

The Role of the Resting Zone in Growth Plate Chondrogenesis

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In mammals, growth of long bones occurs at the growth plate, a cartilage structure that contains three principal layers: the resting, proliferative, and hypertrophic zones. The function of the resting zone is not well understood. We removed the proliferative and hypertrophic zones from the rabbit distal ulnar growth plate *in vivo*, leaving only the resting zone. Within 1 wk, a complete proliferative and hypertrophic zone often regenerated. Next, we manipulated growth plates *in vivo* to place resting zone cartilage ectopically alongside the proliferative columns. Ectopic resting zone cartilage induced a 90-degree shift in the orientation of nearby proliferative zone chondrocytes and seemed to inhibit their hypertrophic dif-

ferentiation. Our findings suggest that resting zone cartilage makes important contributions to endochondral bone formation at the growth plate: 1) it contains stem-like cells that give rise to clones of proliferative chondrocytes; 2) it produces a growth plate-orienting factor, a morphogen, that directs the alignment of the proliferative clones into columns parallel to the long axis of the bone; and 3) it may also produce a morphogen that inhibits terminal differentiation of nearby proliferative zone chondrocytes and thus may be partially responsible for the organization of the growth plate into distinct zones of proliferation and hypertrophy. (*Endocrinology* 143: 1851–1857, 2002)

THE GROWTH PLATE is a layer of cartilage found in growing long bones between the epiphysis and the metaphysis. Longitudinal bone growth occurs at the growth plate by endochondral ossification, in which cartilage is formed and then remodeled into bone tissue (1).

The mammalian growth plate is composed of three principal layers: the resting, proliferative, and hypertrophic zones. Chondrocytes in the resting zone are irregularly scattered in a bed of cartilage matrix, whereas chondrocytes in the proliferative and hypertrophic zones are arranged in columns parallel to the long axis of the bone (1).

The proliferative zone plays a crucial role in endochondral bone formation; it is the region of active cell replication (2). When a proliferative zone chondrocyte divides, the two daughter cells line up along the long axis of the bone (3). As a result, clones of chondrocytes are arranged in columns parallel to this axis. This spatial orientation directs growth in a specific direction and is thus responsible for the elongate shape assumed by many endochondral bones. The mechanism by which proliferative chondrocytes recognize and line up along this axis is unknown.

The hypertrophic zone also plays a key role in endochondral bone formation. Hypertrophic chondrocytes are generated by terminal differentiation of the proliferative zone chondrocytes farthest from the epiphysis. These cells cease dividing and then enlarge, contributing substantially to the growth process (4, 5). The hypertrophic zone also initiates

ossification by attracting vascular and bone cell invasion from the adjacent bone (6).

In contrast to the proliferative and hypertrophic zones, the function of the resting zone is unknown. We hypothesized three possible roles for the resting zone. First, the resting zone might contain stem-like cells capable of generating new clones of proliferative zone chondrocytes. This possibility has been suggested previously (1) but, to our knowledge, has not been tested empirically (7).

Second, the resting zone chondrocytes might produce a morphogen that directs the alignment of proliferative clones into columns parallel to the long axis of the bone. Currently, the mechanism underlying this spatial organization is unknown.

Third, we hypothesized that the resting zone produces a factor that inhibits hypertrophic differentiation. Proliferative chondrocytes close to the resting zone might be exposed to a higher concentration of this factor, preventing hypertrophic differentiation, whereas proliferative chondrocytes farther down the columns might be exposed to lower concentrations, allowing hypertrophic differentiation. Thus, this morphogen could explain the organization of the growth plate into distinct zones of proliferation and hypertrophy.

Materials and Methods

Studies to identify the stem-like cell

Experimental protocols were approved by the Animal Care and Use Committee, National Institute of Child Health and Human Development. Four-week-old male New Zealand White rabbits (Covance Laboratories, Inc., Denver, PA) were administered general anesthesia (8). After exposing the distal ulnar growth plate, a longitudinal incision was

Abbreviations: GPOF, Growth plate-orienting factor; IHH, Indian hedgehog.

made through the periosteum, which was then reflected back along one third of its circumference. The metaphyseal margin of the growth plate was broken from the shaft by manual pressure (9). The cartilaginous growth plate was then excised by making an incision along its epiphyseal margin.

Under the dissecting microscope, each growth plate was cut perpendicular to the longitudinal axis of the bone, dividing the disk-shaped growth plate into two thinner disks. The incision was made in the resting zone, near the border with the proliferative zone (Fig. 1A). To determine the precise location of the resting zone incision, the piece of the growth plate containing the proliferative and hypertrophic zones was examined histologically. The piece containing resting zone cartilage was returned to its original bed, maintaining the original spatial orientation (Fig. 1B). A 30-gauge hypodermic needle was inserted diagonally through the metaphysis, the reimplanted resting zone, and epiphysis to unite these structures. The periosteum and skin were closed by suturing. The animals were killed with pentobarbital at 1, 2, or 3 wk after surgery, and distal ulnae were removed.

Several variations of this procedure were employed. To improve the precision of the resting zone incision, some of the excised growth plates were first cut in half, parallel to the longitudinal axis of the bone. The resting zone was isolated from each of these pieces, and then the two pieces of resting zone were reunited by suturing. In some cases, the excised resting zone was returned to its original bed; in other cases, it was transplanted into the distal ulna of a syngeneic animal (Covance Laboratories, Inc.). In some animals, the recipient proliferative/hypertrophic zones were intentionally left in place. However, histological analysis showed that the remaining proliferative/hypertrophic zone was replaced by bone tissue and had no appreciable effect on the inserted resting zone. Because none of these procedural variations seemed to affect the outcome, we report the results in aggregate.

Studies of chondrocyte orientation

Distal ulnar growth plates were surgically excised as described above. Under the dissecting microscope, the epiphyseal surface of the growth plate was then scraped to remove any remaining bone tissue. The growth plate was cut parallel to the longitudinal axis of the bone to divide the growth plate into three approximately equal pieces. One or more of these pieces were rotated 90 degrees to bring the resting or hypertrophic zone alongside the proliferative zone of the adjacent piece (Fig. 1, D–F). The three pieces were pinned together with a 30-gauge needle and tied together with polypropylene monofilament suture material. The reassembled growth plate was then inserted back into the original bed. An additional pin was inserted diagonally through the metaphysis, reassembled growth plate, and epiphysis to unite these structures. The periosteum and skin were closed by suturing. The animals were killed 6–10 d after surgery, and the distal ulnae removed.

Histology

Distal ulnae were fixed in 10% neutral-buffered formalin for 24 h, transferred to 70% ethanol, decalcified with EDTA, and embedded in paraffin. Five-micrometer sections were taken parallel to the long axis of the bone and stained with Masson trichrome stain. For the stem cell studies, the portion of the growth plate that was not reimplanted was sectioned similarly, except that step-sections of the entire specimen were obtained every 100 μm .

Results

Studies to identify the location of the stem-like cell

Distal ulnar growth plates were surgically excised and cut perpendicular to the long axis of the bone in an attempt to separate the resting zone from the proliferative zone. To determine the precise location of the growth plate incision, the piece containing proliferative and hypertrophic zones was examined histologically. The piece containing the resting zone was placed back into the original bed in its original orientation. One to 3 wk after surgery, growth plate histological structure was assessed (Fig. 1, A–C).

In 13 growth plates, the incision was not completely within the resting zone but traversed into proliferative zone. Thus, some proliferative chondrocytes were reinserted into the ulna along with resting zone cartilage. In 7 of these growth plates, a well-organized growth plate regenerated. In the other 6, proliferative columns regenerated, but the resulting growth plate was poorly organized or lacked a hypertrophic zone.

In 16 growth plates, the incision was located completely within the resting zone. We further classified these growth plates, based on the precise location of this incision. At this stage of development, the resting zone contains distinct types of cartilage. Although the nomenclature used in the literature is inconsistent (1), we refer to these regions as the reserve cartilage and the epiphyseal cartilage. The reserve cartilage lies closest to the proliferative zone and contains flattened chondrocytes (foreshortened in the dimension parallel to the long axis of the bone). The reserve chondrocytes are often grouped in pairs and higher multiples aligned parallel to the

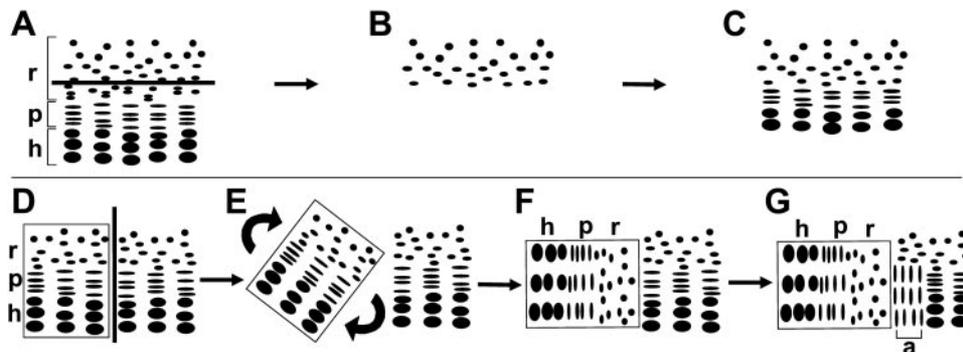


FIG. 1. Diagram of growth plate manipulations. A–C, Surgical procedure to locate the stem-like cells. A, Diagram of growth plate, including resting zone (r), proliferative zone (p), hypertrophic zone (h). The dark horizontal line represents the surgical incision through the resting zone. B, The majority of the resting zone was reimplanted into the distal ulna. The proliferative and hypertrophic zones (not shown) were examined histologically to confirm the location of the incision. C, We hypothesized that the reimplanted resting zone would regenerate new proliferative/hypertrophic columns. D–G, Surgical procedure to investigate spatial orientation. D, Diagram of growth plate. The dark vertical line represents the surgical incision. E, One of the resulting pieces (boxed) was rotated 90 degrees to bring the resting zone alongside the proliferative zone of the adjacent piece. F, The reassembled growth plate was then inserted back into the original bed. G, We hypothesized that the ectopic resting zone would induce a shift in orientation of the adjacent proliferative zone chondrocytes (a). During the surgery, two incisions were actually made in each growth plate, allowing two interfaces to be analyzed per growth plate.

long axis of the bone. The epiphyseal cartilage lies farther from the proliferative zone and thus closer to the bony epiphysis than the reserve cartilage. The epiphyseal cartilage contains round or elliptical chondrocytes usually scattered individually in the cartilage matrix.

The surgical incision was located in the reserve cartilage in 12 growth plates (Fig. 2B). In 5 of these distal ulnae, a complete growth plate regenerated, indicated by the presence of all zones of the growth plate, vascular invasion at the metaphyseal edge of the hypertrophic zone, and morphological signs of active osteogenesis in the primary spongiosa (Fig. 2C). In 5 other bones, proliferative columns were generated, but the resulting growth plate was poorly organized or lacked a hypertrophic zone. In the remaining 2 ulnae, no proliferative columns were observed.

The surgical incision was located in the epiphyseal cartilage in four growth plates (Fig. 2D). Proliferative columns were not generated in any of these four ulnae (Fig. 2E). However, in all four ulnae, the reimplanted resting zone showed changes; after 1–2 wk, the region located closest to the metaphysis developed flat chondrocytes grouped in pairs, triplets, and higher multiples, often aligned parallel to the long axis of the bone (Fig. 2F).

Growth plates were examined 1, 2, or 3 wk after surgery. We noticed a tendency for more complete regeneration in growth plates analyzed after a longer time period. A complete growth plate regenerated in 1 of 11 growth plates analyzed after 1 wk, in 7 of 12 growth plates analyzed after 2 wk, and in 4 of 6 growth plates analyzed after 3 wk.

Studies of chondrocyte orientation

Distal ulnar growth plates were surgically manipulated to bring resting zone cartilage into an ectopic position, alongside columns of proliferative zone chondrocytes. Growth plates were examined histologically, 6–10 d after surgery (Fig. 1, D–G). In 21 growth plates, the resting zone cartilage had been successfully placed alongside the proliferative zone. In 19 of these 21 growth plates, the proliferative chondrocytes near the ectopic resting zone had undergone an approximately 90-degree shift in orientation. These chondrocytes were now aligned in columns perpendicular to the adjacent surface of the ectopic resting zone and thus also perpendicular to the long axis of the bone (Fig. 3, A–D). In contrast, the proliferative chondrocytes distant (more than ~200 μm) from the ectopic resting zone were aligned in columns with the normal orientation parallel to the long axis of the bone (Fig. 3, A–C). Chondrocytes that were approximately equidistant from the ectopic and eutopic resting zones typically assumed an intermediate alignment approximately 45 degrees from the long axis of the bone (Fig. 3B).

The 90-degree shift in orientation also involved the shape of the individual chondrocytes. Proliferative zone chondrocytes distant from the ectopic resting zone were flattened in the normal orientation (thinnest in the dimension parallel to the long axis of the bone) (Fig. 3, B and C). In contrast, proliferative zone chondrocytes close to the ectopic resting zone were thinnest in a dimension perpendicular to the long axis of the bone (Fig. 3, C and D).

We also observed that growth plate chondrocytes adjacent

to the ectopic resting zone did not undergo hypertrophic differentiation (Fig. 3, A and B). Thus, the ectopic resting zone seemed to inhibit hypertrophic differentiation and ossification of the adjacent proliferative zone cartilage.

As a control, growth plates were divided and rotated 90 degrees to bring the hypertrophic zone ectopically alongside the proliferative zone. Unlike the ectopic resting zone, this ectopic hypertrophic zone did not induce a shift in the spatial organization of the proliferative columns ($n = 7$, Fig. 3E). As an additional control, growth plates were divided, but not rotated, and then reattached, thus placing the proliferative zone back in a normal position alongside other proliferative zone cartilage. No shift in orientation occurred ($n = 8$ pieces of cartilage).

In the pieces of growth plate that were rotated 90 degrees relative to the overall bone anatomy, chondrocyte columns were placed alongside bone tissue. These columns did not show evidence of reorientation after surgery (Fig. 3F). Thus, the bone itself did not seem to influence chondrocyte orientation.

Discussion

Studies to identify the location of the stem-like cell

Based on the cellular architecture and kinetics of the growth plate, it has been hypothesized that there exists a population of stem-like cells, analogous to the stem cells found in the epidermis and the intestinal epithelium, capable of generating the columnar clones of chondrocytes found in the proliferative and hypertrophic zones. However, the identity of the putative growth plate stem-like cell is not known (7). Some cell types are not candidates for the stem-like cell. For example, the hypertrophic chondrocyte is terminally differentiated and thus could not serve in this role (10). Similarly, most of the proliferative zone chondrocytes proliferate only briefly and then differentiate into hypertrophic chondrocytes (11).

However, the proliferative chondrocyte that lies uppermost in the chondrocyte column (*i.e.* closest to the resting zone) is a reasonable candidate for the stem-like cell (12). When this cell divides, the upper daughter may continue to serve as a stem-like cell, whereas the lower daughter may proliferate further to populate the entire proliferative/hypertrophic column. This hypothesis suggests that the resting chondrocytes do not participate directly in the growth process (12).

Alternatively, the topmost cell in the proliferative columns may have a limited ability to divide, and thus the columns may have a limited life span. Resting zone chondrocytes might instead serve as the stem-like cells (1). These cells divide only occasionally. When they divide, one of the daughter cells might sometimes transform into a proliferative zone chondrocyte and found a new column to replace exhausted proliferative cell columns (1). Although both stem cell hypotheses have been discussed in the literature (1, 7, 11–13), it is not known which, if either, is correct (7).

Our findings support the latter hypothesis, that resting zone chondrocytes serve as stem-like cells in the growth plate. We surgically removed the proliferative and hypertrophic zones, leaving only the resting zone. When the in-

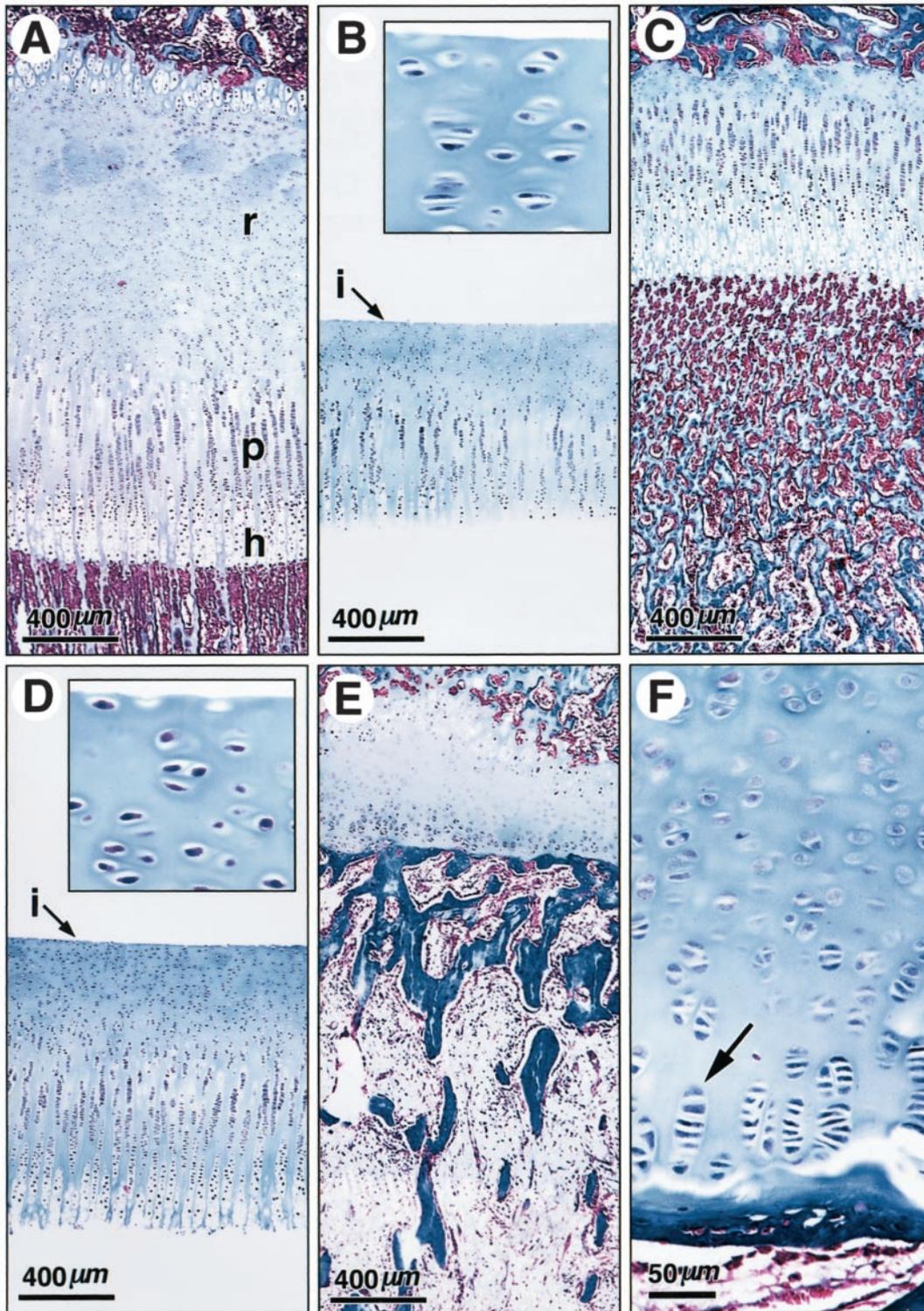


FIG. 2. Representative photomicrographs of rabbit distal ulnar growth plates, surgically manipulated to identify the stem-like chondrocytes. Growth plates (A, appearance before surgery) were excised and cut to separate the resting zone (r) from the proliferative (p) and hypertrophic (h) zones. To determine the precise location of the growth plate incision (i), the pieces containing proliferative and hypertrophic zones were examined histologically (B and D). In some cases, the incision was located low in the resting zone, in the reserve cartilage, which contains flattened chondrocytes often grouped in pairs and higher multiples aligned parallel to the long axis of the bone (B; *inset*, higher magnification of incision edge). In these distal ulnae, a complete growth plate was often generated (C, 2 wk after surgery). In other cases, the incision was located higher in the resting zone, in the epiphyseal cartilage, which contains round or elliptical chondrocytes usually scattered individually in the cartilage matrix (D; *inset*, higher magnification). In these distal ulnae, only partial regeneration occurred (E, 1 wk after surgery; F, higher power magnification, showing chondrocyte columns, *arrow*). Five-micron longitudinal sections were prepared from decalcified samples and stained with Masson trichrome.

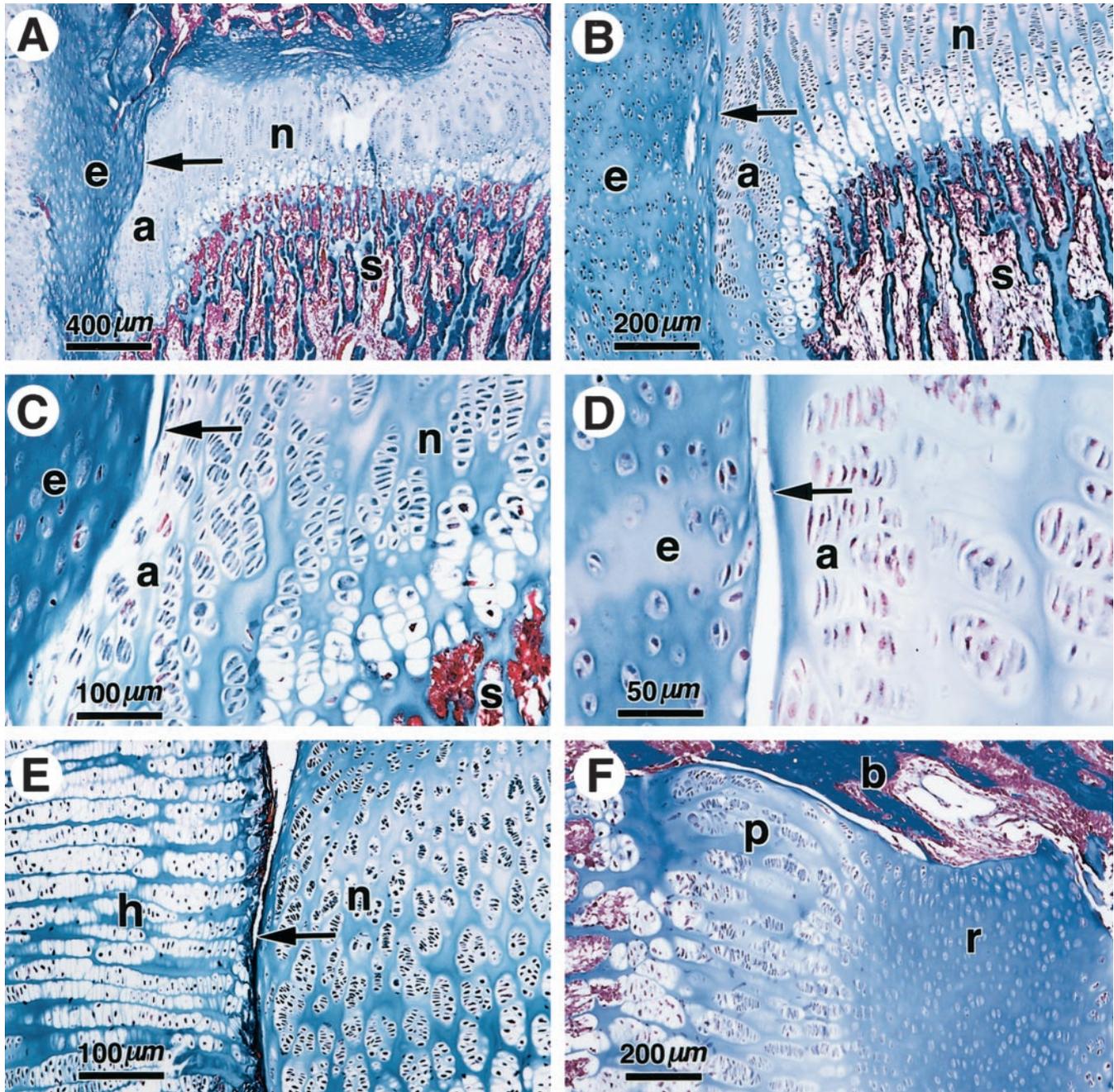


FIG. 3. Photomicrographs of rabbit distal ulnar growth plates, surgically manipulated to study spatial orientation of proliferative chondrocytes. Growth plates from 4-wk-old male New Zealand white rabbits were surgically manipulated to place either resting zone (A–D), or hypertrophic zone (E) ectopically alongside proliferative zone chondrocyte columns. Ten days after surgery, the distal ulnae were dissected. Five-micron longitudinal sections were prepared after decalcification and stained with Masson trichrome. A–D, Increasing magnification, showing the interface (arrows) between ectopic resting zone (e) and proliferative chondrocytes. Proliferative chondrocytes distant from the ectopic resting zone showed a normal orientation (n), whereas proliferative chondrocytes close to the ectopic resting zone showed an altered orientation (a). No shift in orientation was seen in columns adjacent to ectopic hypertrophic zone (E). In the rotated piece of growth plate cartilage (F), proliferative zone columns (p) were placed alongside bone tissue (b); 2 wk later, no shift in orientation had occurred.

cision was made within the reserve cartilage, a complete proliferative and hypertrophic zone often regenerated. Because the cells in this region are sometimes found in pairs and triplets resembling a small proliferative column, it has been speculated that these cells can generate new proliferative columns (1). Our data support that hypothesis. We speculate that the failure of some implants to produce a complete

growth plate may be attributable to technical problems with the surgery. For example, failure to approximate bone and cartilage tissues might lead to hypoxia and cell death in the autograft.

When the incision was made in the epiphyseal cartilage, regeneration of the growth plate was incomplete. However, the metaphyseal edge of the reimplanted epiphyseal carti-

lage did seem to transform into reserve cartilage. Because reserve cartilage chondrocytes can transform into proliferative chondrocytes, these findings, taken together, suggest that chondrocytes in the epiphyseal cartilage have the potential to generate proliferative columns and thus represent stem-like cells. We speculate that the epiphyseal cartilage transplants may not have produced a complete growth plate because of the experimental conditions employed. For example, the epiphyseal cells may require more time than reserve cells to produce proliferative/hypertrophic clones because they are earlier in the chondrocyte differentiation sequence. Alternatively, the failure to produce a complete growth plate might be attributable to the fact that the tissue mass and cell number of the epiphyseal transplants was less than that of the transplants containing reserve zone.

Although our data indicate that resting zone chondrocytes can differentiate into proliferative zone chondrocytes after growth plate manipulation, they do not prove that this process occurs under physiological conditions and thus do not completely exclude the possibility that the uppermost proliferative zone chondrocytes represent the stem-like cells.

In the mammalian embryo, the long bones form initially out of cartilage (12). The epiphyseal cartilage found in postnatal long bones represents a remnant of the embryonic cartilage that has not yet been replaced by bone tissue (1). Our data suggest that this cartilage retains its embryonic potential to generate an organized growth plate.

A stem cell is defined by three properties: 1) it must not be terminally differentiated; 2) it must be able to divide without limit; and 3) when it divides, each daughter can either remain a stem cell, or it can embark on a course leading irreversibly to terminal differentiation (14). Although the resting zone cells may fulfill the first and third of these properties, they probably do not completely satisfy the second property; the proliferation rate in the growth plate approaches zero as the animal ages (15). To reflect this issue, we suggest the term: stem-like cells.

Studies of chondrocyte orientation

In the experiments designed to identify the stem-like cell, the reimplanted resting zone generated new clones of proliferative and hypertrophic cells. These clones assumed an orientation parallel to the long axis of the bone, thus recreating the normal spatial organization of the growth plate. The mechanism by which growth plate chondrocytes recognize and line up along this axis is unknown. We hypothesized that resting chondrocytes produce a growth plate-orienting factor (GPOF), a morphogen that diffuses into the proliferative zone, setting up a concentration gradient that guides the orientation of proliferative columns.

To test this hypothesis, we surgically manipulated rabbit distal ulnar growth plates to place resting zone cartilage ectopically alongside the proliferative columns. Ectopic resting zone cartilage induced a 90-degree shift in the orientation of nearby proliferative zone chondrocytes, involving both the shape of the individual cells and the alignment of the cells into columns. The effect was transmitted across the surgical incision, suggesting that the effect was attributable to a soluble factor. This observation also suggests that the reoriented

cells were derived from the preexisting proliferative zone and not the ectopic resting zone.

Thus, the observations are consistent with our hypothesis that resting zone cartilage produces a GPOF that guides the spatial orientation of proliferative zone chondrocytes. The change in orientation was not seen in proliferative zone chondrocytes more distant from the ectopic resting zone, indicating that the putative GPOF acts only over a short distance. Chondrocytes that were located approximately equidistant from the ectopic and eutopic resting zones showed an intermediate orientation that may reflect the net chemical gradient of GPOF produced from the two pieces of resting zone. Growth plate organization did not seem to be influenced by the orientation of the cartilage relative to the surrounding bone, suggesting that bone itself does not produce a GPOF. When the hypertrophic zone was placed ectopically alongside the chondrocyte columns, no change in orientation occurred, suggesting that the hypertrophic zone does not produce the GPOF. The shift in orientation could have been caused by realignment of existing proliferative zone chondrocytes or by alignment of new daughter cells produced by mitoses after surgery. Our data do not distinguish between these possibilities. Alternatively, the ectopic resting zone might act not by producing a morphogen but rather by binding a morphogen produced elsewhere in the growth plate, thus altering a local concentration gradient. Our hypothesis also provides a possible explanation for the spatial organization that develops spontaneously when growth plate chondrocytes are cultured in cell pellets (16, 17).

The identity of the putative GPOF is not known. PTH-related protein, a potent regulator of chondrocyte differentiation, is expressed in the resting zone (18). However, in the postnatal growth plate, it is also expressed in the early hypertrophic zone (19, 20). Furthermore, PTHrP-deficient mice rescued with a transgene encoding a constitutively active PTH/PTHrP receptor should lack a PTHrP gradient, yet show reasonably normal growth plate structure (21). Deficiency of Indian hedgehog (IHH), another important regulator of chondrocyte differentiation, does cause disorganization of the growth cartilage, but IHH is preferentially expressed in the early hypertrophic zone, not the resting zone (22).

Control of hypertrophic differentiation

Proliferative chondrocytes adjacent to the ectopic resting zone failed to undergo hypertrophic differentiation. This observation raises the possibility that the resting zone might produce a factor that, directly or indirectly, inhibits terminal differentiation of proliferative chondrocytes into hypertrophic chondrocytes. However, the band of proliferative chondrocytes adjacent to the ectopic resting zone was narrower than the normal width of a proliferative zone. This discrepancy could be attributable to subnormal thickness or function of the ectopic resting zone. Alternatively, the band of proliferative zone chondrocytes adjacent to the ectopic resting zone could be attributable not to a hypertrophy-inhibiting factor but rather might be secondary to the shift in spatial orientation of the columns.

The switch to the hypertrophic phenotype may be regu-

lated by several paracrine factors, including PTH-related protein (23), IHH (24), bone morphogenetic proteins (25, 26), fibroblast growth factors (27, 28), and retinoids (29, 30). The putative signal(s) arising from resting cartilage that inhibits terminal differentiation could be one of these factors or could regulate expression of these factors. This growth plate hypertrophy-inhibiting factor could be identical with the GPOF.

Conclusions

Our findings suggest that resting zone cartilage makes important contributions to endochondral bone formation at the growth plate: 1) the resting zone contains stem-like cells that give rise to clones of proliferative chondrocytes; 2) the resting zone produces a GPOF, a morphogen that directs the alignment of the proliferative clones into columns parallel to the long axis of the bone; and 3) the resting zone may also produce a morphogen that inhibits terminal differentiation of nearby proliferative zone chondrocytes and thus may be partially responsible for the organization of the growth plate into distinct zones of proliferation and hypertrophy.

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