteoblast proliferation, differentiation and function (Canalis and Delany, 2002a; Cooper et al., 1999; Eijken et al., 2005).

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## 2. Materials and methods

### 2.1. Cell culture

SV-HFO cells were cultured in  $\alpha$ MEM (GIBCO, Paisley, UK) supplemented with 20 mM HEPES, pH 7.5 (Sigma, St. Louis, MO); streptomycin/penicillin; 1.8 mM  $\text{CaCl}_2$  (Sigma); and heat-inactivated FCS (GIBCO) at 37 °C and 5%  $\text{CO}_2$  in a humidified atmosphere. Thawed cells were precultured for 1 week in the presence of 10% FCS. In this preculture, cells were seeded in a density of  $5 \times 10^3$  vital cells/cm<sup>2</sup> and were subcultured every week. During this preculture, SV-HFO cells remained in an undifferentiated stage. After preculturing, cells were seeded in a density of  $10 \times 10^3$  vital cells/cm<sup>2</sup> in the presence of 2% charcoal-treated FCS supplemented with 10 mM  $\beta$ -glycerophosphate (Sigma). Medium freshly supplemented with hormones was replaced every 2–3 days. Dexamethasone was purchased from Sigma and  $1\alpha,25\text{-(OH)}_2\text{D}_3$  was generously provided by Dr. L. Binderup (Leo Pharmaceuticals, Ballerup, Denmark). For analysis, medium was collected and stored at –20 °C and cells were scraped from the culture dish in PBS containing 0.1% Triton X-100 and stored at –80 °C. Cell lysates were sonicated on ice in a sonifier cell disrupter for  $2 \times 15$  s before analysis.

### 2.2. DNA content

For DNA measurements, 100  $\mu$ l SV-HFO cell lysates were treated with 200  $\mu$ l heparin (8 IU/ml in PBS) and 100  $\mu$ l ribonuclease A (50  $\mu$ g/ml in PBS) for 30 min at 37 °C. This was followed by adding 100  $\mu$ l ethidium bromide solution (25  $\mu$ g/ml in PBS). Samples were analyzed on the Wallac 1420 victor2 (Perkin-Elmer, Wellesley, MA) using an extinction filter of 340 nm and an emission filter of 590 nm. For standards, calf thymus DNA (Sigma) was used.

### 2.3. Alkaline phosphatase (ALP) activity

ALP activity was assayed by determining the release of paranitrophenol from paranitrophenylphosphate (20 mM in 1 M diethanolamine buffer supplemented with 1 mM  $\text{MgCl}_2$  at pH 9.8) in the SV-HFO cell lysates for 10 min at 37 °C. The reaction was stopped by adding 0.06 M NaOH. Absorption was measured at 405 nm. Results were adjusted for DNA content of the cell lysates.

### 2.4. Mineralization

For quantification of the mineral content cell lysates were incubated overnight in 0.24 M HCl at 4 °C. Calcium content was colorimetrically determined with a calcium assay kit (Sigma) according to the manufacturer's description. Results were adjusted for DNA content of the cell lysates.

For Alizarin Red S staining cell cultures were fixed for 60 min with 70% ethanol on ice. After fixation, cells were washed twice with PBS and stained for 10 min with Alizarin Red S solution (saturated Alizarin Red S in demineralized water adjusted to pH 4.2 using 0.5% ammonium hydroxide).

### 2.5. Apoptosis assay

Apoptosis was measured through the binding of annexin V and uptake of propidium iodide (PI) by flow cytometry using a Apoptest-FITC kit (Nexins research, Kattendijke, The Netherlands). For analysis 10,000 osteoblasts were counted using a FACScalibur (Becton Dickinson). Percentage total apoptotic cells was calculated by counting vital (unlabeled), apoptotic (annexin V stained), necrotic (PI stained) and late apoptotic cells (double stained).

### 2.6. Proliferation assay

Proliferation was examined by [<sup>3</sup>H]-thymidine uptake measurements. SV-HFO cells were seeded into 96-well plates, in differentiating (100 nM DEX) and non-differentiating conditions. After culture for 6 days [<sup>3</sup>H]-thymidine (Amersham, UK) was added to a concentration of 1  $\mu$ Ci/ml and incubated for 7 h. Cells were then harvested onto glass fibre mats (TomtecHarvester96; Hamden, CT) and radioactivity measured with a Wallac MicroBeta scintillation counter.

### 2.7. Osteocalcin production

Osteocalcin was determined in the cultures medium. Osteocalcin was assayed by radioimmunoassay by incubating overnight with <sup>125</sup>Iodide labeled human osteocalcin and anti-human osteocalcin, kindly provided by Dr. C.M. Gundberg, Department of Orthopaedics and Rehabilitation, School of Medicine, New Haven, Connecticut, USA. Results were adjusted for DNA content of the cell lysates.

### 2.8. 11 $\beta$ -HSD1 activity

Cortisone to cortisol conversion in SV-HFO cells was measured as described previously (Eijken et al., 2005).

### 2.9. Quantification of mRNA expression

Total RNA was isolated using RNA-Bee solution (Tel-Test, Friendwood, TX) according to the manufacturer's protocol. To remove calcium (derived from extracellular matrix), RNA was precipitated by overnight incubation with 4 M LiCl and 50 mM EDTA at –20 °C. After precipitation and centrifugation for 30 min at 14,000 rpm and 4 °C, the RNA pellet was washed four times with 70% EtOH and dissolved in H<sub>2</sub>O. The total amount of RNA was quantified using the RiboGreen RNA Quantitation Kit (Molecular Probes, Eugene, OR). One microgram total RNA was reverse transcribed into cDNA using a cDNA synthesis kit and according to the protocol of the manufacturer (MBI Fermentas, St. Leon-Rot, Germany), using 0.5  $\mu$ g oligo(dT)<sub>18</sub> and 0.2  $\mu$ g random hexamer primers. Quantitative real-time PCR was carried out using an ABI 7700 sequence detection system (Applied Biosystems, Foster City, CA). Reactions were performed in 25  $\mu$ l volumes using a qPCR core kit (Eurogentec, Seraing, Belgium). Reaction mixes contained 20 ng cDNA, 5 mM  $\text{MgCl}_2$ , 200  $\mu$ M dNTPs, and 0.025 U/ $\mu$ l Hot GoldStar enzyme. Primer and probe sets were designed, using the Primer Express software (version 1.5; Applied Biosystems), amplicons overlapped at least one exon boundary. Cycling conditions were 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min.

### 2.10. Statistics

Data presented are the results of at least two independent experiments performed in triplicate. Values are the means  $\pm$  S.E.M. Significance was calculated using the Student's *t*-test.

## 3. Results and discussion

### 3.1. The essential role of glucocorticoids for proper osteoblast function

To demonstrate the importance of GCs for human osteoblast differentiation in more detail we used the pre-osteoblast model SV-HFO (Chiba et al., 1993), which is a well-characterized osteoblast cell-line showing a controlled GC-induced differentiation process in a 3-week period. During this differentiation process an extracellular matrix (ECM) is formed and the process of mineralization is initiated around day 14 (Fig. 1A). Moreover, GC-induced differentiation leads to increased sensitivity to mechanical loading (Jansen et al., 2004). GC-induced differentiation was dose dependently increased by DEX as demonstrated by increased alkaline phosphatase (ALP) activity (Fig. 1B) and mineralization. Matrix mineralization was induced at a minimal dose of 10 nM (data not shown). When we used mesenchymal stem cells derived from bone marrow GC treatment was as important for the induction of osteogenesis and proper matrix formation/mineralization (data not shown), which is also

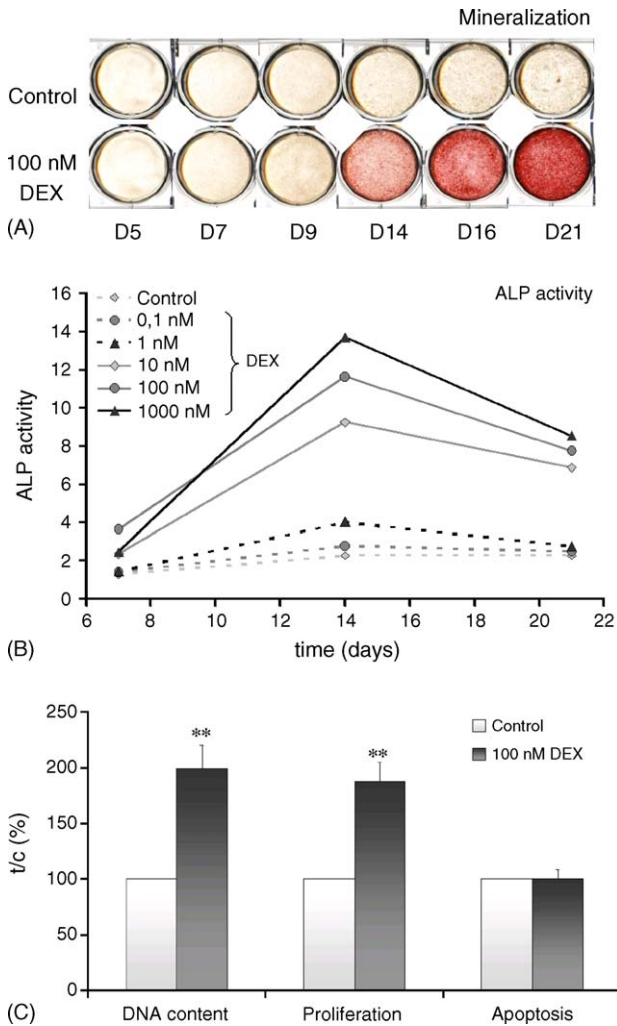


Fig. 1. (A) DEX-induced matrix mineralization by SV-HFO osteoblasts. Cells were cultured in the presence and absence of 100 nM DEX. Cultures were stained for calcium deposition using Alizarin Red S. (B) Dose dependent increase of ALP activity (nmol/μgDNA/min) by DEX at days 7, 14 and 21 of SV-HFO culture, data shown represent a typical SV-HFO culture. (C) Effects of DEX on cultures DNA content (day 14), proliferation (day 6) and apoptosis (day 14). Values are means ± S.E.M. \*\*  $p < 0.01$  compared to control.

demonstrated in several other studies (D’Ippolito et al., 1999; Diefenderfer et al., 2003).

Studies using human osteosarcoma cells and mouse osteoblasts show that GC treatment inhibits osteoblast cell num-

ber and proliferation (Chen, 2004; Engelbrecht et al., 2003; Pereira et al., 2001; Song, 1994). In contrast, other experiments show increased cell number and proliferation after GC treatment (Atmani et al., 2003; Bellows et al., 1987; Jorgensen et al., 2004), which is supported by our observation of increased proliferation and cell number after GC-induced differentiation in human osteoblasts. Furthermore the number of apoptotic cells was not changed. This is shown in Fig. 1C where we show increased cell number measured by the cultures DNA content, higher proliferation measured by thymidine incorporation and rate of apoptosis measured by annexin V staining.

### 3.2. Osteoblasts need to be directed by GCs at the early stages of development

Data clearly show that osteoblasts need to be directed by GCs to differentiate and mineralize. Moreover osteoblasts need to be triggered by GCs in a specific time-window during the early stages of development. This is demonstrated in Fig. 2 where we treated osteoblasts with DEX in different time periods followed by quantification of mineral deposition at day 21. Continuous DEX treatment resulted in strong mineralized cultures as shown in Fig. 1A and in the upper line in Fig. 2. Interestingly, shortening the treatment until days 16 and 12 also resulted in strongly mineralized cultures. When the treatment was further shortened (to day 9 or 7) matrix mineralization was decreased but still occurred. In contrast, cultures that were only treated with DEX starting at day 12 or later failed to initiate mineralization. In summary, osteoblasts are directed by GCs at the beginning of development to differentiate, which eventually leads to matrix mineralization. Once the cells have entered the osteoblast differentiation pathway the mineralization process seems to be independent of GCs.

### 3.3. GC-induced differentiation results in differential expression of genes involved in osteoblast differentiation and bone formation

Besides clear phenotypic characteristics like matrix mineralization, GC-induced differentiation regulates a wide variety of genes that control proper osteoblast differentiation and matrix mineralization. Here we show several examples of genes that are regulated after GC-induced differentiation. In these experiments we analyzed gene expression (mRNA) in the first, second and third week of culture in both differentiated (DEX treated) and

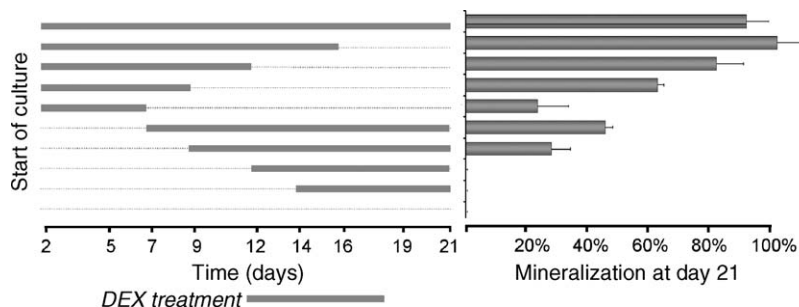


Fig. 2. Time dependent induction of mineralization by DEX. SV-HFO cultures were treated with DEX for different time periods indicated by the horizontal bold gray lines. Mineralization was measured at day 21.

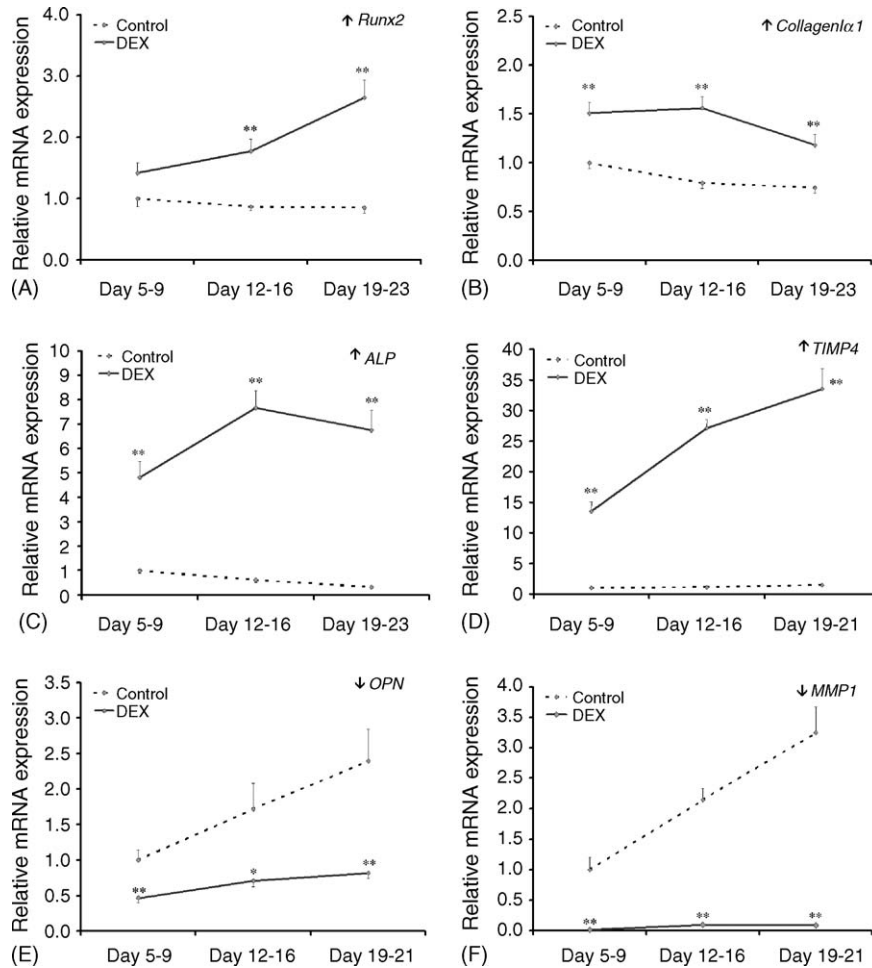


Fig. 3. Q-PCR data (mRNA expression) from non-differentiating (control) and differentiating (DEX treated) SV-HFO cultures at weeks 1–3. (A) Runx2/Cbfa1, (B) collagen $\alpha$ 1, (C) alkaline phosphatase, (D) TIMP4, (E) osteopontin and (F) MMP1. Values are means  $\pm$  S.E.M. \* $p$  < 0.05; \*\* $p$  < 0.01 compared to control.

non-differentiated cultures (non-treated control). GC-induced differentiation resulted in up-regulation of osteoblast markers like Runx2, collagen $\alpha$ 1 and ALP (Fig. 3A–C). In contrast, the non-collagenous ECM protein osteopontin (OPN) was decreased by GC-induced differentiation together with the collagenase MMP1 (matrix metalloproteinase-1), which showed a strong suppression by DEX (40-fold reduction) (Fig. 3E and F). MMPs are extracellular proteins involved in ECM degradation and tissue turnover (Pardo and Selman, 2005; Shapiro, 1998). MMPs in turn are modulated by tissue inhibitors of metalloproteinase (TIMPs). Interestingly, GC-induced differentiation resulted in a strong (30-fold) induction of TIMP4 (Fig. 3D). TIMPs are the major endogenous inhibitors of the MMPs and four TIMPs have been identified, although their specific inhibitory activity towards the MMPs are not specified (Nagase and Woessner, 1999; Rahkonen et al., 2002; Shapiro, 1998). Together, this indicates that during osteoblast differentiation and bone formation when matrix synthesis is needed, matrix degradation is suppressed by decreasing MMP1 expression and increasing TIMP4 expression.

A more complex regulation of gene expression by DEX is shown for osteocalcin (OCN). OCN is the most abundant non-collagenous protein in bone matrix, which gene contains a

Vitamin D responsive element (VDRE) (Morrison et al., 1989) resulting in increased levels of OCN mRNA and protein after 1,25-dihydroxyvitamin D (vitD) treatment (10 nM, Fig. 4). At basal unstimulated conditions DEX did not influence OCN expression, however, vitD-induced OCN expression was significantly reduced by DEX (Fig. 4) (day 14 of culture).

Both OCN and OPN are markers of osteoblast differentiation and OCN is used as a serum marker for bone turnover. Nevertheless these genes are down regulated by GC-induced differentiation. At first sight this seems to contradict a positive role of GCs in osteoblast differentiation. However, despite their use as markers of bone formation, the exact role of OPN and OCN in the bone matrix is not fully understood. In contrast, they seem to act as negative regulators of the mineralization process. OPN and OCN-deficient mice show increased amounts of mineral in their bones (Boskey et al., 1998, 2002; Ducy et al., 1996) and in vitro mineralization studies show that they directly inhibit hydroxylapatite crystal formation (Boskey et al., 1993; Hunter et al., 1994, 1996).

In summary during GC-induced osteoblast differentiation genes are either up-regulated or down-regulated to stimulate osteoblast differentiation and proper matrix development. The genes discussed here are only a small set of the genes that account

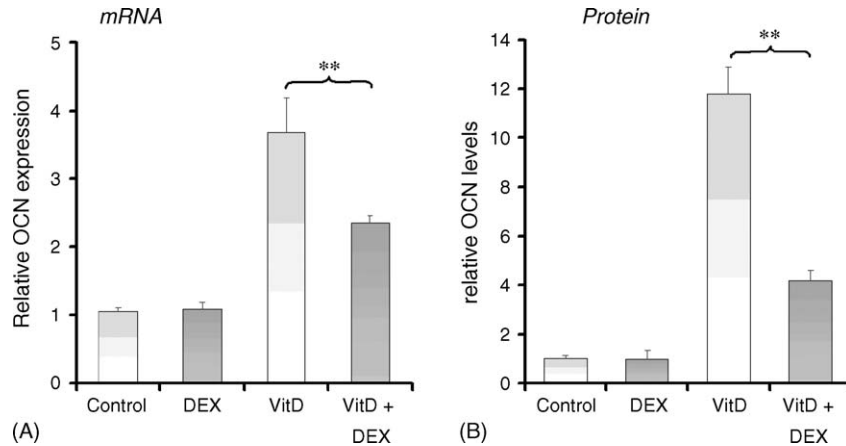


Fig. 4. DEX inhibition of vitD-induced osteocalcin (OCN) expression. (A) OCN mRNA expression after DEX treatment in basal and vitD stimulated cultures (day 14). (B) OCN protein levels in the cultures supernatant after DEX treatment in basal and vitD stimulated cultures (day 14). Values are means  $\pm$  S.E.M. \*\*  $p < 0.01$  compared to vitD stimulated cultures.

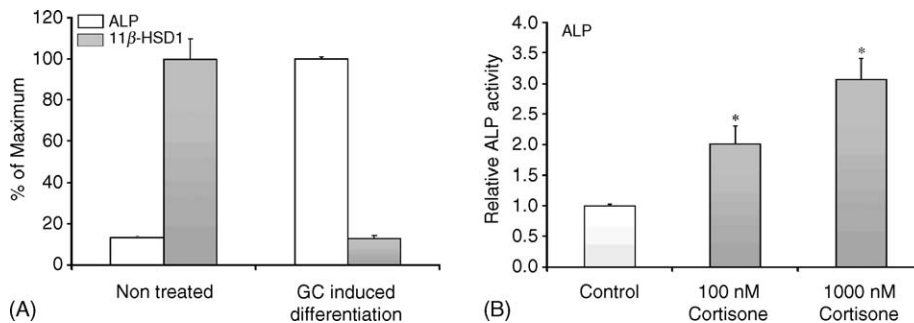


Fig. 5. Significance of 11 $\beta$ -HSD1 expression in human osteoblasts. (A) ALP activity (nmol/ $\mu$ gDNA/min) and 11 $\beta$ -HSD1 activity (cortisone to cortisol conversion per  $\mu$ gDNA) in non-differentiating and differentiating osteoblasts at day 21 of culture. (B) SV-HFO cultures continuously incubated with 100 and 1000 nM cortisone after which ALP activity was measured at day 30 of culture. Values are means  $\pm$  S.E.M. \*\*  $p < 0.01$  compared to control.

for osteoblast differentiation and matrix mineralization. In the literature also other genes are described that are regulated by DEX treatment in osteoblasts like, TGF $\beta$ 1, bone sialoprotein, osterix and others (Igarashi et al., 2004; Oursler et al., 1993). GC-induced osteoblast differentiation probably results in differential expression of a large set of genes influencing pathways and processes involved in osteoblast differentiation and bone formation.

#### 3.4. Osteoblasts produce cortisol via 11 $\beta$ -HSD1 expression

The importance of GCs for proper osteoblast differentiation and bone forming function is emphasized by osteoblastic expression of 11 $\beta$ -HSD1. Expression of 11 $\beta$ -HSD1 in osteoblasts provides an efficient mechanism for the local activation of GCs in bone. In a previous study we showed that 11 $\beta$ -HSD1 is regulated in a differentiation dependent manner in osteoblasts (Eijken et al., 2005). This differentiation dependent regulation is summarized in Fig. 5A, which shows that non-differentiated osteoblasts have low ALP activity but have strong increased levels of 11 $\beta$ -HSD1 to compensate for the lack of cortisol. Their differentiating counterparts have high ALP activity but low 11 $\beta$ -HSD1. Fig. 5B summarizes our previous results demonstrating the significance of 11 $\beta$ -HSD1 for osteoblast differentiation.

High 11 $\beta$ -HSD1 activity induced ALP activity by converting cortisone into biologically active levels of cortisol. This process eventually leads to matrix mineralization (Eijken et al., 2005). Pre-receptor hormone regulation is not unique for GCs. Local levels of androgens and estrogens are also regulated by enzymes present in target tissue (Gruber et al., 2002). Our previous work also showed that human osteoblasts express all the enzymes involved in the production of estradiol, e.g. aromatase, sulfatase and 17 $\beta$ -HSD isoenzyme (Janssen et al., 1999).

#### 4. Conclusion

The major aim of this study was to underscore the significance of GCs for proper human osteoblast differentiation. GCs are essential for human osteoblast differentiation, which is in marked contrast to the widely studied murine osteoblast differentiation. The human GC-induced differentiation process is summarized in Fig. 6A. In the beginning of osteoblast development precursor cells need to be directed by GCs to differentiate into bone forming osteoblasts. During this process several genes are regulated which either stimulate or inhibit bone formation (Fig. 6B). The importance of GCs on osteoblast differentiation is emphasized by the increasing conversion of inactive cortisone into active cortisol via 11 $\beta$ -HSD1 when cells are refrained from

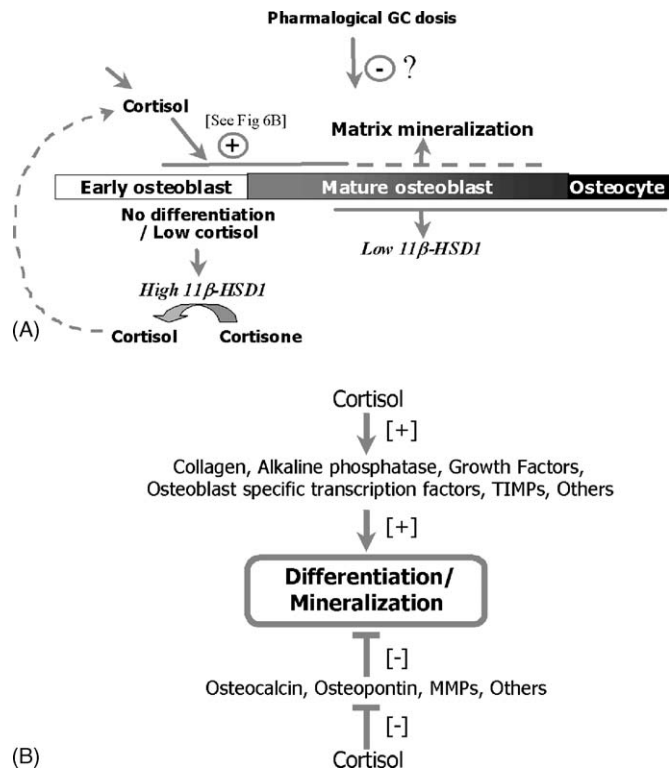


Fig. 6. (A) Schematic diagram depicting the role of GCs and 11 $\beta$ -HSD1 on osteoblast differentiation. (B) Regulation of gene expression by GCs on genes having either a positive or negative effect on bone formation.

GCs. This process might then act as a feed-back mechanism by the cells for delayed differentiation.

The detrimental effects on bone after clinical use of GCs is in this respect unclear but might be caused by various potential mechanisms. It might be duration (i.e. long term) or concentration dependent. However, in our studies we examined a range of concentrations and treated the cells continuously. GC effect might be dependent on osteoblast differentiation stage and act inhibitory at very late stages of osteoblast differentiation and osteocytes (Canalis and Delany, 2002b; Weinstein et al., 1998). Alternatively, the predominant negative effect of GCs could be mediated by the osteoclasts instead of the osteoblasts. Alternatively, in vivo interaction of GCs with other hormones/factors might be important. Moreover several secondary effects (e.g. at the intestine, kidney, parathyroid glands) after GC treatment could impair proper bone formation and might not reflect the normal in vivo role of GCs in bone. To address these issues and to pinpoint the mechanism of the negative effect of GCs on bone extensive additional research is needed. Nevertheless the current study in conjunction with other data in literature unequivocally demonstrates the significance of GCs for proper osteoblast differentiation.

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