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Osteoprogenitor Cell Frequency in Rat Bone Marrow Stromal Populations: Role for Heterotypic Cell–Cell Interactions in Osteoblast Differentiation

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Abstract Glucocorticoids, notably dexamethasone (Dex), have been reported to be a requirement for osteoprogenitor cell differentiation in young adult rat bone marrow stromal cell populations. We have reinvestigated the requirement for Dex and analyzed the frequency of osteoprogenitor cells present. Stromal cells were grown as primary or first subcultures in the presence or absence of Dex and their expression of osteogenic markers (alkaline phosphatase activity, hormone responsiveness, and matrix molecules, including type I collagen, osteopontin, bone sialoprotein, and osteocalcin), as well as their functional capacity to differentiate to form a mineralized bone nodule, were assessed. Dex increased, but was not an absolute requirement for, the expression of osteogenic markers. Bone nodule formation was plating cell density dependent and occurred under all combinations of treatment with or without Dex but was maximal when Dex was present in both the primary and secondary cultures. Dex increased CFU-F by ~2-fold, but increased CFU-O (osteoprogenitor cells; bone nodule forming cells) by 5- to 50-fold depending on the cell density and duration of treatment. Neither CFU-F nor CFU-O expression followed a linear relationship in limiting dilution analysis until very high cell densities were reached, suggesting cooperativity of cell types within the population and a multitarget phenomenon leading to osteoprogenitor differentiation. When a large number of nonadherent bone marrow cells or their conditioned medium was added to the stromal cells, osteoprogenitors comprised approximately 1/100 of plated adherent cells and their expression followed a linear, single-hit relationship. By contrast, rat skin fibroblasts or their conditioned medium totally inhibited bone nodule formation. These data support the hypothesis that in marrow stroma, as in other bone cell populations such as those from calvaria, there are at least two classes of osteoprogenitor cells: those differentiating in the absence of added glucocorticoid and those requiring glucocorticoid to differentiate, that more than one cell type is limiting for stromal osteoprogenitor differentiation suggesting a role for heterotypic cell–cell interactions in osteogenesis in this tissue, and that Dex may be acting directly and/or indirectly through accessory cells in the bone marrow to alter osteoprogenitor cell expression. J. Cell. Biochem. 72:396–410, 1999.

Key words: osteoprogenitors; limiting dilution; bone marrow stroma; dexamethasone; cooperativity; cell nonautonomous behavior

Osteogenic cells are present in the bone marrow stroma of various animals including rodents and human and their ability to produce a bone-like mineralized tissue has been demonstrated both in vivo, i.e., in diffusion chambers loaded with bone marrow cells [Ashton et al., 1984; Friedenstein, 1990; Owen, 1988] and in vitro, where under suitable culture conditions bone-like tissue is synthesized by various marrow stromal cell populations [Benayahu et al., 1991; Bennett et al., 1991; Falla et al., 1993; Haynesworth et al., 1992; Kasugai et al., 1991; Leboy et al., 1991; Malaval et al., 1994; Maniatopoulos et al., 1988; McCulloch et al., 1991; Satomura and Nagayama, 1991]. The osteoprogenitors present in both fetal rat calvaria-derived [Bellows et al., 1990] and young adult rat bone marrow stroma-derived [McCulloch et al., 1991] populations appear to have a limited proliferative lifetime and limited self-renewal capacity,

Abbreviations used: Dex, dexamethasone; CFU-F, colony-forming unit-fibroblast; CFU-O, colony-forming unit-osteoblast; α-MEM, α-minimum essential medium; AP, alkaline phosphatase; OPN, osteopontin; BSP, bone sialoprotein; Coll. I, collagen type I; ON, osteonectin; OC, osteocalcin; PTH, parathyroid hormone; PGE_2, prostaglandin E_2; IPT, isoproteronol.

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although glucocorticoids, notably dexamethasone (Dex), extend the lifetime of at least some of the calvaria-derived progenitors [Bellows et al., 1990; reviewed in Aubin et al., 1993]. Both mass population studies [Bellows et al., 1987, 1990] and limiting dilution analyses [Bellows and Aubin, 1989] suggested that a subpopulation of cells in rat calvaria populations is dependent on Dex or natural glucocorticoids for expression of bone nodule formation. Immunoselection with antibodies against rat alkaline phosphatase (AP) demonstrated that the osteoprogenitors could be separated into two "classes" or differentiation stages: progenitors at one stage form bone in vitro without exogenous Dex stimulation (AP-positive; more mature progenitor), and those at the other stage form bone in vitro only in its presence (AP-negative; more primitive progenitor) [Turksen and Aubin, 1991]. Given the frequent suggestion that Dex is an absolute requirement for osteogenesis in rat marrow stromal cultures [Kasugai et al., 1991; Leboy et al., 1991; Maniatopoulos et al., 1988], it is tempting to speculate that most osteoprogenitors in stromal cultures may be of the relatively immature phenotype.

In addition to osteoprogenitors, bone marrow stroma contains other cell types, including fibroblasts, adipocytes, and endothelial cells [Benayahu et al., 1991; Dexter, 1982; Hasthorpe et al., 1990; McIntyre and Bjornson, 1986; Owen, 1988; Wang et al., 1990]. The term *fibroblast* not only refers to the cells involved in forming the supporting connective tissue, but also specifies a general category of bone marrow stromal cells which are undifferentiated and colony-forming unit-fibroblast (CFU-F) refers to colonies that may include a mixture of cell types [Wang et al., 1990]. Hemopoietic cells are closely associated with stromal cells in bone marrow in vivo and in vitro [Dexter, 1982; Whetton and Dexter, 1993], but when stromal cells are cultured, most well-differentiated hematopoietic cells are normally removed by selective washing and adherence techniques. However, in the course of investigating osteogenesis in rat bone marrow stromal cultures, we noted striking morphological heterogeneity in the cell types present [Malaval et al., 1994; McCulloch et al., 1991] and confirmed that only a proportion of cells were associated with well-developed bone-like nodules [Malaval et al., 1994]. Since it is known that the culture conditions have a significant effect on what lineages proliferate and differentiate, we recently quantified colony types and cells present in rat bone marrow stromal cultures grown under the culture conditions supporting, and over the time course of, osteogenic differentiation. We found that Dex altered the population makeup of the stromal cultures; concomitant with stimulation of AP positive colonies and bone nodules, macrophage differentiation was stimulated, and the proportion of cells with leukocyte common antigen was reduced [Herbertson and Aubin, 1995]. These data, which support the subpopulation heterogeneity of rat stromal cells grown under conditions promoting osteogenesis, together with reports that Dex is an absolute requirement for osteogenesis in rat stromal cultures, led us to undertake the current limiting dilution analyses to assess the frequency of osteoprogenitors in bone marrow stromal populations in vitro in the presence and absence of Dex. Since some data also indicate that differentiation of osteoprogenitor cells in the marrow stroma can be regulated by co-culturing with other cell types (e.g., stimulation by the conditioned medium from rat calvaria osteogenic populations [Hughes and McCulloch, 1991] or inhibition by fibroblastic cells [Ogiso et al., 1991, 1992; Zhang et al., 1991]), and Dex alters the subpopulations present, we also asked whether osteoprogenitor differentiation was cell autonomous in the stroma or accessory cells influenced differentiation in this system.

MATERIALS AND METHODS

Rat Marrow Stromal Cell Cultures

Rat bone marrow stromal cells were prepared and cultured essentially as described [Herbertson and Aubin, 1995; Malaval et al., 1994]. Briefly, young (40–45 day) adult male Wistar rats were killed by cervical dislocation and the femurs were removed under sterile conditions and immersed in α-minimum essential medium (α-MEM) with antibiotics, including 100 μg/ml penicillin G (Sigma, St. Louis, MO), 50 μg/ml gentamycin (Sigma), 300 ng/ml fungizone (Flow Laboratories, Mississauga, ON). After removal of the femoral heads, the marrow was collected by flushing repeatedly through the shafts with a syringe containing α-MEM supplemented with antibiotics as above and 15% heat-inactivated fetal calf serum (FCS), and sieving the cell suspension to remove cell aggregates. Recovered cells were cultured in

the same medium supplemented additionally with ascorbic acid (2.8×10^{-4} M), β-glycerophosphate (10 mM) and with or without Dex (10^{-8} M). Except where noted, analyses presented were done on secondary cultures prepared after 7 days of primary cell growth to permit removal of the nonadherent fraction and more accurate quantification of viable stromal cell numbers. To prepare secondary cultures, primary cultures were harvested with a 1:1 (v/v) mixture of trypsin (0.01% w/v in citrate saline) and collagenase (3 mg/ml collagenase, 4.5 U/ml elastase, 9.7 U/ml DNase, 0.12 mM chondroitin sulfate, 100 mM sorbitol, 111.2 mM KCl, 1.3 mM $MgCl_2$, 13 mM glucose, 21.3 mM Tris-HCl, pH 7.4, 0.5 mM $ZnCl_2$) [Rao et al., 1977]. In both primary and secondary cultures, cells were grown in supplemented medium as above and medium was changed every 2 days. Cultures were maintained for the times indicated with particular experiments, ranging from a few days to ≤35 days.

Cell Growth

For analysis of cell growth, cells were plated at 5×10^4–10^5 cells/35-mm dish in supplemented medium as above with or without 10^{-8} M Dex. At times indicated, cell layers were rinsed with phosphate-buffered saline (PBS) and cells released with trypsin alone or a trypsin–collagenase mixture as above (after matrix deposition was extensive); the harvested cells were counted on an electronic counter (Coulter Electronics, model Zf, Hialea, FL).

Formation of Bone Nodules

Ability of primary or secondary stromal populations to form bone nodules was done in either 96-well microtiter trays (for limiting dilution analysis) or in 35-mm dishes (for mass population analysis) over appropriate cell densities as indicated in each experiment. In both cases, cells were grown in supplemented medium as above with ascorbic acid and β-glycerophosphate with or without Dex (10^{-8} M). For analysis of osteoprogenitors in primary cultures, a viable (Trypan blue-excluding) nucleated cell count was determined immediately after flushing marrows and sieving as described above. Approximately 2×10^7 nucleated cells were recovered per rat (two femurs) and these were plated at densities ranging from 10^3–10^5 cells/well of a microtiter tray; 96 wells were plated per cell density. For secondary cultures, cells were plated at 5×10^3–10^5 cells/dish or from 12.5–750 cells/well in microtiter wells. For quantification of nodule formation, dishes or wells were fixed and stained by the Von Kossa technique, and bone nodules were counted on a grid (dishes) or by visualizing the whole well under low-power microscopy as described [Bellows and Aubin, 1989; Bellows et al., 1986]. For whole population analysis, dish experiments are plotted as the mean number of nodules ±SD of a minimum of triplicate dishes up to six dishes per cell density. For limiting dilution analysis of osteoprogenitor cell number in mixed stromal populations, we used the techniques and analyses we reported earlier for rat calvaria cells [Bellows and Aubin, 1989]. Briefly, the frequency of osteoprogenitor cells was determined by quantifying the fraction of wells not containing bone nodules at each cell density tested. From a plot of the fraction of empty wells against cell number plated per well, the osteoprogenitor cell number was determined from $Fo = e^{-x}$, where Fo is the fraction of empty wells, and x is the mean number of osteoprogenitors per well; assuming a Poisson distribution, $Fo = 0.37$ is the dilution at which one osteoprogenitor is present per well. In limiting dilution experiments, a minimum of 96 wells were plated for each cell density tested and the actual fraction of wells without bone nodules was counted and plotted ±95% confidence limits.

Fibroblasts and the nonadherent fraction of freshly isolated bone marrow were tested for their effects in the limiting dilution experiments. Fetal rat skin fibroblasts were isolated by explant culture from 21 days Wistar rat fetuses. Cells from primary to tertiary cultures were trypsinized, resuspended and plated at 10^5 cells/well in microtiter trays. The next day, marrow stromal cells were plated as above into wells with or without fibroblasts and the cultures maintained as above for up to 21 days, after which bone nodules were quantified and the number of wells lacking bone colonies at each cell density determined. Alternatively, the nonadherent fraction of marrow cultures isolated and cultured as above were collected at each medium change through the first 7 days of primary culture. At each collection, cells were collected by centrifugation and resuspended in medium as above containing 10% dimethyl sulfoxide (DMSO) and frozen at −70°C. When needed, pools of collected cells were thawed, resuspended in supplemented growth medium

as above and added at 10^5 cells/well to stromal cells preplated into microtitre trays at the densities indicated. Care was taken not to disturb the nonattached cells at each medium change thereafter. In other experiments, 24- or 48-h conditioned medium from the nonadherent fraction of cells was prepared; conditioned medium was filtered through a 0.22-µm filter before use. Cultures were maintained and nodule number or fraction of empty wells determined as above.

Analysis of Hormone Responsiveness

Cells were plated in supplemented medium as above with or without Dex at 5×10^4 cells/35-mm dish and assayed at confluence. The effects of parathyroid hormone (bPTH (1–84), 2,200 U/mg, donated by Dr. T. Murray, University of Toronto; tested at 2.5 U/ml), 1-Isoproterenol (IPT; Sigma Chemical; tested at 5×10^{-4} M) and prostaglandin E_2 (PGE_2) (Upjohn, Kalamazoo, MI), tested at 2.5 µg/ml, on intracellular cyclic adenosine monophosphate (cAMP) were analyzed as described previously [Grigoriadis et al., 1988].

RNA Preparation and Northern Blots

For RNA extraction, cells were plated at 2.4×10^3 cells/cm^2 in 100-mm dishes. At day 5, 8, and 12, cells were washed with PBS, harvested with trypsin and collagenase, and lysed in LiCl and urea to prepare total RNA [Chomczynski and Sacchi, 1987]. From each time point, 20-µg aliquots of RNA were fractionated on 1% agarose formaldehyde gels. RNA was transferred to 0.2-µm Biotrans™ nylon membranes (ICN Biomedical Canada Ltd., Mississauga, ON) and immobilized by baking at 80°C for 2 h.

The probes used were as follows. Bone sialoprotein (BSP) cDNA (pBSP1) and rat osteocalcin (OC) cDNA (pOC9) were isolated and subcloned from cDNA libraries made from RNA of cultured fetal rat calvaria and ROS 17/2.8 cells by using PCR amplification with specific primers and confirming specific product by sequencing [Malaval et al., 1994]. The insert of pBSP1 is from nucleotide 15–1823 of rat BSP mRNA. pOC9 contains an insert from nucleotide 153–480 of rat osteocalcin mRNA. A rat AP cDNA was provided by Dr. G.A. Rodan (Merck Sharp and Dohme Research Laboratories, West Point, PA) [Thiede et al., 1988]; a cDNA for rat osteopontin (OPN) was provided by Dr. B. Mukherjee (McGill University, Montreal, Québec); a mouse SPARC/osteonectin (ON) cDNA was provided by Dr. B.L.M. Hogan (Vanderbilt University, Nashville, TN) [Mason et al., 1986]; and a mouse 18S rRNA was provide by Dr. L. Bowman (University of South Carolina, Columbia, SC).

The membranes were prehybridized and hybridized in 5× Denhardt's solution, 5× SSC, 50 mM sodium phosphate, 50% formamide, 250 µg/ml salmon testes DNA, and 25 µg/ml polyU and polyC at 42°C for 16–20 h. The membranes were washed twice in 2× SSC and 0.1% SDS, and twice in 0.1× SSC and 0.1% SDS for 30 min each at temperatures ranging from room temperature to 60°C. X-Omat AR radiographic films (Kodak, Rochester, NY) were exposed to the membranes, using two intensifying screens, for various times, either at −70°C or at room temperature. Quantification of signal and loading were standardized against 18S ribosomal RNA (rRNA), by laser densitometric scanning of developed films on an Ultrascan XL (Pharmacia, Uppsala, Sweden).

To assess OC production in cultures lacking Dex, we used RT-PCR with primers based on rat OC sequence [Yoon et al., 1988]: 5′ primer: 5′AGGACCCTCTCTCTGCTCAC3′ and 3′ primer: 5′AACGGTGGTGCCATAGATGC3′.

Alkaline Phosphatase

The histochemical stain for AP is a modification of Pearse's (1960). Cells were rinsed once with cold PBS and fixed in 10% cold neutral buffered formalin for 15 min, rinsed with distilled water, and left in distilled water for 15 min. Fresh substrate consisting of 10 mg naphthol AS MX-PO4 (Sigma) dissolved in 400 µl N,N-dimethylformamide, then added to 50 ml distilled water and 50 ml Tris-HCl (0.2 M, pH 8.3) and then 60 mg Red Violet LB salt (Sigma), was filtered through Whatman's No. 1 filter directly onto the dishes, and incubated for 45 min at 20°C. The dishes were then rinsed in tap water, drained and stained with 2.5% silver nitrate for 30 min at room temperature (Von Kossa stain). After rinsing twice in distilled water, toluidine blue was applied for 2 s; the dishes were then rinsed 3 times with tap water. The dishes were finally air dried.

Statistics

Data were analyzed with the InStat software package and the analysis of variance (ANOVA), Student's t-test, and the Welch test were employed as indicated in particular figures and tables.

RESULTS

As previously described, rat bone marrow stromal cell cultures grown for 7 days in the presence of medium supplemented with ascorbic acid, β-glycerophosphate, and Dex (10^{-8} M), and then passaged into the same supplemented culture medium, over a time period of approximately 21 days form three-dimensional nodular structures with the morphological and histological (Fig. 1), immunocytochemical [Malaval et al., 1994] and ultrastructural appearance of bone [Maniatopoulos et al., 1988]). Given that exogenously added Dex has been said to be a requirement for osteogenic differentiation (bone nodule formation and presence of osteoblast-associated markers) in rat bone marrow stromal cells, while it is not a requirement for populations isolated from rat calvaria although it is stimulatory, we compared the ability of stromal cell cultures to form bone nodules when they are treated in primary cultures with or without Dex and then plated as secondary cultures at different cell densities and grown in the presence or absence of Dex. Stromal cell cultures formed bone nodules under all combinations of treatment, but the highest number of bone nodules per plated cell formed in those populations cultured with Dex in both the primary and secondary cultures, followed by those treated with Dex at the secondary culture stage (Fig. 2 and Table I). Nodules formed, but only at high plating densities, in cultures never treated with Dex (Table I).

Consistent with the number of bone nodules formed, when cultures treated with or without Dex were assessed for expression of osteoblastic markers, those treated with Dex at both the primary and secondary stages followed by those treated with Dex during secondary culture only expressed the highest responsiveness to added PTH, PGE_2, and IPT (Table II); while both osteoblast and many nonosteoblast cells respond to PGE_2 and IPT, acquisition of high levels of PTH receptor/responsiveness to PTH

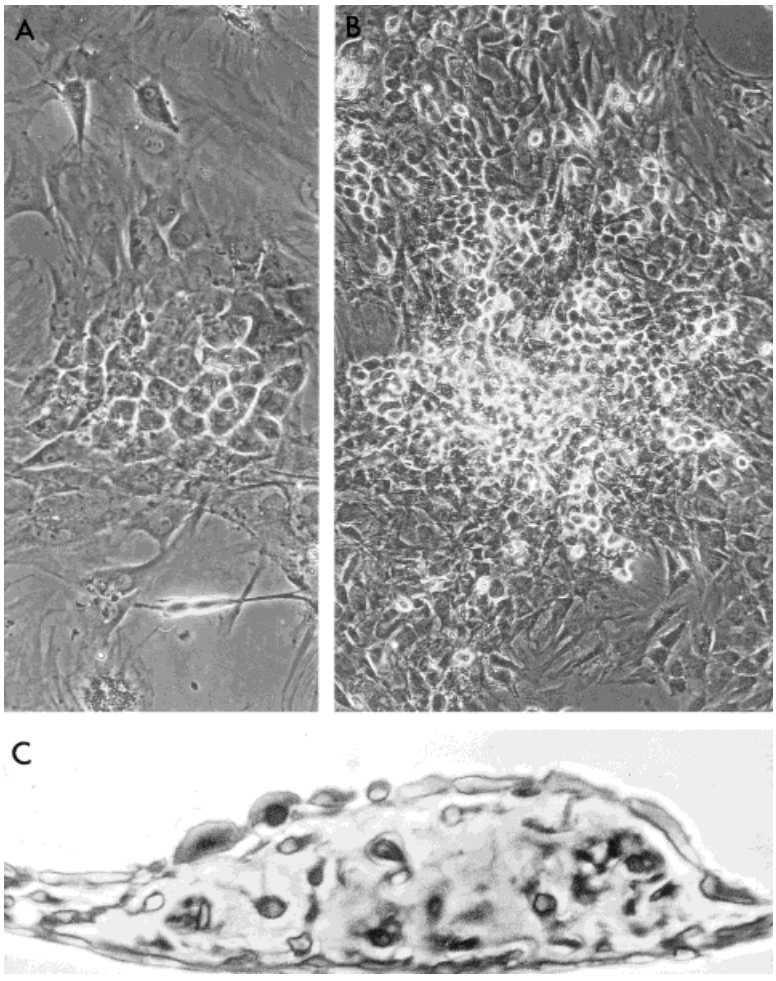

Fig. 1. Morphology of osteoblastic cells and the bone nodules formed in rat bone marrow stromal populations. Phase-contrast photomicrographs of stromal cells grown for 7 days in primary culture and then for various times in secondary cultures in medium supplemented with 10^{-8} M Dex. **A:** Colony at day 7 comprises cells that have assumed a pronounced cuboidal shape and are surrounded by a refractile matrix. **B:** At day 15, much larger colonies of cuboidal cells and surrounding refractile matrix are evident. **C:** Cross section of a bone nodule from a day 21 culture; frozen section, stained with hematoxylin & eosin (H&E). Note the three-dimensional shape of the nodule, the dense collagenous matrix, the cuboidal layer of osteoblastic cells on top of the nodule, and the osteocyte-like cells embedded in matrix.

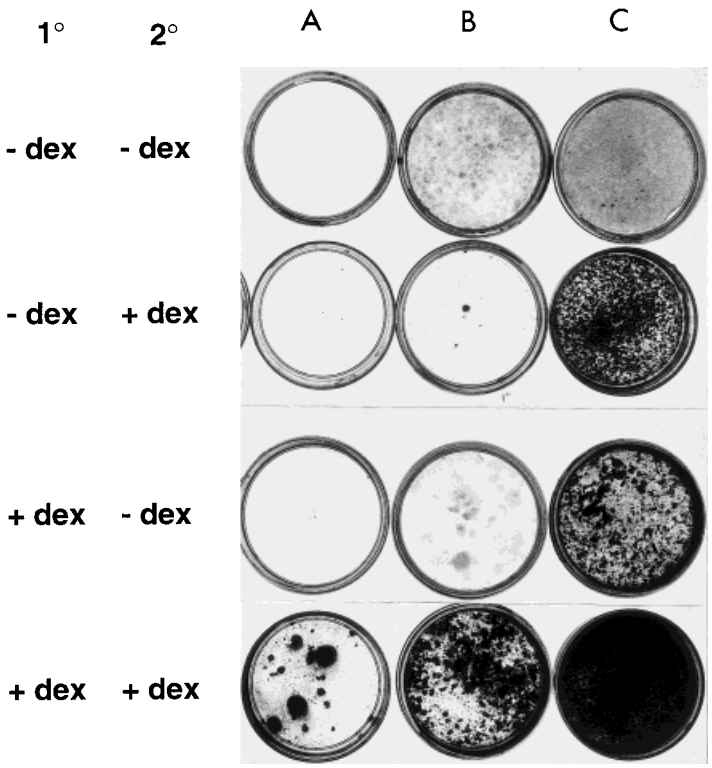

Fig. 2. Rat bone marrow cells were grown for the first 7 days in supplemented medium in the absence (−Dex) or presence (+Dex) of 10^{-8} M Dex. The stromal cells were then collected and replated at various densities in supplemented medium with (+Dex) or without (−Dex) 10^{-8} M Dex. At day 21, the cultures were fixed and stained by the Von Kossa technique for determination of presence of mineralized bone-like nodules. Black, nodules; gray, fibroblastic colonies or nonmineralized nodules. Nodule formation was plating cell density dependent, nodules formed in the absence of Dex, but the presence of Dex stimulated the number of nodules formed at all densities. **A:** 5×10^3 cells/dish. **B:** 10^4 cells/dish. **C:** 5×10^4 cells/dish.

TABLE I. Quantitation of the Number of Bone Nodules Formed at Different Cell Densities in Cells Grown at the Primary (1°) or Secondary (2°) Stage of Culture in the Presence or Absence of Dexamethasone (Dex)[a]

		No. of nodules/dish[b]					
		Cell no./dish					
1°	2°	10^3	2.5×10^3	5×10^3	10^4	2.5×10^4	5×10^4
−Dex	−Dex	0.3 ± 0.3	0	0	0	0	8.2 ± 2.3
−Dex	+Dex	1.0 ± 0.4	4.0 ± 1.0	6.6 ± 1.2	10.0 ± 2.1	41.8 ± 9.6	ND[c]
+Dex	−Dex	0.4 ± 0.2	0.7 ± 0.3	0.5 ± 0.3	2.0 ± 1.3	34.6 ± 5.1	ND[c]
+Dex	+Dex***	2.4 ± 1.0	11.8 ± 2.7	25.0 ± 2.8	69.0 ± 5.0	ND[c]	ND[c]

[a]The −Dex−Dex growth condition was compared with all other +Dex containing conditions at each cell density by an ANOVA and multiple comparison post-test. +Dex conditions are significantly different from −Dex at all densities tested, $P < 0.001$.
[b]Numbers are the means of five dishes ±SD.
[c]ND—not determined; nodule numbers were too high to discriminate individual nodules.
***, +Dex+Dex is significantly different from −Dex−Dex, −Dex+Dex, and +Dex−Dex, $P < 0.001$.

in particular is considered a feature of the mature osteoblast phenotype [Rodan and Rodan, 1988]. Concomitantly, the number of AP-positive colonies was higher in cultures treated with Dex than in those without, and only a proportion of these were coincident with bone nodules [Herbertson and Aubin, 1995] (Fig. 3). Northern analysis was used to assess mRNA expression over the time course of osteogenesis in these rat bone marrow cultures; some blots were overexposed to show that lower, but detectable expression of osteoblast-associated markers occurred in cultures without Dex as compared with cultures with Dex. In the experiment shown, nodules were heavily mineralized in cultures without Dex at the last time point, but some nodules were still forming and mineralizing in the plus Dex cultures. In both Dex-treated and non-Dex-treated cultures, the mRNAs for AP, osteopontin (OPN), type I collagen (Coll. I), osteonectin (ON), and bone sialoprotein (BSP) were detectable. Osteocalcin (OC) mRNA was detected on the Northern blots only from cultures grown with Dex; RT-PCR

TABLE II. Effects of Treatment with Dexamethasone (Dex) on Hormone Responsiveness of Rat Bone Marrow Stromal Cells[a]

Experiment No.	1°	2°	cAMP (fold stimulation) (treated/control) PTH	PGE_2	IPT
1	−Dex	−Dex	1 ± 0	4 ± 1	1 ± 0
	−Dex	+Dex	15 ± 5	21 ± 6	8 ± 2
	+Dex	−Dex	14 ± 3	17 ± 3	2 ± 1
	+Dex	+Dex	61 ± 12	33 ± 6	26 ± 5
2	−Dex	−Dex	4 ± 0	7 ± 2	2 ± 1
	−Dex	+Dex	72 ± 6	50 ± 7	16 ± 3
	+Dex	−Dex	14 ± 3	14 ± 3	3 ± 1
	+Dex	+Dex	55 ± 5	18 ± 2	12 ± 2

[a]Cells were treated (+) or not (−) at the primary (1°) and secondary (2°) stages of culture with 10^{-8} M Dex. Values are the means of triplicates ±SD. Fold stimulation is treated/control values of cAMP in pmoles/10^6 cells.

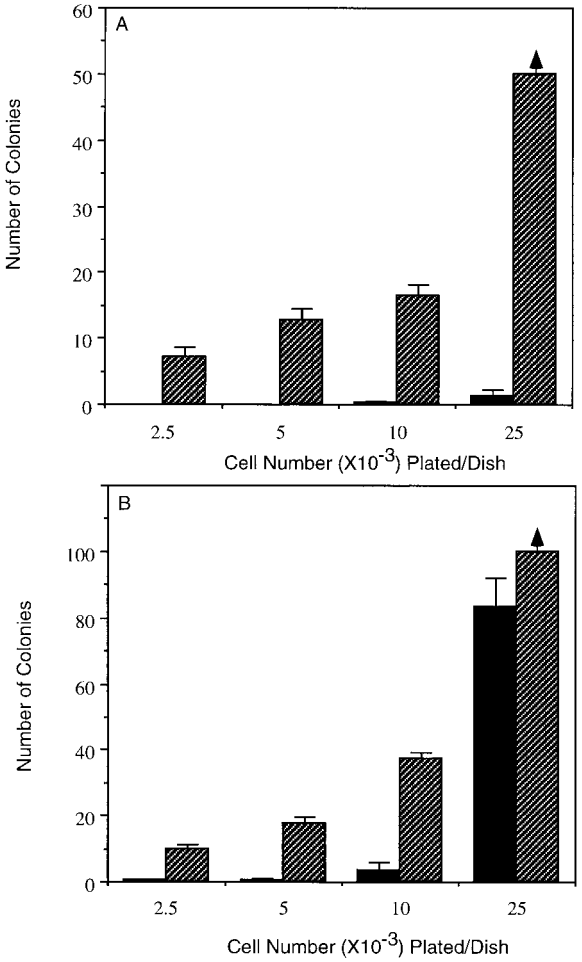

Fig. 3. The number of AP-positive colonies (▨) and number of bone nodules (■) that had formed by day 21 when rat bone marrow stromal cells were plated at various densities and grown without **(A)** or with **(B)** 10^{-8} M Dex. Both AP-positive colonies and bone nodules were stimulated by Dex at all cell densities ($P < 0.02$) and only a proportion of AP-positive colonies were coincident with bone nodules; arrow at 25 × 10^3 cells/dish indicates that the number was greater than that indicated but could not be counted accurately because colonies merged.

clearly showed, however, that OC mRNA was present albeit at low levels in cultures without Dex (Fig. 4). Consistent with our previous report with cultures containing Dex [Malaval et al., 1994], AP, BSP, OPN, and OC increased over the time course of bone nodule formation, while changes were not detectable in ON or Coll. I. The same trends were evident in cultures without Dex, but notably when nodules became heavily mineralized, a reduction in mRNA for Coll. I, AP, BSP, and OC were seen as reported before in marrow stromal cultures [Malaval et al., 1994] and in rat calvaria cultures [reviewed in Stein and Lian, 1993]. Previously, we have reported that protein expression detected by enzyme activity where applicable, enzyme-linked immunosorbent assay (ELISA), and immunocytochemistry parallels mRNA expression in the rat bone marrow system [Malaval et al., 1994]. These data support the conclusion that osteoprogenitors differentiate in rat bone marrow cultures in the absence of Dex, but that the frequency of nodule formation/amount of osteogenesis is stimulated by Dex in keeping with the stimulatory effect of Dex in other osteogenic populations including calvaria-derived cells [Aronow et al., 1990; Bellows and Aubin, 1989; Bellows et al., 1987, 1990].

The initial primary culture period for rat bone marrow cultures has been used to reduce hematopoietic cell contamination and remove dead cells and cell debris resulting from marrow aspiration [Maniatopoulos et al., 1988]. Given that cell–cell interactions between the stromal cell layer and hematopoietic cells affect the differentiation of hematopoietic cells [Dexter, 1982; Whetton and Dexter, 1993], it seemed possible that such interactions reciprocally might alter the ability of osteoprogenitor cells in the stroma to differentiate. Therefore, we then analyzed in more detail the frequency of osteoprogenitors giving rise to bone nodules or bone colonies (CFU-O) in relationship to plating cell density and in relation to fibroblastic colonies or CFU-F in both primary and secondary stromal cultures. In these experiments, CFU-F refers to any fibroblastic or pleiomorphic, non-bone colonies; CFU-O were easily dis-

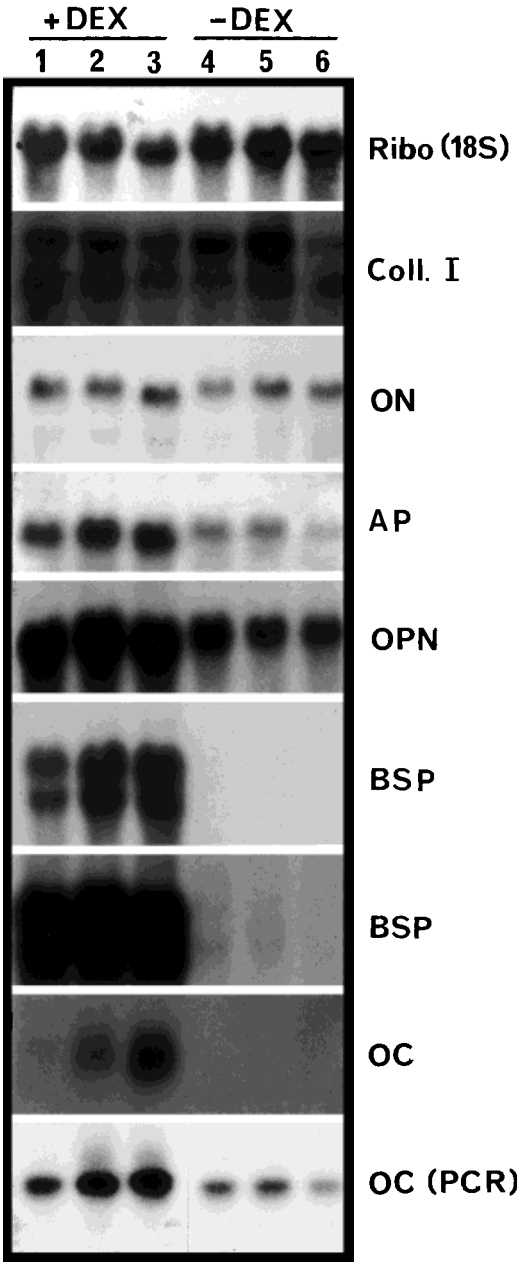

Fig. 4. Expression of mRNAs for osteoblast-associated markers. Rat bone marrow stromal cells were grown with (+Dex) or without (−Dex) 10^{-8} M Dex and mRNAs collected at day 7 **(lanes 1,4)**, day 14 **(lanes 2,5)**, and day 21 **(lanes 3,6)**. Northern blots were probed for 18S ribosomal RNA, or the mRNAs for Coll. I, ON, AP, OPN, BSP, or OC. Note that the lower BSP blot is the same as the upper blot but has been overexposed to show the BSP in the −Dex lanes. The lower OC blot is a Southern with reverse transcription-polymerase chain reaction (RT-PCR)-amplified cDNA prepared with OC-specific primers and probed for OC.

tinguishable from CFU-F by cell morphology (cuboidal cells surrounded by refractile matrix that mineralized and labeled black with Von Kossa staining). Under none of the culture combinations of Dex in primary and/or secondary cultures did the number of bone nodules or CFU-O in secondary cultures follow a linear relationship with plated cell number (e.g., Fig. 3; Table I). Because the numbers of bone nodules formed increased with increasing plating cell density, but in an apparently nonlinear fashion, a limiting dilution analysis was done. In this analysis, either cells were plated as primary cultures directly after isolation from the marrow cavities at densities ranging from 10^3 to 10^5 nucleated cells per microtiter well or cells were grown at the primary culture stage in Dex and then were re-plated at first subculture over a range of low densities from 12.5 to 500 cells per microtiter well. The data are shown only from cultures replated and grown in Dex, since bone nodule formation at limiting dilutions was too low in the absence of Dex for statistical analysis. When either primary (Fig. 5) or secondary (Fig. 6) stromal populations were analyzed, neither the fraction of wells without bone nodules or CFU-O (Figs. 5, 6A) nor the fraction of wells without CFU-F (Fig. 6B) analyzed in the same microtiter wells was linear until high cell densities were reached, suggesting a multitarget phenomenon. Extrapolation of the apparently linear portions of the plots suggested that at least two cell types were limiting under these conditions. Quantitation of the actual numbers of CFU-O or the CFU-F formed was done (Fig. 7); often CFU-F and CFU-O were present and distinguishable morphologically in the same wells. Again, a nonlinear relationship resulted; estimating frequencies over several cell densities, approximately 1/300–1/500 (+Dex) to <1/1,000 (−Dex) plated cells were CFU-O (osteoprogenitors forming bone nodules), while roughly 1/50–1/100 (+Dex) to 1/100–1/250 (−Dex) plated cells were CFU-F in secondary cultures. More CFU-F were present than CFU-O at all plating densities and in either the presence or absence of Dex (Fig. 7). In primary cultures, approximately 1/30,000–1/100,000 nucleated cells were CFU-O in these assays.

In many protocols for studying osteogenesis in marrow stromal cultures, the non-adherent fraction of the bone marrow is removed during the 7-day primary culture period. Given the

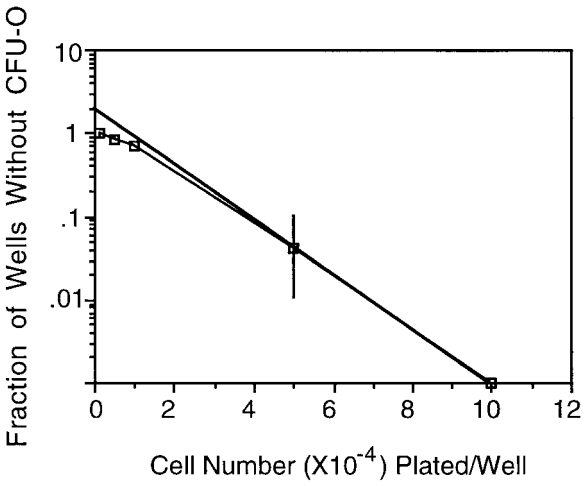

Fig. 5. Primary stromal cells were plated over a range of densities as indicated; 96 wells were plated per cell density. Cells were maintained in fully supplemented medium and 10^{-8} M Dex for 21 days, after which they were fixed, stained by the Von Kossa technique and each well was screened for the presence or absence of CFU-O (bone nodules). The fraction of wells not containing CFU-O for each density was calculated and plotted against the number of cells plated/well; error bars represent 95% confidence limits. An extrapolated line is indicated.

apparent multitarget phenomenon observed, which was more striking (deviated further from a straight line at low density) in secondary than in the primary populations, we next analyzed the effects of adding a fixed, large excess of particular cell types back to the microtitre wells. An excess of skin fibroblasts completely inhibited bone nodule formation at all densities tested (Fig. 8A) consistent with other reports of the inhibitory effects of fibroblasts on osteogenic differentiation [Ogiso et al., 1991, 1992]. By contrast, the addition of the nonadherent fraction of bone marrow cultures (Fig. 8B) or the conditioned medium from the nonadherent fraction of cells (Fig. 8C) stimulated the number of bone nodules formed, shifting the limiting dilution curve to an apparently single target line, from which an approximate frequency of CFU-O was calculated as 1/75–1/100 of plated cells.

DISCUSSION

It is now well established that amongst the cells present in adult rat bone marrow stroma are osteoprogenitors capable of proliferating and differentiating in vitro to form mature osteoblasts making bone (references in Introduction; reviewed in [Aubin and Herbertson, 1997]). Notably, osteoprogenitors in rat bone marrow stroma have been suggested to require exogenously added glucocorticoids, e.g., Dex, to differentiate and form bone in vitro [Kasugai et al., 1991; Leboy et al., 1991; Maniatopoulos et al., 1988], and so Dex is now routinely present in most studies on rat and other species. However, we found that there are precursor cells in rat bone marrow stromal populations which can differentiate to form bone in either primary or secondary cultures in the absence of Dex; this was true whether differentiation was assessed by bone nodule counts or by assessment of osteoblast-associated marker expression (e.g., hormone responsiveness, AP-positive colonies, Northerns for mRNA expression). Clearly, however, Dex stimulates bone nodule formation, increasing the frequency of bone nodule formation in stromal cells exposed to Dex >5–50 times over populations not exposed to Dex. These data suggest that in rat stromal populations, as in rat calvaria-derived populations, there are two pools of osteoprogenitors: ones that differentiate in the absence of exogenously added glucocorticoids and ones that do so only in its presence, although the number of the former type is relatively low and so detectable only at relatively high plating cell densities. By analogy with the fetal rat calvaria-derived progenitors [Turksen and Aubin, 1991], the implication is that the majority of the progenitors in stroma are of the immature category that require a stimulus, e.g., Dex, to differentiate in vitro. Whether these progenitors are identical in other features to the progenitors in calvaria remains to be assessed rigorously.

The stromal cell population of rat bone marrow comprises a heterogeneous mixture of cells, even under conditions in which osteogenesis is stimulated [Herbertson and Aubin, 1995; Malaval et al., 1994; Maniatopoulos et al., 1988], and only a proportion of the cells express the osteoblast-associated markers AP and bone matrix proteins [Malaval et al., 1994]. Consistent with these reports, we found that a relatively small fraction of the stromal cell layer of bone marrow comprises CFU-O or osteoprogenitors capable of differentiating to mature osteoblasts in vitro, and indeed that only a fraction of the AP-positive colonies terminally differentiated to bone nodules. Given the nonlinearity of the limiting dilution analyses, quantitation of absolute osteoprogenitor number is difficult under

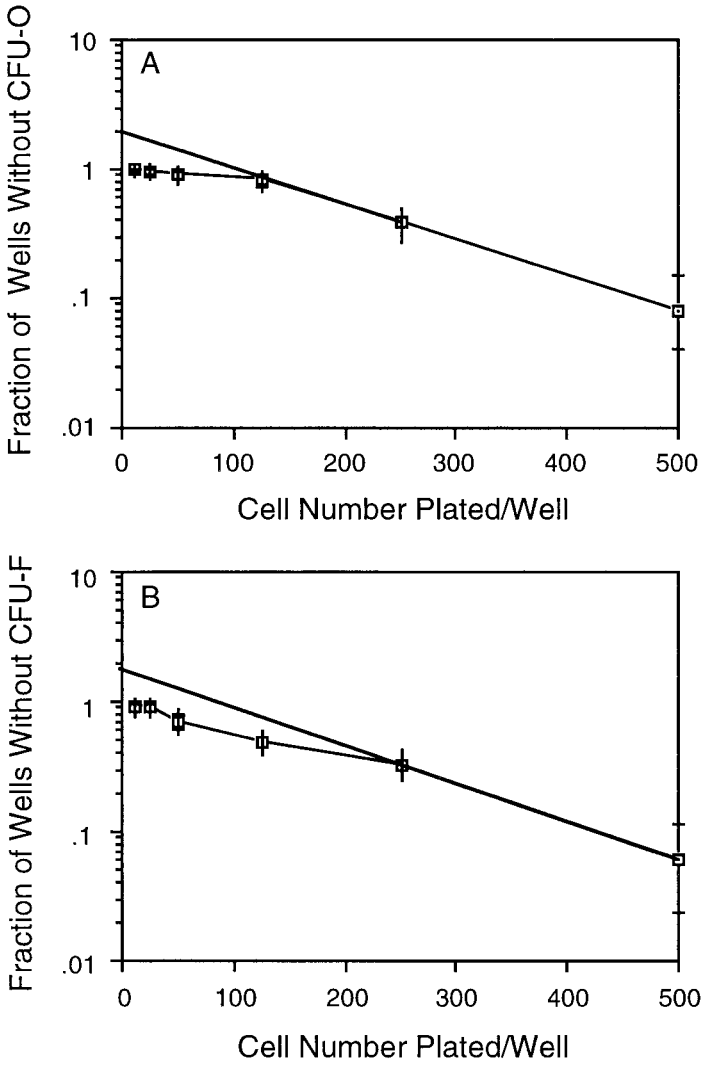

Fig. 6. Secondary stromal cells were plated over a range of cell densities as indicated; 96 wells were plated per cell density. Cells were maintained in fully supplemented medium and 10^{-8} M Dex for 21 days, after which they were fixed and stained by the Von Kossa technique; each well was screened for the presence or absence of CFU-O. **A:** The fraction of wells not containing CFU-O for each density was calculated and plotted against the number of cells plated/well. **B:** A similar analysis was done for CFU-F in the same wells. Neither CFU-O nor CFU-F followed a linear relationship until high cell densities were reached. The shapes of the curves suggested a multitarget phenomenon, and extrapolation of the apparently linear portions of the curves suggested that at least two cell types were limiting in these experiments. The points are the fraction of wells without CFU-O or without CFU-F at each density and bars are the 95% confidence limits.

control conditions. However, as a proportion of viable nucleated cells plated in primary cultures and assessed for bone nodule formation, approximately 1/30,000–1/100,000 cells is an osteoprogenitor. When the nonadherent fraction of bone marrow is reduced through the primary culture stage, osteoprogenitor frequency in the stromal population again varies with density but ranges from approximately 1/1,000 cells (in the absence of Dex) to 1/300–1/500 cells (in the presence of Dex). This number can be compared with an overall clonogenicity or CFU-F number of 1/100–1/250 (in the absence of Dex) to 1/50–1/100 (in the presence of Dex), where CFU-F is defined as any colony of fibroblastic or pleiomorphic, non-bone-forming cell. It is also clear that at least under the culture conditions tested, CFU-O are less frequent than CFU-F or CFU-AP, suggesting caution on the use of assays in which conclusions are reached on osteoprogenitor cell number and regulation based on counts/assessment of AP alone (see also Discussion in [Aubin and Herbertson, 1997; Herbertson and Aubin, 1995]). Of course, at the present time, we cannot exclude the possibility that some or all of the CFU-F are precursors for CFU-O or are CFU-O that have not yet matured to terminal differentiation stages recognizable morphologically. On the other hand, the heterogeneity of individual CFU-F in functional assays (e.g., ability to support various hemopoietic lineages or give rise to differentiated tissue types in diffusion chambers [Owen and Friedenstein, 1988]) does suggest that not all CFU-F are identical or contribute to the CFU-O pool. With respect to osteoprogenitor frequency among total nucleated cells recovered from primary bone marrow,

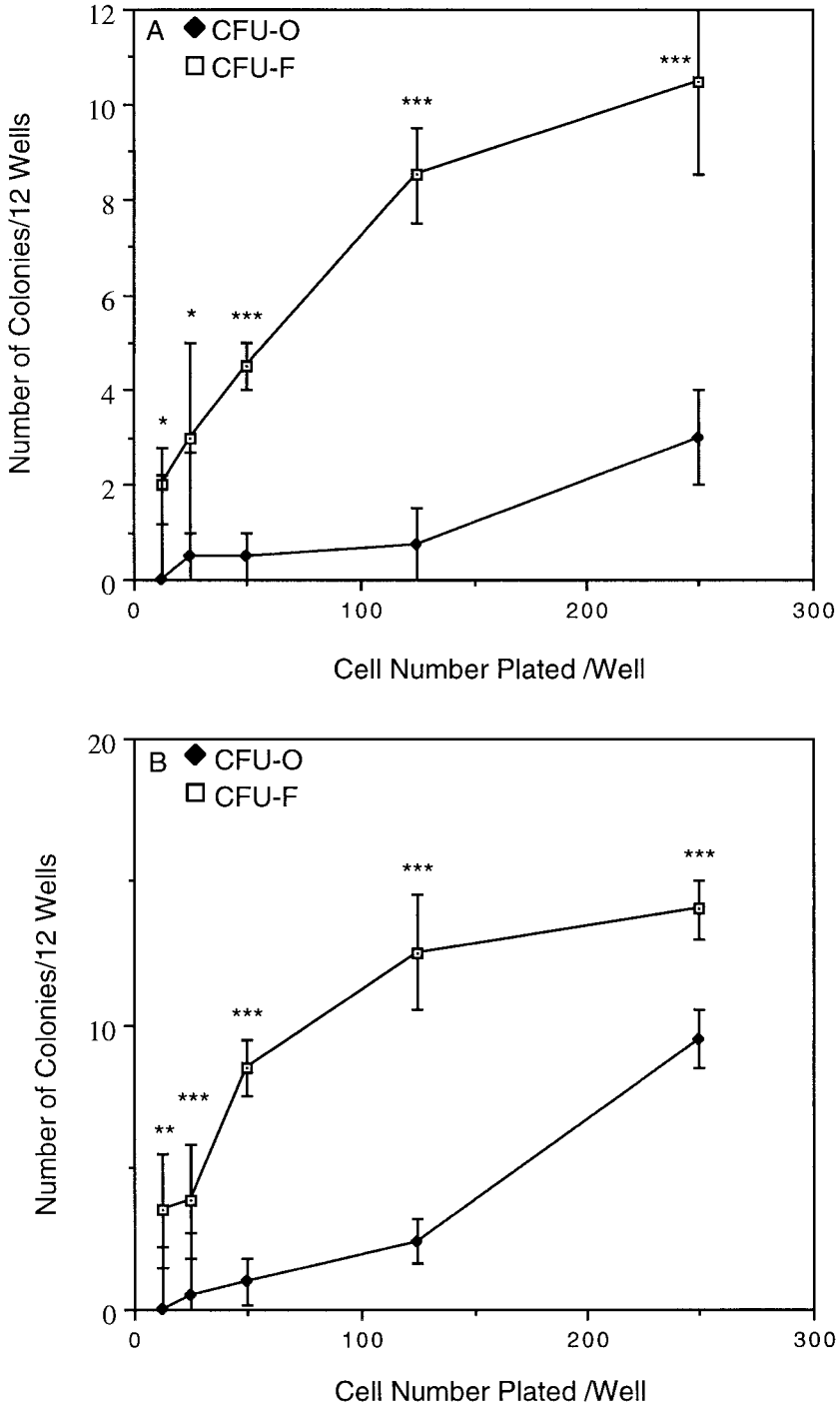

Fig. 7. The number of CFU-O (bone nodules; ◆) or CFU-F (fibroblastic colonies; ⊡) formed at each density plated in the limiting dilution analysis was also quantitated, in the absence **(A)** or presence **(B)** of Dex. Discrete CFU-O and CFU-F were often present in the same wells; numbers were counted in 12 wells (one row of a 96-well microtiter tray) and the mean and SDs determined over the 8 rows at each cell density. CFU-F were present at higher frequency than CFU-O at every density analyzed; $*P < 0.05$; $**P < 0.005$; $***P < 0.001$ by Student's t-test or the Welch test.

Falla et al. [1993] reported that in murine stroma, osteoprogenitor differentiation was linearly related to plated cell density and the osteoprogenitor frequency was approximately $1/2 \times 10^5$ cells in the primary cell isolate. While the frequency is similar to that which we measured (approximately $1/10^5$) in primary cell cultures of rat stroma, in rat we find no evidence of a linear relationship until high cell density is reached. Since Falla and colleagues did not test or did not report densities below 4×10^4 cells/well, a density at which the relationship has already reached linearity in rat, it is possible that similar accessory cell function is operative in mouse, and other species, but this will require testing over appropriate density ranges. It should also be noted, however, that these investigators reported osteogenesis in mouse

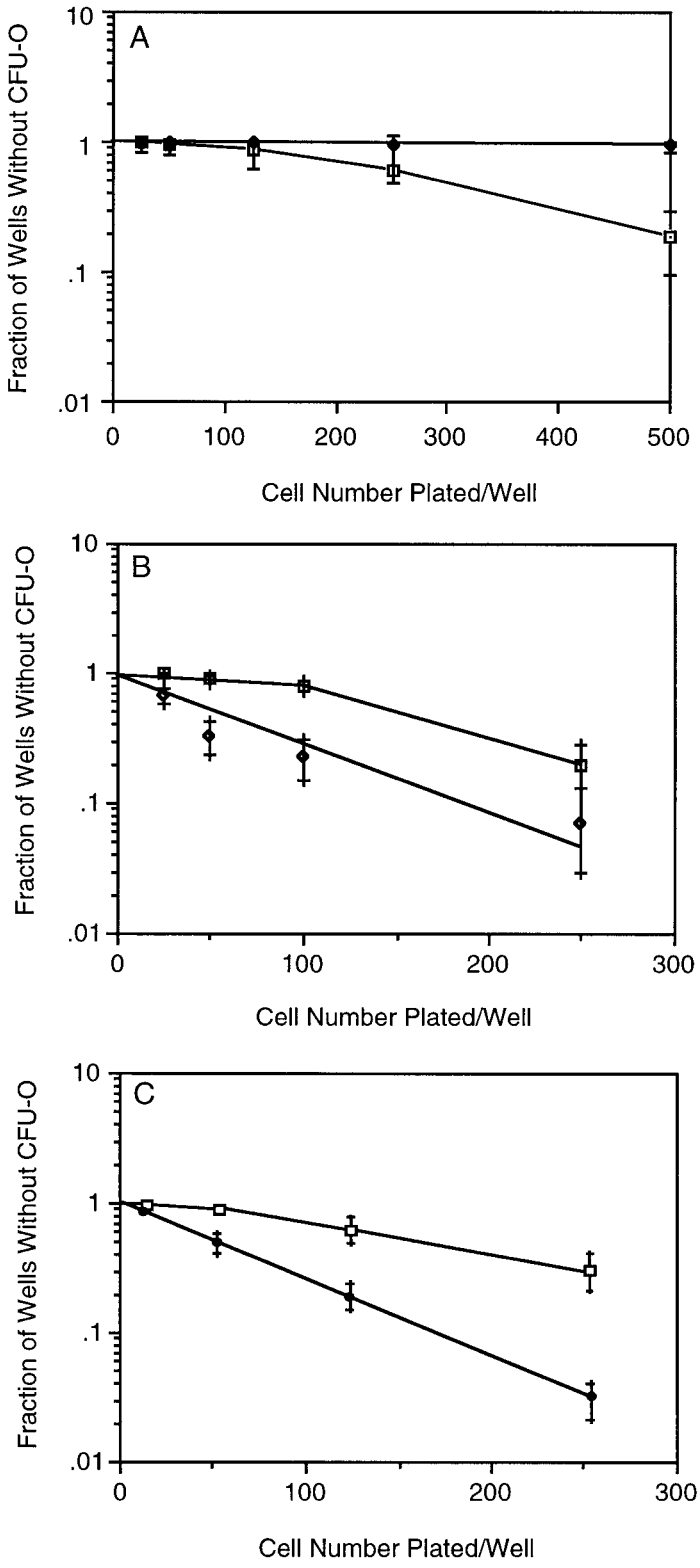

Fig. 8. **A:** The addition of a fixed number (10^5) of rat skin fibroblasts to each well in the limiting dilution analysis completely abrogated the ability of osteoprogenitors to make bone. Stromal cells alone, (⊡); stromal cells + rat skin fibroblasts (◆). At least 96 wells/cell density/per condition were plated. The points are the fraction of wells without bone at each density and bars are the 95% confidence limits. **B:** When a fixed number (10^5) of nonadherent (hematopoietic) cells was added to each well, the expression of osteoprogenitor cells followed a single-target line and the number of detectable osteoprogenitors was increased to approximately 1/100. Stromal cells alone, (⊡); stromal cells + nonadherent cells, (◇). The points are the fraction of wells without bone at each density and bars are the 95% confidence limits. **C:** When the conditioned medium (1:2) of nonadherent cells was added to each well, the expression of osteoprogenitor cells also followed a single-target line and the number of detectable osteoprogenitors was also increased to approximately 1/100. Stromal cells alone (□); stromal cells + conditioned medium of nonadherent cells, (●).

marrow stroma in the absence of added glucocorticoids, and indeed found that Dex was inhibitory to the process in mouse. Thus, further studies are required to compare the types and hormonal requirements of the osteoprogenitors in rat versus mouse versus other species. Notably, the nonlinearity of the limiting dilution data when cells were grown under control conditions obscured whether CFU-O or osteoprogenitors giving rise to bone nodules are likely to

be clonal in the rat marrow system. The latter was addressed, in part, by adding the conditioned medium of nonadherent cells to the assay system. Under this condition, CFU-O followed a linear relationship to plated cell number, suggesting that the production of bone nodules in rat stroma is a clonal event with single osteoprogenitors giving rise to a bone nodule. The frequency of assayable CFU-O under this condition of one limiting cell type is higher and approximately 1/100 of the plated adherent cell population.

Although isolated fetal rat calvaria cells are also clearly not a homogeneous population [Aubin et al., 1982, 1993], limiting dilution analysis of osteoprogenitor cells in these populations has indicated that only one cell type is limiting in such cell populations for osteoprogenitor cell expression, and the data are consistent with the limiting cell type the osteoprogenitor itself [Bellows and Aubin, 1989]. As indicated, the rat bone marrow stromal cell system, on the other hand, achieves linearity only at high cell densities, suggesting a cell nonautonomous behavior to differentiation in this system. This raises two possibilities. One possibility is that of a *community effect*, i.e., that the establishment of a group of differentiated osteoblasts may be dependent on cell interactions that occur only when a critical number of cells is reached [Gurdon et al., 1993a, b]. Our preliminary data suggest that once densities comparable to those in the linear part of the curves are reached, if there are community effects they are saturated, while at the lower cell densities community effects may be playing a role in osteoprogenitor differentiation (unpublished data), but we are currently testing this hypothesis more rigorously. A second possibility consistent with the extrapolated line of the limiting dilution experiments is that heterotypic cell–cell interactions occur, i.e., that osteogenesis is regulated by the nonosteogenic lineages in the bone marrow. Consistent with some earlier reports [Ogiso et al., 1991, 1992; Zhang et al., 1991], we found that fibroblasts from a heterologous source, i.e., skin, inhibited marrow stromal osteogenesis completely. On the other hand, when a large number of cells from the nonadherent fraction of bone marrow was added to the stromal cultures, osteogenesis was stimulated and an increased number of bone nodules formed. Several recent reports have suggested that a class of osteoprogenitor is present in the nonadherent fraction of bone marrow in human [Long et al., 1995] and rat [Scutt and Bertram, 1995]. To determine whether they contributed to the increased osteoprogenitor number we measured, we confirmed that not only the nonadherent cells themselves, but also their conditioned medium, increased the frequency of bone nodules, suggesting that it is not a progenitor from the nonadherent fraction of bone marrow but a secreted product(s) from cells within the population that is stimulatory under these conditions. Given that Dex stimulates monocyte to macrophage differentiation in rat stromal cultures [Herbertson and Aubin, 1995] concomitant with osteogenesis, it is possible that it is cells of this lineage that modulate osteogenesis in these cultures, consistent with their being a generally a rich source of multiple cytokines. In addition, however, both platelets and megakaryocytes have been reported to stimulate differentiation of murine osteoprogenitors [Friedenstein et al., 1992], while IL-10 a product of helper T (type II, Th 2) cells is potently inhibitory in the same species [Van Vlasselaer et al., 1993]. Thus, it is likely that osteoprogenitor cell differentiation in the bone marrow stroma may be under the influence of a complex hierarchy of cells and factors not unlike the hierarchy controlling differentiation of the hematopoietic cells, including cells of the osteoclast lineage [Manolagas and Jilka, 1995]. In this regard, there are considerable data indicating a role for stromal cell subpopulations in regulation of hematopoietic cells [Dexter, 1982; Whetton and Dexter, 1993] and formation of osteoclasts, cells that derive from monocyte-macrophage lineage cells [Suda et al., 1995]. Our data suggest that the cross-talk between cells of hemopoietic origin and those of stroma may be operative on the stromal cells also.

The data support the hypothesis that in marrow stroma, as in other bone cell populations such as those from calvaria, there are at least two classes of osteoprogenitor cells, those differentiating in the absence of exogenously supplied glucocorticoid and those requiring glucocorticoid to differentiate, that more than one cell type is limiting for osteoprogenitor cell differentiation in the stroma suggesting a role for heterotypic cell–cell interactions in osteogenesis in this tissue, and that Dex may be acting directly and/or indirectly through accessory cells present in the bone marrow to alter osteoprogenitor cell expression.

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