THE EFFECTS AND MECHANISM OF PROLACTIN ON BONE REMODELING

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THE EFFECTS AND MECHANISM OF PROLACTIN ON BONE REMODELING

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ABSTRACT

Hyperprolactinemia-induced osteoporosis is believed to be caused 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. More recently, the presence of prolactin (PRL) receptor in human osteosarcoma cell lines and primary osteoblasts supported the hypothesis of a direct PRL effect 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.

MG-63 (as a model of osteoblasts from adult human) and hFOB (as a model of osteoblasts from human fetus) were used to study the effect of PRL at concentrations which mimic the physiological levels in non-pregnant women (1, 10 ng/ml), lactating period (100 ng/ml) and pathological hyperprolactinemia (1000 ng/ml) on osteoblast proliferation, differentiation and activity. The results showed that high doses of PRL i.e., 100 and 1000 ng/ml, significantly decreased MG-63 activity as indicated by alkaline phosphatase activity and differentiation as shown by osteocalcin mRNA expression, while having no effect on cell proliferation. Furthermore, the osteoclastogenic function of MG-63 was stimulated by a reduction of OPG, the decoy receptor of RANKL, and an increase of RANKL, resulting in a higher RANKL/OPG ratio, as indicator of bone resorption. In hFOB, only at 1000 ng/ml PRL significantly decreased the alkaline phosphatase activity and mRNA expression, while PRL at 100 and 1000 ng/ml significantly enhanced osteoblast cell differentiation. The osteoclastogenic function of osteoblast was suppressed as indicated by a decrease in the ratio of RANKL/OPG, both mRNAs and proteins. Furthermore, by using the inhibition of PI3 kinase, LY294002, and ALP activity as a target action of PRL, the present study demonstrated that PI3K was involved in PRL signal transduction in both MG-63 and hFOB.

In conclusion, PRL had different effects on human adult and fetal osteoblasts. PRL suppressed differentiation and stimulated osteoclastogenesis in adult osteoblasts, while enhancing cell osteoblast differentiation and suppressing osteoclastogenesis in young osteoblast. The intracellular signaling pathway of PRL action on human osteoblasts was involved in PI3 kinase.

KEY WORDS: PROLACTIN/ OSTEOBLAST/BONE REMODELING
109 pp

ผลและกลไกการออกฤทธิ์ของโพรแลคตินต่อการสร้างและสลายกระดูก (THE EFFECTS AND MECHANISM OF PROLACTIN ON BONE REMODELING)

ดุษมณี เสรีวัฒนาชัย 4637351 SCPS/D

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คณะกรรมการควบคุมวิทยานิพนธ์: นทีทิพย์ กฤษณามระ, ปร.ด, ตวงพร สุทธิพงษ์ชัย, ปร.ด, สมนึก ดำรงกิจชัยพร, พ.บ., สินี ดิษฐาบรรจง, พ.บ.

บทคัดย่อ

การลดลงของมวลกระดูกอันเนื่องมาจากภาวะโพรแลคตินสูงในเลือดส่วนใหญ่เชื่อว่ามาจากผลของภาวะฮอร์โมนเพศต่ำแต่มีรายงานพบการลดลงของมวลกระดูกในผู้ป่วยภาวะโพรแลคตินสูงมีฮอร์โมนเพศปกติ นอกจากนี้มีรายงานพบว่าตัวรับโพรแลคตินที่เซลล์มะเร็งกระดูกและเซลล์ปกติของหนู ซึ่งแปลว่าโพรแลคตินอาจออกฤทธิ์โดยตรงต่อเซลล์กระดูกได้ ดังนั้นงานวิจัยนี้จึงจะศึกษาผลและกลไกการส่งสัญญานของโพรแลคตินต่อการสร้างและสลายกระดูก

การวิจัยใช้เซลล์ MG-63 (เลียนแบบเซลล์สร้างกระดูกในผู้ใหญ่)และ hFOB(เลียนแบบเซลล์สร้างกระดูกในทารก)เลี้ยงในสารละลายที่มีโพรแลคตินเลียนในความเข้มข้นคล้ายในภาวะร่างกายได้แก่ ระดับปกติ: 1,10 นก./มล., ขณะให้นม: 100 นก./มล. และภาวะพยาธิสภาพเช่นมะเร็งต่อมพิทูอิทารี: 1000 นก./มล. พบว่าในเซลล์MG-63 โพรแลคตินที่ 100 1000 นก./มล.มีผลลดการทำงานของเซลล์และการแสดงออกของยีนส์ออสติโอคาลซินซึ่งเป็นยีนส์บ่งชี้ภาวะการเจริญเต็มที่ของเซลล์(differentiation) ทั้งยังเพิ่มความสามารถในการกระตุ้นการเจริญเติบโตของเซลล์สลายกระดูกโดยลดการแสดงของยีนส์โอพีจี (ตัวรับหลอกของแรงส์ไลแกนด์) ในขณะเดียวกันก็เพิ่มแรงส์ไลแกนด์ทำให้สัดส่วนของแรงส์ไลแกนด์ต่อโอพีจีสูงขึ้นซึ่งจะมีผลต่อการกระตุ้นการสลายกระดูก ส่วนในเซลล์ hFOB ซึ่งเหมือนเซลล์กระดูกในทารก โพรแลคตินที่1000 นก./มล.ลดการทำงานของเซลล์ขณะที่โพรแลคตินที่ 100 และ 1000 นก./มล. เพิ่มการแสดงออกของยีนส์ออสติโอคาลซินแต่ลดความสามารถในการกระตุ้นการเจริญเติบโตของเซลล์สลายกระดูกโดยลดสัดส่วนของแรงส์ไลแกนด์ต่อโอพีจีทั้งในระดับการแสดงออกของยีนส์และโปรตีน นอกจากนี้การวิจัยยังพบว่าโพรแลคตินออกฤทธิ์ที่เซลล์กระดูกโดยยส่งสัญญานผ่านกลไกฟอสโฟอิโนสิไทด์ 3-ไคเนส

จากผลการทดลองสามารถสรุปได้ว่า โพรแลคตินออกฤทธิ์ต่อเซลล์สร้างกระดูกแตกต่างกันในเซลล์สร้างกระดูกจากผู้ใหญ่ โพรแลคตินยับยั้งการแสดงออกของยีนส์บ่งชี้ภาวะเจริญเต็มที่ของเซลล์แต่เพิ่มความสามารถในการกระตุ้นการเจริญเติบโตของเซลล์สลายกระดูกจึงคาดว่าจะมีผลกระตุ้นการสลายกระดูก ส่วนในเซลล์สร้างกระดูกจากทารกโพรแลคตินกระตุ้นแสดงออกของยีนส์บ่งชี้ภาวะเจริญเต็มที่แต่ยับยั้งความสามารถในการกระตุ้นเซลล์สลายกระดูก ทำให้มีผลลดการสลายกระดูก ส่วนการออกฤทธิ์ของโพรแลคตินที่เซลล์กระดูก ใช้กลไกการส่งสัญญานทางฟอสโฟอิโนสิไทด์ 3-ไคเนส

109 หน้า

CONTENTS

CONTENTS (Cont.)

LIST OF TABLES

LIST OF FIGURES

LIST OF FIGURE (Cont.)

LIST OF FIGURE (Cont.)

LIST OF ABBREVIATIONS

$1,25(OH)_2D_3$	1alpha,25-dihydroxyvitamin D_3
%	Percent
α	Alpha
β	Beta
δ	Delta
ε	Epsilon
γ	Gamma
λ	Lambda
μ	Micro
$\mu A. cm^{-2}$	micro-ampere per centimeter-square
μM	micromolar
θ	Theta
ALP	alkaline phosphatase
ANOVA	analysis of variance
AP-1	activator protein-1
bp	base pairs
BMP	bone morphogenic protein
BSP	bone siaoloprotein
°C	degree celsius
Ca^{2+}	calcium ionized form
Ca^{2+} ATPase	calcium-adenosine triphosphatase
CATK	Cathepsin K
cDNA	complementary deoxyribonucleic acid
Cl^-	chloride ionized form
cm	Centimeter

LIST OF ABBREVIATIONS (Cont.)

cm2	Centimeter-square
CO2	carbon dioxide
Col-I	Collagen type I
Col-II	Collagen type II
Col-X	Collagen type X
CRH	Cytokine receptor homology
Cys	cysteine amino acid
DA	Dopoamine
DAG	diacyl glycerol
DEX	Dexamethasone
DNA	deoxyribonucleic acid
DMSO	Dimethyl sulfoxide
E	Exon
ECL	Enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
et al.	and coworkers
h	Hour
H	Hormone
H^+	hydrogen ion
FBS	fetal bovine serum
g	Gram
GH	growth hormone
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
PRL	Prolactin
PRLR	Prolactin receptor

LIST OF ABBREVIATIONS (Cont.)

i.e.	Including
IGF	insulin-like growth factor
IL	Interleukin
JAK	janus kinase
JAM	Junctional adhesion molecule
K+	Potassium ion
kb	kilo-base pairs
kDa	kilo-Dalton
LY294002	2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one
M-CSF	macrophage colony stimulating factor
MEM	Minimum essential medium
mg	Milligram
mg/day	Milligram per day
min	Minute
mM	millimolar
mRNA	messenger ribonucleic acid
mV	Millivolt
n	number of samples
nM	nanomolar
Na^+	Sodium
Na^+/K^+-ATPase	sodium-potassium-adenosine triphosphatase
NCX	sodium-calcium exchanger
ng/mL	nanogram per milliliter
nM	nanomolar
OC	osteocalcin
OD	optical density

LIST OF ABBREVIATIONS (Cont.)

OPG	osteooptegerin
Osx	Osterix
PBS	Standard phosphate buffer solution
PGE	Prostaglandin
PRL	Prolactin
PRLR	Prolactin receptor
r2	coefficient of determination
	correlation coefficient
R	Receptor
RANKL	Receptor activator of NF-kB ligand
RANK	Receptor activator of NF-kB
rhPRL	recombinant human prolactin
RIPA	radioimmunoprecipitation assay
ROCK	rho-associated coiled-coil-containing protein kinase
S	Second
SDF-1	stromal cell-derived factor 1
SDA-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SE	Standard error
Ser	serine amino acid
SGLT	sodium-glucose cotransporter
siRNA	small interference ribonucleic acid
STAT	signaling transducer and activator of transcription
TGF	transforming growth factor
TNF	tumor necrosis factor
TRAP	tartrate-resistant acid phosphatase

LIST OF ABBREVIATIONS (Cont.)

TRANCE	TNF-related activation induced cytokine
Tyr	Tyrosine amino acid

CHAPTER I
INTRODUCTION

Hyperprolactinemia caused by tumors, antipsychotic drugs, or prolonged lactation, was associated with alteration in bone physiology in both animals and human. Women with hyperprolactinemia resulting from pituitary adenomas have been found to have a 17% decrease in cortical (Klibanski et al., 1980) and a 15-30% decrease in trabecular bone density (Cann et al., 1984; Koppelman et al., 1984; Schlechte et al., 1987). Similarly, hyperprolactinemia in men results in osteoporosis, significant loss of bone mineral density in forearms and vertebrae (Greenspan et al., 1986). Rat with chronic hyperprolactinemia demonstrated a reduction in bone density (Adler et al., 1998) and 18% reduction in vertebral calcium content (Krishnamra et al., 1994). Schizophrenic patients treated with antidopaminergic drugs were found to have more than 5 fold serum prolactin (PRL) increase which was associated with a 14% reduction in forearm bone mineral density compared to the age-and sex-matched controls (Marken et al., 1992; Borison et al., 1996).

Another line of evidence came from investigation of physiological hyperprolactinemia and the associated reduction in bone mineral density in prolonged lactation (Cross et al., 1995; Lopez et al., 1996; Krebs et l., 1997; Tojo et al., 1998). This association appeared to be mediated by hypogonadism resulted from sustained PRL elevation (Greenspan et al., 1989; Schlechte et al., 1995; Halbreich et al., 1995). However, Biller and his group (1991) reported that the severity of bone loss in hyperprolactinemic women with normal estrogen levels was significantly less than that in hyperprolactinemia with estrogen deficiency suggesting that hyperprolactinemia-induced bone loss might not be entirely due to lack of estrogen. This proposal was recently supported by a report of severe osteoporosis in a middle-aged schizophrenic patient treated with antipsychotic (antidopaminergic) drug, who had hyperprolactinemia as the only risk factor for his osteoporosis (Meaney and Keane,

2003). Moreover, other studies showing the presence of PRL receptors in human osteosarcoma cell line (Saos-2 and MG-63) and primary bone cell culture from mouse calvariae (Bataille-Simoneau et al., 1996; Clement-Lacroix et al., 1999; Coss et al., 2000) futher supported the hypothesis that PRL also had a direct effect on bone cell.

More evidence on possible relationship between PRL and bone metabolism was provided by experiments in animals. PRL receptor (PRLR) knockout mice were found to have a decrease in mineral apposition rate in the trabeculae and cortical long bone and a 60% decrease in bone formation rate measured by bone histomorphometry (Clement-Lacroix et al., 1999). These data supported the hypothesis that PRL was necessary for normal bone formation and maintenance of bone mass. In addition, Coss and his group (Coss et al., 2000) who studied the effects of PRL on osteoblasts in developing rat pups reported that PRL reduced calvarial bone thickness, endochondral ossification and bone alkaline phosphate. On the other hand, our laboratory found from the in vivo long term study that PRL increased bone turnover with a net gain in calcium content in tibia of young rats (Krishnamra et al., 1994), whereas the PRL-induced increase in bone turnover in adult rats resulted in net bone loss (Krishnamra and Seemoung, 1996). These conflicting results from experiments using different techniques and different age groups of animals raised a number of questions regarding PRL actions on bone cell metabolism. The major question was whether reduction in bone density was also due to a direct action of PRL on bone itself besides an effect secondary to hyperprolactinemia-induced hypogonadism. Furthermore, if PRL did have a direct effect on bone cells, its signal transduction pathway(s) would need to be elucidated.

Hypothesis

PRL could regulate bone remodeling by acting directly on the osteoblast.

Objective

To elucidate the role of PRL and its signal transduction pathway (s) in the regulation of bone remodeling by using two human cell models, MG-63 and hFOB.

Specific objectives

1. To verify the presence of PRLR in osteoblasts
2. To examine the effect of PRL on the osteoblast proliferation, osteoblast differentiation and osteoblast activity
3. To evaluate the action of PRL on bone resorption by determining the production of RANKL and osteoprotegerin
4. To elucidate the intracellular signaling pathway of PRL action in osteoblasts

CHAPTER II
LITERATURE REVIEW

A. Prolactin (PRL)

PRL is a polypeptide hormone which contains 199 amino acids with a total molecular mass of 23 kDa, its gene encoding is located on chromosome 6. PRL is present in all vertebrates and DNA encoding PRL from several species have been isolated and sequenced (Cooke et al., 1980 and 1981; Nicoll et al., 1986; Miller et al., 1981) with the exception of fish, all PRL identified consist of 197-199 amino acids, with cysteines forming three intramolecular disulphide bonds (Rentier-Delrue et al., 1989). PRL identity is variable among species, Bovine PRL and human PRL share 74 % identity, whereas carp and human PRL share only 36 % of similarity which is probably due to the posttranslational modification of mature PRL including glycosylation, phosphorylation or proteolytic cleavage (Sinha et al., 1995; Walker et al., 1994). PRL is synthesized and secreted from the anterior pituitary cells called lactotrophs, which make up 10 to 25 % of the pituitary cell population. Synthesis of PRL starts from prehormone, the N-terminal signal peptide of which is cleaved and transient glycosylation takes place before the compound arrives in the golgi apparatus, where the hormone molecules are destined for storage or release.

A1. PRL secretion

Prolactin is continuously secreted at low basal rate throughout life (Table 1). The secretion is increased at night during sleep with the first peak appearing 60 -90 minutes after an onset of slow-wave sleep. Consistent with its essential roles in lactation, PRL secretion increases steadily during pregnancy and remains high during lactation (Table 1).

The PRL production and secretion can be controlled by chemical, emotional and physical stimulation. For physical stimulation, suckling is a potent stimulator of PRL secretion.

Table1: Normal values of serum PRL in human (Masaoka et al., 1984; Soldin et al., 1995) and female rats (Boass et al., 1992).

Pathological and Physiological conditions	Concentration (ng/ml)
Human	
Newborn (1-7 day)	30-250
Prepubertal child	< 10
Pregnant female, third trimester	100-250
Typical prolactinoma patient	> 100
After 30 min of suckling	300.9 ± 25.9
Female rat	
Diestrus	7.5 ± 3.4
Proestrus-estrus-metestrus	19.7 ± 6.6
Pregnant	115.8 ± 20.3
Lactating	201.3 ± 37.0

PRL is the only pituitary hormone that is secreted at unrestrained high levels when lactrotrophs are completely isolated from the negative trophic influences of the hypothalamus (Lea et al., 1986). Pituitary PRL secretion is under the regulation of hypothalamic hormones such as dopamine (inhibitory) and thyroid releasing hormone (TRH) (stimulatory). Its production and secretion is tonically controlled by the inhibitory effect of dopamine (DA) through the D2 subclass of dopamine receptors present in lactrotrophs ((Melnikova et al., 1998)). This receptor inhibits the activity of adenyl cyclase via coupling to G_i protein. Besides, D2 receptor stimulation activates

K^+ channels and decreases the intracellular concentration of Ca^{2+}. These effects inhibit both the release and synthesis of PRL.

The release of PRL is under the inhibitory dopaminergic tone or from dopamine regulation on anterior pituitary gland. A physical stimulation such as, suckling, can stimulate PRL secretion very rapidly which can produce serum levels up to 650 ng/ml (Arbogast et al., 1998). Other factors such as estrogens also have an indirect effect on PRL secretion which contributes towards growth and development of the mammary gland cells for lactation (Figure1). Consequently, excessive estrogen can induce lactrotroph hyperplasia. Specific receptors for PRL in various cell types and tissues provide diverse effects in various systems, namely, reproduction, osmoregulation, growth, endocrinology and metabolism.

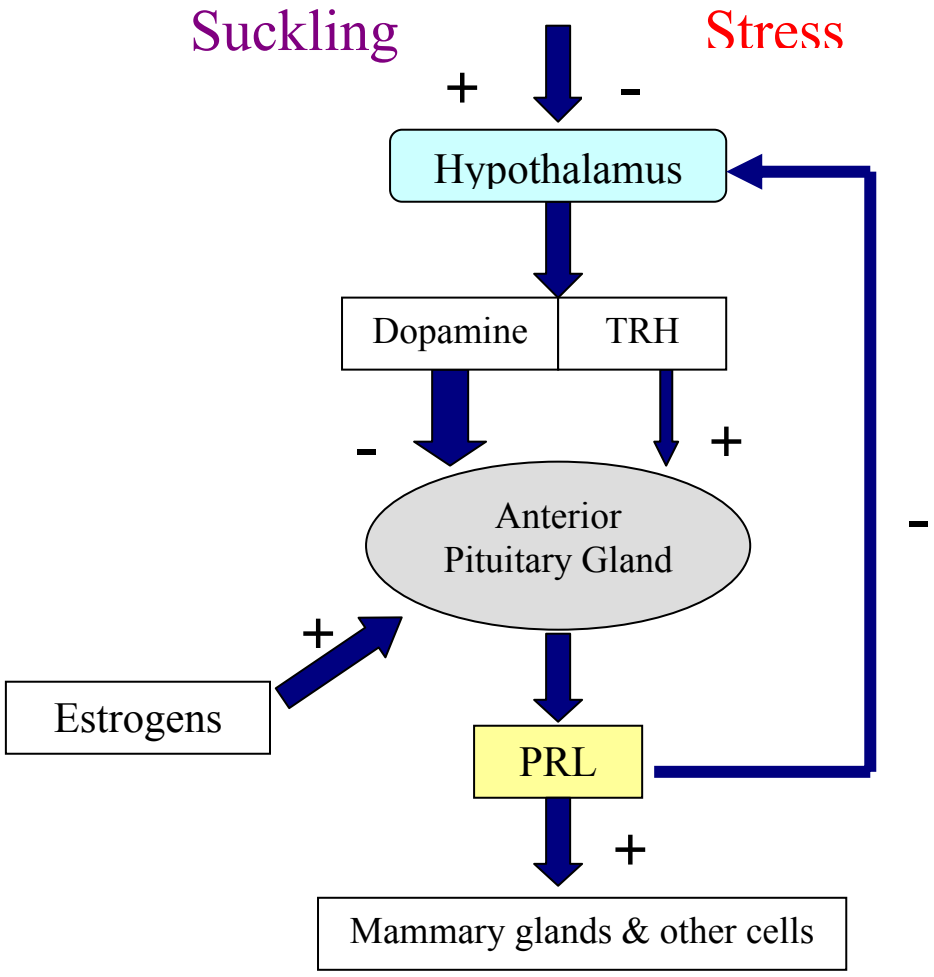


Figure 1: Diagram showing the regulation of PRL secretion (modified from http://cal.man.ac.uk/student_projects/2000/mnby6kas/prl.htm)

A2. PRL receptor (PRLR)

The PRLR was identified more than two decades ago. It is a specific, high affinity and membrane-anchored protein (Posner et al., 1974; Kelly et al., 1974). It is closely related to growth hormone receptor. Both are single-pass transmembrane chains, sharing several structure and functional features (Kelly et al., 1991; Goffin et al., 1996; Ferrag et al., 1998). PRLR is termed as Class I Cytokine Receptors family which includes receptors for several interleukins (Gushchin et al., 1995), granulocyte–colony stimulating factor (G-CSF) and granulocyte macrophage-colony stimulating factor (M-CSF), growth hormone (Gouilleux et al., 1995), and leptin (Feuermann et al., 2004).

The human PRLR gene is encoded on chromosome 5 and its length is around 100 Kb (Arden et al., 1990). PRLR has been classified into 4 isoforms which result from alternative spicing of the primary transcription. These different PRLR isoforms differ in length and the composition of their cytoplasmic tails, and are referred to as the short, intermediate, or long PRLR with respect to their sizes. The rat PRLR isoforms contain 206 (soluble), 291 (short), 393 (intermediate), or 591 (long) amino acids (Figure 2).The extracellular domain is composed of ~ 200 amino acids, referred to as cytokine receptor homology (CRH) regions. The transmembrane domain is a single–pass membrane chain like all cytokine receptors. The intracellular domain contains two regions called box1 and box 2 (Kelly et al., 1991; Murakami et al., 1991). Box 1 is a membrane-proximal region composed of 8 amino acids highly augmented with proline and hydrophobic residues and, is required for JAK2 binding.

The second consensus region, box 2, is much less conserved than box 1 and contains a series of hydrophobic negative charges. While box1 is found in all membrane PRLRs isoforms, box 2 is not found in short isoforms (Kelly et al., 1991; Goffin et al., 1997).

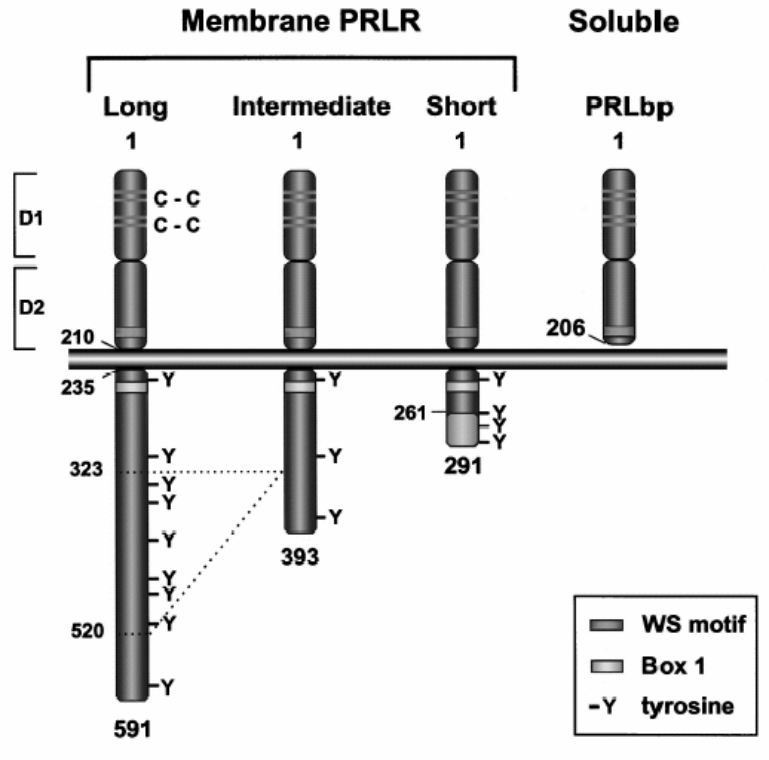


Figure 2: The schematic diagram of the human and rat PRLR (Bole-Feysot et al., 1998) (Box 2 region not shown in this figure).

A3. PRL signal transduction pathway

Upon PRL binding, two PRLR dimerize with at least two regions of the PRLR molecules involved in the binding of the hormone to the PRLR. The activation of PRLR is a ligand-induced sequential receptor dimerization (Figure 3). Firstly, PRL interacts with binding site 1 of monomer PRLR leading to the formation of an inactive complex (H1:R1). The formation of this complex is necessary for PRL binding site 2 to interact with another PRLR. In this way, a trimeric complex (H1:R2), composed of one hormone and one receptors homodimer is achieved.

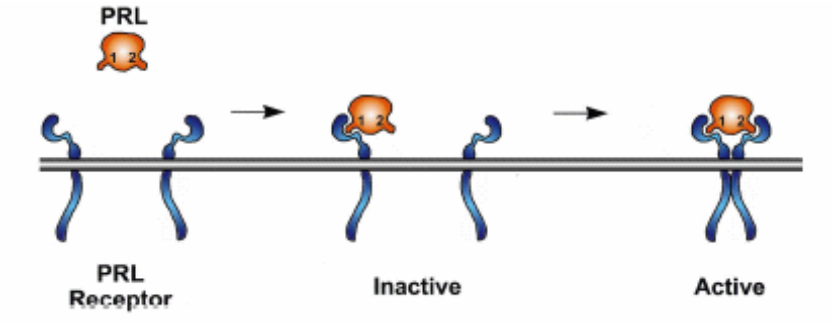


Figure 3: PRL-induced dimerization of PRLRs (Bole-Feysot et al., 1998).

Dimerization causes an activation of cytoplasmic signaling. The signaling transduction is effected via activation of cytoplasmic tyrosine kinase (Janus kinase), mainly JAK2. Activation of JAK2 brings two JAK2 molecules close to each other. Active JAK2 further phosphorylates tyrosine (Tyr) residues of the PRLR, served as the potential binding/docking sites for the adapter or transducer molecules containing SH2 domains (Freeman et al., 2000). Although phosphorylation of JAK2 occurs in all active PRLR isoforms, Tyr phosphorylation of the receptor itself does not occur upon activation of the PRLR-S, despite the presence of four Tyr residues in its intracellular domain (Goupille et al., 1997). After receptor activation, further activation of several intracellular signaling occurs to mediate PRL action in target cells.

As described above, after activation, the phosphorylated JAK2 furthers phosphorylates at a specific Tyr residue in the C-terminus of PRLR. This mechanism occurs only with the PRLR-Long isoform (Ali, 2000). This isoform is the only PRLR form able to activate the JAK2/STAT5 pathway, which is essential for PRL-induced transcription of milk protein genes, differentiation of normal epithelial cells, and initiation and maintenance of lactation. In contrast to the long isoform, PRLR-Short isoform cannot mediate transcriptional activation of the β-casein gene promoter induced by PRL (Lesueur et al., 1991). Instead, this isoform exerts dominant-negative effects on PRL-induced activation of transcription by the PRLR-Long isoform when coexpressed in transfected cells (Hu et al., 2001). PRLR-short isoform was formed to inhibit the PRL-induced long isoform promoting mammary epithelial cell proliferation (Meng et al., 2004). Moreover, it was reported that the PRLR-Short isoform could exert inhibitory effect by forming heterodimer with the long isoform in the ligand-

independent manner (Qazi et al., 2006). Therefore, it has been suggested that the inhibitory role of the PRLR-Short isoform may have physiological significance because the expression of the long and short PRL isoforms of PRLR is regulated in a tissue-specific manner (Lesueur et al., 1991).

The other associated proteins, namely signal transducer and activator of transcription STAT proteins (STAT) are 750-850 amino acid proteins that contain six structurally and functionally conserved domains (Paukku and Silvennoinen 2004). So far, PRL has been shown to regulate the target genes via activation of STAT1, STAT3, STAT4, STAT 5a and STAT5b with STAT5 as the main mediator (Liu et al., 1997; Goffin et al., 1998; Jabbour et al., 1998). After being activated, STAT5 becomes dimerized and the dimer translocates into the nucleus and binds with consensus specific DNA motif called GAS (for γ-IFN-activated sequence) (Horseman et al., 1994; Ihle et al., 1996). Besides JAK/STAT pathway, the other important signaling pathway of PRL signaling transduction is the mitogen-activated protein kinase pathway (MAPKs). Phosphotyrosine residues of the activated PRLR can serve as docking sites for adaptor proteins (Shc/Grb2/SOS) connecting the receptor to the MAPK cascade (Avruch et al., 1994; Erwin et al., 1995; Das and Vonderhaar et al., 1996). Intraperitoneal PRL administration to female rat caused a rapid stimulation of hepatic MAPK activity (Piccoletti et al., 1994). It was referred that MAPK pathway plays an important role in PRL-stimulated mitogenesis, proliferation, differentiation, and survival in PRL target cells, e.g., mammary gland cells (Acosta et al., 2003).

Phosphatidylinositol-3'-kinase (PI3 kinase) is considered as a crucial signal transduction for several cytokine-activated biological processes (Hirsch et al., 2007). There are three classes of PI3K signaling, class I, II, and III that have been identified based on levels of homology of their relevant catalystic subunit. It has been reported that class Ia PI3K is an important signaling pathway of PRL-mediated cellular response of the target cells (Kelly et al., 2001; Clevenger et al., 2003). The PI3K pathway mediates PRL action in tissues such as rat liver, lymphoma, mammary gland cell and duodenal epithelium (Bole-Feysot et al., 1998; Jantarajit et al., 2007). PI3 kinase and a member of the Src kinase family (Fyn) also activate JAK2 in rat

lymphoma cell (Clevenger et al., 1994) and hepatocyte (Berlanga et al., 1995), Figure 4.

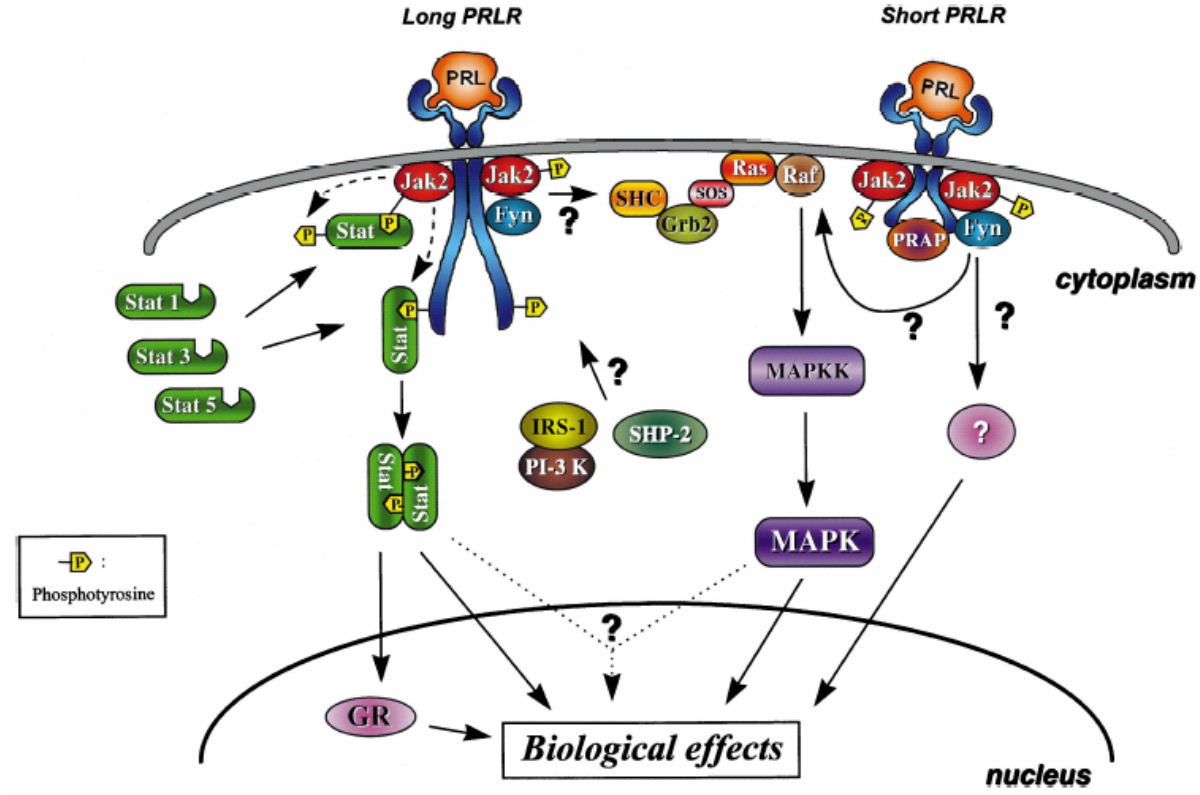


Figure 4: The schematic diagram of the PRL Jak2/Stat5 signal transduction pathways (Bole-Feysot et al., 1998).

The inactive H1:R1 complex versus the active H1:R2 complex formation offers an explanation for the biphasic action of PRL in many cell types. One of the most studied actions of PRL is on the function of testicular steroidogenesis in male reproductive system. In the in vivo study, hyperprolactinemia from pituitary tumor in human and rodent was associated with hypogonadism characterized by testicular atrophy, low serum testosterone and infertility (Bartke et al., 1980). In contrast, Thomus and coworkers (Thomas et al., 1976) studying a leydig cell-enriched rat testicular cell culture found that PRL at low concentration (< 3 ng/ml) increased the gonadotropin-stimulated production of androgen. Previous work in our laboratory also demonstrated the biphasic effect of PRL on duodenal calcium transport. PRL at 200

and 600 ng/ml were found to increase the mucosa to serosa calcium transport 2 to 3 folds, whereas higher concentration of PRL at 800 and 1000 ng/ml returned the flux to a control value (Tanrattana et al., 2004).

B. Bone physiology

Bone is composed of collagen type I (90 %), noncollagenous proteins (10 %) and minerals. It is structurally classified into two types: cortical or compact bone and cancellous or trabecular bone.

The cortical bone forms the middle 80 % of all long bones of the body, including the tibia and fibula in the lower leg, the femur in the upper leg, the radius and ulna in the lower arm, and the humerus in the upper arm. The orientation of the collagen fibers alternates in adult bone from layer to layer, giving a lamellar structure. The density of the cortical bone is four to six times higher than that of cancellous bone.

Cancellous bone, approximately 70 % porous and 15-25 % calcified, contains high vascularization and connective tissues and consists of loosely formed matrix. The function of trabecular bone is mainly for bone remodeling and the buffering of blood pH. Bone can exchange H^+ for Ca^{++}, Na^+ and K^+ (ionic exchange) or release of HCO_3^-, CO_3^- or HPO_4^{-2}. In acute metabolic acidosis, uptake of H^+ by bone in exchange for Na^+ and K^+ release is involved in buffering and this can occur rapidly without any bone loss (Bushinsky et al., 1987).

B1. Bone cells

1.1 Osteoblast

Osteoblast is a bone lining cell which originates from the mesenchymal stem cell (bone marrow stromal cell or connective tissue mesenchymal stem cell). It is responsible for bone formation. Osteoblast produces collagen type I which accounts for 90 % of the total bone proteins and ground substances. The other 10 % of proteins

include osteonectin, osteocalcin and cytokine factors such as TGF-β, OPG, RANKL, M-CSF, the last of which is necessary for growth and differentiation of osteoblast and osteoclast. Under light microscope, osteoblast is seen with a round shaped nucleus at the base of the cell which is opposite to bone surface (Figure 5). The cytoplasm is strongly basophilic, and is seen with prominent golgi complex. Plasma membrane of the osteoblast is rich in alkaline phosphatase, which is used as bone formation marker. The osteoblast has receptors for PTH, 1,25 $(OH)_2$ D_3 and estrogen.

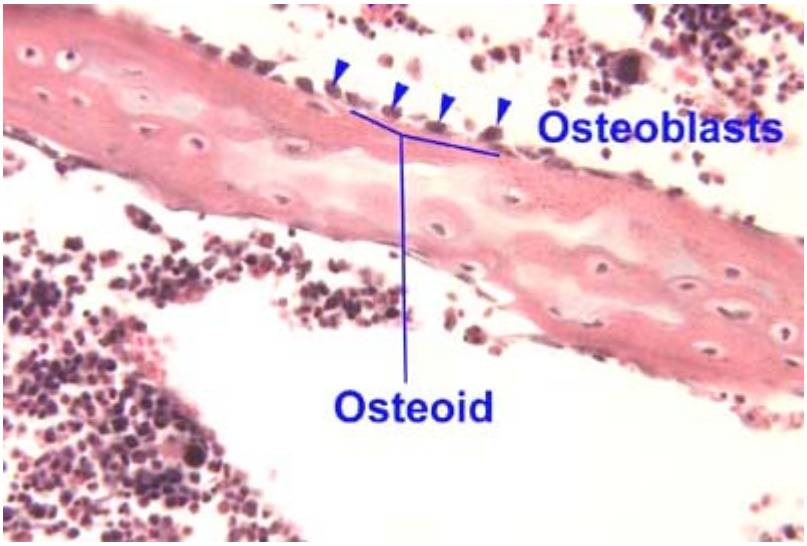


Figure 5: The H&E staining of femur cortical mineralization, blue arrows indicate osteoblasts on bone surface (modified from http://www.answer/topic/osteoid).

Genetic studies in mice have also provided new insights into the transcriptional regulation of osteoblast differentiation. The important transcription factor which regulates phenotype-specific gene expression is A runt-related transcription factor 2 (Runx2), also known as Cbfa1, Osf 2 and AML3 (Ducy et al., 1997; Komori et al., 1997; Otto et al., 1997). It is essential for osteoblast differentiation and is also involved in chondrocyte maturation (Figure 6). Osterix (Osx), another regulator of osteogenesis, (Nakashima et al., 2002) a zinc-finger containing protein, the production of which is induced in myoblasts in response to bone morphogenic protein (BMP).

Multiple transcriptional factors and cofactors can interact with Runx2. AJ18, a zinc finger-containing factor, inhibits Runx activity by competing for its DNA-binding sequence (Jheon et al., 2001). Distal-less homeobox 5 (Dlx5) and msh homeobox homologue 2 (Msx2) are homeobox-containing transcription factors which can be found at an early stage of osteoblast differentiation. They help to maintain normal ossification in murine and human bone structure (Bendall et al., 2000).

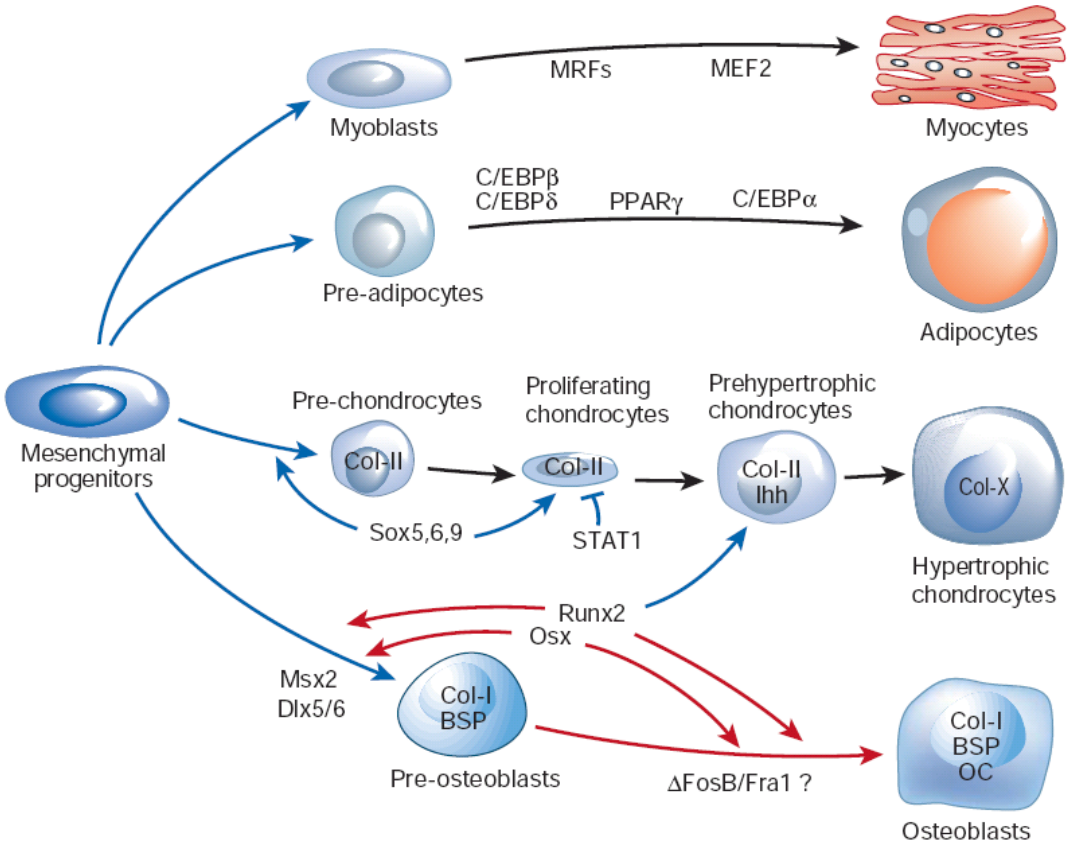


Figure 6: Transcriptional controls of osteoblastic, chondrocytic, adipocytic and myocytic differentiation (Harada et al., 2003).

1.2 Osteocyte

Osteocyte which originates from osteoblast, becomes embedded deep in the calcified bone. The cell has plentiful cell processes that extend through caliculi and come into contact with the processes of other osteocytes and osteoblasts lining cells through gap junctions. The space between osteocyte and bone matrix in lacunae and

canaliculi, called periosteocytic space, is filled with the extracellular fluid (bone ECF). The osteocyte appears to act as a sensor of signaling strain in the skeleton, and releases signaling molecules such as prostaglandins and nitric oxide, which modulate the function of neighboring bone cells. In addition, it has been reported that osteocyte are metabolically and electrically coupled through gap junction protein complexes by connexin 4. Gap junction formation is essential for osteocyte maturation, activity and survival (Schiller et al., 2001; Furlan et al,. 2001; Plotkin et al, 2002).

Lining cells, also of osteoblast origin, are found on cancellous and endocortical bone surfaces. They appear as elongated, flattened cells with darkly stained nuclei. The localization and initiation of remodeling is likely to involve these cells.

1.3 Osteoclast

Osteoclast originates from the hematopoietic stem cells. It is a giant multinucleated cell, formed by fusion of up to twenty cells. Mature osteoclast is around 50 to 100 μm in diameter, with abundant mitochondria, numerous lysosomes and free ribosomes. Multinucleated osteoclasts after being recruited by CSF-1 and RANKL, adhere to bone and undergo differentiation into mature osteoclast. RANKL stimulates osteoclast activation by inducing secretion of protons and lytic enzymes into a sealed resorption vacuole formed between the basal surface of the osteoclast and bone surface.

The most remarkable morphological feature of an active osteoclast is the ruffle border, a complex system of finger-shaped projections of the membrane, which is completely surrounded by another specialized, clear zone. The ruffle border contains bundles of actin-like filaments, which can attach and seal off a distinct area lying underneath the osteoclast. This particular action is necessary for separating the bone-resorbing environment from the surrounding ECF. Acidification of this compartment by secretion of protons (created by proton pump located at the ruffled border membrane) leads to the activation of tartrate-resistant acid phosphatase (TRAP) and cathepsin K (CATK) , which are the two main enzymes responsible for the degradation of bone mineral and collagen matrices together with metalloproteinases and collagenase. The high amount of the phosphohydrolase enzyme, TRAP is

commonly used for the morphological detection of osteoclast in bone specimen (Udagawa et al., 1990).

B2. Bone remodeling

During childhood and the beginning of adulthood, bone becomes larger, heavier and denser by the process of bone modeling, which formation exhibits a higher rate of resorption. Even after bones have attained the adult shape and size in a fully grown body, renewal of bone is essential for maintenance of bone strength throughout life. Old bone is removed (resorption) and new bone is created (formation) all the time, and the process is known as bone remodeling. The **Basic Multicellular Unit (BMU)** is a wandering team of cells that carries on bone remodeling by dissolving a pit in the bone surface and then filling it with new bone. Two main types of cells are responsible for bone renewal: the **osteoblasts** involved in bone formation and the **osteoclasts** involved in bone resorption. On a normal bone surface, the BMU remodeling sequences are as follow.

Origination

After microdamage to bone, or following mechanical stress, or at random, a BMU will originate. Various cytokines and growth factors are released and stimulate the neighboring cells. Once a BMU originates, it travels along the surface of bone.

Osteoclast activation

The activation is an event in the remodeling sequence in which bone surface is converted from origination stage to one in which circulating mononuclear cells of the hematopoetic lineage begin to congregate and fuse together to form differentiated osteoclast. The exact mechanism and specific site of activation are still not understood. One possibility is that osteoclast precursors recognize some changes (mechanical strain, microdamage or hypermineralization) in bone surface and send signals to activate osteoclasts. The final activation of the osteoclast may occur because of interactions that occur between integrins on osteoclast cell membrane with protein in bone matrix that contains RGD (arginine-glycine-asparagine) amino acid sequences such as osteopontin (Miyauchi et al., 1991). Moreover, the lining cells can be

stimulated by endocrine signals such as PTH, PTHrP and 1,25$(OH)_2$ D_3 to generate local paracrine signals for promoting osteoclast differentiation.

Resorption

The mature osteoclasts resorb bone by releasing acid (HCl), lysosomal (TRAPase) and nonlysosomal enzymes (collagenase, gelatinase) into the extracellular bone-resorbing compartment, the area beneath the ruffled border membrane. The residues from this extracellular digestion are either internalized or transported across the cell, possibly induced by calcium sensing receptor, to the bone-resorbing compartment (Dvorak and Riccardi, 2008).

The hydroxyapatite crystals are mobilized by digestion of their noncollagenous proteins, and dissolved by the high acid condition in the bone-resorbing area. The residual collagen fibers are digested either by collagenase or cathepsins at low pH. Since collagen type I is enriched in hydroxyproline and pyridinodine links, the concentrations of hydroxyproline and pyridinodine in the urine and N-terminal collagen peptides in serum can be used as indirect markers of bone resorption in humans.

Osteoblast formation

After resorption, the osteoclasts will be removed, by apoptosis, and this probably attracts osteoblasts. The cytokines and growth factors that are involved in origination and bone-derived growth factors that are released by resorption also attract osteoblasts. The active secreting osteoblasts found lining the layer of bone matrix produce collagen and noncollagen proteins, called osteoid tissue which later becomes calcified. Toward the end of the secreting period, the osteoblasts become either a flat lining cell or an osteocyte.

Mineralization

During bone formation, osteoid begins to mineralize with appearance of about 6 micron thick, spindle or plate shaped crystals of hydroxyapatite ($3Ca_{10}(PO_4)_6$ $(OH)_2$) that tend to be oriented in the same direction as the collagen fibers. The ground substance is primarily composed of proteoglycans and glycoprotein. These high anionic complexes have a high ion-binding capacity and are thought to play an important part in the calcification process and the fixation of hydroxyapatite crystals

to the collagen fiber. The important factor that plays a role in mineralization is vitamin D, which promotes the mineralization of osteoid by maintaining the extracellular calcium and phosphorus concentrations within normal range, which inturn results in the deposition of calcium hydroxyapatite into bone matrix (Holick et al., 1995; DeLuca et al., 1988).

Quiescence

The final osteoblasts turn into surface lining cells which participate in the minute-to-minute release of calcium from bone. Some of the osteoblasts also become osteocytes which remain in the interior of bone, connected by long cell processes which play an essential role in bone adaptation to mechanical stress.

B3. Regulation of bone remodeling

Some of the systemic and local regulators such as PTH, PGE, TGF-β, fibroblast growth factor and RANK ligand, can stimulate both bone formation and resorption through osteoblastic and osteoclastic functions mostly via the cellular pathway called RANKL/OPG pathway.

3.1 OPG and RANKL protein

Osteoprotegerin (OPG) is a peptide of 380 amino acids, that is secreted as a soluble protein from osteoblast. It is a tumor necrosis factor receptor-related protein with mRNA expression in a variety of tissues including kidney, lung, heart, liver, stomach, intestine, thyroid gland, brain and spinal cord, and bone. The potential action of OPG in these other tissues beside bone remains unclear. Major biological role of OPG is to block bone resorption by inhibiting osteoclast differentiation and activity both in vivo and in vitro study.

In the RANKL/OPG pathway, OPG is a decoy receptor of receptor activator of NF-kB ligand (RANKL), blocking RANKL binding to its receptor, RANK on the membrane of osteoclast precursor, thus it consequently blocks bone resorption (Figure 8). OPG overexpression blocks osteoclast production and development, leading to

osteopetrosis in mice, whereas its deletion gives rise to enhanced bone turnover and decrease in bone mass (Wagner et al., 2001; Yasuda et al., 1998).

RANKL is a member of TNF ligand family, originally identified as TNF-related activation induced cytokine (TRANCE). To date, it has been recognized in two forms, a 40 t0 45 kDa cellular membrane-bound form and a 31 kDa soluble form caused by cleavage of full molecules. Activation of RANK by its ligand, RANKL, leads to the expression of specific genes which in turn induces differentiation of osteoclast precursor into mature osteoclasts (Figure7).

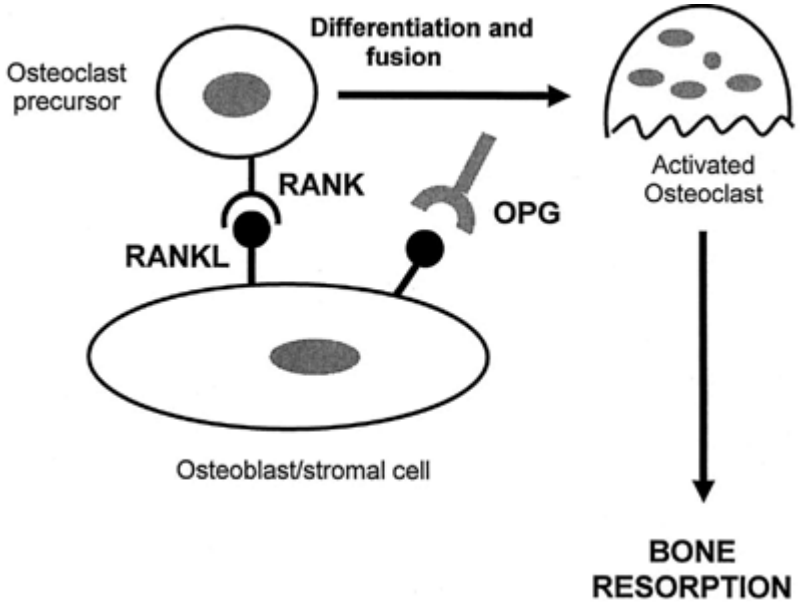


Figure 7: Preosteoblastic/stromal cell regulation of osteoclastogenesis is mediated by the RANK/OPG pathway.(http://medscape.com/viewarticle/479893_2)

The RANKL/OPG ratio is influenced by hormones and cytokines. Treatment of osteoblasts, both cell line and primary cell, with pro-resorptive and calciotropic factors such as 1, 25$(OH)_2$ D3, sustained PTH, PTHrP, prostaglandin E2 [PGE2], and interleukin-1 and -6 (IL-1, IL-6), TNF, PRL and corticosteroid induce up-regulation of RANKL mRNA expression (Udagawa et al., 2000; Schoppet et al., 2002). Conversely, production of OPG can be induced by anabolic or anti-resorptive factors such as

estrogen, calcitonin, bone morphogenetic protein-2 and 4 (BMP 2/4), TGFβ, IL-17, platelet derived growth factor (PDGF) and calcium.

3.2 Hormonal regulation of bone remodeling

3.2.1. Estrogen

The cause of bone loss in postmenopausal osteoporosis is estrogen deficiency (Lindsay et al., 1980), which results in increased osteoclast formation, leading to an imbalance between bone formation and resorption. Estrogen treatment has long been known to inhibit bone loss in postmenopausal women by decreasing bone resorption and increase bone density. The molecular mechanisms of estrogen action on bone are based on the balance between osteoblast and osteoclast. Increased OPG and decreased RANKL mRNA expression lead to less activation of the RANK signaling pathway, thus less activated osteoclasts. There is a large body of evidence to support the regulatory role of estrogen in bone remodeling. For instance, OPG secretion was highest in osteoblasts containing the largest number of estrogen receptors (Hofbauer et al., 1999). In another study, OPG expression by mouse bone marrow stromal cells (precursors of osteoblasts) was upregulated after estrogen treatment. On the other hand, estrogen withdrawal dramatically down-regulated the expression of OPG by these cells (Saika et al., 2001). In addition, estrogens may mediate their effects on osteoclast indirectly by suppressing the production of the bone-resorbing cytokines such as IL-1 and IL-6 (Pacifici et al., 1989; Jilka et al., 1992; Girasole et al., 1992).

3.2.2. Parathyroid hormone (PTH)

Administration of PTH leads to the release of calcium from a rapidly turn over pool for calcium near the surface of bone (TALMAGE & ELLIOTT, 1958). The effect of PTH on osteoblast is mediated through the complex of PTH/PTHrP receptor (Juppner H, 2005). Continuous PTH administration enhanced bone resorption in human, probably via the induction of PGE, SDF-1 and IL-6 acting with or through RANKL to stimulate the differentiation and activity of osteoclasts. In murine bone marrow cultures, PTH stimulates RANKL and inhibits OPG expression, thus lowering

the OPG-RANKL ratio which indicates increase in bone resorption (Horwood et al., 1998; Lee & Lorenzo, 1999).

Intermittent PTH therapy has an anabolic effect on bone by stimulating bone formation. It also increases the proliferation of osteoblast precursors, stimulates the differentiation of sub-populations of osteoblasts, and delays apoptosis of mature, matrix-secreting osteoblasts.

3.2.3. Glucocorticoids

The glucocorticoid receptors are found in most cell types, including bone cells (Beavan et al., 2001) and glucocorticoid response elements (GREs) are present in many genes. In addition, glucocorticoid effects may be mediated both directly and indirectly via GREs and the transcription factor "AP1". Responses to glucocorticoids can also occur by non-genomic mechanisms, involving the glucocorticoid receptor or the steroids molecules themselves (Patschan et al., 2001).

Dexamethasone, a synthetic glucocorticoid, at concentration of 10^{-7} M and 10^{-8} is commonly used to induce human bone marrow stromal cells (BMSC) into differentiated cells exhibiting osteoblast phenotype (Kasugai et al., 1991; Scutt et al., 1996). Glucocorticoids can also modulate the transcription of many of the genes responsible for the synthesis of matrix constituents by osteoblasts, such as type 1 collagen and osteocalcin, and influence the synthesis and activity of many cytokines i.e., interleukin-1 and interleukin-6, and growth factors, especially the insulin-like growth factors (IGF-I and IGF-II) and several of the IGF-binding proteins.

3.2.4. 1, 25$(OH)_2$ D_3

1, 25$(OH)_2$ D_3 is a potent stimulator of osteoclastic bone resorption like PTH. It tends to increase the recruitment of osteoclasts by stimulating osteoclast progenitors to differentiate and fuse (Roodman et al., 1985). 1, 25$(OH)_2$ D_3 also plays an important role in the mineralization of bone matrix. A lack of 1, 25$(OH)_2$ D_3 results in osteomalacia (impaired mineralization) while excessive 1, 25$(OH)_2$ D_3 entails bone loss. In vitro fetal rat calvaria culture, 1, 25$(OH)_2$ D_3 inhibited DNA synthesis of

collagen type I and osteopontin (Ishida et al., 1993). The effect of 1, 25$(OH)_2$ D_3 on osteoblast differentiation was also found in rat primary osteoblast, in which it stimulates the alkaline phosphatase activity up to 50 % after 8 h incubation suggesting a genome activation and de novo protein synthesis (Manolagas et al., 1983). These results show that 1, 25$(OH)_2$ D_3 has distinct effects and enhance on human marrow stromal cell proliferation and differentiation in to osteoblast.

3.2.5. Calcitonin

Calcitonin is a polypeptide hormone that is a potent but transient inhibitor of osteoclastic bone resorption (Wener et al., 1972). It downregulates mRNA of its receptor in osteoclast and induces cytosolic contraction of osteoclast membrane, which has been correlated with inhibition of resorption (Chambers et al., 1982). In addition, calcitonin inhibits osteoclast proliferation and differentiation (Granholm et al., 2007).

B4. Local hormone regulation of bone remodeling

4.1 Parathyroid Hormone-Related Protein (PTHrP)

PTHrP is widely expressed in normal tissues, and can act in paracrine and autocrine fashion. PTH and PTHrP share the same sequence at the N terminal of the mature molecules. The key control of PTHrP production/secretion mostly relies on its mRNA expression in the cell while PTH production is influenced by plasma calcium level (Philbrick et al., 1996). PTHrP was found to be expressed in many fetal tissues, and was believed to mediate skeletal development in the process of embryogenesis (Kronenberg et al., 2003). The general deletion of PTHrP encoding gene caused a lethal skeletal dysplasia in mouse fetus (Karaplis et al., 1994). In addition, the specific deletion of PTHrP gene in osteoblast also caused an inability to form a new bone tissue, which is due to a failure of osteoblast survival and differentiation suggesting an anabolic role of PTHrP in osteogenesis (Miao et al., 2005).

4.2 Prostaglandin E2 (PGE2)

Prostaglandins are produced from fatty acid precursor arachidonic acid in several kinds of cells including osteoblasts. Prostaglandin E2 ($PGE2_)$ has pronounced effects on bone in both anti-resorptive and proresorptive functions depending on the concentrations and experimental models. PGE2 stimulates osteoclast formation in bone marrow cultures (Akatsu et al., 1989), up-regulates RANKL mRNA expression, and down-regulates OPG mRNA expression in primary human bone marrow stromal cells (Brandstrom et al., 2001). Therefore, the effects of PGE2 on OPG and RANKL will ultimately lower the OPG-RANKL ratio that could eventually lead to a decrease in bone mass.

4.3 Interleukin-1 (IL-1) and Interleukin 6 (IL-6)

Interleukin 1 is released by activated monocytes, osteoblasts and tumor cells. IL-1 is a potent stimulator of osteoclast proliferation and differentiation (Pfeilschifter et al., 1988). The effects of IL-1 may be mediated by prostaglandin.

Interleukin 6 is a pleiotropic cytokine, expressed and secreted by normal bone cells including osteoclast. It was implicated in bone loss associated with estrogen depletion in mouse (Jilka et al., 1992). Since both estrogen and androgen suppress the production of IL-6, as well as the expression of its receptor (Boulton et al., 1994), the level IL-6 is elevated in bone marrow and in the peripheral blood of estrogen deficient mice, rats and humans (Miyaura et al., 1995; Cheleuitte et al., 1998).

4.4 Lymphotoxin and tumor necrosis factor (TNF)

Lymphotoxin and TNF are molecules that are related functionally to IL-1. They share the same receptor which is distinct from IL-1 receptor. Many of their biologic properties overlap with those of interleukin-1 however, their effects on bone are synergistic with those of IL-1. Lymphotoxin release by activated T lymphocytes, and TNF, is activated by macrophage. They cause osteoclast resorption and

hypercalcemia by stimulating proliferation of osteoclast progenitors, and cause fusion of committed precursors to form multinucleated osteoblasts (Bertolini et al. 1986).

4.5. TGF-β

TGF-β is related to epidermal growth factor (EGF). It is a powerful stimulator of osteoclastic bone resorption (Tashjian et al., 1986). TGF-β is produced by osteoblast, osteoclast and cancer cells. It stimulates osteoclast proliferation and its progenitor proliferation by acting on immature multinucleated cells. Its action on osteoclast is comparable to those of macrophage colony-stimulating factor (M-CSF) on the other hematopoetic cells (Raisz et al., 1969). This regulator has an important role in bone metastasis by stimulating tumor production of osteolytic factors.

CHAPTER III
MATERIALS AND METHODS

The hypothesis of this dissertation was that PRL regulated bone remodeling by acting directly on osteoblast. Therefore the overall aim was to show the presence of PRLR and to elucidate the role of PRL and its signal transduction pathway in the regulation of bone remodeling by using human osteoblasts MG-63 and hFOB as the study models. The investigation was divided into specific experiments as shown below.

A. EXPERIMENTAL DESIGN

1. Verification of PRLR mRNA and protein expressions in MG-63 and hFOB

1.1 Determination of PRLR mRNA and protein expression in MG-63 in the medium containing with

0.2 % fetal bovine serum (FBS)

10 % FBS

0.2 % FBS and dexamethasone

0.2 % FBS and 1, 25$(OH)_2D_3$

0.2 % FBS, dexamethasone and 1, 25 $(OH)_2D_3$

1.2 Determination of PRLR mRNA and protein expression in hFOB in the medium containing with

0.2 % fetal bovine serum (FBS)

10 % FBS

0.2 % FBS and dexamethasone

0.2 % FBS and 1, 25$(OH)_2D_3$

0.2 % FBS, dexamethasone and 1, 25 $(OH)_2D_3$

2. To examine the effect of PRL on osteoblast proliferation, differentiation and activity of MG-63 and hFOB as the study models

2.1 To examine the effect of PRL on osteoblast activity using alkaline phosphatase (ALP) activity as a marker

2.1.1 Dose response study (0, 1, 10, 100 and 1000 ng/ml PRL)

2.1.2 Time response study (0, 0.5, 3, 6, 12, 24 and 48 h)

2.2 To examine the effect of PRL on the osteoblast proliferation and osteoblast differentiation

2.2.1 Determination of osteoblast proliferation using the MTT assay

2.2.2 Determination of osteoblast differentiation as determined by the osteoblast differentiation marker (osteocalcin), and bone formation marker (ALP) mRNA expression

2.2.2.1 Dose response study (0, 1, 10, 100 and 1000 ng/ml PRL)

2.2.2.2 Time response study (0, 0.5, 3, 6, 12, 24 and 48 h)

3. To evaluate the action of PRL on bone resorption by determining the production of RANKL and osteoprotegerin

3.1 Osteoblast RANKL and OPG mRNA expression

3.2 Osteoblast RANKL and OPG protein expression

4. To elucidate the intracellular signaling pathway of PRL action in osteoblast

5.1 To determine the optimal concentration of PI3-K inhibitor (LY294002) that was not toxic to cells.

5.2 To study the effect of PI3-K inhibitor (LY294002) alone or with PRL on the osteoblast activity

B. CHEMICALS

Dulbecco's Modified Eagle Medium with nutrient mixture F-12 (DMEM/F-12), alpha minimum essential medium (α-MEM), fetal bovine serum (FBS), Trypsin-EDTA and antibiotic-antimyotic were purchased from GIBCO (Life Technologies, Gaithersburg, MD). Trypan blue was purchased from Merck (Darmstadt, Germany). Dimethyl sulfoxide (DMSO) was purchased from BDH (Poole, Dorset, UK). 1, 25-$(OH)_2D_3$, dexamethasone, *p*-nitrophenyl phosphate (PNPP), substrate of ALP and PI3-K inhibitor (LY294002) were purchased from Sigma (St. Louis, MO). Human recombinant prolactin was purchased from R & D systems (Inc.,Minneapolis, MN). Na_2CO_3 and NaOH in the assay solution in ALP activity experiment were purchased from Merck. The primary antibody probes for PRLR, RANKL and β-actin, were purchased from Santacruz (Biotechnology, Santa Cruz, CA). The primary anti-OPG antibody was purchased from R&D Systems (Inc.,Minneapolis, MN). Horseradish-linked anti-rabbit IgG and anti-goat IgG were purchased from Santacruz (Biotechnology, Santa Cruz, CA), and anti-mouse IgG1 was purchased from Zymed (South San Francisco, CA). The alexa Fluor 488 conjugated anti-rabbit IgG antibody was obtained from Molecular Probes (Eugene, OR). Bradford protein reagent was purchased from Bio-Rad (Hercules, CA), and bovine serum albumin (BSA) was purchased from Sigma (St. Louis, MO). A protease inhibitor cocktail tablet was purchased from Roche, and proteins were run on nitrocellulose membrane obtained from Schleicher & Schuell Co Ltd. The chemiluminescence (ECL) detection reagents were purchased from Amersham (Amersham, United Kingdom).

C. CELL CULTURE AND CELL COUNTING

To determine a possible direct effect of PRL on bone remodeling, human osteoblast-like cell (MG-63) and human normal fetal osteoblasts (hFOB) were used in the experiments. Human osteosarcoma cell line, MG-63 kindly given by Dr. Suttatip Kamolmatyakul (Prince of Songkla University, Thailand) were propagated in α-MEM, while human fetal osteoblast 1.19, hFOB, purchased from ATCC (ATCC, No.CRL-11372) were propagated in D-MEM/F-12, supplemented with 2% or 10 % FBS and 1 % of antibiotic-antimycotic solution containing penicillin, streptomycin and amphotericin B. Cells were cultured in 75-cm^2 T flasks at 37 °C in a 5 % CO_2 constant humidity environment. Culture media were changed 2-3 times weekly, and were split when reached 80 % confluence at a 1:10 split ratio using 0.05 % trypsin-0.02 % EDTA. The experiments were performed in passage 12-18 for MG-63, and passage 4-10 for hFOB. Cells were counted using a hemocytometer and trypan blue dye exclusion.

D. EXPERIMENTAL PROCEDURES

1. Cell proliferation assay

MG-63 and hFOB (5000 cells/well) were inoculated into 96-well tissue culture plate. MG-63 was treated with 1, 25-$(OH)_2$ D_3 for 16 h to stimulate PRLR expression prior to PRL treatment (Batille et al., 1998). The recombinant human PRL was reconstituted in BSA and HCl following the manufacturer's instruction before being diluted in α-MEM or DMEM/F12. After a 48-h incubation with PRL at 1, 10, 100 or 1000 ng/ml concentration, unattached cells were then removed by PBS wash. Cells treated with media under similar conditions served as the controls. 3-[4, 5-Dimethylthiazol-2-yl]diphenyltetrazolium bromide (MTT; Sigma St. Louis, MO) was prepared as a stock solution of 5 mg/ml in PBS pH 7.4. Treated medium was then replaced by medium containing 10 % MTT stock solution. Plates were incubated for 3 h at 37°C, after which the medium was replaced with DMSO. Viability of attached MG-63 or hFOB was then determined by the MTT assay (Mossman 1983; Pellen-

Mussi et al., 1997) i.e., by determining the activity of the mitochondrial dehydrogenase. The absorbance of each tissue culture plate was read with a Multiskan EX355 plate reader (Thermo Labsystems Instruments, Helsinki, Finland) at 540 nm. The absorbance of control cells was considered to be 100 %. The relative proliferation of cells was calculated by the formula:

(OD of experimental sample / OD of control cells) x 100 %

Experiments were performed in five replications, and the relative viability was averaged from three independent experiments.

2. ALP activity assay

MG-63 and hFOB were plated in 6-well tissue culture plate at 100,000 cells/well. MG-63 was treated with 1, 25-$(OH)_2$ D_3 for 16 h to stimulate PRLR expression prior to PRL treatment. Both cells were then treated with PRL at concentration of 0, 1, 10, 100 or 1000 ng/ml for 48 h. ALP activity was determined by the conversion of *p*-nitrophenyl phosphate to *p*-nitrophenol (29). Briefly, cells were washed twice with PBS pH 7.4 and incubated for 2 h for MG-63 (Lynda et al., 1992) and 1 h for hFOB with 2 ml of alkaline phosphatase (ALP) reaction buffer containing 100 mM Na_2CO_3, 10 $MgCl_2$ and 20 mM *p*-nitrophenol phosphate, pH 10.3. The reaction was mixed with 1 ml of 5 M NaOH, and 200 µl was then pipetted into 96-well plate for absorbance measurement. Color development was quantified immediately at 410 nm by Multiskan ELISA microplate-reader (Thermo-Labsystems, Helsinki, Finland).

3. RNA purification and reverse transcription-polymerase chain reaction (RT-PCR)

RT-PCR technique was used to investigate the mRNA expression of osteoblast differentiation marker genes, such as ALP and osteocalcin as well as the markers of bone resorption, such as RANKL and OPG. MG-63 and hFOB were plated on round

dish cell-culture at 1 x 10^6 cells/dish. After 24 h, MG-63 was incubated with 1, 25-$(OH)_2 D_3$ for 16 h to stimulate PRLR expression prior to PRL treatment. Then both cells were treated with various concentrations of PRL (1, 10, 100 or 1000 ng/ml) at 37 ºC. Cells treated with media under similar conditions served as the control. After 48 h of incubation, the unattached cells were removed by washing with PBS pH 7.4 twice. Total cellular RNA was extracted using RNeasy mini kit (Qiagen, Valencia, CA) according to the manufacturer's instruction. Briefly, the 350 µl RLT lysis buffer with β-ME was added to cell pellets and the mixture was mixed well before being added to a volume of 700 µl, then applied to the RNeasy spin column and centrifuged for 15 sec. The filtrate was discarded; RW 1 wash buffer was added before the mixture was centrifuged. Then RPE washing buffer was added and the mixture was centrifuged again. This process was repeated twice. RNA was eluted with 50 µl Dep-CC, and then centrifuged.

The extracted RNA was then applied to the SuperScript III first-strand synthesis system for RT-PCR (Invitrogen, Life Technologies, Carlsbad, CA). 2 µg RNA was prepared with 1 µl 10 mM dNTP, and then mixed with 1 µl oligo (dT)-20 before incubating at 65 °C for 5 minutes. After the RNA mixture was plated on ice for at least 1 minute, the 7 µl reaction mixture (4 µl 5x RT buffer, 1 µl 0.1 M DTT, 1 µl SuperScript TM III RT and 1 µl RNase inhibitor) was added. The solution was further incubated at 50 °C for 60 minutes. The reaction was terminated by exposure to 70 °C for 15 minutes, followed by a chill on ice. After a brief centrifugation, 1 µl of RNase-H was added to the mixture which was then incubated for a further 20 minutes at 37 °C. Each PCR reaction (25 µl) contained 1U Taq polymerase, $MgCl_2$, dNTP, the concentration and volume of which were shown in Table 3 for MG-63, and Table 5 for hFOB. Each primer (Table 2) and each 3 µl of cDNA (hPRLR) or 1 µl of cDNA (ALP, OC, RANKL, OPG and GAPDH), were then generated by one cycle denaturating at 95 °C for 5 minutes followed by the amplification steps. The detail of each gene was shown in Table 4 for MG-63 and Table 6 for hFOB. Afterwards, a final extension was performed at 72 °C for 10 min. The PCR amplified fragments were visualized in a 1 % agarose gel stained with 1µg/ml ethidium bromide. The intensity of each band was quantified by the densitometer (Bio-Rad, Hercules, CA) The

expression of each gene in control cells without PRL treatment was considered as 100 %.

Table 2. Oligonucleotide primers used in RT-PCR for MG-63 and hFOB

Target gene	Nucleotide sequence	Size in bp	References
ALP	5'-ACGTGGCTAAGAATGTCATC-3'	475	Rickard et al., (1996)
	5'-CTGGTAGGCGATGTCCTTA-3'		
RANKL	5-GCCAGTGGGAGATGTTAG-3'	486	Granchi et al., (2002)
	5'-TTAGCTGCAAGTTTTCCC-3'		
OPG	5'-GCTAACCTCACCTTCGAG-3'	324	Granchi et al., (2002)
	5'-TGATTGGACCTGGTTACC-3'		
OC	5'-GGCCAGGCAGGTGCGAAGC-3'	25	Robbins et al., (2000)
	5'-GCCAGGCCAGCAGAGCGACAC-3'		
GAPDH	5'-CACCCACTCCTCCACCTTTG-3'	110	
	5'-CCACCACCCTGTTGCTGTAG-3'		
hPRLR	5'-AAATGTGGCATCTGCAACCGTTTTCAC-3'	1790	Bataille et al., (1996)
	5'-GCACTTGCTTGATGTTGCAGTGAAGTT-3'		

RT-PCR, reverse transcription-polymerase chain reaction; ALP, alkaline phosphatase; RANKL,receptor activator of nuclear factor-kB ligand; OPG, osteoprotegerin; hPRLR, human prolactin receptor; OC, osteocalcin.

Table 3. PCR mixture/1 reaction for **MG-63**

Gene	Each primer (mmole)	dNTP (mM)	MgCl2 (mM)	Tag (ul)
hPRLR	10	0.2	1.5	0.125
ALP	10	0.2	2.5	0.125
Osn	10	0.2	1.5	0.125
OPG	10	0.2	1.5	0.125
RANKL	10	0.2	2.5	0.125
GAPDH	10	0.2	1.5	0.125

Each cDNA was generated by one cycle denaturating at 95 °C for 5 minutes followed by the amplification steps, the detail of which was shown for each gene in Table 4.

Table 4. The amplified condition of each gene for **MG-63**

Gene	denaturation	annealing	extension	PCR cycle
hPRLR	94 °C: 1 min	62 °C: 2 min	72 °C: 2 min	30
ALP	94 °C: 30 s	55 °C: 2 min	72 °C: 2 min	32
Osn	95 °C: 30 s	70 °C: 30 s	72 °C: 30 s	30
OPG	94 °C: 30 s	55 °C: 30 s	72 °C: 1 min	22
RANKL	94 °C: 30 s	55 °C: 30 s	72 °C: 1 min	33
GAPDH	95 °C: 1 min	54 °C: 1 min	72 °C: 1 min	20

Table 5. PCR mixture/1 reaction for **hFOB**

Gene	each primer (mmole)	dNTP (mM)	MgCl2 (mM)	Tag (ul)
hPRLR	10	0.2	1.5	0.125
ALP	10	0.2	1.5	0.125
Osn	10	0.2	1.5	0.125
OPG	10	0.2	1.5	0.125
RANKL	10	0.2	2.5	0.125
GAPDH	10	0.2	1.5	0.125

Each cDNA was generated by one cycle denaturating at 95 °C for 5 minutes followed by the amplification steps, the detail of which was shown for each gene in Table 6.

Table 6. The amplified condition of each gene for **hFOB**

Gene	denaturation	annealing	extension	PCR cycle
hPRLR	94 °C: 1 min	62 °C: 2 min	72 °C: 2 min	30
ALP	94 °C: 30 s	55 °C: 2 min	72 °C: 2 min	32
Osn	95 °C: 30 s	70 °C: 30 s	72 °C: 30 s	30
OPG	94 °C: 30 s	55 °C: 30 s	72 °C: 1 min	22
RANKL	94 °C: 30 s	55 °C: 30 s	72 °C: 1 min	30
GAPDH	95 °C: 1 min	54 °C: 1 min	72 °C: 1 min	21

4. Immunofluorescent staining

To verify and localize the PRLR proteins in osteoblasts, MG-63 and hFOB were cultured on cover slips at 100,000 cell/slip. MG-63 and hFOB were plated on the cover slip at 100,000 cells/slip. Then MG-64 was incubated with 1, 25-$(OH)_2 D_3$ whereas hFOB was incubated with 0.2 % FBS for 16 h. Unattached cells were then removed by washing with PBS pH 7.4 twice. The cover slips were removed from the incubator and fixed with paraformaldehyde with 2 % sucrose for 10 min at room temperature. Cells were then washed three times with PBS, and permiabilized with 0.5 % Triton-X100 in PBS for 5 min at room temperature. Then cells were washed with PBS twice, and the nonspecific proteins were blocked with 10 % fetal bovine serum for 30 min at room temperature. Samples were then incubated with the 1:300 rabbit polyclonal anti-PRLR primary antibodies overnight at 4 °C. Cells were further incubated with diluted 1:200 of alexa Fluor 488 and conjugated anti-rabbit IgG antibody as a secondary antibody for 1 h at room temperature. Cells were then washed three times in PBS, and then sealed with a small amount of nail polish. Anti-PRLR was digitally captured by using inverted fluorescence microscopy (Bio-Rad MRC1024 MP scanning system mounted on a Nikon Eclipse TE300 fluorescence microscope).

5. Immunoblotting analysis

To evaluate the action of PRL on bone resorption, the expression of RANKL and OPG proteins were determined by the immunoblotting technique. Similar to the earlier procedures, cells were plated in round dish cell culture at 1 x 10^6 cells/dish. MG-63 was incubated with 1, 25-$(OH)_2 D_3$ for 16 h to stimulate PRLR expression prior to PRL treatment. Then MG-63 and hFOB were treated with various concentrations of PRL (1, 10, 100 or 1000 ng/ml) at 37 ºC. Cells treated with media under similar conditions served as the controls. After 48 h of incubation, unattached cells were removed by washing with PBS pH 7.4 twice. After the incubation with PRL, cells were washed with PBS, and cell lysates were collected after dissolving cells in 5 ml of ice-cold RIPA buffer (150 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.1 % SDS, 1 % Triton X-100, 1 % Nonidet P-40, 1X protease inhibitor, 1 M NaF, 1 M β-

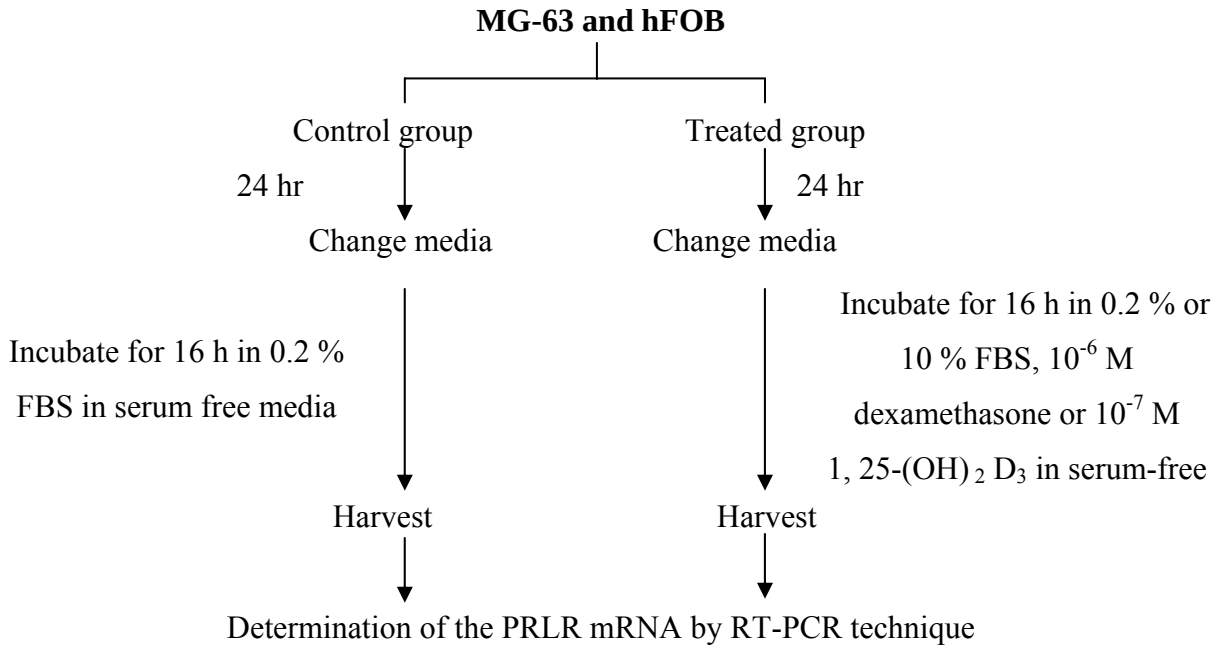
glycerophosphate, 0.5 M Na_3VO_4, 1 M DTT, 1 % sodium deoxycholate, and 5 mM EDTA). Extracted samples were incubated on ice for 30 min and centrifuged at 12,000 rpm for 10 min.

Extracted proteins were treated with SDS sample buffer, followed by boiling for 5 min. The protein concentrations were quantified by a Bovine Serum Albumin. Cytosolic proteins (100 μg for RANKL and 80 μg for OPG) were resolved on 10 % polyacrylamide gels. Electrophoresis was performed at 120 volts in SDS-PAGE buffer pH 8.3 (25 mM Tris base, 192 mM gtycine, 0.1 % SDS). Proteins were electrophoretically blotted onto the nitrocellulose membranes for 2 h at 120 volts, 4 ° C the in transfer buffer (25 mM Tris base pH 8.8, 200 mM glycine, 20 % methanol). The nonspecific proteins on the membrane were blocked by 5 % skim milk in TBS containing 0.1 % Tween-20 (TBST) for 1 h at room temperature. Then RANKL and OPG proteins were immunodetected using 1:2000 goat anti-RANKL polyclonal antibody and 1:2000 goat anti-OPG polyclonal antibody, conjugated with HRP-Rabbit against goat in 3 % skim milk in TBST. β-actin used as an internal control, was immunodetected by rabbit IGg1 monoclonal antibody against mouse. The intensities of the immunoreactive bands were determined using an ECL Plus chemiluminescence detection kit. (Amercham Biosciences, Buckinghamshire UK). The expression of each protein in the absence of PRL was considered as 100 %.

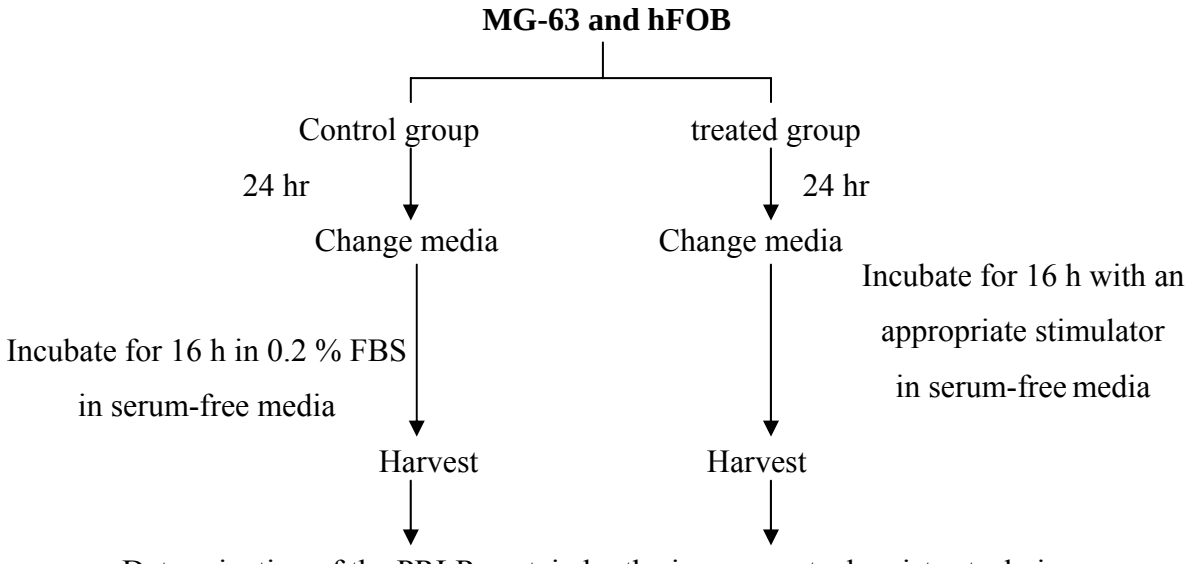
E. EXPERIMENTAL PROTOCOLS

A. Verification of the optimal condition for stimulating PRLR expression in MG-63 and hFOB

To study a direct effect of PRL on bone remodeling through osteoblast, first of all, PRLR mRNA and protein were essentially verified in both MG-63 and hFOB. Cells were plated to a round culture dish at 1 x 10^6 cells/dish, and were then treated with 0.2 % FBS, 10 % FBS, 1 x 10^{-6} M of dexamethasone, 1 x 10^{-7} M of 1, 25-$(OH)_2$ D_3, or a combination of dexamethasone and 1, 25-$(OH)_2$ D_3 prior to PRL treatment. Cells treated with media under similar conditions served as the control. PRLR mRNA expression was determined by using the RT-PCR and gel electrophoresis.



After the optimal conditions for stimulating PRLR mRNA expression in MG-63 and hFOB were obtained, PRLR protein expression and localization in both cells were verified using the immunocytochemistry as shown in the diagram below.



This present investigation showed that PRLR mRNA and protein expression in MG-63 cells, were stimulated after 16 h incubation of 1, 25-$(OH)_2$ D_3. Therefore, 1 x 10^{-7} M of 1, 25-$(OH)_2$ D_3 was added prior to PRL administration in following MG-63

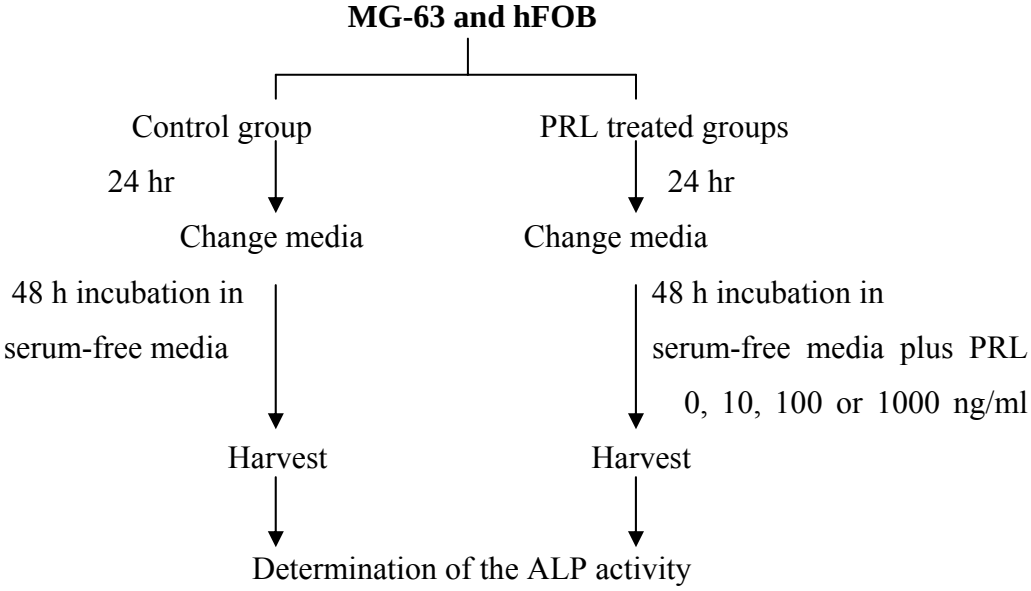
experiments. On the other hand, hFOB were cultured in normal medium prior to PRL administration.

Objective 2. To examine the effect of PRL on the osteoblast proliferation, differentiation and activity MG-63 and hFOB as the study models

2.1 To examine the effect of PRL on the osteoblast activity

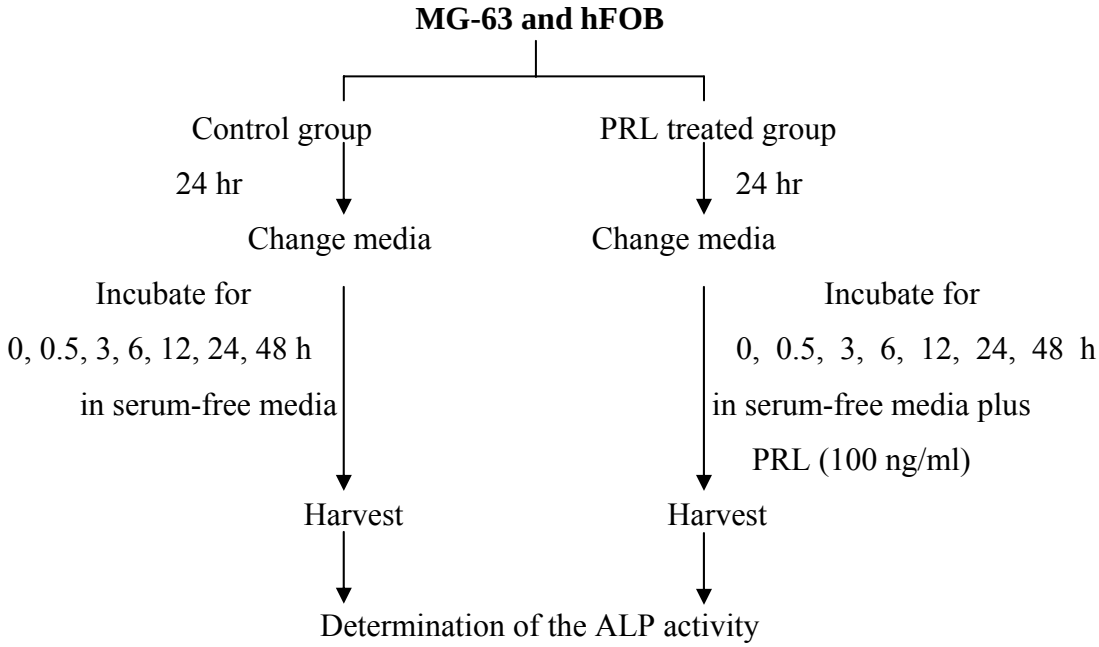
2.1.1 Dose response study: ALP activity

ALP, an enzyme abundantly found on the membrane of osteoblast, provides phosphate for bone formation. Its activity is commonly used as a marker of the osteoblast activity. The following protocol applied to both MG-63 and hFOB. Cells were divided into 5 PRL-treated groups at approximately, 1 x 10^5 cells/group. For the dose response studies, the concentrations of PRL to mimick the PRL levels in various physiological conditions were i.e., 0 (control group), 1 and 10 ng/ml (normal plasma PRL), 100 ng/ml (lactation or pregnancy) and 1000 ng/ml (pathological hyperprolactinemia such as prolactinomas). The obtained information was used to plan the time response study of PRL effect on the osteoblast activity.



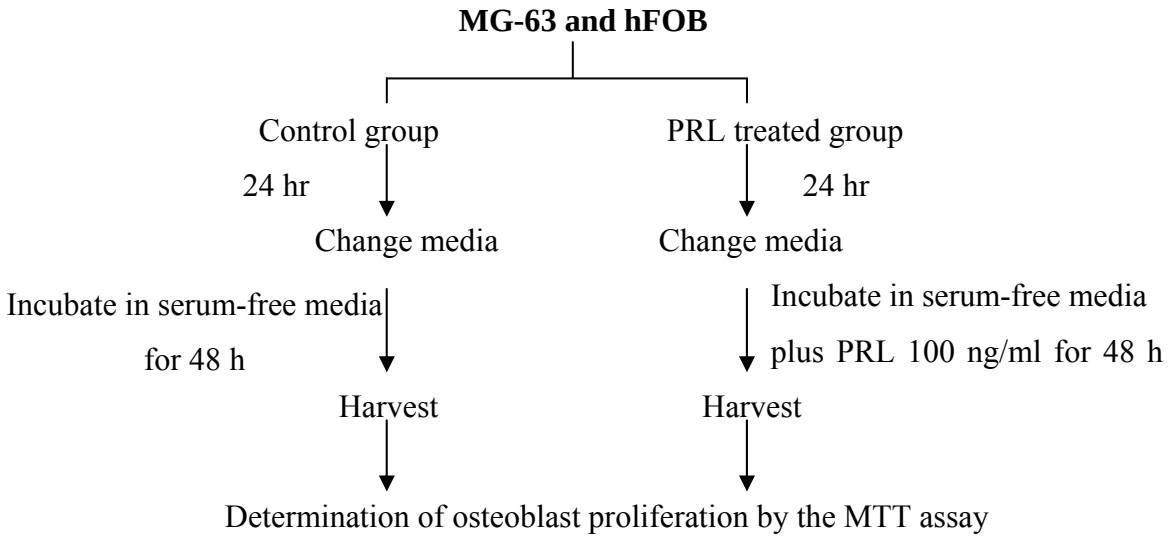
2.1.2 Time-response study: ALP activity

The protocol was applicable to both MG-63 and hFOB. Cells were divided into 7 groups at approximately 1 x 10^5 cells/group. They were incubated with 100 ng/ml of PRL for 0, 0.5, 3, 6, 12, 24 or 48 hrs, as shown in the diagram below. The appropriate incubation time obtained in this experiment would be used to plan the following dose response study of PRL effect on the osteoblast proliferation and differentiation.



2.2 The effect of PRL on osteoblast proliferation using the MTT assay

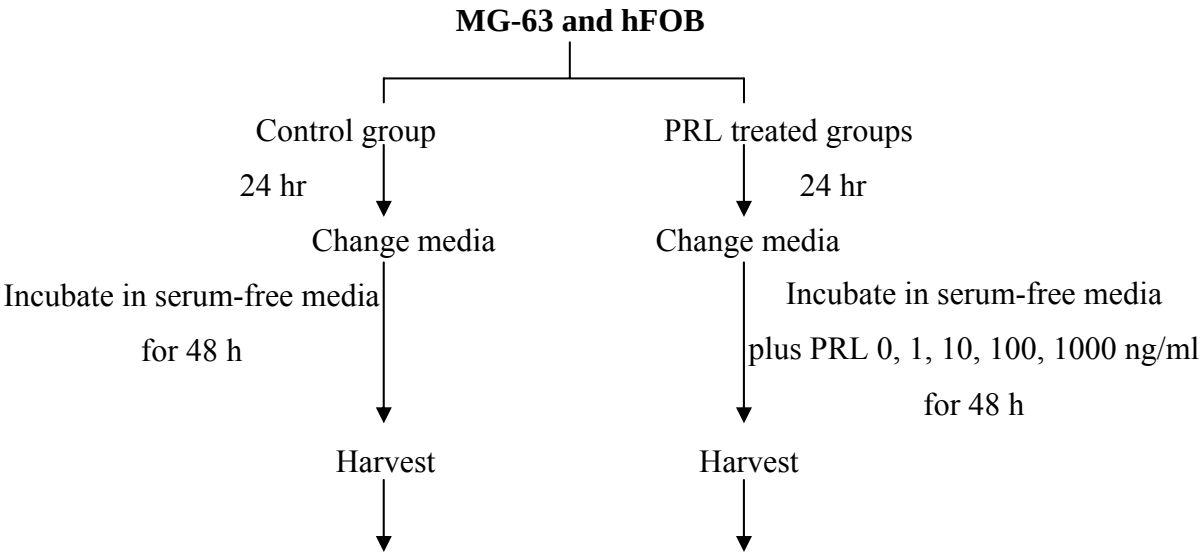
To evaluate the osteoblast viability and proliferation, the tetrazolium MTT (3-(4, 5- dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) was used since it is normally reduced by a mitochondrial enzyme, dehydrogenase, in a metabolically active cell. The chemical reaction generated reduces equivalents such as NADH and NADPH resulting in the intracellular formazan purple. Formazan could be solubilized by DMSO and can be quantified by spectrophotometric technique (Multiskan ELISA microplate reader, Thermo-Labsystems, Helsinki, Finland).



2.3 Osteoblast differentiation as determined by the osteoblast differentiation marker (osteocalcin), and bone formation markers (ALP) mRNA expression

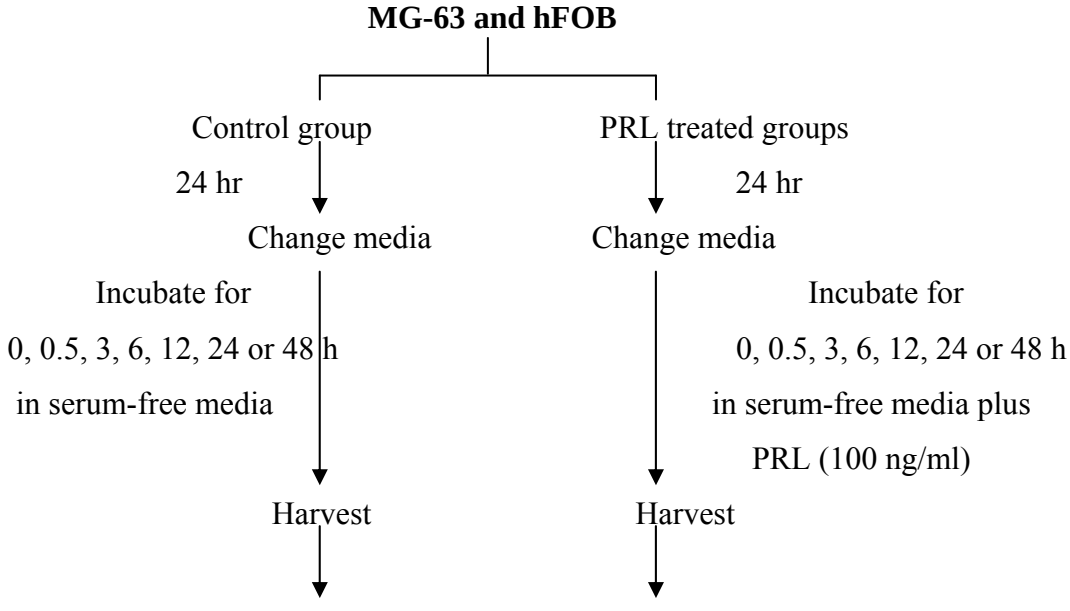
2.3.1 Dose-response study: mRNA expression of osteocalcin and ALP

Both MG-63 and hFOB was each divided into 5 PRL-treated groups, at approximately, 1 x 10^6 cells/group, for the dose-response study. Different PRL concentrations i.e., 0, 1, 10, 100 or 1000 ng/ml were used to mimick the PRL levels in various physiological conditions. The effective concentration of PRL obtained here would be used in the following time response study of the effect of PRL on the osteoblast differentiation.



2.3.2 Time-response study: mRNAs expression of osteocalcin and ALP

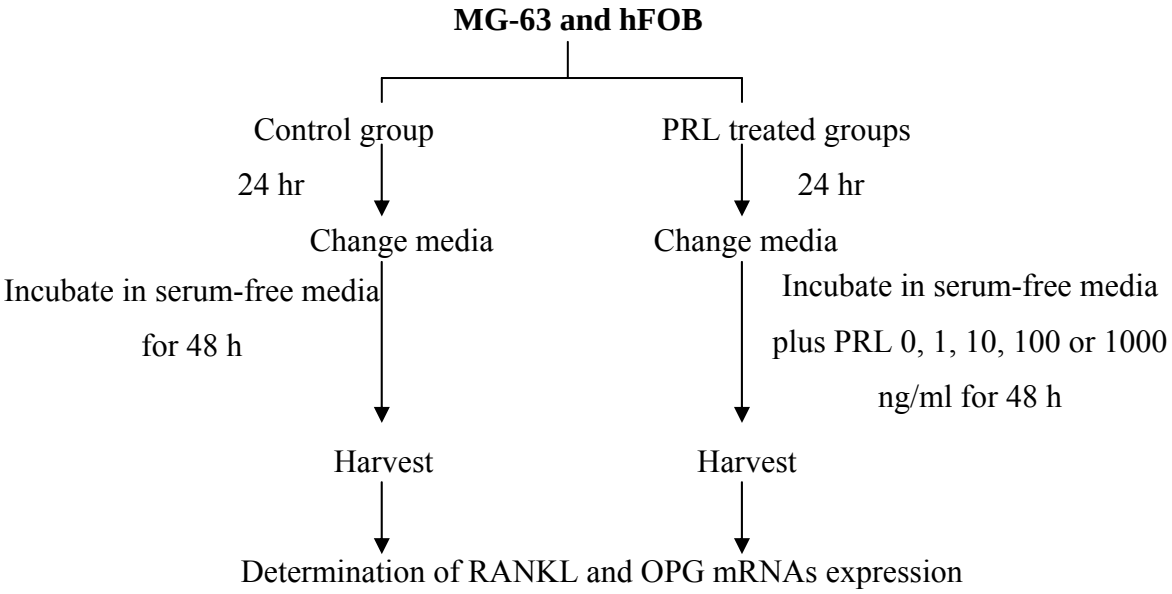
The protocol was applicable to both MG-63 and hFOB. Cells were divided into 7 groups and incubated with 100 ng/ml PRL for a duration of 0, 0.5, 3, 6, 12, 24 or 48 h, as shown in the diagram below.



Objective 3. To evaluate the action of PRL on bone resorption by determining the production of RANKL and osteoprotegerin

3.1 The effect of PRL on RANKL and OPG mRNA expression

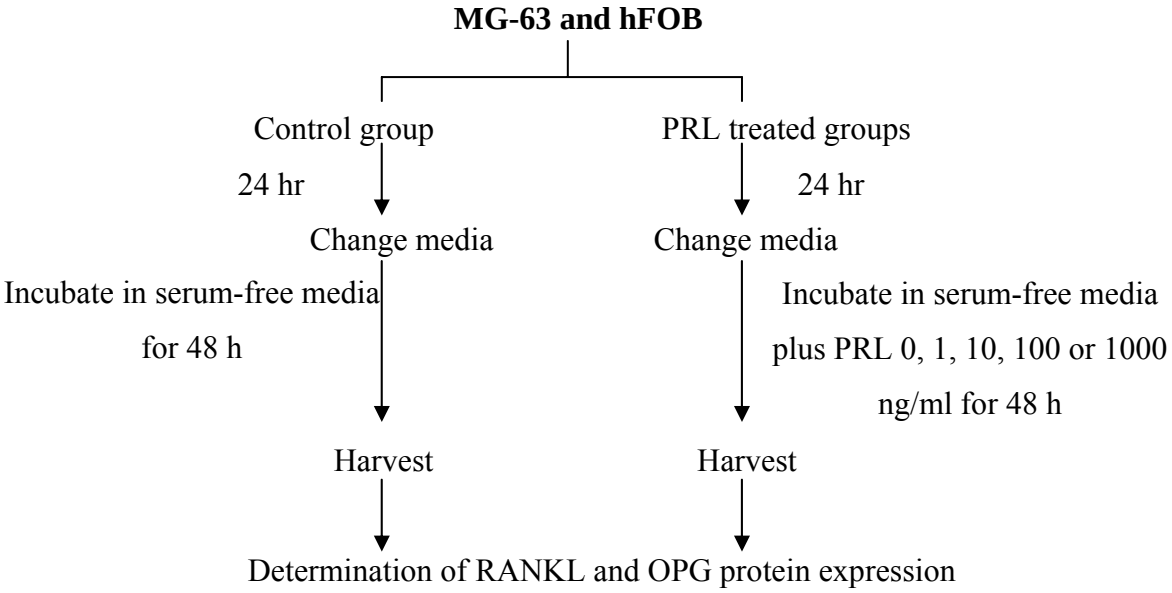
The protocol was applicable to both MG-63 and hFOB. Cells were divided into 5 PRL-treated groups at approximately 1 x 10^6 cells/group. Different PRL concentrations i.e., 0, 1, 10, 100 or 1000 ng/ml, were used to mimick the PRL levels in various physiological conditions. The RANKL and OPG mRNA expressions were examined using the RT-PCR technique and each band was quantified by densitometer as described in the experimental procedures.



3.2 The effect of PRL on RANKL and OPG protein expression

The protocol was applicable to both MG-63 and hFOB. Cells were divided into 5 PRL-treated groups at approximately 1 x 10^6 cells/group. Different PRL concentrations i.e., 0, 1, 10, 100 or 1000 ng/ml, were used to mimick the PRL levels in various physiological conditions. The RANKL and OPG proteins were detected by

using the immunoblotting technique and enhanced chemiluminescence (ECL), detail was described in the experimental procedures.



Objective 4. To elucidate the intracellular signaling pathway of PRL action in osteoblast

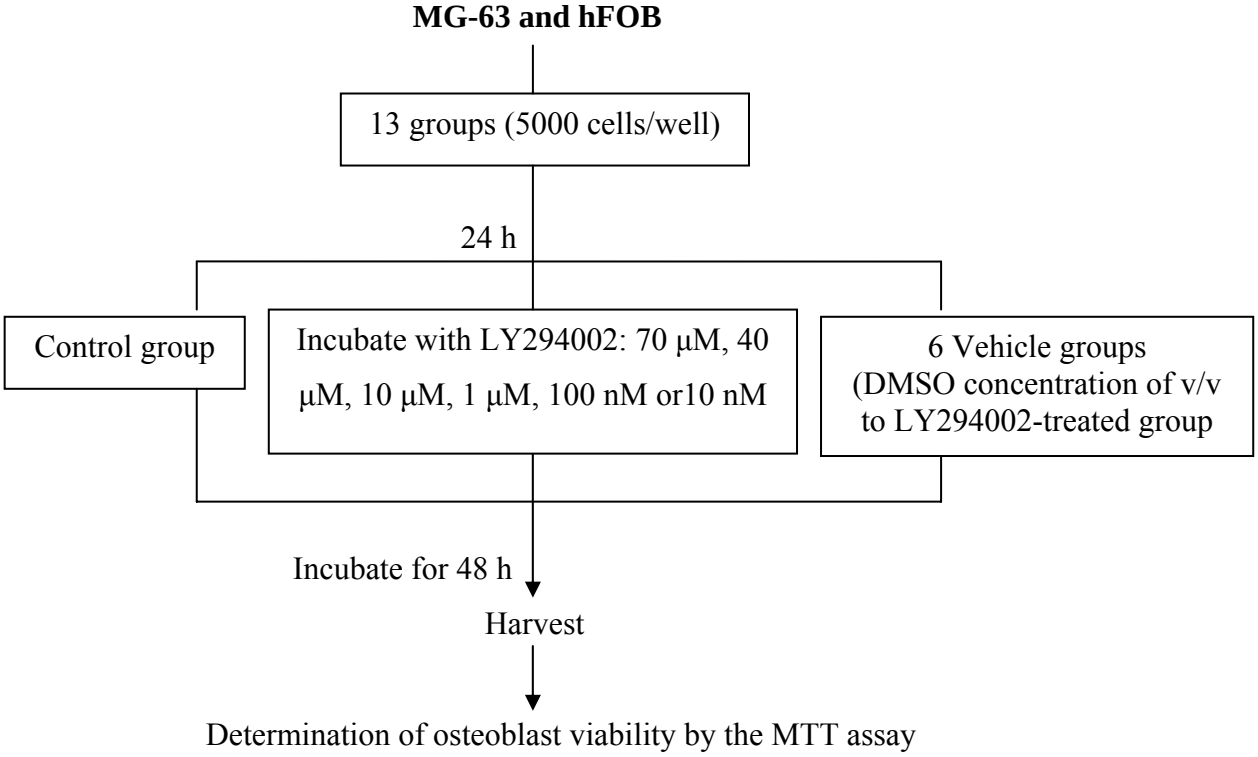
Previous studies have shown the presence of PRL receptor in bone tissue, bone cell lines (Saos-2 and MG-63) and osteoblast culture from mouse calvariae (Bataiille et al. 1996, Clement-Lacroix et al., 1999 and Coss et al., 2000). They provided the evidence supporting the hypothesis that PRL had a direct effect on bone cell. In addition, the mechanisms of action of PRL have been shown to vary depending on cell types. For instance, the MAPKs pathway and PI3 kinase (PI3-K) pathway were reported in rat lymphoma Nb2 cell (AL-Sakkaf et al., 1997). However, the PRL signaling pathway involved in bone cell metabolism had not been investigated.

PI3-K is a lipid kinase that catalyzes the transfer of the γ- phosphate from ATP to the 3`-hydroxyl group of phosphatidylinositol and its derivatives (collectively called phosphoinositides). It plays a crucial role in various important cellular regulatory mechanisms such as cell growth, proliferation and survival/ apoptosis. In mammalian cells, both non-genomic and genomic actions have been found to be mediated via PI3-K activation, for instance, protein synthesis, glucose metabolism,

transformation and differentiation of many cell types (LC et al., 2002) including osteoblast (Nandini Ghosh-Choudhury et al., 2002). Therefore, a possibility of PI3-K involvement in PRL action was investigated.

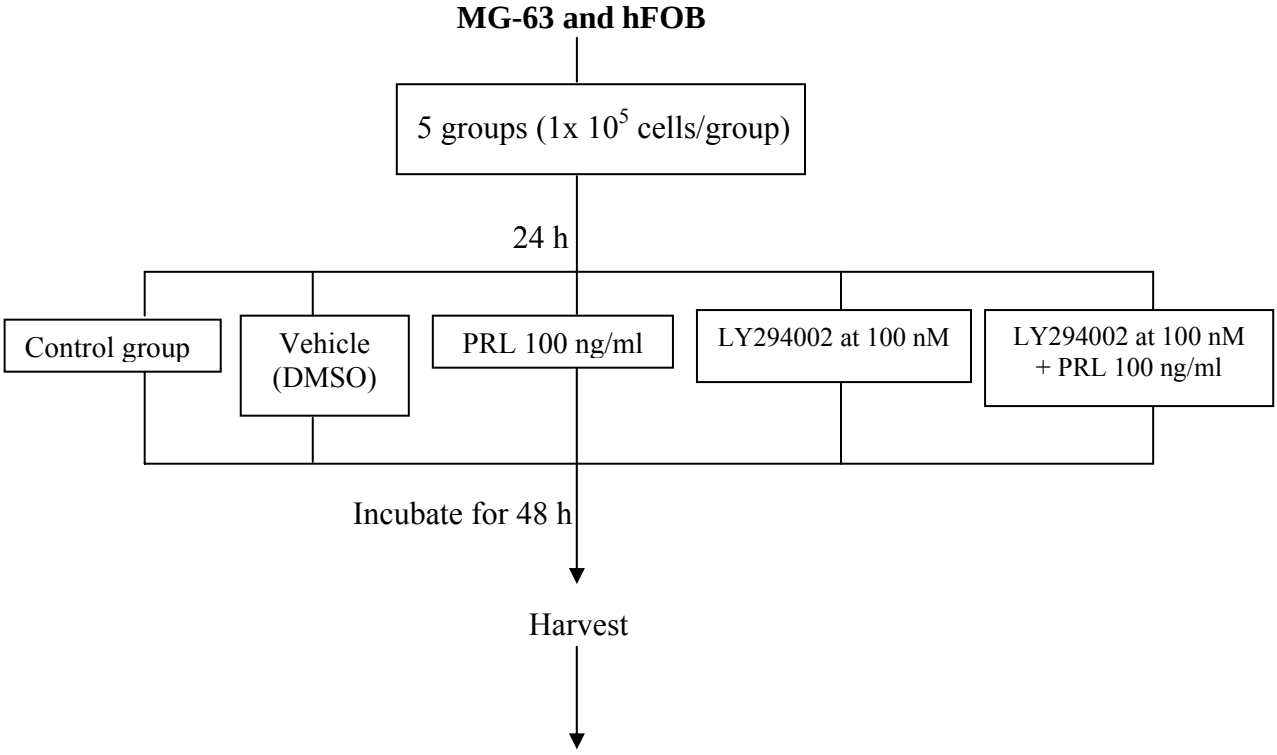
4.1. To determine the optimal concentration of PI3-K inhibitor (LY294002) that was not toxic to cells

Since PI3-K is involved in a number of pathways critical for cell survival, using PI3-K inhibitor, one had to be concerned about its toxicity to the cells. Therefore, the first part of this experiment was to find the optimal concentration of PI3-K inhibitor, LY294002, which was not harmful to both types. MG-63 and hFOB were divided (5000 cells/well) into 13 groups and incubated for 48 h in an absence of LY294002 (control group) or with 70 μM, 40 μM, 10 μM, 1 μM, 100 nM or 10 nM of LY294002. An additional vehicle group was also included. Afterward, cell viability was tested by using the MTT assay. The appropriate concentration of LY294002 was used in the following intracellular signaling study.



4.2 To study the effect of PI3-K inhibitor alone or with PRL on the osteoblast activity, as indicated by the ALP activity as a marker

After the optimal concentration of PI3-K (LY294002) was obtained from experiment 4.1, the intracellular signaling pathway of PRL action in the osteoblasts (MG-63 and hFOB) was determined. Cells were divided into 5 groups and incubated for 48 h in the absence of PRL and LY294002 (< 1 μM) as a control group, vehicle group, PRL-treated, LY294002-treated and the combination of PRL and LY294002-treated groups.



Determination of ALP activity as an indicator of the osteoblast activity

F. Statistic analyses

Values were reported as mean ± SEM. Differences between groups were analyzed by One-way ANOVA followed by Newman-Keuls multiple comparison tests, using GraphPad Prism software.

CHAPTER IV
RESULTS

1. The expression of human prolactin receptor (hRPLR) mRNA and protein in MG-63 and hFOB

The overall objective of these experiments was to investigate the direct effect of PRL on bone remodeling through osteoblast function. First of all, the presence of hPRLR mRNA and protein in MG-63 and hFOB were verified by the RT-PCR and immunocytochemistry techniques. Fetal bovine serum (FBS), dexamethasone and 1, 25 $(OH)_2 D_3$ were used to stimulate the osteogenic differentiating gene expression including that of hPRLR (Bataille-Simoneau et al., 1996)

In MG-63, hPRLR mRNA was slightly expressed in 0.2 and 10 % of FBS whereas it was strongly expressed after incubating for 16 h with 10^{-7} M 1, $25(OH)_2 D_3$ and 10^{-6} dexamethasone. With a combination of 1, $25(OH)_2 D_3$ and dexamethasone, hPRLR mRNA expression was not further increased i.e., no additive effect of the two agents, as shown in Figure 8. In addition, the presence of hPRLR protein , as represented by the immunocytochemistry staining, was shown to be expressed after 16 h incubation with 10^{-7} M 1,25-$(OH)_2 D_3$ (Figure 9A). Negative control i e., in the absence of anti-hPRLR antibody staining, was shown in Figure 9B. Therefore, 16 h incubation of 10^{-7} M 1,25-$(OH)_2 D_3$ was used to stimulate PRLR expression in following MG-63 experiments, prior to PRL administration.

In contrast to MG-63 cells, hPRLR mRNA was highly and equally expressed in hFOB in all conditions as shown in Figure 10. Together it was found that the hPRLR protein was constitutively expressed in normal condition as shown in Figure 11A. Negative control was shown in Figure 11B.

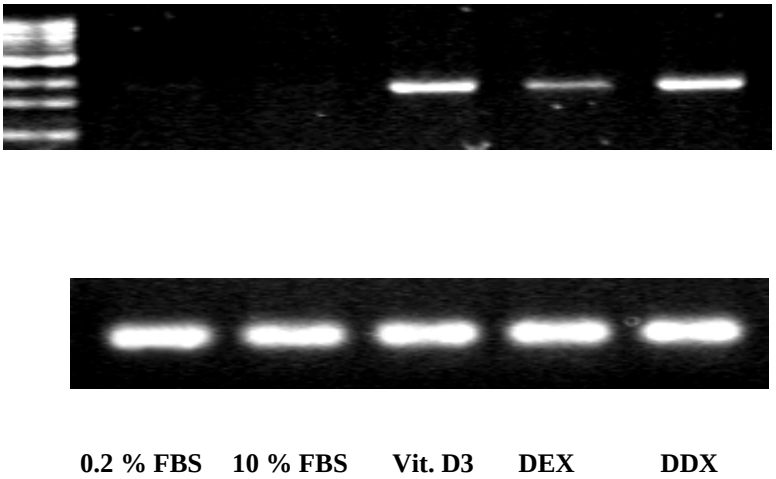
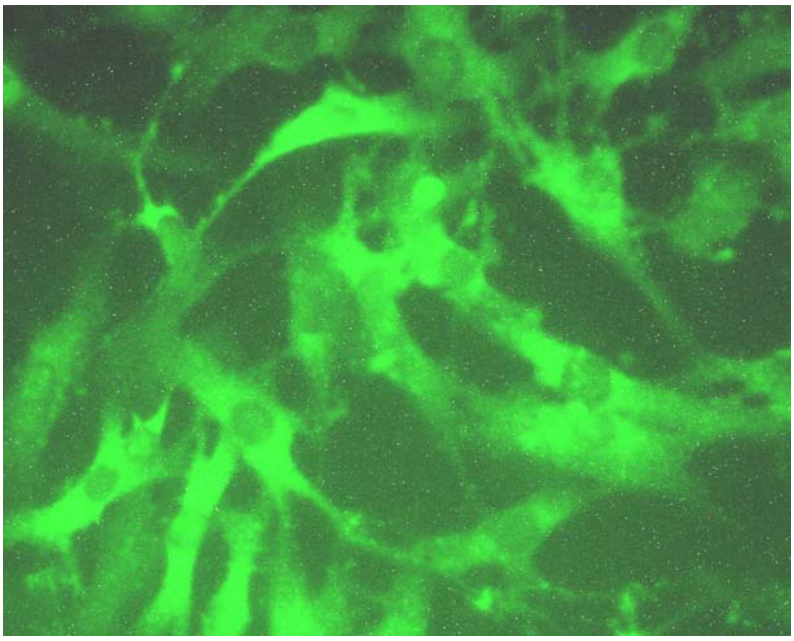


Figure 8 Conventional RT-PCR analyses of human prolactin receptor (hPRLR and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a house keeping gene in MG-63 which were incubated for 16 h in the presense of 0.2 % fetal bovine serum (FBS) as a control group, 10 % FBS, 10^{-7} M 1, 25 $(OH)_2 D_3$ (Vit. D3), 10^{-6} M dexamethasone (DEX), or a combination of 10^{-7} M 1, 25 $(OH)_2 D_3$ and 10^{-6} M dexamethasone (DDX).Results were obtained from three independent experiments; each was triplicated.

A



B

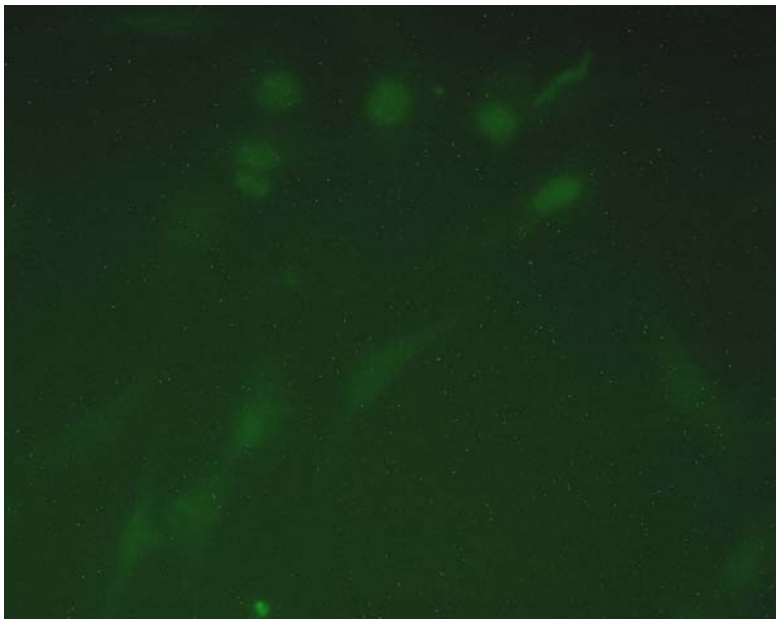


Figure 9 Immunocytochemistry staining of hPRLR protein in MG-6 after incubating with 10^{-7} M 1, 25 $(OH)_2$ D_3 for 16 h (A), and the negative control (no anti-hPRLR antibody staining) (B). The green color represents hPRLR.

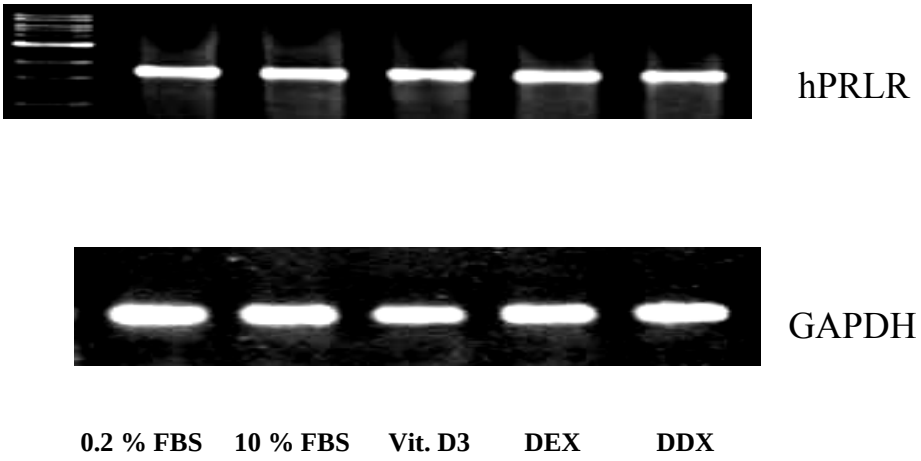
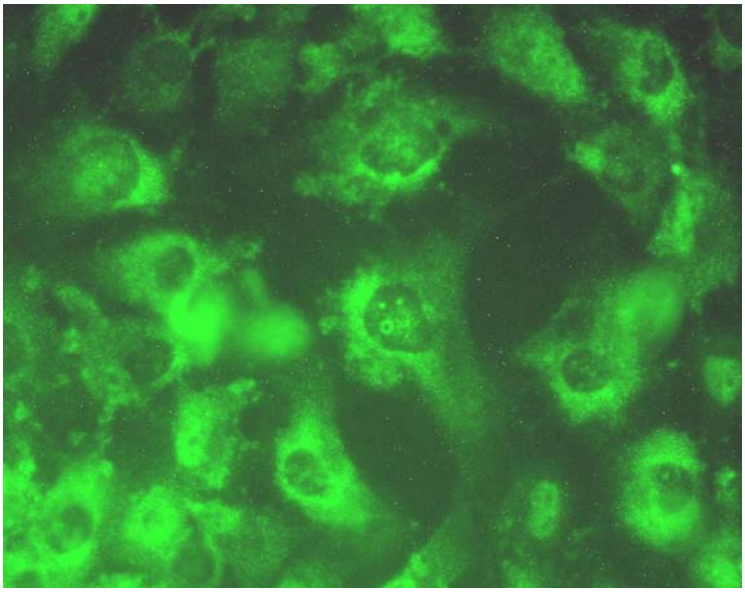


Figure 10 Conventional RT-PCR analyses of human prolactin receptor (hPRLR) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a house keeping gene in hFOB which were incubated for 16 h in the presense of 0.2 % fetal bovine serum (FBS) as a control group, 10 % FBS, 10^{-7} M 1, 25 $(OH)_2 D_3$ (Vit. D3), 10^{-6} M dexamethasone (DEX), or a combination of 10^{-7} M 1, 25 $(OH)_2 D_3$ and 10^{-6} M dexamethasone (DDX). Results were obtained from three independent experiments; each was triplicated.

A



B

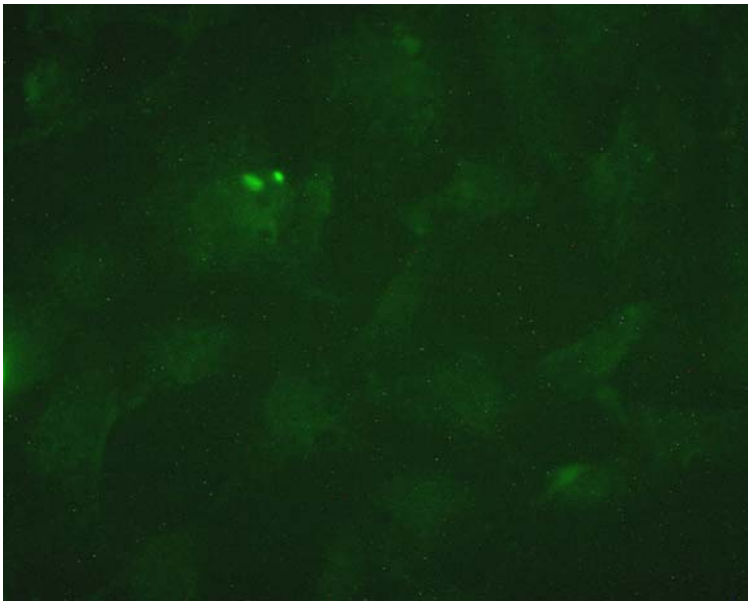


Figure 11 Immunocytochemistry staining of hPRLR protein in hFOB after incubating with 0.2 % FBS for 16 h (A) and the negative control (no anti hPRLR antibody staining) (B). The green color represents hPRLR.

2. The effect of PRL on the osteoblast activity

Various concentrations of human recombinant PRL were used to mimick the physiological PRL levels. An absence of PRL was represented by the control group, 1 and 10 ng/ml of PRL represented the normal levels of serum PRL, 100 ng/ml of PRL represented the pregnant and lactating stages, and 1000 ng/ml of PRL represented the pathological condition such as prolactinomas. The activity of alkaline phophatase, a marker of osteoblast activity, was then determined.

2.1 Dose response study

48 h incubation, PRL at concentrations of 100 and 1000 ng/ml slightly but significantly decreased MG-63 activity from 100.0 ± 1.74 % in the control group to 87.5 ± 2.9 % ($P<0.05$) and 84.2 ± 2.8 % ($P<0.01$) respectively, in MG-63 as shown in Figure 12A. Similar to MG-63, hFOB activity was significantly reduced by 100 and 1000 ng/ml PRL to 83.3 ± 3.0 % ($P<0.05$) and 77.7 ± 5.1 % ($P<0.01$), respectively, as shown in Figure 12B.

2.2 Time response study

To determine the onset of PRL action on osteoblast activity, MG-63 and hFOB were incubated with 100 ng/ml PRL (an effective physiological concentration from the dose response study) with varying incubating time i.e., 0, 0.5, 3, 6, 12, 24 and 48 h. As shown in Figure 13A, although MG-63 showed a tendency for a decrease in ALP activity from 12 h, only a 48 h incubation significantly decreased the osteoblast activity to 85.9 ± 2.2 % ($P<0.01$). As for hFOB, 24 h and 48 h incubation markedly decreased the osteoblast activity to 72.4 ± 5.0 % ($P<0.001$) and to 70.3 ± 0.3 % ($P<0.001$), respectively as shown in Figure 13B. It was clear that PRL at a high physiological concentration required more than 12 h to induce a decrease in the activities of both cells, and that the response of hFOB to PRL was faster and greater than that of MG-63.

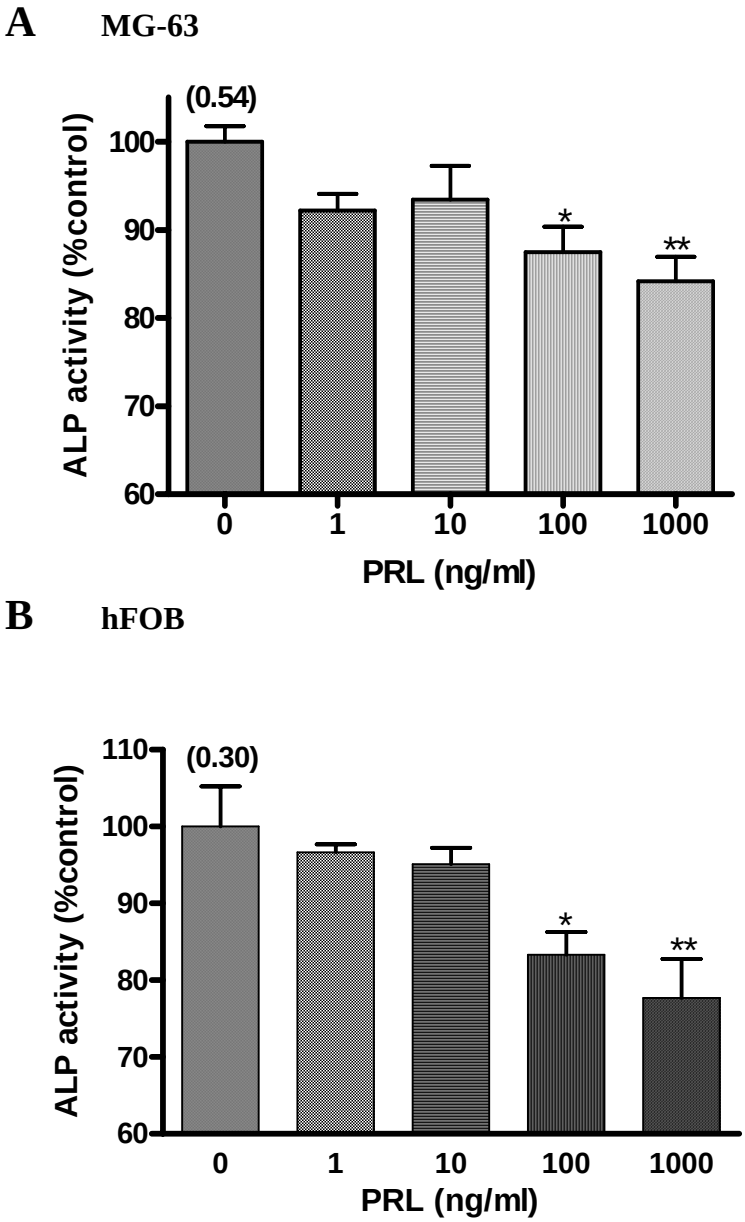


Figure 12 Effects of various concentrations of PRL on the osteoblast activity using ALP activity as a marker, in MG-63 (A) and hFOB (B). Data, presented as a relative activity in percent of control, are means ± SEM from five (MG-63) and four (hFOB) independent experiments; each was replicated five times, * $P<0.05$ and ** $P<0.01$.

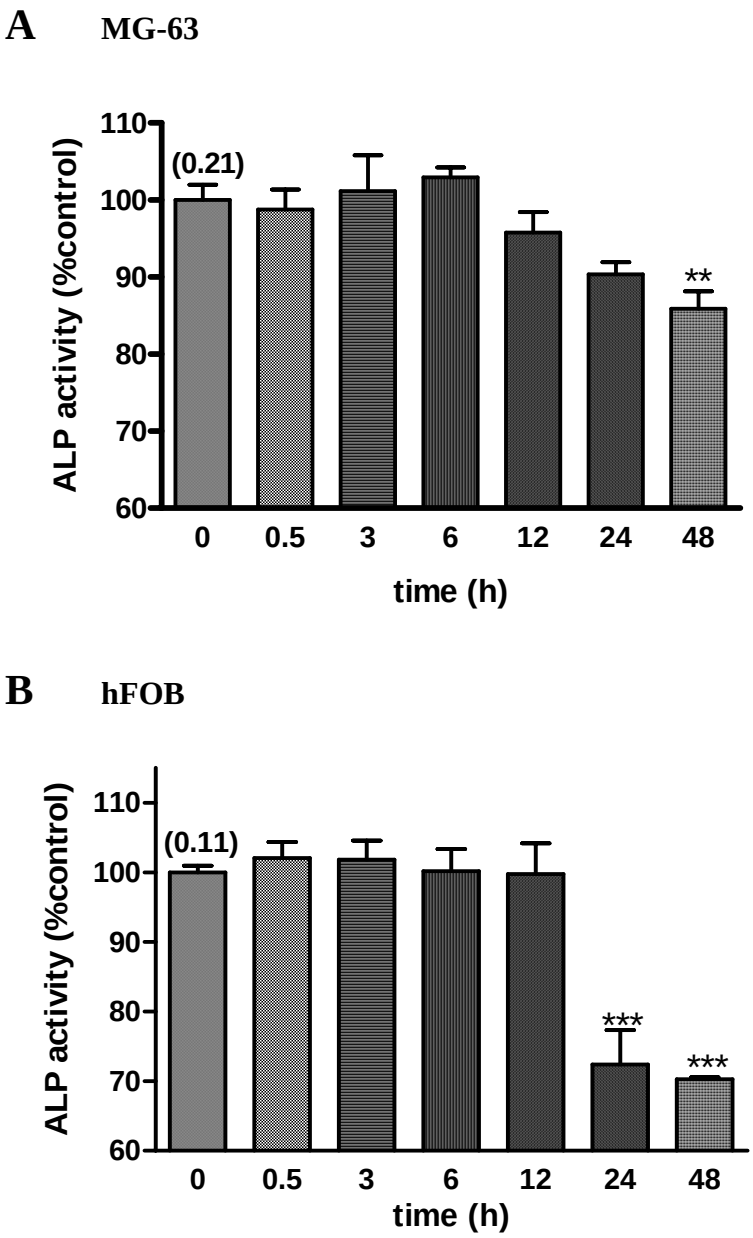


Figure 13 Effects of varying the incubating time with 100 ng/ml PRL on the osteoblast activity using ALP activity as a marker, in MG-63 (A) and hFOB (B). Data, presented as a relative activity in percent of control, are means ± SEM from seven (MG-63) and four (hFOB) independent experiments; each was replicated five times, ** $P<0.01$ and *** $P<0.001$.

3. The effect of PRL on the osteoblast proliferation and differentiation

3.1 The effect of PRL on osteoblast proliferation using the MTT assay

To investigate the effect of PRL on the osteoblast proliferation, the number of cells was determined by the MTT assay. PRL at 0, 1, 10, 100 and 1000 ng/ml had no effect on osteoblast proliferation in both MG-63 and hFOB as shown in Figures 14A and 14B.

3.2 The effect of PRL on osteoblast differentiation as determined by the osteocalcin and ALP mRNA expression

3.2.1 Dose response study

ALP mRNA expression was slightly but significantly reduced by PRL at 10, 100 and 1000 ng/ml from 100.0 ± 3.5 % in the control group to 88.6 ± 3.0 % ($P<0.05$), 85.4 ± 2.6 % ($P<0.05$) and 83.1 ± 2.6 % ($P<0.01$), respectively, as shown in Figure 15A. In contrast to MG-63, as shown in Figure 15B, hFOB differentiation was much less responsive to the suppressive action of PRL and the ALP mRNA expression was slightly reduced from 100.00 ± 3.3 % in the control group to 82.8 ± 2.7 % only by 1000 ng/ml PRL which is a pathological concentration. Interestingly, when the mRNA expression of osteocalcin, a specific marker for bone formation and osteoblast differentiation was determined, MG-63 and hFOB responded to PRL in an opposite manner. For MG-63, osteocalcin mRNA expression was significantly reduced from 100.0 ± 2.1 % in control group to 81.7 ± 3.4 % ($P<0.05$) by 1000 ng/ml PRL as shown in Figure 16A. In contrast, osteocalcin mRNA expression in hFOB was significantly enhanced by 100 and 1000 ng/ml PRL from 100.0 ± 5.7 % in the control group to 155.3 ± 13.0 % ($P<0.05$) and 174.7 ± 12.1 % ($P<0.01$), respectively (Figure 16B).

3.2.2 Time response study

The effect of 100 ng/ml PRL on ALP mRNA expression in MG-63 showed a significant reduction after 24 and 48 h incubation i.e., from 100.0 ± 4.0 % to 77.2 ± 1.59 % ($P<0.01$) and 78.1 ± 2.2 % ($P<0.01$), respectively, as shown in Figure 17A. For osteocalcin, the action of PRL took longer and a significant response was observed after 48 h incubation with the mRNA expression being reduced from 100.0 ± 5.5 % to 76.8 ± 3.6 % ($P<0.05$), as shown in Figure 17B.

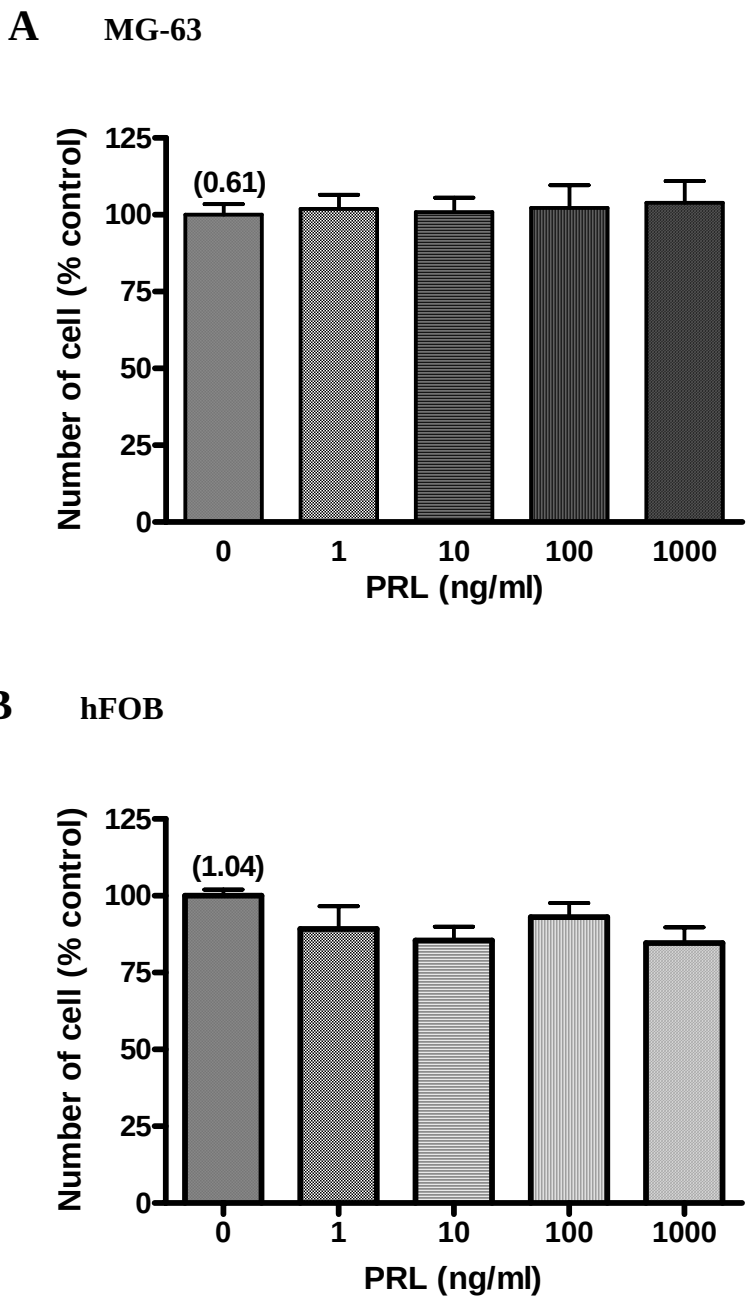


Figure 14 Effects of various concentrations of PRL on MG-63 (A) and hFOB (B) proliferation as determined by the MTT assay. Data, presented as relative values in percent of control, are means ± SEM from five (MG-63) and four (hFOB) independent experiments; each was triplicated.

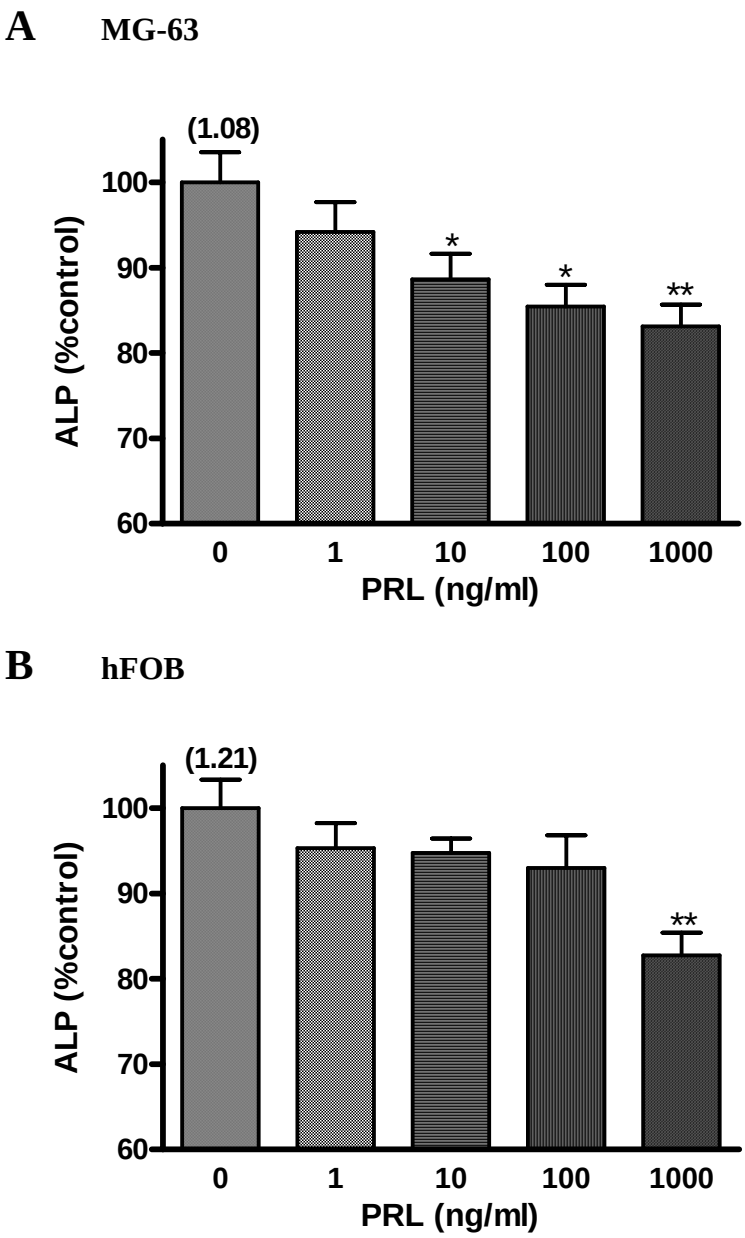


Figure 15 Effects of various concentrations of PRL on the ALP mRNA expression, in MG-63 (A) and hFOB (B). Data, presented as relative values in percent of control, are means ± SEM from seven (MG-63) and four (hFOB) independent experiments; each was triplicated, $^{*}P<0.05$ and $^{**}P<0.01$.

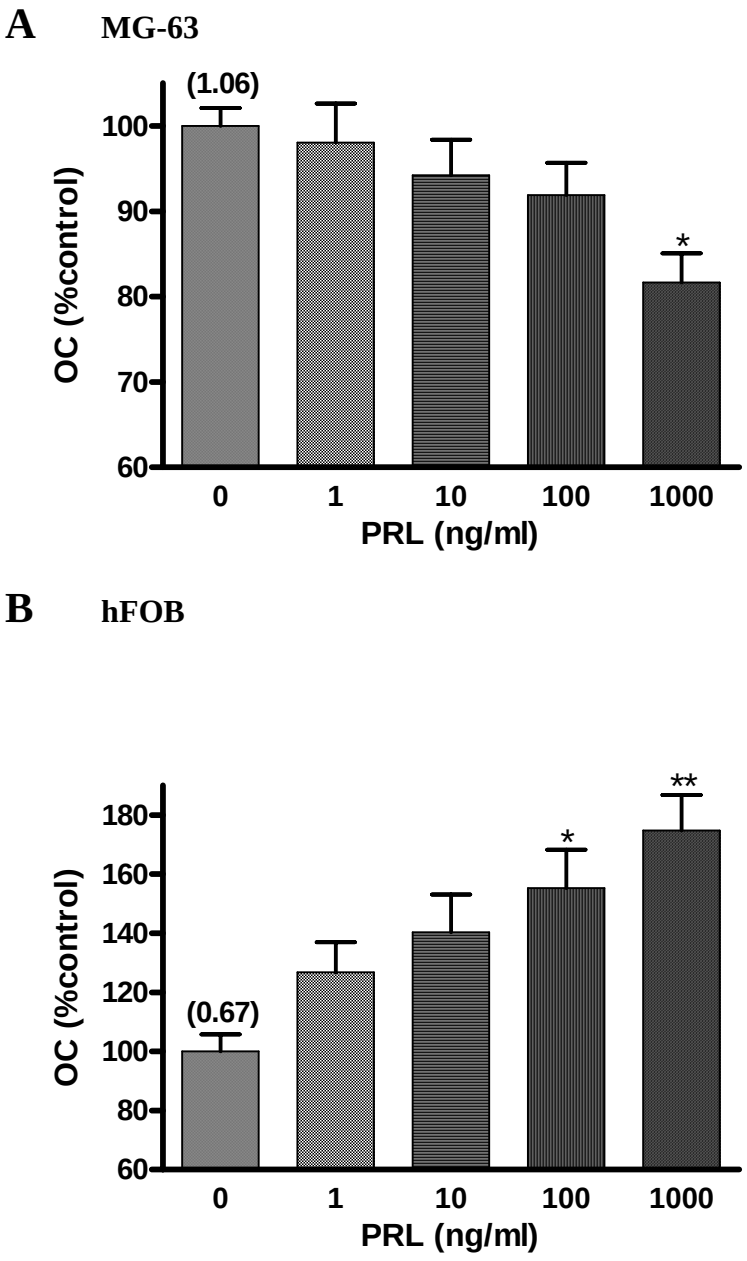


Figure 16 Effects of various concentrations of PRL on osteocalcin (OC) mRNA expression, in MG-63 (A) and hFOB (B). Data, presented as relative values in percent of control, are means ± SEM from five (MG-63) and three (hFOB) independent experiments; each was triplicated, $^{*}P<0.05$ and $^{**}P<0.01$.

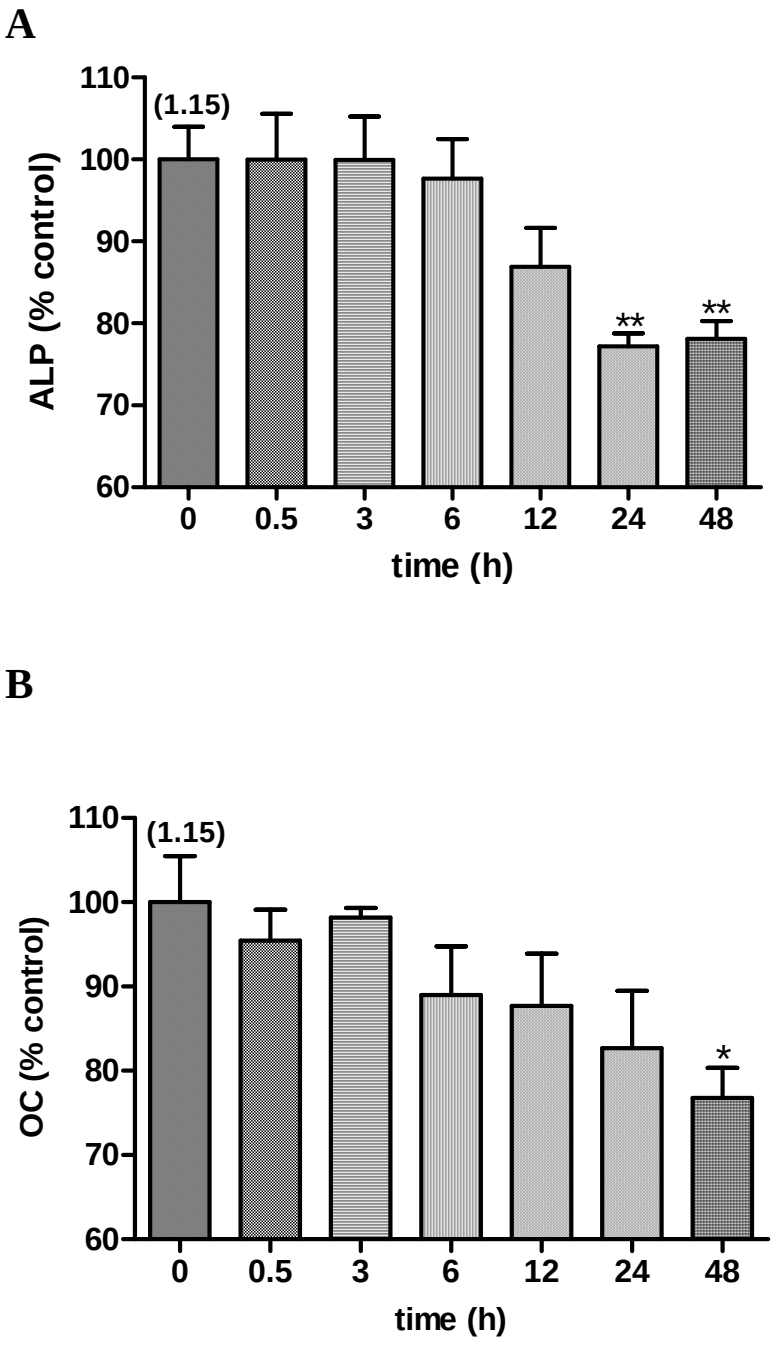


Figure 17 Effects of varying the incubating time with 100 ng/ml PRL on the MG-63 mRNA expressions of ALP (A) and osteocalcin (OC) (B). Data, presented as relative values in percent of control, are means ± SEM from four independent experiments; each was triplicated, $^{*}P<0.05$ and $^{**}P<0.01$.

4. The effect of PRL on bone resorption by determining the production of RANKL and osteoprotegerin

Bone remodeling can be regulated by both systemic and local factors, both of which modulate the cellular mechanism through the common RANKL and OPG pathway. OPG or osteoprotegerin, a decoy receptor of RANKL, synthesized and secreted from the osteoblast can bind to RANKL, thus preventing the binding of RANKL to its receptor, RANK on the osteoclast, and consequently preventing the osteoclast differentiation and bone resorption. The ratio of RANKL and OPG (RANKL/OPG) has been commonly used as an indicator of the osteoclastogenic function of osteoblast that may lead to bone resorption. The mRNA and protein expressions of RANKL and OPG determined in this experiment were used to determine the effect of PRL on the regulation of bone resorption though the RANKL and OPG pathway.

4.1 The effect of PRL on RANKL and OPG mRNA expression

As shown in Figure 18A, 10, 100 and 1000 ng/ml PRL significantly decreased the OPG mRNA expression in MG-63 from 100.0 ± 2.8 % in the control group to 85.8 ± 4.5 % ($P<0.05$), 82.7 ± 1.1 % ($P<0.05$) and 85.4 ± 4.4 % ($P<0.05$), respectively. However, PRL had no effect on RANKL mRNA expression as shown in Figure 18B. Therefore, the RANKL/OPG ratio which was the indicator of bone resorption, was increased by 10, 100 and 1000 ng/ml from 1.00 in the control group to 1.34 ± 0.07 ($P<0.05$), 1.43 ± 0.11 ($P<0.05$) and 1.31 ± 0.12 ($P<0.05$), respectively (Figure 18C).

As for hFOB, PRL at 10, 100 and 1000 ng/ml significantly decreased the RANKL mRNA expression from 100.0 ± 5.8 % in control group to 76.5 ± 5.0 % ($P<0.05$), 76.8 ± 4.0 % ($P<0.05$) and 57.6 ± 7.7 % ($P<0.01$), respectively, as shown in Figure 19A, while having no effect on the OPG mRNA expression (Figure 19B). Therefore, the RANKL/OPG ratio was decreased in the 10, 100 and 1000 ng/ml PRL-treated groups from 1 in the control group to 0.76 ± 0.02 ($P<0.05$), 0.79 ± 0.06 ($P<0.05$) and 0.56 ± 0.06 ($P<0.001$), respectively, as shown in Figure 19C.

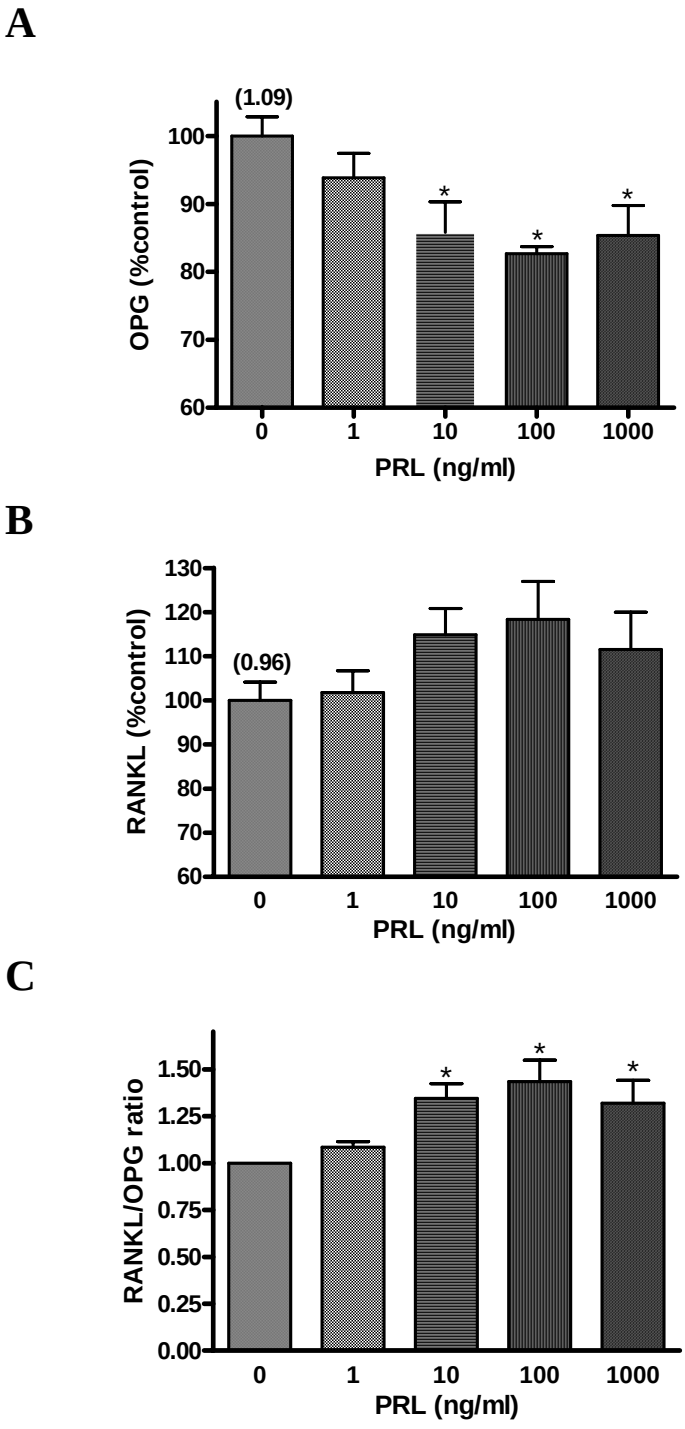


Figure 18 Effects of various concentrations of PRL on OPG (A) and RANKL (B) mRNA expression, and the RANKL/OPG ratio (C) in MG-63. Data, presented as relative values in percent of control, are means ± SEM from five independent experiments; each was triplicated, $^{*}P<0.05$.

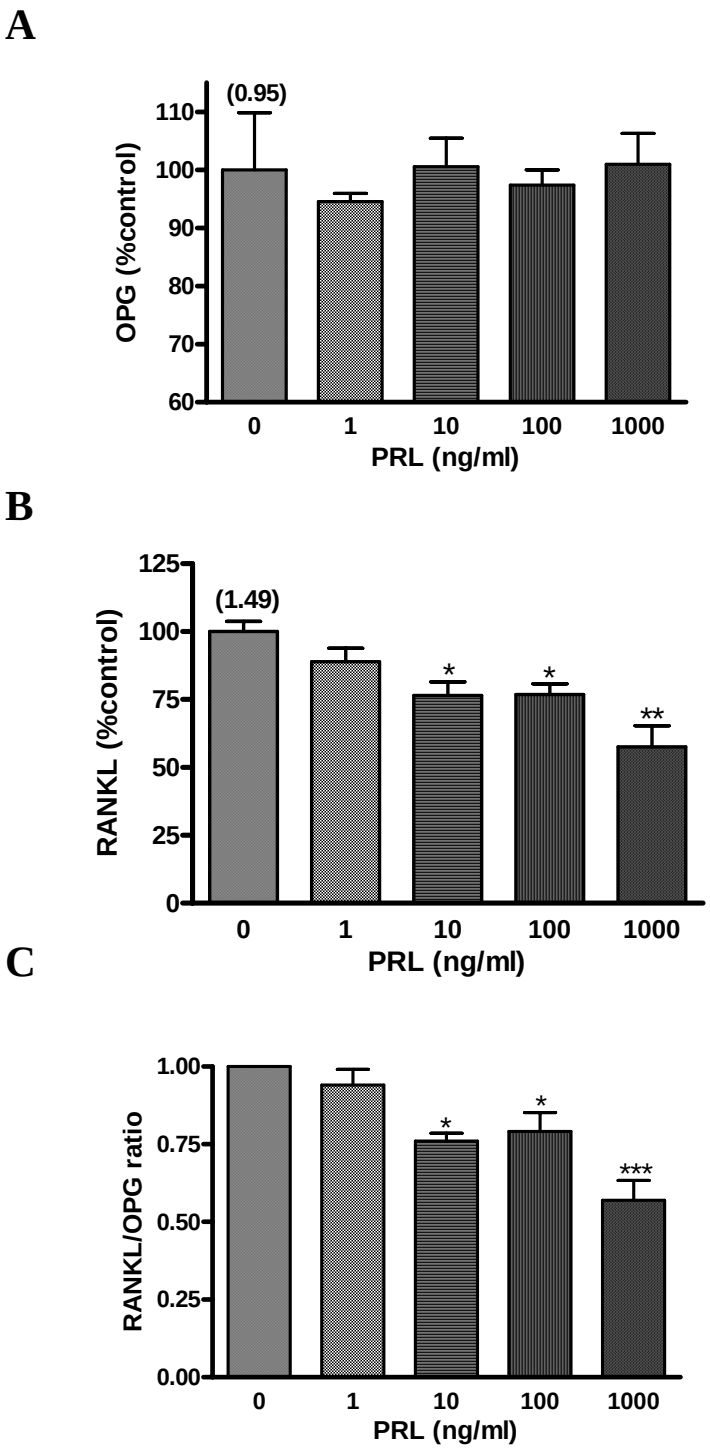


Figure 19 Effects of various concentrations of PRL on OPG (A) and RANKL (B) mRNA expression, and the RANKL/OPG mRNA ratio (C) in hFOB. Data, presented as relative values in percent of control, are means ± SEM from three independent experiments; each was triplicated, $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$.

4.2 The effect of PRL on RANKL and OPG protein expression

The effects of various concentrations of PRL on the protein expression of RANKL and OPG were studied by the immunoblotting assay. The primary antibodies of human RANKL and OPG were used to approximate the whole cell RANKL and OPG proteins.

In MG-63, PRL at 1000 ng/ml significantly decreased OPG protein expression from 100.0 ± 5.6 % to 69.2 ± 7.6 % ($P<0.05$) as shown in Figure 20A, but increased RANKL protein expression from 100.0 ± 5.4 % in the control group to 147.3 ± 9.2 % ($P<0.01$) as shown in Figure 20B. These led to an increase of the RANKL/OPG protein ratio from 1 in the control group to 1.80 ± 0.25 ($P<0.05$) and 2.26 ± 0.19 ($P<0.001$) in 100 and 1000 ng/ml PRL-treated groups (Figure 20C).

On the other hand, in hFOB, PRL at 100 and 1000 ng/ml significantly increased OPG protein expression from 100.0 ± 6.1 % in the control group to 123.0 ± 5.4 % ($P<0.05$) and 140.1 ± 7.5 % ($P<0.001$), respectively as shown in Figure 21A, while decreasing RANKL protein expression from 100.0 ± 5.8 % in the control group to 71.5 ± 2.8 % ($P<0.01$) and 64.8 ± 6.5 % ($P<0.01$), respectively as shown in Figure 21B. Consequently, these led to a decrease in the RANKL/OPG protein ratio from 1 in the control group to 0.59 ± 0.04 ($P<0.01$) and 0.44 ± 0.06 ($P<0.01$) in the 100 and 1000 ng/ml PRL-treated groups, respectively, as shown in Figure 21C.

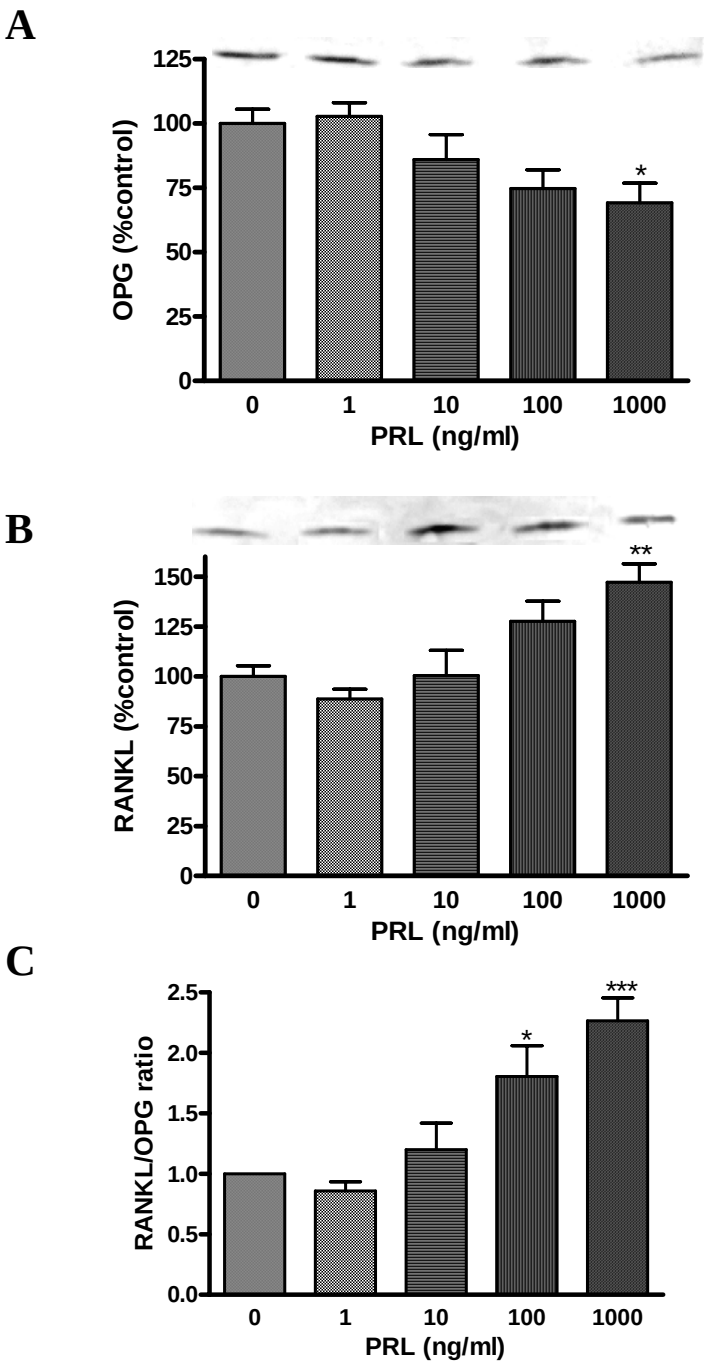


Figure 20 Effects of various concentrations of PRL on OPG (A) and RANKL (B) protein expression, and RANKL/OPG protein ratio (C) in MG-63. Data, presented as relative values in percent of control, are means ± SEM from six (RANKL) and five (OPG and RANKL/OPG ratio) independent experiments; each was triplicated, $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$.

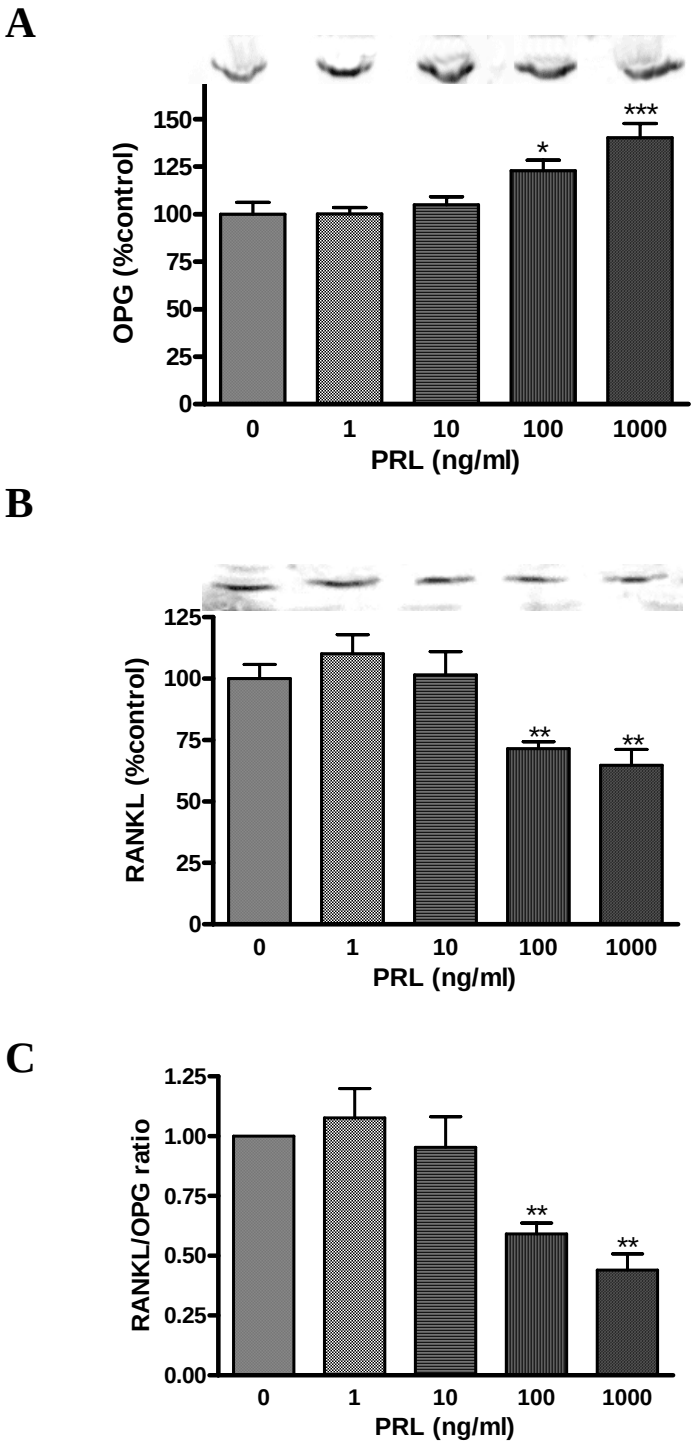


Figure 21 Effects of various concentrations of PRL on OPG (A) and RANKL (B) proteins expression, and RANKL/OPG protein ratio (C) in hFOB.
Data, presented as relative values in percent of control, are means ± SEM from seven (RANKL) and five (OPG and RANKL/OPG ratio) independent experiments; each was triplicated,
$^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$.

5. The intracellular signaling pathway of PRL action in osteoblasts

The first part of this experiment was to find the optimal concentration of PI-3 kinase inhibitor (LY294002) that was not harmful to the osteoblasts (MG-63 and hFOB). Figure 22A shows that the number of MG-63 being significantly decreased from 100.0 ± 3.8 % in control group to 40.8 ± 2.0 % ($P<0.001$), 37.8 ± 1.7 % ($P<0.001$) and 26.5 ± 4.2 % ($P<0.001$) by 10, 40 and 70 μM LY294002, respectively. Figure 22B shows the number of hFOB being decreased from 100.0 ± 1.7 % in control group to 53.9 ± 10.7 % ($P<0.05$) and 41.2 ± 9.2 % ($P<0.01$) by 40 and 70 μM LY294002, respectively. Therefore, the concentrations of LY294002 that were not toxic to MG-63 and hFOB were less than 1 μM.

In the investigation of the intracellular signaling pathway of PRL action in osteoblasts, LY294002 at a final concentration of 100 nM was used in the 1 h incubation prior to PRL administration, after which the action of PRL on osteoblast was determined through changes in ALP activity. Results from both types of cells confirmed that PRL at 100 ng/ml significantly decreased the ALP activity from 100.0 ± 1.8 % in control group to 81.7 ± 2.6 % ($P<0.001$) in MG-63, and 63.7 ± 5.3 % ($P<0.01$) in hFOB. This suppressive action of PRL on the alkaline phophatase activity was completely abolished by preincubating the cells with PI-3 kinase inhibitor. As seen in Figures 23A and 23B, while the vehicle alone or LY294002 alone had no effect on the alkaline phophatase activity, the suppressive effect of PRL was totally abolished by preincubation with LY294002. The results thus indicated that the action of PRL on osteoblast activity was mediated via the PI-3 kinase pathway.

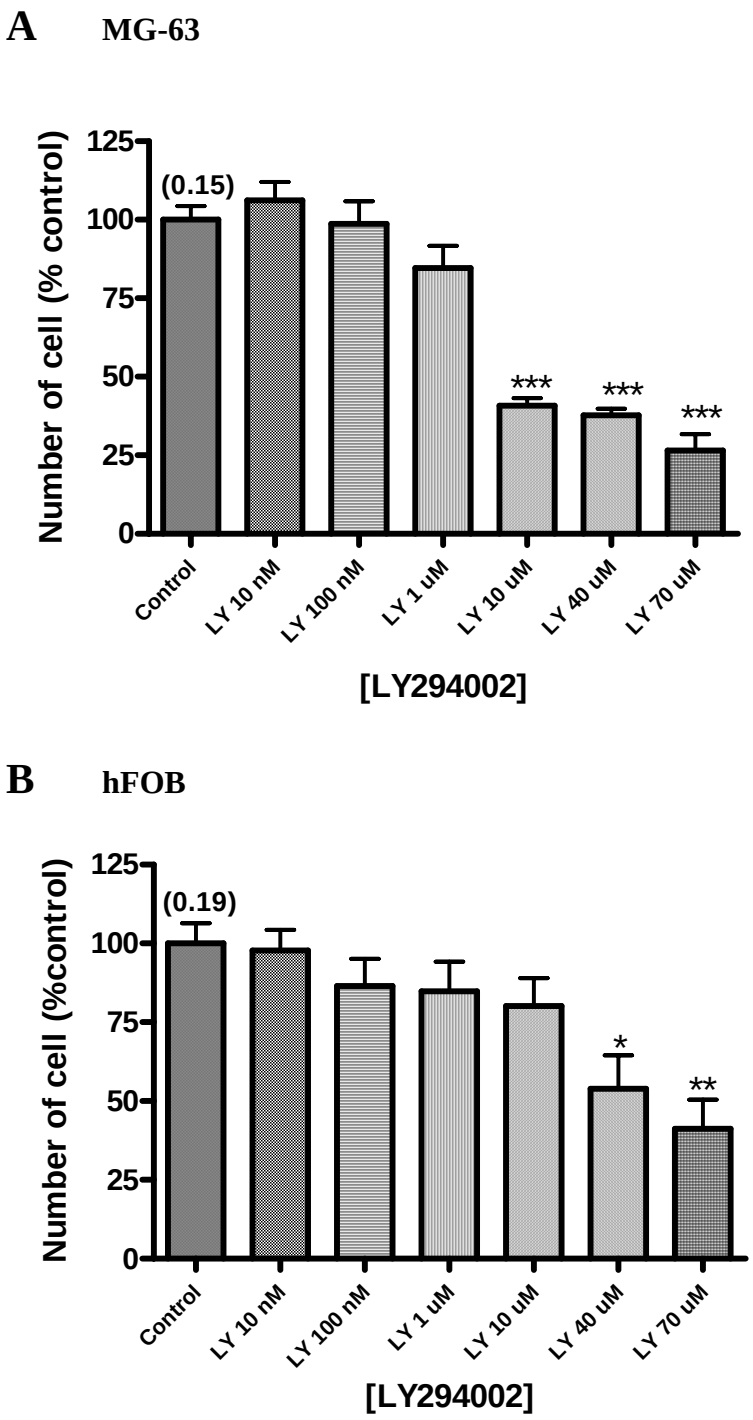


Figure 22 Effects of various concentrations of PI-3 kinase inhibitor, LY294002, on the survival of MG-63 (A) and hFOB (B). Data, presented as relative values in percent of control, are means ± SEM from three independent experiments; each was replicated five times,
$^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$.

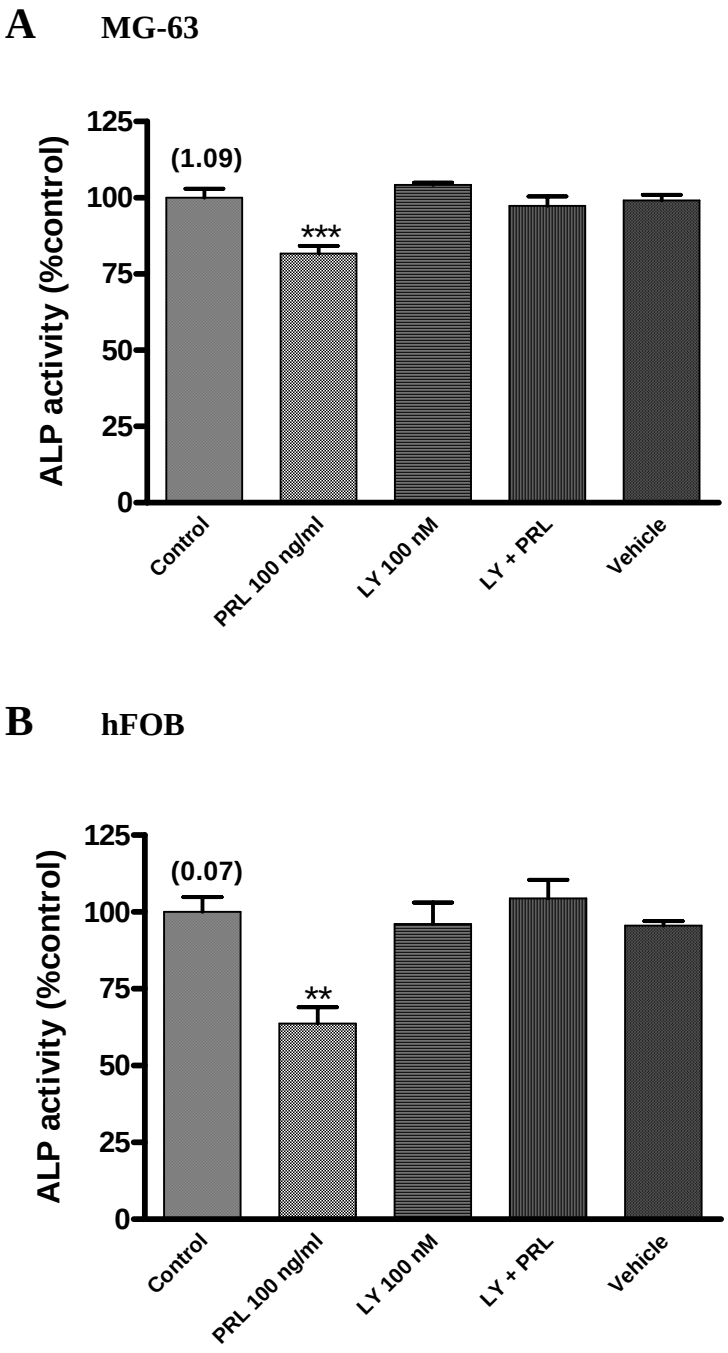


Figure 23 Effects of PI-3 kinase inhibitor (LY294002 at 100 nM) alone or with PRL on the suppressive action of PRL on the alkaline phophatase activity (using as a marker of osteoblast activity) in MG-63 (A) and hFOB (B). Data, presented as relative values in percent of control, are means ± SEM from four independent experiments; each was replicated five times, ** $P<0.01$ and $^{***}P<0.001$.

CHAPTER V
DISCUSSION

Hyperprolactinemia is one of the risk factors of osteoporosis which has been believed to result from hyperprolactinemia-induced suppression of GnRH which in turn causes hypogonadism. However, Biller and his group (1991) reported bone loss in hyperprolactinemic women with normal estrogen levels but the severity of bone loss was significantly less than that in hyperprolactinemia with estrogen deficiency. More recently, the presence of prolactin receptors (PRLR) in human osteosarcoma cell lines (Saos-2 and MG-63) and primary osteoblasts cultured from mouse calvariae (Bataille-Simoneau et al., 1996; Clement-Lacroix et al., 1999; Coss et al., 2000) supported the hypothesis of a direct PRL effect on bone cell. Therefore, the aim of this study was to investigate the direct role of PRL and its signal transduction pathway in the regulation of bone metabolism. To achieve this objective, human osteoblast-like cell (MG-63) and human immortalized normal fetal osteoblast 1.19 (hFOB) were used as experimental models.

MG-63 was derived from osteosarcoma of adult bone tissue (Billiau et al., 1977).This osteosarcoma cell line when cultured in osteogenic medium, exhibited more osteoblastic markers compared to other human and rat osteosarcoma cell lines i.e., Saos-2 cells or U-2OS cells. MG-63 also produced the extracellular matrix required for bone formation such as collagen type I and osteocalcin. It was concluded that this osteosarcoma cell line was suitable as osteoblast model for the study of bone metabolism (Pautke et al., 2004). As for hFOB, the human fetal osteoblasts derived from fetal bone tissue, they also exhibited bone related genes such as Cbfa1 (early osteoblast precursor gene), osteocalcin and PTH receptor. At the surface of hFOB, specific markers of bone marrow mesenchymal stromal stem cells (BMMSCs) i.e., CD13, CD29, CD73/SH3/SH4 were found (Pittenger et al., 1999), whereas the multiple hematopoietic markers such as CD14, CD34 were absent. hFOB was thus characterized as osteoprogenitor with plasticity property of stem cells similar to that of

embryo (Pittenger et al., 1999; Yen et al., 2007). However, obvious discrimination between MG-63 as an adult cell, and hFOB as a fetal cell has not been documented. Therefore, the degree of the overlapping of fetal and adult characteristics and their respective specific marker(s) remained to be sorted out.

A. PRLR mRNA and protein expression in MG-63, an adult human osteoblast model, and hFOB, a fetal human osteoblast model.

PRLR was first identified in 1974 as a specific, high-affinity, membrane-anchored protein (Kelly et al., 1974; Posner et al., 1974). Gene encoding PRLR is located on chromosome 5 that contains at least 10 exons for an overall length exceeding 100 kB (Arden et al., 1990). Distribution of PRLR has been demonstrated in a number of cells including bone cells (Bole-Feysot et al., 1998). Indeed, PRLR has been reported in MG-63 and Saos-2, the human osteosarcoma cell in which PRLR could be upregulated by the stimulatory effect of osteotropic factors such as 1,25-$(OH)_2D_3$ and dexamethasone (Bataille-Simoneau et al., 1996). Since primary osteoblasts obtained from neonatal calvaria also expressed PRLR (Clement-Lacroix et al., 1999), a direct action of PRL on osteoblasts was postulated.

PRLR has been classified into 4 isoforms, one soluble PRLR and three membrane-anchored isoforms, which result from alternative splicing of the primary transcription. These different membrane anchored PRLR isoforms differ in the length and composition of their cytoplasmic tails and are referred to as short, intermediate, and long PRLR with respect to their size. For instance, in rat, PRLR isoforms contain 291 (short), 393 (intermediate), or 591 (long) amino acids (Bole-Feysot et al., 1998). The long isoform is the only PRLR form able to activate the JAK2/STAT5 pathway, which is essential for PRL-induced transcription of milk protein genes, differentiation of normal epithelial cells, and initiation and maintenance of lactation. In contrast to the PRLR-long isoform, PRLR-short isoform cannot mediate the transcriptional activation of the β-casein gene promoter induced by PRL (Lesueur et al., 1991). On the other hand, PRLR-short isoform was suggested to inhibit the PRL-induced PRLR-long isoform activation the promotes mammary epithelial cell proliferation (Meng et al.,

2004). Moreover, it was reported that the PRLR-short isoform exerted inhibitory effect by forming heterodimer with the long isoform in a ligand-independent manner (Qazi et al., 2006). Therefore, it has been suggested that the inhibitory role of the PRLR-short isoform may have physiological significance because the expression of the long and short PRL isoforms of PRLR is regulated in a tissue-specific manner (Lesueur et al., 1991). In the present study, only the long form of PRLR was investigated since this isoform possess a cytoplasmic tail (C terminal) which is required for the intracellular signal transduction (Bole-Feysot et al., 1998). A pair of primers used in the present study was designed to bind to the encoding gene from residue number 322 to residue number 2120, which gave rise only to the PRLR-long isoform mRNA (Figures 8 and 10). Furthermore, PRLR antibody used for determination of PRLR localization aggregated to amino acids which contributed to the non-overlapping protein of the long isoform with other isoforms (Figures 9 and 11).

PRLR mRNA in MG-63 and hFOB cells was verified by using RT-PCR and gel electrophoresis, and PRLR protein localization was demonstrated by immunocytochemistry. The results demonstrated that MG-63 cells normally expressed a very low level of PRLR. However, consistent with the previous study (Bataille-Simoneau et al., 1996), PRLR expression was significantly upregulated after 16 h incubation with dexamethasone or 1,25-$(OH)_2D_3$. The combination of dexamethasone and 1,25-$(OH)_2D_3$, on the other hand, had no additive effect, suggesting a possibility of the two hormones acting via a common pathway. This result was consistent with that in mouse calvaria demonstrating no further increased in VDR mRNA expression after treating with their combination, however, the underlying mechanism was unknown (Swanson et al., 2006). In hippocampal cells, crosstalk of dexamethasone and 1,25-$(OH)_2D_3$ were observed, and was proposed for two mechanisms. First, VDR and GR may compete for the common co-factors. However, it should be noted that the composition of cofactor may be specific in variety of cell. Second, holo-VDR and holo-GR may directly interact, resulting in impaired transactivation potential of (one of) the transaction functions (Obradovic et al., 2006). In addition, it was reported that dexamethasone could stimulate mRNA expression and its activity of 24-hydroxylase, which was the enzyme that hydrolyzed 1,25-$(OH)_2D_3$ into an inactive form of vitamin D, in osteoblastic cell line, UMR-106. This observation suggested that by enhancing

24-hydroxylase expression, dexamethasone favored catabolism of $1,25(OH)_2D_3$, and thus counteracted the anabolic effect of $1,25(OH)_2D_3$ in osteoblasts (Kurahashi et al., 2002).

Our laboratory has recently demonstrated that primary rat osteoblasts, bone tissues from tibia, femur, calvaria and L5-6 vertebrae of adult female rats expressed both short and long isoforms of PRLR. It was shown that all four bones expressed the long-isoform PRLR mRNA more than the short-isoform PRLR. This data confirmed that both cortical and trabecular sites were potential targets of PRL (Charoenphandhu et al., 2007; Seriwatanachai et al., 2008a).

For the first time, this study verified the presence of the long isoform of PRLR mRNA and protein localization in human fetal osteoblasts, hFOB. Interestingly, in contrast to MG-63 cell line, the mRNA of PRLR in hFOB was highly expressed in normal culture medium and was not affected by the 16 h incubation with dexamethasone, $1,25\text{-}(OH)_2D_3$ or their combination. The expression of PRLR mRNA and protein in hFOB cells, similar to that in the primary rat osteoblast (Seriwatanachai et al., 2008a), was constitutive and independent of both hormones. This implicated that hFOB and primary rat osteoblast shared similar characteristics of normal osteoblasts, in contrast to MG-63 which was derived from osteosarcoma of adult bone tissue. The present data from hFOB experiment also lent support to the previous reports of mRNA expression of long and short forms of PRLR in fetal tissues such as adrenal cortex, renal tubules, small intestine, adipose tissue and bone (Bole-Feysot et al., 1998). Furthermore, the expressions of PRLR mRNA and protein have been known to increase in vivo in late gestation in fetal rat (Royster et al., 1995). Although, the PRLR mRNA expression in fetal osteoblasts in this study was not affected by $1,25\text{-}(OH)_2D_3$ or dexamethasone, hormonal regulation of PRLR expression during the embryogenesis could not be excluded. Moreover, there appeared to be an age dependent effect on a number of tissues. For instance, the mRNA and protein expression of both long and short isoforms PRLR in the olfactory bulb showed less PRLR expression in adult tissues than in the embryo (Freemark et al., 1996). This study postulated a novel role of PRL in olfactory differentiation and development and may provide new mechanism by which PRL regulated neonatal behavior and maternal-internal interaction. It was suggested that PRLR expression was differentially expressed in various tissues and the

expression showed distinct phases during the maturation of individual tissue. Based on the evidence of PRLR expression in bone cells as shown by the present study and previous in vitro and in vivo studies, it was suggested that PRL had a potential role in the regulation of fetal skeletal development and bone remodeling in adult skeleton.

B. The effect of PRL on MG-63, an adult human osteoblast model

Accelerated bone turnover is a common feature of physiological and pathological hyperprolactinemia (Naylor et al., 2000). Hyperprolactinemia in physiological conditions such as during pregnancy and lactation that produce physiologically high concentration of PRL of ~75-100 and ~200-300 ng/ml, respectively, (Prentice, 2000; Ritchie et al., 1998) is accompanied by a high bone turnover, that, in turn, is to provide rapid supply of calcium for fetal growth and milk production (Kovacs, 2005). Our previous histomorphometric study in lactating rats showed that suppression of the high secretory rate of endogenous PRL by bromocriptine suppressed maternal bone turnover by reducing calcium deposition rate and calcium resorption rate in femur, tibia, and vertebrae. Furthermore, such reduction of both bone formation and bone resorption could result in a net bone loss in the bromocriptine-treated lactating rats (Lotinun et al., 2003).

The pathological hyperprolactinemia, in contrast, mostly results from PRL secreting tumor or chronic uses of dopamine-related antipsychotic drugs (Haddad & Wieck, 2004; Jung et al., 2006). A conventional use of antipsychotic drug in schizophrenia patients elevates PRL levels by blocking dopaminergic inhibition (Naidoo et al., 2003). Important adverse effects of pathological hyperprolactinemia in women are galactorrhea and hypoestrogenism resulting in amenorrhea, impeding of normal cyclic ovarian function and loss of libido. In men, hyperprolactinemia results in impotence, loss of libido and hypospermatogenesis (Petty, 1999). The serum PRL concentration up to ~1000 ng/mL of PRL which is considered pathological hyperprolactinemia not only stimulates bone turnover but also produces a massive calcium loss, overt osteopenia, and osteoporosis (Biller et al., 1992; Naliato et al., 2005). From the human studies of pathological hyperprolactinemia, several investigators suggested that PRL-accelerated bone turnover was likely due to estrogen

deficiency, since hyperprolactinemia suppressed gonadotrophin secretion (Biller et al., 1992; Schlechte, 1995). However, the PRLR expression in osteoblasts (Clement-Lacroix et al., 1999; Coss et al., 2000; Seriwatanachai et al., 2008a; Seriwatanachai et al., 2008b) and the expression of both short and long isoforms of PRLR in tibiae, femora and vertebrae of adult female rats (Charoenphandhu et al., 2007), as discussed earlier, suggested that osteopenia or osteoporosis could be due to a direct action of PRL on bone cells. This study provided supportive evidence that high physiological (>100 ng/ml) levels of PRL could directly regulate bone remodeling via the osteoblasts.

Because the plasma membrane of osteoblast is rich in alkaline phosphatase (ALP), the activity of ALP is thus commonly used in both clinical and laboratory investigations to represent the osteoblast activity (Farley and Baylink, 1986; Leung et al., 1993; van Straalen et al., 1991). Osteoblasts also synthesize and secrete collagen type I and non collagenous proteins such as osteocalcin, osteopontin and osteonectin. Osteocalcin is a small protein that becomes part of bone matrix in the later phase of osteoblast differentiation (Qi et al., 2003). Both ALP and osteocalcin are thus used as indicators of the osteoblast activity and differentiation (Coss et al., 2000; Ducy et al., 2000). Therein, this present study examined the effect to PRL on ALP activity, ALP and osteocalcin mRNA expression in term of time and dose responses. It was found that PRL at 100 ng/ml significantly decreased the ALP activity after 48 h incubation (Figure 13). This result was consistent with the onset of action of PRL on the reduction of ALP and osteocalcin mRNA expression (Figure 17). PRL at 100 and 1000 ng/ml also significantly decreased the ALP activity and osteocalcin mRNA expression in MG-63. On the other hand, neither low nor high concentration of PRL had effect on osteoblast proliferation as measured by MTT assay (Figure 14). This result agreed with the previous study of 48 h incubation with varying concentrations of PRL up to 1000 ng/ml on osteoblast proliferation (Coss et al., 2000). The adverse action of PRL on ALP activity and its mRNA as well as osteocalcin mRNA expression without increasing the number of osteoblasts suggested a reduction of osteoblast function which could also lead to a decrease in bone formation.

It is known that osteoblasts express the receptor activator of nuclear factor κB ligand (RANKL) and osteoprotegerin (OPG) (Kostenuik et al., 2005). Binding of

RANKL to its receptor on the osteoclast progenitor cells induces bone resorption, whereas binding of RANKL to its decoy receptor OPG inhibits bone resorption. Thus, the ratio of RANKL/OPG expression by osteoblast is an important determinant of osteoclastogenesis and osteoclast activity as well as bone resorption. For instance, higher RANKL/OPG ratio indicates an induction of bone resorption, and lower RANKL/OPG ratio indicates a reduction of bone resorption (Kostenuik et al., 2005). Herein, the present study demonstrated, for the first time, that PRL slightly upregulated the expression of RANKL mRNA while downregulating that of OPG in MG-63 cells, thus leading to an increase in the RANKL/OPG mRNA ratio (Figure 18). Moreover, it was shown that the translational levels of OPG and RANKL (Figure 20) also responded to PRL in a similar manner to that of mRNA expression. Downregulation of OPG mRNA expression in primary rat osteoblasts from adult rats and a decrease in serum OPG levels in anterior pituitary transplantation rats (Seriwatanachai et al., 2008a) were consistent with the present findings. Furthermore, the concentration of PRL may be an important determinant of bone loss, because PRL regulated the expression of bone markers, including osteocalcin, ALP, RANKL and OPG, in a dose-response manner. It was noted that a pathological level of PRL (1000 ng/ml) markedly changed all measured parameters of bone turnover towards bone resorption, whereas 100 ng/ml PRL had less effect on the osteoblastic function.

The study of PRLR knockout in adult mice using double calcein labeling, demonstrated a decrease in bone formation rate and a reduction of bone mineral density as measured by dual energy x-ray absorptiometry (Qi et al., 2003). However, serum estradiol, progesterone and testosterone levels in the knockout mice were decreased, whereas PTH level was increased. Incidentally, changes in the hormone profile could be responsible for the low bone mineral density in PRLR knockout adult mice. Therefore, it was not possible to rule out the indirect systemic effects of PRL via interactions with other hormones implicated in the regulation of bone metabolism. Indeed, impaired ossification in developing calvaria was also observed in neonatal mice of the PRLR knockout dams, however, it was not determined whether there were changes in the hormone levels. Although, these in vivo data did not provide evidence for the exact role of PRL in the regulation of bone metabolism, they clearly showed that the basal circulating level of endogenous PRL (~7–10 ng/ml) must be essential for

the maintenance of normal bone growth and remodeling (Clement-Lacroix et al., 1999). In the present study, 10 ng/ml PRL was found to maintain a slightly elevated expression ratio of RANKL/OPG mRNA, which was apparently appropriate for normal bone function. On the other hand, the pathological levels of PRL often resulted in overt osteopenia similar to estrogen deficiency (Haddad 2004; Jung et al., 2006), which leads to increases in the skeletal remodeling, ultimately resulting in bone loss, and the increased risk of osteoporotic fractures. Estrogen deficiency probably causes bone loss by activating new bone remodeling sites and exaggerating the imbalance between bone formation and resorption (Lindsay and Cosman, 2001). At cellular level, estrogen inhibits osteoclasts, which are responsible for bone resorption, and stimulates osteoblasts that mediate bone formation. Estrogen may also play an important role in determining the life span of bone cells through controlling the rate of apoptosis. Hence, in low estrogen state, the lifespan of osteoblasts may be decreased and that of osteoclasts increased. In adolescents and young women, sustained production of estrogen is thus essential for the maintenance of bone mass (Lindsay et al., 1992).

It is well extablished that in pathological hyperprolactinemic patients, bone turnover is accelerated with bone resorption rate exceeding bone formation rate, and in vivo studies showed high bone turnover in compact bones of mature rats acutely treated with PRL ng/ml (Krishnamra and Seemoung et al., 1996). Interestingly, PRL has been found to be involved in the intestinal calcium transport both in the in vivo and vitro studies (Tanrattana et al., 2004; Charoenphandhu et al., 2006 and 2007; Jantarajit et al., 2007; Thongon et al., 2008). Of note was our previous finding that 1000 ng/mL PRL exerted a typical biphasic action on the small intestine in adult rat. PRL at 200-800 ng/ml significantly promoted the solvent drag-induced duodenal active calcium transport in a dose-response manner. However, the enhanced calcium absorption was diminished to the basal level by the pathological concentration of PRL of 1000 ng/ml (Tanrattana et al., 2004; Jantarajit et al., 2007), aggravating a negative calcium balance. Thus, it was postulated that the massive bone loss in pathological hyperprolactinemia could well result from the PRL-enhanced bone resorption without matching increase in the intestinal calcium absorption.

It was also apparent from our recent reports that decreases in bone formation and bone mass may be prevented by the concurrent stimulation of the intestinal

calcium absorption with ~100 ng/mL PRL (Tudpor et al., 2005; Charoenphandhu et al., 2007). In vitro finding in the present study i.e., suppression of osteoblast activity by PRL was contrary to the histomorphometric data of hyperprolactinemic (induced by the anterior pituitary transplantation) rats that exhibited enhanced mineral apposition and bone formation (Seriwatanachai et al., 2008a). The in vivo increase in bone formation could be due to the coupling of bone formation to enhanced bone resorption, which was absent in the in vitro experiments. Under the physiological condition i.e., in vivo Basic Multicellular Unit (BMU), a wandering team of cells are responsible for coupling bone formation to bone resorption i.e., osteoclasts dissolving bone surface into resorption pit which are subsequently filled with new bone by osteoblasts. Furthermore, the cytokines released from bone resorbing-matrix, local paracrines and cell to cell interactions that play crucial roles in the coupling of bone formation to bone resorption were obviously absent in the in vitro condition. Therefore, the uncoupling of bone formation from bone resorption in the in vitro study condition could account for the differential response of bone cells to PRL in the in vitro and in vivo conditions. Since, PRLR are present only on the surface membrane of osteoblasts but not osteoclasts (Clement-Lacroix et al., 1999; Coss et al., 2000; Seriwatanachai et al., 2008a; Seriwatanachai et al., 2008b), the stimulatory action of PRL on osteoclastic bone resorption as previously reported in vivo was likely via the osteoclastogenic function of osteoblasts. Alternatively, an increase in bone formation could have resulted from the increased calcium supply from the concurrent PRL-stimulated intestinal calcium absorption as a compensatory mechanism (Piyabhan et al., 2000; Tudpor et al., 2005). As bone formation is a complex process consisting of osteoid formation and mineralization, availability of calcium in the plasma is crucial for mineralization, and could increase the rate of bone formation even in the vitamin D-deficient rats (Weinstein et al., 1984). Further investigations are required to demonstrate the effects of PRL on the intricate interactions between bone and intestine.

It could be concluded at this point that demonstration of PRLR and the suppressive effects of high concentrations of PRL on MG-63 cells confirmed the hypothesis that hyperprolactinemia-induced bone loss could be due in part to the direct action of PRL. The present study also showed that PRL could potentially enhance

bone resorption by stimulating the osteoclastogenic function of osteoblast i.e., increasing the RANKL/OPG ratio. However, differential responses of osteoblasts in the in vivo versus in vitro conditions as discussed above must be taken into consideration when extrapolating the in vitro findings to explain in vivo observations. Nevertheless, the present results were able to explain the clinical findings of the hyperprolactinemia-induced high bone turnover and osteopenia.

C. The effect of PRL on hFOB, a human fetal osteoblast model

In adult animals, high bone turnover is a characteristic of both physiological and pathological hyperprolactinemia (Krishnamra et al., 1997; Lotinun et al., 2003; Meaney et al., 2004). Furthermore, PRL exposure in adult animals and humans, depending on the PRL concentrations, could lead to bone loss (Biller et al., 1992; et al., 2003). However, under certain conditions such as during pregnancy, high physiological PRL of ~75-100 ng/ml did not produce a significant bone loss, and only transient osteopenia was reported after 3 months of lactation when plasma PRL ranged between 200 and 350 ng/mL (Prentice et al., 2000; Ritchie et al., 1998). Interestingly, young rats were more responsive to PRL than adult and aging rats. Krishnamra and Seemoung (1996), using the in vivo ^{45}Ca kinetic study, reported that young rats responded differently to PRL compared to adult rats (more than 8 weeks of age), i.e., by enhancing bone gain instead of bone loss despite the presence of high bone turnover. Therefore, PRL stimulated calcium deposition and induced net bone gain in the femur, tibia and sternum of 3-week-old young rats. It was likely that the effects of PRL on bone varied with age. Along these lines of thought, it was therefore hypothesized that, unlike its action in MG-63, the adult human derived osteoblasts, PRL may increase the cellular activities of osteoblasts derived from immature human cells leading to bone formation. The present study thus used differentiated hFOB cells, which had minimal chromosome aberration and exhibited the matrix-producing properties of normal differentiated osteoblasts (Harris et al., 1995; Subramaniam et al., 2002), as the second study model. In addition, the undifferentiated hFOB cells have recently been shown to possess multilineage differentiation potential (Yen et al., 2007), suggesting that they retained the characteristics of fetal cells.

The present results showed that PRL at 100 and 1000 ng/ml stimulated osteocalcin mRNA expression in hFOB cells (Figure 16B) without affecting cell proliferation (Figure 14B). This effect of PRL agreed with the action of other hormones, such as leptin which enhanced osteoblast differentiation but not proliferation (Thomas et al., 1999). This effect was in contrast to PRL effect in MG-63 cells derived from adult humans which showed a decrease in osteocalcin expression after a 24-h PRL exposure. Similar to the primary neonatal rat osteoblasts (Coss et al., 2000) and MG-63 cells in the recent finding, PRL-exposed hFOB cells manifested a decrease in ALP activity (Figure 12B) which was supported by the reduction of its mRNA expression (Figure 15B). Although ALP is a classical marker of bone formation (Stein et al., 1996), its expression depends on the developmental stage of osteoblasts (Owen et al., 1990). Basically, osteoblasts have important roles in all three steps of bone formation, i.e., cell proliferation, extracellular matrix maturation, and mineralization (Owen et al., 1990). But the various actions including responses of osteoblast proliferation, gene expression and enzyme activities to various humoral factors all depend on the stage of development of the cells. For example, transforming growth factor β and its downstream protein Smad3 inhibited osteoblast proliferation, but enhanced ALP activity, mineralization, and expression of bone matrix proteins (Sowa et al., 2002). Generally, ALP expression is increased immediately after cessation of cell proliferation, while the expression of osteocalcin, which is important for the formation of hydroxyapatite crystal lattices (Hoang et al., 2003), is increased later during matrix maturation near the onset of mineralization (Owen et al., 1990). Therefore, a disparate relationship between osteocalcin expression and ALP activity was not surprising and could be observed during the development of osteoblasts.

The previous findings of the PRL-induced increase in the RANKL/OPG ratio in MG-63 cells and decrease in the OPG expression in primary osteoblasts from adult rats supported the in vivo report of net bone loss in adult hyperprolactinemic rats. Hence, it was possible that hFOB cells may respond to PRL by decreasing the RANKL/OPG expression ratio. The decrease in transcription and translation level of RANKL/OPG ratio by 10, 100 and 1000 ng/ml-PRL (Figure 19 and Figure 21) in the present study implied that PRL could potentially suppress bone resorption, thus supporting the earlier report of greater calcium deposition in bones of PRL-treated

young rats (Krishnamra and Seemoung, 1996). Since PRL enhanced osteocalcin expression in the matrix maturation step (Figure 16B) without affecting the in vitro mineralization of primary rat osteoblasts (Charoenphandhu et al., 2008), it was suggested that PRL increased bone calcium deposition in young rats by downregulating RANKL and upregulating OPG, thereby decreasing the RANKL/OPG ratio. Similar to PRL, growth hormone which increases bone turnover and bone gain (Schlemmer et al., 1991; Brixen et al., 2000; Landin-Wilhelmsen et al., 2003) also stimulates OPG synthesis in hFOB cells (Mrak et al., 2007).

In conclusion, the present study demonstrated that hFOB cells strongly and constitutively expressed PRLR. PRL directly increased osteocalcin mRNA expression, and thus decreased the RANKL/OPG ratio in these cells, indicating the stimulatory effect on bone formation and suppressive effect on bone resorption, respectively. The in vitro study supported the previous in vivo findings that, unlike in mature rats, PRL enhanced bone calcium deposition and bone gain in young rats (Krishnamra and Seemoung, 1996).

MG-63 was derived from osteosarcoma in adult bone tissue (Billiau et al., 1977). The actions of PRL on MG-63 mostly were anti-anabolic action i.e. decreasing osteoblast activity and differentiation while enhancing osteoclastogenesis. On the other hand, hFOB, a human fetal osteoblast derived from fetal bone tissue, was characterized as an osteoprogenitor which retained the plasticity of stem cells similar to that of embryogenic cells. The action of PRL on hFOB were mostly anabolic actions that led to bone gain i.e. increasing osteoblast differentiation and decreasing osteoclastogenesis. These age dependent differences in PRL effects were similarly observed in the in vivo study. PRL actions in young rats eventually resulted in net bone gain whereas its actions in adult rats caused net bone loss (Krishnamra and Seemoung, 1996). So far, the underlying mechanism has not been elucidated. Nevertheless, the present evidence of differential responses of fetal osteoblasts and adult derived osteoblasts supported the reported actions of PRL on bone remodeling as occurred in vivo. Therefore, these two types of osteoblasts could be used as osteoblast models for cell from adult and young animals. However, since the age dependent characteristic features of MG-63 and hFOB have not been properly documented, it was suggested that the overlapping window of fetal and adult statuses could be

distinguished further by using more specific markers. Indeed, insulin-like growth factors (IGFs) were known to control local tissue growth and differentiation during embryonic and postnatal mammalian growth (Sara & Carlsson-Skwirut, 1986). Although, human IGF II cDNA probes have been used to determine the late phase of the first trimester in human embryo (Scott et al., 1985; Hyldahl, Engstrom, & Schofield, 1986), however, the level of expression seemed to vary in different organs, from very high in liver to very low in brain (Schofield & Tate, 1987). Moreover, it was further reported that IGF II transcription, especially the exon E1-containing transcripts, was not confined to the fetus, but was also found in other adult organs, with an exception of liver (Schofield et al., 1987). Therefore, the specific marker for determining the fetal from adult status remains to be further clarified.

D. The intracellular signaling pathway of PRL action on human osteoblasts (MG-63 and hFOB)

After binding of PRL to PRLR, dimerization of two PRLR causes an activation of the cytoplasmic signaling. The signaling transduction is effected via activation of cytoplasmic tyrosine kinase (Janus kinase), mainly JAK2, which then phosphorylates other associated proteins, namely signal transducer and activator of transcription (STAT). In the mammary epithelia, PRL-PRLR complex uses the JAK2 signaling pathway in the stimulation of milk production (Bole-Feysot et al., 1998). Besides JAK/STAT pathway, the most important signaling pathway of PRL signaling transduction, at least three other signaling pathways have been reported to be involved in PRLR-associated intracellular signaling pathway i.e., the activation of mitogen-activated protein kinase (MAPK). Phosphatidylinositol-3'-kinase (PI3K) and PKC signaling pathways are reported in a number of tissues such as lymphoma tissue, duodenum and colon (Bole-Feysot et al., 1998; Freeman et al., 2000).

Since the direct actions of PRL in osteoblasts have only been recently demonstrated, nothing was known regarding its signaling pathway. It was recently shown that PRL directly stimulated the duodenal calcium absorption (Jantarajit et al., 2007), and inhibited the colonic Ca^{2+}-dependent $Cl^-$and K^+ secretion via the PI3K

pathway (Puntheeranurak et al., 2007). Furthermore, our laboratory recently reported that, via modulation of tight junction, PRL increased the passive calcium transport via PI3K pathway, and PRL exerted its action through PKC signaling to enhance the active calcium transport in Caco-2 monolayer (Thongon et al., 2008). Therefore, signal transduction of PRL in osteoblasts may also occur via the PI3K. The objective of this part of study was to investigate whether PRL signaling in human osteoblasts involved the PI3K pathway by using the ALP activity as the target of PRL action in MG-63 and hFOB.

Because a potent specific inhibitor of PI3K, LY294002 was used in the study, the induction of apoptosis due to the inhibition of PI-3K activity must be avoided by first selecting the lowest effective concentration of LY294002 for PI3-K inhibition, that did not induce cell death. Therefore, the experiments started with varying concentrations of LY294002 from 10 nM to 70 μM, and osteoblast survival was determined by MTT assay. The appropriate concentration of LY294002 which did not produce an apoptotic effect, was found to be 100 nM, therefore this concentration was used for cell incubation in the PRL-signaling experiment (Figure 22). The present study showed that the suppressive effect of PRL on membrane ALP activity in hFOB and MG-63 cells was completely abolished (Figure 23). Therefore, this indicated that the PI3K pathway was involved in the signal transduction of PRL in human osteoblasts, especially in its suppressive action on ALP activity. The involvement of PI3K in PRL action has been reported in many types of cell such as neurons, mammary cells, T lymphoma Nb2 cell line and colonic (CACO2) cells. PRL also acted through PI3K and Rho/ROCK signaling pathway to stimulate the paracellular passive calcium transport in enterocytes (Thongon et al., 2008). Furthermore, it was shown that PRL induced a rapid tyrosine phosphorylation of IRS-1 and that the 85-kDa subunit of the PI3K and IRS-1 appeared to be associate with the PRLR activation (Bole-Feysot et al., 1998). In addition, in mammary gland epithelial cells, PRL initiated c-Src-mediated PI3K pathway in the control of the expression of c-Myc and cyclin D1, which are important regulators involved in cell proliferation of T47D and MCF7 breast cancer cells. However, the present study did not detect any of such effect of PRL on osteoblast proliferation (Acosta et al., 2003). Therefore, the detailed

signaling cascade of PRL signal transduction pathways in osteoblasts, however, remained to be further investigated.

CHAPTER VI
CONCLUSIONS

The aim of the present study was to investigate the effect(s) and the intracellular signal transduction of PRL action on bone remodeling in osteoblasts. Two experimental models, MG-63 and hFOB, were used to achieve the objectives in this study. The main findings could be divided into four aspects as follow.

1. **mRNA and protein of PRLR (long isoform) expressed in human osteoblasts was specific to a maturation of cell.**

    1.1 MG-63 expressed PRLR long isoform which could be upregulated by dexamethesone and 1,25-$(OH)_2D_3$.

    1.2 hFOB expressed PRLR long isoform constitutively and abundantly without being affected by dexamethesone and 1,25-$(OH)_2D_3$.

2. **By using MG-63 as osteoblast model of adult animals, PRL at high physiological (100 ng/ml) and pathological (1000 ng/ml) concentration exerted catabolic effect on bone metabolism suggesting a possible direct contribution action of PRL in providing calcium in lactation and loss of bone mass in hyperprolactinemic condition.**

    2.1 PRL decreased osteoblast activity and differentiation as represented by ALP activity and osteocalcin, recpectively.

    2.2 PRL increased osteoclastogenic function of osteoblast as indicated by the increased ratio of RANKL/OPG mRNA and protein.

3. **By using hFOB as on osteoblast model of young animals, PRL at high concentrations was found to exert anabolic actions on bone metabolism, suggesting a direct positive effect on of PRL on bone development in young animals.**

    3.1 PRL increased osteoblast differentiation as indicated by elevated level of osteocalcin mRNA expression.

    3.2 PRL decreased osteoclasteogenic function of osteoblast as indicated by the decreased ratio of RANKL/OPG mRNA and protein.

4. **By using ALP activity as a target of PRL action on MG-63 and hFOB, the data indicated that the PI3K pathway was involved in the signal transduction of PRL in human osteoblasts especially, in its suppressive action on the ALP activity.**

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APPENDIX

APPENDIX A
RECOMBINANT HUMAN PROLACTIN PREPARATION

A. Principle

Recombinant human prolactin (rPRL) is derived from Escherichia coli. This expresses DNA sequence the mature human PRL sequence (amino acid 29-227). It is recommended that rhPRL is dissolved in 4 mM HCL containing 1 mg/ml BSA.

B. Reagents

1. rhPRL 50 μg
2. sterile 3 mM HCL containing 1 mg/ml BSA
3. sterile distilled H2O grade 2A
1. sterile standard phosphate buffer (PBS)

C. Procedures

1. Preparation of 80 mM HCL

To prepare 80 mM HCL, 66 μl of HCL (37% HCL analytical grade) is added into 100 ml volucmic flask and then adjusted with autoclaved distilled H2O grade 2A to obtain the final volume of 100 ml. The 80 mM HCL is kept in light protection bottle at room temperature.

2. Preparation of 2 mg/ml BSA

20 mg BSA is dissolved in 10 ml autoclaved PBS in 15 ml centrifuge tube, then sealed and kept at 4 ºC. The solution is prepared on the same day as preparation of 4 mM HCL containing 1 mg/ml BSA solution.

3. Preparation of 4 mM HCL containing 1 mg/ml BSA solution

10 ml of 80 mM HCL and 10 ml of 2 mg/ml BSA are mixed gently in a 25 ml centrifuged tube, after being mixed well, the solution is filtered through a 0.2 μ filter membrane and kept at 4 ºC.

4. Preparation of 10 μg/ml rhPRL

500 μl of sterile 4 mM HCL containing 1 mg/ml BSA solution is gently added into the bottle containing 50 μg of powder, then gently mixed before the dissolved rhPRL solution is removed and placed in a 15 ml centrifuge tube. This process is repeated 10 times to completely removed rhPRL and to obtain a final volume of 5 ml in the 15 ml tube. The final 10 μg/ml rhPRL stock solution is stored at -20 ºC. This preparation is performed in a biosafty condition.

APPENDIX B
PROTEIN DETERMINATION WITH BRADFORD'S ASSAY

A. Reagent

Reagent A Bradford stock solution

Mixed 100 ml of 95% ethanol, 200 ml of 88% phosphoric acid and 350 g of Serva Blue G together and kept at room temperature.

Reagent B Bradford working solution

Mixed 30 ml of reagent A with 1.5 ml of 95% ethanol, 30 ml of 88% of phosphoric acid and obtained to 500 ml. Filter the solution through Whatman No. 1 paper and stored at room temperature for several weeks.

B. Standard

Protein standard solution was prepared by dissolving 0.05 g of bovine serum albumin (BSA) from Sigma Chemical, MO in 100 ml of distill water.

C. Procedures

1. The sample and standard proteins were diluted to appropriate concentration with distill water to obtain a total volume of 100 µl.
2. The added 0.9 ml of reagent B and mixed by vortex immediately.
3. Incubated for at least 5 min at room temperature.
4. Read optical density at 595 nM by spectrophotometer.
5. Protein concentration of sample was calculated using intercept and slope factors of standard curve.

D. Reference

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