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TGF-β1 Modifications in Nuclear Matrix Proteins of Osteoblasts During Differentiation

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Abstract Nuclear matrix protein (NMP) composition of osteoblasts shows distinct two-dimensional gel electrophoretic profiles of labeled proteins as a function of stages of cellular differentiation. Because NMPs are involved in the control of gene expression, we examined modifications in the representation of NMPs induced by TGF-β1 treatment of osteoblasts to gain insight into the effects of TGF-β on development of the osteoblast phenotype. Exposure of proliferating fetal rat calvarial derived primary cells in culture to TGF-β1 for 48 h (day 4–6) modifies osteoblast cell morphology and proliferation and blocks subsequent formation of mineralized nodules. Nuclear matrix protein profiles were very similar between control and TGF-β–treated cultures until day 14, but subsequently differences in nuclear matrix proteins were apparent in TGF-β–treated cultures. These findings support the concept that TGF-β1 modifies the final stage of osteoblast mineralization and alters the composition of the osteoblast nuclear matrix as reflected by selective and TGF-β–dependent modifications in the levels of specific nuclear matrix proteins. The specific changes induced by TGF-β in nuclear matrix associated proteins may reflect specialized mechanisms by which TGF-β signalling mediates the alterations in cell organization and nodule formation and/or the consequential block in extracellular mineralization. J. Cell. Biochem. 69:291–303, 1998.

Key words: nuclear matrix; TGF-β1; bone; osteoblast differentiation; mineralization

Nuclear matrix proteins (NMP) represent a highly diverse group of factors with architectural and/or gene regulatory properties [Penman et al., 1997; Stein et al., 1996]. For example, a variety of transcription factors have been shown to preferentially localize to the nuclear matrix and perhaps may mediate transient chromatin–nuclear matrix associations [Lindenmuth et al., 1997; Getzenberg et al., 1991, 1996; Landers et al., 1994; Sun et al., 1996; Merriman et al., 1995; van Wijnen et al., 1993; van Steensel et al., 1995]. For example, two nuclear matrix–bound transcription factors, AML3/CBFβ1 [Guo et al., 1997; Lindenmuth et al., 1997; Banerjee et al., 1997; Merriman et al., 1995], regulate bone-specific transactivation and vitamin D–dependent enhancer activity of the osteocalcin gene. The interaction of these factors at their DNA regulatory sequences is related to modifications in nucleosomal organization and increased DNase I hypersensitivity [Montecino et al., 1994, 1996a,b] when the osteocalcin gene is activated in osteoblasts.

The protein composition of the nuclear matrix is unique to specific tissues, cell types, and stage of cellular differentiation [Fey and Penman, 1988; Nakayasu and Berezney, 1991; Mattern et al., 1996]. The biological significance of NMP specificity is supported by the observation of distinct nuclear matrix proteins in cancerous cells compared to their normal healthy counterparts (e.g., in bladder [Getzenberg et al., 1996], prostrate [Getzenberg et al., 1991], breast [Pienta and Coffey, 1991], colon [Keesee et al., 1994], and bone [Bidwell et al., 1994b] tissues). Of particular interest to defining subtleties in the control of cellular phenotype are the changes observed in nuclear matrix protein profiles during three developmental stages of osteoblast differentiation: the periods of growth, matrix production, and mineralization [Dworetzky et al., 1990]. Furthermore, osteoblast cultures

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treated with parathyroid hormone (PTH), a known regulator of osteoblast-expressed genes, modifies the nuclear matrix–intermediate filament protein profile and induces the appearance of a unique 200 kd NMP [Bidwell et al., 1994a]. Because transforming growth factor β1 (TGF-β1) influences development of the osteoblast phenotype in vitro [Antosz et al., 1989; Harris et al., 1994; Iwasaki et al., 1993] and in vivo [Beck et al., 1993; Marcelli et al., 1990; Noda and Camilliere, 1989], we examined modifications in nuclear matrix proteins that are associated with the action of this regulator of growth and differentiation on bone cells.

TGF-β1 has multiple effects on bone which include upregulating the synthesis of extracellular matrix proteins in osteoblasts. Cultured rat osteoblasts that are treated with TGF-β1 initially exhibit morphologies that are clearly distinct from the untreated cultures [Breen et al., 1994]. Later in the developmental sequence, as the control cells begin to differentiate, the TGF-β–treated cells are unable to form mineralized nodules and will not express a mature osteoblast phenotype. These observations suggest TGF-β1 may selectively inhibit proliferation of one nodule forming cell or alter its phenotypic properties. Because TGF-β influences both cell structure and gene expression, we investigated a potential role for the participation of nuclear architecture in accommodating these cellular alterations as reflected by modifications in the nuclear matrix protein composition. This was accomplished by evaluating nuclear matrix protein profiles, visualized by high resolution two-dimensional gel electrophoresis, of TGF-β–treated and untreated cultures of rat calvaria derived osteoblasts at multiple time points during the development of the osteoblast phenotype. We addressed if TGF-β treatment, under conditions which prevent osteoblast differentiation, results in modifications in the composition of the nuclear matrix–intermediate filament (NM-IF) scaffold concomitant with morphological modifications of the osteoblast phenotype. This finding provides further support for functional interrelationships between the nuclear matrix–dependent regulation of gene expression and progression of osteoblast differentiation.

MATERIALS AND METHODS

Cell Culture

Rat osteoblasts were isolated from Sprague-Dawley fetal rat calvaria (Taconic, Georgetown, NY) at 21 days gestation as described previously [Breen et al., 1994; Dworetzky et al., 1990; Aronow et al., 1990]. The cells were subjected to three sequential digestions of 8, 12, and 26 min at 37°C in 1 mg/ml Type II collagenase (Worthington, Freehold, NJ). Cells released from the third digest were passed through a sterile swinex filter, collected by centrifugation, and plated at 0.7×10^6 cells per plate in 100 mm plates (Corning, Corning, NY) or at 0.05×10^6 cells per well in six-well plates (Corning, Corning, NY) etched with glass coverslips (Fischer, Fair Lawn, NJ). The cultures were fed minimal essential media (MEM) supplemented with 10% fetal calf serum (FCS) three times a week. On the first day of confluency, cells also received 25 μg/ml ascorbic acid. At the next feeding, the medium was additionally supplemented with 50 μg/ml ascorbic acid and 10 mM β-glycerol phosphate (β-GP). On day 11, the media was changed to BJGb containing 10% FCS, 200 mM penicillin-streptomycin (Sigma, St. Louis, MO) and 500 mM L-glutamine (Sigma).

In some experiments primary preconfluent cultures were subcultivated on day 5 by digestion in 0.25% Trypsin-EDTA (Gibco, Grand Island, NY) followed by a 10 min incubation at 37°C. Completed media (5 ml) was added, and cells were counted and plated at 1×10^6 cells per 100 mm plate. These cultures received 10^{-7} M dexamethasone (Dex) for the first three feedings to promote osteoblast differentiation of the passaged cells. A subset of these cells were grown in the absence of Dex, ascorbic acid, and β-GP, serving as a nonmineralized control.

Cells were treated with 2.5 ng/ml TGF-β in acetic acid to a final concentration of 0.25 ng/ml as described previously [Breen et al., 1994]. The cultures were always treated prior to monolayer confluency. Primary or passaged cultures grown on 100 mm plates were treated with TGF-β for 1) 96 h and collected immediately on day 8 or later on day 14 (matrix maturation period) or 2) 48 h from days 3–5 and collected immediately on day 5 or later on day 23 (mineralization period). In addition, passaged cells were chronically treated with TGF-β from days 4–23 and collected. Cells were also grown on glass coverslips and treated with TGF-β 24 h during the proliferating period to examine the effects of TGF-β on cell structure by phalloidin staining of actin filaments. On day 6, the cells were washed with phosphate buffered saline (PBS) twice and fixed in 4% formaldehyde for

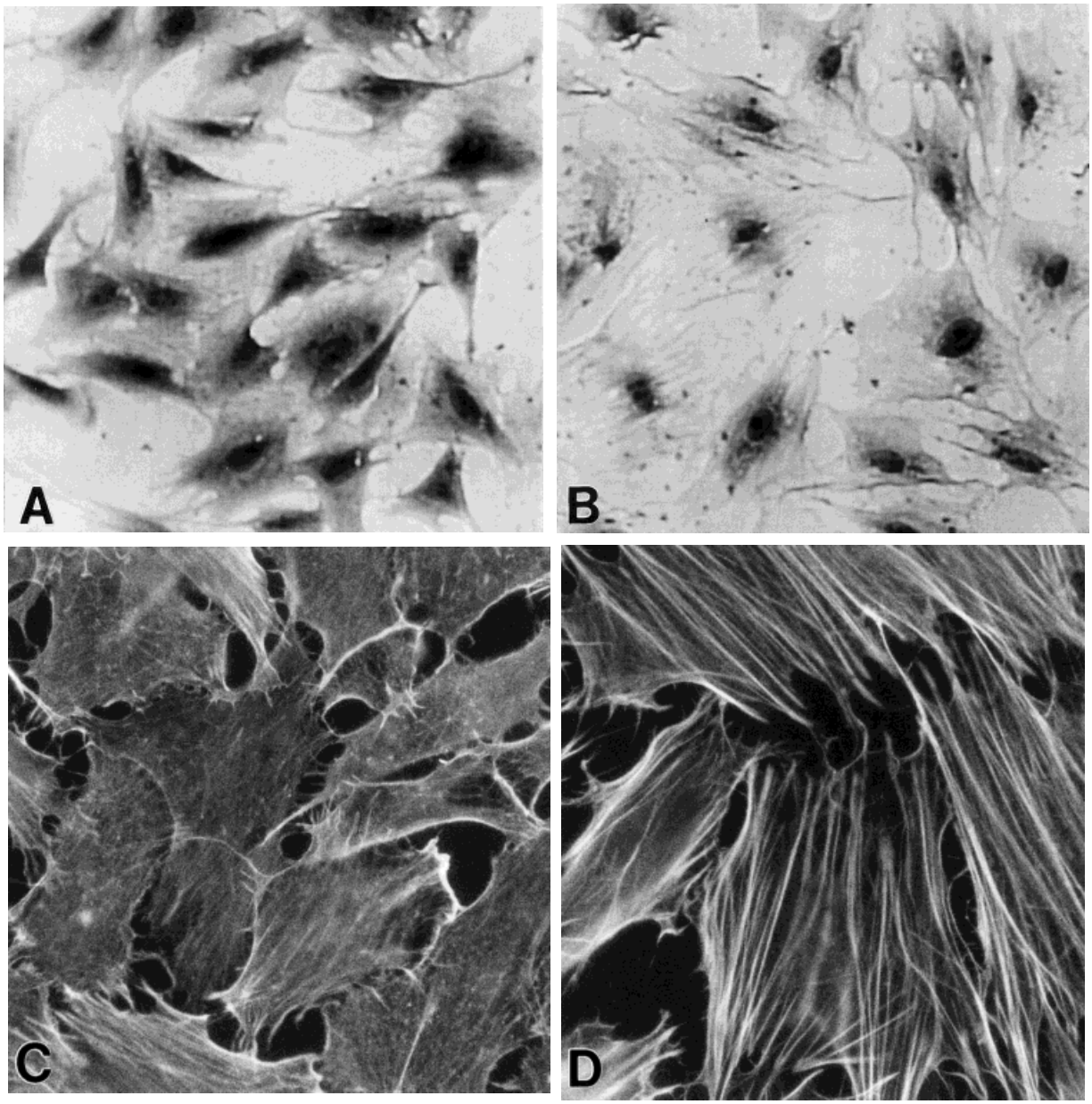

Fig. 1. Light microscopy of primary rat calvarial osteoblasts on day 6 following fixation and staining with toluidine blue (**A,B**) and phalliodin (**C,D**) for control cells (A,C) and cells treated with TGF-β (B,D) from days 4–6 during the proliferative stage. A,B: ×40. C,D: ×80.

10 min at room temperature and stained with phalloidin (Molecular Probes, Eugene, OR) according to the manufacturer's procedure. Some coverslips were also stained with 0.1% toluidine blue for 5 min.

Determination of Osteocalcin Serum Concentrations

Serum concentrations of osteocalcin (OC), a marker of osteoblast differentiation, were determined by radioimmunoassay using ^{125}I-labeled rat osteocalcin using standard procedures [Gundberg et al., 1984].

Nuclear Matrix–Intermediate Filament Preparation

Isotopically labeled nuclear matrix proteins were isolated from two to ten plates (100 mm diameter). The plates were washed with PBS for 24 h and then 2 ml of F12 medium (free of L-glutamine, L-methionine, and L-cysteine [Gibco-BRL, Gaithersburg, MD]) supplemented

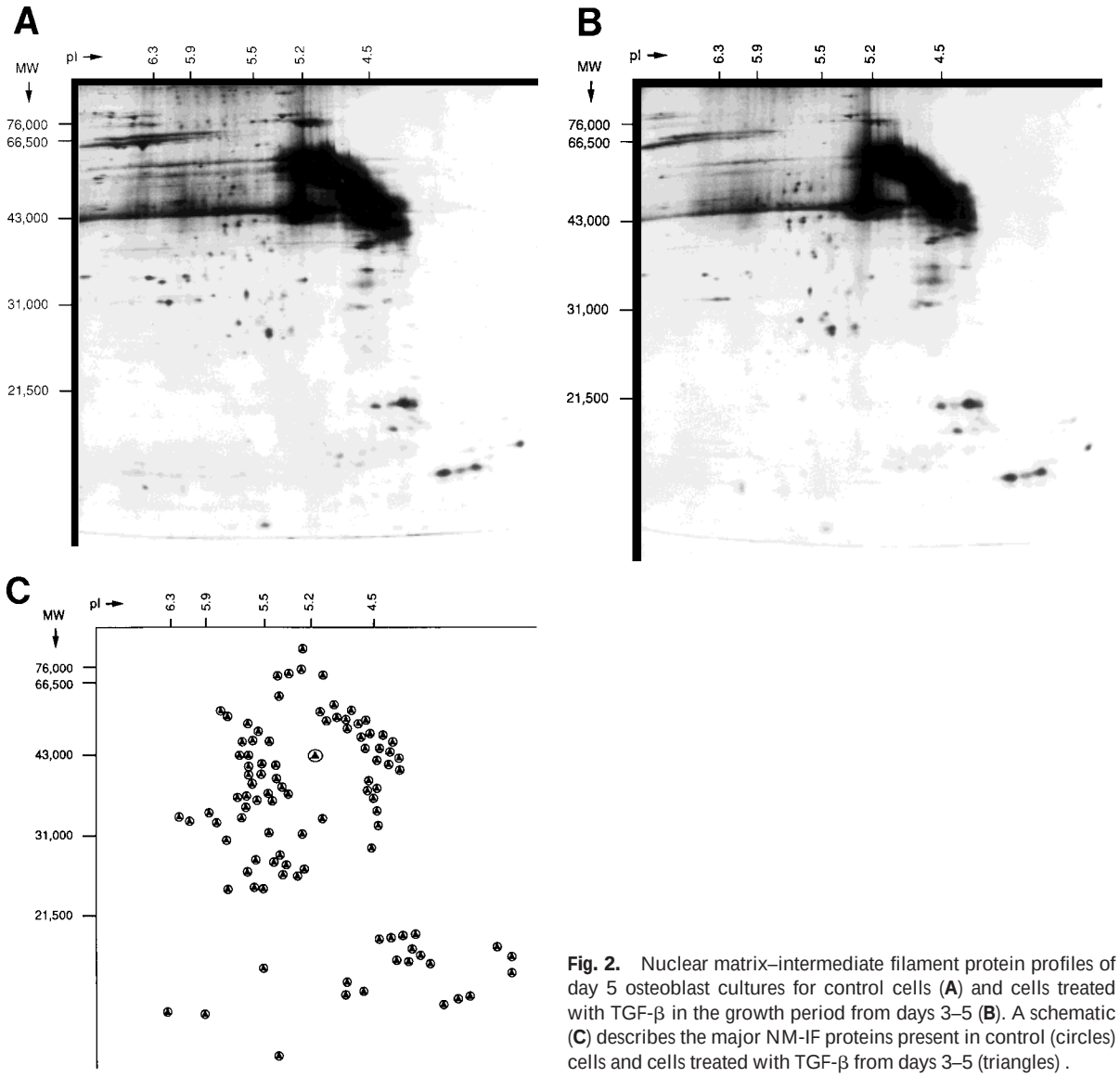

Fig. 2. Nuclear matrix–intermediate filament protein profiles of day 5 osteoblast cultures for control cells (**A**) and cells treated with TGF-β in the growth period from days 3–5 (**B**). A schematic (**C**) describes the major NM-IF proteins present in control (circles) cells and cells treated with TGF-β from days 3–5 (triangles) .

TABLE I. Osteocalcin Synthesis in Rat Calvarial Osteoblasts During Differentiation in Control and TGF-β–Treated Cultures*

	Osteocalcin Concentration, ng/ml media			
Days in culture	Control cultures	TGF-β–treated, 48 h (days 4–6)	TGF-β–treated continuously (days 4–23)	Nonmineralized[a]
8	ND	ND	NA	NA
14	58 ± 13	5.8 ± 3.1	ND	ND
23	690 ± 172	148 ± 25	55 ± 6.6	8.6 ± 2.7

*NA, not assayed; ND, nondetectable.
[a]β-glycerophosphate not included in medium.

with 5% dialyzed FCS, containing 25 µCi/ml ^{35}S-labeled L-methionine and L-cysteine (NEN-Dupont, Boston, MA) was added to each plate. Cultures were incubated for 3 h at 37°C. Nuclear matrix–intermediate filament proteins (NM-IF) were isolated using the Penman method [Fey et al., 1984] with minor modifications [Merriman et al., 1995]. Cultures collected during the differentiation period were subjected to homogenization using a Teflon homogenizer at-

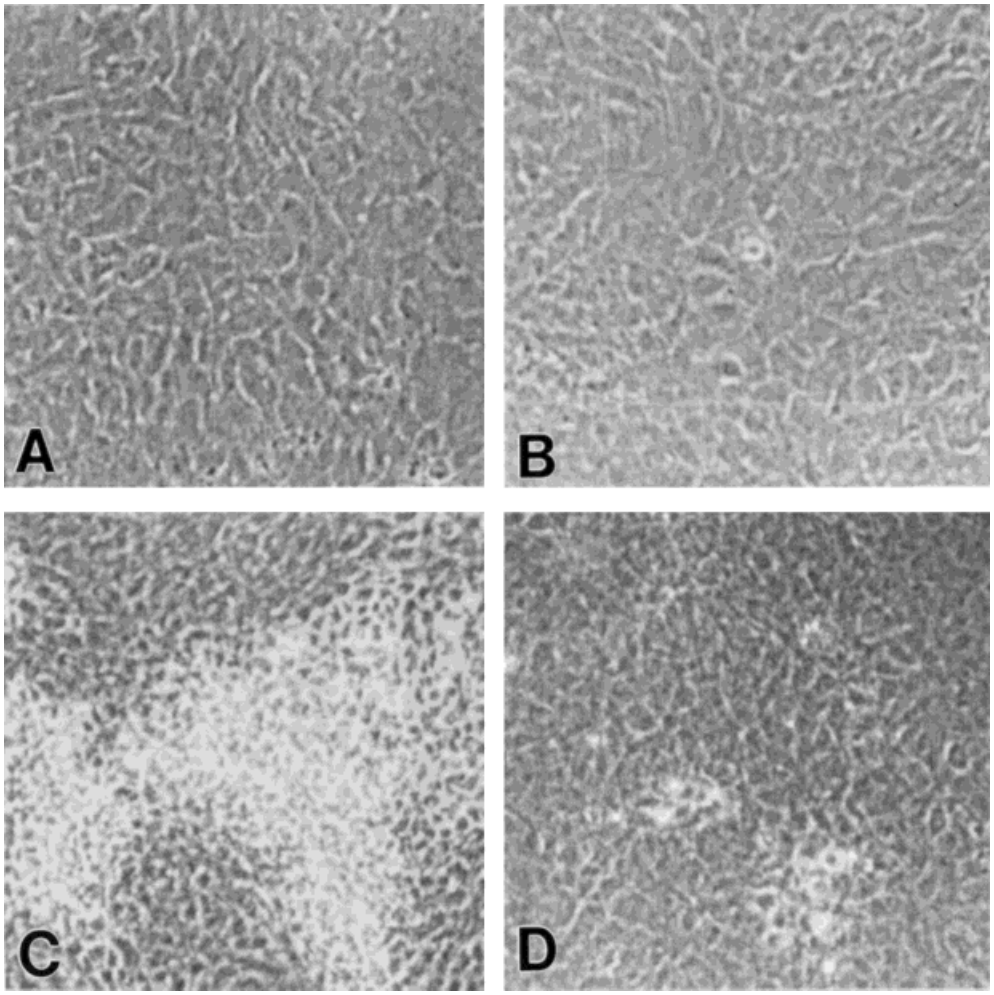

Fig. 3. Phase contrast photographs of osteoblast cultures on day 8 (**A,B**) and day 14 (**C,D**) for control cells (A,C) and cells treated with TGF-β (B,D) from day 4–7. ×40.

tached to an electrical drill following addition of CSK buffer (100 mM NaCl, 300 mM sucrose, 10 mM PIPES/pH 6.8, 3 mM $MgCl_2$, 1 mM EGTA, 0.5% Triton X-100). CSK buffer included just prior to use a broad spectrum of protease inhibitors (Boehringer Mannheim, Indianapolis, IN) and additives (0.2 mM PMSF, 0.5 µg/ml leupeptin, 0.7 µg/ml pepstatin, 10 µg/ml Trypsin Inhibitor, 2 µg/ml TPCK, 40 µg/ml bestatin, 17 µg/ml Calpain Inhibitor, 1 µg/ml E64, 1.0 mM EGTA, 0.2 mM EDTA, 1 mM DTT, 0.75 mM spermidine, 0.15 mM spermine) [Fey et al., 1984].

Two-Dimensional Electrophoresis

High resolution two-dimensional gels were run using the ELSA 2D Gel Apparatus (Millipore, Marlboro, MA) according to the instructions of the manufacturer. NM-IF protein fractions were solubilized in 2-D sample buffer (9.0 M urea, 2.0% (40%) ampholytes [pH = 3–10]). Equal counts, totaling 100,00–300,000 cpm of ^{35}S-labeled protein of each sample were loaded on 4% tube gels and subject to isoelectric focusing for 18,000 volt hours. Samples were electrophoresed in the second dimension on 12% slab gels. Slab gels were fixed in 20% methanol and 7% acetic acid for 1 h. This was followed by 30 min incubations in both Entensify A and B (NEN-Dupont), an autoradiographic enhancer. Control and treated samples to be compared were run in the same set together, along with a two-dimensional electrophoretic protein standard (Bio-Rad, Richmond, CA) which was visualized by Coomassie staining.

RESULTS AND DISCUSSION

Developmental Influences of TGF-β on Osteoblast Nuclear Matrix Proteins

Figure 1 shows toluidine blue and phalloidin staining, respectively, of day 6 primary rat osteo-

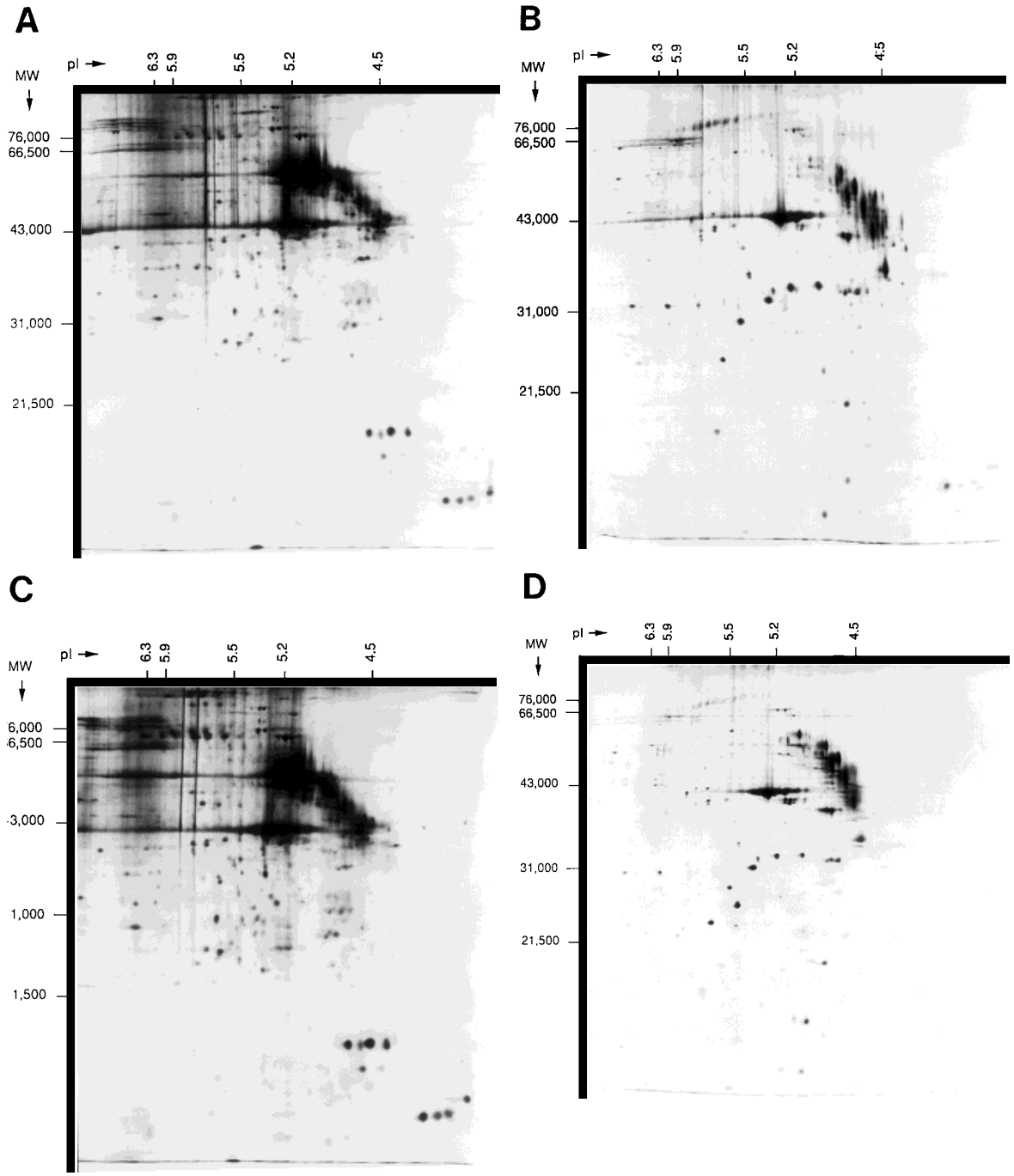

Fig. 4. Nuclear matrix–intermediate filament proteins prepared from day 8 (**A,C**) and day 14 (**B,D**) control osteoblast cells (A,B) and osteoblast cells treated with TGF-β from days 4–7 (C,D), respectively. Schematics describe the most abundant proteins from the TGF-β treated from days 4–7 (triangles) and untreated (circles) protein profiles on days 8 (**E**) and 14 (**F**).

blast cell cultures for control (Fig. 1A,C) and TGF-β–treated (48 h) cells (Fig. 1B,D) during the growth period. Both stains reveal that distinct morphological changes occur in the osteoblast in response to TGF-β during this early stage of development. The TGF-β–treated cells have a more spread appearance compared to the untreated cells. The spreading is most pronounced in the extensions that outline the cytoplasm. Phalloidin staining shows that the TGF-β treatment increases the number of actin filaments and alters the structure of actin in these cells. The actin fibers are thicker in the TGF-β–treated cells and are highly organized,

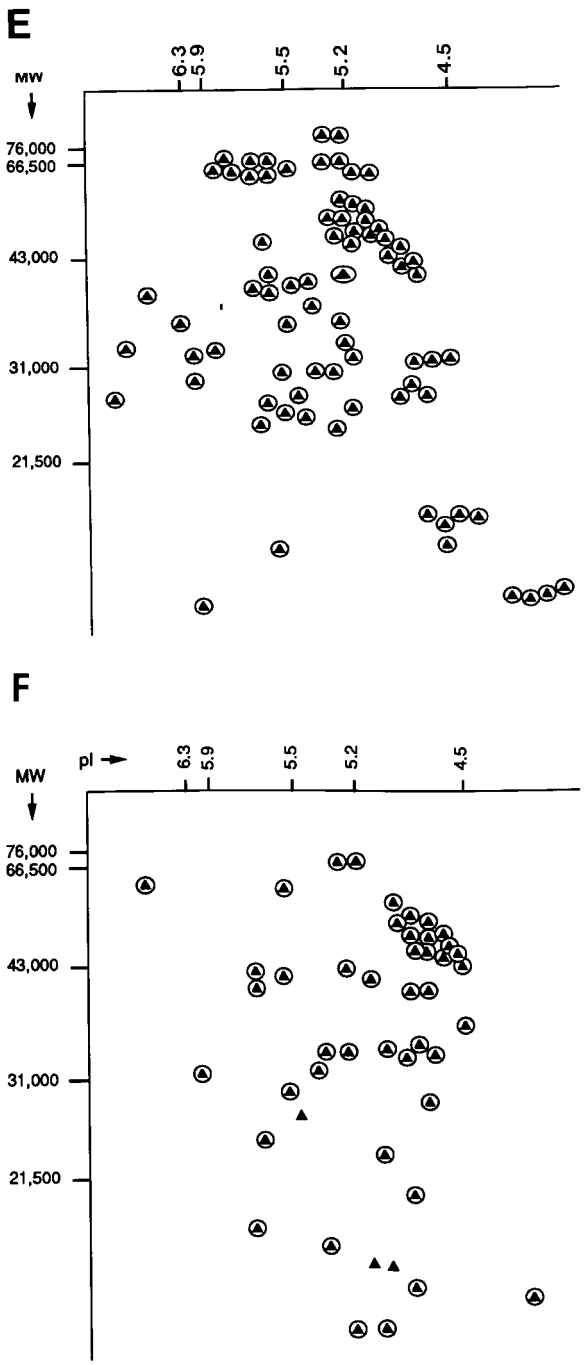

Fig. 4 (Continued)

often in parallel compared to the random distribution in the control cells. These findings are consistent with those described by others in osteoblasts [Breen et al., 1994].

To address potential interrelationships between parameters of nuclear architecture and TGF-β–mediated modifications in nuclear structure and gene expression, we initially examined the protein compositions of NM-IF preparations from day 5 proliferating primary osteoblast cells at the conclusion of a 48 h treatment with TGF-β (Fig. 2). A schematic (Fig. 2C) describes the most abundant proteins present in the NM-IF protein profiles of both the TGF-β–treated (Fig. 2B) and untreated cells (Fig. 2A) and reveals that significant differences are not detected between the two NM-IF protein patterns. The similarities in ^{35}S-methionine and ^{35}S-cysteine labeling of NM-IF proteins between control and TGF-β–treated cells at this stage suggest that the TGF-β–induced alterations in cell morphology (observed by phalloidin staining) occur in the absence of immediate changes in the nuclear matrix.

We verified that TGF-β treatment during the proliferating developmental stage modified expression of the osteoblast phenotype. The effect of TGF-β on synthesis of the bone-specific marker, osteocalcin, was assayed. Table I describes the serum osteocalcin concentrations on the day of harvest for both the TGF-β–treated (48 h) and untreated cultures in nanograms per milliliter. Osteocalcin is secreted at increasing concentrations as the cells develop a mature osteoblast phenotype. On day 8, osteocalcin concentrations are below the level of detection in both the control and TGF-β–treated cultures. In addition, photographs taken on day 8 show that the TGF-β–treated and untreated cultures are morphologically indistinguishable. By day 14, however, the osteocalcin concentration is significantly higher in the control cultures than the TGF-β–treated cultures, indicating that while the control cultures are maturing normally, differentiation in the TGF-β–treated cultures has been delayed. This is further supported by light microscopic observations shown in Figure 3. Here, the TGF-β cultures have a distinct appearance. By day 14, the control cells have multilayered and initiated formation of nodules. The nodules in the TGF-β–treated cultures are very sparse and smaller in size. Thus, the TGF-β–treated cells appear to be at an earlier stage of the developmental sequence than the control cells.

We investigated these late acting effects of TGF-β, which blocks expression of the mature osteoblast phenotype, on modifications of the nuclear matrix. NM-IF protein profiles of primary osteoblast cultures were examined on days 8 and 14 for both untreated cells and following treatment with TGF-β (96 h). Figure 4 shows

the NM-IF protein patterns of control cells on days 8 (Fig. 4A) and 14 (Fig. 4B). Note that the NM-IF protein composition changes as the cells progress through the osteoblast developmental sequence. This is in agreement with findings initially described by the Dwortzkey group [Dworetzky et al., 1990]. In this study, the high resolution capability of the Millipore apparatus is used to enhance the protein separation specifically in the pH range observed by Dworetzky et al. [1990] for these developmentally regulated modifications in NM-IF protein composition. Figure 4 also shows NM-IF protein profiles of cultures that were treated with TGF-β for 96 h during the proliferative period and harvested later on days 8 (Fig. 4C) and 14 (Fig. 4D) together with respective control osteoblasts (Fig. 4A,B). Schematics drawn in Figure 4 compare the most abundant proteins present in the NM-IFs prepared from control cells to those present in the NM-IFs of cells treated with TGF-β (96 h) after either 8 (Fig. 4E) or 14 (Fig. 4F) days in culture. On day 8, no differences are detected between the NM-IF protein profiles of the control and the TGF-β–treated (96 h) cells. Also, biochemical and morphological analyses show that the TGF-β–treated cells resemble the control cells on day 8. Therefore, any TGF-β–induced effects on the cells at this stage are not reflected in the composition of the NM-IF proteins resolved by 2-D gel electrophoresis. By day 14, we began to observe an effect of the TGF-β treatment which inhibits nodule development. There are two proteins that are present in the NM-IF protein preparations from the TGF-β–treated cultures that are absent from the control cell NM-IF protein preparations. Hence, the delay in the maturational sequence of osteoblast differentiation is accompanied by differences in the composition of NM-IF proteins in the TGF-β–treated cells.

Absence of Nodule Formation and ECM Mineralization Contribute to NM-IF Protein Differences Observed in Control and TGF-β–Treated Cells

We addressed if the NM-IF protein modifications resulting from the inhibition of development induced by the treatment with TGF-β (48 h or continuous) are representative of the NM-IF protein modifications that accompany the absence of mineralization. Figure 5 shows photographs taken after 23 days in culture of the untreated (Fig. 5A), TGF-β–treated (48 h) (Fig. 5B), TGF-β–treated (continuously) (Fig. 5C), and the nonmineralized cultures that were grown in the absence of dexamethasone, ascorbic acid, and β- glycerophosphate (Fig. 5D). Cultures that were continuously treated with TGF-β and cultures that were grown under nonmineralizing conditions are similar morphologically with respect to the absence of mineralizing nodule and biochemically. Secreted osteocalcin is barely detectable for both the nonmineralized control cultures and the cultures continuously treated with TGF-β, indicating that neither of these cultures was able to initiate differentiation. However, there still remains a distinct difference in cell shape and size between the two cultures. Furthermore, the chronically treated TGF-β cells are smaller and tightly packed in an extracelluluar matrix (ECM) as a result of the mitogenic effect of the TGF-β. In contrast, the cells grown under nonmineralizing conditions remain cuboidal and monolayered. These findings are consistent with earlier reports that in vitro TGF-β–treated osteoprogenitors are inhibited in their final differentiation.

To determine if the phenotypes induced by the TGF-β treatments are accompanied by modification in the representation of nuclear matrix proteins, we examined the NM-IF protein profiles of day 23 secondary osteoblast cultures from the control (Fig. 6A) and three treatment groups (Fig. 6B–D). By day 23, no unique NM-IF proteins, induced by the 48 h treatment with TGF-β in the growth period, remained (Fig. 6B). In contrast, when comparing the NM-IF profiles of the control mineralized cultures (Fig. 6A) to continuously treated TGF-β cultures (Fig. 6C), we observed differences in the representation of nuclear matrix proteins. There are two proteins present in the NM-IF protein preparations from cultures that were treated with TGF-β (continuous) that are not evident in the control NM-IF preparations. While it appears that these proteins may also be upregulated in the TGF-β (48 h) profile, the upregulation is not significant enough to draw any conclusions. There are also two NMPs in control NM-IF that are not observed in the TGF-β (continuous) treated NM-IF. Thus, while both the acute and chronic treatments with TGF-β inhibit osteoblast differentiation, the NM-IF modifications observed between control and continuous TGF-β1–treated cultures are not identical to modifications observed as a

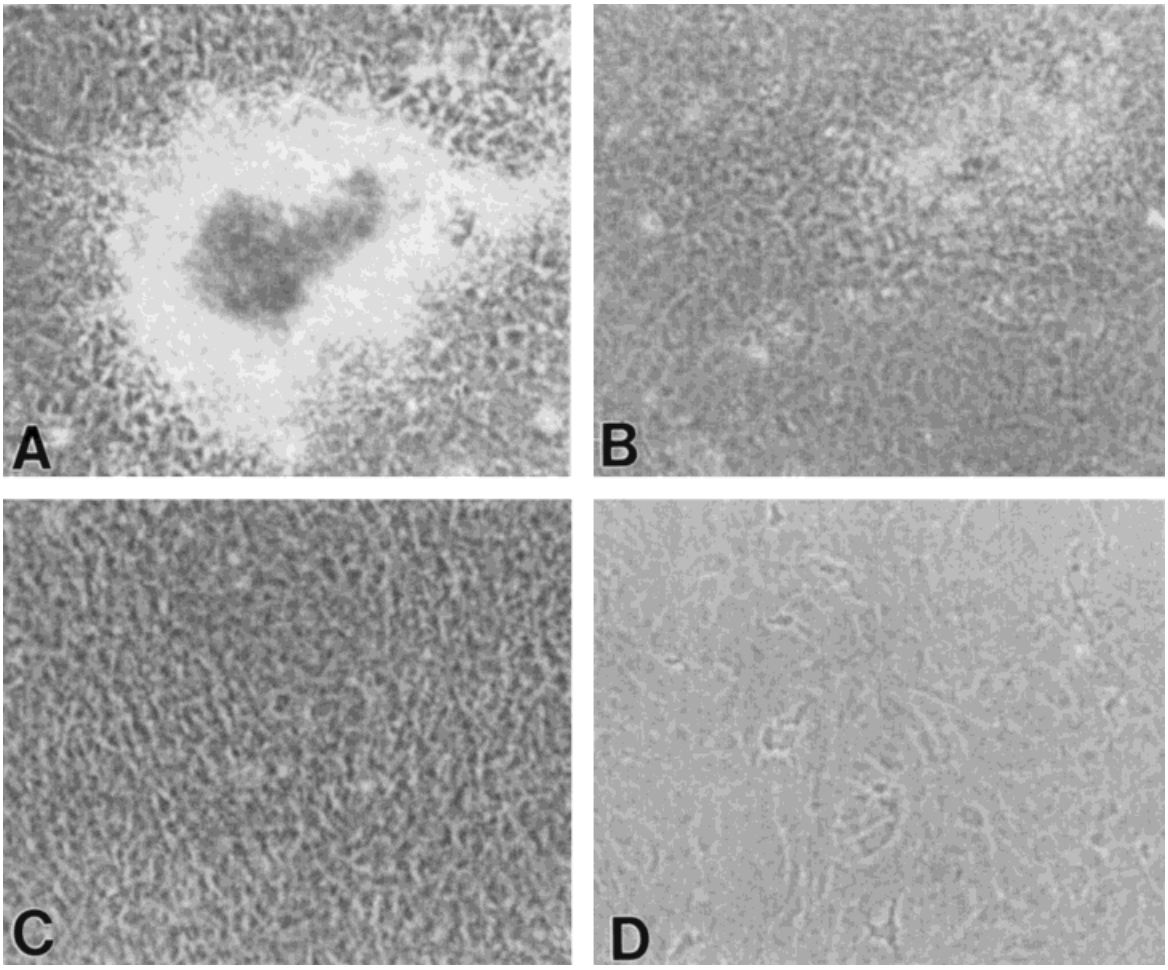

Fig. 5. Light microscopy of osteoblast cultures on day 23 for control cells (**A**), cells treated with TGF-β from days 4–6 (**B**), cells treated with TGF-β from days 4–23 (**C**), and nonmineralized cells (**D**) grown in the absence of ascorbic acid, Dex, and β-GP, requirements for differentiation. ×40.

consequence of the acute TGF-β treatment. Therefore, the changes in NMPs in treated cultures may either reflect the extent of developmental inhibition (lack of nodules) to which the cells have been subjected or be related to TGF-β–regulated proteins.

NM-IFs Responsive to TGF-β1 Can Be Identified

We further investigated the extent to which observed differences in NM-IF proteins were linked to a TGF-β1 treatment and/or the block in mineralized nodule formations. We compared the NM-IF protein profile of the cultures treated with TGF-β (continuously) to the nonmineralized control cultures (Fig. 7A–C). Clusters of protein unique to both the mineralized and nonmineralized control cultures are observed in the 31–36 kD and 62–76 kD ranges, supporting the concept that specific nuclear matrix proteins accompany the absence of mineralization. Cultures continuously treated with TGF-β1 and nonmineralized control cultures reveal that the NM-IF proteins in the 62–76 kD range of the TGF-β–treated cultures represent those present in the mineralized cultures and not the nondifferentiated osteoblasts cultured in the absence of ascorbic acid and β-glycerol phosphate. In addition, several NM-IF proteins unique to the TGF-β–treated cells can be identified that may be a consequence of a direct effect or the modified cell phenotype. The NM-IF protein profile of the control cells more closely resembles the NM-IF protein profile of cells treated continuously with TGF-β than the NM-IF protein profile of the nonmineralized cultures. The observation that cells treated continuously with TGF-β have the capacity to multilayer, while the nonmineralized control cells

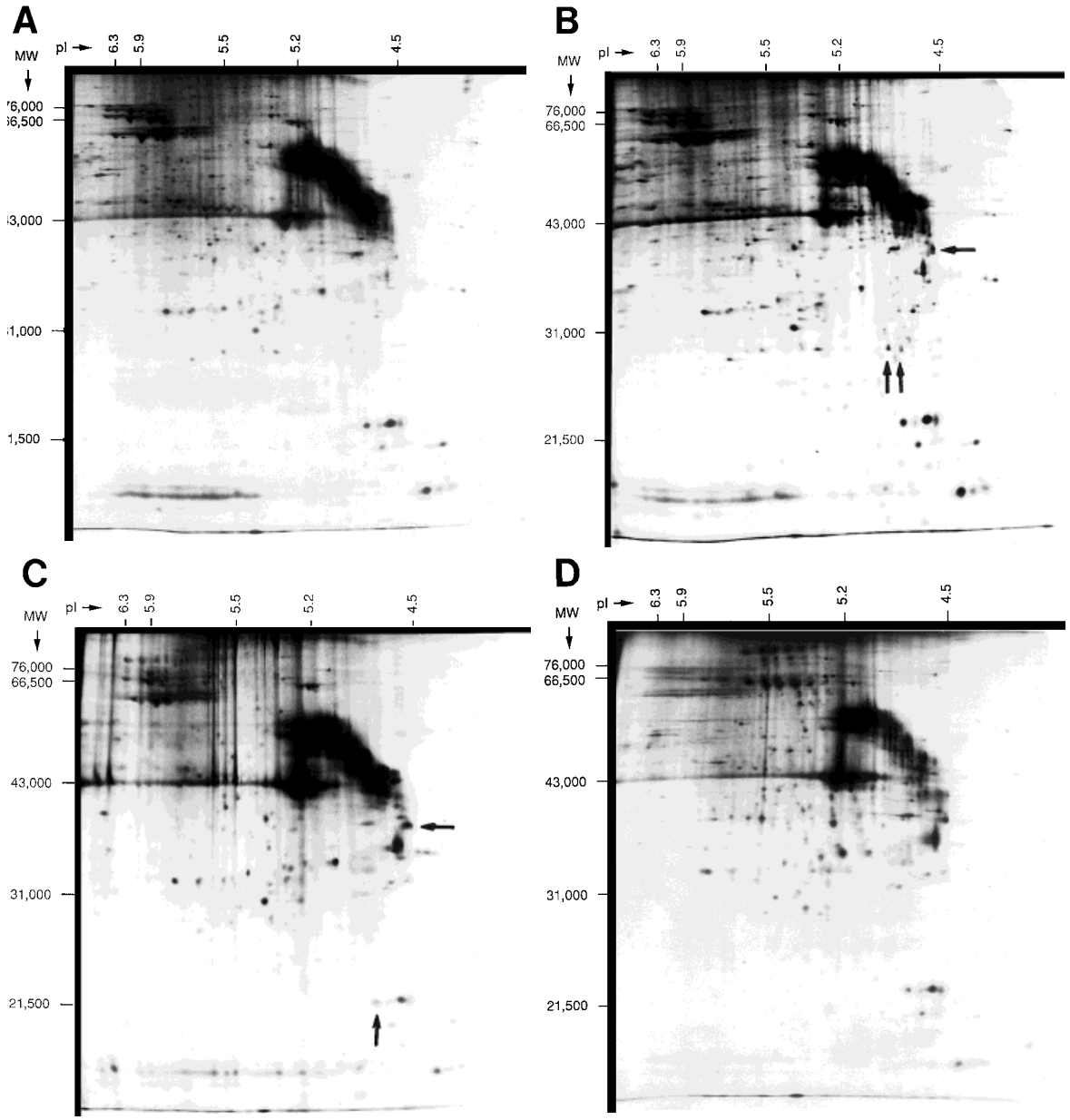

Fig. 6. Nuclear matrix protein profiles from day 23 osteoblast cells for control cells (**A**), cells treated with TGF-β from days 4–6 (**B**), cells treated with TGF-β from days 4–23 (**C**), and nonmineralized cells (**D**) grown in the absence of ascorbic acid, Dex, and β-GP. Arrows on NM-IF protein profiles from TGF-β–treated cultures (B,C) point out differences between the respective TGF-β profile and the control (A).

only monolayer and therefore remain at an even earlier stage of development, may in part provide an explanation for this finding.

In summary, the findings of these studies indicate that TGF-β can induce several unique nuclear matrix proteins that are a consequence of the modification in phenotypic properties which are associated with chronic exposure of osteoblasts to TGF-β. Nuclear matrix intermediate filament protein profiles from osteoblasts producing mineralized nodules differ from those observed in osteoblasts allowed to reach monolayer confluency but prevented from nodule formation and mineralization. The representation of NMPs must be interpreted within the context of our observations that striking differences in TGF-β modifications of NMPs occur only when the cultures had reduced numbers of nodules and not immediately following treatment. NMPs are architectural and/or func-

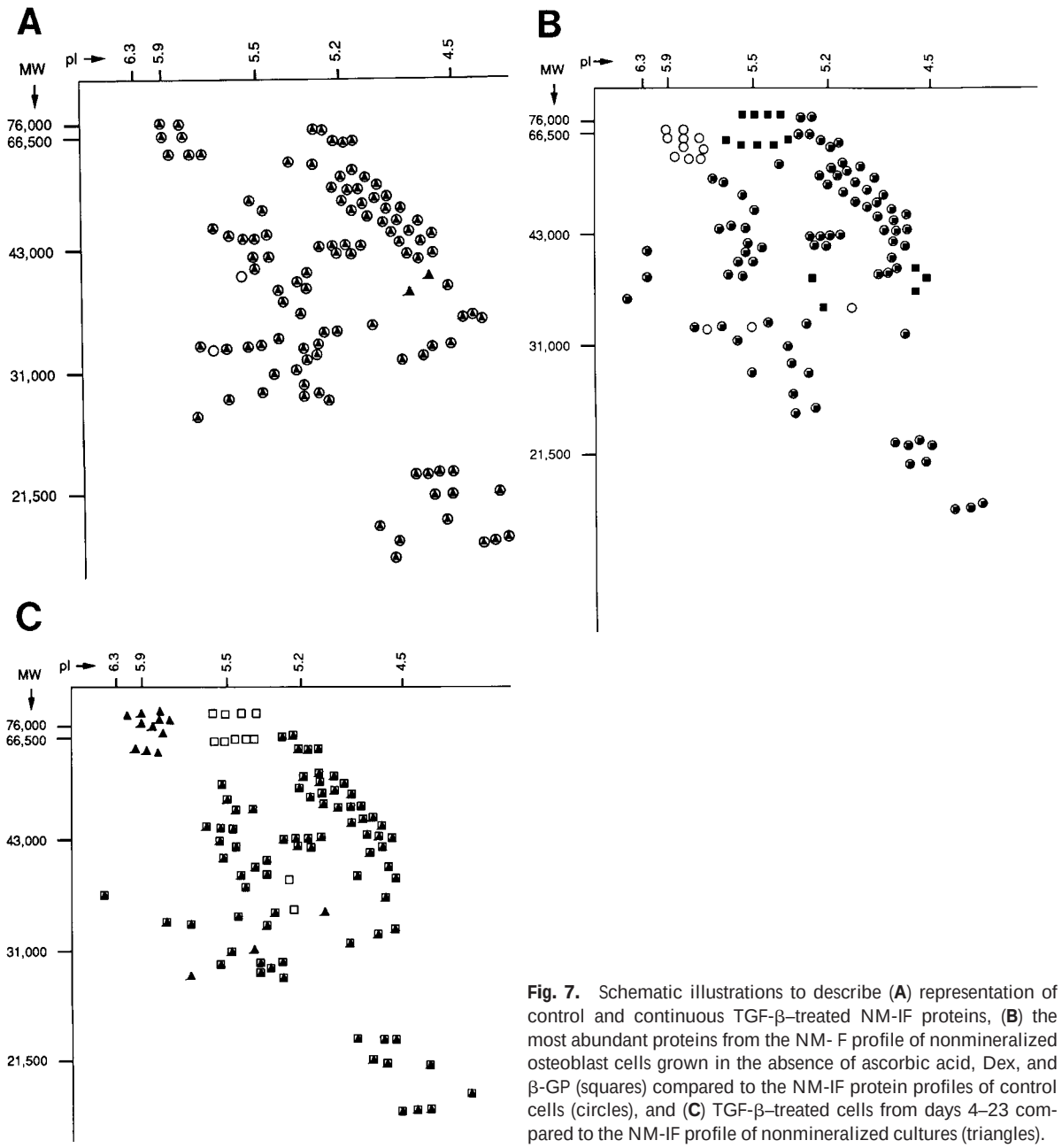

Fig. 7. Schematic illustrations to describe (**A**) representation of control and continuous TGF-β–treated NM-IF proteins, (**B**) the most abundant proteins from the NM- F profile of nonmineralized osteoblast cells grown in the absence of ascorbic acid, Dex, and β-GP (squares) compared to the NM-IF protein profiles of control cells (circles), and (**C**) TGF-β–treated cells from days 4–23 compared to the NM-IF profile of nonmineralized cultures (triangles).

tional components of the nuclear matrix. However, gene regulatory factors controlling phenotypic genes are present in low abundance. Acute responses to TGF-β which indirectly regulate gene transcription may not result in detectable differences of these low abundance NMPs. The documentation of striking changes in NM and NM-IF profiles between normal and tumor cell lines and cells from different tissues supports the value of NM-IF protein profiles in discriminating cellular phenotypes [reviewed in Getzenberg et al., 1990; Stein et al., 1996]. Although we observe an obvious decrease in formation of bone nodules and mineralization, there are limited and specific TGF-β–induced modifications in nuclear matrix proteins. Taken together, this suggests that TGF-β does not acutely modify the osteoblast phenotype but compromises competency for ECM mineralization. One can speculate that those NM-IF proteins which are unique to TGF-β–treated cultures may be related to suppression of the final mineralization stage of the osteoblast and that inherently the cells retain

osteoblast phenotypic properties of immature bone cells. The selective modifications of the nuclear matrix may reflect architectural or gene regulatory mechanisms that support the consequential effects of TGF-β signalling on osteoblast maturation.

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