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Growth hormone induces multiplication of the slowly cycling germinal cells of the rat tibial growth plate

(epiphyseal plate)

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ABSTRACT **To study the effect of locally infused growth hormone (GH) or insulin-like growth factor I (IGF-I) on slowly cycling cells in the germinal cell layer of the tibial growth plate, osmotic minipumps delivering 14.3 μCi of [^{3}H]thymidine per day were implanted s.c. into hypophysectomized rats, and GH (1 μg) or IGF-I (10 μg) was injected daily through a cannula implanted in the proximal tibia. The opposite leg served as a control. After 12 days of treatment, the osmotic minipumps were removed, and three rats in each group were given GH (20 μg/day, s.c.) for an additional 14 days to chase the labeled cells out of the proliferative layers. Labeled cells remained in the germinal layer, in the perichondrial ring, and on the surface of the articular cartilage close to the epiphyseal plate. GH administered together with labeled thymidine significantly increased the number of labeled cells in the germinal cell layer compared to that in the control leg (ratio = 1.95 ± 0.13), whereas IGF-I showed no stimulatory effect (ratio = 0.96 ± 0.04). Therefore GH but not IGF-I stimulates the multiplication of the slowly cycling (label-retaining) cells in the germinal layer of the epiphyseal plate. IGF-I acts only on the proliferation of the resulting chondrocytes.**

The distal parts of the long bones consist of several structures: the epiphyseal growth plate, the perichondrial ring, the articular cartilage, and the bony epiphysis. These structures are developmentally regulated and cannot be distinguished during fetal life. The epiphysis is seen as a bone nucleus at the time of birth in most species, and the growth plate and articular cartilage are gradually separated by accumulating bone. The temporal development of these structures is well characterized, but its regulation is poorly understood. Both the formation of chondrocytes and their subsequent proliferation in the growth plate are important in the process of longitudinal bone growth. The rate of longitudinal bone growth is equal to the rate of production of new cells per cartilage column multiplied by the maximum size of hypertrophied cartilage cells (1). It has previously been shown in rats, mice, chickens, and rabbits, using [^{3}H]thymidine labeling and autoradiography, that cells close to the epiphysis (the germinal layer) divide less frequently than cells in the center of the growth plate (proliferative zone; refs. 2–5). With [^{3}H]thymidine pulse labeling and histomorphometry, it has been shown that 1 cell division in the germinal layer subsequently gives rise to ≈30 hypertrophic chondrocytes. Thus, each germinal cell that enters the cartilage compartment results in an accumulated long bone growth of ≈0.9 mm, assuming that the average size of the largest hypertrophic chondrocytes is 30 × 30 μm. As a result, ≈40 cell divisions in the germinal layer would be required for the complete growth of a rat tibia (6, 7), if for each division 1 cell enters the cartilage compartment.

Earlier it was considered that growth hormone (GH) stimulated longitudinal bone growth mainly through circulating somatomedins produced by the liver (8). However, later it was shown that local injection of GH as well as of insulin-like growth factor I (IGF-I) gives unilateral bone growth (9–11). Furthermore, GH can induce local IGF-I production in the epiphyseal plate at the level of both mRNA and protein (12–14). *In vitro* studies of epiphyseal chondrocytes indicate that GH and IGF-I have different target cells: they bind to different cell populations (15) and give rise to different clonal types in soft agar suspension cultures (16, 17). However, *in vivo*, the target cells for GH and IGF-I have not yet been identified. In pulse-labeling studies with [^{3}H]thymidine on hypophysectomized rats, Kember found that GH increased staining in all layers of the epiphyseal plate with no preferential effect on the germinal layer (18, 19). Rigal (20), on the other hand, showed that pretreatment of rabbits with GH increased [^{3}H]thymidine labeling in the germinal layer of growth plate fragments *in vitro*.

Cotsarelis *et al.* (21) have developed a technique for specific labeling of slowly cycling cells in the cornea. Animals are given [^{3}H]thymidine continuously for 2 weeks. After this period the label is progressively reduced by dilution in the proliferating cells. A long time after the [^{3}H]thymidine infusion, only cells cycling at a low rate remain strongly labeled. This technique has not been used in the epiphyseal plate of growing rats.

The aim of the present study was to identify slowly cycling cells in the proximal tibia and to investigate if GH and/or IGF-I could stimulate cell division of slowly dividing cells in the germinal layer of the rat epiphyseal plate.

MATERIAL AND METHODS

Animals. Sprague–Dawley rats (Alab, Stockholm) were housed under controlled conditions with constant temperature (24–26°C) and humidity (60%) and with a 14-hr light/10-hr dark cycle. The animals were given standard pellet food and tap water ad libitum. In the first experiment a pregnant rat at a gestational age of 10 days was used. At partus, 12 days later, 13 healthy rats were delivered. In the second and third experiments, 17-day-old male rats were used. Animals were killed by cervical dislocation.

[^{3}H]Thymidine Labeling. In the first experiment, two Alzet osmotic minipumps (model 2002; Alab) were implanted s.c. on the back of an ether-anesthetized pregnant rat (gestational day 10). Each pump delivered 14.3 μCi (1 Ci = 37 GBq) of [^{3}H]thymidine (1 mCi/ml; TRK 686, batch 72; Amersham) per day. At delivery 12 days later, the pumps were removed from the mother. The rats were then killed 5 or 21 days after delivery. In the second experiment, four 18-day-old rats each

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Abbreviations: GH, growth hormone; IGF-I, insulin-like growth factor I.

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received one osmotic minipump containing [^{3}H]thymidine implanted s.c. on the back. Fourteen days later two of the animals were killed and the pumps were removed from the remaining two rats, which were killed 14 days later. In the last experiment ten 18-day-old rats were hypophysectomized by the standard parapharyngeal approach (22). Completeness of hypophysectomy was determined by inspection of the sella region at autopsy and by monitoring the body weight after hypophysectomy. None of the animals used in the present study were incompletely hypophysectomized, as determined by this procedure. Eight days after hypophysectomy, osmotic minipumps containing [^{3}H]thymidine were implanted, and daily local injections of GH, IGF-I, or saline were started. Twelve days later, the infusion was terminated by removing the osmotic minipumps. Four animals were then killed while the remaining six animals received another osmotic minipump containing human GH. After another 14 days the remaining six animals were killed.

The tibias of the killed rats were trimmed free of soft tissue, immediately fixed in 4% (wt/vol) paraformaldehyde, and decalcified for 7 days in analytical grade cation-exchange resin (AG 50W-X4; Bio-Rad); 5-μm paraffin sections were prepared of the proximal part of the tibia. The sections were deparaffinized and dipped in nuclear track emulsion (Ilford K-2) diluted 1:1 with distilled water at 40°C. The slides were air-dried and exposed for 42 days at 4°C in light-tight boxes, developed at 20°C in Kodak D-19 (Eastman Kodak) for 3 min, fixed for 5 min, and washed. All sections were analyzed by two independent investigators. A cell with six or more grains over its nucleus was considered labeled, and a maximally labeled cell had 10–12 grains (Fig. 1).

Hormone Treatment. *Local hormone administration.* The method described by Isgaard *et al.* (10) was used for the local hormone administration. One day before the start of the daily local injections, the animals were anesthetized with ether, and small incisions were made in the skin of both hindlimbs at the lateral part of the knee joint. A 6-mm-long tip of an injection needle with an outer diameter of 1.2 mm (Terumo, Louvain, Belgium) was inserted 3 mm into the bone epiphysis of the tibia above the epiphyseal plate. Daily local injections were given under ether anesthesia through the implanted cannulas. The right leg was injected with 1 μg of recombinant human GH or 10 μg of IGF-I in 10 μl of saline while the contralateral leg was injected with the same volume of saline. The selected doses for GH and IGF-I injections had earlier been demonstrated not to influence the level of IGF-I in serum (23).

Systemic hormone administration. In the third experiment, six animals were given systemic infusions with recombinant human GH (20 μg/day) through an osmotic minipump implanted s.c. on the back of the animals. This treatment followed the [^{3}H]thymidine infusion with the aim to promote the clonal expansion of cells in the proliferative cell layer of the epiphyseal plate (thus enable a chase/dilution of the [^{3}H]thymidine in cells with a high cell turnover).

Hormones. Recombinant human GH (Somatonorm; 2 units/mg; lot 64003) and recombinant human IGF-I (0.95 mg/ml; lot DS 911) were kindly provided by KabiPharmacia, Stockholm.

Statistical Procedures. Values are given as the means ± SEM. The significance of differences between means was calculated by ANOVA with randomized complete blocks followed by Student–Neuman–Keul's multiple range test.

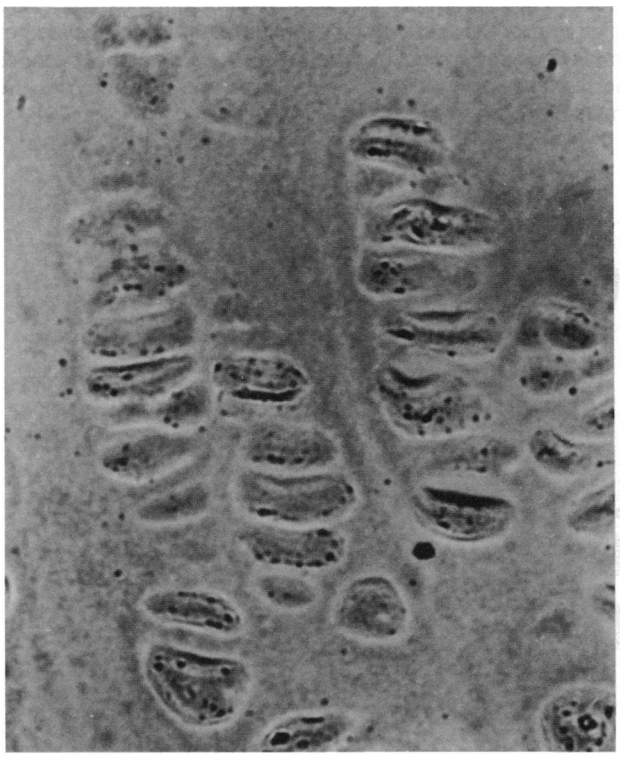

FIG. 1. Labeled chondrocytes in the proliferative zone of the tibia epiphyseal plate of a 28-day-old male rat directly after 14 days of continuous [^{3}H]thymidine infusion. Labeling and autoradiography were according to *Materials and Methods* for experiment 2. (×210.)

RESULTS

Developmental Pattern of the Proximal Tibia from 5 to 25 Days After Birth. Fig. 2 *Upper* shows the tibia of a rat that received [^{3}H]thymidine *in utero* and was killed 5 days after birth. Most cartilage cells are labeled. Tibias from rats killed 25 days after birth were labeled in the proximal part of the epiphyseal plate, in the perichondrial ring, and on the surface of the articular cartilage close to the epiphyseal plate (Fig. 2 *Lower*) while all other cartilage structures were unlabeled. These findings give the spatial localization at different times after birth of cells labeled before birth. To study the division

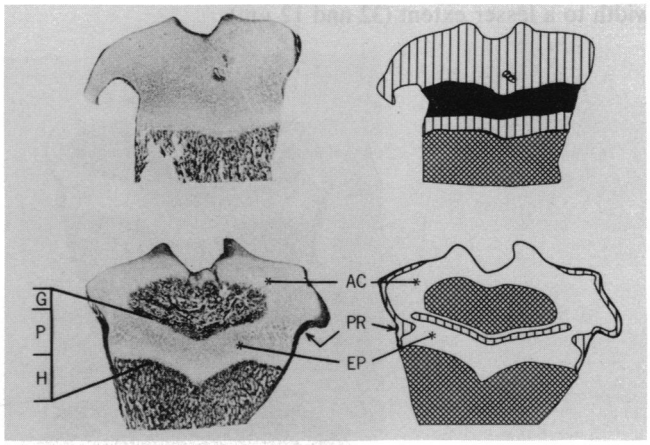

FIG. 2. Localization of labeled cells resulting from [^{3}H]thymidine administration before birth, but examined 5 (*Upper*) and 25 (*Lower*) days after birth. Labeling and autoradiography were according to *Materials and Methods* for experiment 1. (*Left*) The tissue sections were stained with alcian blue/van Giesson and are consecutive to sections chosen for autoradiography and counting. (*Right*) Computer-drawn reproductions of the sections in *Left*. Percent labeled cells in indicated areas: □, 0–9%; ▥, 10–29%; ■, 30–100%; ▨, bone tissue. AC, articular cartilage; EP, epiphyseal growth plate; PR, perichondrial ring. (*Inset*) The size difference between the germinal layer (G), proliferative layer (P), and hypertrophic layer (H) is an approximation of the relative width of the three layers in the indicated section of the epiphyseal growth plate. (×4.)

of these cells in different compartments after birth, it was necessary to label the cells during a later period.

Localization of Slowly Cycling Cells in the Proximal Tibia of Growing Rats. To determine the localization of slowly cycling cells in prepubertal growing rats, tibias were labeled by a continuous [^{3}H]thymidine infusion between days 18 and 32 after birth. The rats were killed on completion of the labeling, and their tibias were examined. The cells were labeled in all layers of the epiphyseal plate and also in most cells in the articular cartilage (Fig. 3 *Upper*). If a 14-day chase period was allowed between completion of [^{3}H]thymidine infusion and killing of the rats, the labeling pattern was similar to that of tibias labeled *in utero* and examined 25 days after delivery (Fig. 3 *Lower*): the label-retaining cells were located in the proximal part of the epiphyseal plate, the perichondrial ring, and on the surface of the articular cartilage.

GH but Not IGF-I Increases the Number of Slowly Cycling Cells in the Germinal Layer of Hypophysectomized Rats. The tibias of different animals infused with the same amount of [^{3}H]thymidine were variably labeled; this made group comparisons more difficult. Therefore, in order to see if GH or IGF-I could change the number of slowly cycling cells, we decided to inject either GH or IGF-I through a cannula to one proximal tibia and use the other tibia as a control. Ten 18-day-old rats were hypophysectomized. Seven days later, cannulas were implanted in their proximal tibia. This was followed by continuous systemic [^{3}H]thymidine infusion and daily injections of GH (five animals) or IGF-I (five animals) to one tibia and saline to the other, for a period of 12 days. Directly after this (day 0), four animals (two GH and two IGF-I treated) were killed while the remaining six animals were given a systemic GH infusion for 14 days to chase labeled cells having a high turnover rate. These six animals (three GH and three IGF-I treated) were then killed (day 14), and the proximal tibias were prepared for autoradiographic examination. The mean difference in epiphyseal width between the treated and untreated side after local GH infusion at day 0 was 128 μm and 96 μm for the two animals, respectively. Locally infused IGF-I increased epiphyseal width to a lesser extent (32 and 12 μm).

Fig. 4 shows that the rats killed at day 0 had a strongly labeled epiphyseal plate with most label in the proliferative cell layer (Fig. 4 *A* and *B*), whereas tibias of rats exposed to systemic GH infusion following labeling (day 14) were labeled only in the germinal layer (Fig. 4 *C* and *D*). Most label-retaining cells were found in the five most proximal cell positions, which we regarded as the germinal cell layer. GH but not IGF-I significantly increased the number of labeled cells in this cell layer (Fig. 5).

DISCUSSION

In the present study slowly cycling (label-retaining) cells of the proximal tibia of prepubertal rats were shown to be labeled in the germinal layer, and it was demonstrated that GH but not IGF-I increases the number of label-retaining cells in that location.

The finding that label-retaining cells were located at the top of the columns in the epiphyseal growth plate 25 days after birth of animals labeled *in utero* confirms earlier studies by Kember and coworker (3, 24). Furthermore, the high frequency of labeled cells in the perichondrium supports an earlier study by Tonna (25) suggesting that the growth in diameter of the growth cartilage could be due to differentiation of cells within the perichondrial ring into chondrocytes.

By using the labeling technique of Cotsarelis *et al.* (21), we were able to localize slowly cycling cells in the proximal tibia of growing prepubertal rats. This technique was a prerequisite to study the effect of GH and/or IGF-I on the number of slowly cycling cells in the germinal cell layer of the epiphyseal plate. Directly after the 14-day labeling period, ≈50% of the cells in the proliferative layer were stained. Probably, even more cells were labeled since the β-rays produced by [^{3}H]thymidine are only effective in producing grains in the upper 1–2 μm of the tissue section (our sections were 5 μm thick). Thus, if grains are seen over a nucleus, they are positive proof that it contains labeled DNA, but absence of grains does not provide proof of the absence of label because the nucleus may lie at the bottom of the section from the emulsion (3).

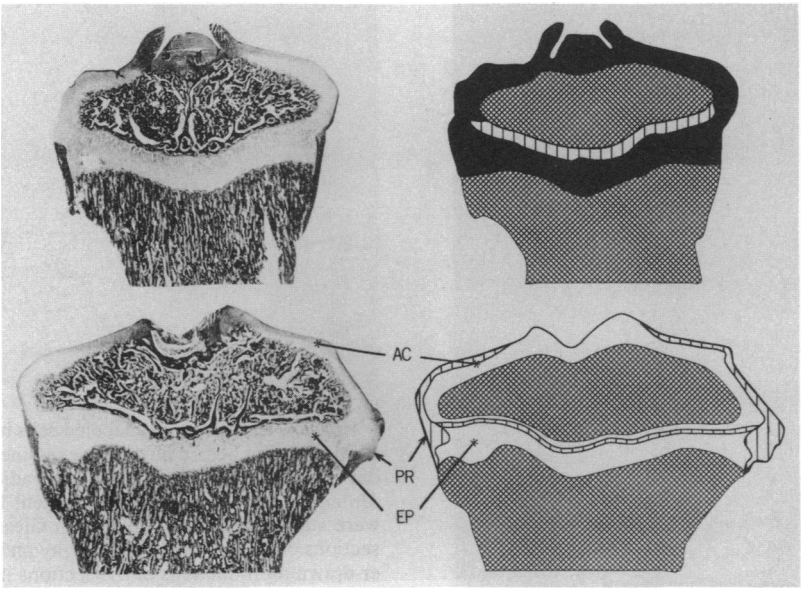

FIG. 3. Localization of labeled cells in the proximal tibia of growing rats labeled continuously for 14 days. Rats were killed directly (*Upper*) or 14 days (*Lower*) after [^{3}H]thymidine infusion. Labeling and autoradiography were according to *Materials and Methods*. (*Left*) The tissue sections were stained with alcian blue/van Giesson and are consecutive to sections chosen for autoradiography and counting. (*Right*) Computer-drawn reproductions of the sections in *Left*. Percent labeled cells in indicated areas: □, 0–9%; ▥, 10–29%; ■, 30–100%; ▩, bone tissue. AC, articular cartilage; EP, epiphysial growth plate; PR, perichondrial ring. (×4.)

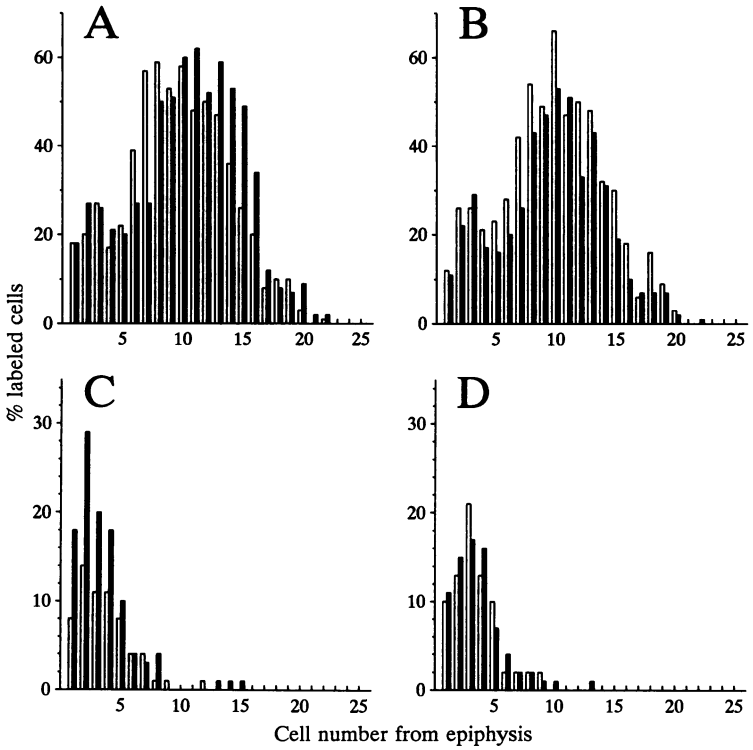

FIG. 4. Labeling pattern in the epiphyseal plate after local GH (*A* and *C*) or IGF-I (*B* and *D*) treatment of hypophysectomized rat infused with [^{3}H]thymidine for 12 days. Four rats were killed immediately (*A* and *B*), and six rats were killed 14 days after (*C* and *D*) termination of [^{3}H]thymidine infusion. Labeling, hormone administration, autoradiography, and counting were according to *Materials and Methods* for experiment 3. Each panel shows results from one representative animal of each group. The cell closest to the epiphysis in each column is regarded as cell number 1, followed by increasing numbers in the direction of the metaphysis. In each cell position, the accumulated number of stained cells (after counting 100 cell columns) is indicated. For each cell position, the open bar is the saline-treated side (control leg) and the filled bar is the GH- or IGF-I-treated side.

The similarity in staining pattern between the *in utero* labeled rats 25 days after birth and growing rats killed 14 days after [^{3}H]thymidine infusion suggests that a stem cell population, established during fetal life, is located in a defined region of the proximal tibia between the articular cartilage and the diaphysis. Cells of this population cycle slowly and probably are progenitors of the cells of the growth plate as well as the periosteum and perichondrium. However, with the labeling technique used in the present study, we were only able to identify the location of label-retaining cells, with no relation to the cellular relationships between individual cells within the growth plate.

The labeling pattern in the growth plate resulting from the systemic GH stimulation after the long infusion of [^{3}H]thymidine is probably a consequence of both a chase and a dilution of labeled cells. The chase of labeled cells is apparent in the late proliferative and hypertrophic cell layers, where cells have stopped dividing and after hypertrophy will degenerate and leave the growth plate in the process of enchondral ossification. The process of dilution, on the other hand, is a result of multiple cell replications where the labeled DNA is divided between daughter cells. Consequently, the dilution is more pronounced in the proliferative than in the germinative cell layer.

In earlier studies with rabbit epiphyseal chondrocytes, cultured in soft agar suspension, it was shown that GH increases the number of clones from cells dissected from the germinal layer, whereas IGF-I increases the number of clones from cells dissected from the proliferative and hypertrophic layer of the epiphyseal plate (23). This is consistent with our present result that GH but not IGF-I stimulates the multiplication of label-retaining cells located in the top layer of the epiphyseal plate. The fact that locally infused IGF-I is able to increase epiphysial width (11, 26) as well as longitudinal bone growth (10), together with the finding in our study that IGF-I was unable to increase the number of label-retaining cells in the germinal layer, indicates that the target cells for IGF-I are located in the proliferative cell layer. The cells of the germinal layer may be regarded as the stem cells of the growth plate and the proliferative chondrocytes as "transient amplifying" cells. The growth demonstrated by local (10, 11) or by systemic (27) IGF-I administration could probably be explained by a stimulation of the proliferative cell layer, where extra cell divisions within the transient amplifying cells could

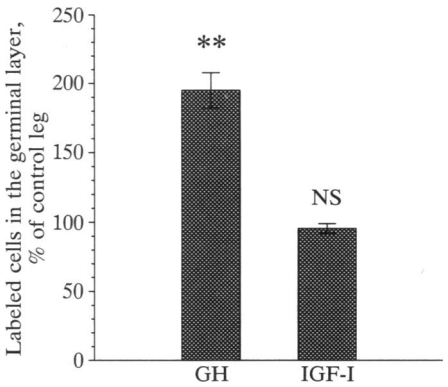

FIG. 5. Effect of GH and IGF-I on the number of label-retaining cells in the germinal layer of the epiphyseal plate of hypophysectomized rats. Labeling, hormone administration, autoradiography, and counting were according to *Materials and Methods* for experiment 3. We regarded the five most proximal cells as the germinal layer. One hundred columns of the control leg and the GH- or IGF-I-treated leg were counted on each animal. Bars show the means ± SEM of three GH- and three IGF-I-treated animals expressed as the percentage of the control leg. **, $P < 0.01$; NS, not significant vs. control leg.

result in significant growth. The results from this study support the theory presented by Green *et al.* (28) and by Isaksson *et al.* (29) that GH stimulates a low differentiated stem cell-like population to start dividing, which gives rise to a clonal expansion, and that IGF-I, on the other hand, enhances the clonal expansion of an already GH-primed cell population.

In conclusion, our study identified the localization of slowly cycling cells in the proximal tibia of both *in utero*-labeled rats and of labeled prepubertal growing rats. We also showed that GH, but not IGF-I, increases the number of slowly cycling cells in the germinal layer of the epiphyseal plate, showing that the two hormones have different target cells.

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