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Expression of the Parathyroid Hormone Receptor and Correlation with Other Osteoblastic Parameters in Fetal Rat Osteoblasts

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Abstract. Primary fetal rat calvarial cell cultures were examined for the expression of different osteoblastic parameters at the single cell level and in the whole population. The presence of the parathyroid hormone (PTH) receptor was studied by employing receptor autoradiography. After 3 days of culture, 10% of the cells expressed the PTH receptor. Immunolocalization of osteocalcin in 3-day-old cell cultures was found to be strongly correlated with the presence of the PTH receptor. Alkaline phosphatase (APase) localization in 3-day-old cultures correlated with only 69% of the PTH receptor expressing cells. Our results show that in 3-day-old rat calvarial cell cultures, only about 10% of the cells show markers of osteoblastic differentiation. The presence of the PTH receptor is strongly correlated with the presence of osteocalcin, but less with the presence of APase, indicating that it is the mature osteoblast that expresses the PTH receptor. After 7 days of culture, most receptor labeling, APase, and osteocalcin expression was found in multilayered areas of cells (nodules).

Key words: PTH receptor — Osteocalcin — Alkaline phosphatase.

Parathyroid hormone (PTH) is a powerful inducer of bone resorption, a process carried out by osteoclasts. PTH receptors, however, are not found on osteoclasts but on osteoblasts. These cells, after being stimulated by PTH, transmit signals to osteoclasts, which in turn start to resorb the calcified bone matrix [1]. We have been studying the mechanism of action of PTH on osteoblasts by analyzing signal transduction pathways [2–4] and the modulation of mRNA levels of osteoblastic parameters [5]. For our studies we used cultured fetal rat calvarial osteoblastic cells (ROBs) which exhibit a temporal expression of markers of the osteoblast phenotype. After a period of proliferation, extracellular matrix (ECM) is formed as inferred from the increased expression of collagen type I and fibronectin mRNAs. ECM maturation begins then with the increased expression of alkaline phosphatase (APase). Finally, the ECM mineralizes which coincides with the expression of osteocalcin and osteopontin mRNA [6]. The time-course of this sequence is dependent on medium components, seeding density, and whether primary or secondary cultures are used [7]. During the culture, discrete multilayered areas of cells become visible (nodules), which are the sites where mineralization occurs. Ultrastructural analysis revealed that these nodules resemble true, woven bone [8]. In our hands, ROB cultures exhibit features of differentiation, such as osteocalcin production and sensitivity for PTH and 1,25-dihydroxyvitamin D_3, in only a few days [5, 9, 10]. Primary osteoblastic cell populations are heterogeneous at any time, due to differences in maturation or differentiation state between the individual cells or to the existence of different subpopulations of cells in one culture [11]. To investigate the mechanism responsible for PTH effects on osteoblastic parameters in such cultures, it would be of great help to define the precise makeup of the cell culture. In order to determine which cells respond to PTH we studied the expression of the PTH receptor at the single cell level by autoradiography using ^{125}I-PTH-related protein (PTHrP). This compound is more stable than PTH and binds to the same receptor [12]. We also studied immunolocalization of osteocalcin and APase expression and correlated the expression of the PTH/PTHrP receptor with these parameters at the single cell level in order to investigate in which stage of the differentiation sequence the PTH/PTHrP receptor is present.

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Materials and Methods

Materials

Goat anti-rat osteocalcin IgG was a generous gift from Dr. PV Hauschka from Enders Res. Labs., Boston, USA. Chicken (c) [^{125}I-Tyr^{36}]PTH-related protein (1-36) amide came from ANAWA Laboratorien AG, Wangen/Zürich, Switzerland. Fetal calf serum (FCS) and alpha minimal essential medium (α-MEM) were obtained from Gibco-Europe BV, The Netherlands. Hy clone-defined equine serum (horse serum) came from Greiner BV, The Netherlands. Naphtol AS-MX phosphate, paranitrophenyl-phosphate, Fast Red TR salt, 4,6 diamidino-2-phenylindole (DAPI), and rabbit anti-goat FITC-conjugated IgG were from Sigma Chemical Company Inc., St. Louis, MO, USA. Bovine (b) PTH (1-34) was obtained from Peninsula Laboratories Inc, St. Helens, UK. Rabbit anti-goat IgG was from Nordic Immunology, Tilburg, The Netherlands, and donkey anti-rabbit indocarbocyanine (CY3)-conjugated IgG from Jackson Immunoresearch Laboratories, West Grove, PA, USA. Kodak NBT-2 emulsion came from Technorama AG, Wallisellen, Switzerland. All other chemicals were of the best grade available.

Cell Culture

ROB were obtained from 20-day-old fetal rat calvariae by a se-

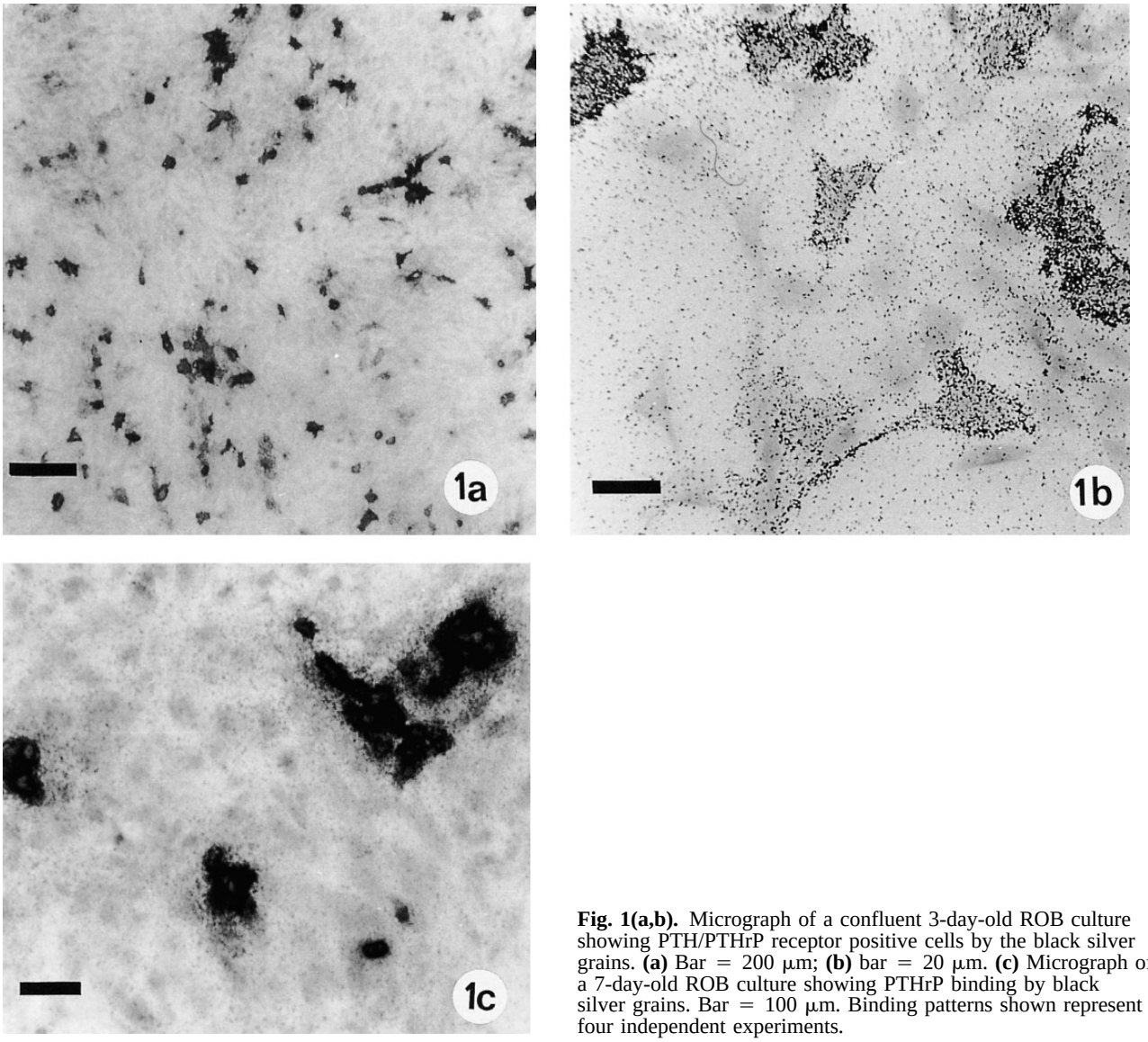

Fig. 1(a,b). Micrograph of a confluent 3-day-old ROB culture showing PTH/PTHrP receptor positive cells by the black silver grains. **(a)** Bar = 200 μm; **(b)** bar = 20 μm. **(c)** Micrograph of a 7-day-old ROB culture showing PTHrP binding by black silver grains. Bar = 100 μm. Binding patterns shown represent four independent experiments.

quential collagenase digestion as described previously [13]. Cells from the first 10-minute digest were discarded and cells from the second and third 30-minute digest were pooled, seeded at 25,000/cm^2, and cultured for 24 hours in α-MEM + 1 mg/ml glucose + 89 μg/ml gentamycin + 10% FCS. Then the medium was replaced by a similar one except that FCS concentration was lowered to 2%. Cells were further cultured in this medium for an additional 2 or 6 days.

PTH/PTHrP Receptor Autoradiography

Cells, cultured on microscope slides or on 10-mm cover glasses, were washed with binding buffer (BB) (50 mM Tris/HCl, 100 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 0.5% heat-inactivated FCS, and 5% heat-inactivated horse serum (pH 7.5)), and incubated for 3 hours at 15°C in BB containing 0.1 nM c[^{125}I]-PTHrP(1-36)NH_2. Nonspecific binding was measured in the presence of 1 μM bPTH(1-34). Only when co-expression with osteocalcin was studied, was binding done in α-MEM + 5% FCS at 37°C for 30 minutes. This procedure did not alter PTHrP binding patterns, but gave much better results for the visualization of osteocalcin. After two washings with BB, cells were fixed with 4% formaldehyde in 80 mM sodium phosphate, pH 7 (neutral formalin) for 10 minutes. When cells were cultured on cover glasses, the glasses were glued onto microscope slides which were dipped twice into Eastman-Kodak NBT 2 emulsion diluted 1:1 with 0.6 M NH_4-acetate at 43°C and exposed for 7 days. After development, the slides were counterstained with hematoxylin and examined by light microscopy. When co-localization was studied, staining for APase or osteocalcin was performed after fixation, before applying the photo emulsion.

Histochemical Determination of APase Activity

After fixation of the cells with neutral formalin for 10 minutes, cells were washed in 0.9% NaCl and incubated for 15 minutes at 37°C with 35 μM naphtol AS-MX phosphate in 0.9% NaCl supplemented with 85 mM sodium borate, 2.7 mM $MgCl_2$ and 0.09% Fast Red TR salt (pH 8.5).

Immunocytochemical Localization of Osteocalcin

Cells were fixed in neutral formalin for 10 minutes and postfixed

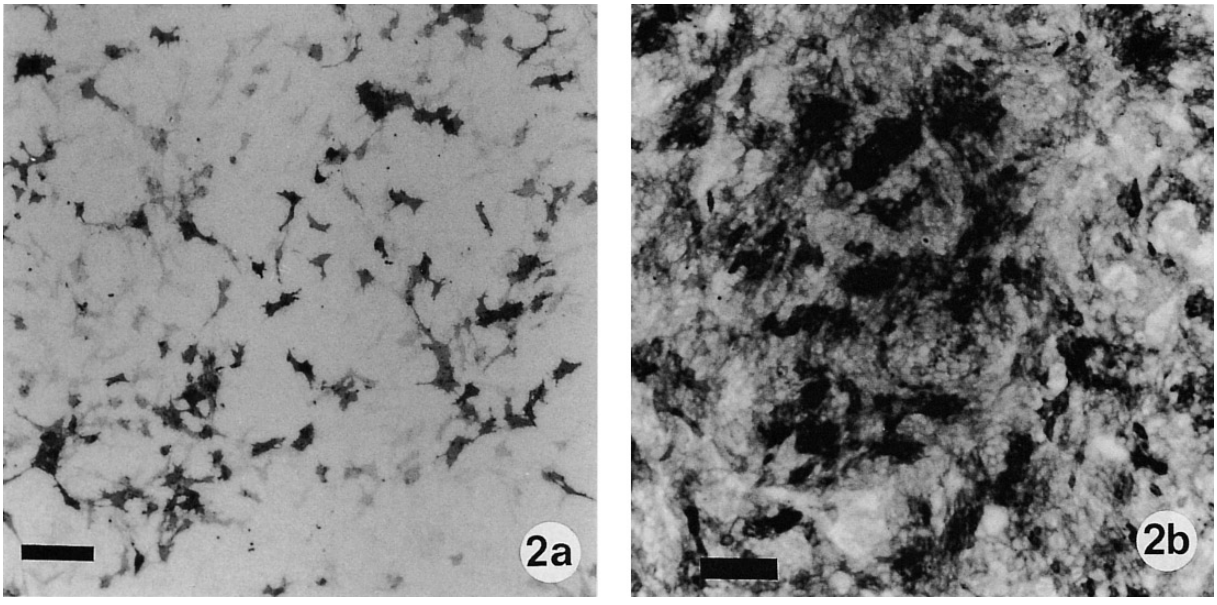

Fig. 2. Micrograph of a ROB culture stained for APase. **(a)** Three-day-old culture; **(b)** 7-day-old culture. Bars represent 200 μm. Staining patterns shown are representative of four independent experiments.

in methanol at −20°C for 20 minutes. After rinsing with PBS + 0.25% BSA (PBS/BSA), cells were incubated at room temperature for 45 minutes with 1:50 goat anti-rat osteocalcin antibody in PBS/BSA. Then cells were incubated with 1:100 rabbit anti-goat IgG for 30 minutes and subsequently with 1:500 donkey anti-rabbit CY3 conjugated IgG for 30 minutes. Antibodies were diluted in PBS/BSA. For cell counting, nuclei were finally stained with 60 ng/ml DAPI for 15 minutes.

Cell Counting

Cells were counted in five randomly selected microscope fields per slide using a 20× objective. This represented approximately 50 cells per field. Unless otherwise indicated, three slides per experiment were counted and data of three independent experiments were pooled.

Results

Expression of the PTH/PTHrP Receptor in ROB Cultures

We applied PTH/PTHrP receptor autoradiography in 3-day-old cultures of fetal ROBs where individual cells can still be distinguished. As can be seen in Figure 1A, ROBs show considerable heterogeneity in the expression of the PTH/PTHrP receptor. Only 10 ± 2% of the cells is positive for this parameter. Control experiments in the presence of 1 μM bPTH(1-34) showed only background silver grains, just like the negative cells in Figure 1A. We also observed heterogeneity in the amount of silver grains per cell. (Fig. 1B). When ROBs are cultured for longer time periods, nodules become visible. When cells are cultured for 7 days, most of the PTHrP label is associated with the nodules (Fig. 1C). When 1 μM PTH was also present (nonspecific binding), a homogeneous light background pattern of silver grains was seen with no background increase at the site of the nodules.

Table 1. Co-localization of APase and the PTH/PTHrP receptor

AP+/PTH-R+	*AP+/PTH-R−*	*AP−/PTH-R+*
69 ± 6%	22 ± 5%	9 ± 1%

Cells expressing one or both parameters were counted and classified in the following groups:
AP+/PTH-R+: positive for both APase and PTH/PTHrP receptor
AP+/PTH-R−: positive for APase but no PTH/PTHrP receptor expression
AP−/PTH-R+: negative for APase but positive PTH/PTHrP receptor expression
Data are means ± SD of percentages, calculated from three independent experiments

Localization of APase Activity in ROB Cultures

Three-day-old ROB cultures exhibit APase activity in a very heterogenous way (Fig. 2A): 15 ± 4% of the cells showed APase activity. We examined the correlation between localization of APase and the PTH/PTHrP receptor in these cultures (Table 1). We did not find a correlation between the intensity of the APase stain and the presence of the PTH/PTHrP receptor, i.e., we found receptor expression in cells low or even negative for APase as well as in cells showing high APase levels. When 7-day-old cultures were examined, strong APase activity was observed in the nodular areas with almost no internodular staining (Fig. 2B).

Immunolocalization of Osteocalcin in ROB Cultures

Also for this parameter, 3-day-old ROB cultures showed considerable heterogeneity, as shown in Figure 3A. In two experiments we found 4% and 10% positive cells, respectively. Note the intense staining at the site of the Golgi apparatus (Fig. 3B). Correlation studies between PTH/

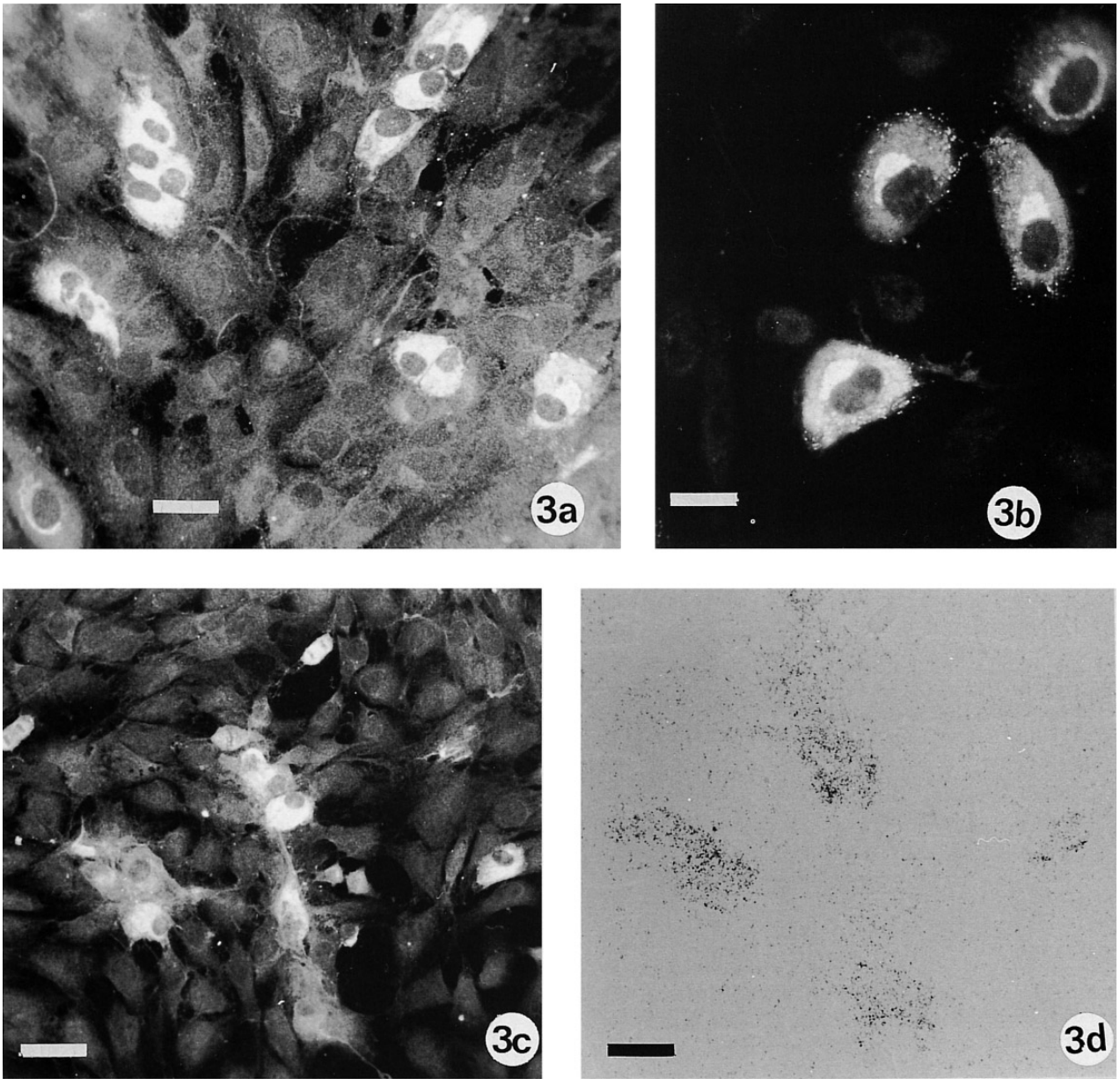

Fig. 3(a,b). Fluoro-micrographs of 3-day-old ROB cultures showing immunostaining of osteocalcin. **(a)** Bar = 30 μm; **(b)** bar = 20 μm. **(c,d)** Co-localization of osteocalcin and the PTH/PTHrP receptor. Image from the same microscopic field. **(c)** Fluoro-micrograph showing osteocalcin; **(d)** micrograph showing PTH/PTHrP receptor by the silver grains. Bars represent 40 μm. Binding and staining patterns are representative of three independent experiments.

PTHrP receptor and osteocalcin expression revealed that both parameters were well correlated, as shown in Table 2 and Figure 3 C and D. In 7-day-old cultures nearly all osteocalcin-positive cells were found in the nodules (Fig. 4).

Table 2. Co-localization of osteocalcin and the PTH/PTHrP receptor

OC+/PTH-R+	*OC+/PTH-R−*	*OC−/PTH-R+*
92 ± 3%	7 ± 4%	0.5 ± 1%

Cells expressing one or both parameters were counted and classified in the following groups:
OC+/PTH-R+: positive for both osteocalcin and PTH/PTHrP receptor
OC+/PTH-R−: positive for osteocalcin but no PTH/PTHrP receptor expression
OC−/PTH-R+: negative for osteocalcin but positive for PTH/PTHrP receptor expression
Data are means ± SD of percentages, calculated from three independent experiments

Discussion

In this study we found that primary cultures of fetal rat calvarial osteoblasts are heterogeneous in the expression of the PTH/PTHrP receptor, of osteocalcin and of alkaline phosphatase activity; these parameters are found in only about 10% of the total cell population. The presence of the PTH/PTHrP receptor was strongly correlated with the presence of osteocalcin but there was less correlation between

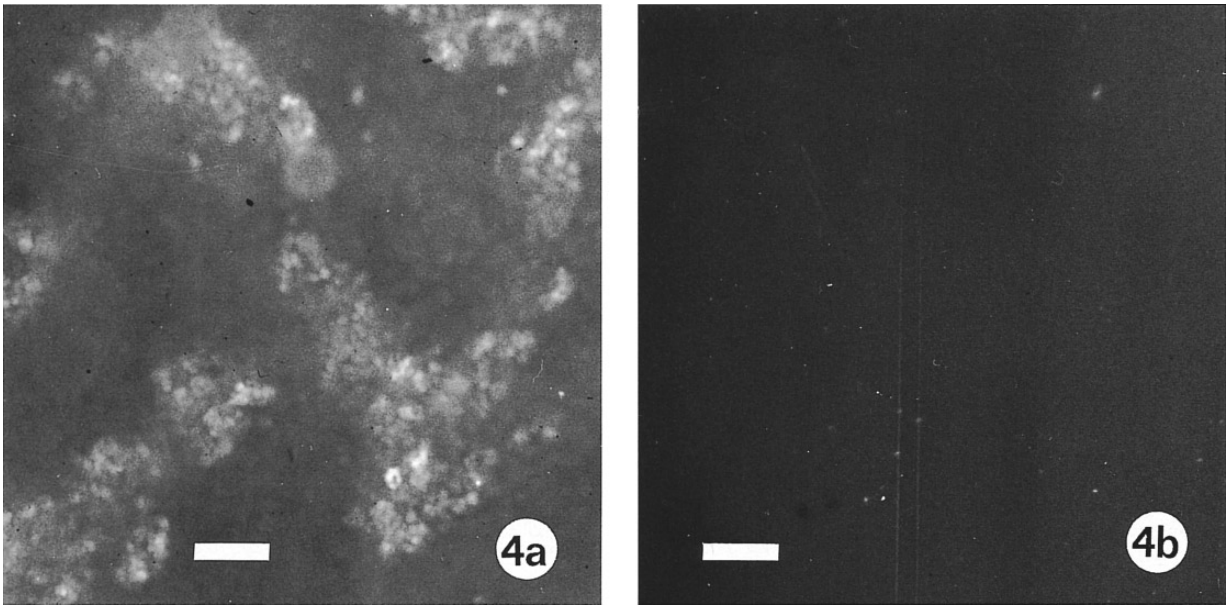

Fig. 4(a,b). Fluoro-micrographs of 7-day-old ROB cultures. **(a)** Specific staining in the presence of anti-osteocalcin antibody; **(b)** aspecific stained culture without anti-osteocalcin antibody. Bars represent 100 μm. Staining patterns are representative of three independent experiments.

the presence of APase activity and the PTH/PTHrP receptor at the single cell level.

Controversy exists at what stage of osteoblastic differentiation the PTH/PTHrP receptor is expressed. Rouleau et al. [14, 15] postulated that the highest number of receptors is on a relatively undifferentiated cell, localized at some distance from the bone surface. Only few receptors were found on the mature osteoblast in their *in vivo* studies with ^{125}I-PTH(1-34) autoradiography. Ureña et al. [16] and Lee et al. [17], however, found most expression of PTH/PTHrP receptor mRNA in osteoblasts at the bone surface. Our results suggest that the PTH/PTHrP receptor is present mainly on the mature osteoblast since we observed a strong correlation between receptor and osteocalcin expression, which is a late marker of osteoblastic differentiation. Moreover, in 7-day-old cultures we found almost all PTHrP binding in the nodular areas where the most mature osteoblasts are found [18].

The correlation between PTH/PTHrP receptor presence and APase activity was less obvious. APase is an early marker of osteoblast differentiation, so it could be that less mature cells which already show APase activity do not yet express the PTH/PTHrP receptor. On the other hand, APase activity is not an exclusive osteoblastic marker and can also be present in fibroblastic cells. APase levels are known to decrease in the course of the differentiation of the osteoblast, leading eventually to an almost APase-negative osteocyte. Therefore cells expressing the PTH/PTHrP receptor without any detectable APase may be very mature cells.

The finding that only 10% of the cells contain a PTH/PTHrP receptor might have implications for the interpretation of experiments performed in primary osteoblastic cell cultures. Receptor number can be estimated by Scatchard analysis in these cultures as approximately 70,000 per cell [19] but with the current findings, receptor number can be estimated at 700,000 per PTH/PTHrP receptor-expressing cell. This number is very high compared with receptor number per cell in an osteoblastic cell line such as UMR 106 (25,000 receptors/cell [20]). Receptor number per cell affects the efficiency of signal transduction in response to the ligand [21], so the present findings are important in the comparison of PTH-induced second messenger systems in primary versus transformed osteoblasts. One also has to consider that when effects of hormones or growth factors on PTH action are studied in these cultures, their final effect can be accomplished via non-PTH/PTHrP receptor-containing cells in the culture.

Our results also show that the cells remain heterogeneous throughout the culture period. We did not particularly investigate the nature of the internodular cells that do not express features of differentiated osteoblasts. Pockwinse et al. [22] proposed that these cells may be immature osteoblasts, not yet differentiated by lack of the appropriate signal, a signal which they suggest may be an organized, mineralized, extracellular matrix. Possibly the heterogeneity observed in these cultures reflects the heterogeneity of the osteoblastic population *in vivo* at the time of isolation. One could speculate that the nodular cells had already reached such a state of maturity that they are able to continue their differentiation process *in vitro;* the internodular cells, maybe less mature, may be unable to differentiate much further because of lack of the right signal. The finding that glucocorticoid treatment of these cultures leads to formation of more nodules [23, 24] suggests that osteoblastic differentiation of at least a subset of cells can be enhanced. To further investigate the nature of the internodular cells, more studies at the single cell level are needed.

In summary, our results demonstrate the makeup of fetal rat calvarial osteoblastic cell cultures with respect to the presence of the PTH/PTHrP receptor, osteocalcin, and APase activity. A few days after seeding, these parameters are expressed in only about 10% of the cells. The presence of the PTH/PTHrP receptor is strongly correlated with the presence of osteocalcin, indicating that the receptor is present on mature cells. Correlation between PTH/PTHrP receptor and APase is less; this might indicate that the ex-

pression of these parameters is not correlated in a time-dependent manner. After 7 days of culture, when nodules are formed, all studied differentiation parameters are strongly expressed in the nodular areas.

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References

1. Rodan GA, Martin TJ (1981) Role of osteoblasts in hormonal control of bone resorption—a hypothesis. Calcif Tissue Int 33:349–351
2. Van Leeuwen JPTM, Bos MP, Herrmann-Erlee MPM (1989) Parathyroid hormone-induced ornithine decarboxylase activity in fetal rat osteoblasts. J Bone Miner Res 4:485–491
3. Bos MP, Van Leeuwen JPTM, Van der Meer JM, Herrmann-Erlee MPM (1991) Modulation of responsiveness to cAMP stimulating agonists by phorbol ester in fetal rat osteoblasts. J Cell Physiol 147:87–92
4. Bos MP, Van der Meer JM, Herrmann-Erlee MPM (1994) Regulation of protein kinase C activity by phorbolester, thrombin, parathyroid hormone and transforming growth factor-β_2 in different types of osteoblastic cells. Bone Miner 26:141–154
5. Jongen JWJM, Bos MP, Van der Meer JM, Herrmann-Erlee MPM (1993) Parathyroid hormone-induced changes in alkaline phosphatase expression in fetal calvarial osteoblasts: differences between rat and mouse. J Cell Physiol 155:36–43
6. Stein GS, Lian JB, Owen TA (1990) Relationship of cell growth to the regulation of tissue-specific gene expression during osteoblast differentiation. FASEB J 4:3111–3123
7. Aronow MA, Gerstenfeld LC, Owen TA, Tassinari MS, Stein GS, Lian JB (1990) Factors that promote progressive development of the osteoblast phenotype in cultured fetal rat calvarial cells. J Cell Physiol 143:213–221
8. Bhargava U, Bar-Lev M, Bellows CG, Aubin JE (1988) Ultrastructural analysis of bone nodules formed in vitro by isolated fetal rat calvaria cells. Bone 9:155–163
9. Groot CG, Danes JK, Van der Meer JM, Herrmann-Erlee MPM (1985) Osteocalcin antigenicity in cultured osteoblast-like cells after stimulation with 1,25-vitamin D_3 Cell Biol Int Rep 9:258
10. Pols HAP, Schilte HP, Herrmann-Erlee MPM, Visser ThJ, Birkenhäger JC (1986) The effects of 1,25-dihydroxyvitamin D_3 on growth, alkaline phosphatase and adenylate cyclase of rat osteoblast-like cells. Bone Miner 1:397–405
11. Aubin JE, Turksen K, Heersche JNM (1993) Osteoblastic cell lineage. In: Noda M (ed) Cellular and molecular biology of bone. Academic Press, New York, pp 1–45
12. Jüppner H, Abou-Samra AB, Uneno S, Gu WX, Potts JT Jr, Segre GV (1988) The parathyroid hormone-like peptide associated with humoral hypercalcemia of malignancy and parathyroid hormone bind to the same receptor on the plasma membrane of ROS 17/2.8 cells. J Biol Chem 263:8557–8560
13. Herrmann-Erlee MPM, Van der Meer JM, Löwik CWGM, Van Leeuwen JPTM, Boonekamp PM (1988) Different roles for calcium and cyclic AMP in the action of PTH: studies in bone explants and isolated bone cells. Bone 9:93–100
14. Rouleau MF, Mitchell J, Goltzmann D (1988) In vivo distribution of parathyroid hormone receptors in bone: evidence that a predominant osseous target cell is not the mature osteoblast. Endocrinology 123:187–191
15. Rouleau MF, Mitchell J, Goltzmann D (1990) Characterization of the major parathyroid hormone target cell in the endosteal metaphysis of rat long bones. J Bone Miner Res 5: 1043–1053
16. Ureña P, Lee K, Weaver D, Kong XF, Brown D, Bond AT, Abou-Samra AB, Segre GV (1992) PTH/PTHrP receptor mRNA expression as assessed by Northern blot and in situ hybridization (abstract) J Bone Miner Res 7 (suppl 1):S119
17. Lee K, Deeds JD, Bond AT, Jüppner H, Abou-Samra AB, Segre GV (1993) In situ localization of PTH/PTHrP receptor mRNA in the bone of fetal and young rats. Bone 14:341–345
18. Pockwinse SM, Lawrence JB, Singer RH, Stein JL, Lian JB, Stein GS (1993) Gene expression at single cell resolution associated with development of the bone cell phenotype: ultrastructural and in situ hybridization analysis. Bone 14:347–352
19. Bos MP, Van der Meer JM, Feyen JHM, Herrmann-Erlee MPM (in press) Down-regulation and differential restoration of cAMP responses upon transient phorbol ester treatment of primary osteoblastic cells. Cell Signalling
20. Ikeda K, Sugimoto T, Fukase M, Fujita T (1991) Protein kinase C is involved in PTH-induced homologous desensitization by directly affecting PTH receptor in the osteoblastic osteosarcoma cell. Endocrinology 128:2901–2906
21. Bringhurst FR, Jüppner H, Guo J, Ureña P, Potts JT Jr, Kronenberg HM, Abou-Samra AB, Segre GV (1993) Cloned, stably expressed parathyroid hormone (PTH)/PTH-related peptide receptors activate multiple messenger signals and biological responses in LLC-PK_1 kidney cells. Endocrinology 132: 2090–2098
22. Pockwinse SM, Wilming LG, Conlon DM, Stein GS, Lian J (1992) Expression of cell growth and bone specific genes at single cell resolution during development of bone tissue-like organization in primary osteoblast cultures. J Cell Biochem 49:310–323
23. Bellows CG, Heersche JN, Aubin JE (1990) Determination of the capacity for proliferation and differentiation of osteoprogenitor cells in the presence and absence of dexamethasone. Dev Biol 140:132–138
24. Pockwinse SM, Stein JL, Lian JB, Stein GS (1995) Developmental stage-specific cellular responses to vitamin D and glucocorticoids during differentiation of the osteoblast phenotype: interrelationship of morphology and gene expression by in situ hybridization. Exp Cell Res 216:244–260