# Global amplification polymerase chain reaction reveals novel transitional stages during osteoprogenitor differentiation

**Fina Liu[1], Luc Malaval[1,*] and Jane E. Aubin[1,2,‡]**

[1]Department of Anatomy & Cell Biology, and [2]Department of Molecular & Medical Genetics, University of Toronto, Toronto, Ontario, M5S 1A8, Canada

*Present address: INSERM Unit 403, Hôpital E. Herriot, Lyon, 69437, France
‡Author for correspondence (e-mail: jane.aubin@utoronto.ca)

*Accepted 21 January 2003*
*Journal of Cell Science 116, 1787-1796 © 2003 The Company of Biologists Ltd*
*doi:10.1242/jcs.00376*

## Summary

**Mesenchymal stem cells give rise to osteoprogenitors that proliferate and differentiate into identifiable preosteoblasts, osteoblasts, bone lining cells and osteocytes. To identify and establish a molecular profile for the more primitive and uncharacterized cells in the lineage, relatively rare (<1%) osteoprogenitors present in primary cultures of fetal rat calvaria cell populations were identified by a replica plating technique. Since the cell number was limited in each colony sampled, we used global amplification PCR to analyze the repertoire of genes expressed in osteoprogenitors. We established a molecular fingerprint and a developmental sequence based on simultaneous expression patterns for both known osteoblast-associated markers (collagen type I, alkaline phosphatase, osteopontin, bone sialoprotein, PTH1R and osteocalcin) and potential regulatory molecules (i.e. FGF-R1, PDGF-Rα and PTHrP). By analysis of 99 osteoprogenitor and osteoblast colonies captured by replica plating at different developmental stages, we found: (1) a recognizable cohort of cells considered more primitive than committed osteoprogenitors; (2) a cohort of early progenitors transiently expressing bone sialoprotein; and (3) that mRNAs for FGF-R1, PDGF-Rα and PTH1R were expressed earlier than other markers and tended to increase and decrease in relative concert with the osteoblast-specific markers. The observations suggest that within the osteoblast differentiation sequence both discrete stages and continua of changing marker expression levels occur with variation in expression for any given marker. This combined approach of replica plating and global amplification PCR allows molecular fingerprinting of definitive primitive osteoprogenitors and will aid in identifying novel developmental stages and novel differentiation stage-specific genes as these cells progress through their differentiation sequence.**

Key words: Osteoprogenitors, Osteoblasts, Differentiation, PCR, Replica plating

## Introduction

According to current understanding, multipotential mesenchymal stem cells give rise to different lineages, one of which is the osteogenic lineage (for reviews, see Aubin and Liu, 1996; Aubin and Triffitt, 2002). Osteogenic cells originate from committed osteoprogenitors that proliferate and differentiate into cells that by morphological, histochemical, immunohistochemical and molecular criteria are identifiable as preosteoblasts, mature osteoblasts, quiescent bone lining cells, and terminally differentiated osteocytes. To identify more primitive cells in the lineage has been difficult because their morphological characteristics are not known to be different from fibroblasts, unique molecular markers are not yet known, and they are relatively rare compared with their more differentiated progeny. Some clonal multipotential and osteoblastic cell lines have been isolated whose phenotypes are consistent with their being relatively immature, but significant differences amongst representative lines suggest that none faithfully represents authentic progenitors (Aubin et al., 1993; Rodan and Noda, 1991). Further, while they have been useful models in which to investigate some aspects of regulation of osteoblast maturation and macromolecular synthesis, their apparently aberrant expression of at least some markers has suggested that other models are required for detailed characterization of the molecular nature of the more primitive osteoprogenitors. To this latter end, we devised an alternative strategy that relies on the use of primary cultures of fetal rat calvaria (RC) cell populations. The well established RC model contains osteoprogenitors that can divide and differentiate in vitro to form bone nodules, although their number is low, i.e. <1% of an RC population under standard culture conditions (Bellows and Aubin, 1989). In this system, the osteoprogenitors present are morphologically indistinguishable from other pleiomorphic or fibroblastic cells making up the majority of the population, but they can be identified indirectly or retrospectively by their ability to give rise to more differentiated progeny, up to and including mature osteoblasts forming the easily identifiable colony type of a mineralized bone nodule (multilayer of cuboidal cells with abundant osteoid and deposited hydroxyapatite). In this proliferation-differentiation sequence, genes associated with proliferative stages (e.g. histones, and proto-oncogenes such as *c-fos* and *c-*

*myc*) characterize the first phase, while expression of the most frequently assayed osteoblast-associated genes collagen type I (COLL-I), alkaline phosphatase (ALP), osteopontin (OPN), osteocalcin (OCN) and bone sialoporotein (BSP) is increased in a well established temporal sequence as osteoblasts develop and bone forms (reviewed by Aubin and Liu, 1996; Aubin and Triffitt, 2002; Stein et al., 1996). Briefly, the differentiation sequence is defined by an increase in COLL-I synthesis, acquisition and increase in ALP activity, and acquisition of expression of several non-collagenous bone matrix proteins, such as OPN, followed by BSP and finally OCN as the latest marker of the mature osteoblast. However, many discrepancies to this expression profile have been reported in this and related models, suggesting that lineage analysis in these heterogeneous populations leads to significant ambiguity.

Earlier, while investigating changes in levels of expression of the osteoblast-related macromolecules in RC colonies classified morphologically as comprising early or more mature osteoblasts versus ones designated 'fibroblastic', we found evidence to suggest that developmental stages more primitive than those recognizable by the presence of cuboidal cells and prior to upregulation of any of the known osteoblast markers were accessible for analysis in this model (Liu et al., 1994). Our solution to the problem of identifying the low frequency primitive osteoprogenitors was to use replica plating on dishes plated at low density and sampled early in the development of colonies. Replica plating has been found to allow screening of large numbers of individual mammalian cell clones for the phenotype of interest while still maintaining a master copy of the colonies; use of polyester cloth, in particular, provides high fidelity copies for a variety of cell types (Esko, 1989; Raetz et al., 1982). Since cell number was limited in each colony sampled, we applied global amplification or poly(A)-PCR (Aubin et al., 2002; Brady et al., 1995; Brady and Iscove, 1993) to determine simultaneous expression profiles of the mRNAs of interest. These included both the osteoblast-related mRNAs and mRNAs for potential regulatory molecules. We now report that this approach not only allows molecular fingerprinting of definitive primitive osteoprogenitors but also reveals novel transient developmental stages of such cells as they progress through their differentiation sequence.

## Materials and Methods

### Cell culture

Cells were isolated by a five-step sequential digestion procedure of 21 day Wistar rat calvariae with a collagenase enzyme mixture, as described previously (Bellows et al., 1986). Cells obtained from the last four of the five digestion steps (populations II-V) were pooled and plated in T-75 flasks containing α-MEM, 15% FBS, 100 μg/ml penicillin G, 50 μg/ml gentamycin and 0.3 μg/ml fungizone, and incubated at 37°C in a humidified atmosphere of 95% air and 5% $CO_2$. After 24 hours, cells were trypsinized (0.01% trypsin in citrate saline), counted, replated at 10-25 cells/$cm^2$ per 100 mm culture dish, and cultured in standard medium described above supplemented with 50 mg/ml ascorbic acid, 10 mM sodium β-glycerophosphate and 10 nM dexamethasone (Liu et al., 1994).

### Replica plating

Replica plating was done essentially as described (Esko, 1989; Raetz et al., 1982) but with minor modifications. Briefly, a disc of 1 μm pore size polyester cloth (HD7-1; B&SH Thompson, Scarborough, ON) was floated above the cells and weighted down by a monolayer of 4 mm glass beads; replica cloths were placed on cells at day 1 or day 4 and were removed on day 5 or day 11, respectively, and transferred into a new dish. The master dish and polyester disc were each rinsed with PBS and fed with supplemented medium as above. The replica disc was incubated at 37°C; whereas, the master dish was incubated at either 25°C or 30°C to stall the proliferative and differentiation activities of colonies. The medium was changed every 2-3 days.

In preliminary experiments to determine the transfer efficiency and fidelity of transfer for colonies of fetal RC cells, replica cloths were fixed on day 25 with 10% neutral buffered formalin and stained with the von Kossa technique. Also on day 25, master dishes were transferred from the lower temperature incubators to a 37°C incubator. After 14 days at 37°C, these master dishes were fixed and stained for the presence of bone nodules as described above. For statistical analysis (Fig. 1), the data were expressed as means and standard deviations.

### Osteoblastic colony isolation and cDNA preparation

After two weeks at 37°C, the replica cloth was fixed in 10% neutral buffered formalin and stained with the von Kossa technique to identify bone nodules. The master dish was transferred to 37°C for 5-9 hours. After this time, the replica disc was matched up with the master dish to localize primitive osteoblast colonies, which were then marked. In addition, single isolated osteoblast colonies containing plump cuboidal cells with unmineralized osteoid or mature osteoblast colonies containing mineralizing osteoid were marked and collected from dishes that were not cultured with polyester discs. Dishes were rinsed with PBS and a cloning ring was placed around the marked colonies. The cells from each colony were released with 0.01% trypsin (when matrix lacked mineral) or a 1:1 mixture of 0.01% trypsin and collagenase (when osteoid was mineralizing), and the enzyme(s) neutralized after cell release by adding α-MEM containing 15% FBS. Total RNA was extracted using a mini-guanidine thiocyanate method as described previously and cDNA was synthesized by oligo(dT) priming, poly(A)-tailed, and amplified by PCR with oligo(dT) primer (Brady and Iscove, 1993; Liu et al., 1994). The 108 colonies reported here were collected from 12 independent cell isolations and replica plating experiments.

### Southern blots and hybridization

Amplified cDNA (5 μl) was run on 1.5% agarose gels, transferred onto 0.2 μm pore size nylon membrane (ICN, Costa Mesa, CA), and immobilized by baking at 80°C for 2 hours. Prehybridization and hybridization were performed as described (Liu et al., 1994). After hybridization, the blots were washed at 65°C for 1 hour each in 2× SSC/0.1% SDS and in 0.5× SSC/0.1% SDS. The blots were then exposed to phosphorimager screens (Molecular Dynamics, Sunnyvale, CA), and digitized images obtained and quantified with the IPLab Gel program (Signal Analytics Corp., Vienna, VA). After quantifying the data, the signal intensity for each probe was standardized against total cDNA (see below). For preparation of comparative histograms of relative expression profiles, we next determined maximal expression value for each message; the largest value was divided by 5 to obtain five ranges of values or categories. Samples were then given a rank of 1, 2, 3, 4, or 5 depending on where their values fell within each range or were given a rank of 0 if the intensity of signal was not detectable. For statistical analysis of relative expression levels (Fig. 4), the actual standardized expression levels for each probe in each sample were used to calculate means and standard deviations within categories of populations. Levels of statistical significance were calculated by the Welch *t*-test.

Labeled probes were used at an activity of $10^6$ cpm/ml. cDNA probes were labeled with [$^{32}$P]dCTP using an oligolabeling kit

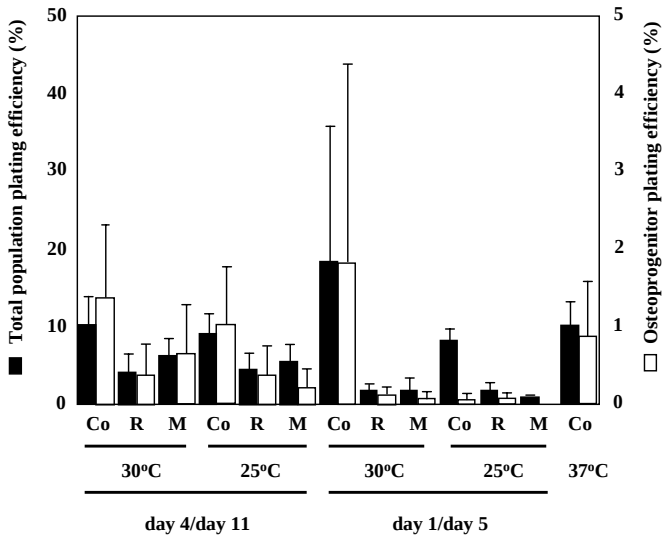



**Fig. 1.** Plating efficiencies/colony forming efficiencies of bone and non-bone colonies in controls (Co), master dishes (M) and replica cloths (R) under different replication conditions. Bars are means and standard deviations of colony counts from a range of 4-34,100 mm dishes depending on the condition. The days indicated correspond to when the replica cloths were placed over/removed from the colonies in master dishes as described in Materials and Methods.

(Pharmacia, Uppsala, Sweden). Total cDNA probe was prepared as described (Sambrook et al., 1989) from poly(A)+ mRNA isolated (Auffray and Rougeon, 1980) from mass populations of fetal RC cells grown in the presence of dexamethasone in which bone nodules were beginning to mineralize. For optimal detection of poly(A)-PCR-amplified cDNA, the probes required sequences at or close to the extreme 3′ ends of the native transcripts. Labeled probes for the cDNAs listed above were generated as described previously (Liu et al., 1994). The rat cDNA probes used were for COLL-I [(Genovese et al., 1984) a gift of D. Rowe, Farmington, CT], bone/liver/kidney ALP [(Noda and Rodan, 1987) gift of G. A. Rodan, West Point, PA], OPN (a gift of R. Mukherjee, Montreal, PQ), BSP and OCN [prepared by generating specific probes by PCR, screening an osteoblast library and then confirming isolated cDNAs by sequencing (see also Liu et al., 1994)]. Rat PDGF-Rα [(Lee et al., 1990) a gift of R. R. Reed, Baltimore, MD] was a 400 bp cDNA *Pst*I fragment obtained by digesting full length cDNA with *Hin*dIII to remove 6 kb of the 5′ region and religating the sticky ends. Mouse FGF-R1 [(Mansukhani et al., 1990) a gift of C. Basilico, New York, NY] was a 400 bp cDNA *Hin*cII-*Pst*I fragment. Rat PTHrP [(Yasuda et al., 1989) a gift of D. Goltzman, Montreal, PQ] was a 700 bp cDNA *Sma*I fragment, and rat PTH/PTHrP receptor [PTH1R (Abou-Samra et al., 1992) a gift of G. V. Segre, Boston, MA] was a 800 bp cDNA *Hin*dIII-*Xba*I fragment.

## Results

### Replica plating

The replica technique as applied to mammalian cells has been used mainly with rapidly growing, and often transformed, cell lines. To validate it for identification of osteoprogenitor cells in primary RC populations, we first did a quantitative analysis of colony formation on master dishes and replicas, and compared total population plating efficiencies and bone colony plating efficiencies of RC cells plated at low density, the efficiency of faithful colony replication and bone nodule formation when replica cloths were added to cultures on day 1/removed on day 5 or added on day 4/removed on day 11, and the best low temperature for stalling colony growth on master dishes while maintaining osteoprogenitor viability, relative immaturity, and subsequent recovery with ability to differentiate intact. Bone and non-bone colonies were identified by well established morphological characteristics and confirmed by immunocytochemical and molecular characteristics as shown elsewhere [data not shown (see Liu et al., 1994; Malaval et al., 1999)]. Consistent with our earlier limiting dilution analyses (Bellows and Aubin, 1989) and other low density plating experiments (Liu et al., 1994), total population plating efficiency/colony forming efficiency of primary RC populations was ~10% and of these the fraction of colonies that were bone was 10%, i.e. ~1% of the starting cell population at 37°C (Fig. 1). Control experiments in which both replica cloth and master dish were maintained at 37°C confirmed that replication of both bone and non-bone colonies worked with essentially 100% efficiency in these low density cultures on both master and replica (Fig. 1). Stall temperatures of 4°C or lower gave extremely poor cell recovery from the master dish so this was not used further. Replica cloths placed over colonies on day 4 (many cells entering log phase growth) and removed a week later (day 11) allowed sufficient numbers of cells to attach to cloth and master dish to achieve ~95% transfer efficiency when the master dish was stalled at 30°C (Fig. 1). In control experiments, we found that a few colonies did not replicate faithfully either because the whole colony transferred to the cloth or little or none of the colony transferred. Several other protocols used [e.g. stalling the master dishes at 25°C to maintain colonies at even lower growth rates or by placing replicas for shorter periods earlier (day1-5)] led to considerably lower replication efficiencies, due to a combination of poor recovery of cells on the master dish at lower temperature and/or overall lower colony efficiencies on replica and master dishes when colonies were replicated only during their early growth phases (Fig. 1).

By using optimal conditions and selecting for fingerprinting only colonies in which cells had a robust appearance, and ones discrete/well separated from and so not contaminated by cells from other colony types, we were able to isolate over 80 definitive osteoprogenitor colonies. Visual inspection indicated that, on average, the osteoprogenitors on the stalled master dishes were relatively quiescent and had undergone only 0-1 additional population doublings; a few which had a senescent or dying appearance were discarded from the analysis. All osteoprogenitor colonies selected for analysis comprised cells with a pleiomorphic morphology indistinguishable from non-bone colonies on the same dishes, indicating that none had yet acquired the cuboidal shape characteristic of differentiated osteoblasts. That cells were well recovered prior to mRNA isolation is indicated by the fact that they were relatively mitotically active after 5 hours and 9 hours from dishes originally stalled at 30°C and 25°C, respectively (not shown). Estimated colony size judged by microscopic viewing on a grid ranged from 500 to 3000 cells per colony, colony size did not directly correlate with any specific phenotypes observed by fingerprinting.

### Global amplification poly(A)-PCR

Previously, we used poly(A)-PCR to establish molecular

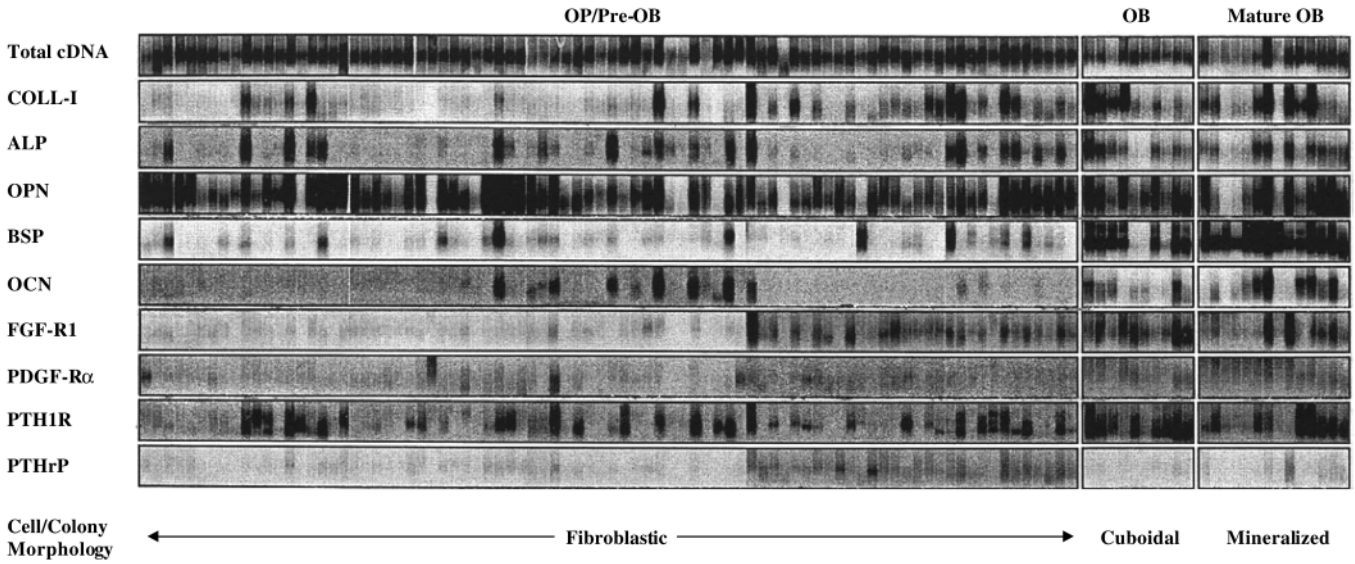



**Fig. 2.** Phosphorimage cDNA expression profiles of osteoprogenitor/preosteoblast (OP/Pre-OB) colonies. OP/Pre-OB colonies were identified by the replica plating technique, and early and mature osteoblast colonies were identified morphlogically by the cuboidal shape of osteoblasts and deposition of osteoid (OB) and mineralization of osteoid (Mature OB), respectively. The mRNA from each sample was reverse transcribed into cDNA and amplified by the poly(A)-PCR method. The resulting cDNA amplified from each colony was then probed for the expression of total cDNA (a control for the amplification procedure), the various bone-related proteins (COLL-I, ALP, OPN, BSP and OCN, PTH1R, PTHrP), and cytokine receptor messages (FGF-R1 and PDGF-Rα) as specified. Each vertical lane is the cDNA from the same colony. The exposure is the same for all colonies for each probe. These colonies are in random order within each category.

profiles for osteoblastic colonies and single cells that had differentiated to different extents as recognized morphologically (i.e. early, intermediate, and mature osteoblast) (Liu et al., 1997; Liu et al., 1994) and we included a few such advanced colonies here. These (labeled OB and Mature OB; 24 colonies total) were subjected to the same poly(A)-PCR manipulations and Southern blotting as colonies identified by replica plating (labeled OP/Pre-OB; 84 colonies total) (Fig. 2). A total cDNA probe was used as a control to assess first strand synthesis and amplification amongst the 108 colonies (Fig. 2); this proved relatively constant and its signal strength was used to standardize relative abundance of all other messages for comparison amongst colonies. Of all messages probed, OPN was most uniformly and abundantly present in virtually all colonies analyzed. PTHrP, PTH1R, FGF-R1 and PDGF-Rα mRNAs were also detectable in virtually all colony types but their abundance was generally lower and heterogeneous from one colony to another (Fig. 2). Consistent with our previous data, most of the mature osteoblast colonies expressed all markers, but to various extents; a few osteoprogenitor colonies also expressed all markers to some degree, but most a much more restricted repertoire and to limited levels (Fig. 2).

Immature progenitor colonies or more mature osteoblastic colonies analyzed in the Southern blot shown in Fig. 2 are in random order, reflective only of the order of processing. To determine whether immature progenitors could be placed in a rank order of more primitive or less primitive cells, we developed a paradigm for their comparison. Relative expression levels of all messages in all colonies sampled were determined by normalization of their signal strengths against that for total cDNA; colonies in which total cDNA was not well detected were eliminated from our subsequent analyses. As described in Materials and Methods, the remaining colony samples were then assigned categories of relative expression from low (0-1) to high (5). This classification smoothed out the small but not the large variations in expression levels such that overall patterns of gene expression became more obvious. Based on numerous previous studies and techniques, it is known that ALP expression rises as osteoblasts mature and then declines as osteoid becomes heavily mineralized, while OCN expression is acquired latest and essentially is diagnostic of post-proliferative osteoblasts (Malaval et al., 1999). Therefore, we rank-ordered colonies manually based on ALP and OCN expression (Fig. 3), which imposed an order for all other genes probed: the mature end (extreme right; COLL-$I^+$/$ALP^+$/$OCN^+$), followed in order to the left by colonies expressing fewer and fewer markers in the order COLL-$I^+$/$ALP^+$ colonies, then COLL-$I^+$/$ALP^-$ colonies, then COLL-$I^-$/$ALP^-$ (i.e. colonies expressing no osteoblastic markers are on the extreme left). Among the COLL-$I^+$/$ALP^-$ colonies, surprisingly a few were $BSP^+$. Since a few COLL-$I^-$/$ALP^-$ colonies were also $BSP^+$, we grouped all these $BSP^+$ colonies together at the COLL-$I^+$/$ALP^-$COLL-$I^-$/$ALP^-$ border. By using categories established in Fig. 3, we also calculated the means and standard deviations of corrected (standardized against total cDNA) expression levels for each probe within categories of populations and looked for statistically significant changes during the osteogenic differentiation sequence (Fig. 4).

These analyses allowed us to discern whether other unexpected patterns existed, for example, in the hormone and growth factor receptors (Figs 3, 4). First, there is a recognizable cohort of cells captured by the replica and considered very primitive (left; no osteoblast-specific mRNAs

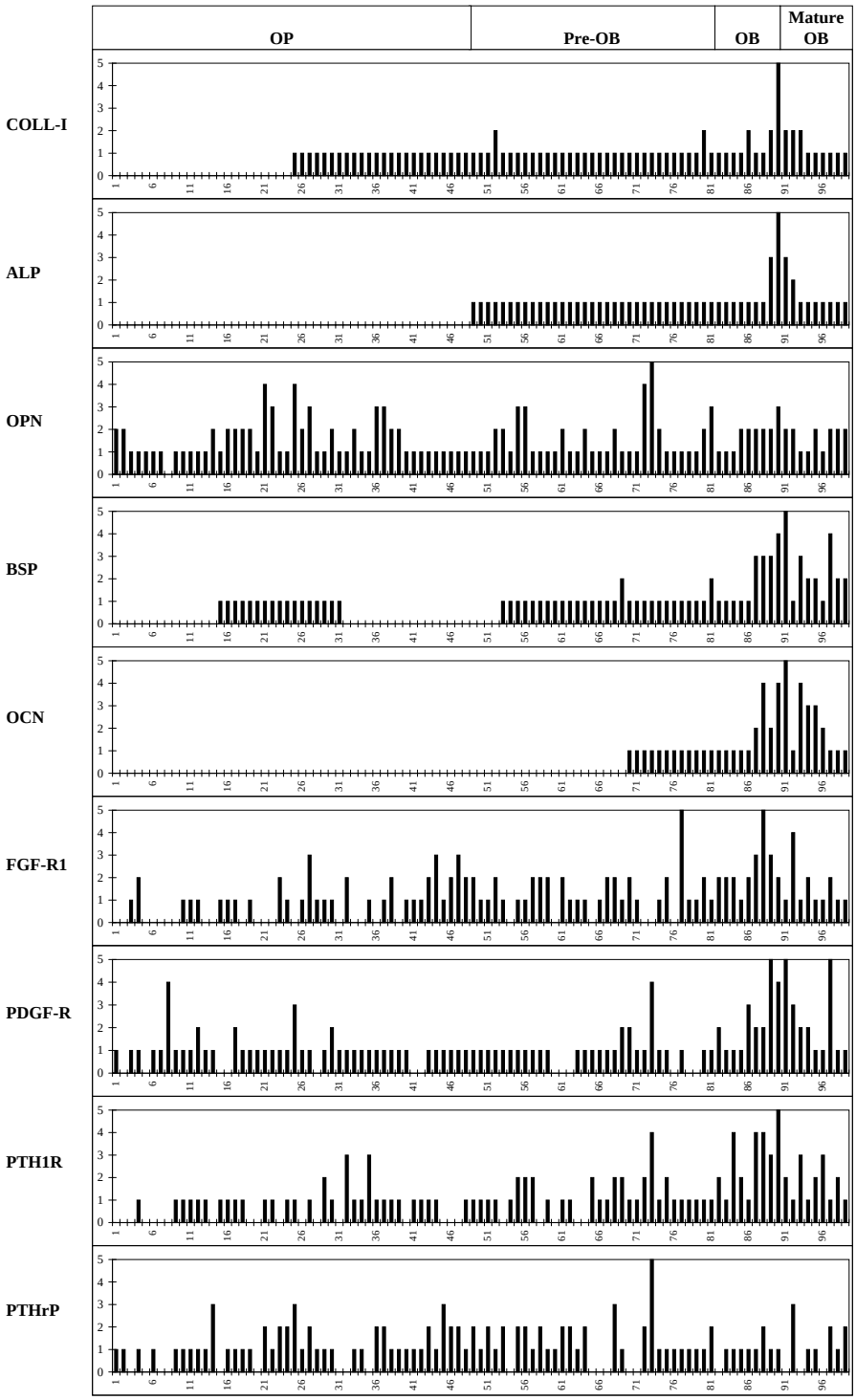
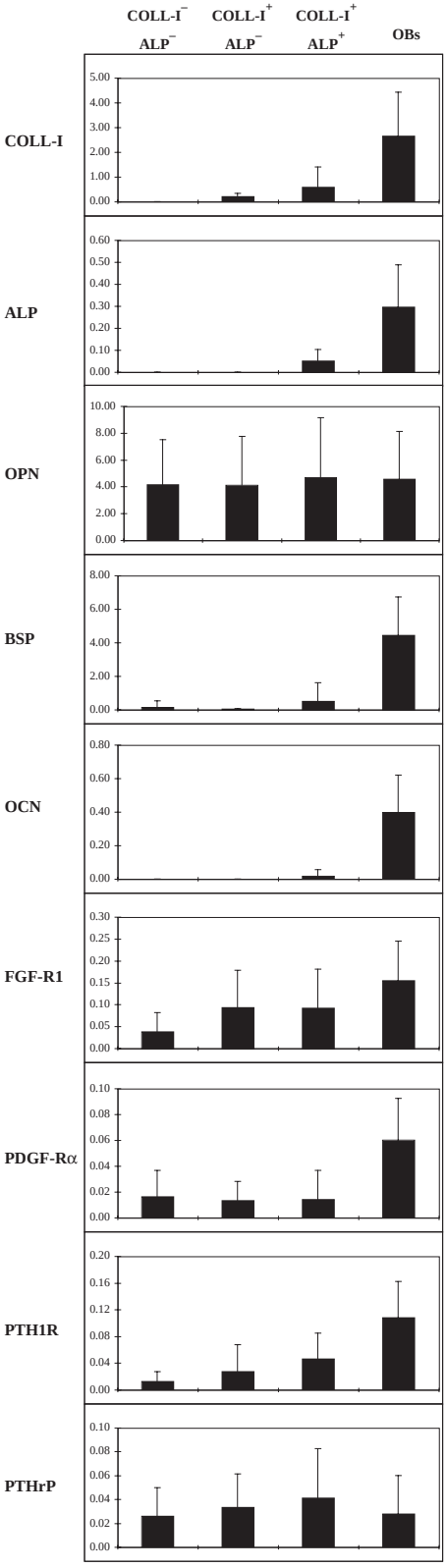



**Fig. 3.** Rank order profiles of normalized cDNA expression by the colonies characterized in Fig. 2. Phosphorimage signal intensities for each probe were quantified and standardized against total cDNA. Immature osteoprogenitor colonies identified by replica plating that did not express detectable levels of ALP were classed as immature or primitive osteoprogenitors (OP) compared to more mature osteoprogenitors/preosteoblasts (Pre-OB) that did express low levels of ALP message; OBs and Mature OBs are defined as in Fig. 2. All colonies were rank-ordered according to the paradigm outlined in the Results.

expressed); these are not quiescent since they express low-intermediate levels of mRNAs for OPN, PTHrP, PTH1R, FGF-R1 and PDGF-Rα. Second, a group of relatively primitive (expressing no other osteoblast-associated mRNAs) progenitors transiently expressing BSP emerges as a distinct developmental stage. Third, during osteoprogenitor differentiation, BSP mRNA expression undergoes a second significant upregulation prior to that of OCN, and relatively early after upregulation of ALP (Fig. 3). Fourth, FGF-R1 and PTH1R mRNA is significantly upregulated prior to that of PDGF-Rα and earlier than upregulation of ALP; a second significant upregulation occurs late in the differentiation sequence as cells acquire other features of differentiated osteoblasts including OCN. Fifth, PDGF-Rα mRNA is maintained at a relatively constant level until it is upregulated similarly to FGF-R1 and osteoblast-associated markers late in the differentiation sequence. Sixth, amongst the more mature cells in the lineage (expressing all osteoblast-associated




| | COLL-I⁺/ALP⁻ | COLL-I⁺/ALP⁺ | OBs |
|---|---|---|---|
| **COLL-I⁻/ALP⁻** | nt | nt | nt |
| **COLL-I⁺/ALP⁻** | | **p<0.05** | **p<0.01** |
| **COLL-I⁺/ALP⁺** | | | **p<0.05** |

| | COLL-I⁺/ALP⁻ | COLL-I⁺/ALP⁺ | OBs |
|---|---|---|---|
| **COLL-I⁻/ALP⁻** | nt | nt | nt |
| **COLL-I⁺/ALP⁻** | | nt | nt |
| **COLL-I⁺/ALP⁺** | | | **p=0.01** |

| | COLL-I⁺/ALP⁻ | COLL-I⁺/ALP⁺ | OBs |
|---|---|---|---|
| **COLL-I⁻/ALP⁻** | p>0.05 | p>0.05 | p>0.05 |
| **COLL-I⁺/ALP⁻** | | p>0.05 | p>0.05 |
| **COLL-I⁺/ALP⁺** | | | p>0.05 |

| | COLL-I⁺/ALP⁻ | COLL-I⁺/ALP⁺ | OBs |
|---|---|---|---|
| **COLL-I⁻/ALP⁻** | p>0.05 | p>0.05 | **p<0.0001** |
| **COLL-I⁺/ALP⁻** | | **p<0.05** | **p<0.0001** |
| **COLL-I⁺/ALP⁺** | | | **p<0.001** |

| | COLL-I⁺/ALP⁻ | COLL-I⁺/ALP⁺ | OBs |
|---|---|---|---|
| **COLL-I⁻/ALP⁻** | nt | nt | nt |
| **COLL-I⁺/ALP⁻** | | nt | nt |
| **COLL-I⁺/ALP⁺** | | | **p<0.0001** |

| | COLL-I⁺/ALP⁻ | COLL-I⁺/ALP⁺ | OBs |
|---|---|---|---|
| **COLL-I⁻/ALP⁻** | **p=0.01** | **p<0.05** | **p<0.01** |
| **COLL-I⁺/ALP⁻** | | p>0.05 | p>0.05 |
| **COLL-I⁺/ALP⁺** | | | p>0.05 |

| | COLL-I⁺/ALP⁻ | COLL-I⁺/ALP⁺ | OBs |
|---|---|---|---|
| **COLL-I⁻/ALP⁻** | nt | nt | nt |
| **COLL-I⁺/ALP⁻** | | **p<0.05** | **p<0.09** |
| **COLL-I⁺/ALP⁺** | | | **p<0.13** |

| | COLL-I⁺/ALP⁻ | COLL-I⁺/ALP⁺ | OBs |
|---|---|---|---|
| **COLL-I⁻/ALP⁻** | nt | nt | nt |
| **COLL-I⁺/ALP⁻** | | nt | nt |
| **COLL-I⁺/ALP⁺** | | | **p=0.02** |

| | COLL-I⁺/ALP⁻ | COLL-I⁺/ALP⁺ | OBs |
|---|---|---|---|
| **COLL-I⁻/ALP⁻** | p>0.05 | p>0.05 | p>0.05 |
| **COLL-I⁺/ALP⁻** | | p>0.05 | p>0.05 |
| **COLL-I⁺/ALP⁺** | | | p>0.05 |

**Fig. 4.** Expression levels for each probe within categories of population. The means and standard deviations (bars on the left) of corrected (standardized against total cDNA) expression levels were calculated and subjected to the Welch *t*-test for statistically significant changes as cells differentiate (table on the right). Categories were defined as COLL-I$^{-}$/ALP$^{-}$ colonies, COLL-I$^{+}$/ALP$^{-}$ colonies, COLL-I$^{+}$/ALP$^{+}$ colonies, and OB colonies (COLL-I$^{+}$/ALP$^{+}$/BSP$^{+}$/OCN$^{+}$) (nt, not testable).

markers) PTHrP, PTH1R, FGF-R1 and PDGF-Rα mRNAs tended also to decrease in relative concert with the osteoblast-specific markers (Fig. 3). Seventh, on average, BSP expression remained higher than did other osteoblast markers in the most mature colonies in the analysis.

## Discussion

Our data provide the first molecular characterization of definitive normal osteoprogenitors committed to terminal differentiation and bone formation in vitro. The profiles established to date are only a first step towards understanding

primitive cells in this lineage, based as they are on analyzing the simultaneous expression profiles of nine mRNAs – six known osteoblast-associated markers (PTH1R, COLL-I, ALP, OPN, BSP and OCN), two receptors for potential regulatory factors (FGF-R1 and PDGF-Rα), and a regulator of skeletal cells (PTHrP) known to be made by osteoblasts and other cell types. Our data indicate that the approach of replica plating combined with poly(A)-PCR provides new insights into the osteoblast developmental sequence, establishing landmarks for new early developmental stages, and clarifying other issues for which discrepant data exist. We have extended characterization of osteoprogenitor cells to cells more primitive than those yet expressing the earliest established markers (i.e. COLL-I and ALP) of committed osteoprogenitors, have identified a cohort of early progenitors transiently expressing BSP, and have characterized distinct stages of upregulation of two different growth factor receptors and a developmentally critical hormone receptor (PTH1R), all of which contribute new understanding of early developmental stages in this skeletal lineage.

The replica technique we applied to the RC population worked with the fidelity (~95%) required for the studies undertaken (i.e. to identify retrospectively osteoprogenitors early in their developmental lifetime). The replicas were made in low density cultures from discrete colonies well separated from other contaminating cell and colony types and early in their developmental sequence while progenitor cells were still proliferative. The progenitor cells stalled at lower than physiological temperature on the master dishes were essentially quiescent or very slowly proliferating, but were able to resume their proliferation and differentiation capabilities ultimately to form mineralized bone nodules when transferred back to 37°C. To date, we have used this replica approach to identify and isolate over 80 osteoprogenitor and preosteoblast colonies, all of which comprised cells with pleiomorphic morphology and none of which displayed the cuboidal morphology of differentiated osteoblasts depositing and mineralizing osteoid. Our reason for selection of large numbers of colonies was to confirm the validity of the approach, that is, each phenotype was expected to be sampled more than once, and to acquire information on the differentiation process itself (i.e. whether cells traversed a continuum of changing expression profiles or traversed the differentiation sequence in quantal leaps). Our data suggest that both patterns are characteristic of and define the osteoblast developmental process (see below).

Previously, we showed that the technique of poly(A)-PCR was a powerful and discriminating tool to establish molecular profiles for osteoblastic colonies and single cells that had differentiated to different extents as recognized morphologically (i.e. early, intermediate and mature osteoblast) (Liu et al., 1994). When mRNA expression in the replica colonies analyzed was quantified by this technique and co-expression profiles of a variety of messages for bone-related macromolecules and growth factor receptors determined, we found marked differences amongst colonies. Elsewhere we have discussed heterogeneity of marker expression amongst single cells at the same developmental stages in terms of a stochastic process contributing to osteoblast plasticity or heterogeneity (Liu et al., 1997; Liu et al., 1994). In the present analysis to seek transition points and landmarks representative of cells as much more or less primitive, we have analyzed expression across larger developmental boundaries and averaged amongst small cohorts of sibling cells (colonies) in which single cell stochastic variations would be averaged and thus minimized as a contribution to the profiles achieved. A few colonies displayed features consistent with their having already reached a preosteoblast or early osteoblast phenotype, i.e., simultaneously expressing all osteoblast-associated markers analyzed (PTH1R, COLL-I, ALP, OPN, BSP, and OCN), as did the few morphologically defined later stage osteoblast colonies used here for comparison and validation. In marked contrast, other replica colonies expressed none of these mRNAs to a detectable levels, while others expressed different combinations of these messages. Expression levels for all markers analyzed with the exception of OPN, which was relatively uniformly and highly expressed in virtually all colonies (see below), covered a range of expression levels from undetectable (off) to detectable (on) but very low to intermediate to very high. This suggests that to some extent, and at least as represented by those osteoblast markers analyzed, there is a quantal change (off-on), but that once a marker is on/acquired its expression as populations of osteoprogenitor cells differentiate represents a continuum from detectable expression through gradually higher expression until mature osteoblasts express most markers analyzed at very high levels.

The rank order profile (Fig. 3) was generated and based on temporal expression of ALP (expression increases with osteoblast maturation and then decreases with osteoid mineralization) and OCN (expressed by post-proliferative osteoblasts) as observed in several previous studies of the bone nodule model (for reviews, see Rodan and Noda, 1991; Aubin and Liu, 1996; Stein et al., 1996) and detailed by Malaval et al. (Malaval et al., 1999), where the kinetics of osteoprogenitor and osteoblast differentiation in vitro was correlated with the expression of several bone matrix proteins. Based on ALP, OCN and other markers, previously the proliferation-differentiation sequence for osteoprogenitors has been categorized mainly into three stages comprising proliferation, matrix production and matrix maturation and mineralization (Stein et al., 1996). In keeping with these previous analyses, we found a sequential upregulation of expression of bone-related macromolecules beginning with COLL-I, followed by ALP and then BSP, and finally by OCN (for reviews, see Aubin and Liu, 1996; Rodan and Noda, 1991; Stein et al., 1996). That virtually all early osteoprogenitor and preosteoblast colonies expressed moderate to high levels of OPN is not surprising since, as outlined above, these colonies had high numbers of cycling cells; OPN is known to be high in proliferating cells, and it was originally cloned as a cell-cycle-related molecule induced in cells in vitro by tumor promoters and growth factors (Nomura et al., 1988; Smith and Denhardt, 1987). Since we performed the low density and replica plating technique in the presence of dexamethasone, we expected and did capture both the more primitive and more mature osteoprogenitors defined earlier by flow cytometry on the basis of absence (primitive, dex-requiring) or presence (more mature, dex-independent) of ALP expression (Turksen and Aubin, 1991). However, novel information was also obtained. The results support our observations made earlier at the single cell level in which we detected the presence of mRNAs for ALP, COLL-I, OPN, BSP, and/or OCN in a few cells in fibroblastic colonies and confirm

the hypothesis for which we had no definitive proof then that these were probably representative of committed osteoblast lineage cells that had not yet acquired the morphological characteristics of the lineage, rather than cells with leaky or promiscuous expression of mRNAs (Liu et al., 1994). It is also notable that all these markers can be and are expressed in cells that have not yet assumed the cuboidal shape of preosteoblasts and osteoblasts; while these studies have not addressed the presence of deposited protein, they do suggest the possibility that none of these, including the RGD-containing BSP and OPN, might be directly responsible for the cuboidal shape determination characteristic of mature osteoblastic cells, and vice versa that overt cuboidal shape is not itself required for detectable transcription levels of these particular genes.

Strikingly, our data also point towards a much more complex series of transitions during this developmental sequence than the three stages commonly described and suggest a minimum of seven transitions based on the markers assessed here. For example, this analysis of primitive progenitor cells revealed a small group of colonies expressing low but detectable levels of BSP; a few of these colonies also expressed COLL-I, but no other osteoblast-associated molecules. We (Liu et al., 1994) and others (Bianco et al., 1991; Bianco et al., 1993; Chen et al., 1991) have shown that BSP is upregulated as osteoblasts mature in vitro and in vivo at sites of de novo bone formation. While it is generally considered a relatively late stage marker, expressed concomitantly with OCN and just prior to mineralization, where it has been proposed to seed hydroxyapatite crystal formation (Hunter and Goldberg, 1993), we have established that it is upregulated prior to OCN and well before detectable mineralization can be observed (Liu et al., 1994). We have now detected transient BSP expression even earlier, prior to the onset of COLL-I and ALP expression and osteogenesis. We have also previously shown the presence of primitive $BrdU^+$(cycling)/$ALP^-$/$BSP^+$ cells by double label immunocytochemistry in clonogenic bone nodules in vitro (Malaval et al., 1999) indicating that the mRNA results reported here are meaningful also at protein translational level. We have further found evidence for a similar primitive transiently BSP-expressing osteoprogenitor in the sutures of developing rat calvaria (Candeliere et al., 2001). It is interesting to speculate that this early and apparently transient expression of BSP may reflect its role as a cell adhesion molecule through its RGD recognition site for integrins, notably the $\alpha_v\beta_3$ vitronectin receptor (Oldberg et al., 1988); BSP has also been reported to mediate osteoblast cell attachment in vitro (Mintz et al., 1993). This role for BSP may be functionally separate from its role during its second round of upregulation during the later stages of the differentiation sequence when osteoblasts are actively synthesizing other matrix molecules and depositing osteoid. Late during this second developmental window, i.e., when matrix is mineralizing and cells achieve osteocyte and/or lining cell status, it is also interesting that BSP expression remains on average higher than that of other bone matrix molecules and ALP.

While levels of PTHrP remained relatively constant from very primitive to more mature stages, PTH1R, FGF-R1 and PDGF-Rα mRNAs followed the same trends as the bone matrix molecule messages, i.e., underwent a significant increase as osteoblasts matured. However, it is also notable that the most primitive colonies expressed all the receptors analysed at easily detectable levels and prior to upregulation of any of the known osteoblast-associated molecules. PTH1R, FGF-R1 and PDGF-Rα play roles in normal skeletal development. Analysis of knockout mice has shown that FGF-R1 is essential for cell proliferation and axial patterning in mouse development (Deng et al., 1994) and mutations in FGF-R1 are associated with skeletal abnormalities in humans (Muenke et al., 1994). In homozygous PDGF-Rα null mouse mutants, mesenchymal cell proliferation is affected (Bowen-Pope et al., 1991), resulting in growth retardation and deficiencies in mesodermal structures (Schatteman et al., 1992). While early mesodermal cells are affected, cells traversing the osteoblast lineage specifically also respond to these growth factors. Growing evidence supports the hypothesis that FGF has considerable anabolic effects on bone (Dunstan et al., 1999; Kawaguchi et al., 2001; Liang et al., 1999; Zhang et al., 2002). PDGF has also been shown to enhance proliferation of osteoblastic cells, but its effects on differentiation have been inconsistent in different models in vitro, although in general inhibition is observed (reviewed by Canalis et al., 1992). While these studies have shown that osteoblastic populations respond to FGF and PDGF, there is little information on which subpopulations of osteoblastic cells express the receptors and whether receptor numbers change as a function of differentiation stage. Our results show that mRNAs for both growth factor receptors are expressed continuously through the lineage from very early progenitor to mature osteoblast and that both tend to peak as osteoblasts reach maturity and then decline concomitant with downregulation of the osteoblast-specific messages. However, they are differentially regulated in the earlier progenitors in the lineage, which may allow for important regulatory differences early in development. Our finding that FGF-R1 goes through the first of its two stages of upregulation relatively early in the differentiation sequence and prior to upregulation of ALP suggests that amongst target populations for FGF spanning multiple developmental stages, FGF may have a special role in very early committed and proliferatively active osteoprogenitors. The second special target stage for both receptors that peak at late developmental stages concomitant with other markers such as BSP and OCN appears to be the matrix synthesizing mature osteoblast cell. There is also a trend towards more rapid downregulation of FGF-R1 than PDGF-Rα in mineralization phase (Fig. 3), suggesting that the latter may play a unique role at this terminal differentiation stage. These data provide evidence that the growth factor receptors can be used as additional markers for osteoprogenitors at different developmental stages and provide some clues as to target genes for their activities.

Many discrepancies exist in earlier determinations of when during osteogenic developmental PTHrP and PTH1R are expressed (reviewed by Aubin and Liu, 1996). In this study, we found that PTHrP is continuously expressed at a relatively constant level throughout the lineage in keeping with most observations (Kartsogiannis et al., 1997; Suda et al., 1996) and is not specifically localized to immature osteoblasts as indicated by others (Karmali et al., 1992). Our results also show that mRNA for PTH1R is expressed continuously through the lineage from early progenitor to mature osteoblast and that it tends to peak as osteoblasts reach maturity and then declines concomitantly with downregulation of osteoblast-

specific messages. These observations are different from studies reporting that the highest number of receptors is on relatively undifferentiated osteoblasts, with relatively few on the terminally differentiated mature osteoblast (Rouleau et al., 1988), but are in agreement with other studies in which PTH1R has been demonstrated to be high in the mature osteoblast population (Bos et al., 1996; Suda et al., 1996), and with earlier in situ studies showing the receptor to be present on osteoblasts and its immediate precursors (Lee et al., 1994; Silve et al., 1982). The fact that we find both molecules expressed already in immature progenitors through to more mature osteoblasts support the idea of a widespread autocrine/paracrine function for these molecules during osteoblast development (Lanske and Kronenberg, 1998; Suda et al., 1996).

Clearly, the combined approach of single colony isolation and poly(A)-PCR offers a means by which to determine a molecular fingerprint of normal (i.e. non-established, non-transformed) primitive osteoprogenitors through to differentiating osteoblasts and mature osteoblasts. The observations suggest that within the osteoblast differentiation sequence both discrete stages and a continuum of changing marker expression levels occur with much variation in expression for any given marker. We have identified novel developmental stages not previously known and characterized by expression of known osteoblast-associated markers such as BSP; we also predict that more stages may be uncovered as more genes are added to this analysis either through analysis as done here or through use of these amplified pools on DNA microarrays. We have also demonstrated that the mRNA expression for certain hormone and growth factor receptors is modulated during osteoblast differentiation and the sequential expression of different receptors appears to provide markers for cells earlier in the lineage than those already expressing ALP and helps to shed light on the cellular targets mediating the diverse effects of overexpression or under-expression of these families of molecules. Moreover, these gene expression relationships have allowed us to statistically examine the developmental flexibility of clonal stem or progenitor cell differentiation (Madras et al., 2002). Finally, the poly(A)-PCR approach used has allowed us to generate cDNA libraries of multiple osteogenic stages, spanning primitive osteoprogenitor to mature osteoblast, from which we are now isolating novel differentiation stage-specific osteoblast lineage genes (Candeliere et al., 1999).

We thank A. K. Gupta, G. A. Candeliere and K. Turksen for helpful discussions, and S.-W. Fung for technical assistance in preparing cells used in these studies. This work was supported by a CIHR grant (MT-12390) to J.E.A. F.L. was supported by a University of Toronto Open Fellowship, a Harron Trust Graduate Student Stipend and a University of Toronto Life Sciences Degree Completion Award.

## References

**Abou-Samra, A. B., Juppner, H., Force, T., Freeman, M. W., Kong, X. F., Schipani, E., Urena, P., Richards, J., Bonventre, J. V., Potts, J. T., Jr et al.** (1992). Expression cloning of a common receptor for parathyroid hormone and parathyroid hormone-related peptide from rat osteoblast-like cells: a single receptor stimulates intracellular accumulation of both cAMP and inositol trisphosphates and increases intracellular free calcium. *Proc. Natl. Acad. Sci. USA* **89**, 2732-2736.

**Aubin, J. E. and Liu, F.** (1996). The osteoblast lineage. In *Principles of Bone Biology* (ed. J. P. Bilezikian L. G. Raisz and G. A. Rodan), pp. 51-67. San Diego: Academic Press.

**Aubin, J. E. and Triffitt, J.** (2002). Mesenchymal stem cells and the osteoblast lineage. In *Principles of Bone Biology, 2nd edn* (ed. J. P. Bilezikian, L. G. Raisz and G. A. Rodan), pp. 59-81. New York, NY: Academic Press.

**Aubin, J. E., Turksen, K. and Heersche, J. N. M.** (1993). Osteoblastic cell lineage. In *Cellular and Molecular Biology of Bone* (ed. M. Noda), pp. 1-45. New York: Academic Press.

**Aubin, J. E., Liu, F. and Candeliere, G. A.** (2002). Single cell PCR methods for studying stem cells and progenitors. *Methods Mol. Biol.* **185**, 403-415.

**Auffray, C. and Rougeon, F.** (1980). Purification of mouse immunoglobulin heavy-chain messenger RNAs from total myeloma tumor RNA. *Eur. J. Biochem.* **107**, 303-314.

**Bellows, C. G. and Aubin, J. E.** (1989). Determination of numbers of osteoprogenitors present in isolated fetal rat calvaria cells in vitro. *Dev. Biol.* **133**, 8-13.

**Bellows, C. G., Aubin, J. E., Heersche, J. N. M. and Antosz, M. E.** (1986). Mineralized bone nodules formed in vitro from enzymatically released rat calvaria cell populations. *Calcif. Tissue Int.* **38**, 143-154.

**Bianco, P., Fisher, L. W., Young, M. F., Termine, J. D. and Robey, P. G.** (1991). Expression of bone sialoprotein (BSP) in developing human tissues. *Calcif. Tissue Int.* **49**, 421-426.

**Bianco, P., Riminucci, M., Bonucci, E., Termine, J. D. and Robey, P. G.** (1993). Bone sialoprotein (BSP) secretion and osteoblast differentiation: Relationship to bromodeoxyuridine incorporation, alkaline phosphatase, and matrix deposition. *J. Histochem. Cytochem.* **41**, 183-191.

**Bos, M. P., van der Meer, J. M., Feyen, J. H. and Herrmann-Erlee, M. P.** (1996). Expression of the parathyroid hormone receptor and correlation with other osteoblastic parameters in fetal rat osteoblasts. *Calcif. Tissue Int.* **58**, 95-100.

**Bowen-Pope, D. F., van Koppen, A. and Schatteman, G.** (1991). Is PDGF really important? Testing the hypothesis. *Trends Genet.* **7**, 413-418.

**Brady, G. and Iscove, N. N.** (1993). Construction of cDNA libraries from single cells. *Methods Enzymol.* **225**, 611-623.

**Brady, G., Billia, F., Knox, J., Hoang, T., Kirsch, I. R., Voura, E., Hawley, R. G., Cumming, R., Buchwald, M., Siminovitch, K. et al. (**1995). Analysis of gene expression in a complex differentiation hierarchy by global amplification of cDNA from single cells. *Curr. Biol.* **5**, 909-922.

**Canalis, E., Varghese, S., McCarthy, T. L. and Centrella, M.** (1992). Role of platelet derived growth factor in bone cell function. *Growth Regul.* **2**, 151-155.

**Candeliere, G. A., Rao, Y., Floh, A., Sandler, S. D. and Aubin, J. E.** (1999). cDNA fingerprinting of osteoprogenitor cells to isolate differentiation stage-specific genes. *Nucleic Acids Res.* **27**, 1079-1083.

**Candeliere, G. A., Liu, F. and Aubin, J. E.** (2001). Individual osteoblasts in the developing calvaria express different gene repertoires. *Bone* **28**, 351-361.

**Chen, J., Zhang, Q., McCulloch, C. A. G. and Sodek, J.** (1991). Immunohistochemical localization of bone sialoprotein in fetal porcine bone tissues: Comparisons with secreted phosphoprotein I (SPP-1, osteopontin) and SPARC (osteonectin). *Histochem. J.* **23**, 281-289.

**Deng, C.-X., Wynshaw-Boris, A., Shen, M. M., Daugherty, C., Ornitz, D. M. and Leder, P.** (1994). Murine FGFR-1 is required for early postimplantation growth and axial organization. *Genes Dev.* **8**, 3045-3057.

**Dunstan, C. R., Boyce, R., Boyce, B. F., Garrett, I. R., Izbicka, E., Burgess, W. H. and Mundy, G. R.** (1999). Systemic administration of acidic fibroblast growth factor (FGF-1) prevents bone loss and increases new bone formation in ovariectomized rats. *J. Bone Miner. Res.* **14**, 953-959.

**Esko, J. D.** (1989). Replica plating of animal cells. *Methods Cell Biol.* **32**, 387-422.

**Genovese, C., Rowe, D. and Kream, B.** (1984). Construction of DNA sequences complementary to rat alpha 1 and alpha 2 collagen mRNA and their use in studying the regulation of type I collagen synthesis by 1,25-dihydroxyvitamin D. *Biochemistry* **23**, 6210-6216.

**Hunter, G. K. and Goldberg, H. A.** (1993). Nucleation of hydroxyapatite by bone sialoprotein. *Proc. Natl. Acad. Sci. USA* **90**, 8562-8565.

**Karmali, R., Schiffmann, S. N., Vanderwinden, J. M., Hendy, G. N., Nys-DeWolf, N., Corvilain, J., Bergmann, P. and Vanderhaeghen, J. J.** (1992). Expression of mRNA of parathyroid hormone-related peptide in fetal bones of the rat. *Cell Tissue Res.* **270**, 597-600.

**Kartsogiannis, V., Moseley, J., McKelvie, B., Chou, S. T., Hards, D. K., Ng, K. W., Martin, T. J. and Zhou, H.** (1997). Temporal expression of PTHrP during endochondral bone formation in mouse and intramembranous bone formation in an in vivo rabbit model. *Bone* **21**, 385-392.

**Kawaguchi, H., Nakamura, K., Tabata, Y., Ikada, Y., Aoyama, I., Anzai, J., Nakamura, T., Hiyama, Y. and Tamura, M.** (2001). Acceleration of

fracture healing in nonhuman primates by fibroblast growth factor-2. *J. Clin. Endocrinol. Metab.* **86**, 875-880.

**Lanske, B. and Kronenberg, H. M.** (1998). Parathyroid hormone-related peptide (PTHrP) and parathyroid hormone (PTH)/PTHrP receptor. *Crit. Rev. Eukaryot. Gene Exp.* **8**, 297-320.

**Lee, C., Gardella, T. J., Abou-Samra, A. B., Nussbaum, S. R., Segre, G. V., Potts, J. T., Jr, Kronenberg, H. M. and Juppner, H.** (1994). Role of the extracellular regions of the parathyroid hormone (PTH)/PTH-related peptide receptor in hormone binding. *Endocrinology* **135**, 1488-1495.

**Lee, K.-H., Bowen-Hope, D. F. and Reed, R. R.** (1990). Isolation and characterization of the a platelet-derived growth factor receptor from rat olfactory epithelium. *Mol. Cell. Biol.* **10**, 2237-2246.

**Liang, H., Pun, S. and Wronski, T. J.** (1999). Bone anabolic effects of basic fibroblast growth factor in ovariectomized rats. *Endocrinology* **140**, 5780-5788.

**Liu, F., Malaval, L., Gupta, A. and Aubin, J. E.** (1994). Simultaneous detection of multiple bone-related mRNAs and protein expression during osteoblast differentiation: Polymerase chain reaction and immunocytochemical studies at the single cell level. *Dev. Biol.* **166**, 220-234.

**Liu, F., Malaval, L. and Aubin, J. E.** (1997). The mature osteoblast phenotype is characterized by extensive palsticity. *Exp. Cell. Res.* **232**, 97-105.

**Madras, N., Gibbs, A. L., Zhou, Y., Zandstra, P. W. and Aubin, J. E.** (2002). Modeling stem cell development by retrospective analysis of gene expression profiles in single progenitor-derived colonies. *Stem Cells* **20**, 230-240.

**Malaval, L., Liu, F., Roche, P. and Aubin, J. E.** (1999). Kinetics of osteoprogenitor proliferation and osteoblast differentiation in vitro. *J. Cell. Biochem.* **74**, 616-627.

**Mansukhani, A., Moscatelli, D., Talarico, D., Levytska, V. and Basilico, C.** (1990). A murine fibroblast growth factor (FGF) receptor expressed in CHO cells is activated by basic FGF and Kaposi FGF. *Proc. Natl. Acad. Sci. USA* **87**, 4378-4382.

**Mintz, K. P., Grzesik, W. J., Midura, R. J., Robey, P. G., Termine, J. D. and Fisher, L. W.** (1993). Purification and fragmentation of nondenatured bone sialoprotein: Evidence for a cryptic, RGD-resistant cell attachment domain. *J. Bone Miner. Res.* **8**, 985-995.

**Muenke, M., Schell, U., Hehr, A., Robin, N. H., Losken, H. W., Schinzel, A., Pulleyn, L. J., Rutland, P., Reardon, W., Malcolm, S. et al.** (1994). A common mutation in the fibroblast growth factor receptor 1 gene in Pfeiffer syndrome. *Nat. Genet.* **8**, 269-274.

**Noda, M. and Rodan, G. A.** (1987). Type β transforming growth factor (TGFβ) regulation of alkaline phosphatase expression and other phenotype-related mRNAs in osteoblastic rat sarcoma cells. *J. Cell. Physiol.* **133**, 426-437.

**Nomura, S., Wills, A. J., Edwards, D. R., Heath, J. K. and Hogan, B. L. M.** (1988). Developmental expression of 2ar (osteopontin) and SPARC (osteonectin) RNA as revealed by in situ hybridization. *J. Cell Biol.* **106**, 441-450.

**Oldberg, A., Franzen, A. and Heinegard, D.** (1988). The primary structure of a cell-binding bone sialoprotein. *J. Biol. Chem.* **263**, 19430-19432.

**Raetz, C. R., Wermuth, M. M., McIntyre, T. M., Esko, J. D. and Wing, D. C.** (1982). Somatic cell cloning in polyester stacks. *Proc. Natl. Acad. Sci. USA* **79**, 3223-3227.

**Rodan, G. A. and Noda, M.** (1991). Gene expression in osteoblastic cells. *Crit. Rev. Eukaryot. Gene Exp.* **1**, 85-98.

**Rouleau, M. F., Mitchell, J. and Goltzman, 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.

**Sambrook, J., Frisch, E. F. and Maniatis, T.** (1989). *Molecular Cloning, A Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

**Schatteman, G. C., Morrison-Graham, K., van Koppen, A., Weston, J. A. and Bowen-Pope, D. F.** (1992). Regulation and role of PDGF receptor a-subunit expression during embryogenesis. *Development* **115**, 123-131.

**Silve, C. M., Hradek, G. T., Jones, A. L. and Arnaud, C. D.** (1982). Parathyroid hormone receptor in intact embryonic chicken bone: characterization and cellular localization. *J. Cell Biol.* **94**, 379-386.

**Smith, J. H. and Denhardt, D. T.** (1987). Molecular cloning of a tumor promoter-inducible mRNA found in JB6 mouse epidermal cells: Induction is stable at high, but not at low, cell densities. *J. Cell. Biochem.* **34**, 13-22.

**Stein, G. S., Lian, J. B., Stein, J. L., van Wijnen, A. J., Frenkel, B. and Montecino, M.** (1996). Mechanisms regulating osteoblast proliferation and differentiation. In *Principles of Bone Biology* (ed. J. P. Bilezikian L. G. Raisz and G. A. Rodan), pp. 69-86. San Diego: Academic Press.

**Suda, N., Gillespie, M. T., Traianedes, K., Zhou, H., Ho, P. W., Hards, D. K., Allan, E. H., Martin, T. J. and Moseley, J. M.** (1996). Expression of parathyroid hormone-related protein in cells of osteoblast lineage. *J. Cell. Physiol.* **166**, 94-104.

**Turksen, K. and Aubin, J. E.** (1991). Positive and negative immunoselection for enrichment of two classes of osteoprogenitor cells. *J. Cell Biol.* **114**, 373-384.

**Yasuda, T., Banville, D., Rabbani, S. A., Hendy, G. N. and Goltzman, D.** (1989). Rat parathyroid hormone-like peptide: comparison with the human homologue and expression in malignant and normal tissue. *Mol. Endocrinol.* **3**, 518-525.

**Zhang, X., Sobue, T. and Hurley, M. M.** (2002). FGF-2 increases colony formation, PTH receptor, and IGF-1 mRNA in mouse marrow stromal cells. *Biochem. Biophys. Res. Commun.* **290**, 526-531.