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# Role of growth hormone receptor signaling in osteogenesis from murine bone marrow progenitor cells ☆

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## Abstract

Growth hormone (GH) regulates many of the factors responsible for controlling the development of bone marrow progenitor cells (BMPCs). The aim of this study was to elucidate the role of GH in osteogenic differentiation of BMPCs using GH receptor null mice (GHRKO). BMPCs from GHRKO and their wild-type (WT) littermates were quantified by flow cytometry and their osteogenic differentiation in vitro was determined by cell morphology, real-time RT-PCR, and biochemical analyses. We found that freshly harvested GHRKO marrow contains 3% CD34 (hematopoietic lineage), 43.5% CD45 (monocyte/macrophage lineage), and 2.5% CD106 positive (CFU-F/BMPC) cells compared to 11.2%, 45%, and 3.4% positive cells for (WT) marrow cells, respectively. When cultured for 14 days under conditions suitable for CFU-F expansion, GHRKO marrow cells lost CD34 positivity, and were markedly reduced for CD45, but 3- to 4-fold higher for CD106. While WT marrow cells also lost CD34 expression, they maintained CD45 and increased CD106 levels by 16-fold. When BMPCs from GHRKO mice were cultured under osteogenic conditions, they failed to elongate, in contrast to WT cells. Furthermore, GHRKO cultures expressed less alkaline phosphatase, contained less mineralized calcium, and displayed lower osteocalcin expression than WT cells. However, GHRKO cells displayed similar or higher expression of *cbfa-1*, collagen I, and osteopontin mRNA compared to WT. In conclusion, we show that GH has an effect on the proportions of hematopoietic and mesenchymal progenitor cells in the bone marrow, and that GH is essential for both the induction and later progression of osteogenesis.


*Keywords:* Mesenchymal progenitor cells; CFU-F; Osteoblast; Osteogenesis; Growth hormone

During osteogenic development, bone marrow progenitor cells (BMPCs) pass through distinct phenotypic stages characterized by the key regulatory factors and proteins they produce [4,30]. The numbers of progenitors progressing through these osteogenic stages largely determine the rate of bone formation [29], with osteoblast number being controlled by changes in both the production and survival of mature cells [31]. Growth hormone (GH) is a major regulator of postnatal skeletal growth by virtue of its ability to stimulate the differentiation and proliferation of chondrocytes in the growth plate of long bones [33]. However, the significant actions of GH on bone, involving both osteoblasts and osteoclasts, are less well characterized.

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Biochemical markers of bone formation are decreased in humans with GH receptor deletion [24] and clinical studies have detected reduced bone mineral density and concentration in GH deficiency [33]. GH replacement therapy stimulates bone turnover and improves bone mass during childhood and adolescence, and decreases fracture incidence by 3- or 4-fold [51]. GH receptor-deleted mice (GHR−/−) have reduced femoral width and low cortical bone mass associated with a very low mineral apposition rate at 4 weeks of age. The reduced osteoblast surface area in these mutants is consistent with impaired osteoblast proliferation [44]. Similarly, in rats GH induces subperiosteal bone formation associated with increased mechanical strength [1].

IGF-1 is believed to be a major mediating factor in the bone anabolic actions of GH as the reduced cortical bone formation in GHR−/− mice can be restored with IGF-1 treatment [44]. Some of these actions may be mediated through the local induction of BMPs -2 and -4, and BMPR Receptor 1A by both GH and IGF-1 [25,27]. This induction correlates spatially with increases in such early markers of bone formation as osteocalcin and osteopontin [25]. These bone anabolic actions of GH evidently do not require activation of STAT5a or b, since combined deletion of these transcription factors is without effect on cortical bone thickness or mineral apposition rate [44].

We and others have previously shown that GH receptors are present in osteoblasts, and that osteoblast cell lines respond to GH in vitro with proliferation and increased markers of definitive bone formation such as alkaline phosphatase, osteocalcin, and type 1 collagen matrix production [3,18]. We have also shown the induction of key extracellular proteoglycans that regulate bone phenotype (such as decorin and biglycan) by GH in vitro and in vivo [54]. While the anabolic actions of GH on bone have been assumed to result from its actions on osteoblasts, the role of GH in the differentiation of bone marrow precursors along the bone lineage remains unknown. Human bone marrow stromal cells express immunoreactive GH receptors in culture, both in the proliferating progenitor cells and in more differentiated cells [28]. In the only study to date, Kassem et al. [19] reported that GH increased the rate of proliferation of human marrow stromal cells without influencing their differentiation, and that this was synergized by IGF-1 and -2 [23]. Thus, it appears that GH influences the progenitor pool, although its effect on the various progenitor subpopulations remains unclear.

Of the available antibodies that appear to recognize murine lineage-specific antigens, vascular cell adhesion molecule 1 (VAM-1/CD106), a cell adhesion molecule constitutively expressed by bone marrow stoma in vitro and in vivo [7,43] as well as by CFU-Fs [42], has proven effective for the characterization of bone marrow stroma [16]. Further characterization of these stromal elements is often reported in terms of their negative expression for CD34, a marker of hematopoietic stem and progenitor cells [8,21], and CD45/leukocyte common antigen (LCA) present on all cells of hematopoietic origin, except erythroid cells, platelets, and their precursor cells [35,38].

In this study, we have screened freshly harvested and culture-expanded BMPC's from growth hormone receptor knockout (GHRKO) mice for the expression of CD34, CD45, and CD106 cell surface antigens against cells from wild-type (WT) mice. In addition, we examined the potential of the GH receptor-deleted cells to differentiate into osteoblasts by profiling mRNA expression of the osteoblast-related markers *cbfa-1*, collagen-I, osteopontin, and osteocalcin, as well as alkaline phosphatase activity and calcium content. We hypothesized that deletion of the GH receptor would impair the ability of marrow progenitor cells to expand and then commit to the osteoblast lineage, resulting in a decrease in the biochemical markers of osteoblast development. Our results show that GH is not only involved in the later stages of osteoblast differentiation, but also during progenitor expansion and subsequent tissue-specific lineage commitment.

## Materials and methods

*Animals.* 129OLA/BalbC growth hormone receptor knockout (GHRKO) mice completely lack GHR signaling due to a functional disruption of the GHR gene [55]. Although at birth GHRKO animals are indistinguishable from their wild-type (WT) littermates, after the third postnatal week, they have reduced growth (~50% of wild-type at 6 weeks), increased systemic GH and reduced levels of systemic IGF-1 [56]. Litter-matched 129OLA/BalbC mice carrying two normal copies (wild-type) were used as controls for this study. Wild-type and homozygote animals were identified by PCR analysis of tail DNA as previously described [6]. These animals were a generous gift of JJ Kopchick and K Coschigano, Edison Biotechnology Institute, Ohio University.

The University of Queensland's Animal Ethics Committee approved all animal procedures.

*Materials.* Ficoll–Hypaque was purchased from Amersham (Buckinghamshire, UK); glutamine, penicillin, gentamycin, and fungizone were purchased from Life Technologies (Gaithersburg, MD, USA). Fetal bovine serum (FBS) and α-modified essential media (α-MEM) were purchased from JRH Biosciences (Australia) following an extensive selection protocol. Red phycoerythrin (R-PE)-conjugated monoclonal antibodies (mAbs) to CD34 (the RAM34 clone), CD45/LCA (the I3/2.3 clone), and CD106 (the M/K-2 clone), and their isotype-matched controls were purchased from Becton–Dickinson (Mountain View, CA) and Southern Biotechnology (Alabama, USA). Dexamethasone (Dex), sodium β-glycerophosphate (β-GP), L-ascorbic acid-2-phosphate (AsAP), EGF, and sodium pyruvate were purchased from Sigma (St. Louis, MO). Unless otherwise stated, all other chemicals were of analytical grade and purchased from Sigma.

*Isolation of bone marrow progenitor cells and primary culture.* Mouse bone marrow progenitor cells (mBMPCs) were isolated from the femora and tibia of eighteen 8- to 12-week-old GHRKO and WT mice by flushing the marrow cavities using standard culture media (α-MEM supplemented with 15% FBS, 2 mM glutamine, 100 μg/ml AsAP (L-ascorbic acid-2-phosphate), 5 ng/ml EGF, 100 nM sodium pyruvate, 100 μg/ml penicillin G, 50 μg/ml gentamycin, and 300 ng/ml fungizone). Within each GHRKO or WT group, aspirates were randomly assigned to one of three groups, giving a total of 6 animal aspirates per group, in triplicate. Marrow aspirates were then triturated using a 21-gauge syringe and passed through a 70 μm cell strainer (Becton–Dickinson) to remove bone fragments. Cells were separated by density centrifugation through Ficoll–Hypaque at 1000*g* and 15 °C for 45 min. After washing by centrifugation and counting