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THE DEVELOPMENT IN VIVO AND IN VITRO OF THE AVIAN PATELLA.

By

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With 16 figures in the text.

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Introduction.

The part played by extrinsic and intrinsic factors in the development and maintenance of skeletal form is still undetermined and while most authors agree that extrinsic factors such as movement, muscular activity, tension in the limb are essential to the maintenance of skeletal form, there is considerable difference of opinion as to the relative importance of these factors in early development. MURRAY and HUXLEY (1925) and MURRAY (1926) showed, by means of chorio-allantoic grafts, that the limb-bud of the chick embryo from the end of the third day onwards is a self-differentiating mosaic and that the form of the cartilaginous elements of the developing limb, including that of the articular surfaces, self-differentiates and does not depend on the presence of contiguous elements, the nervous system, functional activity, muscular pull, etc. MURRAY (1926) described the results of a large number of experiments in which the graft consisted of limb-buds of avian embryos or fragments of limb-buds, from embryos 3 or more days old onwards. He found that the grafts increased in size and that cartilaginous structures were formed which were very like the normal. He showed conclusively that in the limb-bud isolated from a 4-day embryo, all the segments of the limb skeleton can differentiate independently. FELL and ROBISON (1929) described the development *in vitro* of femora isolated from $5^1/_2$-day and 6-day embryos and found that the femora increased in size and that the articular structures continued to develop in the absence of adjacent parts. MURRAY and SELBY (1930) grafted femora isolated from 6-day embryos on to the chorio-allantois of other embryos. Their results were in general similar to those of FELL and ROBISON. In this paper, the authors review in considerable detail the literature of the subject and

discuss the various factors concerned in the development of the cartilaginous skeleton, with particular reference to the femur. They conclude that while the gross form of the shaft and articular surfaces is brought into existence mainly by the action of forces intrinsic in the femur itself, which demand for their action a certain approach to normality of the surrounding conditions, an important part is played by extrinsic factors of a mechanical nature in modelling the finer details of skeletal form. On the other hand, they state that the form of the bony skeleton in the case of replacing bones depends entirely on conditions extrinsic to the bone; it is primarily dependent on the form of the cartilaginous framework and is very liable to alteration if the mechanical conditions be changed.

As already stated, all observers do not agree that the factors responsible for the primary form of the cartilaginous skeleton are intrinsic in the limb-bud itself. CAREY (1922) considers that the primary form of the cartilaginous skeleton depends entirely on mechanical conditions brought about by differential growth rates of contiguous parts, the passive pull of soft parts retarded in growth, active muscular pull and the weight of the free part of the limb, in support of which view he adduces evidence derived from a study of the normal development. In a later paper, CAREY, ZEIT and MCGRATH (1927) describe the regeneration of the patella. These workers removed the patellas from puppies 6 to 8 weeks old and found that regeneration took place if movement of the knee-joint was permitted, the new patella passing through cartilaginous and bony stages. When arthrodesis of the knee-joint was performed, no regeneration of the patella occurred. They adduce these experiments in support of the mechanical theory of the origin of embryonic and adult bone substance. They conclude that bone and presumably cartilage are formed by modification of the polyvalent mesenchyme cell, both the modification itself and the shape of the resulting element being determined by the mechanical conditions existing in the part.

As MURRAY and SELBY (1930) have pointed out, however, there are at least two problems connected with skeletal development, (1) the development of the skeletal tissue, and (2) the development of the skeletal form. CAREY and his co-workers appear to consider that the same forces act in both cases. In pathological conditions, cartilage and bone are certainly found to arise from mesenchyme which does not normally produce either cartilage or bone. LEVI (1930) has produced cartilage experimentally in the region of the phalanges in guinea-pigs and fowls by intermittent pressure. Recently, FISCHER (1931) has found *in vitro* that fibroblasts isolated from the heart produce cartilage when in contact with skeletal cartilage which has been freed entirely from perichondrium, the skeletal cartilage taking no part in the production of the newly-formed cartilage. It does not necessarily follow, however,

that the factors which operate in regeneration, in the formation of ectopic bone, or in experimental conditions such as described by LEVI or FISCHER act also in normal development. Although there is no direct experimental evidence pointing to the existence, in the embryo, of cells specifically and intrinsically determined for chondrogenesis, there is a certain amount of suggestive indirect evidence such as the experiments of FELL (1928) who found that cartilage differentiated in cultures of 3-day avian limb-bud, and those reported by MURRAY (1926) in which cartilage of all stages of histological development was found in grafts of 2-day limb-bud regions. Concerning the development of skeletal form, it is apparent, as already stated, from the work of MURRAY, that the primary cartilaginous form of an element such as the femur „self-differentiates" when it is in conditions which are not too remote from the normal.

In the experiments recorded by MURRAY (1926) the appearance in grafts of structures resembling the patella is described, but later MURRAY and SELBY (1930) state that the piece of cartilage which most resembled the patella was probably a detached piece of fibula. The patella does not normally begin to develop until the end of the 10th or the beginning of the 11th day, at which time movement is active in the embryo and the muscles are well-developed and arranged in definite groups, that is to say, at a stage when the conditions postulated by CAREY are thoroughly established. Accordingly, it was decided to ascertain whether or not presumptive patellar mesenchyme would differentiate *in vitro* when isolated from the rest of the limb skeleton and to determine as far as possible the factors responsible for its final form.

Histogenesis of the avian patella.

Since no adequate data are available, the normal development of the avian patella was investigated. It is convenient to describe the degree of development of the bones which enter into the formation of the knee-joint. At the beginning of the 9th day of incubation the femur, tibia and fibula are well-developed and exhibit the main characteristics of form which they possess in later life. The shafts of both femur and tibia are ossified, the ossified area occupying at least one third of the total length of the bones. The fibula also shows advancing ossification; it has a well-developed head but ends distally in a fine, tapering bony point. The muscular tissue consists of long thin fibres, arranged in definite bundles, corresponding to their future distribution in the limb. Arising from the pelvic region and passing down on to the anterior surface of the femur is a well-defined mass of muscle which represents the great extensor group of the thigh. This ends at the upper limits of the condyles of the femur in a mass of mesenchyme which is continuous with the mesenchyme on the anterior surface of the tibia and with that lying between the ends of the bones where the knee-joint subsequently

develops. Staining with thionin blue at this stage (Fig. 1), however, shows no trace of chondrogenesis. Round the epiphyseal ends of the bones, the mesenchyme has become condensed to form an actively chondrogenic perichondrium. When the ends of the bones are pulled apart, the mesenchyme often tends to separate from the perichondrium and in fixed and stained preparations, an artificial line of cleavage is often seen between them. Histologically, this mesenchyme consists of fibroblasts separated by a very small amount of intercellular material.

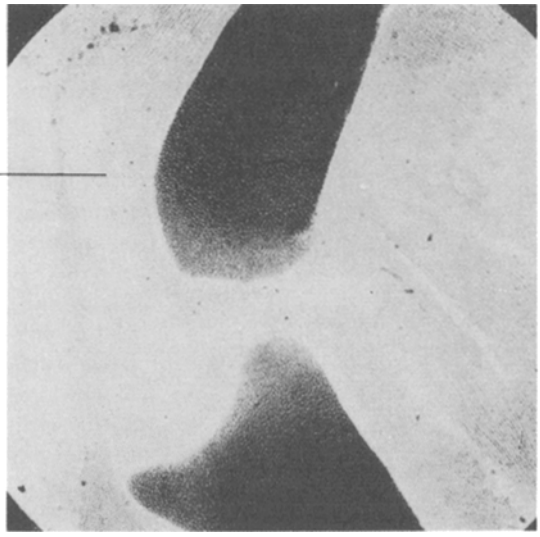

Fig. 1. Section through centre of knee-joint region of limb of other side of same embryo as that from which explant in fig. 3 was derived. The section was stained with thionin blue which demonstrates specifically areas of early chondrogenesis. At the site (*S*) at which the patella develops, no trace of cartilage is seen. ZENKER. × 36.

On the outer side of the limb, however, the tissue is less cellular and contains fine collagen fibrils arranged parallel to the long axis of the limb.

Towards the end of the 10th and the beginning of the 11th day, the epiphyseal perichondrium of both femur and tibia has become much thicker and is separated from the surrounding mesenchyme and the developing intra-articular ligaments by well-defined spaces which represent the cavity of the knee-joint. An area of precartilage in which mitoses are numerous appears in the mesenchyme just below the termination of the extensor muscle. By the end of the 11th day, the mesenchyme surrounding this area of chondrification has become condensed to form a rudimentary perichondrium. By the end of the 12th day, the cartilage cells are separated by narrow but deeply staining partitions of matrix and the chondroblasts are still multiplying by mitotic division. The whole mass is surrounded by a definitive perichondrium. (See Fig. 2.)

Between the 11th and 12th day (see Fig. 11) the developing patella is roughly triangular in shape when looked at from the anterior or posterior aspects. The two sides are unequal in length, the outer or lateral side being shorter than the medial. They meet in a rounded apex which becomes slightly flattened out as development proceeds at the expense of the longer side. The rudiment increases rapidly in size in all directions partly by perichondrial activity and also by the formation of cartilage matrix. In the early stages of development, the anterior and posterior surfaces of the patellar rudiment are convex but during the 16th day of incubation 2 concavities develop on the posterior surface for articulation with the condyles of the femur. These are separated by a ridge which terminates below at the somewhat flattened apex of the patella. The articular surfaces are well-marked at the and of the 17th day and at this stage capillary blood-vessels and fibroblasts begin to penetrate the perichondrium and invade the cartilage. Simultaneously the development of the knee-joint and of the muscular system continues. A band of tough fibrous tissue develops between the lower part of the anterior surface of the patella and the upper anterior surface of the tibia and a mass of fatty tissue intervenes between this and the synovial membrane of the knee-joint. On the outer side, another ligament appears which stretches to the head of the fibula.

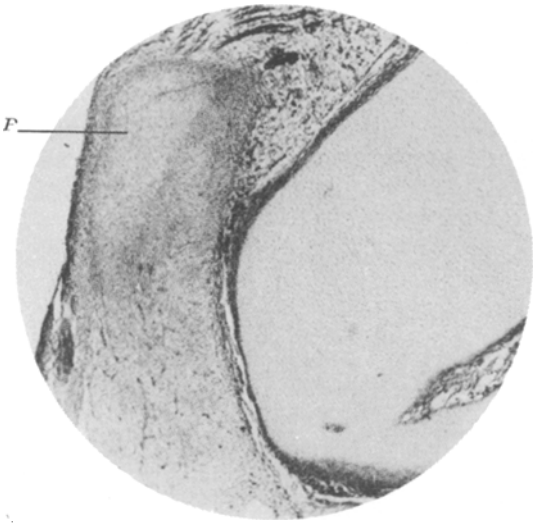

Fig. 2. Section through knee-joint region from 13-day avian embryo, showing formation of cartilaginous patella. *P* patella. BOUIN: MALLORY'S triple stain. × 40.

Histologically, at this stage the chondroblasts of the patellar cartilage are of the small-celled type characteristic of hyaline cartilage and separated by a very abundant matrix. The perichondrium remains chondrogenic but the connective tissue cells which accompany the invading blood vessels also form cartilage. As development proceeds the patella increases in size. The formation of deeply staining cartilage matrix continues but the chondroblasts retain their small-celled character. At the 11th week after hatching, ossification begins. The perivascular fibroblasts cease giving rise to cartilage cells and in the centre of the

patella the cartilage cells around the blood vessels become hypertrophic and vacuolated. Calcium salts demonstrable with Alizarin and silver nitrate are deposited in the matrix in these hypertrophic areas. The fibroblasts surrounding the blood vessels now assume the character of osteoblasts and lay down bone. Erosion of the cartilage takes place rapidly and soon the whole patella with the exception of the articular surfaces becomes replaced by trabecular bone in the interstices of which haematopoiesis is active. This calcification of the hypertrophic cartilage is interesting in view of the fact that in normal osteogenesis of the diaphysis of the long bones of the fowl (femur, tibia, fibula, humerus) calcification of the hypertrophic cartilage of the centre of the shaft does not occur. In mammalian long bone development, it is a constant feature. In both birds and mammals, the hypertrophic cartilage cells appear identical, but as judged by staining reactions the quantity and possibly the quality of the cartilage matrix varies. The cartilage of the long bones of mammals differs from those of birds in the greater quantity of matrix and in the fact that it stains more intensely with aniline dyes; the avian patella, on the other hand, has a very abundant matrix which stains deeply with aniline dyes.

Methods.

The methods employed in this investigation consisted essentially (1) in the isolation from the limb skeleton and cultivation *in vitro* of the tissue in which the patella subsequently develops, and (2) in the cultivation of the entire knee-joint region. The tissue was taken from embryos at the beginning of the 7th, 8th and 9th days of incubation.

The perichondrium of the developing articular surfaces gives rise to cartilage when cultivated *in vitro*, and so great care must be taken to prevent its inclusion in the explant. At the beginning of the 9th day of incubation, this perichondrium is fairly well-defined and it is possible to remove the mass of tissue lying between the condyles of the femur without injuring or tearing it. With embryos of this age, the following procedure was adopted. The limbs were removed from the pelvic girdle and the skin dissected off. A transverse incision was made at the lower end of the extensor group of muscles, avoiding injury to the perichondrium, and another in the region between the femur and the tibia and fibula. The mass of tissue between these two cuts was carefully separated from the long bones and from the surrounding tissue. The future patellar tissue from 7- and 8-day embryos was removed in the same way, but greater difficulty was experienced in preventing injury to the epiphyses. Explants of the whole knee-joint were obtained by removing the skin and cutting through the muscles and bones a little beyond the epiphyses. The tissue thus removed, which was about $1^1/_2$ mm. square in size in the case of 9-day embryos, less in those younger, was then explanted on large coverslips ($1^1/_4$ in. square) so that either the anterior or posterior surface was uppermost. The medium used consisted of equal parts of fowl plasma and embryo extract prepared from a 9-day embryo. After the plasma had coagulated the coverslips were inverted over large hollow-ground slides and sealed with paraffin in the usual way. The cultures were transferred to fresh medium every 48 hours. The zone of outgrowth was removed, the centre of the explant being kept intact. In some cases, the tissue was cultivated by the valuable method which was used by Fell and Robison (1929) for the cultivation of isolated femoral rudiments. The explants

were placed on the surface of a clot, contained in a small watch-glass, consisting of equal parts of plasma and embryo extract. The watch-glass was enclosed in a PETRI dish and was surrounded by cotton-wool saturated with sterile water. As before the cultures were transferred to fresh medium every 48 hours, the zone of outgrowth being removed while the centre of the explant was kept intact. Whole knee-joint cultures were always cultivated on watch-glasses. The cultures were fixed in ZENKER's fluid plus 2 per cent. acetic acid for quarter to half an hour after varying periods of cultivation *in vitro*. In the case of coverslip cultures some of the cultures were sectioned serially either at right angles to or parallel with the plane of the coverslip, i. e. in either the coronal or the sagittal plane, and the sections stained with MALLORY's triple stain or safranin picro-indigo-carmine. Others were mounted whole and stained with thionin blue. Watch-glass cultures were always sectioned serially in the sagittal plane.

The knee-joint region from which the tissue was taken was fixed either in BOUIN's fluid or in ZENKER's fluid plus 2 per cent. acetic acid, and serial sections were examined histologically in order to make sure that chondrogenesis had not begun before explantation. The sections were in many cases stained with thionin blue, a stain which demonstrates chondrogenesis in the very earliest stages. Thus every culture had 2 controls. Wax reconstructions of cultures and of stages in the normal development of the patella were also made.

The Development in vitro of the avian patella.

Since the most interesting results were obtained from cultures of presumptive patellar tissue from embryos at the beginning of the 9th day of incubation, these will be described first.

Appearances in Cultures from 9-day Embryos.

The tissue when first explanted consists of a roughly rectangular mass of mesenchyme about $1^1/_4$ mm. square in size, in the centre of which no structure can be made out. Attached to one side are a few strands of muscle which represent the lower end of the extensor group of muscles. After 24 hours' cultivation *in vitro* the tissue has spread out slightly on the coverslip and there is a considerable emigration of fibroblasts and mononuclear wandering cells into the medium; this emigration increases during the subsequent 24 hours. The muscle grows out in the form of long thin multinucleated strands which show no trace of cross-striation, although the tissue from which it has arisen is already striated. No trace of contraction of muscle strands was ever seen, and after three or four subcultures the muscle is no longer present in the zone of outgrowth. A number of degenerate cells are often present during the first 3 or 4 days' cultivation both in the explant and in the zone of outgrowth. These cells have probably been injured during the process of dissection; as a rule they disappear after the second or third subculture. On the 3 rd day of cultivation, a translucent area is generally seen at that end of the explant to which the muscle is attached. This indicates the commencement of chondrogenesis but the details of this cannot be made out owing to the size and thickness of the explant. As cultivation

proceeds this area increases in size and translucency and by the sixth day of cultivation it has a very definite roughly triangular form (Fig. 3). The shape of the cartilage is well seen in "whole mount" preparations which were stained with thionin blue (Fig. 3). The base of the triangle is slightly concave and to it is attached the remains of the muscle removed during the original dissection. The other two sides are unequal in length, the short one being about one half the length of the longer. They meet in a rounded apex. The longer side is often slightly concave near the base. The cartilage increases in size and the shape of the mass persists as a rule for at least 20 days *in vitro*. After that time, however, it tends to become oval in shape and loses its typical outline, although some cultures have retained their triangular form for at least seven weeks. The cartilage mass is very precisely demarcated from the rest of the explant and does not extend in a diffuse manner into it. Both upper and lower surfaces of the cartilage are slightly convex and show no irregularity of contour during the culture period.

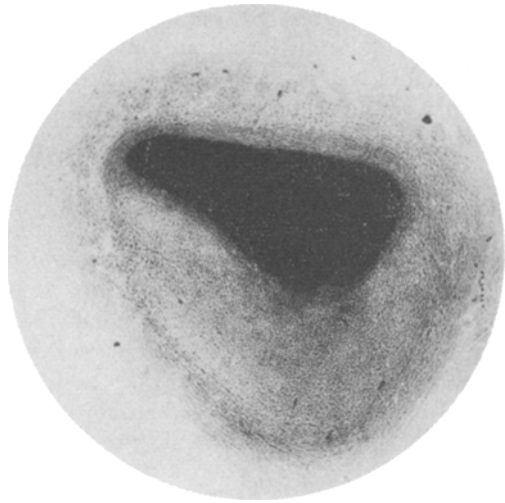

Fig. 3. Whole mount of patella culture 6 days after appearance of cartilage in the explant. Note the triangular shape of the cartilage mass which is sharply demarcated from the surrounding tissue. ZENKER: thionin blue.

Fig. 4. Section through the middle of knee-joint region of limb from which presumptive patellar tissue was removed. In this mass of mesenchyme the cartilage in fig. 3 developed. Epiphyses of the femur and tibia are uninjured and the perichondrium is intact. ZENKER: thionin blue. × 36.

In some cultures, two separate areas of chondrification were found which were quite distinct from one another. One was always triangular in shape and was generally larger than the other which was rounded or oval in form. The triangular mass was always in direct relation to the muscle of the explant and probably represented the patellar rudiment, while the other had no constant position in the explant. In such a culture, the control usually showed that the epiphyseal perichondrium had been injured to a considerable extent. Where the injury was slight, second centres of chondrification were never found. In such cases, the cartilage-forming cells probably emigrated into the medium and formed part of the zone of outgrowth where multiplication was active and the conditions unfavourable for differentiation. As the zone of outgrowth was always removed at each sub-culture and was not transplanted with the original explant they did not become incorporated in the central mass where the circumstances are favourable for chondrogenesis.

Histological Appearances.

As already described, the cultures were fixed after varying periods of cultivation, and examined histologically either as whole mounts or

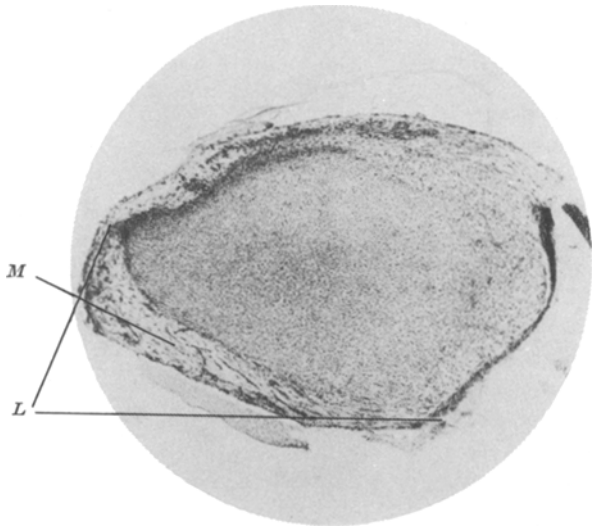

Fig. 5. Culture of presumptive patellar mesenchyme after 3 days' cultivation, showing commencing chondrogenesis. *L* upper limit of future patella; *M* muscle fibres derived from distal end of great extensor muscle of thigh. The area of commencing chondrogenesis is roughly triangular. ZENKER *plus* acetic acid: MALLORY'S triple stain. × 40.

in serial sections. After 3 days' cultivation, an area of early cartilage is generally to be seen. This consists of a zone of compactly arranged cells multiplying by mitotic division and separated by a small amount

of intercellular material. It is roughly triangular in shape, the base of the triangle being always in close relation to the muscle which represents the lower end of the extensor group. At this stage the margins of the area of chondrogenesis are not sharply demarcated by a perichondrium from the surrounding tissue (Fig. 5). A section through the knee-joint region from which this tissue was taken is shown in Fig. 6. As will be seen, the femoral and tibial perichondria are intact and examination of the whole series of sections reveals no injury at any point. Fig. 7 shows the knee-joint region of the other side from which no tissue was taken. The section is through the centre of the situation of the future patella and no trace of cartilage is present. The cartilage, therefore, has apparently differentiated in the culture, from the undifferentiated presumptive patellar tissue.

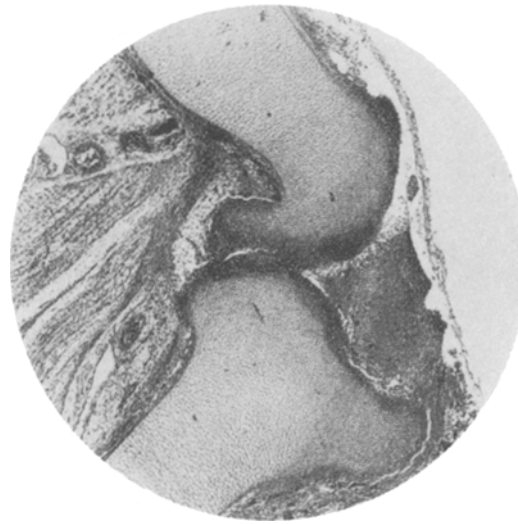

Fig. 6. Section through centre of knee-joint from which tissue for culture shown in fig. 5 was taken. The femoral and tibial epiphyses are undamaged and the perichondrium is intact. BOUIN: MALLORY'S triple stain. × 40.

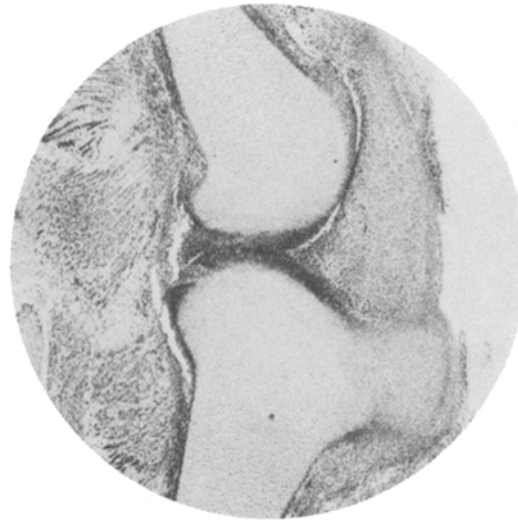

Fig. 7. Section through centre of knee-joint of limb from other side of same embryo as fig. 6. In the region where the patella subsequently develops no cartilage is to be seen. BOUIN: MALLORY'S triple stain. × 40.

In a culture examined after 6 days' cultivation, the cartilage cells are separated by a fairly abundant matrix and the whole area is surrounded by a perichondrium which contains white fibres. From this stage onwards,

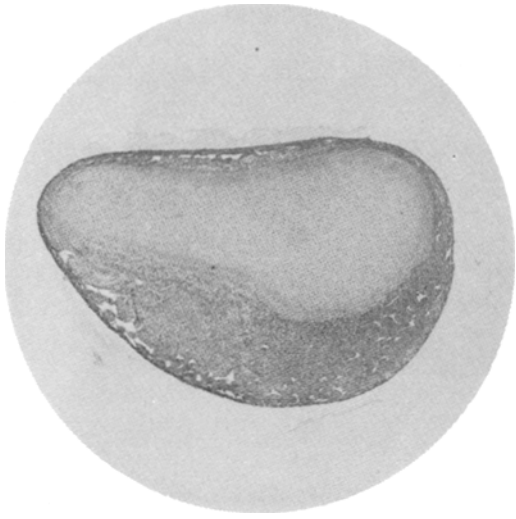

Fig. 8. Section through culture of patella developing *in vitro* after 12 days' cultivation. The cartilage mass, which consists of small-celled chondroblasts, is roughly triangular in shape and sharply demarcated from the surrounding tissue. ZENKER: MALLORY's triple stain. × 55.

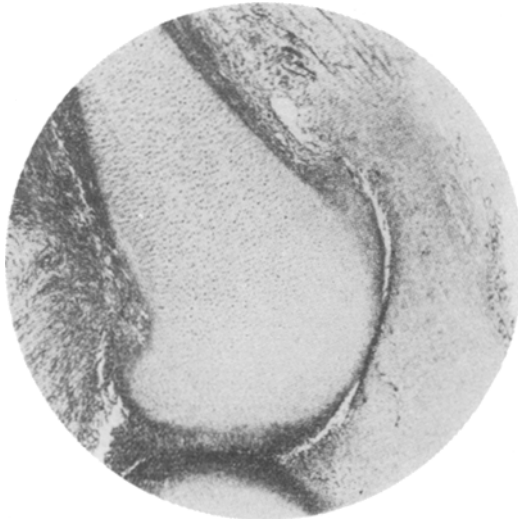

Fig. 9. Section through knee-joint of other side of same embryo as fig. 8. Cartilage formation in the patellar region has not yet begun. BOUIN: MALLORY's triple stain. × 60.

increase in size of the cartilage mass is brought about mainly by the activity of the perichondrium and by the production of cartilage matrix. The chondroblasts themselves only show occasional mitoses. As already mentioned, the cartilage always appears in a definite position in the explant immediately below the muscle. The perichondrium consists of two or three layers of cells and is well demarcated from the rest of the tissue (see Fig. 8). On the longer side, however, the perichondrium is somewhat diffuse externally and tends to fade into the surrounding tissue which consists of fibroblasts separated by fine collagen fibrils and amongst which fine thin-walled vessels persist for a considerable period. The limits of the cartilage, however, are always well defined. The muscular tissue of the original explant does not continue to develop. The muscle substance apparently undergoes some form of degeneration resulting in the formation of bundles of multinucleated cells with somewhat bulky cytoplasm which are separated by fairly large spaces. The exact nature of this change is unknown but the condition is quite obvious after the

8 th day of cultivation. If the presumptive patellar tissue is explanted on a plasma clot in a watchglass the arrangement of muscle, developing patella and the sharp demarcation of the latter from the surrounding tissue is well seen after 6 days' cultivation owing to the manner in which the tissue spreads out on the surface of the clot. In some cultures branched pigment cells appeared in the explant after several days' cultivation. These were not present at explantation and represent a differentiation of previously unpigmented elements.

The cartilage remains healthy for at least 3 weeks *in vitro*. After that time, areas of necrosis are often found in the centre of the cartilage, although in some cases it has been found healthy after 38 days. The degeneration of the cartilage is probably due to the large size of the explants and the formation of a thick connective tissue capsule round the explant which prevents diffusion of nutritive substances from the medium.

A series of cultures was maintained for 8 weeks *in vitro*. The zone of outgrowth remained healthy and was derived from the thick connective tissue capsule which developed round the explant. In most of the cultures, the cartilage mass became oval in shape and was partially or completely necrotic at the end of the culture period, the surviving cartilage cells being of the small-celled type. No invasion of the cartilage by fibroblasts occurred and it is presumed that the presence of blood vessels is necessary for the invasion of the cartilage. No trace of ossification was present; this was to be expected as the length of the period of cultivation was less than that which normally elapses before ossification of the patella begins. The development of fat between the lower part of the posterior aspect of the patella and the synovial membrane of the knee-joint *in vivo* has been mentioned. In the cultures, the region corresponding in part to this area remained very cellular throughout the period of cultivation but no transformation of fibroblasts into fat cells occurred.

In these cultures in which two centres of chondrification appeared, the smaller centre consisted of small-celled cartilage and was always surrounded by a perichondrium which was uniform in arrangement round the nodule, and no connection was ever found between it and the larger triangular mass.

The Form of the Patellar Cartilage which differentiates in vitro.

The triangular form of the cartilage mass which differentiated *in vitro* has been described. In all cultures of tissue isolated from 9-day embryos (81 specimens) the shape of the cartilage was more or less the same at least up to the 20 th day of cultivation. Variations in size were sometimes found, the mass remaining small in some cases. The concavity of the upper margin was slightly variable, being deeper in some cases than in others. Occasionally the difference in length between the

two sides was diminished (see Fig. 16). Apart from these minor differences, however, the form was remarkably constant.

As already mentioned, each culture had 2 controls, the knee-joint region from which the tissue was taken and the intact knee-joint region of the other side. All these controls were examined histologically. In the intact knee-joints no trace of chondrogenesis at the site of the future patella was ever observed, showing that chondrogenesis must have taken place during cultivation *in vitro*. In the knee-joints of the side from which the tissue was taken fairly extensive tearing of the epiphyseal perichondrium of the femur or tibia was present in 18 specimens, out of 81. In only 5 cultures, however, were additional nodules of cartilage present. In 13 specimens, the femoral ortibial perichondrium had been separated but not removed from the cartilage. In none of the corresponding cultures were additional nodules of cartilage found. It is, therefore, to be concluded that the differentiated cartilage represents the patellar rudiment and has not arisen from epiphyseal perichondrium.

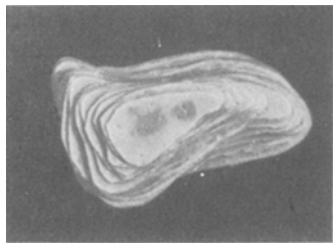

Fig. 10. Wax reconstruction of patella isolated from avian embryo at the end of the 11th day.

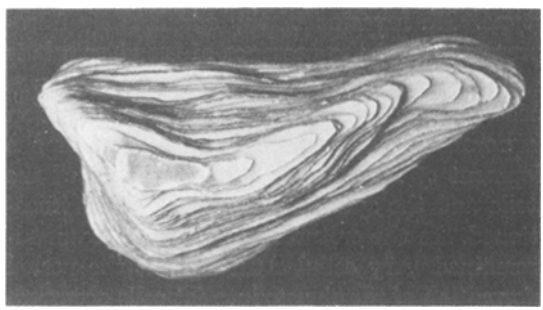

Fig. 11. Wax reconstruction of patella isolated from avian embryo after $14^1/_2$ days' incubation.

In normal development, as already stated, the patellar cartilage remains triangular in shape until the end of the 15th day of incubation (see Fig. 11). The anterior and posterior surfaces are convex, the former being rather more rounded than the latter. Between the 16th and the 17th day, 2 well-marked concavities appear on the upper part of the posterior surface. Wax reconstructions were made from serial sections of the normal patella taken at the end of 11 and of $14^1/_2$ days' incubation. Photographs of these are shown in Figs. 10 and 11. The anterior surface is uppermost and to the upper concave margin the extensor muscle was attached. The inequality of the two sides is obvious and in the older rudiment the smaller (lateral) side is slightly concave. Reconstructions were also made of the patella from 17- and 19-day embryos. These showed the presence of the concavities for articulation

with the condyles of the femur. Fig. 12 and 13 are photographs of reconstructions made from serial sections of cultures after 14 and 20 days' incubation which were chosen at random from the series. The anterior

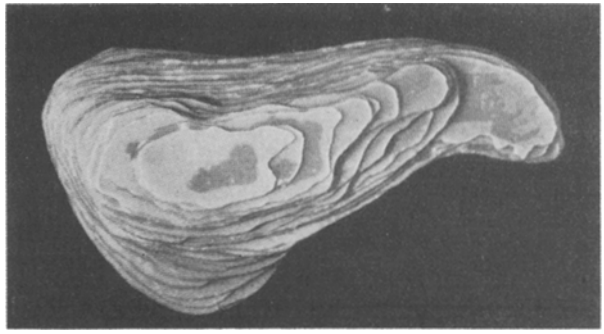

Fig. 12. Wax reconstruction of 20-day culture of avian patella which differentiated *in vitro*.

and posterior surfaces of both are convex, and as will be seen, they are similar in shape and resemble also the reconstructions of the patella from 11- and $14^1/_2$-day embryos. The most obvious difference between them and the patella from the $14^1/_2$-day embryo is the absence in the cultures of a small concavity on the shorter or lateral side. They differ, however, from the reconstruction of the patella from the 17-day embryo in that they show no trace of articular surfaces. In a reconstruction of a 38-day culture, two projections were seen on one surface. Between them was a shallow depression, the depth of which was 70 μ as estimated from the number and thickness of the sections. In this culture, considerable necrosis of cartilage was present. The structures did not correspond in position to the situation of the articular surfaces and it was decided that the condition was due to fixation shrinkage of the necrotic cartilage. The existence of some form of irregularity was detected in the sections by the presence of 2 areas of cartilage. In no other culture, except when an additional nodule of cartilage was present, was a similar appearance found in section.

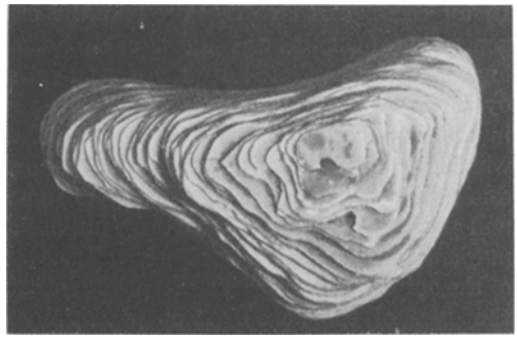

Fig. 13. Wax reconstruction of 14-day culture of avian patella which differentiated *in vitro*.

From these observations, it is apparent that the cartilage has arisen from mesenchyme destined to form the patella and that the shape of the cartilage mass resembles that seen in the primary stages of the development of the patella. The resemblance ceases, however, when the articular surfaces begin to form.

Cultivation of entire knee-joints from 9-day embryos.

Since articular surfaces for the condyles of the femur do not develop in the patellar rudiment which has differentiated *in vitro*, the entire knee-joints from a series of embryos at the beginning of the 9th day were cultivated on watch-glasses in order to ascertain if the presence of the condyles of the femur would bring about the formation of concavities. Degeneration of the explants took place very rapidly, however, owing to their large size and no differentiation of the patella occurred.

Appearances in cultures of tissue from 7- and 8-day embryos.

It was then decided to investigate the earliest stage at which the patellar cartilage would differentiate under the conditions of cultivation *in vitro*. For this purpose, the presumptive patellar tissue was removed from embryos at the beginning of the 7th and 8th days of incubation. In all cases, cartilage appeared in the explants from 8-day embryos, generally on the 3rd or 4th day of explantation. The shape of the cartilage mass was often, however, irregular, sometimes round, sometimes oval. In 50 per cent. of the cultures, however, it resembled that seen in the cultures from 9-day embryos. Two centres of chondrification which were quite distinct from one another often developed. This was due partly to the greater difficulty of removing the tissue at this stage and also to the fact that the epiphyseal perichondrium is not very sharply marked off from the surrounding tissue and the small-celled cartilage of the epiphyses merges imperceptibly into the tissue intervening between the ends of the bones. Thus potentially epiphyseal cartilage-forming tissue was probably removed along with future patellar tissue. It was thus not always possible to say whether or not the cartilage had arisen from future patellar tissue. The triangular form of the patella in the case of tissue from 8-day embryos certainly does not differentiate invariably as it does in the case of tissue removed from 9-day embryos.

In the tissue isolated from the inter-condylar region of 7-day embryos, cartilage always appeared after 2 or 3 days' cultivation. The mass never resembled the shape of the patellar cartilage. Histological examination of the knee-joint region at this stage revealed the fact that the small-celled cartilage of the epiphyses merged imperceptibly into the surrounding tissue, and it was impossible to determine the limit of the tissue destined to form epiphyseal cartilage.

Since indubitable patellar cartilage does not differentiate in the tissue removed from the patellar site of 7-day embryos, the whole future knee-joint region from such embryos was cultivated *in vitro* in order to see if patellar cartilage would differentiate itself from the mass of chondrogenic tissue surrounding the developing epiphyses. The tissue was explanted on watch-glasses so that the anterior surface was upwards, and this position was preserved throughout the culture period. The explants grew rapidly in size but separation of the developing articular surfaces did not take place. The epiphyses fused, the patella did not differentiate and the whole knee-joint, after 8 days' cultivation was represented by an irregular mass of cartilage. Degeneration of the cartilage took place very rapidly owing to the large size of the explants.

Cultivation of Part of the Patellar Mesenchyme.

MURRAY (1926), found that in a number of grafts derived from transverse halves of limb-buds, the cartilaginous shafts are incomplete, ending usually in a blunt point. Histologically the cartilage was of the hypertrophic and vacuolated type found in the centre of the developing skeletal rudiment. He obtained a similar result in a graft of the posterior limb regions of a 2-day embryo and considers that this incompleteness is due to the cut which divided the original bud or tissue having traversed the anlage of the skeletal element concerned. He is of the opinion that the anlagen of the cartilaginous elements of the long bones of the limb-bud are each a mosaic with respect to the kind of cartilage to be produced from each part and that, accordingly, no one part is capable of regenerating the whole. Since, however, the patella develops relatively late in embryonic life and the cartilage remains of the small-celled type for at least 12 weeks after chondrogenesis has begun, it was of interest to determine whether or not in embryos at the beginning of the 9th day of incubation the patella is also a mosaic system.

The future patellar tissue was removed from both limbs of embryos at the beginning of the 9th day, the knee-joint regions being kept as controls. Only those cultures which had controls showing no trace of injury to the epiphyseal perichondrium were studied. The tissue from one side was kept intact and explanted on coverslips in the usual way and that from the other was divided longitudinally into two parts as nearly equal as possible. These parts were explanted separately. In the divided explants, the zone of outgrowth was preserved at subculture for the first three passages. In the entire explants, the cartilage differentiated as usual forming a triangular mass. In the divided explants, a centre of chondrification usually appeared in each. One of these was always larger than the other and was generally oval in shape while the smaller was generally rounded (Figs. 14 and 15). The larger covered an area at

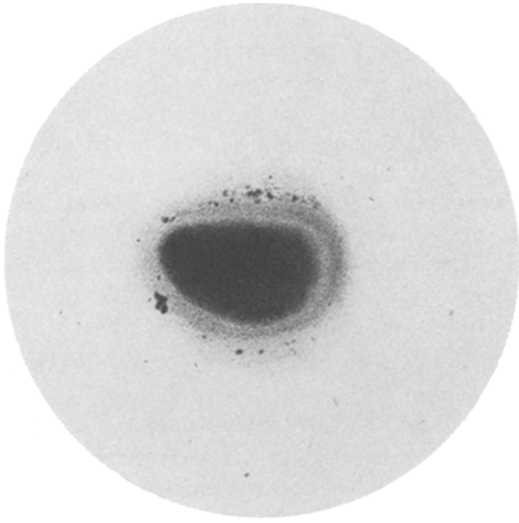

Fig. 14.

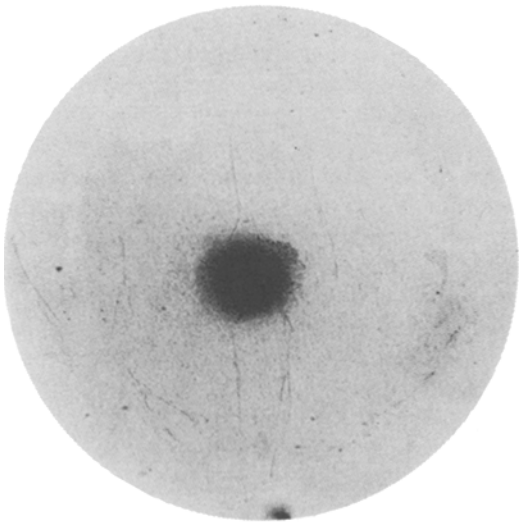

Fig. 15.

Figs. 14 and 15 are cultures derived from the future patellar mesenchyme of the other side of the same embryo as fig. 16. The mesenchyme was divided into two equal parts which were explanted separately. The cartilage in culture shown in fig. 14 is considerably greater in amount than that obtained in the culture shown in fig. 15, but the sum of the cartilage in both cultures is less than that in fig. 16. ZENKER: thionin blue (× 40).

least four times that of the smaller. In no case did the form of the cartilage mass which developed in a divided explant resemble the shape of that which appeared in the undivided explant (Fig. 16). The sum of the total amount of cartilage obtained in the divided explants was always less than that obtained in the undivided explant from the other sides of the embryo. This is probably accounted for by the injury to the cartilage-forming tissue during the cutting. In addition, some of the cartilage-forming cells probably emigrated into the medium during the first passage and may not have become incorporated in the part of the explant where differentiation takes place. The inequality in size may be explained by the division of the tissue into two parts which though approximately equal in size are unequal as far as cartilage production is concerned. In some cases, a centre of chondrification appeared in one explant only. This was always larger than was the case when two centres were found, but was never so large, nor had it the triangular shape of the cartilage seen in the

undivided explant. In such cases it was always noticed at the time of explantation that the parts were unequal in size.

It seems likely in those cases in which cartilage appeared in both explants that the smaller nodule represents that part of the patella medial to a line drawn at right angles to and bisecting the upper margin while the larger mass represents that part of the patella lateral to that line. Since, however, the two pieces when placed together have not the form of the normal patellar cartilage *in vitro*, it cannot be definitely stated that the patella is a mosaic. The only conclusion to be drawn from this experiment is that the tissue in which the patella develops is, as a whole, a mosaic in which the non-cartilage-forming part cannot replace any of the cartilage-forming tissue.

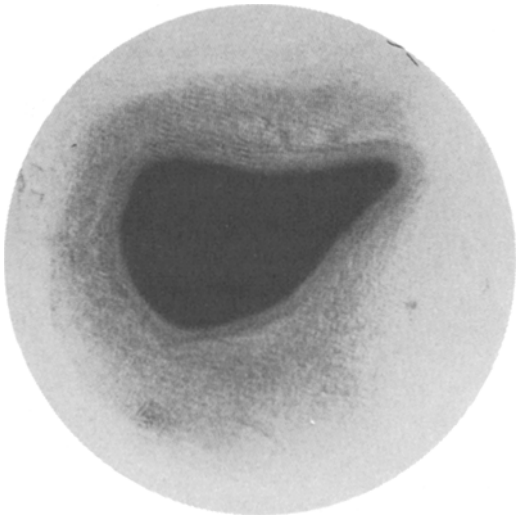

Fig. 16. Whole mount of avian patella which differentiated *in vitro*. 8 days' cultivation ZENKER: thionin blue (× 40).

Discussion.

The patella is still regarded by many workers as a sesamoid bone which develops in tendon and is comparable to the structure found in the neighbourhood of the interphalangeal joints of many animals. The work of DE VRIESE (1909) who made a comparative study of the development of the patella in different animal species showed that the cartilaginous rudiment of the patella develops in a cellular mesenchyme in a manner histologically similar to that of the cartilage of the rest of the limb-skeleton. This finding has been confirmed for the avian patella.

As mentioned in the introduction, MURRAY and SELBY (1930) consider that the gross form of the cartilaginous skeleton and of the articular structures self-differentiate and they consider that these principles are probably applicable to the rest of the limb-skeleton. Concerning the self-differentiation of cartilaginous forms they state, however, that their data deal mainly with the femur and they have no evidence concerning the influences which regulate the development of "negative" (concave) articular surfaces. In addition, these workers show that there is no direct evidence which demonstrates conclusively that cartilage as a tissue differentiates in the absence of extrinsic conditions.

CAREY (1922), from experimental and morphological investigations of a different kind, concludes that the development of cartilage as a tissue and the development of the primary form of the cartilaginous skeleton depends entirely on mechanical conditions present in the part. The results of the present investigations do not give any additional information concerning the conditions determining the differentiation of cartilage. They show, however, that the tissue in which the patella develops is of such a nature that cartilage develops in it when it is isolated from the limb-skeleton and from the mechanical conditions existing therein. It might be considered that the presence of muscle in the explant by exercising tension on the mesenchyme would favour the formation of cartilage. The muscle, however, was scanty in amount and much of it grew out into the medium and was removed at the first subculture: it did not preserve its histological identity and showed no functional activity. In some cultures the muscle was almost completely removed and the cartilage differentiated in the usual way.

The shape of the patellar cartilage which develops *in vitro* resembles closely that seen in the early stages of normal development *in vivo*. Such an almost geometrical form is never seen in nodules of cartilage which develop *in vitro* in cultures of 3-day limb-buds or in cultures of perichondrium. It is apparent, therefore, from the observations recorded that the primary form of the patella "self-differentiates" *in vitro* in the sense stated by MURRAY and SELBY (1930).

A significant feature was the absence of the development of the concave articular structure for articulation with the condyles of the femur. The non-appearance may have been due to the slowing down of the developmental rate which occurs *in vitro*. Even after 7 weeks' cultivation, however, no trace of articular surfaces was seen in patellar cartilages which had preserved their primary form *in vitro*. It appears, therefore, that in the case of the patella the presence of contiguous elements, in this case the condyles of the femur, is necessary for the development of the articular surface. The "negative" surface thus possibly differs from a "positive" surface like the head of the femur in that it requires for its development the presence of the corresponding surface.

The mesenchyme destined to form the patellar cartilage seems to be isolated from the surrounding tissue at a relatively late stage of development. Only after the formation of epiphyseal cartilage begins to be limited to a definite zone of cells can indubitable patellar-forming mesenchyme be obtained. The development of the patella *in vivo* does not appear to take place until almost three days after this has occured.

This work was done during the tenure of a Carnegie Research Fellowship of the University of Glasgow and much of it was carried out in the

Strangeways Research Laboratory, Cambridge. I wish to thank the director of the Strangeways Research Laboratory, Dr. H. B. FELL, for the facilities afforded me there and for her encouragement and help during the course of the work. I am indebted to Dr. P. D. F. MURRAY of the Strangeways Research Laboratory for much valuable advice and criticism. The photomicrographs were prepared by Mr. JOHN KIRKPATRICK of the Pathology Department of the University and Western Infirmary, Glasgow, and by Mr. H. P. HUDSON of the Pathology Department, Cambridge University, to both of whom I have pleasure in expressing my thanks. I also wish to thank the Medical Research Council for their support.

Summary.

1. The normal development of the avian patella has been described. Chondrogenesis begins ot the end of the tenth and the beginning of the eleventh day of incubation. The articular surfaces for the femur do not begin to appear util the sixteenth day. Shortly after this, the cartilage is invaded by capillary blood-vessels and fibroblasts. Many of these perivascular fibroblasts become transformed into chondroblasts and give rise to cartilage. About the twelfth week after hatching, the perivascular formation of cartilage ceases and the cartilage round the blood-vessels hypertrophies; in the matrix of this hypertrophic cartilage calcification takes place. Simultaneously, bone is formed around the blood-vessels by the perivascular fibroblasts which have become transformed into osteoblasts.

2. The mesenchyme from 7-, 8- and 9-day avian embryos from the site in which the patella develops was cultivated *in vitro*. Cartilage appeared in the explants in all cases. In tissue from 9-day and from some 8-day embryos the form of the cartilage mass resembled that seen in the early stages of normal development. This was not the case with the tissue from 7- and some 8-day embryos.

3. Articular surfaces did not appear in the cultures even after 7 weeks' cultivation.

4. In cultures of the knee-joint region, including the ends of the long bones, from 7-day and 9-day embryos, the patella did not differentiate. This is considered to be due to the large size of the explants which prevented adequate nutrition of the tissue.

5. In cultures of parts of patellar mesenchyme, cartilage appeared *in vitro*, but the size of the cartilage mass was smaller and the form did not resemble that which developed in cultures of the undivided patellar mesenchyme. The tissue in which the patella develops is a mosaic of cartilage-forming and non-cartilage-forming tissue and no one part is capable of regenerating the whole.

Zusammenfassung.

1. Die normale Entwicklung der Patella des Huhnes im bebrüteten Ei und nach dem Ausschlüpfen wird beschrieben. Die Chondrogenese beginnt am Ende des 10. und am Anfang des 11. Bebrütungstages. Die Gelenkflächen erscheinen erst am 16. Tage. Kurz darnach dringen kapillare Blutgefäße und Fibroblasten in das Knorpelgewebe ein. Viele von den perivaskulären Fibroblasten wandeln sich in Chondroblasten um und bilden Knorpelgewebe. Etwa in der 12. Woche nach dem Ausschlüpfen hört die perivaskuläre Bildung von Knorpel auf und der Knorpel um die Blutgefäße herum wird hypertrophisch. In der Grundsubstanz dieses hypertrophierten Knorpels findet die Verkalkung statt. Die perivaskulären Fibroblasten wandeln sich in Osteoblasten um und gleichzeitig mit dem Verkalkungsprozeß entsteht Knochengewebe um die Blutgefäße herum.

Bei 7-, 8- und 9-tägigen Embryonen wurde das Mesenchym der Gegend, in der sich später die Patella entwickelt, herauspräpariert und in vitro gezüchtet. In all diesen Explantaten wurde Knorpelbildung beobachtet. Die Gestalt des Knorpels in Explantaten von 9- und von einigen 8tägigen Embryonen ähnelte derjenigen der früheren Stadien der normalen Entwicklung *in vivo*. Bei Explantaten von 7- und von einigen 8tägigen Embryonen war dies nicht der Fall.

3. Zu einer Entwicklung von Gelenkflächen kam es in den Explantaten nicht, obgleich diese 7 Wochen lang *in vitro* am Leben gehalten wurden.

4. In Explantaten von 7- und 9tägigen Embryonen, welche die ganze Kniegelenkgegend (einschließlich der Enden der Knochen) enthielten, fand keine Entwicklung der Patella statt, wahrscheinlich ist daran die Größe der Explantate schuld, die eine hinreichende Ernährung verhindert.

5. In Explantaten von Teilstärken des Patellarmesenchyms entwickelt sich zwar Knorpel *in vitro*, aber im Gegensatz zu den Kulturen der ganzen Patellargegend war die Menge des Knorpels kleiner und die Form war nicht patellaartig. Man darf daraus den Schluß ziehen, daß das Gewebe, in welchem sich die Patella entwickelt, ein Mosaik von knorpelentwickelnden und knorpel-nicht-entwickelnden Geweben darstellt, und daß das Ganze nicht von einzelnen Teilen regeneriert werden kann.

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