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Evidence for Direct Effects of Prolactin on Human Osteoblasts: Inhibition of Cell Growth and Mineralization

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ABSTRACT

Hyperprolactinemia is one of the risk factor of decrease in bone mass which has been believed to be mediated by hypogonadism. However, the presence of prolactin receptor in human osteosarcoma cell line and primary bone cell culture from mouse calvariae supported the hypothesis of a direct prolactin (PRL) action on bone cells. Therefore, the aim of this study was to investigate the role of PRL and its signal transduction pathway in the regulation of bone metabolism via osteoblast differentiation. Human pre-osteoblasts (SV-HFO) that differentiate in a 3-week period from proliferating pre-osteoblasts (days 2–7) to extracellular matrix producing cells (days 7–14) which is eventually mineralized (days 14–21) were used. Concentration of PRL mimicked a lactating period (100 ng/ml) was used to incubate SV-HFO for 21 days in osteogenic medium. Human prolactin receptor mRNA and protein are expressed in SV-HFO. PRL significantly decreased osteoblast number (DNA content) which was due to a decrease in proliferation. PRL increased osteogenic markers, RUNX2 and ALP in early stage of osteoblast differentiation while decreasing it later suggesting a bi-directional effect. Calcium measurement and Alizarin red staining showed a reduction of mineralization by PRL while having neither an effect on osteoblast activity nor RANKL/OPG mRNA ratio. We also demonstrated that PRL action on mineralization was not via PI-3 kinase pathway. The present study provides evidence of a direct effect of prolactin on osteoblast differentiation and in vitro mineralization. J. Cell. Biochem. 107: 677–685, 2009.

KEY WORDS: HYPERPROLACTINEMIA; OSTEOBLAST DIFFERENTIATION; BONE FORMATION; RANKL/OPG

A number of causes have been implicated to physiological and pathological hyperprolactinemia. Pregnancy and lactation produced physiologically high concentration of prolactin (PRL) of ~75–100 and ~200–300 ng/ml [Ritchie et al., 1998; Prentice, 2000], anterior pituitary tumor [Schlechte et al., 1983; Greenspan et al., 1986; Klibanski and Greenspan, 1986] or use of antipsychotic drug in schizophrenia patients elevates prolactin (PRL) levels by dopaminergic inhibition [Naidoo et al., 2003]. Hyperprolactinemia-induced osteoporosis is, so far, believed to be mediated by hypogonadism. However, in hyperprolactinemic women with normal estrogen levels, net bone loss still occurred, but its severity was significantly less than that in hyperprolactinemia without estrogen. PRL is a peptide hormone produced by the lactotrope cells in the anterior pituitary gland and is primarily associated with lactation. We previously found in the in vivo studies that PRL has a significant effect on bone remodeling in lactating rat, stimulating recruitment of calcium for fetal development and breast feeding [Lotinun et al., 1998; Lotinun et al., 2003]. The prolactin receptor (PRLR) knockout mice were shown to have a significant effect on fetal skeletal development suggesting a role for PRL signaling to maintain a normal fetal bone development [Clement-Lacroix et al., 1999]. In addition, the expression PRL receptor (PRLR) in osteoblasts as assessed by PCR and immunohistochemistry suggested a direct effect of PRL on bone remodeling [Bataille-Simoneau et al., 1996; Coss et al., 2000; Seriwatanachai et al., 2008a,b]. However, it is yet not clear whether bone is directly regulated by PRL. The skeletal effects observed in the lactating rats and PRLR knockout mice may still be due to the presence of other hormones in lactating stage and other changes in the PRLR knockout mice. Nevertheless, the expression of PRLR in human fetal osteoblast (hFOB 1.19), osteoblast-like cell, MG-63 and Saos-2 and rat osteoblast suggested a direct effect of PRL on osteoblasts [Bataille-Simoneau et al., 1996; Coss et al., 2000; Seriwatanachai et al., 2008a,b]. There are only few studies focusing on the direct short-term effects of PRL

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on osteoblast [Coss et al., 2000; Seriwatanachai et al., 2008a,b]. None of them has studied the putative role of human PRL on human osteoblast differentiation and bone formation.

The aim of this study was to assess the impact of PRL and PRL signaling, on human osteoblast differentiation related to bone formation. The expression of PRLR and the effect of PRL on osteoblast activity and matrix mineralization were measured together with studies on PRL signal transduction pathway.

MATERIALS AND METHODS

CELL CULTURE

SV-HFO are human pre-osteoblast [van Driel et al., 2004; Eijken et al., 2006] were cultured in α-MEM (GIBCO, Paisley, UK) supplemented with 20 mM HEPES, pH 7.5, streptomycin/penicillin, 1.8 mM $CaCl_2$ (Sigma, St. Louis, MO) and heat-inactivated FCS (GIBCO, Paisley, UK) at 37°C and 5% CO_2 in a humidified atmosphere. Cells were seeded in a density of 5×10^3 vital cells/cm^3 and pre-cultured for 1 week in the presence of 10% FCS. During this pre-culture, SV-HFO cells remained in an undifferentiated stage. After preculture, cells were seeded in density of 10×10^3 vital cells/cm^2 in osteogenic medium consisting of 2% charcoal-treated FCS supplemented with 10 mM β-glycerophosphate (Sigma) and dexamethasone (Sigma).

Recombinant human PRL (R&D Systems, Inc., Minneapolis, MN) was reconstituted in BSA and HCl following the manufacturer's instruction before being diluted in α-MEM. Medium freshly supplemented with and without PRL was replaced every 2–3 days. For biochemical 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 2×15 s before analysis.

IMMUNOCYTOCHEMISTRY

To verify and localize the PRLR proteins in osteoblasts, SV-HFO cells were seeded on cover slips in density of 10×10^3 vital cells/cm^2 and incubated with osteogenic medium for 5 and 12 days. Unattached cells were then removed by washing with PBS pH 7.4 twice. The cover slips were removed from the incubator and were fixed with 4% of paraformaldehyde for 10 min at room temperature. Cells were then washed three times with PBS, and permeabilized with 0.15% Triton-X100 in PBS for 5 min at room temperature. Then cells were washed twice with PBS, and nonspecific proteins were blocked with 10% BSA for 30 min at room temperature. Samples were then incubated with the 1:300 diluted rabbit polyclonal anti-PRLR (Santa Cruz, CA) primary antibody for 1 h at room temperature. Cells were further incubated with 1:200 diluted Alexa Fluor 488 conjugated anti-rabbit IgG antibody as a secondary antibody for 1 h at room temperature. Cells were then stained with nuclear DAPI stain for 2 min. Anti-PRLR was digitally captured by using inverted fluorescence microscopy (Bio-Rad MRC1024 MP scanning system mounted on a Nikon Eclipse TE300 fluorescence microscope).

DNA CONTENT

For DNA measurements, 100 μl SV-HFO cell lysates were treated with 200 μl heparin (8 IU/ml in PBS) and 100 μl ribonuclease A (50 μg/ml in PBS) for 30 min at 37°C. This was followed by adding 100 μl ethidium bromide solution (25 μg/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.

ALKALINE PHOSPHATASE (ALP) ACTIVITY

ALP activity was assayed by determining the release of *p*-nitrophenol from *p*-nitrophenylphosphate (20 mM in 1 M diethanolamine buffer supplemented with 1 mM $MgCl_2$ 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.

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 colorimetrically 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 an anthraquinone derivative, Alizarin Red S solution.

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 apoptotic (annexin V or PI stained) cells.

PROLIFERATION ASSAY

Osteoblast proliferation was examined by using a colorimetric BrdU cell proliferation enzyme-linked immunosorbent assay kit (No. 1 647 229, Roche, Mannheim, Germany). SV-HFO cells were seeded into 96-well plates, in osteogenic medium with and without 100 ng/ml PRL. 100 μM BrdU labeling solution was added to each well giving a final concentration of 10 μM. After 24 h incubation and removal of the culture medium the cells were fixed and DNA was denaturated. Subsequently, the anti-BrdU-peroxidase conjugate was added which binds to the incorporated in newly synthesized DNA. The immune complexes were detected by subsequent reaction with tetramethylbenzidine as substrate for 10 min. The reaction was stopped by addition of 200 μl 1 M H_2SO_4 to each well, and the reaction product was quantified by measuring the absorbance at 450 nm with reference to 690 nm using the Wallac 1420 victor2 (Perkin-Elmer, Wellesley, MA) [Gratzner, 1982].

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_2O. 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 0.2 μg oligo(dT)18 and 0.2 μg random hexamer primers and a cDNA synthesis kit (MBI Fermentas, St. Leon-Rot, Germany).

Quantitative real-time PCR (QPCR) was carried out using an ABI 7700 sequence detection system (Applied Biosystems, Foster City, CA). Reactions were performed in 25 μl volumes using a qPCR core kit (Eurogentec, Seraing, Belgium). Reaction mixes contained 20 ng cDNA, 5 mM $MgCl_2$, 200 μM dNTPs, and 0.025 U/μl 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. The relative gene expression was calculated by the comparative Ct method of which Ct sample is normalized to endogenous house keeping gene, GAPDH, according to the calculation; relative Ct value $= 2^{-[\text{delta}]Ct_{SAMPLE}-[\text{delta}]Ct_{GAPDH}}$

STATISTICS

Data are presented as mean ± SEM and are derived of at least two independent experiments each consisting of at least three cultures. Differences between groups were analyzed by the Student's *t*-test.

RESULTS

EXPRESSION OF HUMAN PROLACTIN RECEPTOR (hPRLR)

hPRLR expression and PRL effects were studied in detail using the SV-HFO osteoblast differentiation model. This human pre-osteoblast model produces an extracellular matrix during culture, which eventually is mineralized in 2–3 weeks time period [Chiba et al., 1993; Eijken et al., 2006], as shown in Figure 1. hPRLR mRNA was found to be expressed in human osteoblasts and the expression remained constant during differentiation (Fig. 2). Incubation with PRL did not significantly change the hPRLR mRNA expression significantly at the various days albeit that at all days tested hPRLR mRNA expression appeared to be lower than in untreated cultures. Also at protein level, the hPRLR was demonstrated in human osteoblasts (Fig. 3).

EFFECTS OF PROLIFERATION AND APOPTOSIS

PRL treatment decreased total cell number as reflected by a decrease in the total amount DNA during osteoblast differentiation (Fig. 4A). The decrease in DNA only became apparent after 7 days of culture. For total protein we found a similar pattern after PRL treatment as observed for total DNA with a reduction from day 7 of culture onwards (data not shown). The reduction in cell number can be due to an increase in apoptosis and/or an inhibition of cell proliferation. PRL did not affect apoptosis of human osteoblasts (Fig. 4B) but inhibited cell proliferation (Fig. 4C).

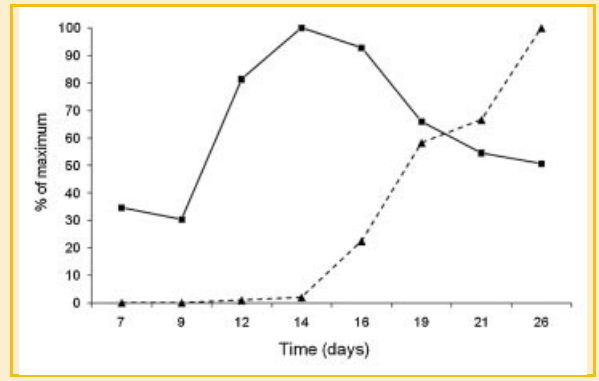

Fig. 1. Temporal pattern of alkaline phosphatase activity (solid line) and mineralization (dashed line) during differentiation of SV-HFO pre-osteoblasts induced by osteogenic medium.

EFFECTS ON HUMAN OSTEOBLAST DIFFERENTIATION

To investigate the role of PRL on osteoblast differentiation, we analyzed the expression of known osteoblast marker genes and the effect of on ALP activity. PRL significantly increased Runx2 at early differentiation, day 5 while decreasing it at the late osteoblast differentiation, day 21 (Fig. 5A). This pattern is similar to that of ALP mRNA expression as shown in Figure 5B. PRL increased collagen type Ia mRNA expression at d5 but did not affect it later on during differentiation (Fig. 5C). ALP activity showed a peak around days 10–12 of culture and in this period the ALP activity in the PRL condition was significantly higher at day 12 while it did not reach significantly at day 10 (Fig. 5D).

PRL EFFECTS ON MINERALIZATION

Next we examined the direct effect of PRL on osteoblast mineralization using Alizarin Red staining at day 14 and day 21.

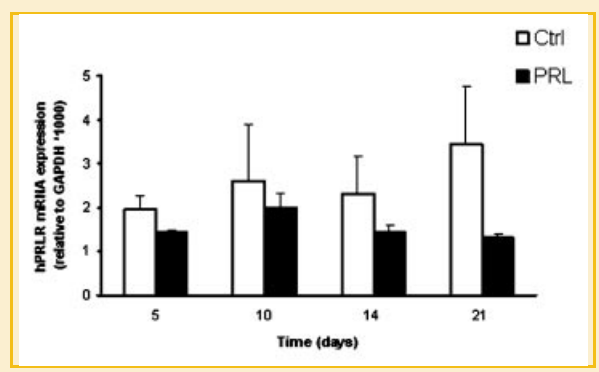

Fig. 2. Expression of hPRLR mRNA (QPCR) during human osteoblast differentiation in control condition (open bars) and after treatment with 100 ng/ml PRL (solid bars).

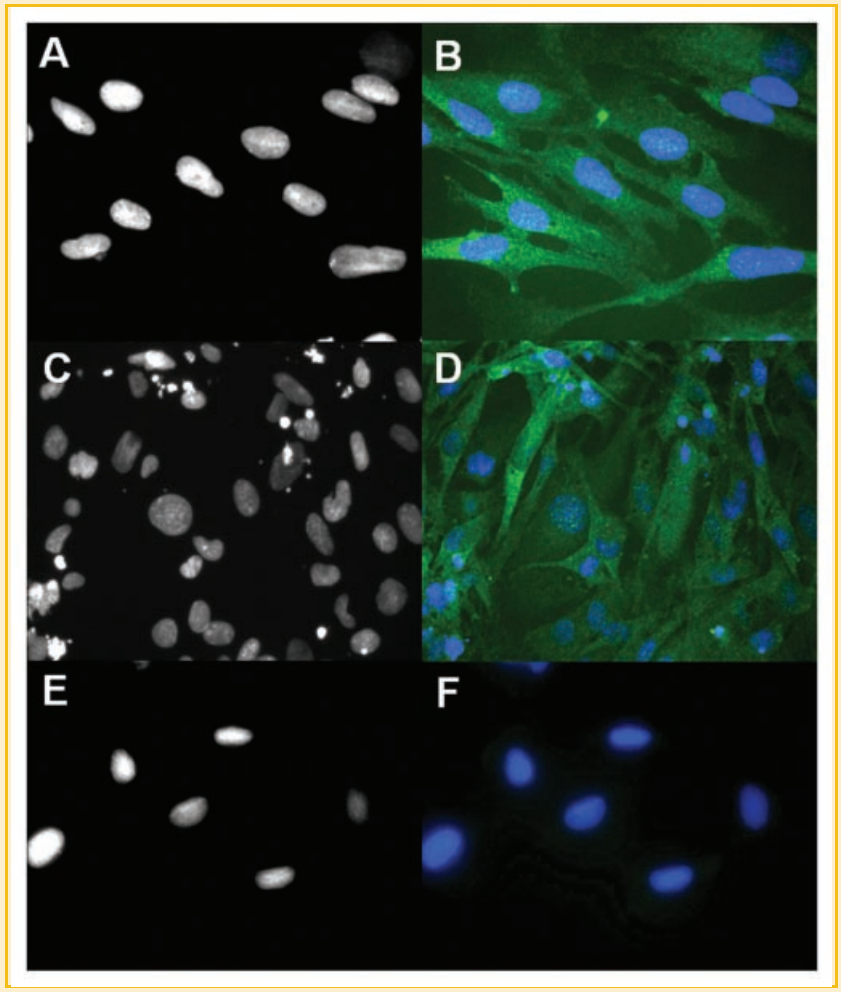

Fig. 3. Immunofluorescent analysis of hPRLR protein expression in SV-HFO cells exposed for 5 days (A,B) and 12 days (C,D) to osteogenic medium (n = 3). A,C,E: DAPI nuclear staining, (B,D,F) double staining with DAPI (blue) and hPRLR antibody conjugated with ALEXA (green). Cells were incubated in the absence (anti-hPRLR) of primary antibody against hPRLR proteins (E,F). Experiments were performed in triplicate, magnification 400×.

We found that PRL visibly reduced the alizarin red staining at day 14 and day 21 (Fig. 6A). In contrast to the control condition, total Ca^{2+} was not yet detectable at d14 in PRL treated group, and at day 21 of culture it was significantly decreased in the PRL condition (Fig. 6B). After correcting the total Ca^{2+} with DNA, it did not change the PRL effect and significance (Fig. 6C).

EFFECTS OF PRL ON RANKL AND OPG EXPRESSION

To elucidate the action of PRL on osteoclastogenesis function of osteoblast, we measured the expression of RANKL and its decoy receptor, OPG. We could not find a significant change in their expression as well as the bone resorption indicator, RANKL/OPG ratio, in PRL-treated group in any given days (Fig. 7A–C).

PI3 KINASE AND PRL ACTION

PI3 kinase pathway is one of the recent pathways reported to be involved in PRL action in many kinds of cells [al Sakkaf et al., 1996; Hugl and Merger, 2007; Jantarajit et al., 2007; Seriwatanachai et al., 2008b]. Our previous report [Seriwatanachai et al., 2008b] also suggested that the shortterm action of PRL on the reduction of osteoblastic ALP activity is via PI-3 kinase activity. We tested the effect of PI3 kinase inhibition by LY294002 in various phases (d0–3, d4–7, and d11–14) during osteoblast differentiation. In general these studies showed that treatment with PRL reduced total DNA (Fig. 8A–C). In none of the phases inhibition of PI-3 kinase alone did effect total DNA level nor did it inhibit the PRL effect (Fig. 8A–C). When LY294002 was added together with PRL at days 11–14 the reduction in DNA was even stronger (Fig. 8C). We confirmed the inhibitory effect of PRL on mineralization (Fig. 8D–F). Inhibition of PI-3 kinase activity in control condition suppressed the mineralization only when added at the early differentiating stage (d0–3 and d4–7) but not when added at the onset of mineralization (d11–14) (Fig. 8D–F). PI-3 kinase inhibition did not block the PRL effect on mineralization, in contrast, when added in the early phases it even augmented the inhibition by PRL (Fig. 8D,E).

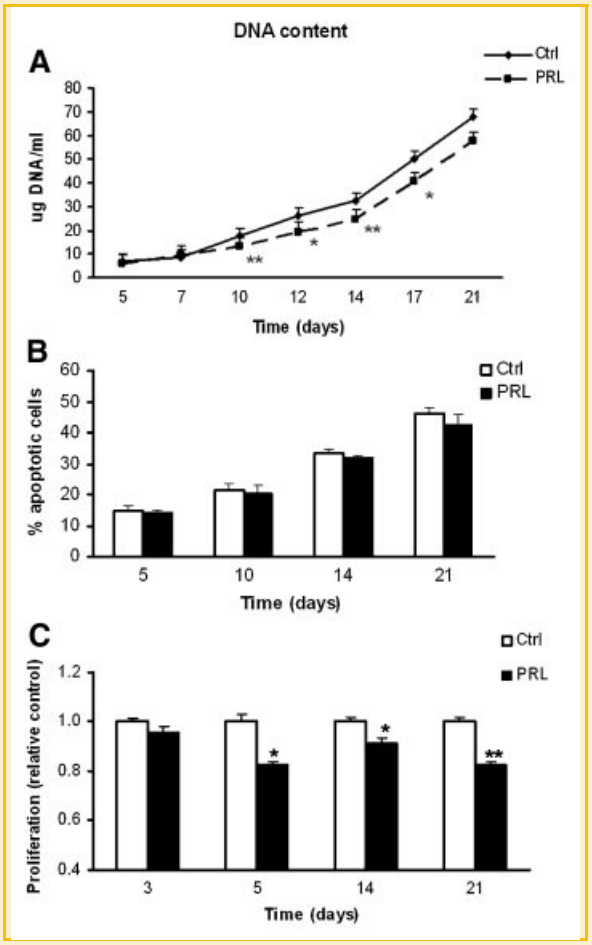

Fig. 4. Effect of PRL treatment on total cell number as reflected by (A) total DNA content, (B) apoptosis, and (C) proliferation. Data shown are mean of two independent cultures performed in triplicate. $^{*}P < 0.05$, and $^{**}P < 0.01$ compared with their respective values of control group.

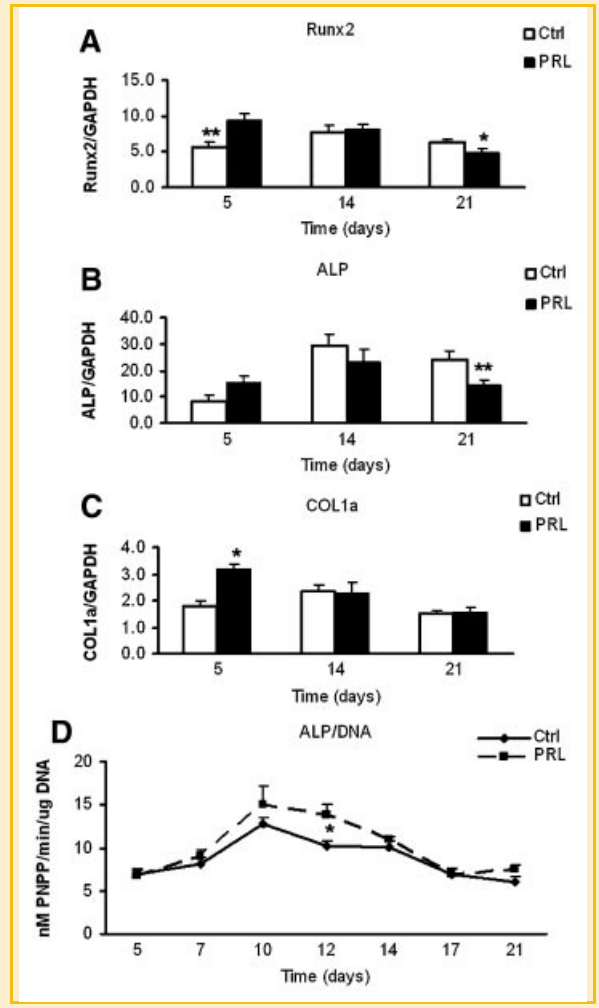

Fig. 5. Effect of PRL on osteoblast differentiation as presented by RUNX2 (A) ALP (B), collagen Type Ia; COL Ia (C) mRNAs expression and ALP/DNA (D). Data shown are mean of two independent cultures performed in triplicate. $^{*}P < 0.05$, and $^{**}P < 0.01$ compared with their respective values of control group.

DISCUSSION

The current study demonstrates for the first time direct effects PRL on human osteoblast differentiation. PRL treatment had a bi-directional effect on osteoblast differentiation and bone formation. PRL reduces human osteoblast proliferation while accelerating its differentiation. However, this acceleration is not accompanied by an enhanced mineralization. Apparently, the accelerated differentiation did not lead to a condition favorable for mineralization. An explanation for this yet elusive but the decrease in total protein may be linked to an insignificant matrix protein production.

Recently, an increased understanding has been acquired with respect to the therapeutic treatment of hyperprolactinemic patients. However, the role of prolactin in the regulation of bone remodeling and the associated bone loss remains largely unclear. Of note was our previous demonstration that PRL receptor is expressed in human fetal osteoblast (hFOB 1.19), osteoblast-like cell (MG-63) and primary rat osteoblast derived from mesenchymal stem cells [Seriwatanachai et al., 2008a,b]. Also other evidence provided osteoblast as a new target of PRL. Deformed skeletal development in PRL receptor knock out pup suggesting an importance role of PRL for maintaining fetal bone development [Clement-Lacroix et al., 1999]. However, so far no data were available on direct effects of PRL on human fetal osteoblast differentiation. We demonstrated hPRLR expression at all stages during differentiation of the human pre-osteoblast cell line, SV-HFO. This model produces an extracellular matrix during culture which eventually is mineralized in 2–3 weeks time period [Chiba et al., 1993; Eijken et al., 2006] therefore, it is suitable for studying PRL effect on human osteoblast differentiation and in vitro bone formation. We presented that in vitro administration of PRL result in a reduction of osteoblast growth. The observation that in PRL-treated osteoblast shows a declinement of osteoblast number as represented by DNA content in a time-dependent manner (Fig. 4A). This finding suggested

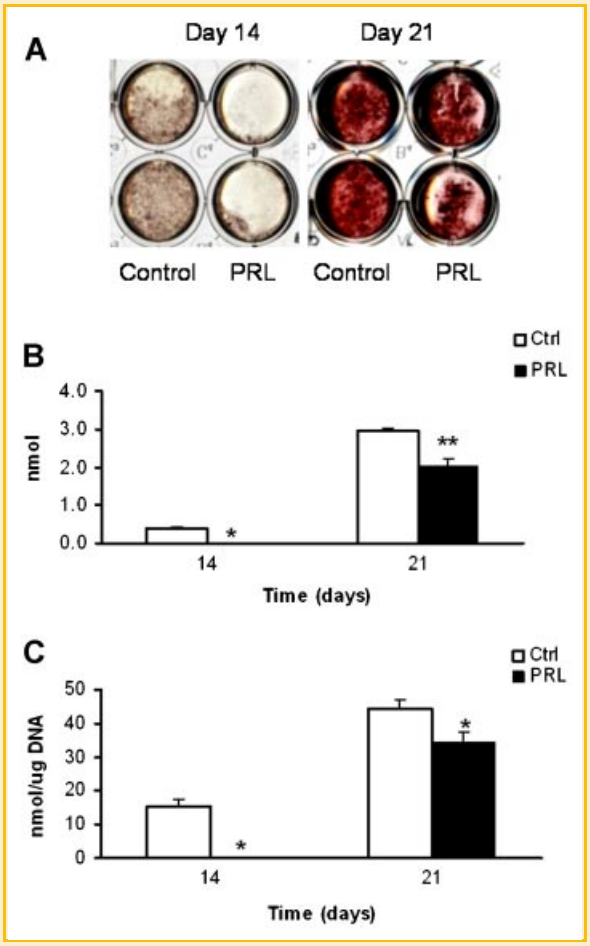

Fig. 6. The effect of PRL on mineralization as presented by mineral Alizarin Red staining (A), total Ca^{+2} (B), and Ca^{+2}/DNA (C). Data shown are mean of two independent cultures performed in triplicate. $^{*}P < 0.05$, and $^{**}P < 0.01$ compared with their respective values of control group. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

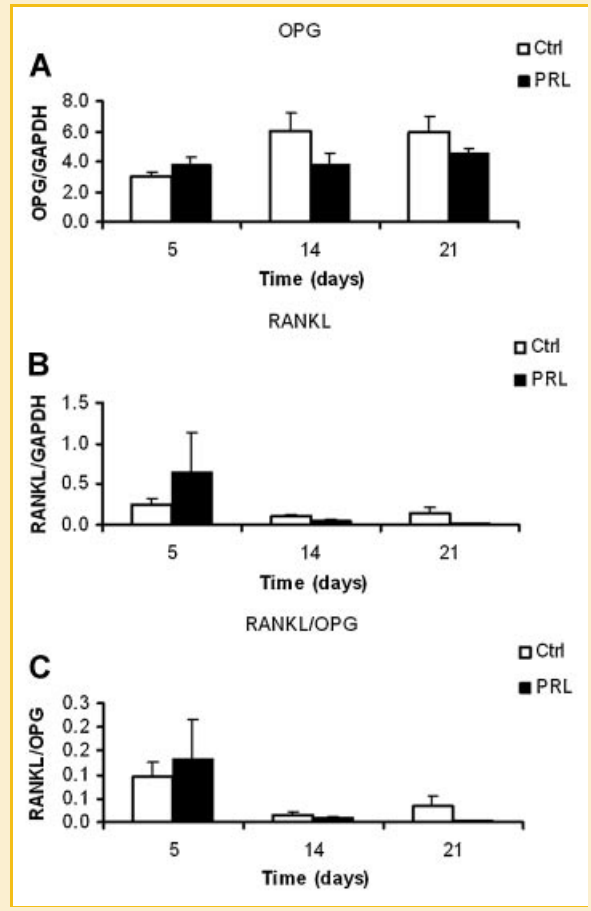

Fig. 7. The effect of 100 ng/ml PRL on the bone resorption-associated parameters which are mRNA expressions of OPG (A) RANKL (B) and the ratios of RANKL/OPG mRNA (C). SV-HFO was exposed to a osteogenic medium (open bar) or with PRL (close bar) for 5, 14, and 21 days. Data shown are mean of two independent cultures performed in triplicate.

that the downregulation of osteoblast quantity is enhanced by PRL action. Therefore, in the next part of the study, we checked whether the reduction of osteoblast quantity regarding the effect of PRL on osteoblast number also could be indirectly manifested through an induction of spontaneous apoptosis or reduction of its proliferation. Indeed, PRL-treated osteoblasts were found reduced proliferation at early phase of osteoblast differentiation, without enhancing a number of apoptosis in PRL treated group. Interestingly, in the present experiment of which tested between vehicle and PRL treated group, and previous observation has found that PRL has no effect on osteoblast-like cell and rat osteoblast proliferation, however, in shortterm PRL administration and in non-osteogenic medium [Coss et al., 2000; Seriwatanachai et al., 2008a,b]. Thus it could simply suggest that change in osteoblast proliferation in bone development influenced by PRL was occurred only in osteogenic medium and in prolonged administration of PRL.

Extensive collagen degradation during a tissue-turnover processes in variety of biological and physiological provides a dynamic cellular activity including proliferation [Hotary et al., 2003; Mott and Werb, 2004; Chun et al., 2006]. Recently, a combined deficiencies in uPARAP/Endo180, a cellular collagen receptor, and MT1-MMP, a principal mesenchymal cell collagenase, mice found to have a severe effect on bone formation and that caused by a proliferative failure and poor survival of bone- and cartilage-forming cells suggesting a necessary of collagen degradation for osteoblast proliferation [Wagenaar-Miller et al., 2007]. In fact, we observed a augmented collagen type I mRNA expression as affected by PRL at early stage of differentiation (d5) which could considerably propose that it could inhibit the early osteoblast proliferation resulting in a certain reduction in DNA accumulating content along culturing period.

The central regulator of bone formation is the Runx2 (also known as Cbfa1/AML3). This transcription factor is required for osteoblastic fate determination, and mediated the complex pathways required for bone formation and turnover [Lian and Stein, 2003; Lian et al., 2004]. We showed that RUNX2 and ALP mRNA were reduced in PRL-treated group in late differentiation suggesting a decrease in

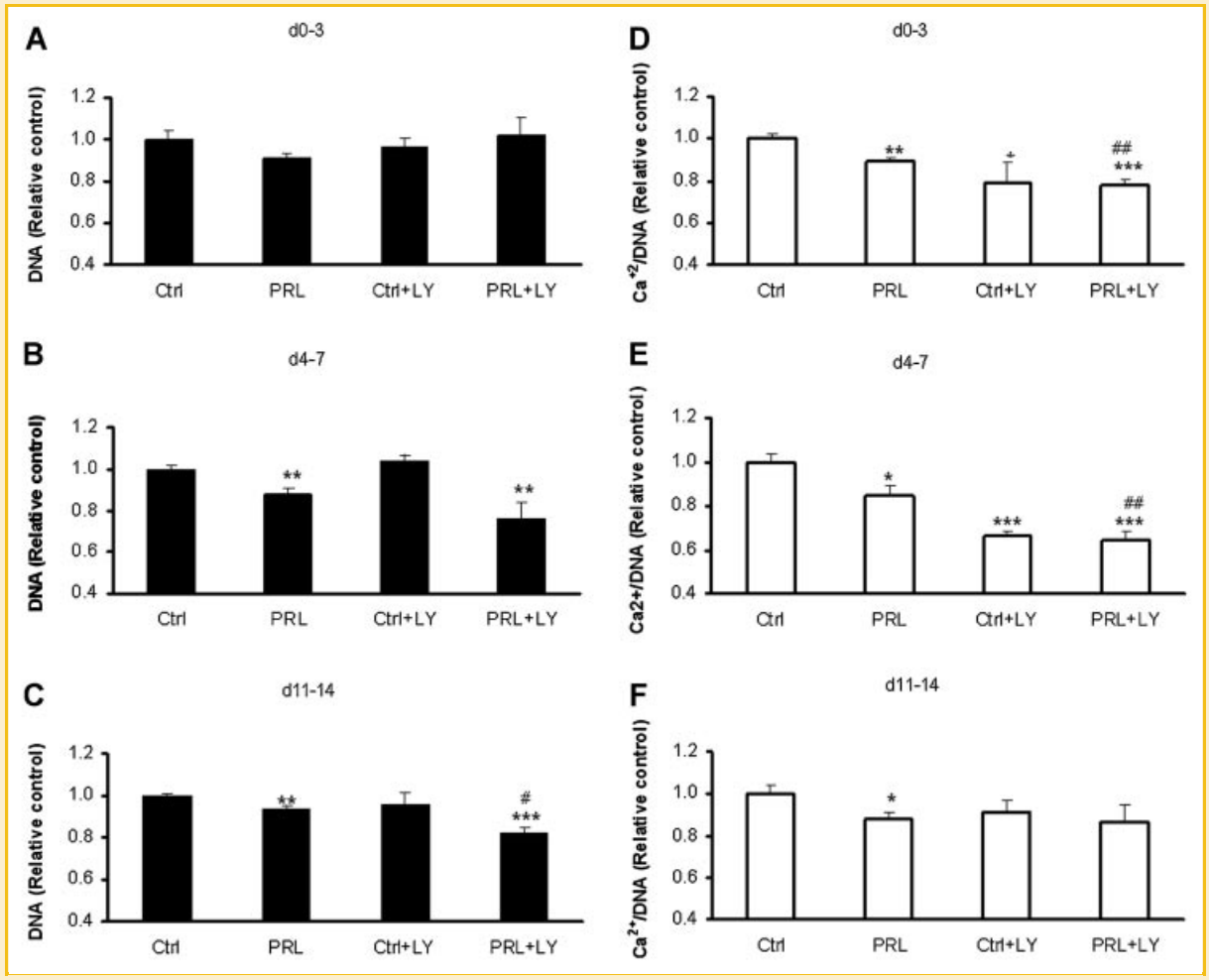

Fig. 8. The combined action of PRL and PI-3 kinase inhibitor (LY294002) on DNA (A–C) and Ca^{2+}/DNA (D–F). LY294002 was added into osteogenic medium with and without PRL at d0–3 (A,D), d4–7 (B,E), and d11–14 (C,F). All plates were harvested at d14 to determine DNA content and Ca^{2+}/DNA. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ compared with control group, $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$, compared with PRL group. Data shown are mean of two independent cultures performed in triplicate.

pre-osteoblast differentiation and bone formation. Albeit this should be investigated more detail as RUNX2 has been shown to exert a complex role in osteoblast differentiation, crucial for osteoblast differentiation in early stages but inhibitory in later stages [Liu et al., 2001; Maruyama et al., 2007]. A number of experiments also observed the reduction of osteocalcin, ALP mRNA effected by PRL [Coss et al., 2000; Seriwatanachai et al., 2008b], consistent with our present finding. ALP activity remained unchanged which is possibly due to a reduction of DNA and protein content. Regardless of the reduction of total protein, we similarly found a decrease in its total activity (data not shown), indeed, we showed a significant reduction of ALP mRNA expression. Interestingly, RUNX2 and ALP mRNA expression was found to be enhanced in PRL-treated group at early stage of differentiation (d5). This observation is consistent with the recent report that shortterm PRL administration increases osteocalcin mRNA, a marker of late osteoblast differentiation, while reducing RANKL/OPG ratio in human osteoblast derived from fetal bone tissue favors osteoblast differentiation and bone formation [Seriwatanachai et al., 2008a]. This contradictory finding implies a bi-directional effect of PRL regarding to the stage of osteoblast differentiation. However, the different incubation period, PRL level and medium condition should be also taken into account.

The next step of experiment was concerned about the role of PRL on mineralization since some evidence found a contradictory action according to different model and technical approaches. We observed a decrease in Ca^{2+} content by biochemical assay, and as shown by Alizarin Red staining, which is truly resulted from a reduction of Ca^{2+} content, not secondary to the decrease in DNA content as it is proved by the reduction of Ca^{2+}/DNA content. Indeed, a reduction of bone ossification and mineralizing rate in PRL receptor knockout murine embryo and 8-week-old mouse [Clement-Lacroix et al., 1999] supports a significance role of PRL in fetal and adult bone mineralization.

Up to date, they have been stated of knowledge concerning signal transduction of the PRLR. Nb2 cells are some of the favored models with which to study PRL actions, as shown that PRL induces a rapid tyrosine phosphorylation of IRS-1 and of the 85-kDa subunit of the phosphatidyl-inositol (PI)-3 kinase [al Sakkaf et al., 1996; Berlanga et al., 1997]. Both PI-3 kinase and IRS-1 appear to associate with the

PRLR in a PRL-dependent manner. It has been proposed that a proliferative effect of PRL activation of PI-3 kinase might be mediated by Fyn in Nb2 cells [al Sakkaf et al., 1997] and beta-cell line [Hugl and Merger, 2007]. We previously demonstrated that a certain concentration of PI3 kinase inhibition reverses PRL reduced ALP activity without effecting cell survival in MG-63 and hFOB suggesting PI3 kinase is one of potential PRL pathways in human osteoblast [Seriwatanachai et al., 2008a]. However, the present study found that shortterm administration of PI3 kinase inhibitor did not reverse the inhibitory action of PRL on DNA and Ca^{2+} content suggesting PI3 kinase involved in bone mineralization and osteoblast number is independent to PRL action. Noted, the induction of apoptotis according to the inhibition of PI-3 kinase was ruled out by optimizing the concentration of LY294002, and was not shown to decrease DNA content. However we found that the combination of PRL and PI3 kinase inhibitor constantly induced a decreased in DNA content in d4–7 and d11–14 but not d0–3 confirming the action of PRL on DNA content is not via PI3 kinase pathway. Moreover, data from Ca^{2+} content demonstrated that shortterm PI3 kinase inhibition at proliferative not mineralizing stage is sufficient for inhibiting the mineralization where as the osteoblast number remains unchanged. This time specific manner can also be found in glucocorticoid (dexamethasone) action of which is capable to decrease bone mineralization once cell have not entered to osteoblast differentiation pathway [Eijken et al., 2006].

The results also demonstrated unchanged in OPG, a decoy receptor of RANKL, and RANKL mRNA expression in osteoblast at entire stage of differentiation leading to non-significance RANKL/OPG ratio. Unlikely, the results from osteosarcoma showed that PRL increases RANKL/OPG ratio after administrating PRL in nondexamethasone medium for shortterm incubation. It, however, suggested the cell type specificity and differentiation dependence. Besides, a number of in vivo studies reported the diverse effect of PRL on bone remodeling in which depends on age of animal, weight bearing and connective structure [Krishnamra and Cheeveewattana, 1994; Krishnamra and Seemoung, 1996; Krishnamra et al., 1997]. The combined dimerization of PRL receptor isoforms through its action on bone development and osteoblast differentiation also is not ruled out. Indeed, evidences show that PRL receptor may be crossly combined among isoforms in which gives variety biological effects in a number of kinds of cell [Perrot-Applanat et al., 1997; Qazi et al., 2006]. In conclusion, despite the issues discussed above, the observations of the present study demonstrate that a prolonged incubation of PRL can have an inhibitory effect on osteoblast number, mineralization and differentiation as altered by a reduction of bone precursor marker.

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