Effect of hypophysectomy on the proliferation and differentiation of rat bone marrow stromal cells

Yeh, James K., Jodi F. Evans, Meng-Meng Chen, and John F. Aloia. Effect of hypophysectomy on the proliferation and differentiation of rat bone marrow stromal cells. Am. J. Physiol. 276 (Endocrinol. Metab. 39): E34–E42, 1999.—Conditions such as estrogen deficiency, skeletal unloading, and aging have all been demonstrated to have various effects on the proliferation and differentiation of bone marrow stroma-derived osteoprogenitor cells. Here we have sought to examine the effects of pituitary hormone deficiency on the proliferation and the differentiation of these osteoprogenitor cells using the hypophysectomized (HX) rat as a model. In the present study, we use an in vitro culture system to examine the effects of HX on the osteogenic potential of rat bone marrow stroma. With the intact animal as a control, we used [3H]thymidine incorporation and cell number as indexes of proliferation. We also measured alkaline phosphatase enzyme activity, relative levels of osteocalcin expression with RT-PCR, and osteopontin and bone sialoprotein steady-state levels by Northern blot to delineate the effect on differentiation. Our results indicate that osteoprogenitor cells exposed to a pituitary hormone-deficient environment in vivo demonstrate an enhanced proliferative capacity and also exhibit an augmented expression of differentiation markers when exposed to an optimal environment in vitro.

pituitary hormone; osteoblasts; osteogenesis; alkaline phosphatase; calcium

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control, we used [3H]thymidine incorporation and cell number as indexes of proliferation. We also measured AP enzyme activity, relative levels of OCN expression with RT-PCR, and OPN and BSP steady-state levels by Northern blot to delineate the effect on differentiation. Our results indicate that osteoprogenitor cells exposed to a pituitary hormone-deficient environment in vivo demonstrate an enhanced proliferative and differentiative capacity in vitro when their environment is again optimal.

MATERIALS AND METHODS

Animal care. Age-matched HX and intact control female, 8-wk-old Sprague-Dawley rats were purchased from Hilltop where the hypophysectomy surgeries were performed. On arrival, 3 days postoperatively, and throughout the experiment, the HX rats were given 3% sucrose water and allowed free access to a standard pelleted chow diet (Rodent Laboratory Chow 5001, Ralston Purina, St. Louis, MO). Animals were maintained in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and animal protocols were approved by the Laboratory Animal Care Committee of Winthrop University Hospital.

Histomorphometric analysis. Rats were labeled with 8 mg/kg of calcein subcutaneously (Sigma, St. Louis, MO) at 3 days before being killed. The left and right tibiae were removed and cut with an Isomet saw (Buehler, Lake Bluff, IL). The proximal section of each right tibia was stained by Villanueva osteochrome bone stain (Polysciences, Warrenton, PA) for 5 days and then processed for methyl methacrylate embedding without decalcification (33). The specimens were destained and dehydrated with sequential changes (70, 95, and 100%) of ethanol solution and xylene and then embedded in methyl methacrylate (Eastman Organic Chemicals, Rochester, NY). Proximal tibiae were frontal-cut longitudinally with a diamond wire histo-saw (Delaware Diamond Knives, Wilmington, DE) at a thickness of 40 µm and then coverslipped with Eukitt (Calibrated Instruments, Hawthorne, NY).

Histomorphometric parameters of cancellous bone of the proximal tibia were measured with a digitizing morphometry system (Osteometrics, Atlanta, GA) and the nomenclature standard (20) as described previously (33). Briefly, the measured parameters included total tissue area, trabecular bone area and perimeter, and calcine labeled perimeters in the secondary spongiosa of the metaphysis in the region 1–4 mm proximal to the growth plate-metaphysial junction. These parameters were then used to calculate cancellous bone volume, total volume, and labeled surface tissue area.

The left tibia was removed and decalcified for 5 wk at 4°C in 10% EDTA containing 7.0% sucrose (pH 7.2). After a thorough wash in PBS solution, the decalcified bones were dehydrated in graded alcohol and embedded in 2-hydroxyethyl methacrylate (JB-4 Plus embedding kit, Polysciences). Polymerization was carried out at 4°C in a cold room overnight. Polymerized blocks were kept in a desiccator at 4°C until sectioning. Sections of 4-µm thickness were cut with a microtome (2040 Reichert-Jung, Cambridge Instruments, Buffalo, NY) and then mounted on glass slides. Tartrate-resistant acid phosphatase was localized with a commercially available kit with α-naphthyl phosphate disodium salt as substrate (Sigma) (33).

Cell culture. Both femoral and tibial bones were removed from the rats at 6 wk post-HX. Age-matched intact rats served as the control group. Under aseptic conditions, the bones were dissected free of soft tissue, and the epiphyseal cartilage was scraped away with a scalpel and discarded. The distal end of the bones was then cut open, and bone marrow was flushed out with 10 ml α-MEM medium (without phenol red) supplemented with 10% FBS, antibiotic-antimycotic, 50 µg/ml ascorbic acid, and 2 mM l-glutamine (basal medium) with an 18-gauge needle for the femur and a 22-gauge needle for the tibia. The bone marrow cells were pooled from each of the four bones of each animal, and a single cell suspension was created by repeated flushing through an 18-gauge needle followed by a 22-gauge needle. For the initial proliferation experiments, cells were seeded at a density of 3.5 × 10^6/cm² and allowed to attach for 3 days before the removal of nonadherent cells. Differentiation experiments were carried out in the above medium supplemented with 10 mM β-glycerophosphate and 10^{-8} M dexamethasone (differentiation medium), and initial plating was at the same density as in the proliferation experiments. Cell numbers were determined with a hemocytometer.

Adherent and nonadherent cell fractions. Bone marrow cells were plated at a density of 3.5 × 10^6/cm². After a 1-day initial attachment period, the nonadherent cells in the medium were removed and cultured in separate dishes, and either basal or differentiation medium was replaced in the adherent culture. The first medium change for the adherent cultures took place 4 days later, and medium was changed every second day thereafter. The nonadherent cultures were allowed a 4-day attachment period before the medium was replaced with fresh basal or differentiation medium. Thereafter, the medium was changed every second day. Another set of dishes was seeded at the same density and allowed to attach for 5 days before the nonadherent cells were removed and the medium was replaced with either fresh basal or differentiation medium. Cell number was determined after trypsinization with a hemocytometer.

[3H]thymidine incorporation. Bone marrow cells were plated as stated above and cultured in basal medium. The medium was replaced after 72 h, the adherent cells were cultured for another 4 days, and the medium was changed every 2 days. One microcurie of [3H]thymidine (37 Ci/mmol, Amersham, Arlington Heights, IL) was added to each well, and the cells were cultured for another 24 h. The medium was then removed, and the cells were washed with PBS and detached with 0.05% trypsin in PBS. The cells were collected by centrifugation, lysed with 0.1 N sodium hydroxide, and [3H]thymidine incorporation was measured with a scintillation counter and expressed in counts per minute.

AP activity. On day 14 of culture, the cells were washed with 1× PBS and fixed with ice-cold 95% ethanol (EtOH). The cells were then washed with distilled H_2O and allowed to equilibrate in 20 mM bicarbonate buffer, pH 8.8, followed by a 20-min incubation at room temperature with 1 mg/ml p-nitrophenylphosphate in bicarbonate buffer with continuous agitation. p-Nitrophenol in the supernatant was then measured by spectrophotometry at 405 nm, and the results were expressed as nanomoles per minute per 10^2 cells. Histological staining for AP was performed with the Sigma B5L-1 kit according to the directions of the manufacturer. Cell number for the AP cultures was obtained by the method of Currie (9). After the AP activity of the cells was measured, the cells were fixed again in 10% Formalin for further mechanical stability. They were then washed once with tap water and twice with borate buffer (10 mM, pH 8.8), stained with 1% methylene blue in borate buffer for 15 min, and then rewarshed several times with tap water followed by borate buffer. Methylene blue-positive colony-forming units (CFU) were used as a measure of total CFU-fibroblast (f) colony numbers. Bound methylene blue was then eluted with 1% HCl, and the
Table 1. Effect of hypophysectomy on body weight and histomorphometric indexes measured in tibial metaphysis

<table>
<thead>
<tr>
<th>Animal Group</th>
<th>Animal Wt.</th>
<th>Total Bone Volume (BV/TV) %</th>
<th>Osteoclasts, cells/mm² growth plate</th>
<th>Osteoclast Surface, mm²/mm²</th>
</tr>
</thead>
<tbody>
<tr>
<td>HX</td>
<td>268 ± 21.4</td>
<td>22.6 ± 4.0</td>
<td>7.38 ± 1.0</td>
<td>1.89 ± 0.46</td>
</tr>
<tr>
<td>Intact control</td>
<td>193 ± 13.5</td>
<td>5.9 ± 2.8</td>
<td>3.26 ± 0.6</td>
<td>0.46 ± 0.29</td>
</tr>
</tbody>
</table>

Values are means ± SD of 10 rats/group. Total bone volume values are tibial cancellous bone volume (BV) as a percentage of total tibial volume (TV). *Significant difference between age-matched intact control and hypophysectomized (HX) rats (P < 0.05) as determined by Student’s t-test.

RESULTS

Body weight and histomorphometric data. At 6-wk postsurgery, as previously demonstrated, the body weight of the HX rats remained within a 5% range of their initial presurgery weight, whereas the age-matched intact control rats experienced a 32% gain in weight (Table 1). The cancellous bone volume of the proximal tibia was significantly decreased by 6-wk post-HX compared with the intact control group. Using calcein labeling to examine the osteoblast population on the bone surface, we found that the osteoblast number was decreased by HX. Furthermore, the osteo-

absorbance was read at 650 nm. Cell number was then determined by comparison with a standard curve.

Calcium. At day 21 of culture, calcium in the cell layers was extracted with 1% HCl, and calcium content was measured with atomic absorbance. Mineralized nodules were histochemically stained at day 18 of culture with alizarin red, pH 6.2, and nodules were counted manually (24).
under differentiation culture conditions stromal cells from intact control and HX rats

Table 3. Proliferation characteristics of bone marrow stromal cells from intact control and HX rats under basal culture conditions

<table>
<thead>
<tr>
<th>Assay</th>
<th>Intact Control</th>
<th>HX</th>
</tr>
</thead>
<tbody>
<tr>
<td>[3H] thymidine incorporation,</td>
<td>1,350 ± 185</td>
<td>2,535 ± 203*</td>
</tr>
<tr>
<td>counts/min</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 8 (×10^3)</td>
<td>4.1 ± 0.7</td>
<td>5.0 ± 0.6</td>
</tr>
<tr>
<td>Day 14 (×10^3)</td>
<td>3.2 ± 0.41</td>
<td>5.8 ± 0.50*</td>
</tr>
<tr>
<td>CFU-f colony, day 14</td>
<td>25.3 ± 2.5</td>
<td>43 ± 8.2*</td>
</tr>
</tbody>
</table>

Values are means ± SD of 6 replicate cultures from 4 different animals/group. Colony-forming unit-fibroblast (CFU-f) colony values are in numbers of colonies. Cells were allowed to attach to culture dishes for 3 days before nonadherent cells were removed with 1st medium change. *Significant difference between age-matched intact control and hypophysectomized (HX) groups as determined by Student's t-test.

decreased to less than one-half of that of the intact animals.

Characterization of bone marrow stromal cell proliferation under basal and differentiating conditions. Total marrow stromal cells recovered from the HX animals were significantly less than total numbers recovered from the intact animals (3.35 ± 0.57 × 10^8 cells HX vs. 4.57 ± 0.77 × 10^8 cells control). These data represent the total number of cells recovered from two femora and two tibiae (n = 10). However, initial plating densities were the same in both groups throughout the experiment. After 7 days of incubation in basal medium, the 24-h [3H] thymidine incorporation was 88% greater in the HX group, but the cell number per dish did not differ from the intact control (Table 2). However, by 14 days of culture in basal medium, the cell number and total CFU-f were both higher (81 and 70%, respectively) in the HX group compared with the intact control. When the initial 3-day attachment period was followed by the addition of differentiation medium, cell number was still greater in the HX group. By day 8 under differentiating conditions, it was already 67% more than the intact control group, and by day 14 it was 90% greater (Table 3). CFU-AP and CFU-f colony numbers, after 14 days of incubation in differentiation medium, were also greater in the HX vs. intact control groups by 58 and 19%, respectively. The percentage of AP-positive colonies was also greater in the HX group. Sixty percent of the total colonies in control cultures were AP positive, whereas 80% of the total colonies were AP positive in the HX cultures.

Recruitment of noncommitted precursor cells. To investigate whether the increased cell number in the HX group is because of an enhanced recruitment of noncommitted precursors, we separated the stromal cells into adherent and nonadherent fractions. The cell fractions were separated and cultured as stated in MATERIALS AND METHODS. Cell numbers were counted at days 8 and 14 in the basal cultures and at day 14 for cultures grown in differentiation medium. At day 8 of basal culture, the cell number in the 1-day adherent fraction of the HX group was only 44% of the intact control, but by day 14 there was no significant difference in cell number (Fig. 1). This implies that the initial committed mesenchymal precursor population in the HX animals is less than that of the intact control, but after exposure to optimal culture conditions, the HX cells surpass the controls in proliferative capacity. In the nonadherent fractions by day 14, the HX group had a 35% greater cell number than the intact control. Moreover, the HX cultures had greater cell numbers at both time points when cells were allowed to attach and recruit other noncommitted mesenchymal precursor cells for 5 days. Therefore, we conclude that there is an enhanced recruitment of noncommitted stromal cells in the HX cultures, and the proliferative capacity of the committed precursor population surpasses control levels after 14 days of optimal culture conditions. In differentiation medium, the results were similar to those with the basal medium in the 5-day adherent fractions, but in the 1-day adherent and nonadherent fractions, the HX cultures had a much higher cell number (+268 and

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Fig. 1. Effect of hypophysectomy (HX) on proliferation of committed and noncommitted stromal cells under basal and differentiating conditions. Cell fractions were separated and cultured in basal (bas) or differentiation (diff) medium as described in MATERIALS AND METHODS, and cell numbers were measured at days 8 and 14. Values are means ± SD of 3 replicate cultures from each of 6 different animals/group. Hypox, hypophysectomy; adh, adherent; nadh, nonadherent. *Significant difference between HX and intact control cultures as determined by Student's t-test.
+218%, respectively) than the intact control cultures. This is possibly an indication that both the committed and noncommitted stromal cells of the HX animals are more responsive to differentiating conditions.

Characterization of bone marrow stroma-derived osteoprogenitor cell differentiation. **CELLULAR INDEXES.** When AP activity was measured at day 14 of culture under differentiating conditions, the HX cultures demonstrated a greater activity (at least +110%) in all cell fractions (Fig. 2). Mineralized nodules were counted at day 18, and calcium content was measured at day 21 of incubation. These differentiation parameters were also greater in the HX cultures vs. the intact control cultures in all fractions analyzed. The most significant difference was found with the 1-day adherent fraction, where the intact control cultures had developed <10% the number of nodules and only 15% of the level of calcium as the HX group (Fig. 3). Again these results indicate that both the committed preosteoblasts and the noncommitted stromal cells in the HX cultures have an enhanced sensitivity to differentiating conditions.

**MOLECULAR INDEXES.** Once we found that the cellular markers of osteoblast differentiation were enhanced in the HX bone marrow stromal cell cultures, we sought to examine a few osteogenic markers to determine whether they too would be enhanced. RT-PCR was used to compare relative levels of OCN mRNA levels. Initially, we analyzed OCN expression levels in 1-day adherent and 5-day adherent cultures after 12 days of incubation. The 12-day time point was used because it has been demonstrated to be near the beginning of matrix maturation and the point at which OCN expression can first be detected in stromal cell-derived osteoblast cultures (16, 32). In both the 1-day and 5-day adherent cultures, the HX group demonstrated higher relative OCN expression at day 12 (3× and 1.7×, respectively) (Fig. 4A). The nonadherent fractions were not analyzed for OCN expression. The significant difference in OCN expression between the control and HX cultures prompted us to investigate temporal differences in expression. After a 5-day initial seed, total RNA was extracted from HX and intact control cultures at days 7, 9, 12, 14, and 16 and analyzed for relative OCN expression (Fig. 4B). No significant difference was seen until day 12, and by day 16, the difference had increased to the point at which HX culture OCN mRNA levels were 2.7 times those of the intact control cultures.

Duplicate cultures were also analyzed by Northern blot to compare steady-state expression levels of OPN and BSP. Levels of OPN expression at day 9 were already 1.5 times intact control levels in the HX cultures (Fig. 5A); BSP levels, however, did not surpass the control until day 12, when they reached a level 3.2 times greater than the intact control cultures (Fig. 5B).
In the HX cultures, expression levels of both of these markers seem to reach peak levels earlier than intact controls, but, by day 16, there is no significant difference between expression levels. Figure 5C is a representative Northern blot depicting steady-state levels of OPN and BSP transcripts in the HX and intact control cultures at days 12 and 14.

**DISCUSSION**

Hypophysectomy is known to result in osteopenia, and the histomorphometric data from these experiments confirm previous conclusions that this osteopenia is, in part, due to a decrease in the osteoblast population on the bone surface (23, 33). Hypophysectomy has also been demonstrated to have a negative effect on hematopoiesis and to result in anemia, leukopenia, thrombocytopenia, and impaired DNA and RNA synthesis in the bone marrow (17). The total number of marrow stromal cells recovered from the HX animals is in parallel with these findings. Total stroma cells recovered from HX animals were significantly less than...
in MATERIALS AND METHODS and cultured them under both conditions. By visual observation, we found significantly greater numbers of CFU-f and CFU-AP per dish under nondifferentiating and differentiating conditions. Under differentiating conditions and a 3-day initial attachment period, the HX cultures show greater cell numbers after only 8 days of culture, indicating a possibly greater sensitivity to dexamethasone. When examining total CFU-f under nondifferentiating and differentiating conditions, we again found significantly greater numbers in the HX cultures; when comparing the percentage of total fibroblastic colonies positive for alkaline phosphatase we also found a greater percentage in the HX cultures vs. the control. This implies that the osteoblastic precursors of the HX marrow are a definite contributor to the enhanced proliferative capacity of the marrow stroma. The HX stromal cells also appear to have superior recruitment potential, as evidenced by the greater total number of CFU-f colonies under both conditions. Through visual observation, we also noted that the area of CFU-f and CFU-AP per dish was also greater in the HX cultures, which supports our hypothesis of enhanced proliferative capacity of both mesenchymal precursors and the preosteoblast population.

To investigate the recruitment potential of the two groups we separated the bone marrow stromal cells into adherent and nonadherent fractions as described in MATERIALS AND METHODS and cultured them under nondifferentiating and differentiating conditions. Under nondifferentiating conditions, we discovered that the initial committed mesenchymal precursor population (1-day adherent fraction) of the HX animal appears to be reduced compared with the intact control. However, after 14 days of optimal culture conditions, this fraction of cells from the HX cultures has greater cell numbers, indicating a greater proliferative capacity. The proportion of nonadherent cells in the two groups cannot be deduced in this manner because other factors like recruitment potential contribute to final cell numbers. We also did not measure the proportion of cells in the different fractions and therefore cannot attribute greater cell numbers in the nonadherent fraction of the HX group to greater starting numbers. If our assumption that the initial committed fraction of cells is less in the HX group than when comparing the 5-day adherent fractions, we can say that the committed progenitor population of the HX animal possesses recruitment potential superior to the intact counterparts.

We have also demonstrated that there is a greater induction of the osteoblast phenotype from the noncommitted progenitor population in the marrow stroma by separating these two fractions of progenitor cells and culturing them in differentiating medium. Under these conditions, all fractions from the HX animal showed greater proliferative and differentiation capacities than the intact control cultures. By 14 days of culture in differentiating medium, cell number in the HX cultures was very significantly greater in all fractions, indicating an extreme sensitivity of these cells to differentiating conditions. After examining differentiation markers at the cellular level in the different fractions, we found that AP activity per cell was greater in all fractions of the HX cultures, confirming a greater sensitivity of these cells to differentiating conditions. Because AP activity is not exclusively a marker of osteoblasts, we examined calcium-containing colony-forming units (CFU-Ca) colony numbers and total calcium content as well. These measurements are indicators of mineralization potential. CFU-Ca colony numbers were significantly greater, and calcium content was at significantly increased levels, in all fractions of the HX cultures. These data correlate with the increases in cell number and AP activity of these fractions under differentiating conditions compared with the intact control. Several factors could be at work here. First, the nonadherent and initial adherent progenitors from the pituitary-deficient animal appear to be more sensitive to the inductive properties of the dexamethasone in the medium. Second, the recruitment potential of the adherent precursor population could be augmented as discussed earlier. It has been hypothesized that when the adherent fraction of precursors is exposed to anabolic stimuli present in the culture medium, they secrete factors such as prostaglandin E2, which has been shown to mediate the transition between nonadherent and adherent preosteoblast cells (24, 25). Because the adherent preosteoblasts from the HX animals have an enhanced response to anabolic stimuli as demonstrated through our proliferation experiments, perhaps they are secreting greater amounts of recruiting factors into the medium and therefore augmenting osteoprogenitor recruitment.

Our examination of molecular markers of differentiation has revealed an earlier progression toward mineralization and enhanced levels of expression in HX cultures. We examined gene expression of two noncollagenous matrix proteins, OPN and BSP. OPN expression has been detected during both the proliferative and differentiation stages of osteoblast development (2, 28, 32), but the role it plays in mineralization has not been clearly defined (32). It has been speculated, however, that its function during the proliferative stage of development is related to the mediation of cell attachment and it conditions the surface of the culture dish in preparation for mineralization (28, 32). As in these other osteoblastic development models, we have also detected this matrix protein during the proliferation stage with considerably higher levels detected in the HX cultures. The second matrix protein of which gene
expression levels were measured, BSP, has been implicated as an influence in the initial formation of mineralized nodules (32). When temporal patterns and peak expression of BSP between the two groups are compared, HX cells not only reach maximal expression earlier than control cells, but peak levels are also greater than those of control cells as well. Using RT-PCR coupled to Southern blot, we also analyzed relative OCN mRNA levels in the two groups. This osteoblastic marker is not only specific to bone but is also highly correlated with the calcification of the ECM (28). By day 12 of culture, there were considerably greater mRNA levels of OCN in the HX cultures, and this difference remained through day 16.

It has been postulated that proliferation is functionally related to the synthesis of the ECM, and the maturation and organization of the ECM contribute to the shutdown of proliferation. This, in turn, promotes the expression of genes that prepare the matrix for mineralization. ECM mineralization or events during the early stages of mineralization are possibly responsible for downregulating genes expressed during ECM maturation and organization (28). Therefore, in this model of osteoblast development, the formation of the ECM is completely related to the stages of differentiation. If this model is applied to the current study, it may help to explain the phenomenon that is occurring in the differentiation of the HX osteoprogenitors. During the first stage, proliferation is elevated, leading to an enhanced deposition of matrix proteins during the ECM maturation stage, as evidenced by increased OPN levels at the end of this stage. This perhaps led to an early shutdown of proliferation and an early enhanced preparation of the matrix indicated by a temporal shift to the left and greater peak levels of BSP. Ultimately, these events sparked the increased expression levels of OCN and subsequently mineralization. This explanation still leaves us to explain the causative mechanism behind the initial increase in proliferation of the HX osteoblast lineage cells. Why do the in vivo and in vitro results seem in such stark contrast?

The elevated response of the bone marrow stromal cells of the HX animal when placed in vitro could be because of a hypersensitivity of these cells to a factor(s) present in the fetal bovine serum and lacking for an extended period of time in vivo. The most obvious of these is growth hormone (GH), a major systemic growth regulator and mitogenic factor that exerts its effects on bone both directly and indirectly via insulin-like growth factor (IGF-I) (3, 10). Circulating GH interacts with certain receptors located mainly in hepatic tissue, resulting in the stimulation of the synthesis and secretion of IGF-I (1). IGF-I also has paracrine-autocrine properties and is locally secreted by osteoblasts in response to GH (10). Perhaps these osteoprogenitors experience an upregulation of the IGF-I receptor because of the acute lack of GH and, subsequently, the lack of systemic and local IGF-I. It has been demonstrated that human patients with reduced serum levels of IGF-I experienced a compensatory upregulation of lymphocytic IGF-I receptor gene expression in response to low circulating IGF-I levels (11). If this were also true with osteoblasts, it would explain why, when these cells are placed in culture with fetal bovine serum, of which bovine GH is a definite component, they are stimulated to divide at an elevated rate. These receptors initiate the Ras/Raf/MEK/mitogen-activated protein kinase (MAPK) signaling cascade and MAPKs have been indicated as transcriptional regulators of proliferation in osteoblasts (27). This is only one possibility, however, because the HX animal lacks more than just GH. Many other hormones with anabolic properties in bone are also lacking in vivo, such as parathyroid hormone and estrogen. Moreover, GH replacement in HX rats cannot completely restore trabecular bone formation (7), indicating the involvement of other pituitary and/or local growth factors in bone formation.

In conclusion, we have observed that once bone marrow stromal cells from a HX animal are cultured in vitro, they experience an enhanced proliferative and differentiative capacity compared with those of the intact animal. The cellular and molecular mechanisms behind this phenomenon have yet to be elucidated. An understanding of these regulatory mechanisms is critical for gaining insight into the pathogenesis of pituitary deficiency-related osteoporosis and other bone-related diseases, as well as for the development of new treatment regimens.

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