

DIFFERENTIATION OF RAT OSTEOBLAST-LIKE CELLS IN MONOLAYER AND MICROMASS CULTURES

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Abstract

During intramembranous bone formation, preosteoblasts condense, differentiate into osteoblasts and deposit bone matrix. We compared the differentiation process of rat calvarial osteoblast-like cells inoculated as micromasses, which mimic the *in vivo* condensation process, with cells inoculated as monolayers. The cells were analysed morphologically at 1, 2 and 3 weeks by light microscopy (alkaline phosphatase activity, mineralization), by transmission electron microscopy, and biochemically (collagen typing, alkaline phosphatase activity, protein and DNA content). The cells inoculated as monolayers formed alkaline phosphatase positive and mineralized nodules during the culture period. The cells inoculated as a micromass formed a large mineralized area consisting of smaller nodules. The ultrastructure of the cells in both culture systems showed the typical features of osteoblasts and osteocytes. The main difference between monolayer and micromass cultures was found after 1 week in culture. The cells inoculated as a micromass formed a multilayer of cells. The cytoplasm contained rER, mitochondria, vesicles and ribosomes. There were abundant collagen fibrils in membrane folds and in the extracellular matrix. This was in contrast to the cells in monolayer culture which showed hardly any collagen fibrils in the extracellular matrix. The promotion of the differentiation was also confirmed by biochemical data showing that the DNA content was lower in the micromass than in the monolayer cultures during the culture period. These results show that micromass, as compared to monolayer, culture promotes the differentiation of rat osteoblast-like cells *in vitro*.

Key Words: Osteoblasts; cell culture; micromass culture; monolayer culture; differentiation; inoculation; light microscopy; ultrastructure; biochemistry

Introduction

The *in vitro* osteoblast developmental sequence can be considered as a re-initiation of events occurring *in vivo*, because the freshly isolated cells have similar levels of bone phenotype-associated transcripts (Owen *et al.*, 1990). There are three principal periods in the differentiation of rat osteoblasts in culture: proliferation, extracellular matrix maturation and mineralization (Owen *et al.*, 1990). Mineralization of the formed nodules occurs after 2-3 weeks in monolayer cultures (Bellows *et al.*, 1991; Collin *et al.*, 1992; Gerstenfeld *et al.*, 1987). In contrast, the mineralization in micromass cultures starts at 1 week and by 2-3 weeks most of the culture consists of mineralized tissue (Masquelier *et al.*, 1990; Zimmermann *et al.*, 1988). In this study we compared the differentiation process of rat osteoblasts *in vitro*, inoculated with the same cell number per dish, either as monolayer cultures or as micromass cultures. To follow the differentiation process, the cells were analyzed at 1, 2, and 3 weeks by observing the ultrastructure of the cells, by histochemical detection of the alkaline phosphatase activity (ALP) and von Kossa stain for mineralization. Furthermore, ALP activity, DNA and protein content were quantified at 1, 2 and 3 weeks.

Materials and Methods

Materials

All tissue culture disposable materials were purchased from Falcon (Becton Dickinson AG, Basel, Switzerland). All growth media and fetal calf serum were purchased from Gibco (Invitrogen, Basel, Switzerland). All chemicals were purchased from Fluka (Buchs, Switzerland) when not otherwise stated.

Methods

Osteoblast cell culture. Parietal and frontal calvariae (4 per animal) were aseptically explanted from 6 day old IcoIbm rats. The calvariae were placed in Tyrode's buffered salt solution calcium- and magnesium-free (TBSS). The periosteum and endosteum were removed enzymatically with 0.05 % trypsin (1:250; Sigma, Buchs, Switzerland) and 0.02 % collagenase A (0.76U/mg; Roche Diagnostics, Rotkreuz, Switzerland) dissolved in TBSS (40 calvaria/20 ml). The calvariae were shaken for 70 min in a water bath at 37°C. After washing with TBSS, they were treated with 0.02 % collagenase A in BGJ₆ Fitton-Jackson modification for 3 h. Afterwards the calvariae were

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washed with BGJ_b supplemented with 10 % foetal calf serum. The calvariae were then transferred into 60 mm culture dishes (4 frontal and 4 parietal/dish). The growth medium supplemented with 10% FCS and 50 µg/ml ascorbate was changed completely every 48 h. The culture was kept for 3 weeks. After 3 weeks the migrated cells and the calvariae were washed with TBSS and 5 ml of TBSS containing 0.05 % trypsin and 0.02 % collagenase A (0.76 U/mg) was added. After 1 h in the incubator the dish was washed several times with culture medium BGJ_b supplemented with 10 % FCS. The cells obtained were filtered through a 40 µm nylon mesh. The suspended cells were centrifuged at 600 g for 5 min. The cell pellet was resuspended in serum containing medium BGJ_b and centrifuged. The viability of the resuspended cells was tested by the 'dye exclusion' of 0.4 % trypan blue and the cells counted in a haemocytometer. The inoculation densities were 2·10⁵/10 cm² for monolayers and 2·10⁵/30 µl/10 cm² for micromasses. The micromass cultures were kept for 1 h in the incubator before 2 ml growth medium was added. All the cultures were kept at 37°C in a humidified atmosphere of 5 % CO₂ and 95 % air. The media were completely changed every 48 h. The growth medium was supplemented with 50 µg/ml ascorbate.

Light microscopy. After 1, 2 and 3 weeks the cultures were stained histochemically for the alkaline phosphatase, using the Sigma Kit no. 85L (Sigma, Buchs, Switzerland). As a result, an insoluble, visible blue pigment is formed at sites of phosphatase activity. At 2 and 3 weeks mineralization was detected by von Kossa staining.

Transmission electron microscopy (TEM). At 1, 2 and 3 weeks, the cell cultures were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer pH 7.4 for 20 min, postfixation in 1% OsO₄ in 0.1 M cacodylate buffer pH 7.4 for 1 h at 4°C, then 2 % aqueous uranyl acetate for 1 h at room temperature; dehydration in an ethanol series and embedding in LR White (London Resin Co, Basingstoke, U.K.). Ultrathin sections through the nodular area of monolayer cultures were cut with a Drukker Diamond knife (Drukker International, Cuijk, The Netherlands) on a LKB III Microtome (LKB, Bromma, Sweden) and stained with 2% uranyl acetate and lead citrate (Reynolds, 1963). Osmium rich regions, i.e., the nodules in the monolayer, and the centers of the micromass cultures, were selected respectively. The sections were examined using a JEOL (Tokyo, Japan) JEM 100CX transmission electron microscope operated at 100kV.

Biochemistry. Whole cultures were washed with phosphate buffered saline and 500 µl 0.25 M sucrose/35 mm dish was added. The cells were scrubbed off the dish and transferred into a cryotube and frozen at -80°C. Immediately before the assays, the cells were thawed and sonicated (3 x 20s) at 70 W and 20 kHz on ice. The samples were analysed in a Perkin Elmer UV/VIS Spectrophotometer Lambda 12 (PerkinElmer Instruments,

Huenenberg, Switzerland) and in a Hoefer TKO 100 Mini-Fluorometer (Hoefer Scientific Instruments, San Francisco, CA) for DNA determination.

Alkaline phosphatase activity (ALP). A Sigma Kit no. 245 (Sigma, Buchs, Switzerland) was used for the quantitative, kinetic determination of alkaline phosphatase activity.

Total protein. The Bio-Rad protein assay kit II (BioRad, Glattbrugg, Switzerland) was used with bovine serum albumin as standards.

DNA. A simple and rapid assay for quantitative DNA determination in crude homogenates was used (Labarca and Paigen, 1980). Each sample was diluted with 3 ml of phosphate buffered saline, pH 7.4 containing 2 M NaCl (DPBS) and sonicated as described above. Then aliquots were mixed with 0.1 µg/ml Hoechst 33258 in DPBS and the fluorescence was read in a Hoefer TKO 100 Mini-Fluorometer.

Collagen. During the culture period, the growth medium was supplemented with 60 mg/ml b-aminopropionitrile to prevent cross linking of the collagen fibrils. Whole cell layers were harvested and processed, as previously described for chondrocyte cultures (Bruckner *et al.*, 1989). After 3 weeks in culture whole culture dishes were frozen (calvaria and the outgrown cell were separated), thawed, homogenized and treated with 25 ml of 0.5 M acetic acid, containing 0.2 M NaCl and 1 mg/ml of pepsin and stirred for 48 h at 4°C. To inactivate pepsin 1/10 vol of 1 M unbuffered Tris was added and the samples were neutralized by addition of 10 M NaOH, adjusted to 1 M in Na⁺-ion concentration by addition of solid NaCl, and extracted for 48 h at 4°C. The undigested residue was centrifuged at 27'000 g. The collagens were precipitated with 176 mg/ml (NH₄)₂SO₄. The pellet was dissolved in 0.4 M NaCl, 0.1 M Tris-HCl, pH 7.4. Aliquots were precipitated with 3 vol ethanol, and dissolved in 0.1 M Tris HCl, 0.8 M urea, 2% SDS, 10% glycerine, pH 6.8. A first aliquot remained unreduced, a second aliquot was reduced with 2 % b-mercapto-ethanol. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by (Laemmli, 1970), using a 4.5 % stacking gel and a 4.5 % - 15 % gradient running gel. Collagen standards were run in parallel with the samples. The electrophoresis was performed at 220 V, followed by staining with Coomassie blue.

Statistics. To compare the treatment, contrast analysis of variance models was performed. Main effects and interaction effects were examined by F-Tests. 'Least Squares Means' were calculated to yield average means accounting for the other variables in the model. LS Means were compared by using Tukey's multiple range test. QQ-Plots of the residuals and Tukey-Anscombe plots (residuals x predicted) were analyzed to check for a normal distribution assumption.

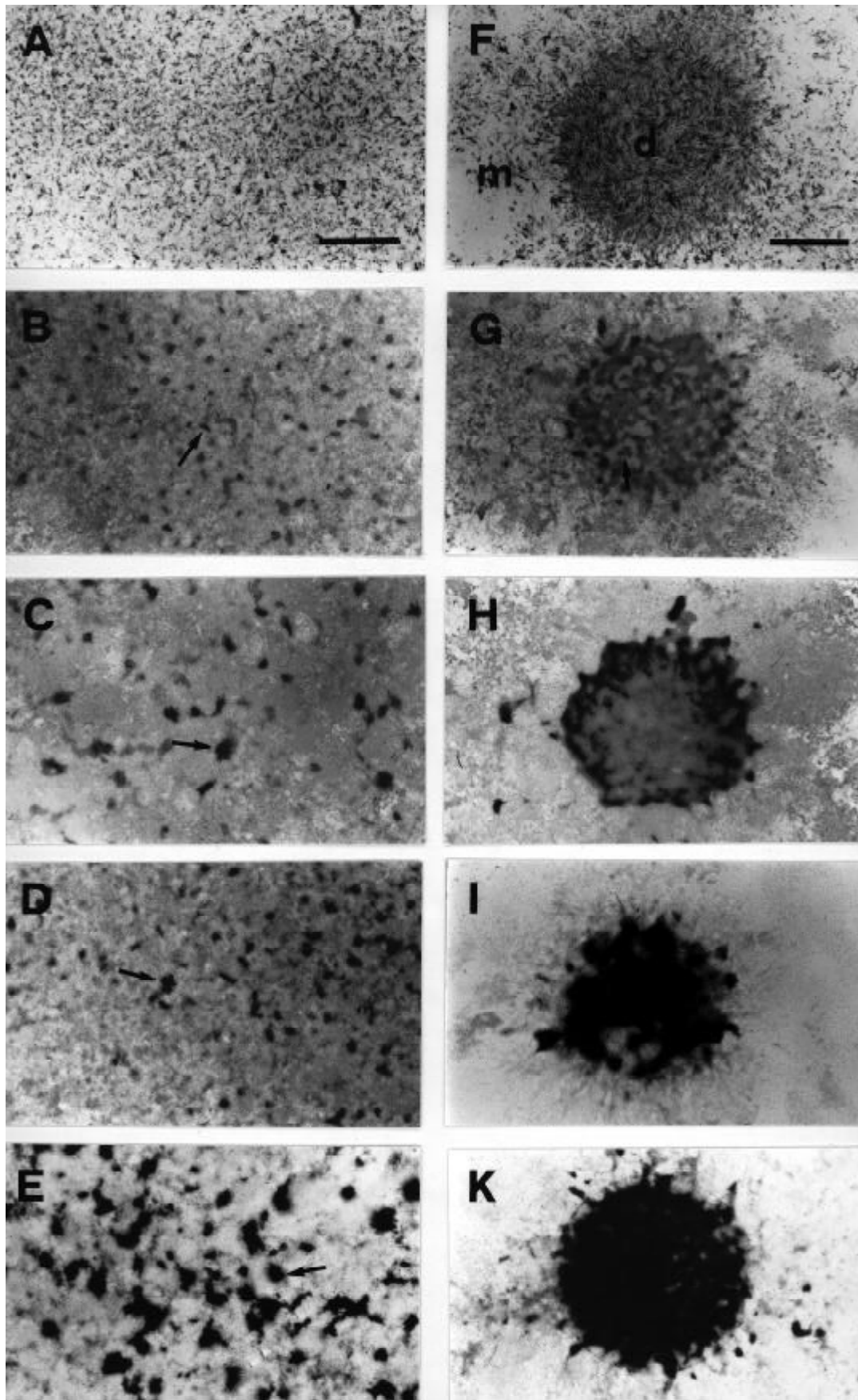


Figure 1. Light microscopical view of monolayer (A-E) and micromass (F-K) cultures at 1,2 and 3 weeks. Same magnification in all pictures, bar scale 3mm. Nodule formation (arrow), originally inoculated drop of cells (d) and migrated cells (m). A) ALP activity at 1 week. B) ALP activity at 2 weeks. C) ALP activity at 3 weeks. D) mineralization at 2 weeks. E) mineralization at 3 weeks. F) ALP activity at 1 week. G) ALP activity at 2 weeks. H) ALP activity at 3 weeks. I) mineralization at 2 weeks. K) mineralization at 3 weeks.

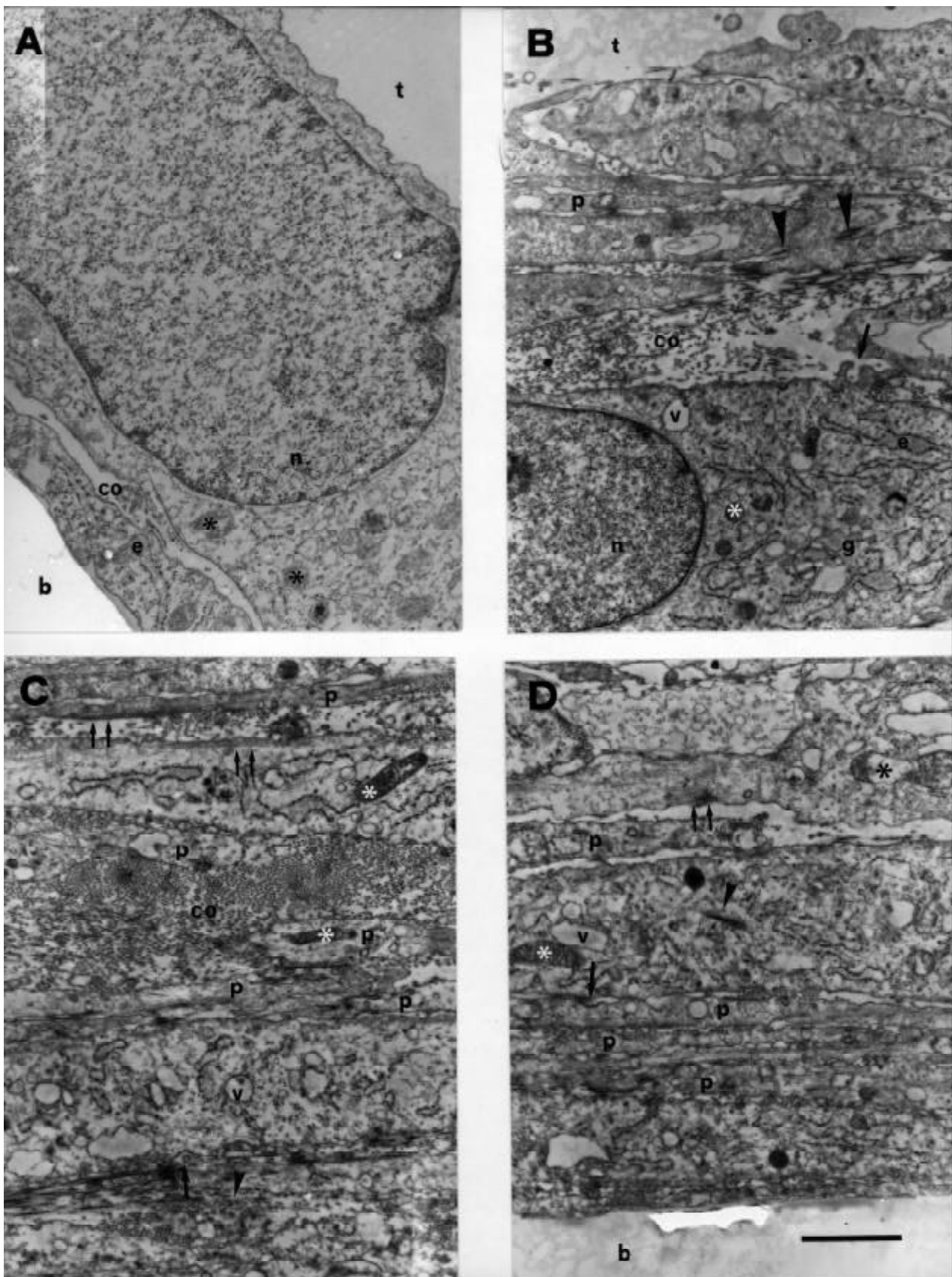


Figure 2. Ultrastructure at 1 week. Same magnification in all pictures, bar scale 2 μ m. Growth medium interface (t), cultures dish surface (b), nucleus (n), endoplasmatic reticulum (e), mitochondria (asterisk), vesicle (v), Golgi area (g), cytoskeletal elements (double arrow), cell processes (p), endo/exocytosis (arrow), collagen fibrils (co), 'extruding' collagen fibrils (large arrow head), details shown in next figure (small arrow head). A) Monolayer. B) Micromass; top cell layers. C) Micromass; middle cell layers. D) Micromass ; lower cell layers.

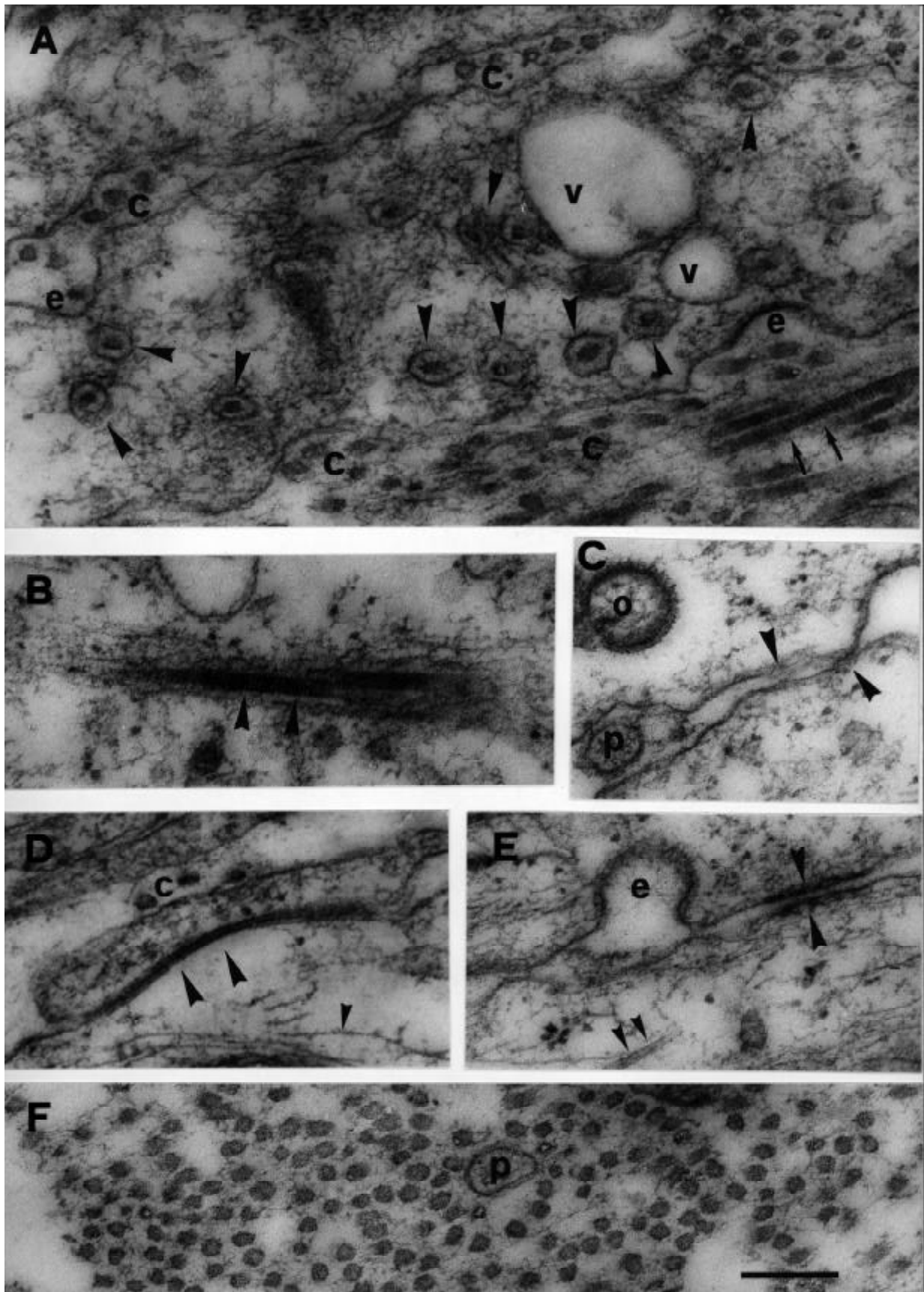


Figure 3. Detail of ultrastructure of micromass cultures at 1 week. Same magnification in all pictures; bar scale 150 nm. Collagen fibrils in cross section (c), endo/exocytosis (e), cell process (p). (A) cross section of collagen fibrils in membrane folds (arrow head), cross striation of collagen fibrils (arrow), vesicle (v). (B) longitudinal section of collagen fibrils (double arrow head) in membrane fold C). Focal contact points (arrow head), annular gap junction (o). (D) gap junction (large arrow head), cytoskeletal element (small arrow head). (E) cell membrane in close apposition (large arrow head), thick cytoskeletal element (small arrow head). (F) Extracellular collagen fibrils in cross section.

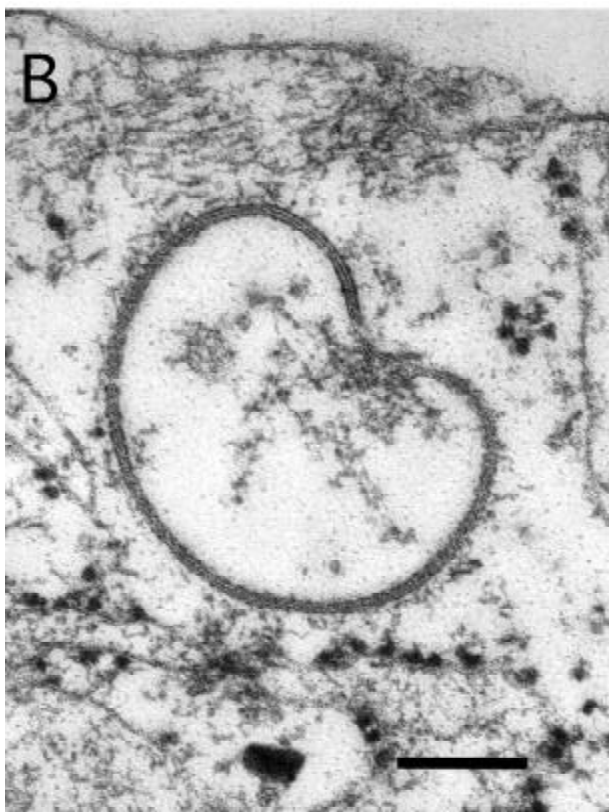
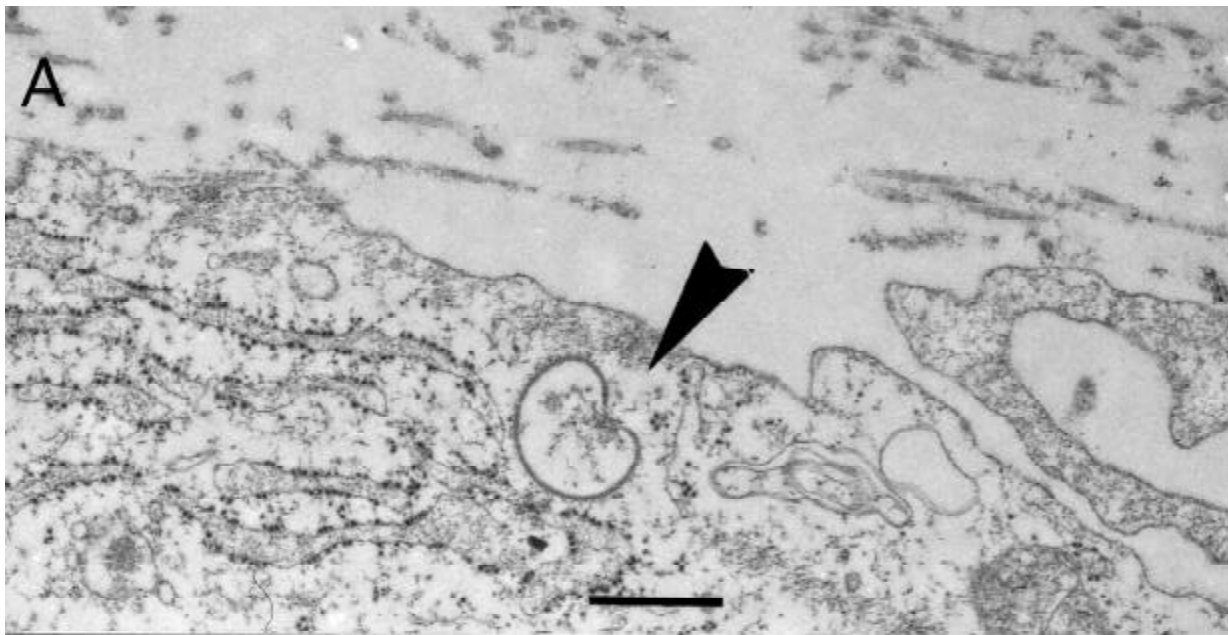


Figure 4. A) Ultrastructure at 1 week of oval gap junction (arrow head), bar scale 500 nm. B) Detail of A; bar scale 75 nm.

ules (Fig. 1G, 1I). At 3 weeks, the original drop of cells was fully mineralized and the ALP positive cells were confluent (Fig. 1H, 1K).

TEM Ultrastructure. Monolayer cultures at 1 week were different from the micromass ones. The monolayer culture had only some areas with more than 2 cell layers (Fig. 2A). There was hardly any collagen between the cell layers of the monolayer. The cytoplasm contained rER, which contained material with medium density, mitochondria, and many vesicles and ribosomes (Fig. 2A). The presence of cytoskeletal elements was not evident with the fixation procedure we used.

The micromass at 1 week consisted of a thick multilayer of elongated cells (Fig. 2B-2D). Between the cells in the middle layers there were abundant orthogonally oriented collagen fibrils and typical cross-striation was seen (Fig. 2B, 2C). These fibrils were also found in membrane folds but the frequency varied from section to section (Fig. 3A, 3B). The collagen fibrils in membrane folds resembled the extracellular fibrils in diameter, staining pattern, and electron density (Fig. 3A, 3F). The diameter of the collagen fibrils was rather uniform at approximately 50 nm. The lower cell layers only had a few collagen fibrils in the adjoining extracellular matrix (Fig. 2D). The cells had many cell-cell contacts either as focal points or with longer cell membrane areas in very close apposition (Fig. 3C-3E). There were gap junctions observed with various appearances such as annular (Fig. 3C), stretched (Fig. 3D) or oval (Fig. 4). The cytoplasm contained abundant rER, which was dilated in some cells, and many free ribosomes (Fig. 2B-2D). The mitochondria were numerous and of various sizes and shapes, accounting for sec-

Results

ALP and mineralization. Most of the cells cultured as monolayers stained positively for ALP after 1 week (Fig. 1A). After 2 weeks there were ALP positive and calcified nodules, which increased in size and number at 3 weeks (Fig. 1B-1E). In micromass cultures at 1 week, the original drop consisted of ALP positive cells and there were many ALP positive cells migrating off the original drop (Fig. 1F). At 2 weeks, the original drop of inoculated cells consisted of smaller ALP positive and mineralized nod-

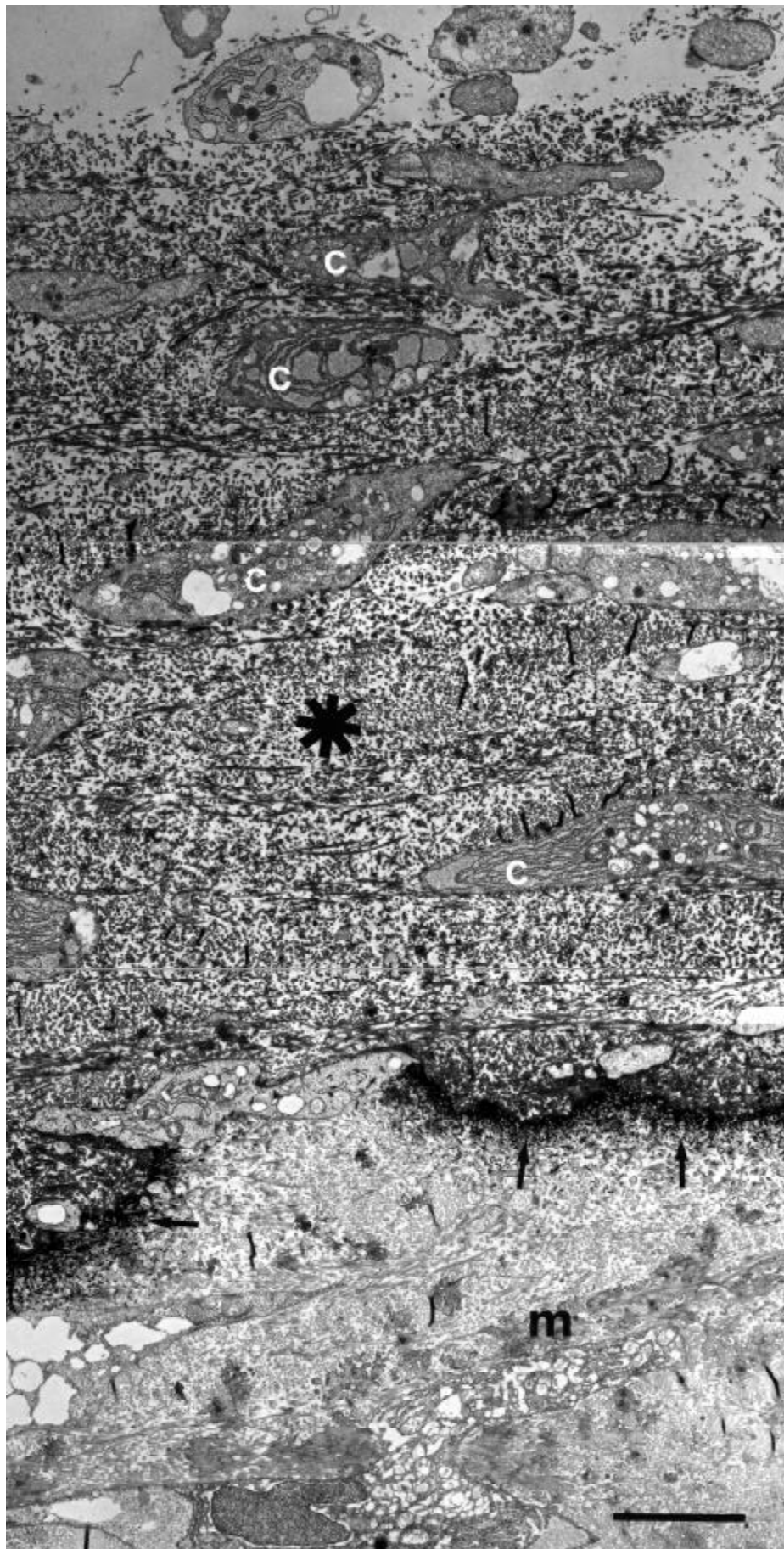


Figure 5. Ultrastructure at 3 weeks. Bar scale 5 mm. Cells (c) separated by collagen fibrils (star), mineralized matrix (m), mineralization front (arrow).

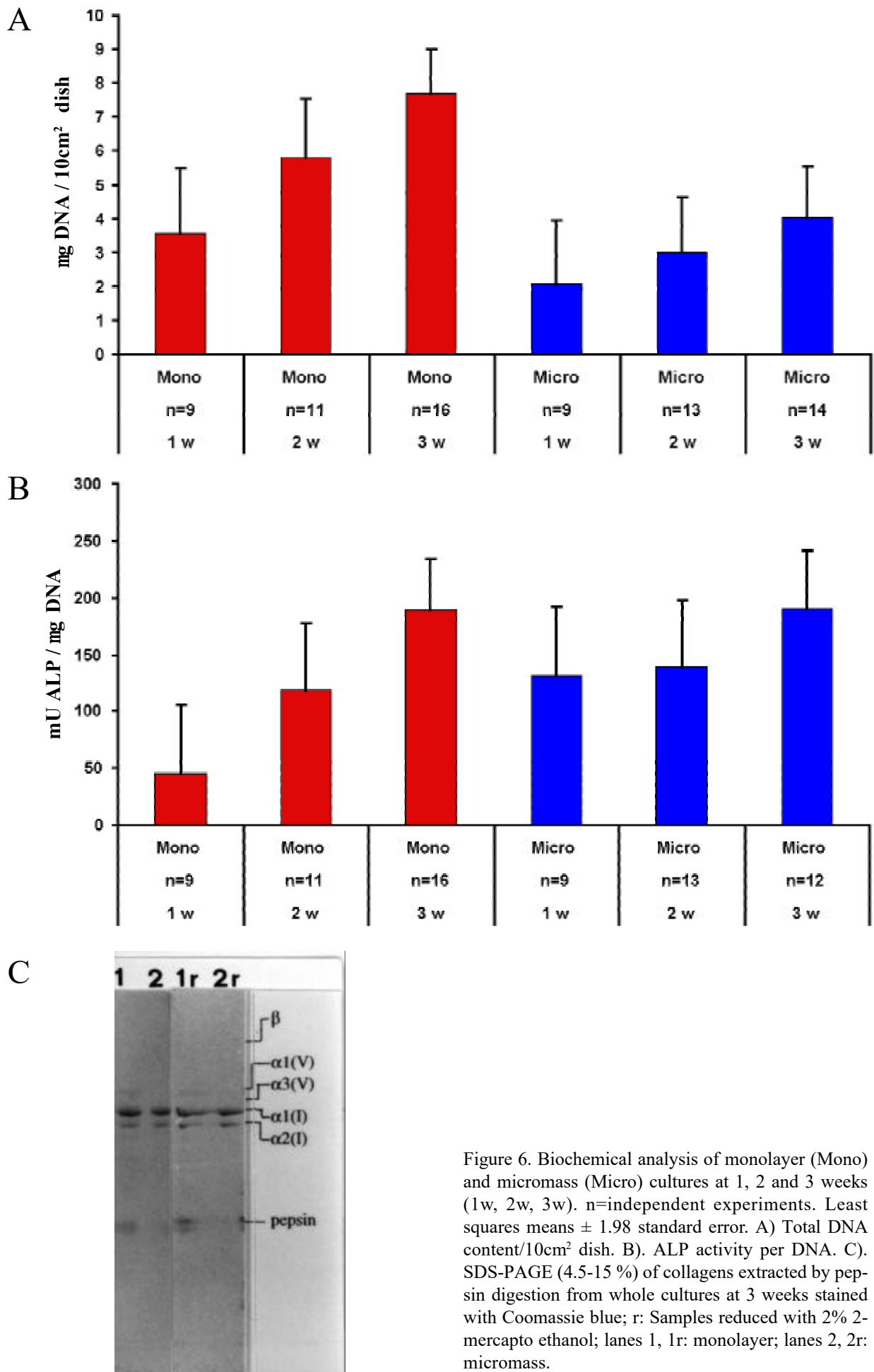


Figure 6. Biochemical analysis of monolayer (Mono) and micromass (Micro) cultures at 1, 2 and 3 weeks (1w, 2w, 3w). n=independent experiments. Least squares means \pm 1.98 standard error. A) Total DNA content/10cm² dish. B). ALP activity per DNA. C). SDS-PAGE (4.5-15 %) of collagens extracted by pepsin digestion from whole cultures at 3 weeks stained with Coomassie blue; r: Samples reduced with 2% 2-mercapto ethanol; lanes 1, 1r: monolayer; lanes 2, 2r: micromass.

tioning effects, ranging from round to banana-shaped (Fig. 2B-2D). The Golgi area and the vesicles were numerous, showing diverse ranges of size, form and electron density (Fig. 2B-2D). There were coated pino/exocytotic vesicles fused with the plasma membrane (Fig. 2B-2D, Fig. 3A, E). Cytoskeletal filaments of varying diameters were present (Fig. 2B-2D; Fig. 3D, E). Many microfilaments were running parallel to the plasma membrane in the cortical region of the cells. Some filaments of larger diameter were also present (Fig. 3E). Many cell processes had prominent densely packed microfilaments lying mainly parallel to the culture dish surface (Fig. 2B-2D).

At 2 and 3 weeks, the ultrastructure of the nodules of the monolayer and the centre of the micromass were similar (Fig. 5). The cells in monolayer and micromass culture were characterized as described for micromass at 1 week. The cells deeper in the multilayer were completely surrounded by a mineralized matrix (Fig. 5). The amount of collagenous matrix further increased as did the mineralized zones which coalesced into a seam. The cells were separated by massive collagen layers (Fig. 5). The bulk of the orthogonally arranged collagen fibrils was found in the middle to upper cell layers. The diameter of collagen fibrils was larger than at 1 week and varied from 50 to more than 100 nm. The collagen fibrils were also found in membrane folds (not shown).

Biochemistry. The observed increase in the cell density was confirmed by the quantification of the DNA content. The monolayer culture had a significantly ($p < 0.015$) higher DNA content at 3 weeks than at 1 week (Fig. 6A). The increase in the DNA content in micromass culture was non significant over time. Furthermore, the micromass cultures had a significantly ($p < 0.015$) lower DNA content than monolayers at 3 weeks (Fig. 6A). This indicated that the cells inoculated as monolayers have a higher proliferation rate than the cells kept as micromass cultures. The total protein content and the total protein content expressed per mg DNA of the monolayer or the micromass cultures did not increase significantly during the culture time (data not shown). The progression of differentiation was also seen on the biochemical level, in such a way that the ALP activity expressed per mg DNA of the monolayer was significantly ($p < 0.008$) higher at 3 weeks than at 1 week while no significant differences in the ALP activity per DNA were observed in the micromass during culture time (Fig. 6B). The micromass cultures had a higher ALP activity per DNA than the monolayer cultures but the differences were non significant. These results provided evidence that the cells in micromass cultures attained a highly differentiated state relatively early. The isolated cells inoculated either as monolayer or micromass produced mainly collagen type I, some collagen type V (Fig. 6C).

Discussion

The results of this study showed that cell-cell contacts, induced by micromass cultures, promoted the formation of differentiated osteoblasts at 1 week. These findings were

based on ultrastructural observations and biochemical analysis.

The cells in micromass cultures formed a multilayer consisting of elongated cells separated by abundant orthogonally oriented collagen fibrils with a rather uniform diameter and the typical cross-striation. The ultrastructure of the cells kept as micromasses showed the typical features of osteoblasts described by others (Hancock and Boothroyd, 1965; Weinger and Holtrop, 1974).

Osteoblasts, cultured in monolayers rather than micromasses, were different. Hardly any collagen was detectable between the cell layers. The cytoplasm also tended to contain less organelles at 1 week, but after 2 weeks there were no detectable ultrastructural differences. Gerstenfeld (Gerstenfeld *et al.*, 1993), describes osteoblasts as being flattened with small nuclei and some cytoplasmic organelles, such as ribosomes, ER, and a few mitochondria and vesicles at early culture times. During the culture time, the progressive increase in size and organelle content correspond to the greater metabolic activity of these cells during their post-proliferative period of active extracellular matrix synthesis (Gerstenfeld *et al.*, 1993).

Furthermore, collagen fibrils were often found within membrane folds. These findings were identical to those described in tendon fibroblasts (Trelstad and Hayashi, 1979). The close contact between some cells by tight junctions, gap junctions or by apposition of their plasma membranes has also been described previously (Arana-Chavez *et al.*, 1995). We also found oval and annular gap junctions (Shapiro, 1997) predominantly in micromass cultures. There is much evidence that the formation of cell-cell contacts is fundamental for the differentiation of osteoblasts. Cells of the osteogenic lineage form a continuous protoplasmic network that extends from the osteocytes to the vascular endothelium, passing through osteoblasts (or bone lining cells) and stromal cells (Palazzini *et al.*, 1998). Gap junctions occur at these sides, enabling metabolic and electric coupling and thus forming a functional syncytium (Palazzini *et al.*, 1998). Gap-junctional communication (GJC) is required for the maturation process of osteoblastic cells MC3T3-E1 in culture (Schiller *et al.*, 2001a). As the cell progress toward a mature phenotype, mRNA and protein for connexin 43 (Cx43) and gap-junctional intercellular communication (GJC) are increased (Schiller *et al.*, 2001b). Such an increased GJC was accompanied by a parallel decrease in ALP activity and by an increase of osteocalcin expression (Romanello *et al.*, 2001). The effect of GJC on proliferation is controversial. On one hand an enhancement of Cx43 expression increases proliferation and differentiation of an osteoblast-like cell line UMR 106-01 (Gramsch *et al.*, 2001). On the other hand, temperature sensitive osteoblastic cell line exhibits an inhibition of proliferation, increasing Cx43 mRNA and protein expression and increased GJC at the permissive temperature (Donahue *et al.*, 2000). Altering gap junctional permeability by manipulating the expression of Cx43 and Cx45 in osteoblastic cells alters transcriptional activity of osteoblast-specific promoters (Lecanda *et al.*, 1998).

GJC is affected by various treatments. Prostaglandin E2 or PTH increases the expression of Cx43 mRNA and the formation of gap junctions (Civitelli *et al.*, 1998; Massas and Benayahu, 1998). The timing of PTH treatment, that increases matrix mineralization, coincides with the highest expression of Cx43 (Schiller *et al.*, 2001b). However, estrogen uncouples osteoblastic cells (Massas *et al.*, 1998). Exposure of osteoblastic cells to magnetic fields leads to an inhibition of the cell growth which is independent of GJC, but ALP activity is dependent on GJC (Vander Molen *et al.*, 2000). Serial passage of MC3T3-E1 cells reduces changes in cell morphology, GJC and responsiveness to TGF-B1 and BMP-2 (Chung *et al.*, 1999). Genetic deficiency in Cx43 causes a delayed ossification, craniofacial abnormalities and osteoblast dysfunction (Lecanda *et al.*, 2000). Cells rendered Cx43 deficient through antisense transfection retained the osteoblastic characteristics but displayed a dramatic attenuation in the cAMP response to PTH (Vander Molen *et al.*, 1996). Furthermore, an inhibition of GJC induces trans-differentiation of osteoblasts to an adipocytic phenotype *in vitro* (Schiller *et al.*, 2001c).

The morphological findings were confirmed by biochemical analysis. Differences between monolayer and micromass cultures were also observed at the biochemical level. The micromass cultures had a higher ALP activity relative to the DNA or protein content than the monolayer cultures at 1 week, but at 2 or 3 weeks these values were similar. The cells inoculated as a monolayer had to proliferate to establish cell-cell contacts. These changes were reflected by a significant increase in the DNA content and ALP activity in monolayer cultures during the second and the third weeks of culture, whereas the DNA accumulation and ALP activity in micromass cultures was non significant over the culture period.

The morphological and biochemical results were consistent with those reported in the literature (Aronow *et al.*, 1990; Aubin 1999; Bellows *et al.*, 1991; Bhargava *et al.*, 1988; Ecarot-Charrier *et al.*, 1983; Gerstenfeld *et al.*, 1993; Masquelier *et al.*, 1990; Nefussi *et al.*, 1997; Owen *et al.*, 1990; Wiestner *et al.*, 1981; Zimmermann *et al.*, 1988; Zimmermann *et al.*, 1991). This indicated that the cells studied were normal, as determined by these criteria.

In conclusion, micromass culture probably promoted the differentiation of osteoblasts by promoting early cell-cell contacts, by increasing the formation of collagen and by decreasing the proliferation. The micromass culture system seemed to mimic the *in vivo* condensation processes, which is a pivotal stage in skeletal development (Hall and Miyake, 2000). During intramembranous bone formation preosteoblast alkaline phosphatase positive cells form before condensation but after the epithelial-mesenchymal interaction upon which preosteoblast formation and condensation depend. Preosteoblasts then condense, transform into osteoblasts and deposit bone matrix. Condensation amplifies the number of preosteoblasts (Dunlop and Hall, 1995). The cells in the condensation have a dramatically increased cell-cell communication, increased cell-cell contact, attain a rounded morphology and increase the number of gap junctions (Civitelli *et al.*, 2002; Coelho

and Kosher, 1991; Marie, 2002; Thorogood and Hinchliffe, 1975). The micromass culture system therefore provides a convenient culture model of the *in vivo* process, upon which further experiments may be possible.

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Discussion with Reviewer

D. Wheatley. The authors claim the importance of the promoting effect of GLCs but this cannot be done from EM or biochemical analysis. All that one can do is note a greater preponderance of GLCs in one case as opposed to another. Anything more is mere speculation..

Authors: The aim of the experiments was not to analyse the effects of gap junctional communications (GJC) *per se*, but to compare monolayer and micromass cultures to investigate the effects of a close neighborhood of the cells shortly after inoculation. We have clear evidence that the micromass culture technique is promoting the differen-

tiation of rat calvarial osteoblast-like cells by allowing cell-cell contact, directly after inoculation of the cells. We found, by TEM, that many different cell-cell contacts, as well as abundant collagen fibrils in the ECM, are present at 1 week. Furthermore, biochemical data clearly showed that the differentiation by means of ALP activity normalized to the DNA content of the cells is increased as compared to that found in the monolayer cultures.

We did not show that GJCs are directly responsible for these observations. As pointed out in the discussion, there is much evidence that GJCs are indeed very important for normal differentiation of bone and cartilage. In addition, cell-cell adhesion molecules (CAM) e.g. N-CAM and cadherins is also crucial for normal osteoblast differentiation and osteogenesis (for review see Civitelli *et al.*, 2002; Hall and Miyake, 2000; Marie, 2002; all references cited in text). Micromass culture technique offers a convenient tool to investigate such important mediators of cellular communication like GJC, CAM and cell surface receptors by blocking them directly by antibodies or by genetic modifications of such molecules etc.