Journal of Cellular Biochemistry 49:310–323 (1992)

Expression of Cell Growth and Bone Specific Genes at Single Cell Resolution During Development of Bone Tissue-Like Organization in Primary Osteoblast Cultures

Shirwin M. Pockwinse, Laurens G. Wilming, Donna M. Conlon, Gary S. Stein, and Jane B. Lian

Department of Cell Biology, University of Massachusetts Medical Center, Worcester, Massachusetts 01655

Abstract Primary cultures of calvarial derived normal diploid osteoblasts undergo a developmental expression of genes reflecting growth, extracellular matrix maturation, and mineralization during development of multilayered nodules having a bone tissue-like organization. Scanning electron microscopy of the developing cultures indicates the transition from the uniform distribution of cuboidal osteoblasts to multilayered nodules of smaller cells with a pronounced orientation of perinodular cells towards the apex of the nodule. Ultrastructural analysis of the nodule by transmission electron microscopy indicates that the deposition of mineral is confined to the extracellular matrix where cells appear more osteocytic. The cell body contains rough endoplasmic reticulum and golgi, while these intracellular organelles are not present in the developing cellular processes. To understand the regulation of temporally expressed genes requires an understanding of which genes are selectively expressed on a single cell basis as the bone tissue-like organization develops. In situ hybridization analysis using ^{35}S labelled histone gene probes, together with ^{3}H-thymidine labelling and autoradiography, indicate that greater than 98% of the pre-confluent osteoblasts are proliferating. By two weeks, both the foci of multilayered cells and internodular cell regions have down-regulated cell growth associated genes. Post-proliferatively, but not earlier, initial expression of both osteocalcin and osteopontin are restricted to the multilayered nodules where all cells exhibit expression. While total mRNA levels for osteopontin and osteocalcin are coordinately upregulated with an increase in mineral deposition, in situ hybridization has revealed that expression of osteocalcin and osteopontin occurs predominantly in cells associated with the developing nodules. In contrast, proliferating rat osteosarcoma cells (ROS 17/2.8) concomitantly express histone H4, along with osteopontin and osteocalcin. These in situ analyses of gene expression during osteoblast growth and differentiation at the single cell level establish that a population of proliferating calvarial-derived cells subsequently expresses osteopontin and osteocalcin in cells developing into multilayered nodules with a tissue-like organization. © 1992 Wiley-Liss, Inc.

Key words: osteocalcin, histone, osteopontin, vitamin D, transcription, oncogene, chromatin structure, nuclear matrix, tumor cells

The ability to culture normal diploid calvarial cells under conditions that support progressive development of the osteoblast phenotype and a bone tissue-like organization has provided a viable in vitro model system for examining the selective expression of genes and physiological signalling mechanisms that mediate osteoblast growth and differentiation [Owen et al., 1990; Aronow et al., 1990]. The tissue-like organization in vitro is reflected by the progressive development of nodules of multilayered cells in a mineralized extracellular matrix with orthogonally organized type I collagen fibrils. The ordered deposition of mineral within the collagen fibers initiates and is primarily associated with the nodule areas [Stein et al., 1990, 1992; Lian et al., 1992]. Both mammalian [Aronow et al., 1990; Owen et al., 1990] and avian [Gerstenfeld et al., 1987, 1988] osteoblast culture systems have been utilized over the past several years to study a broad spectrum of parameters associated with bone cell growth and differentiation that include but are not restricted to steroid hormone responsiveness, growth factor requirements, and expression of genes related to proliferation and properties of the developing and mature osteoblast [Rodan and Noda, 1991].

Received February 13, 1992; accepted March 26, 1992.

© 1992 Wiley-Liss, Inc.

By the combined application of molecular and histochemical approaches, a sequential expression of genes has defined a developmental sequence in primary cultures of normal diploid osteoblasts that contains three principal periods and two transition points where signalling mechanisms in the regulation of osteoblast phenotype expression are operative [Owen et al., 1990; Stein et al., 1990]. Initially, during the first period, actively proliferating osteoblasts express a series of genes supporting cell growth and extracellular matrix biosynthesis, including histone, TGFβ, fibronectin, and collagen. At the first transition point with the down-regulation of proliferation, expression of genes involved with the maturation and organization of an extracellular matrix competent for mineralization is initiated (e.g., alkaline phosphatase mRNA and enzyme activity peaks). Then, at the second principal transition point, extracellular matrix mineralization is initiated, and defines the onset of the third period, at which time expression of genes which include osteopontin and osteocalcin are up-regulated.

Two key questions that necessitate resolution are concerns of the potential heterogeneity of the isolated cells that would generate discrete bone-like nodules and, second, the extent to which temporal expression of genes and peak periods of gene expression coincide with discrete areas of cellular organization or a reflection of a uniform level of expression in all cells throughout the culture. Thus, in order to address the molecular mechanisms operative in primary cultures of osteoblasts as the bone cell phenotype progressively develops, it was necessary to establish the extent to which cell growth and osteoblast-related genes were expressed within the context of the developing bone tissue-like organization. We therefore utilized in situ hybridization to examine histone, osteocalcin, and osteopontin gene expression at the single cell level during key periods of the osteoblast developmental sequence in relationship to modifications in cell ultrastructure and organization as revealed by transmission electron microscopy and scanning electron microscopy. In proliferating normal diploid osteoblasts all cells expressed the cell cycle regulated H4 histone genes; osteocalcin was not observed in proliferating osteoblasts by in situ hybridization. Post-proliferatively, the expression of osteopontin and osteocalcin was restricted to the developing and mature nodules. In contrast to the normal diploid cells, cultured osteosarcoma cells expressed concomitantly both the proliferation and the differentiation-related genes and this occurs in the absence of development of tissue organization. The reciprocal relationship observed between expression of cell growth genes in proliferating diploid cells and expression of osteoblast-related genes during the onset and progression of bone cell differentiation and tissue organization is abrogated in osteosarcoma cells. These findings point to an important biological contribution of in situ hybridization to define the selective expression of genes encoding the osteoblast phenotype in relation to the individual osteoblast during different stages of tissue development.

METHODS

Cell Culture

Calvaria from fetal rats of 21 days gestation were isolated and subjected to sequential digestions of 20, 40, and 90 min at 37°C in 2 mg/ml collagenase A (Boehringer-Mannheim, Indianapolis, IN) with 0.25% trypsin (Gibco, Grand Island, NY). The cells of the first two digests were discarded, and those released from the third digestion were plated in minimal essential medium (MEM; Gibco) supplemented with 10% fetal calf serum (FCS) 50 μg/ml ascorbic acid, and 10 mM β-glycerol phosphate on 22mm Thermanox coverslips (U.S.A. Scientific, Ocala, FL) which were pre-cleaned in 70% ETOH for 24 h. Media was changed every 2 days throughout the 35 day culture period. ROS 17/2.8 cells, provided to us by Drs. Sevgi and Gideon Rodan, were cultured in DMEM supplemented with calcium and 5% fetal calf serum as described by Majeska et al. [1980].

Autoradiography for ^{3}H-Thymidine Labelled Cells

For in situ autoradiography, ^{3}H-thymidine was added to the medium of cells growing on 22 mm glass coverslips to (1 μCi/ml) for 24 h. Cells were then rinsed twice in ice-cold PBS and fixed in absolute methanol (−20°C). Coverslips were then air dried overnight or first stained for alkaline phosphatase mineral by von Kossa stain and 0.5% toluidine blue as previously described [Aronow et al., 1990] and dried. Autoradiography was performed using Ilford K5 emulsion as described by Baserga and Malamud [1969]. Exposures were for 5–7 days at 4°C [Owen et al., 1990].

In Situ Hybridization

Cells were rinsed twice in Hank's Balanced Salt Solution, fixed in 4% paraformaldehyde in PBS for 15 min at RT, rinsed, and stored in 70% ethanol at 4°C for at least 24 h. The probes used for hybridization were rat H4 histone [Grimes et al., 1987], rat osteocalcin [Lian et al., 1989], and rat osteopontin [Oldberg et al., 1986]. Probes were prepared by nick translation, labelled with ^{35}SdCTP, and passed through a G50 Sephadex spin column. The specific activity ranged from 1 to 3 × 10^8 cpm/μg. A sample of each probe was then sized by electrophoresis in a denatured agarose mini-gel.

In preparation for hybridization, the coverslips were rehydrated for 10 min in PBS and 5 mM $MgCl_2$ and either stored in 50% formamide, 2× SSC (0.3 M sodium citrate, 3.0 M sodium chloride pH 7.25) awaiting hybridization or were RNase treated as a negative control. For the RNase controls, RNase A (100 μg/ml in 2× SSC) was boiled for 10 min prior to use. After placing a 25 μl drop of RNase A on a parafilm covered glass plate, the coverslip was placed face down on the drop, and the plate was covered with parafilm and incubated 1 h at 37°C. The coverslips were washed five times in 2× SSC and stored in 50% formamide, 2× SSC until hybridization.

Hybridization was carried out in 5 M dithiothreitol, 50% dextran sulfate, 2% bovine serum albumin, 20× SSC, and 1.0 M sodium phosphate (pH 6.0). An equal volume of hybridization mix was added to the denatured probe, avoiding air bubbles. Working quickly, a 20 μl aliquot of hybridization mix/probe per coverslip was pipetted on a prewarmed (40–50°C) parafilm covered glass plate. A coverslip was placed face down on the drop. The plate was covered with parafilm and incubated at 40°C for H4 histone, 42°C for osteocalcin, and 50°C for osteopontin for 3 h. The coverslips were washed for 30 min in 2× SSC, 50% formamide at 42°, 45°, and 50°C for H4, OC, and OP, respectively. The second and third washes were 15 min in 1× SSC at room temperature on a shaker. Coverslips were dehydrated for 2 min each in 70%, 95%, and 100% ethanol. They were then dried, mounted face up on a 3 × 1 microscope slide and cured overnight.

Autoradiography for ^{35}S Labelled Cells

Following hybridization, coverslips were prepared for autoradiography by melting 10 ml Ilford K5 emulsion at 42°C for 20 min, followed by addition of 1 volume of 0.6 M ammonium acetate. The coverslips were dipped and dried upright for 2 h at room temperature without a safelight. Slides were placed in a light-tight slide box with drierite and exposed for 2–3 weeks at 4°C. The slides were developed in 50% Kodak D19 for 5 min at 15°C, fixed, washed, and dried. Preparations were then stained in 0.5% Toluidine blue, washed in 50 mM $MgCl_2$ then distilled water, and dried.

Transmission Electron Microscopy

Fetal rat calvaria and coverslips with cultured rat osteoblasts were fixed in 3% glutaraldehyde in 0.1 M sodium cacodylate buffer, washed, postfixed in 1% osmium tetroxide, rinsed, dehydrated, and embedded in Poly/Bed 812. Sections were cut, stained in uranyl acetate and lead citrate, and examined in a JeoL 100-S electron microscope.

Scanning Electron Microscopy

Coverslips with cultured rat osteoblasts were fixed with 1% glutaraldehyde in 0.1 M sodium cacodylate buffer, rinsed, postfixed in 1% osmium tetroxide, dehydrated, critical point dried, and coated with gold palladium in a Technics Hummer Sputter Coater (Anatech, Alexandria, VA). Preparations were examined in an ETEC Autoscan scanning electron microscope.

RESULTS

Parameters of Osteoblast Proliferation and Differentiation

To utilize in situ hybridization for assessing the sequential expression of genes during development of the osteoblast phenotype at the single cell level, it was necessary to culture the cells on plastic (Thermanox) coverslips. Therefore, we initially characterized the growth and formation of mineralized nodules on Thermanox coverslips. The temporal expression of alkaline phosphatase followed by osteocalcin with mineralization paralleled that observed on tissue culture plastic. Figure 1A illustrates the expression of genes during the three principal periods of the osteoblast developmental sequence: expression of histone and collagen during the initial period of proliferation and extracellular matrix biosynthesis; expression of alkaline phosphatase postproliferatively during the period of extracellular matrix maturation and organization; and expres-

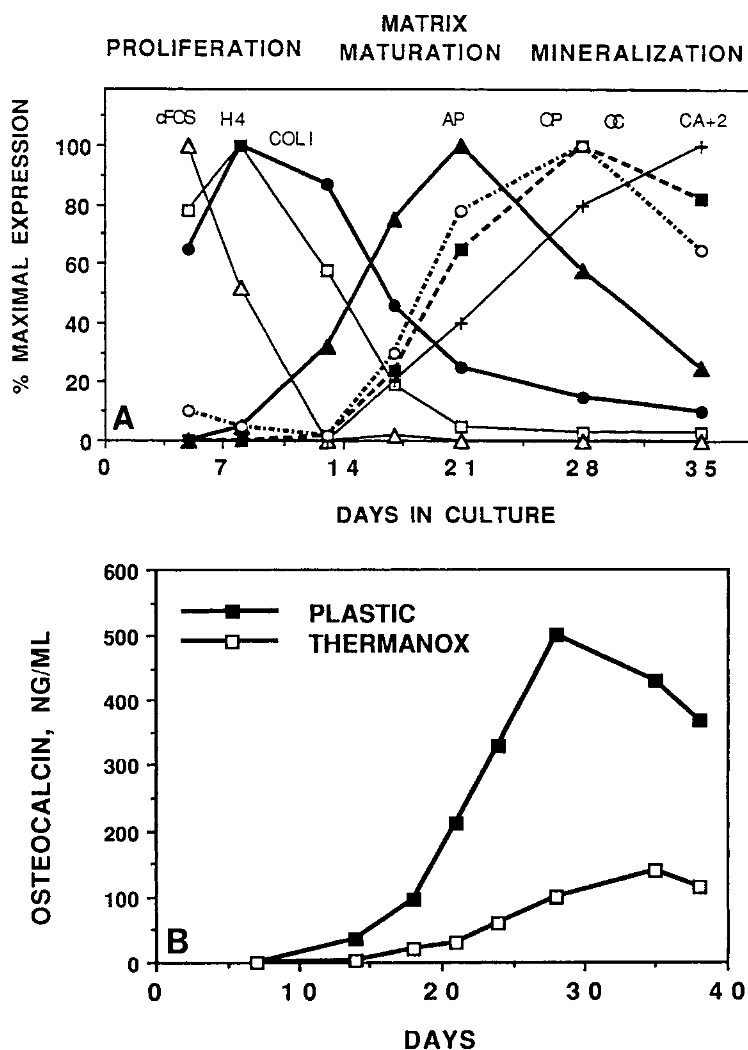

Fig. 1. Temporal expression of cell growth and osteoblast phenotype related genes during development of in vitro formed bone-like tissue by normal diploid rat osteoblasts. **A:** Quantitation of mRNA levels of osteoblast expressed genes. Total cellular RNA was isolated from cultures on days 5, 8, 13, 18, 21, 28, and 35 for hybridization by Northern blot analysis using radiolabelled probes for cell growth H4 histone, extracellular matrix (type I collagen), and osteoblast phenotype related genes (alkaline phosphatase AP, osteopontin OP, osteocalcin OC) as described in Owen et al. [1990]. Mineral deposition was quantitated by calcium content of the cell layer. **B:** A comparison of osteocalcin synthesis, measured by radioimmunoassay of media from cells cultured on tissue culture 24 mm plastic dishes (■) and Thermanox cover slips in 24 mm wells (□).

sion of osteopontin and osteocalcin during the third period when mineralization of the mature osteoblast extracellular matrix is ongoing. On the Thermanox coverslips, cell growth lagged behind that of the tissue culture plastic; however, the same temporal pattern of alkaline phosphatase activity and osteocalcin synthesis was observed and the same morphologic organization of mineralized nodules developed. A comparison of these key parameters of bone cell differentiation is seen in Figures 1 and 2. Figure 1B compares the level of osteocalcin synthesis between the two cultures and reflects the decreased size of mineralized nodules on Thermanox (Figure 2). On Thermanox coverslips nearly every cell became alkaline phosphatase positive and mineralization is initiated in the nodule area in a manner identical to the mineralization observed in cultures on tissue culture plastic (Figure 2).

Transmission Electron Microscopy of Normal Diploid Osteoblast Cultures

Transmission electron microscopy of normal diploid osteoblasts in primary culture indicates that the cells exhibit a morphology character-

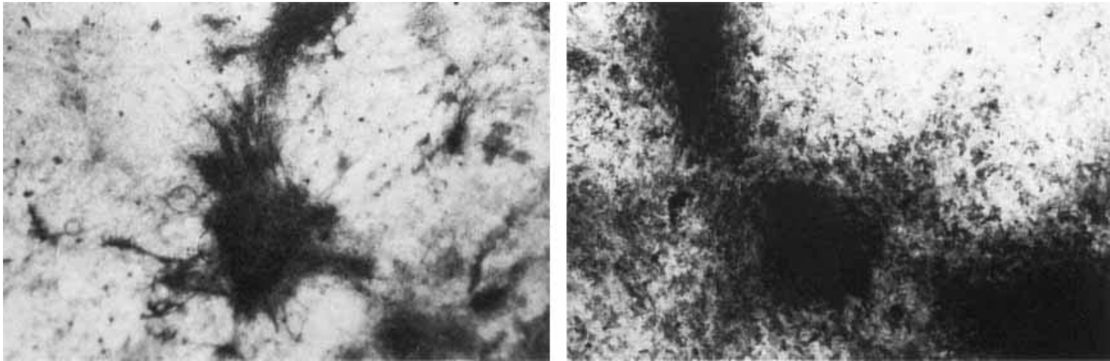

Fig. 2. Comparison of osteoblast growth and differentiation on Thermanox coverslips (**left**) and tissue culture plastic (**right**). Shown are cells cultured 17 days. The combination of alkaline phosphatase histochemical stain with von Kossa silver nitrate and toluidine blue reveals cellular areas and mineralized nodules. Note a similar nodule growth pattern and developing tissue organization on both the Thermanox and tissue culture plastic. However, the nodules are more advanced in size on the dish compared to Thermanox. ×21.

ized by distinct changes in cell shape and orientation in relation to the developing nodule during the progressive differentiation of the osteoblast phenotype. As illustrated in Figure 3A, in day 12 cultures, the majority of the cells isolated from the third enzymatic digest of 21 day fetal rat calvaria exhibit the cuboidal morphology characteristic of osteoblasts in bone [Holtrop, 1990]. Formation of the extracellular matrix is reflected by the presence of fine collagen fibrils being actively secreted by these osteoblasts. Such cells were still observed after 38 days of culture (Fig. 3B), but were confined to the internodule region and exhibited a marked increase in the representation of glycogen-containing vesicles. The cells in the multilayered nodule have undergone extracellular matrix mineralization and have the appearance of a young osteocytic-like cell. They contain abundant and well-organized granular endoplasmic reticulum, a large golgi, lysosomal bodies, and some lipid deposits. Cytoplasmic processes are being initiated and appear lacking the extensive cytoplasmic organelles present in the main cell body but containing microfilaments as observed in vivo [Holtrop, 1990] (Fig. 3C). The overall organization of the culture is reflected by an alignment and orientation of the cells, with respect to the nodules, at the periphery of the mineralized nodule (Fig. 3D). Note that these cells are flattened and elongated with large euchromatic nuclei (i.e., *reflecting* active transcription of genes).

Scanning Electron Microscopy of Normal Diploid Osteoblast Cultures

The surface morphology of the osteoblast cultures is clearly illustrated by the scanning electron micrographs in Figure 4. Typical prophase and telophase mitotic cells are shown. These early stage proliferating osteoblasts have the characteristic cuboidal morphology and the absence of polarity or orientation. Post-proliferatively, as the organization of the extracellular matrix is ongoing and the cell density has significantly increased, the initial orientation of cells with respect to the nodule is visible. Then, in the mature culture undergoing mineralization of the extracellular matrix (Fig. 4C–F) in the multilayered nodules, the orientation of the cells toward the nodule is pronounced. The 10× magnification (Figure 4C) illustrates the overall organization of the mineralizing culture, while the three panels showing the nodule at higher magnifications (Fig. 4D–F) illustrate both the orientation of internodular cells towards the apex of the nodule and the rough surface morphology resulting from deposition of mineral.

In Situ Hybridization Analysis of Cell Growth and Differentiation-Associated Genes in Normal Diploid Osteoblast Cultures

Since in primary cultures, an apparently homogeneous population of proliferating calvarial-derived cells progressively differentiate to cells with characteristic osteocytic morphology within multilayered nodules exhibiting bone tissue-like organization, it is necessary to determine at the single cell level which cells express specific genes characteristic of both the developing and mature osteoblast phenotype during the differentiation process. We therefore carried out in situ hybridization analysis of the cultures using an H4 histone gene probe to monitor proliferation, based upon the tight coupling of histone gene

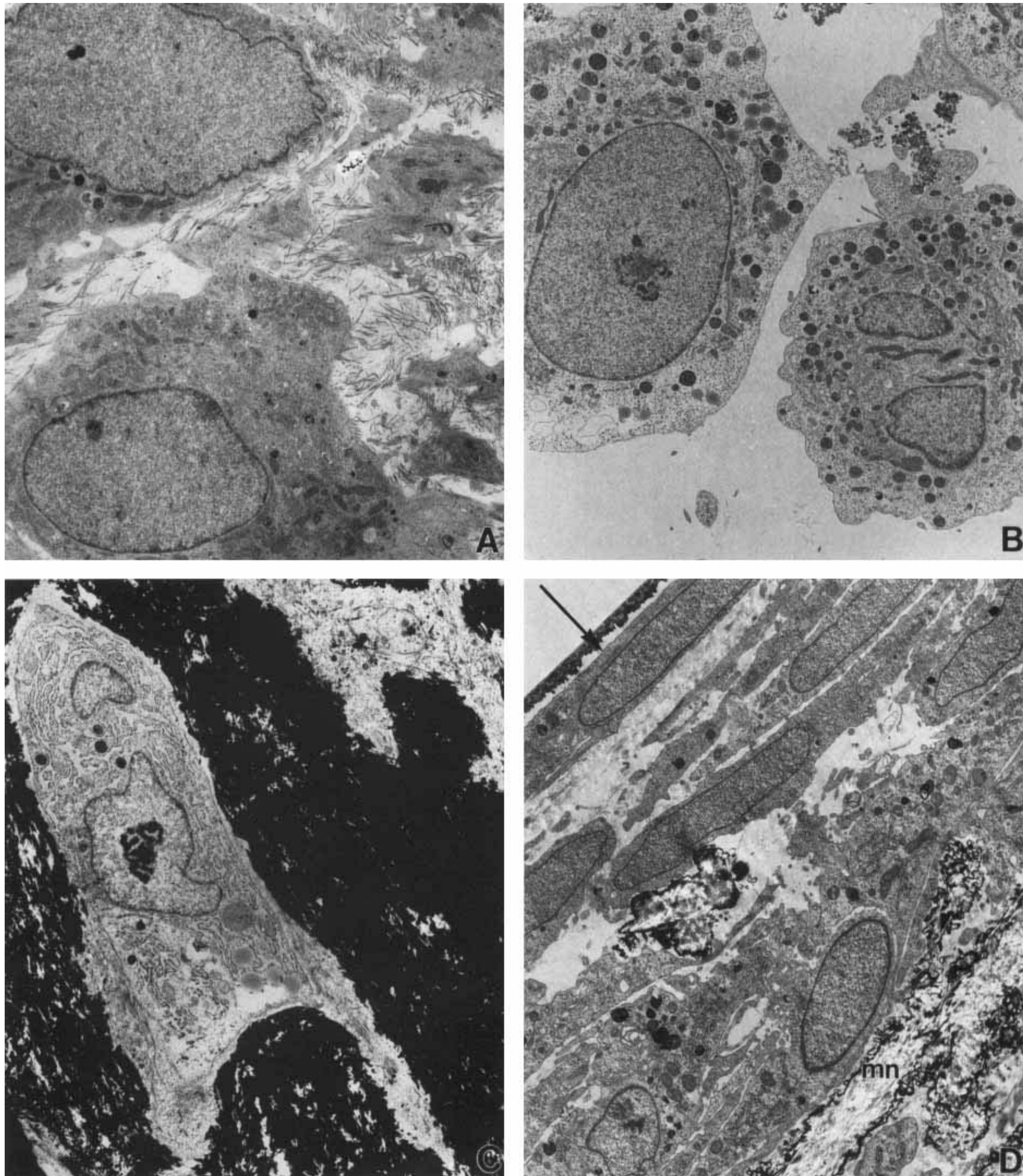

Fig. 3. Ultrastructural detail of cellular differentiation in cultured osteoblasts. **A:** Typical large cuboidal cells which are actively secreting collagen fibrils. ×4,250. **B:** Cuboidal shaped osteoblasts can still be found after 38 days of culture, but are confined to the internodular areas. ×4,373. **C:** In day 38 cultures the multilayered nodules have undergone extracellular matrix mineralization and cells have the appearance of a young osteocytic-like cell. ×6,000. **D:** The cells immediately adjacent to and surrounding a mineralized nodule (mn) are flattened, elongated, and parallel (arrow) with respect to each other. ×7,000.

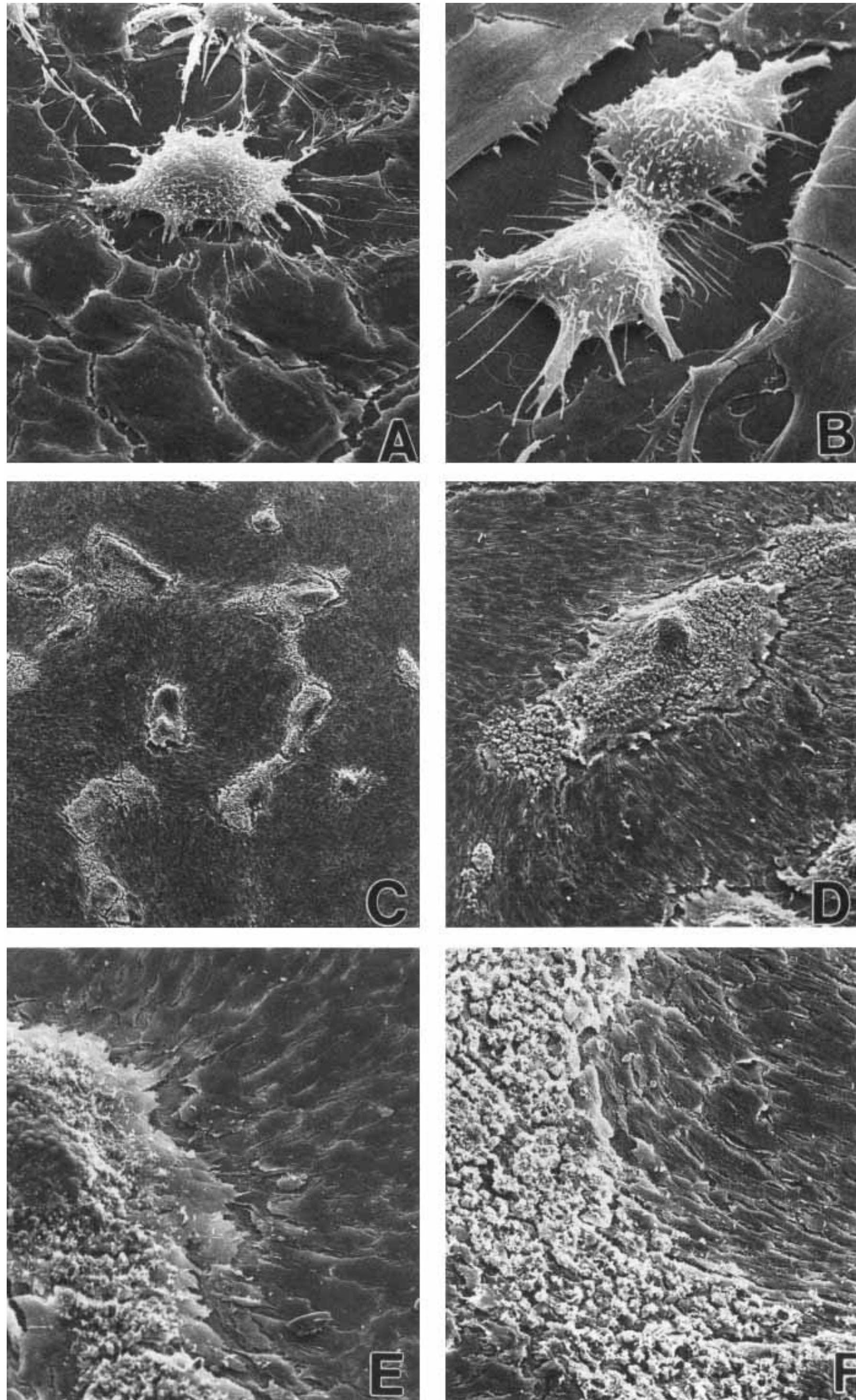

Fig. 4. Surface morphology of rat osteoblast cultures and nodules by SEM. **A,B:** Proliferation period (day 7) cells exhibit a uniform distribution with flattened morphology. Mitotic cells are evident throughout the culture. A, prophase. ×630. B, telophase. Mag = ×1,750. **C:** Overall organization showing formation of the multilayered nodules undergoing mineralization (day 18). ×10. **D,E:** Nodules at higher magnification illustrating the orientation of the cells towards the apex of the nodule. D, ×30. E, ×110. **F:** The rough surface morphology resulting from deposition of mineral is shown. ×110.

expression to DNA synthesis. Similarly, hybridization analysis was carried out using osteopontin and osteocalcin gene probes based on expression of these two genes in association with extracellular matrix maturation and mineralization. These comparisons can provide a direct indication of the spatial distribution of cells expressing specific genes in the developing cultures, in addition to the extent to which expression of these genes is restricted to particular developmental periods. Figure 5A–C demonstrates the extent of proliferative activity during the initial 18 days of the culture period as reflected by in situ hybridization using the cell cycle regulated H4 histone gene probe. It is evident that during the early proliferative period intense cytoplasmic labelling is apparent in 50% of the cells. Day 7 is represented in Figure 5A. Since histone mRNA is present only during the S-phase of the cell cycle, which is 9 h of the 20 h doubling time for these cells, the 50% labelling indicates that 98% of the cells are actively proliferating. In contrast, the absence of proliferative activity on day 18 is indicated by the absence of cells hybridizing with the radiolabelled histone gene probe in either the nodules (Fig. 5B) or internodular region of the culture (Fig. 5C). Since high magnifications are required for visually illustrating in situ hybridization labelled cells, Figure 6 shows ^{3}H-thymidine labelled cells at low magnification to illustrate the initial uniform proliferation of cells throughout the culture dish. At monolayer confluency, day 8, proliferation continues in focal areas producing the nodule (Fig. 6A,B). A few days later, proliferation is subsiding in internodular regions (Figure 6C) and ceases initially in the center of the nodules.

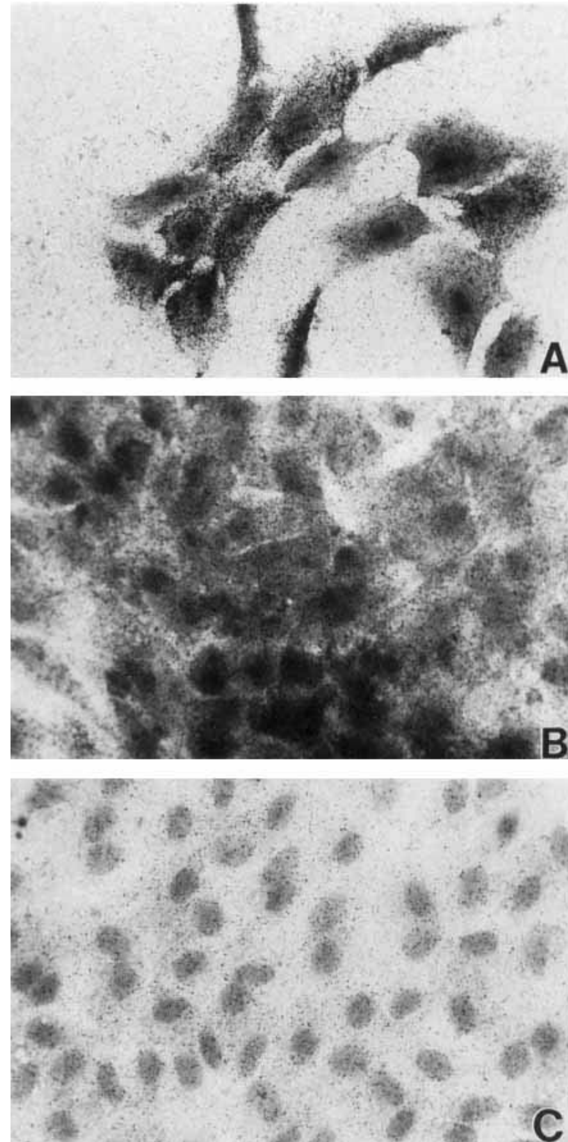

Fig. 5. In situ hybridization of primary rat osteoblast cultures with a histone gene probe. A: Histone H4, day 7 cultures represent the proliferating period, show high intensity of cytoplasmic labelling in 50% of the cells *indicating that 98% are* actively proliferating. B: Day 18 (post-proliferative) cells in early nodule formation and in the non-nodule area (C) show an absence of any labelled cells. The low level uniform distribution of grains over the nucleus, cytoplasm, and entire field represents background. ×336.

Figure 7A–D shows the results from in situ hybridization carried out using a radiolabelled osteopontin gene probe to address the timing of osteopontin gene expression during the osteoblast developmental sequence and the relationship of expression to the organization of the bone-like nodule. Labelling of cells with the osteopontin gene probe was observed during proliferation prior to nodule formation in a few cells. However, as seen in Figure 7A, all cells became intensely labelled on day 17 during the initial stage of nodule formation following hybridization with the osteopontin gene probe. On day 29 (Fig. 7B) when organization of the nodule has further progressed, expression of osteopontin continues at an elevated level. The extent of osteopontin expression with respect to the bone tissue organization associated with mineralizing nodules is additionally illustrated in Figure 7C, where cells at the periphery of the nodule also exhibit expression. All cells in the nodule show labelling with the osteopontin gene probe. In contrast, as indicated in Figure 7D, the internodular cells do not label with the osteopontin probe, indicating that osteopontin gene ex-

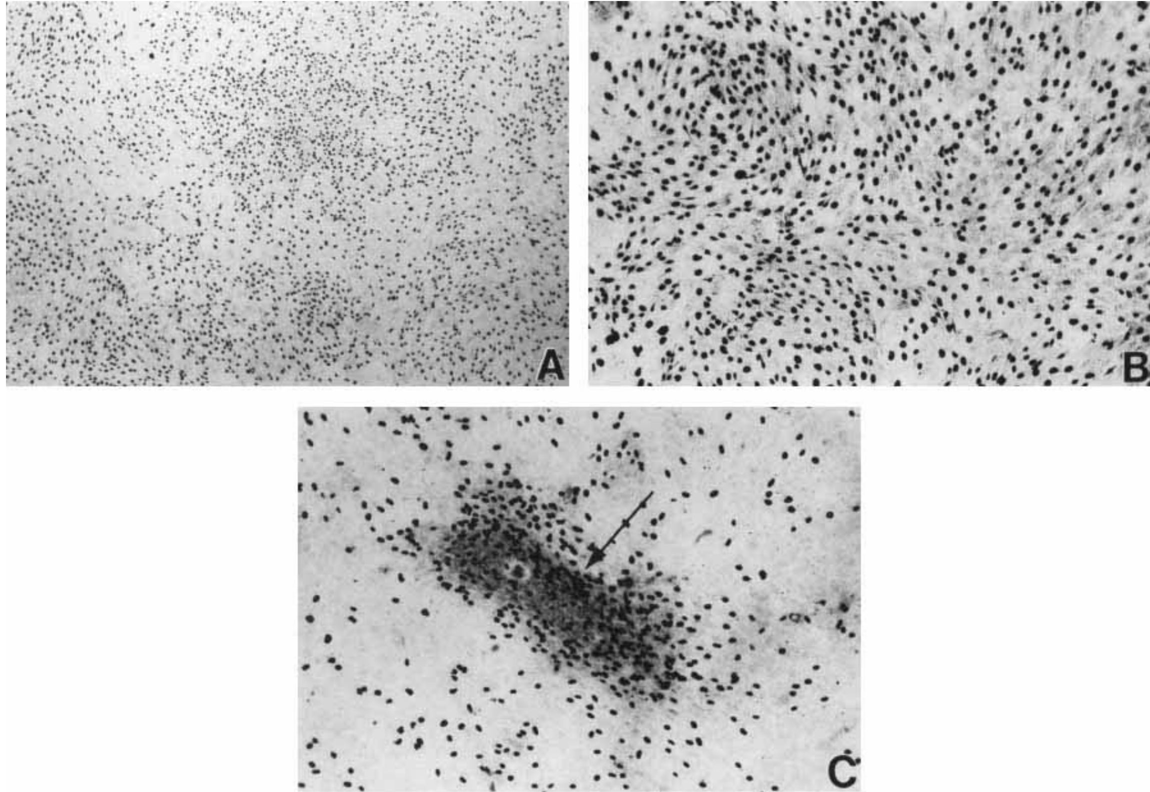

Fig. 6. Cell growth of rat osteoblast cultures demonstrated by autoradiography of ^{3}H-thymidine labelled cells. **A:** 22× and **B** 55× showing cells at monolayer confluency and **C,** several days later, 55×, illustrating proliferative cells in early nodule formation with a decline of proliferative activity in internodular cells. Arrow points to proliferating cells at the periphery of the nodule.

pression is primarily restricted to the nodule and the contiguous cells.

In situ hybridization analysis of the developing osteoblast cultures for osteocalcin gene expression is shown in Figure 8A-D. Osteocalcin gene expression is not observed prior to the initiation of nodule formation. However, in day 17 cultures shown (Fig. 8A) at the early stage of nodule formation, all cells hybridize with the radiolabelled osteocalcin gene probe. Cells at the peripheral region of the nodule that are shown in Figure 8B,C are intensely labelled by the osteocalcin probe. The restriction of osteocalcin gene expression to cells within or immediately associated with the mineralized nodule is supported by the results presented in Figure 8D, which indicate the absence of labelled cells in the internodular region. Taken together, these results indicate that both osteopontin and osteocalcin are expressed in a developmental manner. Both genes are co-expressed during the later period of the osteoblast developmental sequence, in association with extracellular matrix mineralization and the development of bone tissue-like organization.

In Situ Hybridization Analysis of Gene Expression in Osteosarcoma Cells

To further establish the relationship between expression of cell growth and osteoblast-related genes with the onset and progression of differentiation during the development of tissue organization in osteoblasts, we utilized in situ hybridization to examine expression of the histone, osteocalcin, and osteopontin genes in ROS 17/2.8 osteosarcoma cells. ROS 17/2.8 cells express many of the genes associated with the mature osteoblast phenotype including osteocalcin and osteopontin; however, they do so concomitant with proliferation rather than post-proliferatively [Stein et al., 1990, 1992] (Fig. 9). Abrogation of the growth-differentiation relationship in the ROS 17/2.8 osteosarcoma cells is accompanied by an inability of the cells to develop an organized type I collagen extracellular matrix that is characteristic of the normal diploid osteoblast in bone in vivo. These osteosarcoma cells do not develop multilayered nodules with a bone tissue-like organization as seen with cultured normal diploid osteoblasts. This inability of the

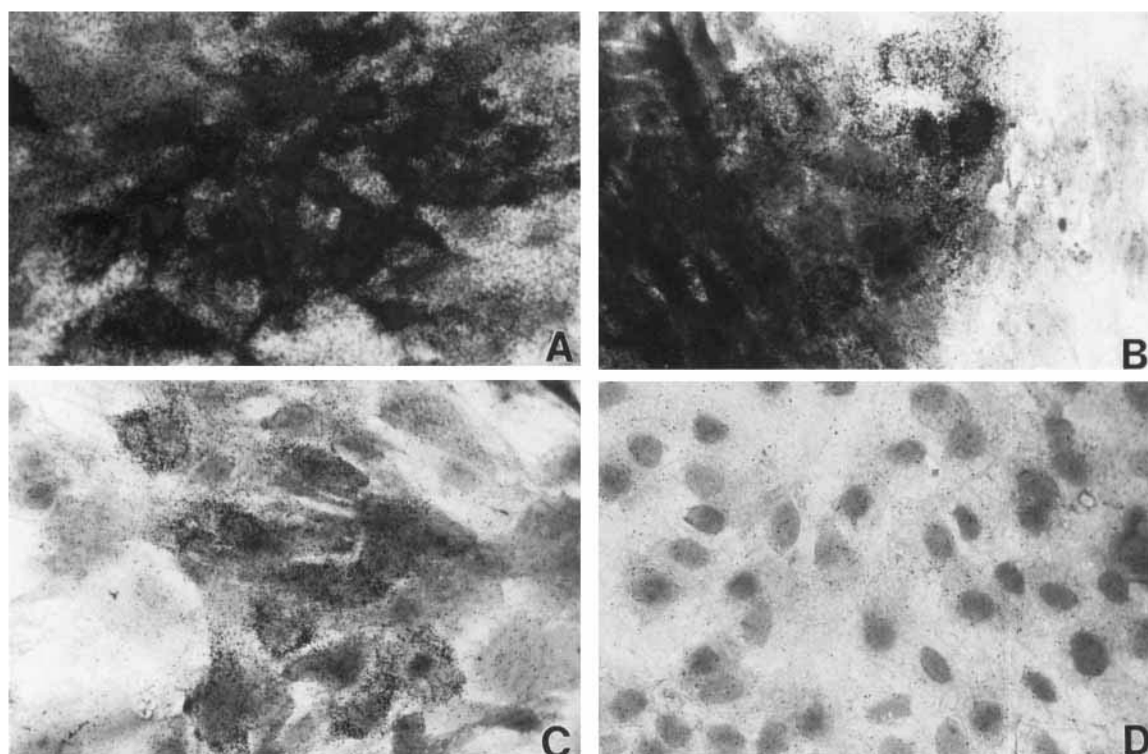

Fig. 7. In situ hybridization for osteopontin expression in rat osteoblast cultures. **A:** Day 17, showing early nodule formation with intensely labelled cells for osteopontin. **B–D:** Day 29 (B) demonstrates OP positive cells at the periphery of the nodule and (C) in the oriented cells aligned toward the mature nodule. D shows an absence of OP label in internodular areas. ×352.

osteosarcoma cells to develop a bone tissue-like organization is the basis for their effectiveness as a model system for further investigating the relationship of bone tissue organization to the expression of specific genes during progressive development of the osteoblast phenotype.

When ROS 17/2.8 cells are actively proliferating (in Fig. 10A exponential growth) 4 days following subcultivation and hybridized with a radiolabelled H4 histone gene probe, 50% of the cells are labelled, indicating that greater than 98% of the cell population are proliferating. On day 8, while the cells do not form nodules, the dense multilayered cultures cease proliferative activity at which time less than 0.1% of the cells hybridize with a histone gene probe (data not shown).

The photomicrographs shown in Figure 10B,C show that a radiolabelled osteocalcin gene probe hybridizes preferentially with cells in the totally confluent regions of the culture. This is consistent with the 3-fold increase in mRNA levels from day 2 to day 8 (Figure 9). The presence of some labelled cells with the osteocalcin gene probe in pre-confluent cultures (day 4; Fig. 10B) indicates that osteocalcin gene expression is ongoing in at least a subset of the osteosarcoma cell population and competency for expression is not related to tissue organization. The variable intensity of osteocalcin labelled cells at confluency (Day 8; Fig. 10C) also suggests a heterogeneity in the extent to which the cells express the osteocalcin gene. Osteopontin, assayed by the in situ hybridization procedure using a radiolabelled osteopontin gene probe, exhibits a low level of activity throughout the ROS 17/2.8 cell cultures (Fig. 10D). This is consistent with the moderate mRNA level of expression on day 8 compared to day 10, as shown in Figure 9. Nonetheless, both genes are expressed in the absence of a regional distribution of cells and the absence of tissue organization in the osteosarcoma cell cultures.

DISCUSSION

These studies demonstrate that growth and the ordered developmental sequence of osteoblast differentiation reflected by a temporal expression of genes of cell growth and bone cell related gene products is related to the develop-

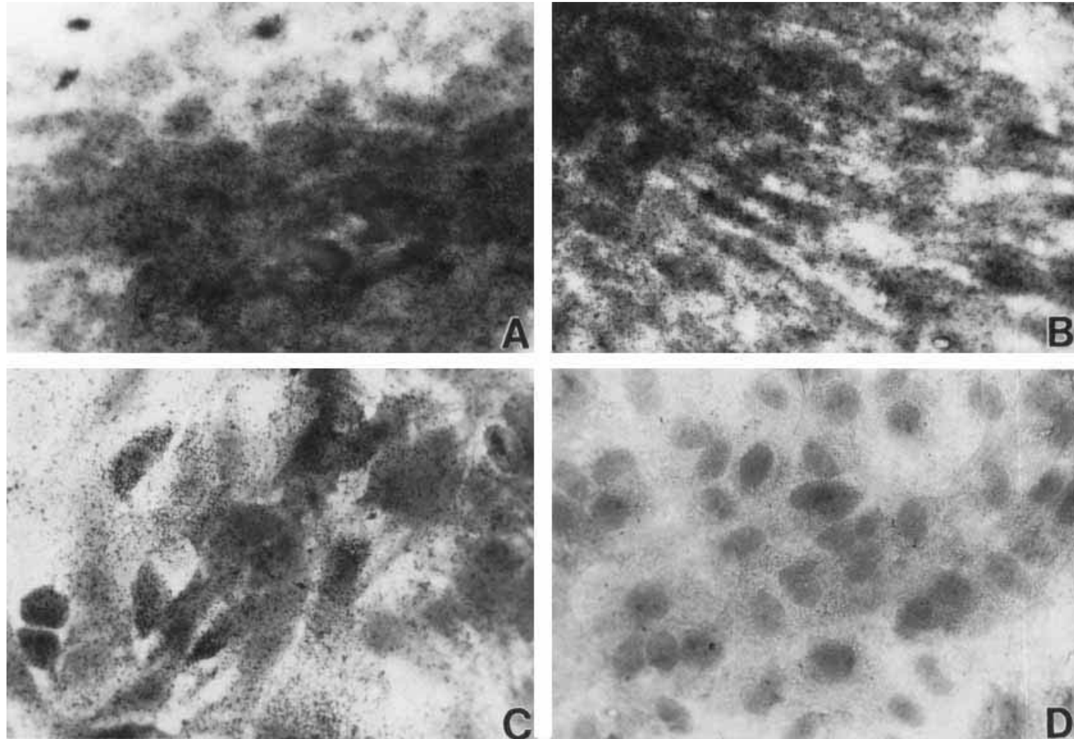

Fig. 8. In situ hybridization for osteocalcin expression in rat osteoblast cultures. **A:** Day 17 cultures show early stages of nodule formation with all cells positive for osteocalcin. **B:** In more developed nodules (day 24), OC cell expression continues. The field shown is the edge of a mineralized nodule. **C:** OC positive cells surrounding the nodule are evident. **D:** OC is not detectable in the internodular area. ×352.

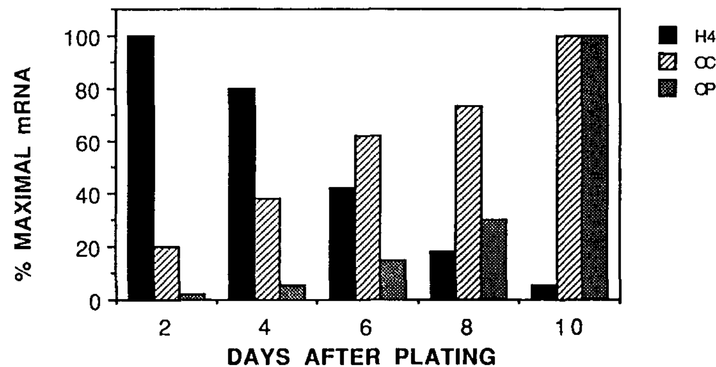

Fig. 9. mRNA expression of histone H4, osteocalcin, and osteopontin in ROS 17/2.8 cells. Histone H4 (solid bars), osteocalcin (hatched bars), and osteopontin (shaded bar) in the ROS 17/2.8 cell culture system are both expressed concomitantly throughout the culture period. Note the increase in OC when H4 has declined at confluency (day 8).

ing tissue organization. Clearly, osteopontin and osteocalcin expression are localized to cells of the post-proliferative developing nodule with maximal expression in cells of the actively mineralizing nodule (Figs. 7, 8). Previously the osteopontin and osteocalcin proteins have been observed to be associated with mineralization in vivo and mineralized areas of osteoblast cultures by immunocytochemistry [Mark et al., 1988; Gerstenfeld et al., 1987]. We now show a selectivity of osteopontin and osteocalcin expression in mature osteoblasts associated with mineralized nodules. Thus, while the protein binds to hydroxyapatite it is also produced by cells in mineralized areas. These in situ hybridization observations are consistent with earlier studies

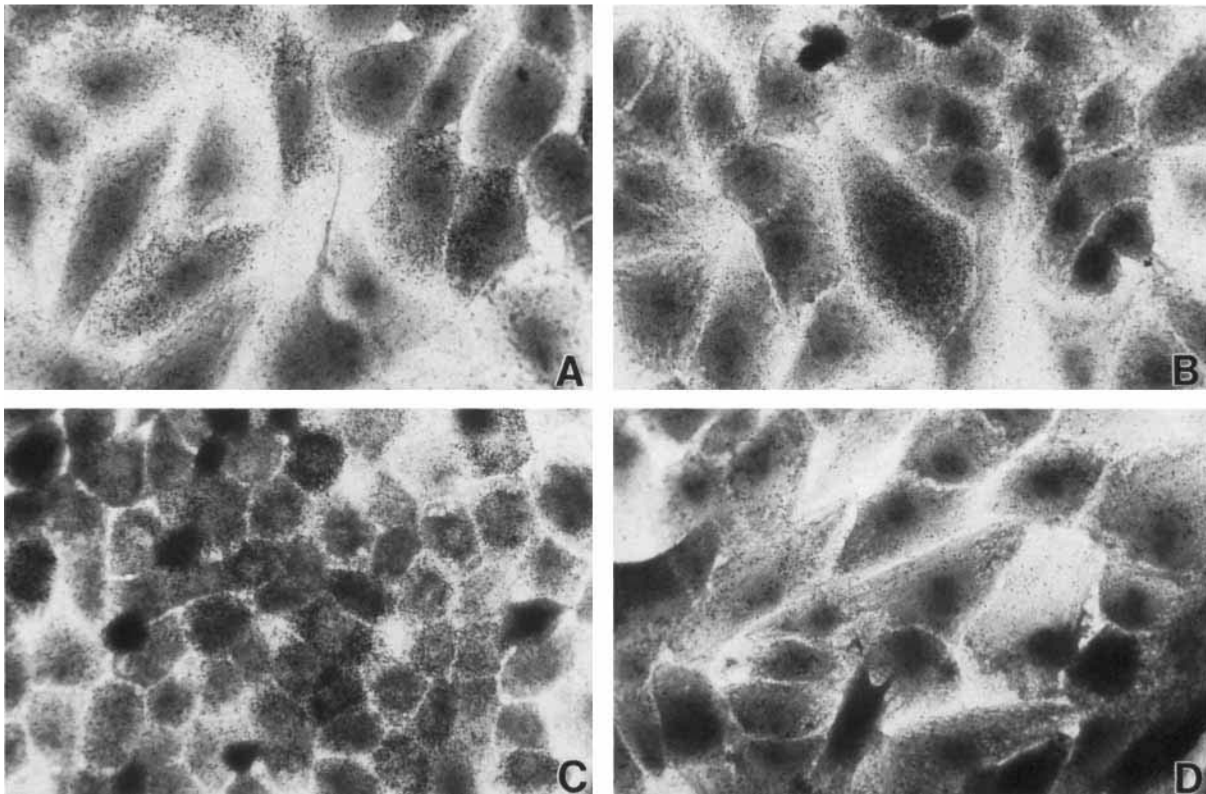

Fig. 10. In situ hybridization of ROS 17/2.8 osteosarcoma cells with histone, osteocalcin, and osteopontin gene probes. **A:** Histone H4, day 4 proliferation period shows high intensity of cytoplasmic labeling in 50% of the cells indicating that 98% are actively proliferating. **B:** Osteocalcin labeled cells at day 4 (preconfluent cultures). Some cells are heavily labeled although all cells exhibit activity above background. **C:** Osteocalcin labeled cells at day 8 (confluency). All cells express OC with an increased representation of heavily labeled cells. **D:** Osteopontin labelled cells at day 8. Uniform but low level of labeling is observed in all cells throughout the culture. ×384.

where levels of osteocalcin and osteopontin gene transcripts were assayed by analysis of cellular mRNA levels. The simultaneous expression of both was apparent [Owen et al., 1990]. Thus, a key question is whether at the single cell level both genes are concomitantly expressed. Indeed the present studies demonstrate concomitant mRNA levels of expression localized to mineralizing nodules. The in situ analysis indicates a selectivity in expression of both genes to cells within the nodule and the perinodular region, but not the internodule region.

The observations from scanning electron microscopy and transmission electron microscopy indicate an orientation of these cells toward the nodule. Such organization of cells with respect to the maturing nodule may be related either to interchange of cellular signals for osteoblast growth and differentiation or to the organization of intracellular matrices for expression of genes related to initiation and/or propagation of mineralization. With respect to the latter possibility, it is evident that both the nuclear matrix [Dworetzky et al., in press] and the cytoskeleton [Egan et al., 1991 a, b] undergo modifications in composition or organization during the osteoblast developmental sequence which correlate with and may be functionally related to changes in gene expression associated with progressive development of the bone cell phenotype. Another possibility that should be considered is that the orientation and polarity of the cells may reflect cellular migration which may contribute to the multilayering of cells in formation of the nodules. Alternatively, the orientation of cells towards the nodule observed by SEM and TEM may represent a polarization aligned with the mineralized nodule. Unquestionably, all cells derived from fetal rat calvaria undergo proliferative activity. This was established by ^{3}H-thymidine labelling and autoradiography. A key question with respect to nodule formation in osteoblast cultures is whether the multilayering of cells is a result of the increased *proliferative*

capacity of some cells that form the nodule or a local migration of cells to create a focal environment for production and/or competency of a matrix competent for mineralization. Even prior to monolayer confluency, distinct areas of increased cellular density were apparent. When cells reached monolayer confluency, internodule areas became apparent as proliferation continued in focal areas for a limited time contributing to the multilayering of cells forming a nodule.

While the extent to which proliferation alone or together with cell migration participates in nodule formation remains to be definitively established, the ^{3}H-thymidine studies indicate further growth and development of the nodule when proliferation between nodular areas has been completed. Within the context of the proliferation/differentiation relationship, in situ hybridization analysis using the cell cycle regulated H4 histone gene probe indicates that proliferation and the expression of genes associated with maturation, organization, and mineralization of the bone cell nodule are mutually exclusive. However, cells in the internodular regions also cease to proliferate, and while they become alkaline phosphatase positive, they do not exhibit induction of osteocalcin or osteopontin gene expression. Thus, it appears that cessation of proliferation is required, but not sufficient for, expression of genes such as osteocalcin and osteopontin, reflecting the mature osteoblast phenotype and the contribution of the organized mineralizing extracellular matrix to osteoblast-related gene expression is reinforced. Such a relationship between cell and extracellular matrix organization is consistent with the restricted ability of nodular cells to support an ordered deposition of mineral within the extracellular matrix in contrast to a precipitation of mineral within the internodular regions of the cultures as reported by Bhargava et al. [1988].

The relationship between proliferation and osteopontin gene expression remains to be definitively established. Low levels of osteopontin mRNA are detected in total cellular RNA from proliferating osteoblasts when assayed by Northern blot analysis in these cultures [Owen et al., 1990] and other systems [Strauss et al., 1990]. These studies show a limited number of labelled cells during the proliferative period. This may reflect the lower level of osteopontin mRNA during proliferation. Alternatively, osteopontin may be present in all cells but below a threshold level for detection by in situ hybridization. While a positive signal denotes definitive expression of a gene, one cannot exclude low but physiologically significant levels of expression by absence of a signal.

In situ hybridization analysis in normal diploid osteoblasts, compared with that observed in osteosarcoma cells where bone-like nodules do not develop, provides insight into the relationship between the expression of osteoblast-related genes and development of bone tissue-like organization. In the normal diploid osteoblast, there is a requirement for shutdown of proliferation and development of an extracellular matrix with a layered orthogonal organization of type I collagen fibrils to support the ordered deposition of mineral that promotes osteoblast differentiation. In contrast, expression of the differentiation genes in osteosarcoma cells occurs in the absence of an organized extracellular matrix and is compatible with proliferation. In both the normal osteoblast and ROS cell cultures, the doubling time of the cells is 20–24 h. Therefore the representation of H4 mRNA in 50% of the ROS and ROB cells directly establishes that almost all cells are undergoing proliferative activity and that the cell cycle parameters are similar. Notably, only some of the cells gave a strong signal for both osteocalcin and osteopontin, suggesting either a heterogeneity in the tumor derived cell line as previously reported [Speiss et al., 1986] or the few positive cells may possibly reflect a cell cycle regulation of osteocalcin and osteopontin. Thus the cells expressing osteocalcin may represent those cells that have completed proliferative activity. Despite the heterogeneity, since all cells are actively proliferating, expression of osteocalcin or osteopontin could occur in actively growing cells. What cannot be evaluated from the present studies is whether osteocalcin and/or osteopontin and H4 are co-expressed in the same cell.

In summary, the biological relevance of the diploid culture system is predicated upon the extent to which osteoblast growth and differentiation are expressed in relation to the bone tissue-like organization of the mineralized nodule. The localization of expression for osteocalcin and osteopontin in the maturing nodule is consistent with both the developmental expression of these genes in fetal rat [Yoon et al., 1987] and the in vivo in situ expression in developing long bone in osteoblasts at the zone of mineralization [Weinreb et al., 1990]. While a broad spectrum of genes expressed in an ordered and

functionally related manner has been established in primary cultures of normal diploid osteoblasts, the rate limiting regulatory mechanisms remain to be determined. To begin to understand the regulatory mechanisms involving bone cell differentiation, the expression of cell growth and phenotype marker genes must be further defined at the single cell level and in relationship to the development of the nodule and the extracellular matrix mineralization process.

ACKNOWLEDGMENTS

Studies reported from the authors' laboratories were supported by grants from the National Institutes of Health (AR33920, AR35166, AR39588, GM32010), and the March of Dimes Birth Defect Foundation.

We are most appreciative of the assistance from Drs. Robert Singer and Jeanne Lawrence and Carol Johnson with the in situ hybridization procedures. The editorial assistance of Patricia Jamieson is also appreciated.

REFERENCES

Aronow MA, Gerstenfeld LC, Owen TA, Tassinari MS, Stein GS, Lian JB: J Cell Physiol 143:213–221, 1990.

Baserga R, Malamud D: "Autoradiography, Techniques and Application." New York: Harper and Row (Hoeber Medical Division), 1969.

Bhargava U, Bar-Lev M, Bellows CG, Aubin JE: Bone 9:155–163, 1988.

Dworetzky SI, Fey EG, Penman S, Stein JL, Lian JB, Stein GS: Proc Natl Acad Sci USA 87:4605–4609, 1990.

Egan JJ, Gronowicz G, Rodan GA: J Cell Biochem 45:101–111, 1991a.

Egan JJ, Gronowicz G, Rodan GA: J Cell Biochem 45:93–100, 1991b.

Gerstenfeld LC, Chipman SD, Glowacki J, Lian JB: Dev Biol 122:49–60, 1987.

Gerstenfeld LC, Chipman SD, Kelley CM, Hodgens KJ, Lee DD, Landis WJ: J Cell Biol 106:979–989, 1988.

Grimes S, Weiss-Carrington P, Daum H III, Smith J, Green L, Wright K, Stein G, Stein J: Exp Cell Res 173:534–545, 1987.

Holtrop ME: In Hall BK (ed): The Osteoblast and Osteocyte. Caldwell, NJ: Teleford Press, 1990.

Lian JB, Stein GS, Owen TA, Tassinari MS, Aronow M, Collart D, Shalhoub V, Peura S, Dworetzky S, Pockwinse S: In Stein GS, Lian JB (eds): "Molecular and Cellular Approaches to the Control of Proliferation and differentiation." San Diego: Academic Press, 1992, pp 165–222.

Lian JB, Stewart C, Puchacz E, Mackowiak S, Shalhoub V, Collart D, Zambetti G, Stein G: Proc Natl Acad Sci USA 86:1143–1147, 1989.

Majeska RJ, Rodan SB, Rodan GA: Endocrinology 107:1494–1503, 1980.

Mark MP, Butler WT, Prince CW, Finkelman RD, Ruch JV: Differentiation 37:123–136, 1988.

Oldberg Å, Franzén A, Heinegård D: Proc Natl Acad Sci USA 83:8819–8823, 1986.

Owen TA, Aronow M, Shalhoub V, Barone LM, Wilming L, Tassinari MS, Kennedy MB, Pockwinse S, Lian JB, Stein GS: J Cell Physiol 143:420–430, 1990.

Rodan GA, Noda M: Crit Rev in Eukaryotic Gene Exp 1:85–98, 1991.

Speiss YH, Price PA, Deftos JL, Manolagas SC: Endocrinology 118:1340–1346, 1986.

Stein GS, Lian JB, Owen TA: FASEB J 4:3111–3123, 1990.

Stein GS, Lian JB, Owen TA, Holthuis J, Bortell R, van Wijnen AJ: In Stein GS, Lian JB (eds): "Molecular and Cellular Approaches to the Control of Proliferation and differentiation." San Diego: Academic Press, 1992, pp 299–341.

Strauss PG, Closs EI, Schmidt J, Erfle V: J Cell Biol 110: 1369–1378, 1990.

Weinreb M, Shinar D, Rodan GA: J Bone Miner Res 5:831–842, 1990.

Yoon K, Buenaga R, Rodan GA: Biochem Biophys Res Commun 148:1129–1136, 1987.