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FEATURE ARTICLES

The diversity of preosteoblastic morphology
− Preosteoblastic response to parathyroid hormone −

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ABSTRACT : The current concept of a preosteoblast is a precursor of an osteoblast, which is regarded as a transient cell type during osteoblastic differentiation. We have previously demonstrated different phenotypes of preosteoblasts expressing Runx2, ALPase, and BrdU incorporation. Transmission electron microscopy revealed following four distinct preosteoblastic cell types : 1) cells rich in rough endoplasmic reticulum (rER) but with a few vesicles and vacuoles (ER-rich/vesicle-poor preosteoblasts), 2) cells extending their cytoplasmic processes connecting distant cells, with a small amount of scattered cisterns of rER and many vesicles and vacuoles (ER-poor/vesicle-rich preosteoblasts), 3) translucent cells showing few dispersed cell organelles and irregular cell shape with a translucent cytoplasm (translucent cells), and 4) small cells without developed cell organelles (small undifferentiated cells). ER-rich/vesicle-poor preosteoblasts were often closely adjacent to mature osteoblasts and therefore appeared to be ready for differentiation into osteoblasts. In contrast, after the administration of parathyroid hormone (PTH), ER-poor/vesicle-rich preosteoblasts rather than ER-rich/vesicle-poor cells significantly increased in number, forming a huge meshwork overlying mature osteoblasts. Thus, ER-poor/vesicle-rich preosteoblasts appeared to respond well to PTH. We also attempted to unveil the cellular behavior of these preosteoblasts against PTH and to dissect the role of osteoclasts on the mediation of PTH anabolic actions. PTH stimulated the proliferation of ER-poor/vesicle-rich preosteoblasts and bone formation in mature osteoblasts. However, an increased population of ER-poor/vesicle-rich preosteoblasts appears to require cell coupling from osteoclasts to differentiate into ER-rich/vesicle-poor preosteoblasts and mature osteoblasts. Without osteoclasts, PTH could induce neither preosteoblastic differentiation into mature osteoblasts nor subsequent bone formation. In this mini-review, we will introduce preosteoblasts in vivo consisting of several cell types with different ultrastructural properties and PTH action on preosteoblasts.

Key Words : *preosteoblast, PTH, ALPase, Runx2, transmission electron microscopy*

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1. Introduction

Preosteoblasts are precursors of mature osteoblasts that are localized on the bone surface[1]. Bone-synthesizing osteoblasts are called mature or active form of osteoblasts[1-4], which can be seen in the regions of stimulated bone modeling and remodeling[4, 5]. Alternatively, flattened inactive osteoblasts covering the bone surface as seen in cortical bone are referred to as bone lining cells. Abundant preosteoblasts with elongated cytoplasmic processes in all directions are observable in the vicinity of bone-forming mature osteoblasts, while only a few preosteoblasts are present close to bone lining cells. Thus, the development of preosteoblastic networks appears to be correlated with the osteoblastic activity of bone formation.

Many investigators have previously attempted to elucidate where osteoblastic precursors exit and the types of functions they have[6-11]. It seems obvious, at least in part, that preosteoblasts are able to proliferate but not synthesize calcified bone matrix, while mature osteoblasts enable the synthesis of calcified bone matrix but do not proliferate. Osteoblastic lineages including preosteoblasts and mature osteoblasts possess enzymatic activity of tissue non-specific alkaline phosphatase (ALPase), which divides pyrophosphate into phosphate ion monomers[12]. Therefore, the presence of ALPase activity appears to be a good hallmark of osteoblastic lineages. It is of interest that preosteoblasts that lack calcification ability possess ALPase enzymatic activity involved in calcification. While much attention has been drawn to the characterization of the cellular properties and functions of mature osteoblasts, preosteoblasts have not been highlighted, probably because they are regarded only as a transient state prior to reaching fully differentiated osteoblasts. In addition, preosteoblasts have not been clearly distinguished from bone marrow stromal cells, presumably because of the overlapping cellular functions common to both cell types. Therefore, the number of osteoblastic phenotypes present in vivo and the function of preosteoblasts are still unknown. In other words, it may be important to clarify whether preosteoblasts are merely precursors of mature osteoblasts or if they have their own function before becoming mature osteoblasts in vivo. While many studies have suggested the diversity of preosteoblastic morphology and function, preosteoblasts are target cells for many osteotropic factors and hormones including parathyroid hormone (PTH) that regulate bone metabolism.

In this mini-review, we would like to provide clues to better understand the histological and functional aspects of preosteoblasts, especially their morphological diversity and response to PTH in bone.

1) Morphological diversity and historical categories of preosteoblasts

Concept of preosteoblasts and osteoprogenitors

Research work was directed around the 1960s to investigate the presence of osteoblastic precursors. The term "preosteoblast" was originally used by Pritchard in 1956[13], and in 1962, Young proposed the term "osteoprogenitor"[14]. However, the criteria of preosteoblasts were still conceptual. In 1967, Scott attempted to localize precursors of osteoblasts by administering ^{3}H-thymidine via electron microscopic autoradiography[15], based on the principal concept that osteoblasts are bone-synthesizing matured cells whereas preosteoblasts are proliferative osteoblastic precursors (this histological category distinguishing mature osteoblast from preosteoblasts is still widely used). Therefore, he classified isotope-labeled proliferating cells into A cells and B cells and concluded that spindle-shaped A cells possessed the characteristics of preosteoblasts, which are located between mature osteoblasts and bone marrow. Based on these ultrastructural analyses, the spindle-shaped cell type between mature osteoblasts and bone marrow tissue have been histologically regarded as preosteoblasts.

Ultrastructural identification of preosteoblasts

Approximately ten years later, Martineau-Doizé *et al*.[16] identified three preosteoblastic profiles in rat femoral metaphysis using quantitative autoradiography for the binding and internalization of ^{125}I-epidermal growth factor (EGF). The identified cell types include endocytic cells, rough endoplasmic reticulum (rER)-rich cells, and undifferentiated cells[16]. Interestingly, these preosteoblasts expressing EGF receptors showed different localization in bone. The endocytic cells were found in the vicinity of the epiphyseal plate and near osteoclasts on the metaphyseal trabeculae. The ER-rich cells were present in the vacated chondrocyte lacunae of the epiphyseal plate. The undifferentiated cells were observed between the metaphyseal trabeculae. Afterwards, Rouleau *et al*. discovered PTH receptor-expressing preosteoblasts by

electron microscopic autoradiography and found that the majority of PTH receptor-expressing preosteoblasts were present as a cell type in the intertrabecular space of the metaphyseal region, which was distinct from the mature osteoblasts[17, 18]. This cell type was named "PT-cell". The PT-cell extended multiple cytoplasmic processes to interface with both the bone matrix and the microvascular osseous circulation, indicating vascular-osseous interactions by PTH.

We have previously attempted to clarify the different phenotypes of preosteoblasts *in vivo* by examining Runx2, ALPase, and BrdU incorporation[19]. TEM observations revealed following four preosteoblasts cell types, 1) cells rich in rER but with a few vesicles and vacuoles (ER-rich/vesicle-poor preosteoblasts), 2) cells extending their cytoplasmic processes connecting distant cells, with a small amount of scattered cisterns of rER and many vesicles and vacuoles (ER-poor/vesicle-rich preosteoblasts), 3) translucent cells showing few dispersed cell organelles and irregular cell shape with a translucent cytoplasm, and 4) small cells without developed cell organelles (small undifferentiated cells). ER-rich/vesicle-poor preosteoblasts are often seen at close proximity to mature osteoblast and therefore appear to be ready for differentiation into osteoblasts. In contrast, ER-poor/vesicle-rich preosteoblasts extended their cytoplasmic processes not only to mature osteoblasts but also frequently to bone-resorbing osteoclasts. Abundant vesicles and vacuoles including lysosomes in this cell type may indicate intracellular vesicular transport rather than being ready for matrix synthesis. Thus, there appears to be a variety of preosteoblasts, implicating that the term "preosteoblasts" does not mean a specific cell type, but represents a general name describing osteoblastic precursors. In addition, preosteoblasts with different states of cell organelle development and distinct localization in bone appear to imply their own specific functions compared to those of other bone cells.

Discovery of Runx2/osterix in osteoblastic differentiation

Twenty years ago, advances in molecular biology and genetic engineering led to the discovery of runt-related transcription factor 2 (Runx2), also referred to as core binding factor α1 (Cbfa1), and osterix as essential transcriptional factors for osteoblastic differentiation[20, 21]. Runx2 has been simultaneously discovered by Komori *et al*.[20], Otto *et al*.[22], Olsen's team[23] and Karsenty's group[24] and was believed to be one of the most reliable hallmarks of osteogenic cells including preosteoblasts and mature osteoblasts[25-28]. For this reason, Runx2 has been broadly used as an adequate differentiation marker to identify osteoblastic lineages. Mice with homologous gene depletion of Runx2/osterix showed markedly diminished ossification and thus, Runx2/osterix were believed to be master genes that introduce undifferentiated mesenchymal cells into osteoblastic lineages. However, it is still unclear whether Runx2-/osterix-expressing preosteoblasts are identical to morphologically classified preosteoblasts, namely endocytic cells, PT-cells, ER-rich/vesicle-poor cells, ER-poor/vesicle-rich cells, and translucent cells. In any case, preosteoblasts appear to be, at least in part, ALPase- and Runx2-/osterix-positive cells with proliferating potential, existing between mature osteoblasts and bone marrow tissue.

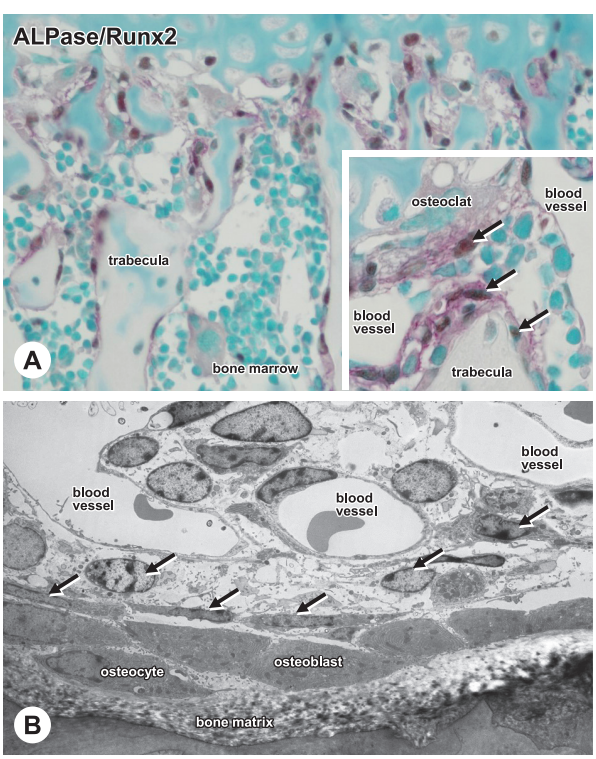

Fig. 1 Detection of ALPase-/Runx2-reactive cells and TEM observation in murine metaphysis

A : Double staining of ALPase (red) and Runx2 (brown). ALP-positive cells (red) covering the metaphyseal trabeculae show Runx2-positivityin their nuclei. An inset: Note that only ALPase-positive cells show Runx2-positivity, which is absent in osteoclasts and bone marrow cells. B : A lower magnified image of cells surrounding metaphyseal trabecular bone. Mature osteoblasts on the bone surface are covered by cells so called "preosteoblasts" (black arrows). Modified from Narimatsu et al[19]

Postulation of trans-differentiation into preosteoblasts/osteogenic cells

Recently, several investigators have suggested that during endochondral ossification, hypertrophic chondrocytes in the growth plate/epiphyseal cartilage could be trans-differentiated into osteogenic cells with signaling of β-catenin, thyroid hormone, and Indian hedgehog[29-32]. This is a new concept regarding the origin of osteoblasts because osteoblasts have been believed to be derived from undifferentiated mesenchymal cells. In our own observation, as mentioned above, we found large translucent cells with characteristics of scattered rER and Golgi apparatus, which resemble hypertrophic chondrocytes. Therefore, the translucent cells in our classification of preosteoblastic phenotype may be derived from hypertrophic chondrocytes. Although previous reports have suggested that all hypertrophic chondrocytes fall into apoptosis at the chondro-osseous junction, some chondrocytes may survive after the invasion of vascular endothelial cells into the cartilage. It seems possible that these hypertrophic chondrocytes would be trans-differentiated into osteogenic cells, *i.e.* preosteoblasts. However, further studies are necessary to clarify this issue.

2) Ultrastructure and distribution of preosteoblasts responsive to PTH

Cellular response to anabolic action of PTH in bone

It is well known that the receptors for PTH are expressed mainly in both preosteoblasts and mature osteoblasts, but not in deeply embedded osteocytes or osteoclasts and their precursors in bone[33]. Bone anabolic effect by intermittent administration of PTH likely involves osteoblasts, preosteoblasts, and osteoclasts, as basic and clinical works have strongly suggested[34-38]. PTH not only promotes preosteoblastic proliferation and osteoblastic bone formation but also accelerates osteoclastic bone resorption, consequently inducing high bone turnover. However, PTH-driven bone formation is predominant to bone resorption, which results in the anabolic effect in bone. We have previously attempted to unveil the cellular response of preosteoblasts/osteoblasts against PTH and to dissect the role of osteoclasts on the mediation of PTH anabolic actions[39]. PTH appears to directly affect preosteoblastic proliferation and indirectly stimulate osteoblastic bone formation by mediating osteoclastic activity, *i.e.* cell coupling. Examination of

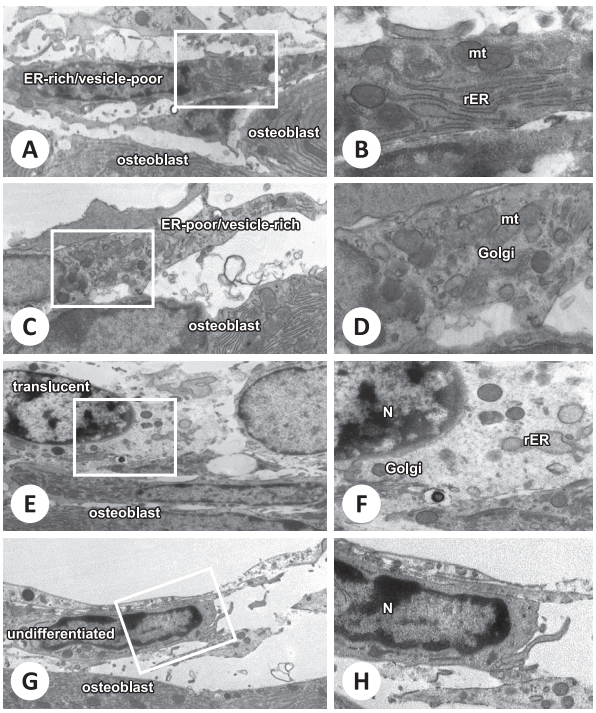

Fig. 2 Preosteoblastic phenotypes assessed by TEM observation

Panels B, D, F, H are higher magnification of boxed areas of A, C, E, G. A : a lower magnified image of an ER-rich/vesicle-poor preosteoblast. B : An ER-rich/vesicle-poor preosteoblast is shown to possess abundant rER. C : a lower magnified image of an ER-poor/vesicle-rich preosteoblast. D : An ER-poor/vesicle-rich preosteoblast develops Golgi apparatus and many vesicles and vacuoles. E : A TEM image of a translucent cell cells. F : A translucent cell shows dispersed rER and scattered Golgi apparatus throughout the translucent cytoplasm. G : An undifferentiated cell often is seen close to blood vessels in the metaphyses. H : An undifferentiated cell has a few cell organelles. N : nuclei, mt: mitochondria, Modified from Narimatsu et al[19]

c-fos$^{-/-}$ mice that lack osteoclasts revealed that PTH successfully increased preosteoblastic numbers but failed to increase osteoblastic bone formation[40]. Therefore, it is likely that PTH directly stimulates preosteoblastic proliferation, but it appears to require cell coupling from osteoclasts for preosteoblastic differentiation into mature osteoblasts and subsequent bone formation[39, 41, 42]. On the other hand, preosteoblasts have been reported to support osteoclastic formation and subsequently their resorption by mediating receptor activator of nuclear factor κB (RANK)/RANK ligand (RANKL) system[43-46]. It was also reported that PTH stimulated RANKL expression through the cAMP/protein kinase A/CREB cascade[47]. Based on these findings, preosteoblasts are apparently key cells for bone metabolism induced by PTH.

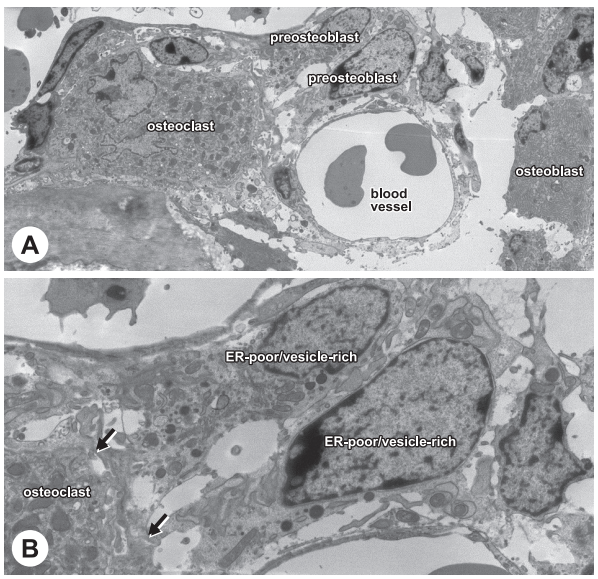

Fig. 3 TEM observations of ER-poor/vesicle-rich preosteoblasts surrounding bone-resorbing osteoclasts

A : ER-poor/vesicle-rich preosteoblasts featuring scattered rER and numerous vesicles and vacuoles are often found close to osteoclasts. B : These osteoblasts make cell-to-cell contacts (arrows) with bone-resorbing osteoclasts. Modified from Narimatsu et al[19]

Preosteoblastic phenotypes responsive to PTH

It appears, therefore, of interest to verify which of the preosteoblastic cell types would respond to PTH for proliferation. As mentioned above, in our own observation using normal mice, the preosteoblastic phenotypes include ER-rich/vesicle-poor cells, ER-poor/vesicle-rich cells, translucent cells, and small undifferentiated cells. TEM observations verified two major preosteoblastic cell types, namely the ER-rich/vesicle-poor and ER-poor/vesicle-rich cells, among our classification of preosteoblasts. ER-rich/vesicle-poor cells were located closely adjacent to the basolateral side of mature osteoblasts, with similar distribution and development of cell organelles, *e.g.* rER and Golgi apparatus, as those of mature osteoblasts. However, ER-poor/vesicle-rich cells were present in the region between blood vessels and ER-rich/vesicle-poor cells/mature osteoblasts, extending their long cytoplasmic processes to not only mature osteoblasts but also osteoclasts and blood vessels. In contrast, after PTH administration, a huge amount of meshwork in ER-poor/vesicle-rich cells was formed overlying mature osteoblasts, involving many bone marrow cells found inside. ER-poor/vesicle-rich cells significantly increased in number, rather than ER-rich/vesicle-poor cells. Thus, ER-poor/vesicle-rich cells but not ER-rich/vesicle-poor cells appeared to be well responsive to PTH for promoting proliferation.

Comparison of PTH-responsive preosteoblasts classified by our and other groups

Thorough classification of preosteoblastic phenotypes was carried out mainly by a Canadian group in the late eighties[16-18]. Martineau-Doizé *et al*.[16] have reported endocytic cells located in the vicinity of osteoclasts on the metaphyseal trabeculae and an ER-rich cell profile present in vacated chondrocyte lacunae of the epiphyseal plate. "ER-rich cells", originally termed by Martineau-Doizé *et al*., appeared to be different from our "ER-rich/vesicle-poor cells". ER-rich/vesicle-poor preosteoblasts categorized by us did not contact the bone surface, while resembling mature osteoblasts in that they were bipolar and possessed substantial amounts of rER cisternae and developed Golgi apparatus. ER-poor/vesicle-rich preosteoblasts, on the other hand, were flat, had many cytoplasmic processes, and had low ER content. These cells were similar to those described by Rouleau *et al*.[17, 18] as a PT-cell, a PTH receptor-expressing subtype. After PTH administration, the number of ER-poor/vesicle-rich cells was substantially higher than that of ER-rich/vesicle-poor preosteoblasts in our electron microscopic observation, and they formed a huge amount of meshwork in the intertrabecular space. Meanwhile, endocytic cells termed by Martineau-Doizé might be the same phenotype as ER-poor/vesicle-rich cells, presumably also as PT-cells. Indeed, endocytic cells and ER-poor/vesicle-rich cells were often observed to be localized close to osteoclasts.

TEM analysis of PTH-injected *c-fos*$^{-/-}$ specimens revealed abundant ER-poor/vesicle-rich preosteoblasts (PT-cells), but mature osteoblasts and ER-rich/vesicle-poor preosteoblasts were barely seen. *C-fos*$^{-/-}$ mice lack osteoclasts and their precursors and therefore, cell coupling between osteoclasts and osteoblasts must be disrupted. Taken together, we postulated the possibility that in a normal state, ER-poor/vesicle-rich cells could differentiate into ER-rich/vesicle-poor preosteoblasts, and then into mature osteoblasts to synthesize bone matrix. However, the differentiation process from ER-poor/vesicle-rich cells and ER-rich/vesicle-poor cells might require coupling factors from osteoclasts.

Cellular response of preosteoblasts in the different regimens of PTH administration

Next, we raised the question of whether different frequencies of human PTH (hPTH(1-34)) administration

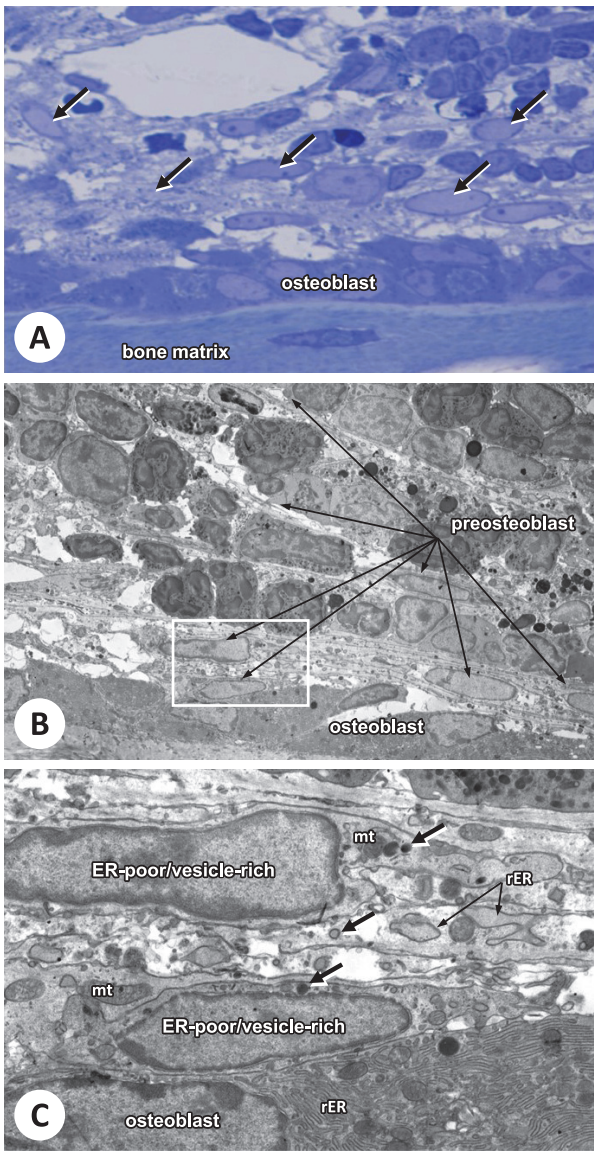

Fig. 4 Light microscopic and electron microscopic observation on PTH-injected murine metaphyses.

A : A semi-thin section of epoxy resin-embedded PTH-treated specimens. A huge amount of meshwork of preosteoblastls (arrows) can be seen. B : TEM observation demonstrates several layers of preosteoblasts overlying mature plump osteoblasts. C : When magnifying a boxed area in panel B, PTH-driven thick layers of preosteoblasts are shown to be composed of many ER-poor/vesicle-rich preosteoblasts. Ultrastructural features of ER-poor/vesicle-rich preosteoblasts overlying a mature osteoblast are easily identified with dispersed and relatively enlarged endoplasmic reticulum cisterns (rER), several vesicles and vacuoles including endosomes and lysosomes (arrows). Modified from Luiz de Freitas et al[39]

would induce bone formation similarly in terms of quantity and quality, as well as exert similar effects on preosteoblasts. To investigate these issues, mice were subjected to different frequencies of PTH administration (low-frequency : 1 time/2 days and 1 time a day, high-frequency : 2 and 4 times a day) at a dose of 20 mg/kg of hPTH(1-34)[48]. Highly frequent PTH administration led to the formation of thin trabeculae, showing a thick preosteoblastic cell layer, several osteoclasts, and scalloped cement lines that indicated accelerated bone remodeling. The thick preosteoblastic layer was composed of mainly ER-poor/vesicle-rich cells and included many bone marrow cells and tartrate-resistant acid phosphatase (TRAP)-positive osteoclasts. Therefore, the degree of development of the preosteoblastic cell layer appears to be correlated to osteoclast formation and subsequent bone resorption activity. On the other hand, lower-frequency PTH administration induced new bone with mature osteoblasts lying on mildly convex surfaces representative of arrest lines, which suggested minimodeling-based bone formation. It is of interest that unlike the high-frequency PTH administration, a preosteoblastic cell layer was not developed over the mature osteoblasts.

One may wonder if the different histologies of the preosteoblastic cell layer between the high and low frequencies of PTH administration are ascribed to how long the signaling of one PTH administration can continue to accelerate cell proliferation and Runx2/osterix expression in preosteoblasts. Though it is preliminary, we have chronologically examined the gene expression of Runx2, RANKL, and osteoprotegerin, as well as ALPase-reactive areas and TRAP-positive cell numbers in murine femora after an injection of 20 mg/kg of hPTH(1-34) (*data not shown*). RANKL was immediately elevated but then decreased to a lower level. On the other hand, osteoprotegerin mRNA was increased and then suddenly decreased. The number of TRAP-positive osteoclasts did not change during the experimental period. In contrast to RANKL, Runx2 expression was gradually increased and continued for a relatively long time after PTH injection. Consistently, with the PTH injection, the ALPase-reactive area was slightly but significantly increased. More interestingly, preosteoblasts overlying the mature osteoblasts came to extend their cytoplasmic processes in all direction, forming their meshwork between the mature osteoblasts and blood vessels. Taken together, PTH administration at long intervals, *i.e.* low frequency, appears to stimulate preosteoblast/osteoblast activity, while PTH administration at short intervals, *i.e.* high frequency, induces osteoclast formation in the huge meshwork of preosteoblasts, *i.e.* ER-poor/vesicle-rich

preosteoblasts.

Concluding remarks

A preosteoblast does not seem to be a single cell type, but a general name of osteoblastic precursors located over mature osteoblasts. There are at least four ultrastructural preosteoblasts cell types : ER-rich/vesicle-poor preosteoblasts that appear to be ready to differentiate into mature osteoblasts, ER-poor/vesicle-rich preosteoblasts, translucent cells resembling hypertrophic chondrocytes, and small undifferentiated cells with few cell organelles. Among these preosteoblastic phenotypes, ER-poor/vesicle-rich preosteoblasts predominantly and directly respond to PTH, proliferating and extending their cytoplasmic processes in all direction, *e.g.* toward surrounding blood vessels, mature osteoblasts, and osteoclast precursors. Taken together, the ER-poor/vesicle-rich preosteoblastic phenotype may be a key cell type that essentially regulates PTH function in bone.

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