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การวิจัยสหสาขาเพื่อพัฒนาศักยภาพการวิจัยด้านแคลเซียมและกระดูก
Multidisciplinary approach to cultivate and strengthen research in
calcium and bone metabolism

หัวหน้าโครงการวิจัย: ศาสตราจารย์ ดร.นทีทิพย์ กฤษณามระ
 ภาควิชาสรีรวิทยา
 เครือข่ายวิจัยด้านแคลเซียมและกระดูก (COCAB)
 คณะวิทยาศาสตร์ มหาวิทยาลัยมหิดล

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1. โครงการวิจัย

- 1.1 โพรแลคติน : บทบาทในฐานะฮอร์โมนควบคุมเมตาบอลิซึมของแคลเซียมและกระดูก
- 1.2 การควบคุมวัฏจักรการสร้าง – สลายกระดูก (Bone Remodeling)

2. คณะผู้ดำเนินการวิจัย

- 2.1 **ชื่อ** ดร. นทีทิพย์ กฤษณามระ
คุณวุฒิ ปรินญาเอก **ตำแหน่ง** ศาสตราจารย์
สถานที่ทำงาน ภาควิชาสรีรวิทยา คณะวิทยาศาสตร์ มหาวิทยาลัยมหิดล
โทรศัพท์ 02-2015629 **โทรสาร** 02-354-7154
E-mail scnks@mahidol.ac.th
หน้าที่ความรับผิดชอบ หัวหน้าโครงการ
- 2.2 **ชื่อ** นพ. ดร. นรัตถพล เจริญพันธุ์
คุณวุฒิ ปรินญาเอก **ตำแหน่ง** รองศาสตราจารย์
สถานที่ทำงาน ภาควิชาสรีรวิทยา คณะวิทยาศาสตร์ มหาวิทยาลัยมหิดล
โทรศัพท์ 02-201-5629 **โทรสาร** 02-354-7154
E-mail naratt@narattsys.com
หน้าที่ความรับผิดชอบ ผู้ร่วมวิจัยหลัก
- 2.3 **ชื่อ** ดร. ชีราพร พันธุ์ธีรานุรักษ์
คุณวุฒิ ปรินญาเอก **ตำแหน่ง** ผู้ช่วยศาสตราจารย์
สถานที่ทำงาน ภาควิชาชีววิทยา คณะวิทยาศาสตร์ มหาวิทยาลัยมหิดล
โทรศัพท์ 02-201-5275 **โทรสาร** 02-354-7161
E-mail sctpt@mahidol.ac.th
หน้าที่ความรับผิดชอบ ผู้ร่วมวิจัย

- 2.4 ชื่อ ดร. ฉัตรศรี เตชะปัญญา
 คุณวุฒิ ปริญญาเอก ตำแหน่ง รองศาสตราจารย์
 สถานที่ทำงาน ภาควิชาสรีรวิทยา คณะแพทยศาสตร์ มหาวิทยาลัยศรีนครินทรวิโรฒ
 โทรศัพท์ 02-649-5374 โทรสาร 02-260-1533
 E-mail chatsri@swu.ac.th
 หน้าที่ความรับผิดชอบ ผู้ร่วมวิจัย
- 2.5 ชื่อ ดร. สพญ. สุทธาสินี ปุญญโชติ
 คุณวุฒิ ปริญญาเอก ตำแหน่ง รองศาสตราจารย์
 สถานที่ทำงาน ภาควิชาสรีรวิทยา คณะสัตวแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
 โทรศัพท์ 081-901-6916 โทรสาร 02-613-6967
 E-mail sutthasinee@gmail.com
 หน้าที่ความรับผิดชอบ ผู้ร่วมวิจัย
- 2.6 ชื่อ ดร. จันทรินา เจริญพันธ์
 คุณวุฒิ ปริญญาเอก ตำแหน่ง อาจารย์
 สถานที่ทำงาน ภาควิชาสหเวชศาสตร์ คณะแพทยศาสตร์ มหาวิทยาลัยธรรมศาสตร์
 โทรศัพท์ 02-926-5725 โทรสาร 02-926-9711
 E-mail jantarima@naratt.com
 หน้าที่ความรับผิดชอบ ผู้ร่วมวิจัย
- 2.7 ชื่อ นางสาวสนา แสงอำนาจ
 คุณวุฒิ ปริญญาตรี ตำแหน่ง นักวิทยาศาสตร์
 สถานที่ทำงาน ภาควิชาสรีรวิทยา คณะวิทยาศาสตร์ มหาวิทยาลัยมหิดล
 โทรศัพท์ 02-201-5629 โทรสาร 02-354-7154
 E-mail scwsg@mahidol.ac.th
 หน้าที่ความรับผิดชอบ ผู้ช่วยวิจัย/เลขานุการโครงการ
- 2.8 ชื่อ นางสาวจิรวรรณ ทองบุญชู
 คุณวุฒิ ปริญญาตรี ตำแหน่ง ผู้ช่วยวิจัย
 สถานที่ทำงาน เครื่องข่ายวิจัยด้านแคลเซียมและกระดูก คณะวิทยาศาสตร์
 มหาวิทยาลัยมหิดล
 โทรศัพท์ 02-201-5629 โทรสาร 02-354-7154
 E-mail jirawan-thong@yahoo.com
- 2.9 ชื่อ นางสาวสุดาทิพย์ พรหมอารักษ์
 คุณวุฒิ ปริญญาตรี ตำแหน่ง ผู้ช่วยวิจัย
 สถานที่ทำงาน เครื่องข่ายวิจัยด้านแคลเซียมและกระดูก คณะวิทยาศาสตร์
 มหาวิทยาลัยมหิดล
 โทรศัพท์ 02-201-5629 โทรสาร 02-354-7154
 E-mail aely_73@yahoo.com

3. Background and Rationale :

At present, there is little basic biomedical research on calcium and bone metabolism in Thailand. One of the reasons for this lack of interest in this field is that metabolic bone diseases are mostly chronic, not easily diagnosed in the early stages, and are not life threatening compared to other diseases such as cardiovascular or infectious diseases. However, incidence of bone diseases has been known to increase dramatically in recent years with prolonged life expectancy and the increase in the elderly population in both developed countries and developing countries including Thailand. Undoubtedly, bone diseases will have a negative socioeconomic impact in Thailand in the very near future. It is thus necessary for Thailand to encourage basic research in the field of calcium and bone metabolism which will ultimately lead to better understanding of the etiology and mechanisms of bone diseases, effective treatment and prevention. Multidisciplinary research approach is the best way to tackle complex research problems because it provides alternative views as well as ways to solve the problem.

Our research group known as the Consortium for Calcium and Bone Research or COCAB was established in 2003 as a research unit in the Center of Excellence of the Faculty of Science, Mahidol University. The present members also include academicians from the Faculty of Medicine, Ramathibodi Hospital, Mahidol University, as well as from other universities. We carry out research together in two major projects; Prolactin, its role in the regulation of calcium and bone metabolism and Regulation of bone remodeling in health and disease.

4. Objectives :

Our aim is to understand the basic mechanisms of hormonal and neural control of the target cells. More emphasis is put on bone metabolism and the mechanism underlying metabolic bone disorders. Our strategy is to continue with our basic research in calcium and bone metabolism in experimental animals (in vivo system), organ and cell culture (in vitro system) using physiological, histological, biochemical, biophysical and molecular approaches.

โครงการวิจัยแบ่งเป็นโครงการย่อย ดังต่อไปนี้

1. Mechanism of prolactin in the regulation of intestinal calcium absorption and bone metabolism
2. Effect of estrogen on the regulation of transport-related functions of prolactin in the endometrium

3. Expression and physiological roles of claudins in bones: evidence that bone-lining epithelium exists.
4. Modulation of nerve innervation and its neurotransmitter in bone related to osteoporosis
5. Anti-osteopenic actions of estrogen in chronic depressive female rats
6. Application of atomic force microscopy (AFM) in characterization of bone surface, structure and investigation of prolactin and estrogen actions.

5. Summary of the Research Finding

5.1 Prolactin: its novel role as calcium regulating hormone

Since female mammals including women lose a large amount of calcium for fetal bone development during pregnancy and for milk production in lactation, it was not surprising that their intestinal calcium absorption shows a marked increase which is seen as an adaptive change to accommodate the high demand for calcium. Although $1,25(\text{OH})_2\text{D}_3$, an active vitamin D hormone, has been known to be a major regulator of intestinal calcium absorption, its circulating levels are not elevated during these reproductive periods. Moreover, vitamin D-deficient pregnant and lactating rats still exhibit markedly increased intestinal calcium absorption. Thus, it seems that $1,25(\text{OH})_2\text{D}_3$ is not crucial for calcium absorption in pregnant and lactating periods. In addition, vitamin D receptor knockout mice show upregulation of the transient receptor potential channel subfamily V, member 6 (TRPV6), an apical calcium channel required for transcellular calcium absorption. Therefore, maternal calcium metabolism appears to be regulated by some other hormones.

Considering prolactin with its elevated level during the reproductive periods and being associated with electrolyte transport in many types of epithelia, **we proposed that prolactin is a calcium regulating hormone in pregnancy and lactation.**

Our present projects are a continuation of the decade-long investigations on the physiological role of prolactin in the regulation of calcium metabolism with special focus on two major target organs, namely, intestine and bone. We are the first to demonstrate this novel role of prolactin and **the summary of our findings has been presented in a recent review "Is prolactin the cardinal calciotropic maternal hormone? "In Trends in Endocrinology and Metabolism 2010; 21: 395-401.**

Prolactin and Intestinal Calcium Absorption

The mechanistic action of prolactin on the intestinal calcium absorption depends on its circulating level. This has been observed in non-mated as well as in pregnant and lactating animals. Briefly, prolactin stimulates the transepithelial calcium transport in a two-step manner. Sustained hyperprolactinemia such as in pregnancy and lactation, induces a long-lasting adaptation in the absorptive cells to elevate the baseline calcium transport by approximately 2-fold (we call this "Step-1"), that is further increased ("Step-2") by transient prolactin surge such as in suckling or nursing. Step-1 changes involve transcription of genes encoded for protein required for calcium absorption, e.g. TRPV6 and calbindin-D9k. Step-2 appears to correlate with milk production, and through the orchestrating actions of prolactin on other target tissues, involves mobilization of calcium from sites in the body such as bone.

Calcium traverses across the intestinal epithelium via two routes, transcellular and paracellular, with the latter being predominant. We have shown that prolactin stimulates both pathways. For the transcellular pathway, prolactin upregulates transporter genes encoded for TRPV 5, TRPV 6, voltage-dependent L-type calcium channel (Ca_v) 1.3, and parvalbumin, as well as increasing the activity of Ca-ATPase-1b ($PMCA_{1b}$) basolateral membrane.

Regarding the paracellular route, prolactin lowers the transepithelial resistance and increases calcium permeability by downregulating the tight junction-associated proteins occludin and ZO-1, resulting in Step-1 paracellular calcium transport. Step-2 paracellular transport is increased by prolactin-induced serine phosphorylation of claudin-15. Prolactin signaling pathway appears to involve phosphoinositide-3-kinase (PI3K) and Rho-associated kinase (ROCK) and not the classical Janus kinase-2 pathway.

We have also shown that the acute effect of prolactin is through activation of prolactin receptor long form (PRLR-L) and its down stream mediators, PI3K and protein kinase C, leading to Step-2 transcellular calcium transport.

Prolactin and Bone Turnover

Mammalian skeletal system shows remarkable adaptation during the reproductive cycles with bone calcium accumulation in early and mid pregnancy, slight bone loss in the third trimester and as high as 10-30 % reduction in bone mineral density in lactation. This bone loss caused by milk production can lead to pregnancy and lactation-associated osteoporosis (PLO) with symptoms like back pain, vertebral fracture and height loss.

To prove a direct action of prolactin on bone, we were able to demonstrate the presence of PRLR in osteoblasts. Moreover, we showed that prolactin by downregulating osteoprotegerin (a decoy receptor for RANKL) and upregulating RANKL, results in

increased bone resorption. Since prolactin downregulates Runx2 (a transcription factor for osteoblast differentiation), it can also decrease the number of differentiated osteoblasts. Prolactin appears to have more effect on trabecular bone than cortical bone. But it is not known what factors determine the site and magnitude of action. PRLR distribution is possibly one of the factors.

We have recently focused our investigation on changes in the microstructure of maternal bone to cope with calcium demand for fetal development in pregnancy and milk production in lactation. We found bone accretion in both cortical and trabecular parts of the long bone in pregnant rat, suggesting the building up of calcium reserved pool. As expected lactation induces progressive trabecular bone loss. Studies in pregnant and lactating rats also showed a marked increase in the length of long bone, leading us to look at changes in the growth plate. We found that chondrocytes in every growth plate zone express PRLR. We are the first to provide evidence on the chondroregulatory action of prolactin in the tibial growth plate of lactating rats. Growth plate changes observed in pregnancy, in contrast, are not dependent on prolactin. The chondroregulatory actions of prolactin is apparent only in mammals with persistent growth plate cartilage. However, in other mammals, including humans, prolactin increases bone resorption and can induce bone loss if intestinal calcium absorption is inadequate.

Although we have already provided evidence showing the remarkable actions of prolactin in orchestrating calcium transport in the intestine and bone with an aim to provide adequate calcium supply for developing fetuses and postnatal milk production, we still have to answer many remaining questions regarding the relative contribution of absorbed calcium versus resorbed calcium from bone to meet calcium demand under different physiological conditions, the cellular and molecular mechanism of action of prolactin in bone cells, and how different PRLR isoforms determine the outcome of prolactin action.

Prolactin and Electrolyte Transport in the Endometrium

As we know, blastocyst implantation and embryo development require optimal intrauterine environment which unquestionably depends on the transport activities of the endometrial epithelial cells. Among a number of hormones, growth factors and cytokines, prolactin as a hormone with markedly elevated levels during pregnancy and lactation, is found to have a regulatory role.

In the present investigation, we used primary cell culture of porcine glandular endometrial epithelial cells to study the action of prolactin. We found that prolactin acutely stimulated anion secretion across the epithelial cells. The action is mediated by activation of the PRLR-short isoform (PRLR-S), which are present on both apical and

basolateral membranes, and the JAK2 signaling pathway. The prolactin-stimulated anion secretion is mostly a result of the activation of DPC- and NPPB- sensitive Cl^- channel, and bumetanide sensitive $\text{Na}^+ - \text{K}^+ - 2\text{Cl}^-$ cotransport. Although the exact role of prolactin in the human endometrium is not known, the pattern of prolactin secretion and expression supports its role in implantation and placentation. Based on the present data and other reports, it is likely that prolactin helps regulate the volume and composition of the fluid within the uterine cavity.

5.2 Regulation of Bone Remodeling

Bone Lining Epithelium

Besides providing support for the body, bone also acts as a huge ion exchange system that is a part of the pH buffering system as well as regulation of ionic composition of the extracellular fluid. Bone extracellular fluid (BECF) itself is probably under regulation since its composition is not the same as the extracellular fluid (ECF), for example it has a higher K^+ concentration than that of the ECF. It has been proposed that osteoblasts and bone – lining cells (formerly thought to be inactive osteoblasts) form bone membrane that actively controls the paracellular ion exchange between BECF and ECF similar to an epithelium. However, there has been no direct evidence to support the existence of this epithelium like bone membrane.

In 2008, we were able to provide evidence in support of this hypothesis. Since one of the most important properties of an epithelium is the formation of tight junctions to hold epithelial cell together as a sheet of cells, we set out to investigate the presence of tight junctions. We found that primary rat osteoblasts and bone tissues express the tight junction associated proteins, namely ZO-1,-2,-3, cingulin, occludin, claudin-1 to -12, -14 to -20, -22 and -23. By using western blot analyses, we showed expression of claudin-5,-11,-14 and -15. Claudin -16 was shown by immunofluorescent study in decalcified tibia section, specifically on the trabecular surface. In addition, expression of claudin -5,-11,-14, -15 and -16 were found in bone lining cells. We also demonstrate that primary osteoblasts cultured in permeable Snapwell formed a monolayer with transepithelial resistance of $\sim 110-180 \Omega \text{ cm}^2$, conferring the presence of barrier functions of the tight junction. We conclude that osteoblasts express several tight junction-associated proteins which are likely to have regulatory role on ion transport across bone membrane.

Nerve Innervations of Bone and Osteoporosis

It is known that bone is regulated by humoral and neural control, the latter via autonomic catecholamine secreting function. The sympathetic nervous system has been shown to suppress osteoblast-induced bone formation and to stimulate osteoclast

differentiation and bone resorption. Many studies further showed β_2 -adrenergic receptor in osteoblast as being responsible for sympathetic nervous system-induced up regulation of RANKL and subsequent bone loss. Moreover, previous investigation showed that osteoblast response may depend on adrenergic receptor (AR) subtypes, eg. α_1 -AR, β_1 -AR, β_2 -AR and β_3 -AR. β_3 -AR transcripts are primarily expressed in adipocytes, heart, skeletal muscle, smooth muscle of the gastrointestinal tract and the urogenital system. Since activation of β_3 -AR could accelerate the differentiation process in several cell types and regulate expression of many transcription factor genes, we investigated β_3 -AR expression in rat osteoblast-like UMR 106 cells.

Our investigation showed that activation of β_3 -AR could modulate expression of several genes related to osteoblast functions and bone remodelling in primary osteoblasts and the osteoblast-like UMR 106 cells. β_3 -AR activation is likely to direct proliferation of pre-osteoblasts toward differentiation process since β_3 -AR agonist induced down regulation of Runx 2, important for proliferation of osteoblast progenitors and upregulations of several osteoblast differentiation markers ie., osterix, osteocalcin, and osteopontin. Furthermore, the low-dose β_3 -AR agonist may provide a protective effect against bone resorption as indicated by a decrease in the ratio of RANKL/OPG expression ratio. Thus, our investigation indicates the significance of β_3 -AR system in the regulation of osteoblast function and bone remodeling. However, further study is required to demonstrate the molecular mechanisms of β_3 -AR and its significance in the in vivo control of bone metabolism.