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Inhibitory effects of pharmacological doses of dexamethasone on mineralization of mesenchymal progenitor cells *in vitro*

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Received March 10, 2009, accepted April 24, 2009

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Pharmazie 64: 674–679 (2009) *doi: 10.1691/ph.2009.9561*

Dexamethasone (Dex), a synthetic glucocorticoid, has a clinical adverse effect on bone acquisition and metabolism at pharmacological doses. To investigate the underlying mechanisms of Dex induced bone loss, we employed calvaria derived mesenchymal progenitor cells (MPCs) to examine the effects of Dex on their osteoblast lineage commitment and mineralization function. MPCs were cultured up to 28 days in the presence or absence of pharmacological doses of Dex. Alkaline phosphatase (ALP) and von Kossa histochemical staining showed that Dex decreased ALP activity and mineralized nodule formation. In addition, Dex treatment led to inhibition of cell proliferation and a decrease of cell numbers as assessed by BrdU incorporation and MTT methods, while it increased apoptosis as shown by flow cytometry of annexin V-stained cells. These effects were associated with a marked reduction of secreted IGF-I levels as indicated by ELISA quantification, raising the possibility that Dex decreased proliferation and promoted apoptosis of MPCs through the inhibition of IGF-I secretion. To further define the effect of Dex on osteoblast lineage commitment, Runx2 and Osx, the key transcription regulators of osteogenesis, were determined at both mRNA and protein levels. Interestingly, no effects were observed on mRNA and protein expression of Osx, while the mRNA and protein levels of Runx2 were inhibited by Dex treatment. Taken together, the inhibition of the expression of IGF-I and Runx2 by Dex in this *in vitro* system may account for the impaired MCP proliferation, osteoblastic differentiation and mineralized matrix deposition. These findings and the *in vitro* MCP system developed will facilitate further mechanistic studies of glucocorticoid induced bone loss.

1. Introduction

Dex is a synthetic glucocorticoid frequently used to inhibit the process of inflammation or autoimmune disorders (Canalis 2005). However, in terms of clinical treatment, high-dose and long-term use of Dex for patients would cause bone loss or severe osteoporosis as an adverse effect (Mikami et al. 2007). The mechanisms of Dex-induced osteoporosis have been investigated by examining skeletal cells, including osteoblasts, osteoclasts and their precursors, in different species, human, rat and mouse, both *in vivo* and *in vitro* (Hong et al. 2008). In osteoblast cultures, Dex exhibited both negative and positive effects on osteoblast differentiation and mineralization, depending on the duration and dose of Dex, the level of cell maturity or density, and the different species (Eijken et al. 2006; Leclerc et al. 2004). The effects of Dex on osteoclastogenesis and the bone resorptive function of osteoclasts may be time- and dose- dependent (Sivagurunathan et al. 2005). For BMSCs, Dex treatment resulted in dose-dependent inhibition of osteoblast precursor cell proliferation in the rat (Scutt et al. 1996), inducing differentiation of human BMSCs (Haasper et al. 2008), and decrease of the attachment and proliferation of murine BMSCs in culture (Chen 2004). In the mesenchymal progenitor cell line ROB-C26, Dex induced differentiation into adipocytes and osteoblasts, but failed to induce terminal osteoblast differentiation (Ito et al. 2007). However, the effects and mechanism(s) of Dex on calvaria-derived MPCs remain poorly understood.

MPC differentiation is controlled by multiple transcription factors at various stages of development. Runx2, the master-regulator of osteogenesis (Komori et al. 1997; Otto et al. 1997) and Osx, a newly identified zinc-finger containing protein, are osteoblast-specific transcription factors (Nakashima et al. 2002). MPCs first differentiate into preosteoblasts and then osteoblasts, which is a process for which Runx2 is needed. The Runx2-expressing preosteoblasts then differentiate in one or several steps into mature osteoblasts, which is a process that requires Osx. It could be concluded that Runx2 and other transcription factors collaborate with Osx in order to produce a bone-specific matrix that subsequently becomes mineralized during the process of bone formation.

In this study, the effects of a pharmacological dose of Dex (Dex concentration ≥ 0.1 µM) on differentiation with mineralization

Abbreviations: Dex, dexamethasone; Runx2, Runt-related transcription factor 2; Osx, Osterix; ELISA, enzyme-linked immunosorbent assay; IGF-I, Insulin-like growth factor-I; MPCs, mesenchymal progenitor cells; BMSCs, bone marrow stromal cells; ALP, alkaline phosphatase; PBS, phosphate-buffered saline; BMP, Bone morphogenetic protein.

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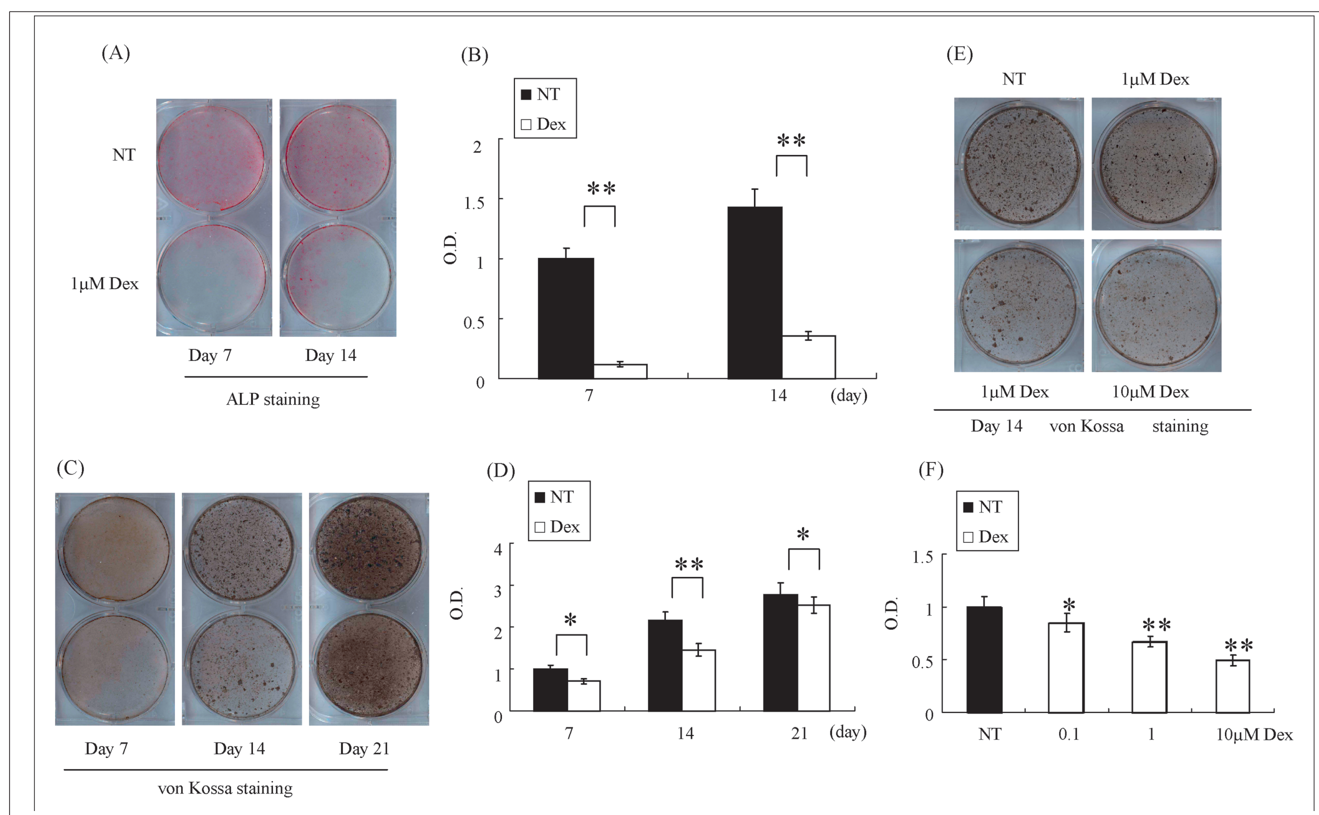

Fig. 1: Effects of Dex on mineralizations in MPC cultures. (A and C) Time-dependent effects of Dex on the mineralization. Cells were cultured for 7, 14, and 21 days in the presence or absence of 1 microM Dex, and then ALP and von Kossa histochemical staining were performed. (E) Dose-dependent effects of Dex on the mineralization. Cells were cultured with or without treatment of indicated concentrations of Dex for 14 days, and then von Kossa histochemical staining was performed. (B, D and F) Densitometric analysis of ALP and von Kossa staining observed in A, C and E respectively using NIH ImageJ 1.41. Data represented mean ± SD (n = 3).
* P < 0.05;
** P < 0.01

in MPCs were investigated, together with implications for the inhibition of mineralization. The results of an expression analysis showed that Dex inhibited the expression of IGF-I and Runx2, which might in turn result in less mineralization.

2. Investigations and results

2.1. ALP and von Kossa staining

To examine the effect of Dex on mineralization in MPCs, ALP and von Kossa staining were performed. A time course study revealed that mineralization was decreased in Dex-treated cells compared with that of control cultures (Fig. 1A and C). Fewer positive ALP and von Kossa nodules were found at days 7, 14, and 21 respectively in Dex-treated cells than in untreated.
MPCs were cultured in the presence of various concentrations of Dex for 14 days (Fig. 1E). This dose-dependent experiment revealed that pharmacological doses (0.1 μM, 1 μM and 10 μM) inhibited the mineralization of MPCs. Dex progressively decreased mineralization nodules at concentrations from 0.1 μM to 10 μM.

2.2. ALP activity

In the presence of Dex, the ALP activity of the Dex-treated group was not significantly changed at days 1 and 4 compared with that of untreated controls. However, compared with untreated controls, Dex reduced ALP activity in the culture by 0.22-fold (P < 0.05) on day 7, and 0.23-fold (P < 0.05) on day 14. As a result, changes between untreated controls and Dex-treated groups were significant when assessed by two-way ANOVA (P < 0.05; Fig. 2).

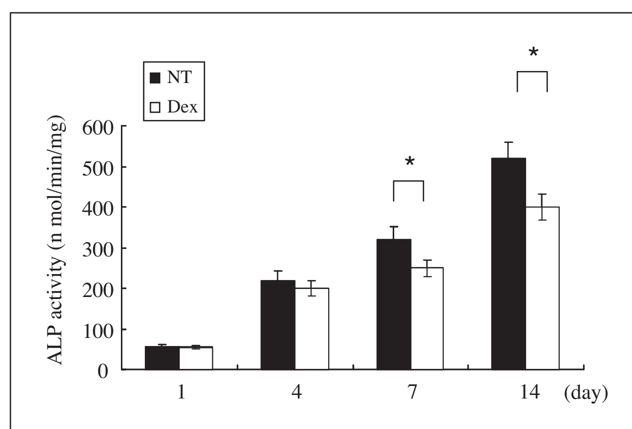

Fig. 2: Effects of Dex on ALP activity in MPC cultures. Cells were cultured for the indicated periods of time with or without treatment of 1 μM Dex. ALP activities were measured and expressed as nanomoles of p-nitrophenol produced per min per mg of protein.
* P < 0.05

2.3. MPC proliferation

MPC proliferation was assessed by flow cytometry of BrdU-stained cells (Fig. 3A) and MTT assay (Fig. 3C). In the presence of Dex, cell proliferation was significantly decreased at 12 h (0.25-fold, P < 0.05) and 24 h (0.20-fold, P < 0.05), although no significant difference was seen at 6 h. Further, MTT assay results for the cell proliferation behavior of cultures grown in the presence or absence of Dex (1 μM) are shown in Fig. 3C. Dex significantly decreased the growth of MPCs in a time-dependent manner during the culture period, compared with that in untreated controls (P < 0.05). Dex decreased the cell

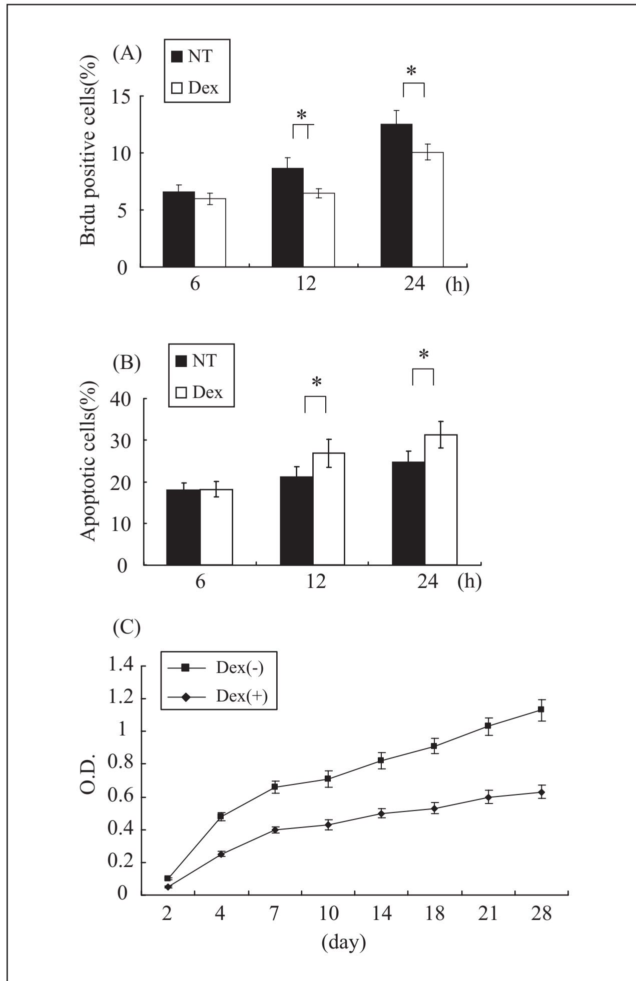

Fig. 3: Effects of Dex on proliferation and apoptosis of MPC cultures. (A) MPC proliferation was assessed by flow cytometry of BrdU incorporation at 6 h, 12 h, 24 h in the presence or absence of 1 μM Dex. (B) MPC apoptosis was assessed by flow cytometry of annexin V-stained cells at the 6 h, 12 h, 24 h in the presence or absence of 1 microM Dex. (C) MPCs were cultured for 2, 4, 7, 10, 14, 18, 21 and 28 days in the complete BGJb medium containing 50 μg/ml ascorbic acid and 5 mM beta-glycerophosphate in the presence or absence of 1 μM Dex. Cell proliferation was decreased in the Dex-treated cells (a diamond dotted line) compared with untreated controls (a square dotted line) ($P < 0.05$, two-way ANOVA).
* $P < 0.05$

numbers in the culture by 0.35-fold ($P < 0.01$) on day 7, 0.35-fold ($P < 0.05$) on day 10, 0.36-fold ($P < 0.001$) on day 14, 0.28-fold ($P < 0.01$) on day 18, 0.28-fold ($P < 0.01$) on day 21, and 0.21-fold ($P < 0.01$) on day 28, compared with untreated controls.

2.4. *MPC apoptosis*

MPC apoptosis was assessed by means of flow cytometry of annexin V-stained cells (Fig. 3B). In the presence of Dex, cell apoptosis was significantly increased at 12 h (0.26-fold, $P < 0.05$) and 24 h (0.26-fold, $P < 0.05$), although there was no significant difference at 6 h.

2.5. *Enzyme-Linked Immunosorbent Assay (ELISA)*

The level of IGF-I synthesized and secreted by cells was measured at 6 h, 12 h, and 24 h (Fig. 4). In the presence of Dex, the quantity of IGF-I was significantly decreased at 12 h (0.28-fold, $P < 0.05$) and 24 h (0.25-fold, $P < 0.05$), but there was no significant difference at 6 h.

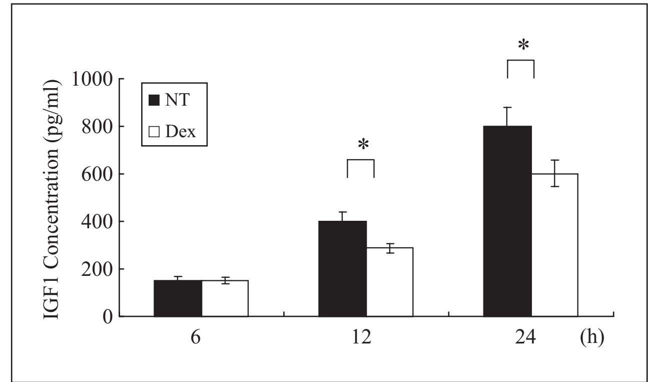

Fig. 4: Effects of Dex on IGF-I secretion of MPC cultures. The level of cell synthesized and secreted IGF-I in the presence or absence of 1 μM Dex was measured at the 6 h, 12 h, 24 h and expressed as mean of pg of IGF-I by ml medium

2.6. *Effect of Dex on Runx2 and Osx expression*

As Runx2 and Osx are the key regulators of osteoblast differentiation, it is necessary to examine whether Dex can affect Runx2 and Osx expression in MPCs. The cells were cultured in the presence or absence of 1 μM Dex for the indicated number of days. The mRNA and protein expression levels of Runx2 and Osx were assayed by real time PCR or Western blot analysis. No effects were observed on mRNA and protein expression of Osx, but the mRNA and protein levels of Runx2 were inhibited by Dex treatment (Fig. 5).

3. Discussion

This study examined mineralization, proliferation, apoptosis, and ALP activity in cultured MPCs in the presence and absence of Dex. Dex demonstrated an inhibitory effect only at pharmacological concentrations of 1 μM and 0.1 μM, but a stimulatory effect at 0.01 μM (Ishida and Heersche 1998). In addition, in laboratory experiments, Dex impaired differentiation of mesenchymal cells towards cells of the osteoblastic lineage and prevented the terminal differentiation of osteoblastic cells (Pereira et al. 2002, 2001), which resulted in a decrease in the number of mature osteoblasts. The decrease in osteoblastic cell differentiation together with an increase in apoptosis caused a decrease in bone-forming cells. As shown in this study, Dex not only significantly increased cell apoptosis at 12 h and the 24 h (Fig. 3B), but also dramatically decreased cell numbers in the culture at 12 h and 24 h (Fig. 3A) and on days 7, 10, 14, 18, 21, and 28 (Fig. 3C), compared with untreated controls, and it was confirmed that ALP activity in Dex-treated cells significantly decreased in comparison with Dex-untreated (Fig. 2). Based on this evidence, it seems that, in cells uncommitted to mineralization, a pharmacological dose of Dex has an inhibitory action on mineralization, reducing proliferation and inducing apoptosis by certain mechanisms.

The IGF-I signaling pathway is unequivocally a major autocrine/paracrine regulator of bone growth (Macrae et al. 2007). It has been shown that IGF-I can stimulate proliferation and protect against Dex-induced apoptosis by activation of MAPK pathways (Avram et al. 2008). On the other hand, Dex can reduce the expression of IGF-I and its binding proteins 3, 4, and 5 (Lafage-Proust et al. 2003). In that study the IGF-I level in culture supernatants was measured by ELISA. Interestingly, the level of IGF-I synthesized and secreted in the cells was significantly decreased at 12 h (0.28-fold, $P < 0.05$) and 24 h (0.25-fold, $P < 0.05$) (Fig. 4) in the presence of Dex. In short, it was the inhibitory level of IGF-I mediated by Dex that resulted in

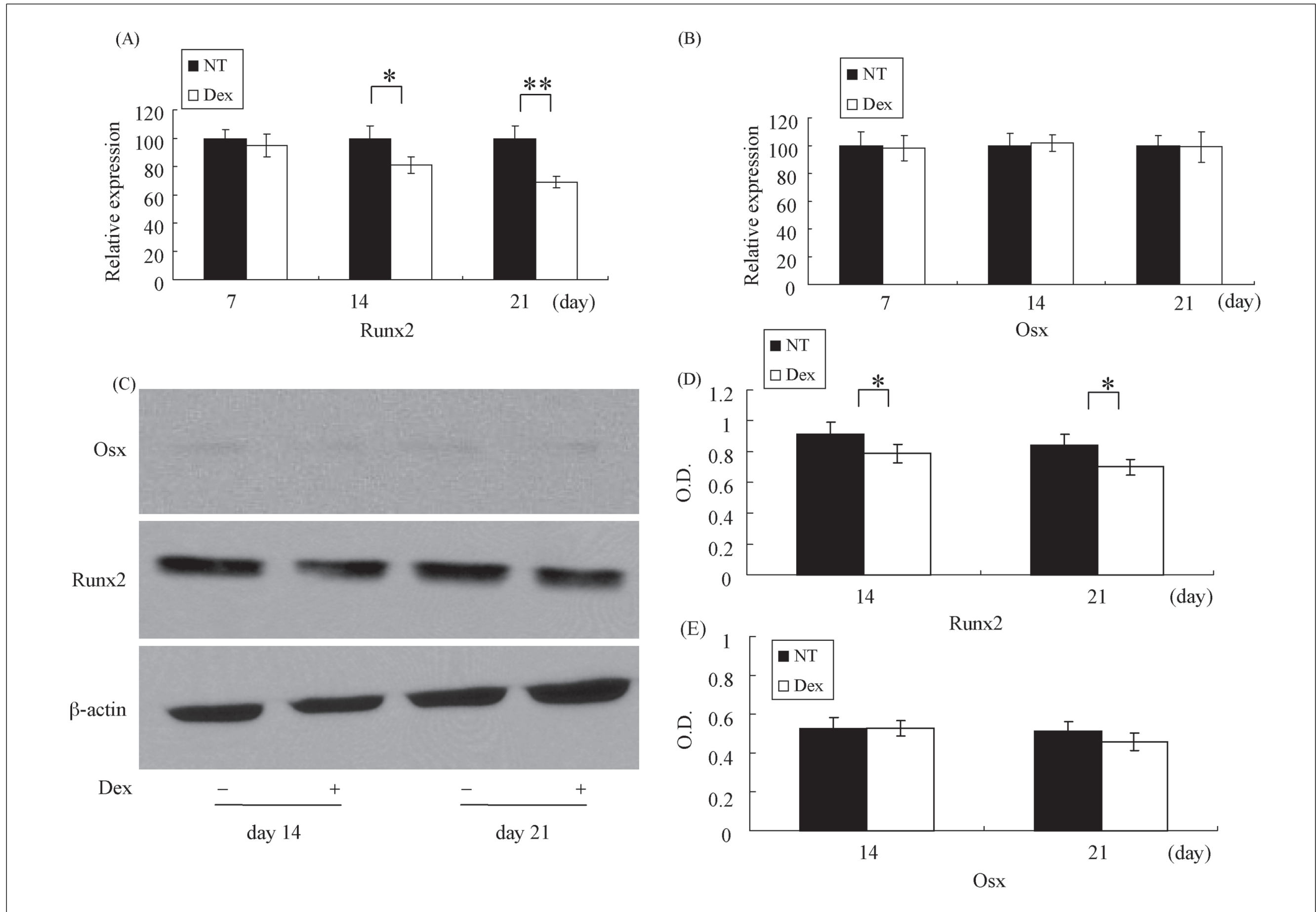

Fig. 5: Effect of Dex on the expressions of Runx2 and Osx in MPCs. Cells were cultured with or without treatment of 1 μM Dex for 7, 14 and 21 days. The mRNA expressions (A:Runx2, B:Osx) were determined by real time PCR, and the protein expressions (C) were assessed by Western blotting with the anti-Runx2 and anti-Osx antibodies. Densitometric analysis of Runx2 (D) and Osx (E) protein expression was shown in a bar graph with Image J software. Data were presented as a ratio of Runx2 to beta-actin, Osx to beta-actin (mean ± SD; n = 3).
* $P < 0.05$;
** $P < 0.01$

decreased proliferation and increased apoptosis and subsequent inhibition of mineralization.

It has been demonstrated that Dex enhances gene expression of Runx2 in cultured bovine vascular smooth muscle cells, and promotes their osteoblastic differentiation (Mori et al. 1999). Dex also promotes early osteoblast differentiation of a mesenchymal progenitor, ROB-C26, in parallel with inductive expression of an osteoblast-specific transcription factor, Runx2 (Mikami et al. 2008). In contrast, Dex suppresses osteoblastic differentiation and activity through dual signaling modulation of both the BMP/Runx2 and Wnt pathways, by enhancing the expression of their antagonists (Hayashi et al. 2009). More importantly, the effect of Dex on a multipotential cell line, D1, which was derived from bone marrow and was capable of differentiating into either the osteoblast or the adipocyte lineage, showed that Dex increased PPARgamma 2 gene expression 2-fold, while Runx2 gene expression decreased by 50-60% in mesenchymal cells *in vitro*, suggesting that Dex might switch uncommitted osteoprogenitor cells in marrow from osteoblastic differentiation into the adipocytic pathway, leading to diminished mineralization (Li et al. 2005). Similarly, our data showed that Dex decreased the mRNA and protein expression of Runx2 in MPCs (Fig. 5A and C), which might mediate the inhibition of mineralization by Dex. Because Osx is a transcription factor for terminal osteoblast differentiation, and it has been reported in *in vitro* studies that Dex induced mineralization with induction of Osx mRNA expression in fetal rat calvarial cells (Igarashi et al. 2004) and human skin-derived precursor cells (Buranasinsup et al. 2006), Osx

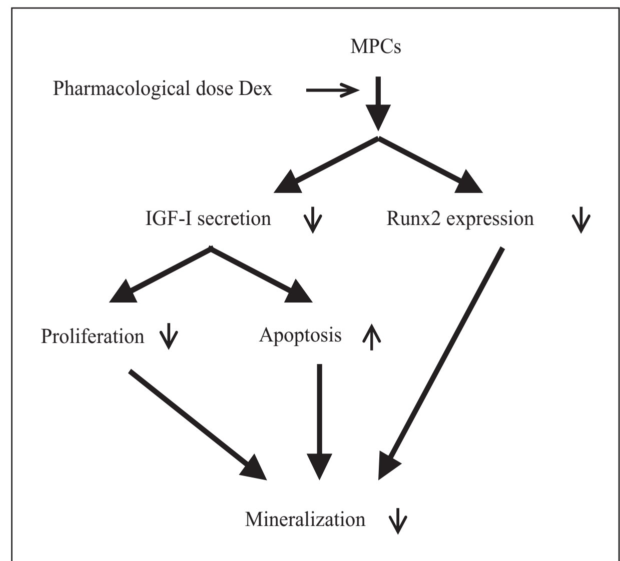

Fig. 6: Effect of pharmacological dose Dex on MPCs mineralization. Dex, on the one hand, reduced proliferation and induced apoptosis of MPCs through the inhibition of IGF-I secretion, and on the other hand, decreased the expression of Runx2, which collectively resulted in subsequent inhibition of mineralization

expression might be critical for differentiation of Dex-induced osteoblast differentiation. The expression of Osx was also examined, but no significant changes were found, probably because of differences of cell type, cell maturity or Dex concentration. In summary, it was demonstrated that pharmacological dose Dex inhibited mineralization in MPCs, and this inhibition of mineralization was mediated, at least in part, by IGF-I- and Runx2- dependent mechanisms (Fig. 6). More studies *in vivo* are needed to confirm this conclusion.

4. Experimental

4.1. Animals

All procedures involving animals were approved by the Institutional Animal Experiment Committee of Shanghai Jiaotong University School of Medicine. Three-month-old adult C57BL/6 mice were purchased from the Chinese Academy of Sciences, China. In the timed pregnancies the day of vaginal plug appearance was defined as E0.5. For harvesting of embryos, timed pregnant female mice were sacrificed by cervical dislocation. The gravid uterus was dissected out and suspended in a bath of cold PBS, and the embryos were harvested after amnionectomy and removal of the placenta.

4.2. MPC cultures

The culture conditions for MPCs were identical to those described previously (Day et al. 2005). Briefly, the developing calvarium between the skin and brain tissues was isolated from the embryonic mouse skull at E12.5 in Hanks Balanced Salt Solution (HBSS, Sigma) and digested in 0.5% collagenase, 0.25% dextrose in HBSS at 37 °C for 1 h. The cells were disassociated by repetitive pupating with a 1 ml micropipette, spun down, resuspended in BGJb medium (Invitrogen) containing 10% FBS, 1 × Antibiotics/Antimycotics (Invitrogen), and plated in 10-cm plates. The cells were grown at 37 °C in a 5% CO_2 and humid atmosphere.

4.3. ALP and von Kossa histochemical staining

The MPCs were cultured in 6-well plates with 2×10^5 cells/well in BGJb medium (Invitrogen) containing 10% FBS and 1× Antibiotics/Antimycotics (Invitrogen) for 4 days until they were confluent. The medium was then changed to osteogenic medium supplemented with 50 μg/ml L-ascorbic acid and 5 mM β-glycerolphosphate to BGJb medium until the stainings were performed. The medium was changed every other day. ALP and von Kossa histochemical staining were performed for the 7-, 14-, and 21-day samples. The cells were washed with cold PBS, fixed in 2% paraformaldehyde/PBS for 10 min, and stored at 4 °C in 100 mM cacodylic acid buffer (pH 7.4). For ALP staining, the fixed cells were incubated at 37 °C with freshly prepared alkaline phosphatase substrate solution (100 mM Tris-maleate buffer, pH 8.4, 2.8% *N*,*N*-dimethyl formamide (v/v), 1 mg/ml Fast Red TR, and 0.5 mg/ml naphthol AS-MX phosphate) until a red color developed. For von Kossa staining, the fixed cells were exposed to UV light in the presence of 3% silver nitrate ($AgNO_3$). The reaction was terminated by removing $AgNO_3$ solution and the monolayer was washed carefully with distilled water. Densitometric analysis of ALP and von Kossa staining was performed using NIH ImageJ 1.41 (http:rsb.info.nih.gov/ij/).

4.4. ALP activity

ALP activity was measured using p-nitrophenyl phosphate as a substrate as described previously (Partridge et al. 1981), and expressed as nanomol of *p*-nitrophenol produced per min per mg of protein. Protein concentration was determined by Bradford protein assay (Bio-Rad).

4.5. MPC proliferation and apoptosis in vitro

The MPCs were plated on 6-well plates at a density of 1×10^5 cells/well, and cultured in BGJb medium (Invitrogen) supplemented with 10% FBS and 1× Antibiotics/Antimycotics (Invitrogen) for 6, 12, 24 h respectively. For the proliferation assay, BrdU (10 μM) was added to the culture medium 2 h before harvesting. Cells were stained with fluorescent anti-BrdU antibody and 7-amino-actinomycin D following the manufacturer's instructions (BD Biosciences). For the apoptosis assay, annexin V-PE (BD Biosciences) and 7-AAD were added for staining. The MPCs were analyzed by FACSCalibur (BD).

4.6. MTT assay

Cell numbers were assessed by MTT (Sigma, USA) assay. The cells were seeded on to 96-well plates (1200 cells/well) and cultured in the previously mentioned culture medium for 2, 4, 7, 10, 14, 18, 21 and 28 days. After incubation of each group in the presence or absence of Dex for the time intervals specified above, the medium was removed and replaced with 20 μl MTT medium (5 mg/ml). After 30 min incubation at 37 °C, the cells were added to 150 μl DMSO/well and shaken for 10 min at low speed to dissolve the crystals. The absorbance was measured at 490 nm, and the data were expressed as cell viability (% of untreated control). The changes in cell proliferation of each group are shown in Fig. 3C.

Table: Primer sequences for genes used in real time PCR

Gene	Primer Sequence	Orientation	Size (bp)
Runx2	5′- gtgtcactgcgctgaagagg -3′	Forward	376
	5′- gaccaaccgagtcatttaaggc -3′	Reverse	
Osx	5′- ggaggtaggtggtagtgtaggtg -3′	Forward	270
	5′- ctgctgaggaaaggaacaggagg -3′	Reverse	
β-actin	5′- ctgtccctgtatgcctctg -3′	Forward	218
	5′- atgtcacgcacgatttcc -3′	Reverse	

4.7. Enzyme-Linked Immunosorbent Assay (ELISA)

For the IGF-I ELISA, 96-well microtiter plates were coated with purified monoclonal anti-mouse IGF-I antibody at 0.5 μg/well in 100 μl of PBS, incubated overnight at RT on a shaker, then washed 3 times followed by 1 h incubation and 3 final washes. Standards were prepared by diluting recombinant DNA derived IGF-I in assay buffer in concentrations ranging from 0 to 25 ng/ml. Prior to IGF-I assay, 0.1 mL acid/ethanol reagent was added to 0.025 ml of each sample. The mixture was incubated at RT for 30 min and microcentrifuged at 10 000 × g for 10 min, and 0.05 ml of supernatant was then neutralized with 0.025 ml 1 M Tris base and diluted with IGF-I assay buffer to a final dilution of 40-100-fold. Standards, controls or treated samples (50 μl/well) and 50 ng/well (50 μl/well) biotinylated goat anti-mouse IGF-I antibody were added to the prepared assay plates and incubated at RT for 2 h on a shaker. Wells were then washed 3 times, followed by addition of streptavidin-HRP conjugate (100 μl/well) and further incubated for 20 min at RT. After 4 washes, 100 μl/well of OPD solution was added to each well and incubated for an additional 10-20 min. The reaction was stopped by the addition of 50 μl/well 2N H_2SO_4 and the absorbance was measured at 450 nm with a Thermo-Max microplate reader.

4.8. Quantitative real-time PCR

Total RNA was extracted from cells following the TRIzol method recommended by the manufacturer (Invitrogen). The RNA concentration was estimated spectrophotometrically, and only pure RNA (A260:A280 ratio ≥1.8) was used for further analysis. First strand cDNA was synthesized using the iScript cDNA Synthesis Kit (Bio-Rad). The cDNA was amplified in the Opticon Continuous Fluorescent Detector (MJ Research, Waltham, MA) by SYBR Green supermix (Bio-Rad) and sequence specific primers. Real time PCRs were performed in triplicate for each cDNA, averaged, and normalized to endogenous β-actin reference transcripts. Primer sequences used are given in the Table.

4.9. Western blotting

The cells were washed twice with ice-cold PBS and resuspended in lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1 mM $MgCl_2$, 1 mM EDTA, 1% Triton X-100, and 10% glycerol). Protease and phosphatase inhibitors (Sigma) were added to the lysis buffer. The cell lysates were homogenized by rotating at 4 °C for 30 min and then centrifuged at 14,000 rpm for 20 min at 4 °C. The supernatant was transferred to a new microcentrifuge tube, and protein concentration was measured by Bradford protein assay (Bio-Rad). For Western Blotting, equal amounts of protein (10 or 20 μg/lane) were solubilized in Laemmli sample buffer and loaded on to an SDS-PAGE system (Bio-Rad). Following electrophoresis, the proteins were transferred to a polyvinylidene difluoride membrane in a Bio-Rad wet transfer system. Protein transfer efficiency was verified by prestained protein markers. The membranes were then blocked with 5% nonfat dried milk for 1 h at RT and subsequently incubated overnight at 4 °C with monoclonal antibodies directed against Runx2 or Osx (Santa Cruz, USA). Signals were detected by a horseradish peroxidase-conjugated secondary antibody, and bound antibodies were visualized using the Supersignal West Dura Substrate (Pierce) and autoradiography. Densitometric analysis of Runx2 or Osx protein expression was performed using NIH ImageJ 1.41 (http:rsb.info.nih.gov/ij/).

4.10. Statistics

The results were expressed as the mean ± SD. Statistical differences were determined by two-tailed Student's t-test or two-way ANOVA, as needed. The statistical significance was defined as $P < 0.05$.

Acknowledgments: This work was supported by an NSFC grant (No. 30672145 and 30872641); Shanghai Science and Technology Commission project (No. 06DZ22020 and 07DZ22008).

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