# Histological Studies on the Developing Femur of the Male Mouse

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**Résumé**

The present study was undertaken in order to clarify histological age changes in the femur of the mouse with special reference to the appearance of ossification centers and the growth of the distal metaphysial region. Observation was performed using 140 mice of the bc strain ranging in age from the 16th day in fetal life to 336 days in postnatal life. Sections were prepared longitudinally as exactly as possible, being stained with hematoxylin and eosin.

The presence of the thin perichondral bone plate and the ossification center in the diaphysis was already noticed as early as the 16th day in fetal age. On the other hand, the formation of the ossification center in the distal epiphysis was observed to begin on 5 days after birth. During the course of development of the epiphysial ossificafion, the epiphysial plate was found to consist of three layers, a) resting cartilage cells, b) proliferating cartilage cells and c) hypertrophic cartilage cells. On 15 and 20 days of postnatal age, the epiphysial plate showed a maximum in width and decreased afterward. Although the resting cartilage cells disappeared on the stage of 60 days and the other cartilage cellular elements in the plate decreased in number progressively from this stage onward, even on 336 days, a time when was the final stage of the observation, a few cartilage cells were still seen remaining and the perforation of the epiphysial plate by the medullary cavity was still not found to occur.

Alizarin red S stain, roentogenologic and histological methods have been extensively applied to the study of bone, and recently WIRTSCHAFTER ('60) published an atlas entitled "Genesis of mouse skeleton" in which the former two methods were employed. Studies on normal age changes of bones by the use of histological method have already been made in mice (CHEN '52; SILBERBERG and SILBERBERG '41; ZORZOLI '48) as well as in rats. These studies, however, have been based on the observation of specimens obtained mainly from the tibia and sternum in mice, and there are some differences in the rate of skeletal growth in different bones. In contrast to the extensive literature on the femur of the rat which was reviewed by PRATT ('57; '59) little attention has been paid to that of the mouse especially in relation to its histological age changes.

Now, the present study was undertaken in order to clarify histological age chan-

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ges in the femur with attention to the appearance of ossification centers and the growth of the distal metaphysial region.

## Materials and Methods

One hundred and forty mice of the bc strain (KONDO, HIMENO, IKOMA, and KATSURAGI '53) were used in this study. Observation was performed at daily intervals from the 16th day to term in fetal age, and at intervals of days from birth to 336 days in postnatal age. At autopsy, the left femur was removed together with surrounding tissues and fixed in 10% formalin or BOUIN's fluid. The fixed materials were decalcified in 5-10% solution of trichloric acetic acid and embedded in paraffin. Sections were cut at 5$\mu$ longitudinally as exactly as possible and were stained with hematoxylin and eosin. Concerning the epiphysis, all the descriptions presented are based on the distal end.

## Observations

16th day-fetus (Fig. 1). The femur on this stage was recognized as a simple cartilagenous model. Vacuolated alveolar cells which had degenerated nuclei were located in the middle of the cartilagenous shaft consisting of transversely spindle-shaped cells arranged in an almost orderly row. However, under the thin inner layer of the perichondrium a thin bone plate was already formed. Beneath the bone plate, lateral parts of the middle portion of the cartilagenous model were disintegrated. Mesenchymal cells originated from the inner layer of the perichondrium penetrated through the thin bone plate into the disintegrated parts. Epiphysial cartilage abundant in round cells was situated on either extremity.

17th day-fetus. The cartilagenous hypertrophied and vacuolated cells in the diaphysis were fragmented. New vacuolated cells were added by transfiguration of the spindle-shaped cells. The part consisting of fragmented cells in its center and vacuolated cells both in its distal and proximal regions expanded further toward both ends of the model, including vacuolizing changes of young cells adjacent to it. The perichondral bone collar presented itself on the periphery of the shaft, and through it vascular mesenchyma entered into the primary medullary cavity, i. e. the core consisting of fragmented cells, where intracartilagenous ossifiation was seen to be beginning.

18th day-fetus. The cells of the perichodrium adjacent to the collar increased in size and osteoblasts were lining along the surface of the collar. Through the spaces formed in the perichondral bone collar, the mesenchymal tissues of the perichondrium accompanying blood vessels penetrated to the developing medullary cavity where thin-walled blood vessels were noticed. The primary bone marrow consisting of mesenchymal tissue and blood vessels was fairly spaciously situated in the middle portion of the diaphysis, and was dotted with coarse bone trabeculae, showing the first sign of hematopoiesis. New bone trabeculae with centrally remained cartilage matrix encircled by osteoblasts were found to be formed in continuity to the part of vacuolated cells. The epiphysial cartilage was depressed to the distal end owing to the remarkably developing medullary cavity, and consequently was seen to be relatively narrower in length than that of 17th day-stage.

19th day-fetus (Fig. 2). Diaphysial ossfication developed further. The zones of epiphysial cartilage, of proliferating cartilage cells (cartilage column), of hypertrop-

hic cartilage cells ( provisional calcification ) and of bone trabeculae became to be well defined. Epiphysial cartilage cells were somewhat round or oval in shape and some of them showed mitotic figures in the epiphysial periphery. Proliferating cartilage cells were spindle-shaped and showed a change to hypertrophic and alveolar cells as the next zone was approached. Thus formed hypertrophic cartilage cells were unstainable, but their outlines alone were distinguished. Fine intercellular cartilage matrixes which were remained afer the fragmentation of cells were extending the branches to the bone marrow, accompanying osteoblasts on their peripheries. Many blood vessels invaded into the part of fragmented cells. Bone trabeculae were coarse in the middle and fine in the periphery of the diaphysis.

1 day of age (Fig. 3). The peripheral collar which had already been formed by the perichondral ossification on the lateral side together with the endochondral ossification on the medial side had many innerspaces including mesenchymal tissue with blood vessels. In the diaphysis, bone trabeculae disappeared in the middle part and shifted to the proximal and distal ends. Since the bone marrow was already occupied by abundant hematopoietic foci, it could be named the secondary or hematopoietic bone marrow.

5 days of age (Fig. 4, 12). In the periphery of the diaphysis the bone collar was constituted largely by thin, compact and continuous bone substance. The broad bone marrow in the diaphysis included very violent hematopoietic foci and few bone trabeculae except near the epiphysis. In the epiphysial plate which existed between the diaphysis and the epiphysis, zone of hypertrophic cartilage cells was wide. The distal end of the femur was still composed of hyaline cartilage. However, at the center of this end, the cells were undergone to the beginning of degenerative change. Somewhat larger and vacuolated cells were separated by increased matrix, and their nuclei swelled and lost a majority of their chromatins. These changes show clearly the first indication of appearance of the secondary (epiphysial) center of ossification.

10 days of age ( Fig. 5 ). The secondary center of ossification was observed more evidently in the center of the distal end. Premature bone trabeculae lined with osteoblasts were located in the mesenchymal tissue in which hematopoietic foci were scattered. Mesenchymal tissue on the articular side seemed to penetrate into the center and change therein to the osteogenic tissue. Around this ossification center, vacuolated and hypertrophied cartilage cells were arranged in all directions. In the epiphysial plate, the zone of proliferating cartilage cells was as wide as that of the preceding stage.

15 days of age (Fig. 6). The epiphysial ossification center of the distal end was well established. Owing to the expansion of the ossification center, hyaline cartilage cell zone surrounding the center has markedly decreased in width. And at the inner margin of this zone, progressive replacement of cartilagenous matrix among hypertrophied and vacuolated cells by bony one with the aid of osteoblasts was observed. The epiphysial plate showed a clear-cut band-like appearance. The bone trabeculae located on the lateral side in the metaphysis (which implies the intermediate part between diaphysis and epiphysis, including epiphysial plate and bone trabeculae directing toward the diaphysis) were connected with the perichondral bone collar, forming cancellous bone in this stage and also in other stages. Moreover in the trabeculae, bone-formation by uninuclear osteoblasts seemed to occur simultaneously with bone destruction by polynuclear osteoclasts.

20 days of age. The epiphysial plate showed a maximum in width on 15 and

20 days. Bone trabeculae were formed more coarsely in the epiphysial end than in the metaphysis, and were provided with many rounded and spindle-shaped osteoblasts on their surfaces. Hematopoietic activity in the epiphysis was vigorous in bone marrow spaces located between the coarse bone trabeculae. The bone trabeculae in the metaphysis were still delicate, but were defined more clearly than those in the previous stages. In the spaces of the cancellous bone of the metaphysis abut on the epiphysial plate, there were many osteoblasts and a few osteoclasts.

30 days of age (Fig. 7, 13). The extent of epiphysial ossification center expanded to the periphery. The hyaline cartilage encircling the epiphysial ossification was thin on the side of epiphysial plate and slightly thick on the side of articular surface. The distinguishable zones of proliferating cartilage cells and of hypertrophic cartilage cells were slightly narrower than those of the previous stage.

60 days of age (Fig. 8). The bone trabeculae in the epiphysis became in their feature to be coarse, thick and sturdy. The bone trabeculae in the metaphysis also manifested a substantial appearance as those in the epiphysis did.

90 days of age (Fig. 9, 14). The bony network of the epiphysis presented an appearance similar to that seen on 60 days of age. In the metaphysis, zone of proliferating cartilage cells and zone of hypertrophic cartilage cells —— especially the latter zone —— were considerably narrower, but were still distinctly distinguishable. The matrix of the zone of proliferating cartilage cells has markedly increased in amount and formed separately a layer between that zone and the epiphysial bone. The bone trabeculae in the metaphysis exhibited thick bands and sometimes were seen to be separated into islands in sections.

180 days of age (Fig. 10, 15). The metaphysis presented an irregularly waved line. Proliferating cartilage cells and hypertrophic cartilage cells arranged in each row were a few in number. On the epiphysial side of the metaphysis a coarse thick bony plate was contacted with the zone of proliferating cells. While, on the diaphysial side coarse thick bone trabeculae forming network were present extensively in direct contact with the zone of hypertrophic cartilage cells.

270 days of age. There were a few remnants of bone trabeculae both in the epiphysis and the diaphysis, only in the latter they were found restrictedly near the epiphysial plate. The bony plate which included medullary cavities was contacted with the diaphysial surface of hypertrophic cell zone in the metaphysis. From this bony plate a few bone trabeculae extended toward the diaphysis.

336 days of age (Fig. 11, 16). In the epiphysial plate, a few proliferating and hypertrophic cartilage cells were still seen to be remained. The perforation by medullary cavity in the epiphysial plate was not yet found. The epiphysial and diaphysial bone marrows in this stage were hematopoietic and not fatty.

## Comments

SILBERBERG and SILBERBERG ('41) stated that there are great differences in the rate of skeletal aging in different strains of mice. The material which constitutes the basis of the present study was obtained from our stock colony of the bc strain albinos.

ZORZORI ('48) described that the lower limb skeleton was entirely cartilagenous and there were areas in which the process leading toward bone formation began on 15 days of gestation in Swiss strain mice. In the present study, the femur of the

16th day-fetuses, which were the earliest materials used, already showed a thin bone plate to be formed under the thin inner layer of the perichondrium. In the center of its shaft, the cartilage cells enlarged and degenerated, and the matrix between these cells seemed to be calcified. A vascular bud with mesenchymal cells derived from the inner layer of the perichondrium penetrated through the thin bone plate into the center of the shaft. It is soundly established that this vascular bud is an osteogenic tissue. From the mesenchymal cells of the osteogenic tissue are derived the chondroclasts, osteoblasts and osteoclasts, the first of which break down and remove the calcified matrix, the second secret the bony substance around the strands of remaining calcified cartilage, and finally the third absorb bone where subsequent occurrence of resorption and rearrangement of the bone is to be observed. The problems on such a pattern of calcification and ossification were very comprehensively investigated by BLOOM and BLOOM ('40) and MCLEAN and BLOOM ('40) using fetal and young bones.

The secondary (epiphysial) centers of ossification were established after birth, and the first indication of the ossification center in the distal epiphysis was found on 5 days of postnatal age and the center appeared in a fashion similar to that of the primary diaphysial one.

WIRTSCHAFTER ('60) determined the time of the appearance of ossification centers in C3H mice by the use of alizarin red S stain method. According to his descriptions various parts of the femur exhibit the first sign of ossification on such days after conception as described in the following; diaphysis on 15 days, distal epiphysis on 25 days, greater trochanter on 30 days, lesser trochanter on 30 days and head on 33 days. These data are almost much the same to ours as far as diaphysis and distal epiphysis are concerned.

By 15 days of age the resting cartiage cells continued to the distal epiphysial cartilage cells without any clear demarcation between them, while from 15 days onward, the epiphysial plate showed a clear-cut band-like appearance because of the establishment of the epiphysial ossifiation center of the distal end.

During the course of development of the bone, three layers of cartilage cells could be distinguished as the constituents in the eipiphysial plate; a) resting cartilage cells (non-oriented), b) proliferating cartilage cells arranged in columns parallel to the long axis of the bone, and c) hypertrophic cartilage cells (similar to those described for the fetal ossification center).

On 15 and 20 days, the epiphysial plate was wider than that in any other later stages (Fig. 6). On 30 days the zone of resting cartilage cells decreased in width owing to the expansion of the epiphysial ossification center (Fig. 7, 13). On 60 days, the epiphysial plate decreased totally in width along with the decrease in width of the zones of proliferating and hypertrophic cartilage cells in it, and presented a gently waved line (Fig. 8). The zone of resting cartilage cells changed into the bone tissue in that the cartilage cellular elements were scarcely found. The bone trabeculae became sturdy and broadened. These changes were evidently recognizable to advance with the age of animals up to the final stage observed. These findings are fairly well paralleled with those of SILBERBERG and SILBERBERG ('40) and of ZORZOLI ('48) in which for some other strains of mice, the decrease in width of the epiphysial plate occurred in the tibia by two months of age.

In the stages of 180, 270 and 336 days, the columnar arrangement of the cartilage cells became more and more irregular. In the final material used (336 days of age) the perforation of the epiphysial plate was still not noticed. SLIBERBERG and

SILBERBERG ('41) showed the slight perforation of the epiphysial plate of the tibia which indicated the very beginning of the union of epiphysis and diaphysis in some instances at the age of a year. It has been said that at the termination of growth in any particular bone the epiphysial plate disappears and the epiphysis unites with diaphysis, but the present study was not designed to determine the growth-termination of the femur and showed no complete perforation of the epiphysial plate of the femur even at the age of about a year which was the final stage observed.

## Summary

1. Developing femurs of bc strain mice during fetal and postnatal stages were histologically examined with special reference to the appearance of ossification centers and the growth of the distal metaphysial region.

2. On the 16th day of fetal stage, the thin bone plate was already formed under the thin inner layer of the perichondrium. Enlargement and degeneration of cartilage cells located in the center of the shaft were accompanied by probable calcification of the matrix among the cells, indicating the appearance of the primary endochondral ossification center.

3. The first indication of the appearance of the ossification center in the distal epiphysis was noted on 5 days of postnatal stage in a fashion similar to that described on the primary center.

4. From 15 days onward, the epiphysial plate consisting of three layers —— resting cartilage cells, proliferating cartilage cells and hypertrophic cartilage cells —— showed a clear-cut band-like appearance because of the establishment of the epiphysial ossification center of the distal end. On 15 and 20 days, the epiphysial plate was wider than that in any other later stages.

5. The resting cartilage cells alone disappeared on the stage of 60 days, and thereafter the other cellular elements in the epiphysial plate remained to progressively decrease in number and to become more and more irregular in their columnar arrangement until the final stage of observation (336 days of age), a time when the perforation of the plate still failed to occur.

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## Explanation of Figures

Photomicrographs of sections of the left femurs of bc strain mice stained with hematoxylin and eosin. Figs. 1-11 were in lower magnification by the use of a 10 x ocular and an 8 mm objective, and Figs. 12-16 in higher magnification by the use of a 10 x ocular and a 4 mm objective. All figures are oriented with the distal epiphysis to the top and the diaphysis to the bottom.

1. 16th day-fetus. The femur is recognized as a simple cartilagenous model in the middle of which hypertrophic cartilage cells are present. Both the thin perichondral bone plate and the primary endochondral ossification center are already formed.

2. 19th day-fetus. The diaphysial ossification center expands to both epiphysial ends. Several distinctions of zone and part can be made in this figure; namely from the top downward, epiphysial cartilage cells, proliferating cartilage cells, hypertrophic cartilage cells, medullary cavity occupied by bone trabeculae and bone marrow.

3. 1 day of postnatal age. The histological structure is shown to be almost similar to that of Fig. 2, but the bone marrow is now occupied by abundant hematopoietic foci.

4. 5 days of age. The histological pattern is roughly almost identical with that of the preceding two figures. The zone of hypertrophic cells is rather widened.

5. 10 days of age. The ossification center in the epiphysis is seen distinctly to the top of this figure.

6. 15 days of age. The epiphysial ossification center occupied by bone trabeculae and by hematopoietic foci expands to the periphery. The epiphysial plate is seen to be very widened. The proliferating cells are arranged in a column.

7. 30 days of age. The resting (hyaline) cartilage cells are seen as a thin layer. The epiphysial plate is distinguishable, but slight narrower than those of the preceding figure.

8. 60 days of age. The zone of the resting cartilage cells is seen changing to the epiphysial bone. The bone trabeculae in the metaphysis manifest a substantial appearance as those in the epiphysis.

9. 90 days of age. The epiphysial plate is further narrowed. Cells decrease in number, and cartilagenous matrix increases in amount.

10. 180 days of age. The columnar arrangement of proliferating cells is still recognizable, only it becomes irregular. The epiphysial plate presents totally an irregularly waved line.

11. 336 days of age. In the epiphysial plate, cartilage cells are seen to be remained in only a few number. The calcification of matrixes between the cells has been advanced. The perforation does not yet occur.

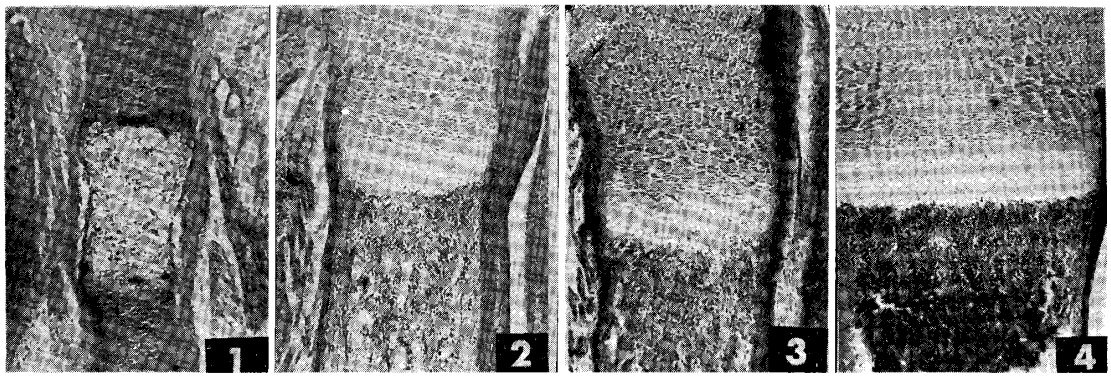
12. 5 days of age. As in Fig. 4.

13. 30 days of age. As in Fig. 7.

14. 90 days of age. As in Fig. 9.

15. 180 days of age. As in Fig. 10.

16. 336 days of age. As in Fig. 11.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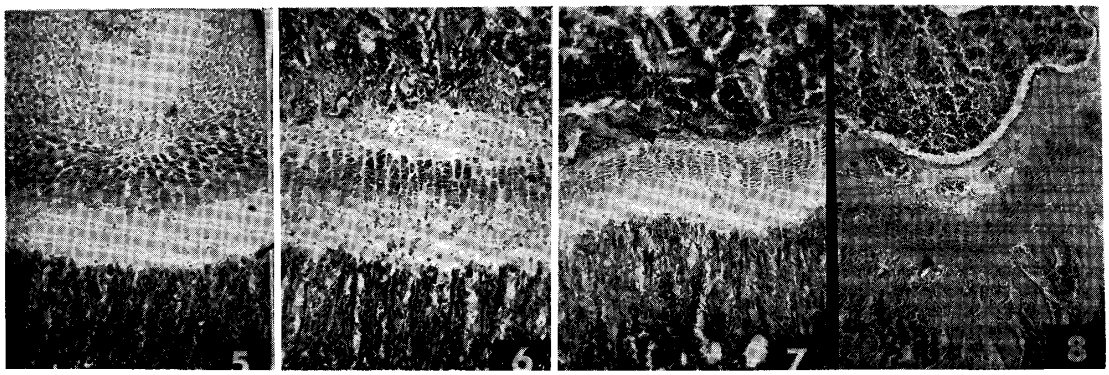
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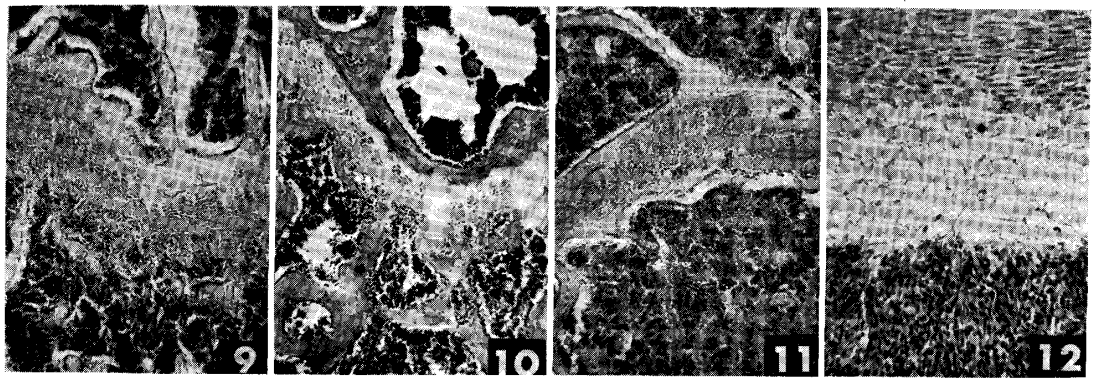
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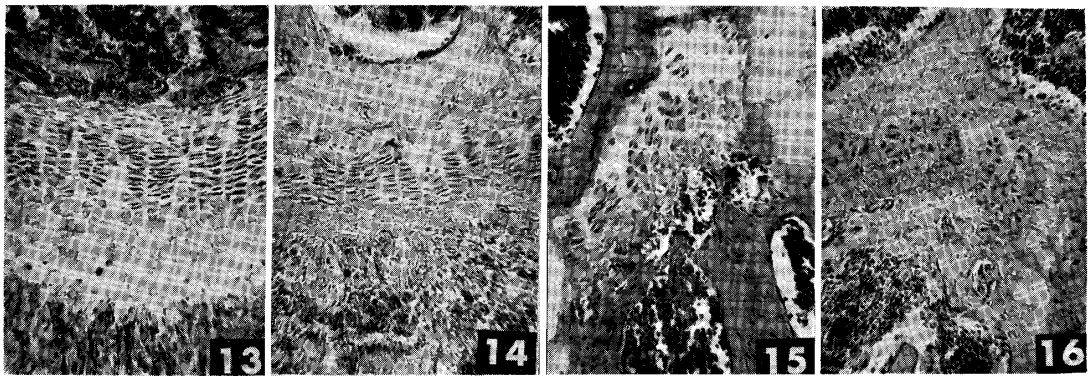
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