Cell Proliferation, Extracellular Matrix Mineralization, and Ovotransferrin Transient Expression during In Vitro Differentiation of Chick Hypertrophic Chondrocytes into Osteoblast-like Cells

Chiara Gentili,* Paolo Bianco,‡ Monica Neri,* Mara Malpeli,* Giuliano Campanile,* Patrizio Castagnola,* Ranieri Cancedda,*§ and Fiorella Descalzi Cancedda*

*Istituto Nazionale per la Ricerca sul Cancro and §Istituto di Oncologia Clinica e Sperimentale, Universita' di Genova, Genova, Italy; and ‡Dipartimento Biopatologia Umana, Sezione di Anatomia Patologica, Universita' La Sapienza, Roma, Italy

Abstract. Differentiation of hypertrophic chondrocytes toward an osteoblast-like phenotype occurs in vitro when cells are transferred to anchorage-dependent culture conditions in the presence of ascorbic acid (Descalzi Cancedda, F., C. Gentili, P. Manduca, and R. Cancedda. 1992. *J. Cell Biol.* 117:427–435). This process is enhanced by retinoic acid addition to the culture medium. Here we compare the growth of hypertrophic chondrocytes undergoing this differentiation process to the growth of hypertrophic chondrocytes maintained in suspension culture as such. The proliferation rate is significantly higher in the adherent hypertrophic chondrocytes differentiating to osteoblast-like cells. In cultures supplemented with retinoic acid the proliferation rate is further increased. In both cases cells stop proliferating when mineralization of the extracellular matrix begins. We also report on the ultrastructural organization of the osteoblast-like cell cultures and we show virtual identity with cultures of osteoblasts grown from bone chips. Cells are embedded in a dense meshwork of type I collagen fibers and mineral is observed in the extracellular matrix associated with collagen fibrils. Differentiating hypertrophic chondrocytes secrete large amounts of an 82-kD glycoprotein. The protein has been purified from conditioned medium and identified as ovotransferrin. It is transiently expressed during the in vitro differentiation of hypertrophic chondrocytes into osteoblast-like cells. In cultured hypertrophic chondrocytes treated with 500 nM retinoic acid, ovotransferrin is maximally expressed 3 d after retinoic acid addition, when the cartilage–bone-specific collagen shift occurs, and decays between the 5th and the 10th day, when cells have fully acquired the osteoblast-like phenotype. Similar results were obtained when retinoic acid was added to the culture at the 50 nM "physiological" concentration. Cells expressing ovotransferrin also coexpress ovotransferrin receptors. This suggests an autocrine mechanism in the control of chondrocyte differentiation to osteoblast-like cells.

THE chick epiphyseal growth plate is a unique site to investigate ontogenic processes leading to the formation of endochondral bone. The sequence of events includes the formation of hypertrophic cartilage, its invasion by blood vessels from the perichondrium, the erosion of the calcified cartilage, and its replacement by bone tissue. It is generally accepted that hypertrophic chondrocytes in the growth plate degenerate at the site of the transition region from cartilage to osteoid and ultimately die. Several authors have reported that in organ cultures hypertrophic chondrocytes start expressing bone markers and they have proposed that hypertrophic chondrocytes may contribute to the formation of a bone matrix (37, 41, 43). We have recently shown that in vitro chick hypertrophic chondrocytes, obtained as single isolated cells after 3 wk in suspension culture, further differentiate to osteoblast-like cells when transferred to anchorage-dependent culture conditions in the presence of ascorbic acid (11). The cells acquire an elongated or star-shaped morphology, start expressing alkaline phosphatase, reorganize their extracellular matrix by discontinuing the production of cartilage-specific proteoglycans and by switching from the synthesis of type II and type X collagens to the synthesis of type I collagen, and express and secrete other specific differentiation marker proteins. Eventually, calcium mineral is deposited on the newly formed matrix. Supplementing the culture medium with 1 mM retinoic acid during the first 5 d in culture results in a dramatic enhancement of the differentiation process. In vitro hypertrophic chondrocytes undergoing further differentiation produce and secrete large amounts of a noncollagenous glycoprotein with an apparent molecular mass of 82 kD in reducing conditions and of 63 kD in unreducing conditions (11).

In the present manuscript, we extend our previous findings, by investigating the cell proliferation rate during the differentiation process and report on the ultrastructural characterization of the osteoblast-like cell cultures. We also report the purification of the 82-kD glycoprotein from conditioned culture medium and its identification as chicken ovotransferrin, by amino acid sequence determination of its NH_2 terminus. This protein is transiently expressed by the differentiating hypertrophic chondrocytes. In cultures supplemented with retinoic acid, both at a high and at a "physiological" concentration, the protein reaches its highest expression level 3 d after retinoic acid addition, when the shift from the cartilage-specific to the bone-specific collagen occurs. Evidence is also given for the expression of ovotransferrin receptors by cultured chondrocytes. The coexpression by the cells of the growth factor and its receptor is suggestive of an autocrine (or self-responsive) mechanism playing a role in the control of chondrocyte proliferation and differentiation during their transition to osteoblast-like cells.

Materials and Methods

Cell Culture

Cultures of chondrocytes were performed as described (3). Dedifferentiated chondrocytes were obtained by plating on culture dishes cells freshly dissociated from 6-d-old chick embryo tibiae. To reinduce differentiation, dedifferentiated chondrocytes, expanded as adherent cells for 3 wk, were transferred to suspension culture for an additional 3–4 wk until a homogeneous population of single isolated hypertrophic chondrocytes was obtained. Osteoblast-like cells were obtained as described by Descalzi Cancedda et al. (11). Hypertrophic chondrocytes were filtered through a nylon filter, digested with hyaluronidase, and plated at confluence (2×10^5 cells in 30-mm dish) in Coon's modified F12 culture medium containing 10% FCS. After 3 d the medium was supplemented with 100 μg/ml ascorbic acid and 10 mM β-glycerophosphate. The medium was changed every other day without cell passaging. When indicated retinoic acid was added to the culture medium at final concentrations between 10 and 500 nM. Fresh retinoic acid was added and the culture medium was changed every day. Cultures of chondrocytes derived from a single cloned cell were obtained according to Quarto et al. (36). Chick embryo osteoblast cultures were obtained according to the procedure described in Manduca et al. (25) from cells grown out from bone chips.

DNA Measurement

Cell layers were scraped in 0.01% SDS in PBS, and digested overnight at 50°C with proteinase K (50–150 μg/ml in 10 mM Tris HCl, pH 7.8, 5 mM EDTA). DNA content was determined in a DNA fluorometer from Hoefer on 0.1 ml sample added to 2 ml dye solution containing 10 mM Tris HCl, pH 7.6, 1 mM EDTA, 0.1 M NaCl, 0.1 μg/ml Hoechst 33258 from Sigma Immunochemicals, St. Louis, MO.

Histochemistry

Alkaline phosphatase activity was determined using the histological kit 86 by Sigma Immunochemicals.

Ultrastructural Analysis

Cultures were sacrificed at indicated days by fixation with 4% formaldehyde (freshly made from paraformaldehyde) in 0.1 M phosphate buffer, pH 7.2, postfixed with 1% OsO_4 for 30 min and routinely ethanol dehydrated and Araldite embedded. Thin sections contrasted with U/Pb were examined in a Philips CM10 operated at 80 KeV.

Cell Labeling and Protein Analysis

Cells were labeled with [^{35}S]methionine as described by Descalzi Cancedda et al. (9). Aliquots of culture media were run for protein analysis by SDS-PAGE in reducing and unreducing conditions (2). Immunoprecipitation of specific proteins was performed as previously described (9).

Western Blot Analysis for Transferrin Receptors

Whole cell lysates were prepared by adding to the cells 0.1% SDS in PBS. Aliquots of samples, containing ~300 μg of proteins, were loaded on a 10% SDS–polyacrylamide gel. Electrophoresis was performed in reducing conditions. After electrophoresis the gel was blotted to a BA85 nitrocellulose membrane (Schleicher & Schuell GmbH, Dassel, Germany) according to the procedure described by Towbin et al. (44). The blot was saturated for 16 h with 2% BSA in TTBS buffer (20 mM Tris HCl, pH 7.5, 500 mM NaCl, 0.05% Tween 20), washed several times with TTBS and incubated with the polyclonal antiserum directed against the oviduct transferrin receptor (αOV-TfR) (15) for 2 h at room temperature. After additional washes the detection was performed by a biotin-conjugated anti-rabbit IgG (H + L) (Jackson Immunoresearch Laboratories Inc., West Grove, PA) and HRP (Jackson Immunoresearch Laboratories Inc.) using 4-chlor-1-napthol (Merck Biochemica, Darmstadt, Germany) as substrate.

RNA Extraction and Northern Analysis

Total RNA was extracted from cells using the guanidinium thiocyanate method (6). For Northern analysis ~10 μg of total RNA were electrophoresed through 1% agarose gel in the presence of formaldehyde and blotted onto Hybond N–membranes. Hybridization and washing conditions were performed as recommended by Amersham Corp. (Arlington Hts. IL). Probes were as follows: BST XI-BST XI fragment of ovotransferrin cDNA (22), pCOL3 for (αI I) collagen (45), pDR5 for Ch21 (10), insert of pCP15 for osteopontin (4), and pXCR7 for rRNA (gift from Dr. F. Amaldi, Universita' di Tor Vergata, Roma, Italy).

Purification of Ovotransferrin

Conditioned culture medium was supplemented with tracer amounts of radioactive medium obtained by metabolic labeling of stage III chondrocytes with [^{35}S]methionine and containing a highly radioactive 69-kD glycoprotein. Medium was freed of collagen by 30% ammonium sulfate precipitation and then concentrated by precipitation with saturated ammonium sulfate. The precipitated proteins were dissolved and dialyzed in 20 mM phosphate buffer, pH 7.6, and loaded on a DE52 column equilibrated with the same buffer. Proteins that did not bind to the column (including the radioactive 69-kD glycoprotein as the only radioactive protein) were dialyzed against 0.5 N CH_3COOH, lyophilized dissolved in 0.1% trifluoracetic acid in H_2O and separated on a Vidac C18 (TP 5μ, 300 Å) reverse phase column by a 0–70% gradient of acetonitrile containing 0.1% trifluoracetic acid. Chromatography was performed in 90 min; the flow rate was 1 ml/min; fractions were collected every minute and sampled for radioactivity determination. The radioactive peak was collected and an aliquot was analyzed by SDS-PAGE followed by silver staining.

Amino-terminal Sequence Determination

The amino-terminal sequence of the purified protein (0.3 mg) was determined with a pulse liquid sequencer from Applied Biosystems Inc. (Foster City, CA) according to the manufacturer's instructions.

Results

Cell Growth during In Vitro Differentiation

To determine whether chondrocyte differentiation to osteoblast-like cells was accompanied by cell proliferation, we directly compared the growth rates, measured as an increase in the culture DNA content, of two hypertrophic chondrocyte cultures, maintained in the presence of ascorbic acid and grown adherent or in suspension (Fig. 1 *A*). Cells were plated sparse (4×10^4 cells per 3-cm dish). In the adherent culture, the cell doubling time was ~7 d. In the control, hypertrophic chondrocyte suspension culture, the cell doubling time was >14 d. Fig. 1 *B* shows results from an independent experiment in which growth rates of hypertrophic

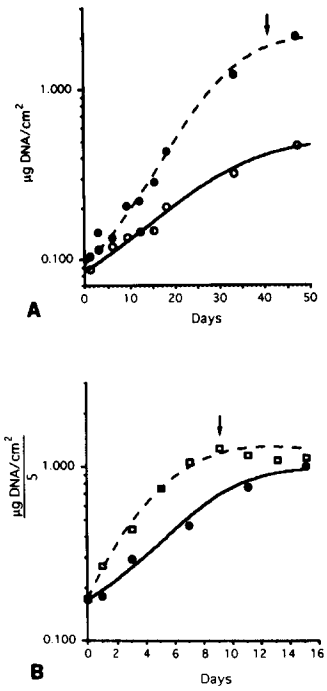


Figure 1. Growth of hypertrophic-cultured hypertrophic chondrocytes. After filtering, hypertrophic chondrocytes were plated as adherent cells (●) or maintained in suspension (○). All culture media contained ascorbic acid; 500 nM retinoic acid was added to the medium of one culture (□). Total DNA was determined in duplicate 3-cm culture dishes over the culture time. Initial cell density was 4 × 10^4 cells/dish in *A* and 2 × 10^5 cells/dish in *B*. Arrows indicate when culture mineralization was first detectable in the adherent culture in *A* and in the retinoic acid supplemented culture in *B*.

chondrocytes grown adherent in the presence and absence of retinoic acid were compared. Cells were plated at confluence (2 × 10^5 cells per 3-cm dish). In the culture not supplemented with retinoic acid the cell doubling time was ~4 d. The addition of 500 nM retinoic acid to the culture medium enhanced the cell doubling time by approximately twofold. Both in the presence and absence of retinoic acid, the growth curves reached a plateau at the time that mineralization of the extracellular matrix started (Fig. 1, *arrows*).

It should be noted that cell proliferation was observed also when cells were plated at confluence (Fig. 1 *B*), since the cells have a tendency to grow in multilayers.

Cell Ultrastructure, Matrix Formation, and Mineralization

The morphology, organization and mineralization of the extracellular matrix produced by osteoblast-like cells were assessed by EM (Fig. 2). Obvious differences were noted compared with the morphology of the cartilage-like matrix produced by tibial hypertrophic chondrocytes in suspension (unpublished results) and cultured vertebral chondrocytes (16). Fibrils exhibiting a 64–70-nm periodicity, consistent with type I collagen, represented the dominant feature observed both in the absence (Fig. 2, *a* and *b*) and presence of retinoic acid (Fig. 2 *c*). No proteoglycan "granules," typical of cartilage and cartilage-like matrix, were ever observed. Fibrils were coaligned and assembled in coherent bundles in older cultures. Early foci of mineralization consisted of clusters of thin mineral crystals, exhibiting a filament or plate-like habit. At the edges of such clusters spreading of mineralization along collagen fibrils was obvious. Individual collagen fibrils with evidence of discontinuous mineralization along their axis were observed in cultures treated with retinoic acid (14 and 16 d), and in control cultures of embryonic osteoblasts grown out from bone chips (Fig. 2 *d*). The mineralization highlighted the fibril periodicity, as is commonly seen in early mineralization of bone matrix in vivo. Such individually mineralized fibrils were not detected in the absence of retinoic acid, in spite of the virtual identity of the morphology of the collagenous matrix produced in such cultures with that seen in retinoic acid-treated cultures.

Ovotransferrin Is Transiently Expressed by Hypertrophic Chondrocytes Undergoing Differentiation to Osteoblast-like Cells

The 63-kD glycoprotein secreted by differentiating hypertrophic chondrocytes was purified from conditioned culture medium by ammonium sulfate precipitation, DEAE cellulose chromatography, and HPLC reverse phase chromatography (Fig. 3). The amino acid sequence determination of its NH_2 terminus was performed by automated Edman degradation. The sequence of the first 25 amino acids was identical to the published sequence of the NH_2 terminus of the chicken ovotransferrin (7) (Fig. 4).

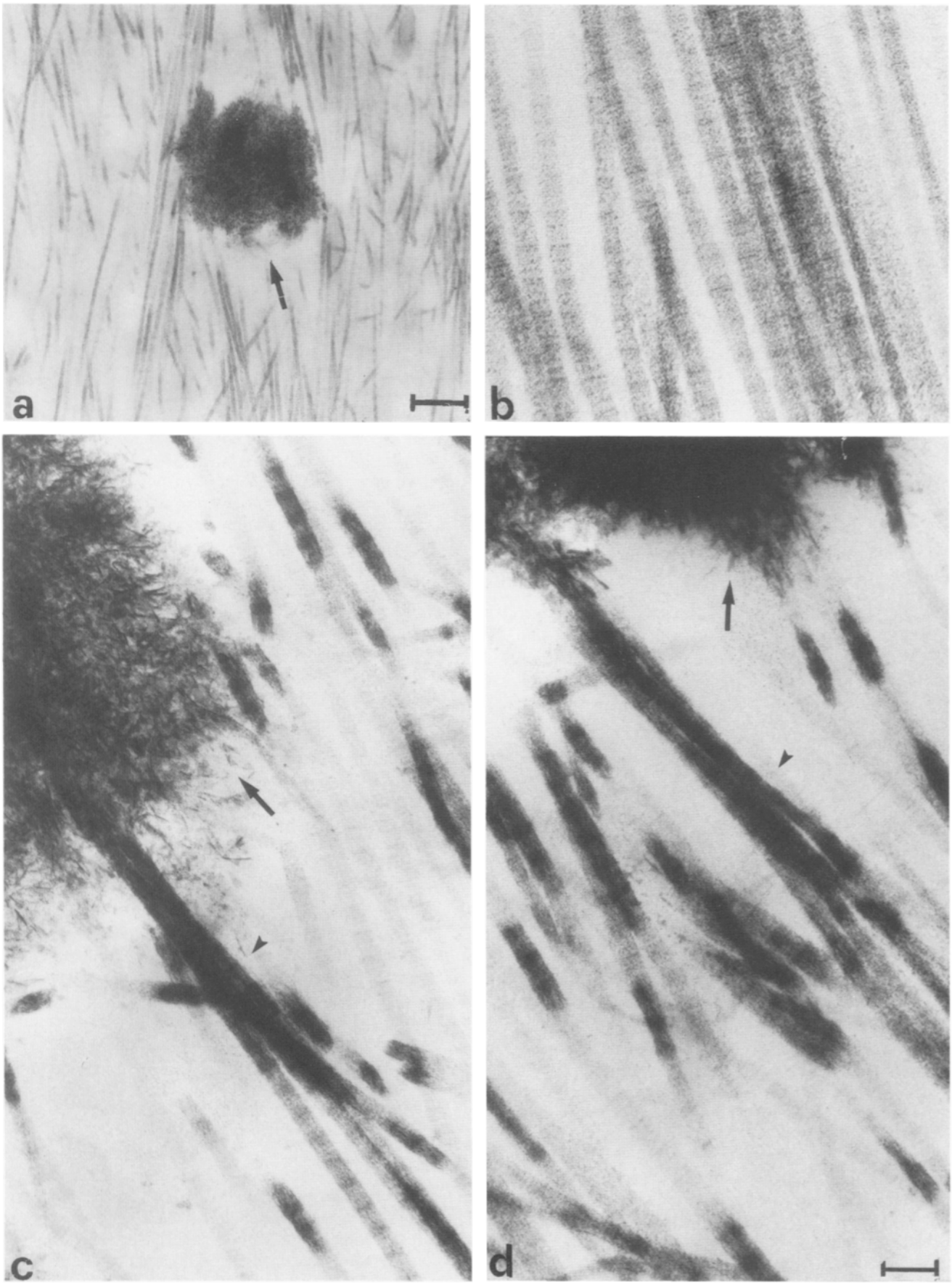
Ovotransferrin mRNA levels in hypertrophic chondrocytes replated as adherent cells and maintained in the presence of ascorbic acid for 0, 20, and 40 d were also determined (Fig. 5). Ovotransferrin mRNA was not detected in the starting hypertrophic chondrocyte population, reached a maximum level after 20 d in adherent culture in the presence of ascorbic acid and was barely detectable at later times. In the same cells, the level of type I collagen mRNA progressively increased while mRNA for Ch21, a carrier protein highly expressed by hypertrophic chondrocytes (9, 10), decreased. Interestingly osteopontin was highly expressed only at late culture times, when mineralization occurred. When the relative amount of the ovotransferrin secreted was investigated in a parallel labeled culture dish, it was found that, after induction, the protein decrease was somewhat slower than expected, based on the mRNA level (not shown). This suggests a possible heterogeneity in the differentiation timing of different culture dishes. Transient expression of the ovotransferrin protein was more clearly observed when chondrocytes were treated with retinoic acid, because this condition gives rise to a synchronous and homogeneous population of differentiating cells (see below).

Differentiation of Osteoblast-like Cells in a Cloned Cell Population

In some experiments, the starting hypertrophic chondrocyte population was derived from a single cloned dedifferentiated cell. The change in the pattern of proteins secreted by these hypertrophic chondrocytes grown adherent in the presence of ascorbic acid (Fig. 6) was the same observed in cultures of chondrocytes derived from primary cultures (11).

Retinoic Acid Enhances Differentiation of Hypertrophic Chondrocytes both at High and Physiological Concentration

We have previously shown that retinoic acid supplemented to the culture medium for 5 d at a relatively high concentration (1 μM) highly accelerated the maturation of hypertrophic chondrocytes to osteoblast-like cells (11). It has been suggested that the addition of retinoic acid at nonphysiological concentrations may cause artefacts and that the observed phenotypic changes may not reflect a true cell differentiation (34). In addition the pulse treatment of the cells may cause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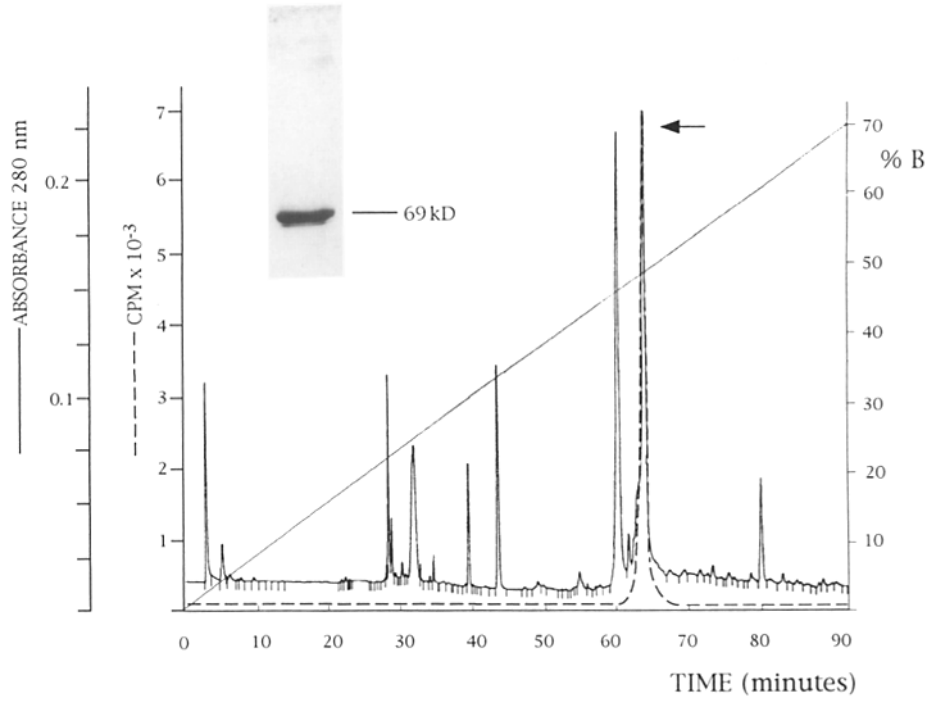

Figure 3. Purification of ovotransferrin from spent culture medium. Continuous line: densitometric profile (280 nm) of HPLC protein separation by reverse phase chromatography. Dashed line: profile of radioactivity. Solvent A: 0.1% trifluoroacetic acid in H_2O; solvent B: 0.1% trifluoroacetic acid in acetonitrile. Arrow indicates the only radioactive peak in the chromatogram. The electrophoretic analysis on 12.5% SDS-PAGE of the peak indicated by the arrow is shown in the insert. Electrophoresis was performed in unreducing conditions. Detection of proteins was performed by silver staining.

reversion of the phenotype when retinoic acid is removed. Therefore, we performed the experiments by keeping retinoic acid constant, at different concentrations, during culture.

The pattern of labeled proteins secreted by chondrocytes continuously treated with 50 nM retinoic acid is shown in Fig. 7. The pattern of proteins secreted by chondrocytes continuously treated with 500 nM retinoic acid is substantially identical, although the transition to the osteoblast-like pattern is slightly accelerated in the latter culture (not shown). Ovotransferrin was clearly transiently expressed in both cell cultures. Expression of ovotransferrin occurred at the switch from the synthesis of type II collagen to the synthesis of type I collagen (Fig. 8). The maximal expression was observed at day 3, when the pro α1 (II) band disappeared, the α1 (II) band was highly diminished and the type I procollagen was already detectable.

In some experiments retinoic acid was added at a 10-nM concentration. In this condition the differentiation of hypertrophic chondrocytes into osteoblast-like cells was not as rapid as in culture treated with 50 or 500 nM retinoic acid. After 15 d type X collagen was still expressed and the persistence of a large number of polygonal cells in the culture dishes was observed (not shown).

Ovotransferrin expression preceded the expression of alkaline phosphatase. In the retinoic acid–treated culture, alkaline phosphatase activity began to be detectable after ~1 wk and reached its maximal values after ~8–10 d (Fig. 9 *B*), 1–2 d before the onset of mineralization. At that time the culture presented a distinct pattern of cell growth with the appearance of a reproducible circular swirling pattern previously described in calvaria-derived osteoblast cultures (17). At the same time in the control untreated cultures the large majority of the chondrocytes presented a polygonal morphology and were alkaline phosphatase negative; the small percentage of cells positive for alkaline phosphatase activity presented a more elongated morphology and had a tendency to group in clusters (Fig. 9 *A*). It is to be noted that, when retinoic acid was present throughout the whole culture, mineralization was already observed after 9–11 d, well before the 3 wk required in the pulse treated cultures (11).

Expression of Ovotransferrin Receptors by Hypertrophic Chondrocytes Undergoing Differentiation to Osteoblast-like Cells

Cell lysates were prepared from hypertrophic chondrocytes plated as adherent cells and maintained in culture for 5 and 14 d in the presence of ascorbic acid. At the same time a lysate was also prepared from a culture maintained for 9 d in the presence of ascorbic acid and retinoic acid. The presence of the tissue (oviduct) specific transferrin receptor was inves-

Figure 2. Ultrastructure of extracellular matrix. (*a*) Overview of the collagenous matrix produced by adherent osteoblast-like cells grown in the absence of retinoic acid at 47-d culture after mineralization occurred. A patch of early mineralization is obvious (*arrow*). (*b*) Matrix produced by osteoblast-like cells in the absence of retinoic acid. Detail of a bundle of collagen fibrils with 64–70-nm periodicity. (*c*) Matrix produced and mineralized by osteoblast-like cells grown in the presence of 500 nM retinoic acid, 14 d. A patch of mineralization is shown (*arrows*), together with evidence of mineralization of individual collagen fibrils (*arrowheads*). (*d*) Demonstrates the pattern of matrix mineralization observed in cultures of chick embryo osteoblasts outgrown from bone chips. Note the identity of the patterns demonstrated in *c* and *d*. Bars: (*a*) 0.2 μm; (*b–d*) 0.1 μm.

1) APPKSVIRWXTISSPEEKKXNNLRD-
2) APPKSVIRWCTISSPEEKKCNNLRD-

Figure 4. Sequence of the amino terminal end of the purified 69-kD protein. (*1*) Amino-terminal sequence of the purified 69-kD protein; (*2*) amino-terminal sequence of chicken ovotransferrin (ref. 7); *X* indicates undetermined residues.

tigated by Western blot analysis (Fig. 10). The presence of a protein recognized by the anti-transferrin receptor and comigrating with the receptor from the oviduct (not shown) was clearly detectable in all three extracts.

Discussion

In the present study, we have extended our previous observation that hypertrophic chondrocytes undergo differentiation to osteoblast-like cells in vitro, when they are transferred to substrate-dependent culture conditions in the presence of ascorbic acid. Here we focused on the modulation of cell proliferation and on the extracellular matrix mineralization. In addition we described the ovotransferrin transient expression during the process.

After their plating on the adhesion permissive substratum, the cell doubling time was enhanced to ~4–7 d depending upon initial cell density. Addition of retinoic acid to the culture medium further enhanced cell proliferation. The measured doubling time was ~2 d. The dependence of cell division on cell shape and anchorage is a well known fact. In experiments where cells of the 3T3 cell line were either held in suspension or allowed to settle on patches of an adhesive material on nonadhesive substratum, it was observed that the patch diameter determines the extent of spreading of individual cells and the probability that they will divide (30).

Differentiating osteoblast-like cells stop proliferating when mineralization occurs. Type I collagen is the only collagen secreted in large amounts at that time. The data presented here demonstrate that the texture and organization of the matrix assembled by the osteoblast-like cells derived

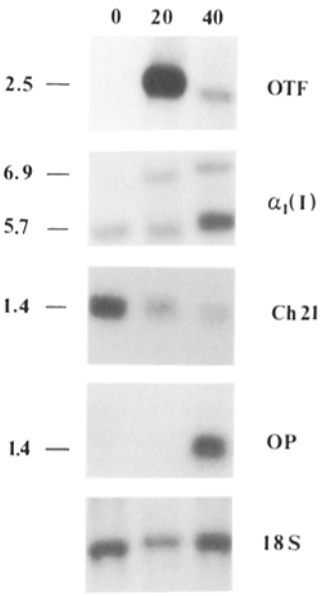

Figure 5. Northern blot of RNAs extracted from cultured chondrocytes. (*0*) Hypertrophic chondrocytes maintained in suspension culture; (20, 40) cells replated at confluence and maintained as adherent cells in the presence of ascorbic acid for additional 20 and 40 d. Numbers on the left refer to RNA size in kb.

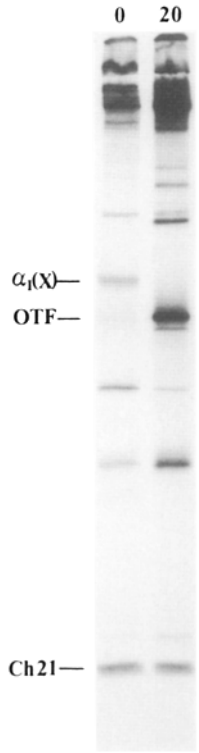

Figure 6. [^{35}S]methionine-labeled proteins secreted by cells derived from a cloned population of chondrocytes. Cells were maintained in medium supplemented with 100 μg/ml ascorbic acid and 10 mM β-glycerophosphate. Numbers refer to days in culture; *0* refers to suspension culture of hypertrophic chondrocytes at the time they were plated. OTF indicates ovotransferrin. Polyacrylamide gel concentration was 12.5%. Electrophoresis was performed in unreducing conditions.

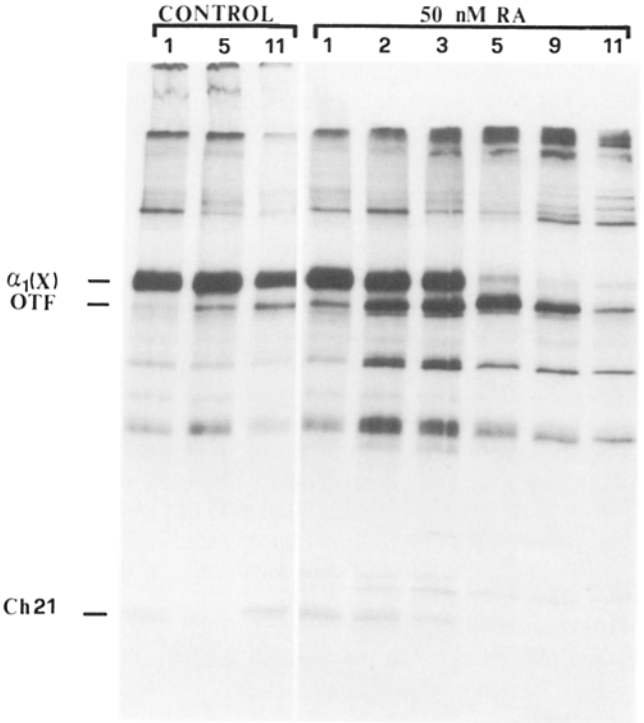

Figure 7. Proteins secreted by cultured chondrocytes treated with retinoic acid. Hypertrophic chondrocytes were replated as adherent cells and maintained in medium supplemented with ascorbic acid and β-glycerophosphate (*control*) or in the supplemented medium with the further addition of 50 nM retinoic acid. Numbers refer to days in culture. *OTF*, ovotransferrin. Polyacrylamide gel concentration was 15%. Electrophoresis was performed in unreducing conditions.

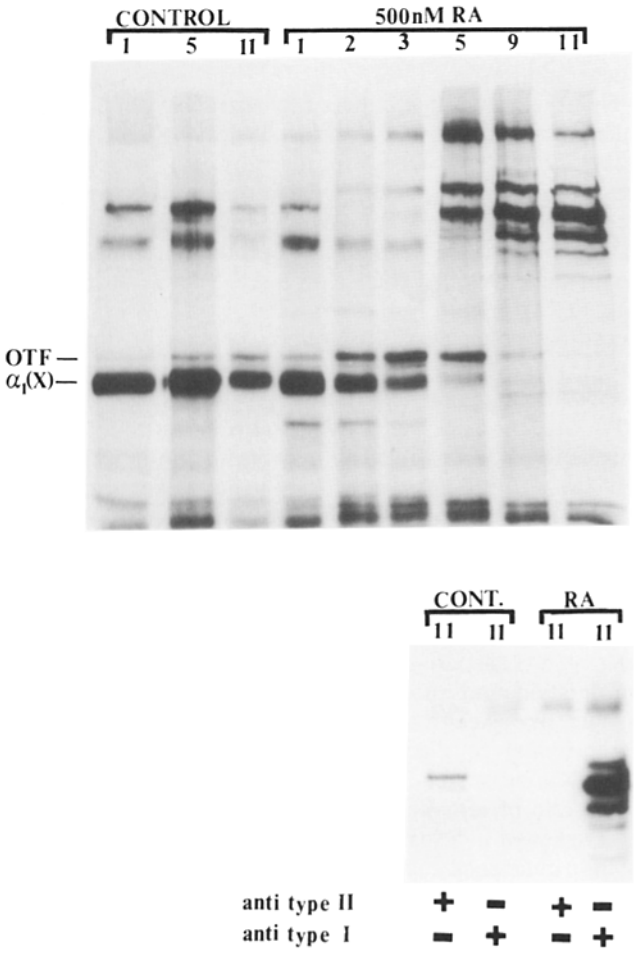

Figure 8. Proteins secreted by cultured chondrocytes treated with retinoic acid. Hypertrophic chondrocytes were replated as adherent cells and maintained in medium supplemented with ascorbic acid and β-glycerophosphate (*control*) or in the supplemented medium with the further addition of 500 nM retinoic acid. In the lower panel aliquots of [^{35}S]methionine-labeled culture media were immunoprecipitated with specific anti-collagen antibodies and run for protein analysis on SDS-PAGE. Numbers refer to days in culture. OTF indicates ovotransferrin. Polyacrylamide gel concentration was 7.5% Electrophoresis was performed in reducing conditions.

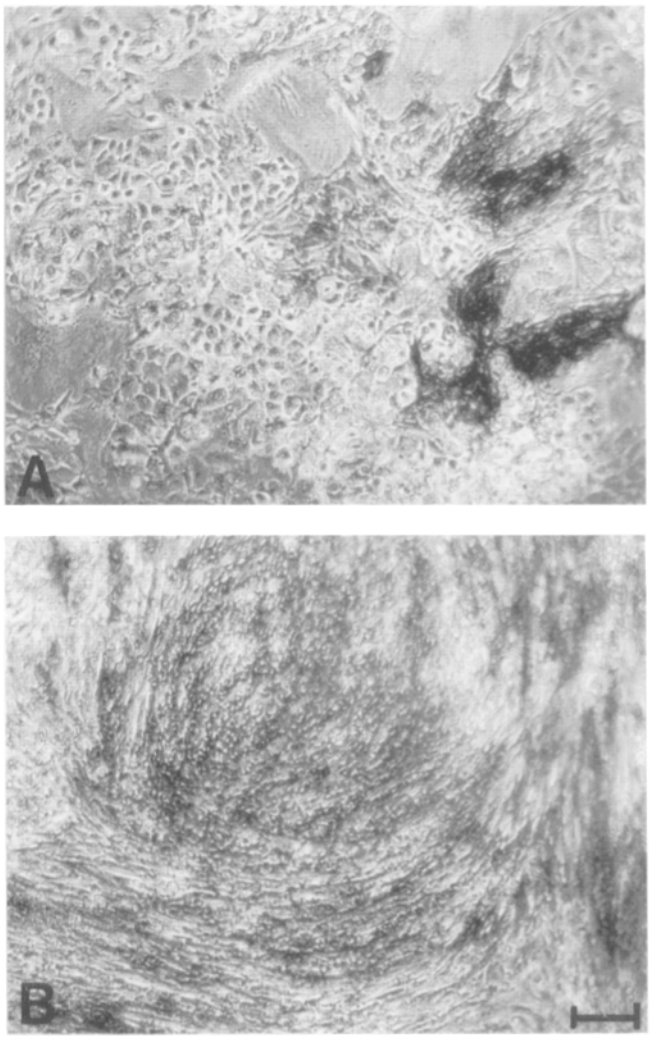

Figure 9. Expression of alkaline phosphatase in a retinoic acid–treated culture. Histochemical staining was performed 9 d after plating on chondrocytes maintained in the presence of 500 nM (*B*) or on control chondrocytes not supplemented with retinoic acid (*A*). Bar, 100 μm.

from hypertrophic chondrocytes closely resemble that observed in cultures of embryonic osteoblast grown out from bone chips (our results) or derived from embryonic calvariae (18), whereas they differ from the cartilage-like matrix observed in suspension cultures of hypertrophic chondrocytes (our own unpublished results) or in cultures of vertebral chondrocytes (16). In cultures performed both in the presence and absence of retinoic acid, patches of mineral aggregates characterize early stages of mineralization. Evidence of mineralization onto and within periodically banded collagen fibrils is detected in both types of culture. Interestingly, such evidence is much less obvious in cultures of osteoblast-like cells in the absence of retinoic acid, in spite of the similar morphology of the collagenous matrix produced with and without retinoic acid. Whether this reflects an effect of retinoic acid on the rate of mineralization per se or, more likely, is the result of the effect of retinoic acid on cell differentiation remains to be determined.

In culture, a major expression of alkaline phosphatase activity is observed before mineralization. In addition, osteopontin is highly expressed at the time mineralization occurs. The same sequence of events, i.e., alkaline phosphatase activation, arrest in cell proliferation, osteopontin expression and deposition of mineral in the extracellular matrix on type I collagen fibers was observed also in cultures of differentiating osteoblasts (17, 31).

The finding that the hypertrophic chondrocyte to osteoblast-like cell transition is detectable in cultures derived from cloned cells, together with the rapid (2–3 d) phenotypic change observed in retinoic acid treated cultures, are in agreement with the existence of an additional maturation step of hypertrophic chondrocytes and strongly against the possibility of a selection during culture of existing cell subpopulations.

Our results demonstrate that hypertrophic chondrocytes undergoing differentiation to osteoblast-like cells transiently express high levels of transferrin. Transferrins are a group of glycosylated, iron binding, 80-kD proteins (7, 8). Trans-

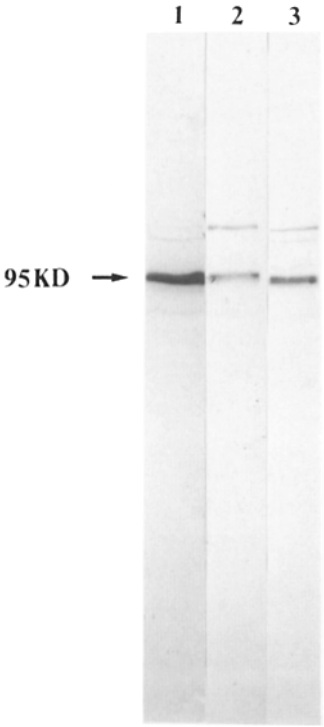

Figure 10. Immunoblot analysis of transferrin receptor. Cell lysate from hypertrophic chondrocytes plated as adherent cells and maintained in the presence of ascorbic acid for 5 (lane *1*) and 14 (lane *2*) d were run on 10% SDS-polyacrylamide gel, blotted to nitrocellulose filter and subjected to immunoblot analysis using αOV-TfR polyclonal antibodies. In the blot the lysate from the same cells grown adherent in the presence of ascorbic acid and with the further supplement of 500 nM retinoic acid was also analyzed (lane *3*). Arrow refers to the characteristic 95-kD oviduct transferrin receptor recognized by the antibodies.

port and delivery of iron to target cells is the first function ascribed to transferrin. Transferrin receptors are associated with virtually all actively proliferating cells, but are expressed at significantly higher levels on certain cell types such as immature erythroid cells, due to the special iron requirement for hemoglobin synthesis (5, 28). We have observed an enhancement of the cell proliferation rate during the differentiation process of hypertrophic chondrocytes to osteoblast-like cells. A role of ovotransferrin in the control of the cell proliferation rate during the process, possibly by autocrine mechanisms, could therefore be postulated. Here, the word autocrine is used to mean a self-regulatory activity and not necessarily a signal transduction since, after the binding of the ligand to transferrin receptors, a signal transduction has not been demonstrated yet.

Transferrin and its receptor play a role in embryonic morphogenesis. Ekblom and colleagues have shown that, in rat organ cultures of both developing kidneys and teeth, transferrin is required for cell proliferation and differentiation (13, 35). Inductor, mesenchyme, and transferrin must all be simultaneously present for acquisition of transferrin responsiveness. Organ-cultured mesenchymal cells are not initially responsive to transferrin, but they acquire responsiveness as a consequence of a short-term inductive tissue interaction; transferrin is required for subsequent proliferation and terminal differentiation (12). In both organs progression of morphogenesis is characterized by changes in the proliferation rate of different cell populations and overt cell differentiation is preceded by active cell proliferation (39, 40). Transferrin is produced by liver and yolk sac and is present in the serum of 11-d-old mouse embryos at the time when kidney differentiation starts (12). A local source for transferrin at this stage of embryogenesis was not shown in kidneys.

During amphibian limb regeneration, the content of transferrin is highly increased in adult sciatic nerves. The growth factor is then axonally transported and distally released by regenerating nerves (24).

We have shown that cultured hypertrophic chondrocytes undergoing transition to osteoblast like cells also express the tissue-specific ovotransferrin receptor. We consider the coexpression by the cells of the ovotransferrin and of its receptor a strong suggestion of autocrine mechanisms controlling hypertrophic chondrocyte proliferation and/or differentiation. Detailed studies on the in vivo developmental regulated expression of ovotransferrin and ovotransferrin receptors, and the possible interference of the in vitro chondrocyte differentiation by the addition of both the factor and the specific antibodies, is currently being studied.

We have evidence that a transition of hypertrophic chondrocyte to osteoblast-like cell may occur also in vivo at the chondro–osseous junction of long bones (Galotto, M., G. Campanile, G. Robino, F. Descalzi Cancedda, P. Bianco, and R. Cancedda, manuscript in preparation). Given the direct effect of transferrin on the proliferation of induced mesenchymes and in tissue morphogenesis, it may be postulated that, by paracrine mechanisms, hypertrophic chondrocytes at the chondro–osseous junction induce or enhance proliferation and osteoblastic differentiation of the embryonic periosteum mesenchyme and/or undifferentiated mesenchymal cells accompanying the ingrowth of metaphyseal blood vessels.

The role of retinoic acid in cartilage differentiation has been reviewed in a variety of articles including ref 11. Here we have confirmed and extended our previous report that at later stages of chondrocyte differentiation retinoic acid promotes maturation of chondrocytes to stage II (hypertrophic) and stage III (osteoblast-like) (11). This is in agreement with the observation made by other authors that retinoic acid induces differentiation in cultured chondrocytes (29, 34). In particular we observed that the continuous presence of retinoic acid at a physiological concentration (i.e., a concentration similar to the concentrations determined in the posterior chick limb bud whereas lower concentration was present in the anterior limb bud) (42) in the culture medium accelerates the chondrocyte differentiation process toward the organization of a mineralized bone-like matrix in a manner comparable with the acceleration observed when high concentrations of retinoic acid are present.

In our culture system, retinoic acid has an inductive effect on ovotransferrin expression. Levels of transferrin and transferrin mRNA are responsive to added retinoids also in Sertoli cells (21). Retinoic acid responsive elements can be identified by computer assisted search on transferrin promoters (19). It is interesting to note that the Ch21 protein, a newly described marker of hypertrophic chondrocytes belonging to the lipocalin family (extracellular transport proteins for small hydrophobic ligands, such as retinoids and some of its metabolites) (9, 10), is maximally expressed by chondrocytes before the highest level of ovotransferrin expression is reached.

Although it is generally believed that hypertrophic cells are terminally differentiated cells unable to undergo any further differentiation, the alternate view that hypertrophic chondrocytes can transdifferentiate to either osteoblasts or marrow stromal cells has long been held. In vitro cultures

of cartilage pieces of different origin have shown that hypertrophic chondrocytes may have osteogenic potential (23, 37, 41, 43, 46). In different species, hypertrophic chondrocytes express in vivo proteins characteristic of the "osteoblast phenotype" (1, 14, 26, 27, 32, 33, 38). A peculiar form of ossification suggestive of a deregulated maturation of chondrocytes into osteoblast-like cells has been described in Thanatophoric Dysplasia, the most common of the lethal neonatal bone dysplasias in man (20).

We have presented the first evidence that hypertrophic chondrocytes in culture undergo differentiation to osteoblast-like cells; however, to prove that the phenotype switch from hypertrophic chondrocytes to osteoblasts occurs in vivo additional experiments are required. The hypothesis can be made that cellular cross-talk between preosteoblasts and hypertrophic chondrocytes, mediated by extracellular matrix and/or secreted factors as ovotransferrin and Ch21, triggers the deposition of the first bone, and qualifies it as a joint endeavor of hypertrophic chondrocytes and osteoblasts.

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