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Withdrawal of parathyroid hormone after prolonged administration leads to adipogenic differentiation of mesenchymal precursors *in vivo*

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ABSTRACT

Intermittent PTH-like drugs are the only approved so-called anabolic agent that increases bone mass in both mice and humans. It is well documented that PTH targets mature cells of the osteoblast lineage, with only indirect evidence of its actions on early cells of the osteoblast lineage. Using a triple transgenic mouse model that allowed labeling of very early cells of the osteoblast lineage, we traced the progeny of these into osteoblast lineage in adult mice. These early cells expressed PTH1R and multiplied when PTH (1–34) was administered daily. We also showed that the early mesenchymal cells showed accelerated differentiation into mature osteocalcin-positive osteoblasts and osteocytes. Rather surprisingly, when teriparatide administration was stopped, these early mesenchymal precursors differentiated into adipocytes. We showed that the adipogenic differentiation is accompanied by a decrease in wnt signaling in osteoblast precursors. In this review, we discuss the possible clinical relevance of this finding and the possible molecular mechanisms that contribute to this phenotype *in vivo*.

1. Introduction

The bone marrow adipocytes were first identified more than a century ago [1]. However, until recently, very little has been known about their roles, development and their regulation by various paracrine factors and hormones *in vivo*. At the time of birth, the bone marrow mainly consists of an active hematopoietic red marrow with very little adiposity [2]. The first set of bone marrow adipocytes develop in the terminal phalanges around the time of birth [3]. In humans, by early adulthood, marrow adipocytes occupy roughly half of the bone marrow volume. Subsequently, marrow adipocytes gradually increase throughout adulthood [4]. Interestingly, men exhibit a higher percentage of marrow filled with adipocytes than women before the menopause; this contrast reverses immediately after menopause, when women begin to exhibit higher bone marrow adiposity than men [5].

For several years, adipocyte differentiation has been studied *in vitro* using less differentiated marrow stromal cells and cell lines derived from these stromal cells. Typically, undifferentiated stromal cells are subjected to adipocyte differentiation media for a period of 2–4 weeks [6,7]. The differentiation of such stromal cells into adipocytes is regulated by a complex and temporally regulated transcription factors that coordinate expression of several proteins that are crucial for establishing the mature fat-cell phenotype. Three principal adipogenic transcription factors, PPARγ and C/EBPα/β, are the master regulators of adipogenic differentiation process. PPARγ is a nuclear receptor and like other PPARs heterodimerizes with RXR and activates target promoters [8]. PPARγ alone is necessary and sufficient to drive adipogenesis. C/EBPα promotes adipogenesis at least in part by inducing PPARγ [9]. *In vivo*, C/EBPβ null mice show an overall reduced total body adipose tissue [10].

2. Skeletal Progenitors

Mammalian bone marrow contains cells capable of forming colonies in culture (CFU-Fs) [11]. Some of these colonies contain, within one colony, cells capable of differentiating into osteoblasts, chondrocytes or adipocytes *in vitro* or after subcutaneous transplantation [12]. These stromal cells, sometimes called mesenchymal stem cells (MSC), have been partially purified and have been studied extensively as potential tools for regenerating bone and other tissues. Their normal fates *in vivo* remain elusive, however, because specific marking of these cells in lineage tracing experiments *in vivo* has only recently been possible. Nestin promoter-driven GFP (*nestin*-GFP) was first used to mark mesenchymal precursors that could become CFU-Fs and differentiate into osteoblasts, chondrocytes or adipocytes *in vitro* [13]. Lineage tracing experiments using *nestin*-creERT2; reporter mice provided evidence that precursors marked with this transgene can become osteoblasts and adipocytes *in vivo*, though others have been unable to reproduce these findings [14]. Other groups have subsequently used a wide variety of transgenes to mark stromal cells and follow their fates, as they

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differentiate into adipocytes and osteoblasts *in vivo*. Transgenes using promoters such as those for Sox9 [15–17], LepR [14], MX1 [18] and Gli1 [19] give rise to both osteoblasts and adipocytes *in vivo*. Interestingly, stromal cells marked using *Gremlin*-creER [20]; reporter mice could become chondrocytes, stromal cells and osteoblasts, but failed to differentiate into adipocytes *in vivo*, illustrating heterogeneity of fates in cells marked with various promoter constructs used to mark skeletal progenitors *in vivo*. We chose to use Sox9 in our studies, because it is required for the formation of the early mesenchymal condensations, the precursors of both chondrocytes and osteoblasts, in fetal life [17], and because Sox9 is expressed in many stem cell types, from hair follicle stem cells [21] to breast [22], liver [23], intestine [24] and pancreatic stem cells [25]. Using Sox9cre-ERT mice and crossing them to tomato reporter mice we were able to mark a subset of cells in the metaphysis, endocortical and periosteal surfaces. A single dose of tamoxifen was enough to induce recombination and label Sox9 expressing cells red.

3. Parathyroid hormone determines fates of skeletal precursors

Parathyroid hormone (PTH) is a major regulator of mammalian calcium homeostasis, partly through actions on bone [26]. When hPTH (1–34), teriparatide, is administered by once daily subcutaneous injection to humans or rodents, bone mass is increased due to an increased number of osteoblasts and an increase in bone formation rate [27]. The cause of the increased number of osteoblasts after teriparatide has been studied extensively. We recently, used the *Sox9*-creERT2 lineage tracing strategy to show that, besides acting on the post-mitotic, mature cells of the osteoblast lineage, teriparatide-mediated increase in bone mass involves action on early osteoprogenitors. Teriparatide suppresses apoptosis of early skeletal precursors, and thus contributes to the overall increase in the number of new osteoblasts. When the PTH receptor was knocked out using *Sox9*-creERT2, then PTH could no longer increase the number of osteoblast precursors, suggesting that at least some of the actions of teriparatide on these early skeletal precursors are direct actions *via* PTH receptors [15].

We also observed that PTH1R is required for the actions of teriparatide to increase the numbers of early cells of osteoblast lineage *in vivo*. When daily PTH administration was prolonged, the number of red *Sox9*-creERT2 cells continued to increase and differentiated into osteoblasts with a higher rate of differentiation than found with controls. After four weeks of administration of a high dose of once daily teriparatide, the increase in bone mass was quite substantial. No adipocytes that descended from *Sox9*-creERT2 cells were observed at the end of four weeks of teriparatide administration. Strikingly, however, cessation of teriparatide administration not only led to a dramatic decrease in bone mass approximately to the level of control skeletons never treated with teriparatide, but, unlike the controls, the bone marrow of the teriparatide-withdrawn mice was full of adipocytes predominantly located in the metaphyseal region extending into the diaphysis. Interestingly, both male and female mice that underwent teriparatide withdrawal developed this phenotype. Because of the design of this experiment (in which tamoxifen was given months before sacrifice of the mice), we could not determine at which stage or stages of differentiation, *Sox9*-creERT2-marked cells or their descendants started moving along the adipocyte lineage. Strikingly teriparatide withdrawal experiments performed in *Ocn*-creERT; reporter mice showed that several adipocytes seen after teriparatide withdrawal were descendants of *Ocn*-creERT+ cells [15].

While it is well established that bone mass falls after cessation of teriparatide treatment in osteoporotic patients, nothing is known about the possible adipocytic accumulation after cessation of teriparatide in humans [28,29]. In our studies, sudden cessation of teriparatide after prolonged administration led to adipocytic differentiation of descendants of *Sox9*-creERT2 cells. Similarly, teriparatide withdrawal experiments performed in *Ocn*-creER-driven reporter mice showed that mature osteoblasts differentiate into adipocytes by a pathway that needs to be characterized, suggesting striking plasticity of post-mitotic, mature osteoblasts. This conversion of *Sox9*-creERT2 or *Ocn*-creER cells to adipocytes did not occur as long as PTH administration continued, suggesting that the option of becoming an adipocyte is suppressed by PTH administration. This observation is consistent with prior work showing that PTH suppresses adipocytic differentiation of human stromal cells *in vitro* [30]. Further evidence of the importance of suppression of adipocyte differentiation by activation of the PTH/PTHrP receptor was found in studies of mice heterozygous of the PTHrP gene; these mice exhibit an increase in marrow adiposity [31].

In the marrow adipocyte pool, the overall contribution of cells that differentiated from *Sox9*-creERT2 or *Ocn*-creER cells ranged between 10 and 30%. In these studies, tamoxifen was administered at the same time as the initiation of teriparatide treatment. Interestingly, if the withdrawal experiment was performed in *Sox9*-creERT2 mice, in which tomato cells were labeled soon after birth, at day P3, tomato cells contributed > 98% of the marrow adipocytes formed after PTH-withdrawal *in vivo*. These findings suggest that precursors of adipocytes, either Sox9-expressing cells or their descendants, expand postnatally. At some point, some of these cells stop expressing *Sox9* and can no longer be marked after tamoxifen administration, but can still become adipocytes under appropriate conditions [15].

4. Wnt signaling regulates fates of skeletal precursors

Wnt/β-catenin signaling plays a crucial role in controlling osteoblast and adipocyte differentiation. Removal of β-catenin from bone marrow stromal cells (BMSCs) *in vitro* causes these cells to more readily differentiate into the adipocytic lineage [32]. *In vitro*, wnt10b supplementation increases osteoblastogenesis and decreases adipogenesis in ST2 cells, a bone marrow-derived stromal cell line [33,34] and similar findings were observed when cultured cells were treated with wnt1 [34,35]. β-catenin is the key component of the canonical Wnt pathway, and it regulates Wnt target gene transcription when this pathway is activated by any of a variety of Wnt ligands (wnt10b, wnt1, wnt3, for example). Thus, β-catenin is a critical target for exploring the function of the canonical Wnt pathway [36]. Stabilization of β-catenin in BMSCs can promote osteoblastogenesis and inhibit adipogenesis, and over-expression of β-catenin in pre-adipocytes can also inhibit their differentiation into mature adipocytes [37]. When β-catenin was deleted *in vivo* from cells expressing osterix and presumably already committed to the osteoblast lineage, a substantial fraction of these cells subsequently became adipocytes [38]. Teriparatide administration, both *in vitro* and *in vivo* increases Wnt/β-catenin signaling [39]. Teriparatide administration also suppresses various Wnt inhibitors such as DKK-1 [40] and sclerostin [41,42]; this leads to an increase in Wnt/β-catenin signaling. Therefore, we examined whether teriparatide withdrawal led to a sudden decrease in Wnt/β-catenin signaling in *Sox9*-creERT2+ cells by examining whether the non- phosphorylated (Active) form of β-catenin accumulated in this setting. PTH-withdrawn mice showed a dramatic decrease of active β-catenin in *Sox9*-creERT2+ cells compared to the levels in mice subjected to continued once daily PTH administration. The cause of this decrease in activation of β-catenin is not known, but it may reflect mechanisms that serve to normally keep bone mass “normal”. There must be mechanisms of skeletal homeostasis that normally increase and decrease bone mass to optimize the stresses on individual osteoblasts/osteocytes. We can speculate that the increase in bone mass in response to once daily injection of teriparatide leads to a higher bone mass than the mouse “needs” and that the bones sense this state. That is, homeostatic mechanisms may be sensed that lead to a decrease in bone mass with cessation of teriparatide administration, until bone mass falls to its basal level. The low level of active β-catenin in *Sox9*-creERT2+ cells after stopping teriparatide may mean that Wnt signaling participates in this homeostatic mechanism. When the number of osteoblasts/osteocytes is artificially high after teriparatide administration, they may each receive insufficient stress from gravity

and muscle pull to activate canonical Wnt signaling. This could lead to a decrease in activation of β-catenin, as seen in this experiment, and then to lower bone mass.

5. Other regulators of fate of skeletal precursors

During development, deletion of PTH receptor in mice leads to substantial marrow adipogenesis [43]. This may reflect direct actions of PTH; since parathyroid hormone suppresses peroxisome proliferator activated receptor-γ promotes adipogenesis. The zinc-finger protein, ZFP521 suppress adipogenesis, while ZF423 promotes adipogenesis both *in vitro* and *in vivo*. PTH is also known to regulate zinc finger proteins ZFP521 and ZFP423 [44,45]. Whether, these zinc finger proteins regulate the fate switch after PTH-withdrawal remains to be explored. Further, whether humans exhibit similar expansion of adipocytes after stoppage of teriparatide remains to be explored.

6. PTH withdrawal and increase in adipocytes *in vivo*

To the extent that PTH signaling in bone *in vivo* reflects activation of the canonical Wnt pathway, our *in vivo* data suggests the importance of the canonical Wnt pathway for determining both osteoblast and adipocyte differentiation. Our data indicates that PTH-mediated increase in Wnt/β-catenin signaling is essential for continuously steering skeletal stem cells into the osteoblast lineage and restrains bone marrow fat formation in adult animals. We also show that *in vivo* lineage tracing using *Ocn*-creER induces several mature osteoblasts to become adipogenic cells following PTH-withdrawal, and that normally such osteoblast-derived adipocytic cells are few in number in the bone marrow microenvironment in young C57B6 mice. This reprogramming of osteoblasts could be explained by three possible cellular mechanisms. First, osteoblasts, upon removal of β-catenin, may trans-differentiate directly into the adipocytic lineage and proceed to become mature adipocytes; alternatively, osteoblasts may de-differentiate into skeletal precursors, which may then differentiate into adipocytes; in addition, osteoblasts may begin to accumulate intracellular lipid droplets [46]. Several lines of evidence indicate that Wnt/β-catenin signaling regulates expression of PPARγ. Transient activation of Wnt signaling suppresses expression of mRNAs encoding PPARγ [47]. Moreover, PPARγ may bind to β-catenin and regulate its activity [48]. Furthermore, in multipotent mesenchymal precursor cells the balance between osteogenic and adipogenic transcription factors may be important for the cells to maintain their quiescence and to determine lineage commitment. Whether PTH keeps in check the adipogenic commitment of precursors using the canonical Wnt pathway or other pathways is still unclear, and if localized production of PTHrP by various cells types contributes to this phenomenon is also not understood.

In summary, PTH-administration controls the fate of osteoblast precursors and the lineage-committed mature osteoblasts, with respect to differentiation into osteoblastic *vs*. adipocytic populations in bone; this correlates with increased expression of Wnt/beta-catenin signaling. This is the first demonstration that sudden cessation of PTH- treatment can actively reprogram pre-osteoblasts from osteoblasts to adipocytes and future studies are required to understand the molecular mechanisms of dedifferentiation of osteoblast precursors and mature osteoblasts into adipocytes. Whether this PTH and Wnt-suppressed adipogenesis in marrow relates to the regulation of adipogenesis that occurs as animals age is an interesting and unanswered question.

Competing financial interests

The authors have no competing financial interests.

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