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# Donor Variation in the Growth Properties and Osteogenic Potential of Human Marrow Stromal Cells

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**Abstract** Human marrow stromal cells (MSCs) were isolated from posterior illiac crest marrow aspirates obtained from 17 healthy donors, ages 19–45 years, with no apparent physical disability. First passage hMSCs exhibited growth rates in vitro that varied up to 12-fold between donors. No correlation between growth rate and the age or gender of the donor was evident ($P \leq 0.05$). When hMSCs were cultured without passage for eight days (subconfluent cultures) or 22 days (confluent cultures) in the absence of any osteogenic agonists, levels of alkaline phosphatase enzyme activity varied 40-fold and 10-fold, respectively, between donors. When exposed to osteo-inductive media, donor populations also showed dramatic differences in levels of bone-specific gene induction. Collectively, these data demonstrate that hMSC cultures are composed of a heterogeneous mixture of cells at various stages of differentiation and with distinct osteogenic potentials. Differences in both growth rate and ALP activity were evident in hMSC cultures established from multiple aspirates obtained over a six month period from the same donors. Therefore, it appears that cellular heterogeneity produced by the method of harvest is propagated within and among different donor populations during culture expansion in vitro. J. Cell. Biochem. 75:424–436, 1999. 

**Key words:** marrow stromal cells; osteogenesis

Bone marrow contains a unique cell population, referred to as colony forming-unit fibroblasts (CFU-Fs), mesenchymal stem cells, or marrow stromal cells (MSCs), that represent multi-potential progenitors of mesenchymal cell lineages. MSCs isolated from a variety of species can be induced to differentiate into osteoblasts [Leboy et al., 1991; Rickard et al., 1996], chondrocytes [Ashton et al., 1980; Johnstone et al., 1998], adipocytes [Lanotte et al., 1982; Bennet et al., 1991], myoblasts [Wakitani et al., 1995; Phinney et al., 1999], and hematopoiesis-supporting stroma [Majumdar et al., 1995] in vitro. They also form bone and cartilage tissue when impregnated into ceramic carriers and implanted in vivo [Goshima et al., 1991; Freidenstein et al., 1987]. In addition, human MSCs (hMSCs), which are readily isolated from posterior illiac crest marrow aspirates, can be culture-expanded in vitro without losing their osteogenic potential [Bruder et al., 1997]. These attributes make MSCs attractive candidates for use in cell therapy, or as vectors for ex vivo gene therapy.

Accordingly, several animal model systems have been employed to ascertain whether MSCs may be used clinically to effect repair of damaged bone and cartilage tissue. Wakitani et al. [1994] showed that implantation of culture-expanded MSCs produce tissue regeneration in full-thickness articular defects in rabbits. MSCs also facilitate bone regeneration when grafted onto surgically induced segmental defects in the radius of rabbits [Niedzwiedzki et al., 1993] and the femurs of rats and dogs [Bruder et al., 1998]. These results indicate that MSCs may be useful cell therapy vectors to treat skeletal injuries as well as a variety of diseases wherein normal bone turnover is impaired, such as osteoporosis, osteogenesis imperfecta, and even normal aging. Several phase I clinical trails have been undertaken to address issues of toxicity, levels of engraftment, and the life span of hMSCs following transplantation into humans as a first step toward bringing these cells to the clinic [Lazarus et al., 1995; Keating et al., 1998].

One important unanswered question regarding the use of hMSCs in clinical applications is whether cells isolated by standard methods rep-

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resent a homogenous population of multi-potential progenitors or a mixture of cells with varying potentials and at different stages of differentiation. Immunological characterization of human bone marrow cultures has shown that MSCs exhibit uniform immunoreactivity to various antibodies, including anti-CD10 (Keating et al., 1982), anti-smooth muscle actin [Charbord et al., 1990] and Stro1 [Simmons and Torok-Strob, 1991]. Moreover, Bruder et al. [1998a] recently showed that activated leukocyte cell adhesion molecule is expressed on the surface of all purified, culture-expanded hMSCs elaborated from the illiac crest. However, clonal variation in the ability of hMSC lines to sustain hematopoiesis in long-term bone marrow cultures [Collins et al., 1987; Deryigina et al., 1994] and in the osteogenic potential of primary hMSCs in vivo [Kuznetsov et al., 1997] indicate that a given cell population is functionally heterogeneous. Large variations between donors in both the yield of hMSCs [Castro-Malaspina et al., 1980] and their levels of alkaline phosphatase (ALP) enzyme activity [Majors et al., 1997; Jaiswal et al., 1997] suggest that such heterogeneity also exists between populations. The latter may have important consequences in the clinical outcome of cell therapy protocols that employ hMSCs.

Over the past two years our center has accumulated hMSC cultures from over 50 donors. In this report we have examined the growth properties and osteogenic potential of a large number of these donor samples. Our data reveals striking differences between hMSC populations with regard to growth rate, endogenous levels of ALP expression, and in the level of bone-specific gene expression following exposure to osteo-inductive (OI) media. Because these differences were evident even between samples obtained at different times from the same donor, we suspect that donor to donor variability is the result of a bias sampling method that introduces heterogeneity in the population at the time of harvest. As a result, careful analysis of hMSC donor populations is necessary before administering cells to patients in gene or cell therapy regimens.

## MATERIALS AND METHODS

### Cell Preparation and Culture

Bone marrow aspirates were obtained from the illiac crest under local anesthesia from healthy volunteers ranging in age from 19 to 45 years after informed consent. The marrow aspirates were diluted 1:1 in α-MEM supplemented with 10% fetal bovine sera (FBS; Atlanta Biologicals lot #6003E). A total of one-third volume of the marrow cells was layered onto two-third volumes of Ficoll-Plaque Plus (Pharmacia; density 1.077 g/ml) and centrifuged at 1,000*g* for 30 min at 4°C. The mono-nuclear cell fraction was collected and centrifuged at 2,000*g* for 30 min at 4°C. The cell pellet was resuspended in α-MEM containing 10% FBS at a concentration of $10 \times 10^6$ cells per milliliter. Cells were plated at a density of $30 \times 10^6$ cells / 9.5 $cm^2$ and cultured at 37°C in a humidified chamber with 5% $CO^2$. After three days the non-adherent cell fraction was removed and the adherent cell layer, which constitutes the MSCs, was cultured an additional 7–10 days with media changes every three days. When cultures reached approximately 90% confluence, cells were harvested by incubation with 0.5% trypsin and 1 mM EDTA for 5 min at room temperature, resuspended in cell freezing media (GIBCO Life Sciences), and stored under liquid nitrogen. In most cases, experiments were conducted using thawed aliquots of these first passage hMSCs. To induce an osteogenic phenotype, cells were continuously cultured in OI media, which was comprised of α-MEM supplemented with 10% FBS, 10 mM β -glycerolphosphate, 50 μg/ml ascorbic acid, and $10^{-8}$ M dexamethasone.

### Cell Proliferation Assays

Growth kinetics of hMSCs obtained from each donor were measured by plating first passage cells obtained from frozen stocks at a density of approximately 12,000 cells/9.5 $cm^2$ in replicate plates and counting the increase in cell number with time using a hemocytometer. All measurements were done in duplicate. In some instances the growth kinetics of serially passaged cultures were also measured. To do so, first passage cells were plated as described above, and the increase in cell number with time was monitored until the cells reached approximately 90% confluence. Then, cells were harvested and replated in replicate plates at a density of 12,000 cells/9.5 $cm^2$ and the growth kinetic measurement repeated. This process of serial subcultivation was repeated until the cells reached senescence. The number of population doublings was then tabulated from the number of cells at the start and finish of each cell passage.

### Alkaline Phosphatase (ALP) Assays

HMSCs were collected by centrifugation at 2,000*g* for 10 min at room temperature. The cell pellets were resuspended in 100–200 µl of lysis buffer [1.5 M Tris(hydroxymethyl)aminomethane, 1 mM $ZnCl_2$, 1 mM $MgCl_2$, 1% Triton X-100, pH 9.2] and incubated at 37°C for 30 min. The protein content of each lysate was measured using a commercial assay kit (Protein Assay; Bio-Rad). Aliquots of the cell extract (5–20 µl) were added to alkaline buffer (Sigma 221) containing 5 mM p-nitrophenyl phosphate (Sigma 104) and the rate of p-nitrophenol production was measured at 410 nm. The specific activity of the cell extract was determined by assuming that 1 O.D. unit at 410 nm is equivalent to 64 nmoles of p-nitrophenol.

### Cytochemistry

Cell monolayers were washed with PBS, stained for 5 min with a 2% (w/v) solution of Alizarin Red S adjusted to pH 4.1 with ammonium hydroxide, and then washed with distilled water. Alkaline Phosphatase expression was detected using a commercial staining kit (Sigma # 85L-2).

### RT-PCR Assays

Total RNA was isolated from hMSCs using a commercial RNA isolation kit (Fluka). Approximately 1–4 µg of total RNA was converted to single stranded cDNA using a commercial cDNA synthesis kit (GIBCO Life Sciences). Aliquots of the cDNA were amplified in 100 µl reactions that contained 25–50 pmoles of a forward and reverse primer, 1× PCR buffer (10× buffer is 100 mM Tris-HCl, 500 mM KCl, 15 mM $MgCl_2$, 0.01% gelatin, pH 8.3 [Sigma]), dCTP, dATP, dGTP, and dTTP each at 0.2 mM, and 0.5 U of *Taq* DNA Polymerase (Sigma). Amplifications for the following human cDNAs were performed using primers described by Rickard et al. [1996] with the exception of bone sialoprotein: β-actin (5′: AGCCATGTACGTTGCTA; 3′: GTCCGCCTAGAAGCA), bone/liver/kidney alkaline phosphatase (5′: ACGTGGCTAAGAATGTCATC; 3′: CTGGTAGGCGAT GTCCTTA), bone sialoprotein (5′: AACGAAGAAAGCGAAGCAGAAGTG; 3′: CTGACCATC ATAGCCATCGTAGCCT), and parathyroid hormone receptor (5′: AGGCCAGCCAGCATAATGGA A; 3′: CTCCCGTTCACGAGTCTCAT). PCR reactions were performed using an Amplitron II thermocycler (PGC) after an initial denaturation at 94°C for 2 min. The same reaction profile was used for each primer set: 94°C for 30 sec, 55°C for 2 min, and 72°C for 2 min with the exception of bone sialoprotein, wherein the reaction profile was: 94°C for 30 seconds, 59.4°C for 45 seconds, and 72°C for 90 seconds. All reactions were terminated by incubating at 72°C for 10 min. For each set of PCR primers, the amount of product accumulation in an amplification reaction was initially measured as a function of cycle number, and the range of cycles where product accumulated at an exponential rate was determined. All subsequent PCR reactions were performed for a specific number of cycles which fell within this exponential range: β-actin = 22, alkaline phosphatase = 36, bone sialoprotein = 34, and parathyroid hormone receptor = 40.

For each PCR amplification, an aliquot of each product was electrophoresed in a 1% agarose gel. The gel was then stained with SYBR Green 1 nucleic acid binding dye (FMC) and quantitated using a fluorescence imager (Storm 480, Molecular Dynamics). The amount of product accumulation for each gene was then normalized to that for β-actin.

## RESULTS

### Growth Kinetics and Osteogenic Potential of Human MSCs

Marrow aspirates (10–15 ml) obtained from the posterior illiac crest and plated at a density of $30 \times 10^6$ mononuclear cells per 9.5 $cm^2$ typically yielded confluent monolayers after 10–14 days in culture. At first passage the cell monolayers were comprised predominantly of fibroblastoid shaped MSCs that uniformly expressed collagen type I, collagen type IV, fibronectin, and vimentin. In contrast, expression of smooth muscle actin was variable, and was typically absent in approximately 20% of the cell population. A few cells in the culture also stained with periodic acid schiff, indicative of the presence of macrophages (data not shown). HMSCs from a representative donor (#6, see Table I) could be expanded to approximately 36 population doublings in vitro (Fig. 1a). Following exposure to OI media, first passage cultures exhibited a marked increase in ALP activity, an enzyme expressed during the early stages of osteoblast differentiation (Fig. 1b). Cytochemical staining of cells revealed that ALP induction was a population wide phenomenon (Fig. 1c), and that cells exposed to OI media also deposited an

**TABLE I. Effect of Donor Age and Sex on Growth Rate and ALP Activity of First Passage MSCs In Vitro**

| Donor # | Age | Sex | Cells/Day ×10,000 | 8 Day ALP | 21 Day ALP |
|---|---|---|---|---|---|
| 6 | 35 | M | 3.92 | 78.5 | 620.6 |
| 12 | 24 | M | 5.6 | 43.6 | 758.1 |
| 13 | 37 | F | 2.25 | 836.6 | 3804 |
| 14 | 22 | F | 4.87 | 152.2 | 965.4 |
| 15 | 40 | M | 6.38 | 221.6 | 400.6 |
| 18 | 19 | F | 8 | 280.6 | 384 |
| 20 | 20 | F | 5.95 | — | — |
| 21 | 31 | F | 2.47 | 279.1 | 394 |
| 25 | 31 | M | 4.13 | 201.6 | 544.4 |
| 26 | 19 | F | 3.56 | 486.4 | 1423.7 |
| 27 | 27 | F | 4.16 | 473.6 | 1042.7 |
| 28 | 31 | M | 3.69 | 780.1 | 1278 |
| 29 | 27 | F | 2.37 | 839.8 | 2601 |
| 30 | 31 | M | 7.16 | 288 | 1264 |
| 31 | 35 | F | 3.30 | 1563 | 3328 |
| 33 | 44 | M | 3.33 | 197.4 | 702.2 |
| 34 | 27 | F | 0.83 | 96.6 | 789.3 |
| 40 | 25 | F | 2.95 | 205.6 | 410.7 |
| 45 | 32 | M | 10.7 | 350.8 | 347.0 |
| 46 | 45 | F | 7.21 | 1696 | 1978 |
| 47 | 23 | F | 2.45 | 745.5 | 1523 |
| 48 | 27 | M | 2.59 | 152.7 | 1136 |

extracellular matrix that stained strongly with Alizarin Red S (Fig. 1d). Therefore, the majority of the hMSC population appeared to differentiate into osteoblasts.

### Growth Kinetics of Human MSC Donor Populations

When the growth rate of first passage hMSCs obtained from 17 different donors (Table 1) was measured, a 12-fold variation between donors was observed (Fig. 2). In three separate instances, significant differences in growth rates were also observed between duplicate hMSC cultures established from the same donor over a period of six months ($P \leq 0.05$; Fig. 3a–c). Moreover, when the growth rates of 22 samples of hMSCs obtained from 17 donors (Fig. 4a) were plotted vs. age of donor (Fig. 4b) or gender (Fig. 4c), no statistical correlation was evident ($P \leq 0.05$). Growth rate also did not appear to correlate with the life span of different donor hMSC populations in vitro (data not shown).

### Variations in ALP Levels Between hMSC Donor Populations

Since cellular differentiation typically entails growth arrest, differences in growth rates may reflect variations in the number of committed cells within each donor hMSC population. To examine this possibility, we measured levels of ALP enzyme activity in each donor population following growth for 8 days or 22 days without passage in basal media. Because ALP expression is upregulated during the early phase of osteoblast differentiation, levels of this enzyme in subconfluent cultures (8 days) should reflect the number of committed osteogenic cells within the population. Alternatively, since culture at high density is known to induce mesenchymal cells to differentiate into osteoblasts [Caplan et al., 1983], ALP levels in confluent cultures (22 days) should reflect the total number of osteoprogenitors. First passage hMSC populations exhibited widely disparate levels of ALP enzyme activity (Fig. 5a). Some populations expressed low levels of ALP at both 8 days and 22 days, some expressed high ALP levels at these time points, and others showed dramatic increases in ALP expression between 8 days and 22 days. Overall, ALP levels among donors varied 40-fold after 8 days and 10-fold after 21 days of continuous culture. The levels of induction between the two time points ranged from 1-fold to 17-fold (Fig. 5b). Levels of ALP expression in hMSCs after 8 days in culture showed no correlation with cell growth (Fig. 5c), but ALP activity at 22 days was inversely correlated with cell growth (Fig. 5d; $P \leq 0.05$).

When ALP activity was continuously monitored over three weeks in culture, significant differences in the induction kinetics of this enzyme were evident among different donor populations (Fig. 6a). Therefore, measurements of ALP activity made at 8 or 21 days accurately reflect changes in enzyme activity within a given population over time. To confirm that donor to donor variability in ALP activity is attributable to cellular heterogeneity, we generated approximately 20 clonal populations each from donor 13 (high ALP expression) and donor 18 (low ALP expression), and then assayed ALP activity in each clone after 8 or 21 days in culture without passage. As anticipated, most of the clonal populations derived from donor 13 expressed high levels of ALP activity at both 8 or 22 days in culture (Fig. 6b). However, some clones showed low levels of ALP activity at both time points (16, 21–23, 30) while others showed large increases between days 8 and 22 (3, 14,

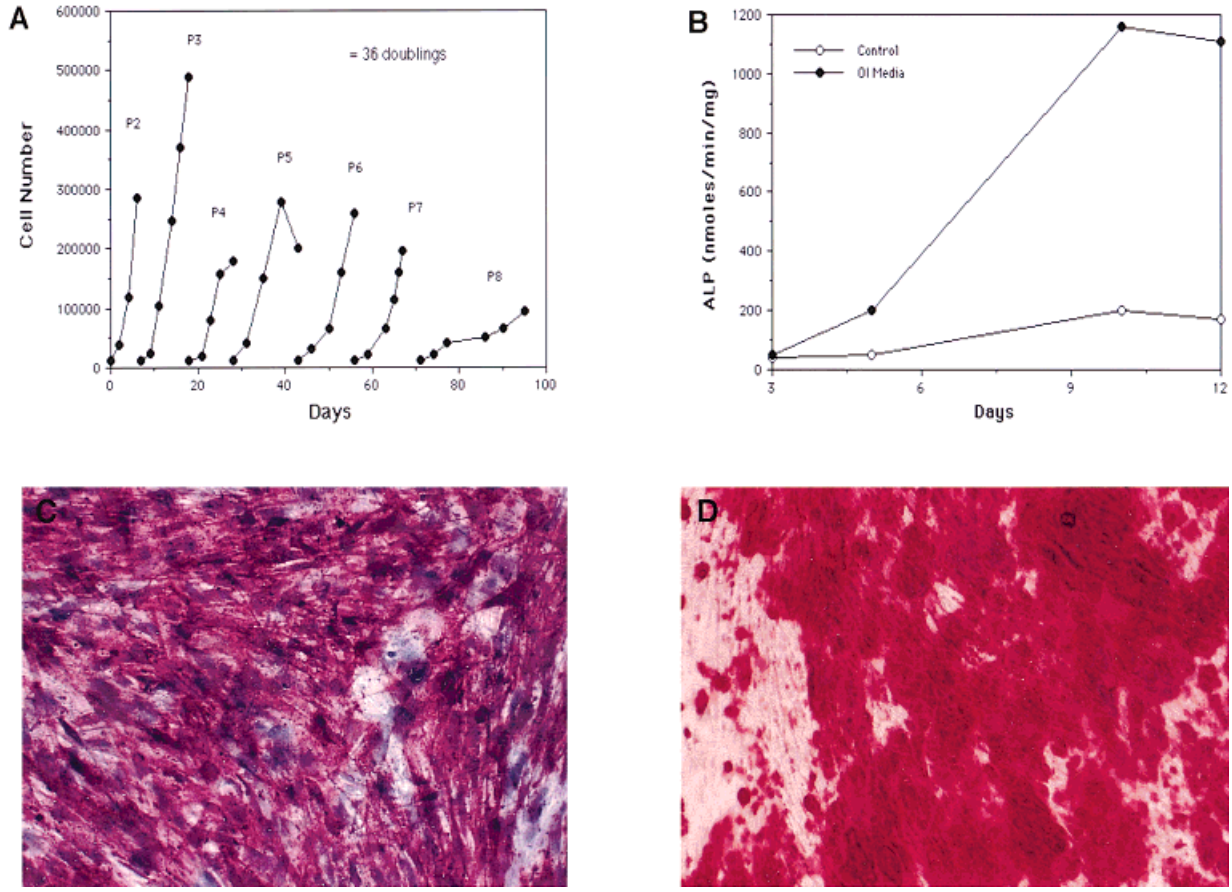


**Fig. 1.** Growth properties and osteogenic potential of human MSCs. **A:** Human MSCs from a representative donor were serially passaged in vitro by successively plating cells at a density of 12,500 cells/9.5cm$^2$ and culturing them until they reached 90% confluence. The number of population doublings was calculated by counting the cumulative increase in cell number at each passage. Cells were estimated to have undergone 10 population doublings prior to first passage. **B:** First passage MSCs from the same donor as in (A) were cultured for two weeks in basal media (○) or in OI media (●) and ALP enzyme activity measured at the indicated time points. After three weeks of continuous culture in OI media cultures were stained for expression of ALP (**C**) or with Alizarin Red S to demonstrate deposition of a mineralized matrix (**D**).

27). In contrast, most of the clonal populations generated from donor 18 expressed minimal ALP activity at these time points (Fig. 6c) except for clone 29, which showed a dramatic increase in ALP activity from 8 to 21 days. These results indicate that hMSC populations are heterogeneous with respect to cellular levels of ALP activity, and suggest that these cultures are comprised of cells at different stages of differentiation and with distinct osteogenic potentials.

### Osteogenic Potential of Human MSC Donor Populations

To further confirm the heterogeneous nature of hMSC populations, we exposed five separate donor populations that exhibited varying levels of basal ALP activity to OI media and then measured the induction levels of several bone-specific genes by RT-PCR. Initially, we empirically determined the number of amplification cycles wherein PCR product accumulation for each gene of interest was exponential (Fig. 7a). All subsequent amplifications performed on hMSC RNA were within these exponential ranges to ensure that differences in the relative abundance of the PCR products reflect that of the corresponding mRNA within the cell. After exposure to OI media for 22 days, first passage hMSC populations derived from donors 15, 18, 27, 34, and 48 possessed widely varying levels of ALP, bone sialoprotein (BSP) and parathyroid hormone receptor (pTHR) mRNA (Fig. 7b,c). In particular, donors 15 and 18 failed to exhibit any significant induction of the three genes. In contrast, donors 27, 34, and 48 showed signifi-

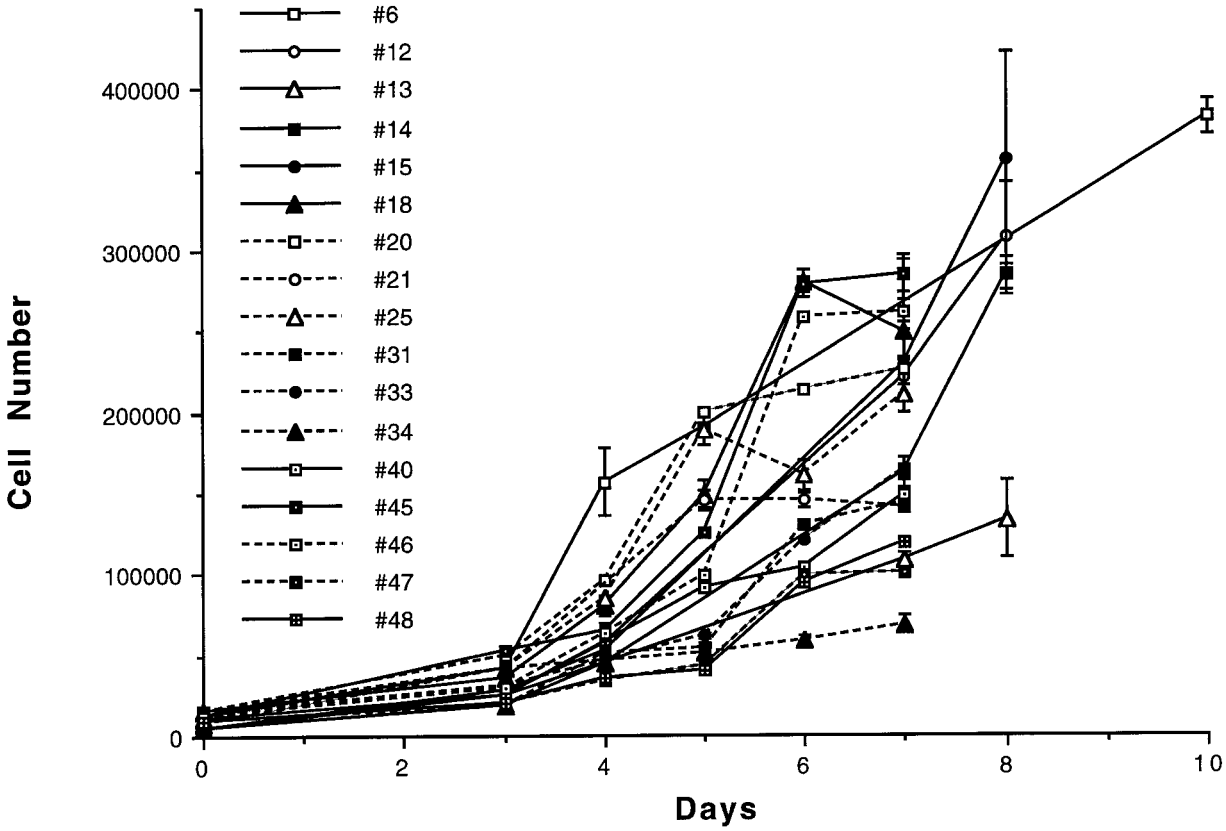


**Fig. 2.** Growth rates of hMSCs obtained from 17 different donors. First passage hMSCs were seeded at a density of 12,000 cells/9.5 $cm^2$ and the increase in cell number as a function of time was measured by counting the number of cells in replicate samples with a hemocytometer. All measurements were done in duplicate and the error bars represent the variance between samples. Growth rates were calculated from the proliferation curves by measuring the slope of each line using data from day 3 to day 8.

cant increases in ALP and pTHR mRNA levels and donors 34 and 48 showed dramatic increases in BSP mRNA levels, as well. HMSCs cultures derived from donors 15, 18, and 27 also showed essentially no staining by Alizarin Red S after 22 days of continuos exposure to OI media. Therefore, lack of expression of BSP, a calcium binding protein secreted by osteoblasts during active bone formation, is consistent with an inability of these cells to deposit a mineralized extracellular matrix. However, cultures derived from donors 34 and 48 stained strongly with Alizarin Red S (data not shown). Finally, basal levels of ALP induction (Fig. 5b) are significantly correlated with levels of bone specific gene expression in cells following exposure to OI media (Fig. 8). Therefore, ALP measurements made in subconfluent and confluent cultures appear to accurately predict the number of osteoprogenitors within a given hMSC donor population. The latter may offer a rapid means to assess the utility of different donor populations in cell therapy for treating bone disease.

## DISCUSSION

In this report we demonstrate that 22 distinct hMSC populations established from 17 donors exhibit widely disparate growth rates in vitro. These cell populations also exhibited marked differences in their expression of ALP enzyme activity after 8 or 22 days of culture in basal media, and in levels of ALP, BSP, and pTHR mRNA levels following exposure to OI media. These findings indicate that there is considerable variability among samples in the composition of hMSC populations established from posterior illiac crest marrow aspirates. Because both growth rate and levels of ALP expression varied significantly in different MSC cultures established from the same donor, we contend that the method of obtaining MSCs by small volume marrow aspirates introduces het-

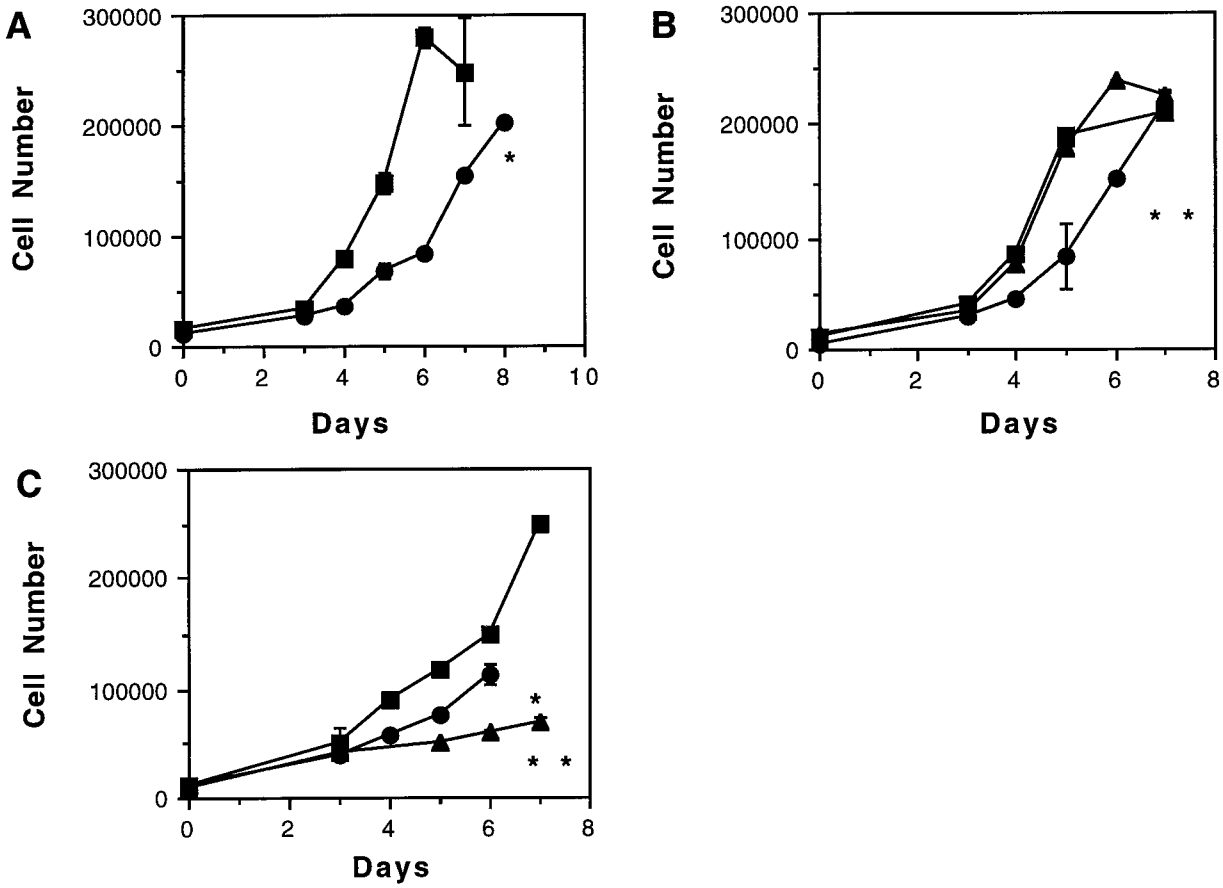


**Fig. 3.** Growth rates of multiple hMSC cultures established from a single donor. Multiple marrow aspirates were obtained from (**A**) a 19-year-old female, (**B**) a-31-year old male, and (**C**) a 27-year-old female over a period of six months. First passage hMSCs established from these aspirates were seeded at a density of 12,000 cells/9.5 $cm^2$ and the increase in cell number was measured in replicate plates as a function of time. All measurement were done in duplicate and the error bars represent the variance between samples. Growth rates were calculated from the proliferation curves by measuring the slope of each line using data from day 3 to day 7. (*$P \leq 0.01$; **$P \leq 0.025$, Student's *t*-test).

erogeneity within a given sample at the time of harvest. This conclusion is substantiated by the fact that other groups have also reported large donor variability in the yield of MSCs from posterior illiac crest marrow aspirates [Castro-Malspina et al., 1980; Lazarus et al., 1995]. Although it is difficult to completely rule out the fact that differences in the physiological status of the donor can account for the variable properties of MSC cultures, we believe this is unlikely because in two instances we have documented large variations in the growth rate of hMSCs obtained from bi-lateral aspirates taken simultaneously from the same donor (data not shown).

One possible explanation to account for how a sampling bias can lead to large variations in growth rates is that the growth rate itself is reflective of the biological age of the cells. We and others [Bruder et al., 1997] have noted that the growth rate of hMSC cultures in vitro decreases as the number of population doublings increases. Therefore, large initial disparities in the number of hMSCs within a given aspirate would necessitate that cells undergo a different number of population doublings to reach a particular density. As a result, at first passage, cells would be of different biological ages and hence proliferate at different rates. Our data suggests this hypothesis is incorrect because we failed to detect a strong correlation between growth rate at first passage and the life span of cells in vitro.

Alternatively, the composition of hMSC cultures may also vary significantly from sample to sample, and this could also effect growth rate. It has been well established that rates of colony formation for fibroblastic cells in culture is highly variable [Matsumura et al., 1979; Mit-

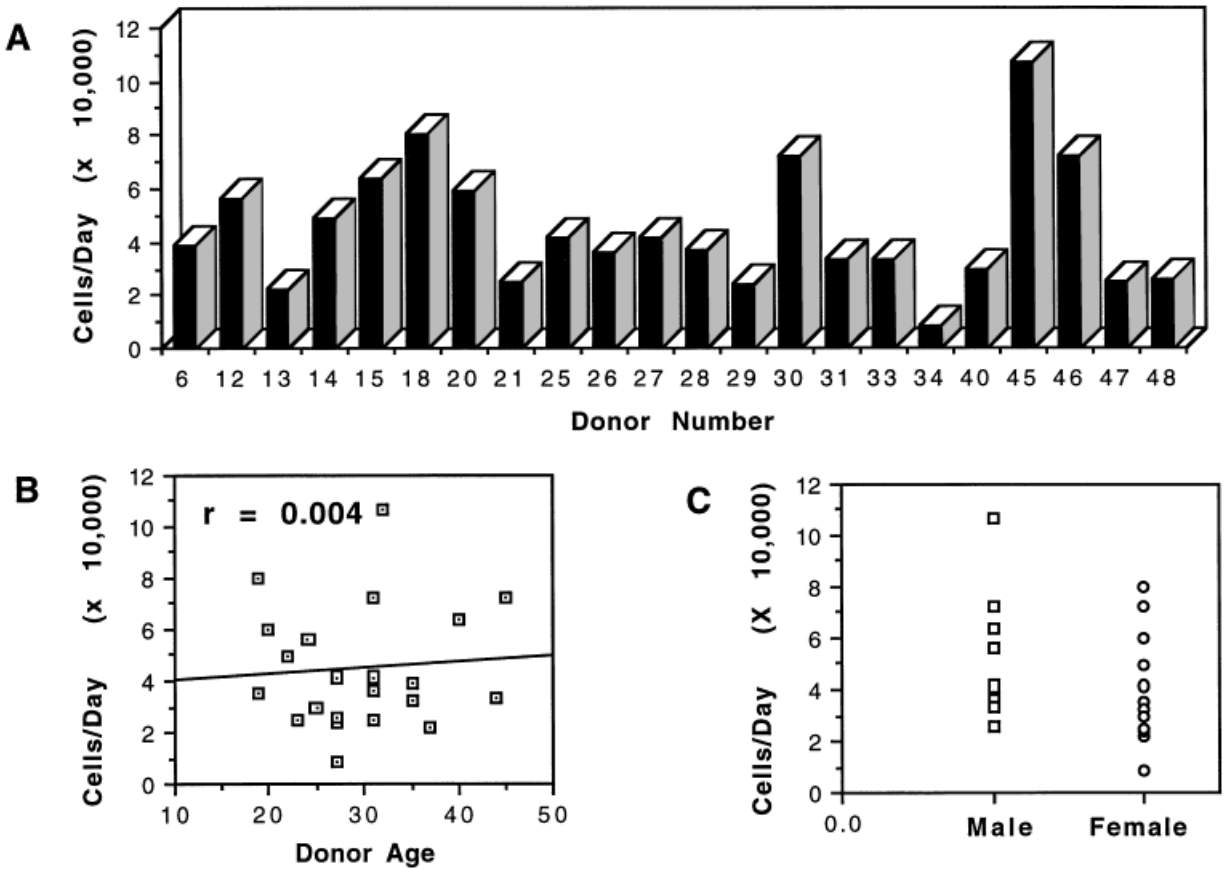


**Fig. 4.** Correlation of MSC growth rate with donor age and gender. Growth rates of 22 separate hMSC samples established from 17 donors (**A**) were plotted against the age (**B**) and gender (**C**) of each donor. No correlation of growth rate with age ($r^2 = 0.004$, $P = 0.05$) or gender ($r^2 = 0.09$, $P = 0.05$) was evident.

sui and Schneider, 1976], although the reason for such heterogeneity is not understood. We and others [Bruder et al., 1997] have noted that different hMSC colonies within a given population also proliferate at different rates. Mets and Verdnok [1981] attempted to explain this phenomenon by classifying hMSCs into two populations; Type I cells that posses a spindle shaped morphology and exhibit a high proliferative capacity and Type II cells that posses a flattened morphology and divided slowly or not at all. Based on these definitions, the authors showed that early passage hMSC cultures were comprised predominantly of Type I cells. However, as the cultures aged, the percentage of Type II cells increased resulting in a concordant drop off in proliferative capacity. Anticipating that these cell types are not randomly distributed in marrow, its possible that aspirates contain different proportions of Type I and Type II cells, resulting in variable growth rates in vitro. Moreover, differences in the rate at which Type I cells become Type II cells may also contribute to variations in growth rates between donor populations.

Although we recognize that hMSC cultures are morphologically heterogeneous, our data reveal that these cell populations are also functionally heterogeneous and are comprised of cells at different stages of differentiation. Such heterogeneity is evident by the fact that levels of ALP enzyme activity varies between samples as much as 40-fold in subconfluent cultures and up to 10-fold in confluent cultures. These results are consistent with that of Jaiswal et al. [1997] who reported that after 8 days in culture, ALP expression varied up to 15-fold in hMSC cultures established from seven different donors. Since ALP is a differentiation marker of early osteoblasts, we propose that changes in its activity under these culture conditions provides a measure of the proportion of non-osteogenic, osteoprogenitors, and committed osteogenic cells within a population. This is

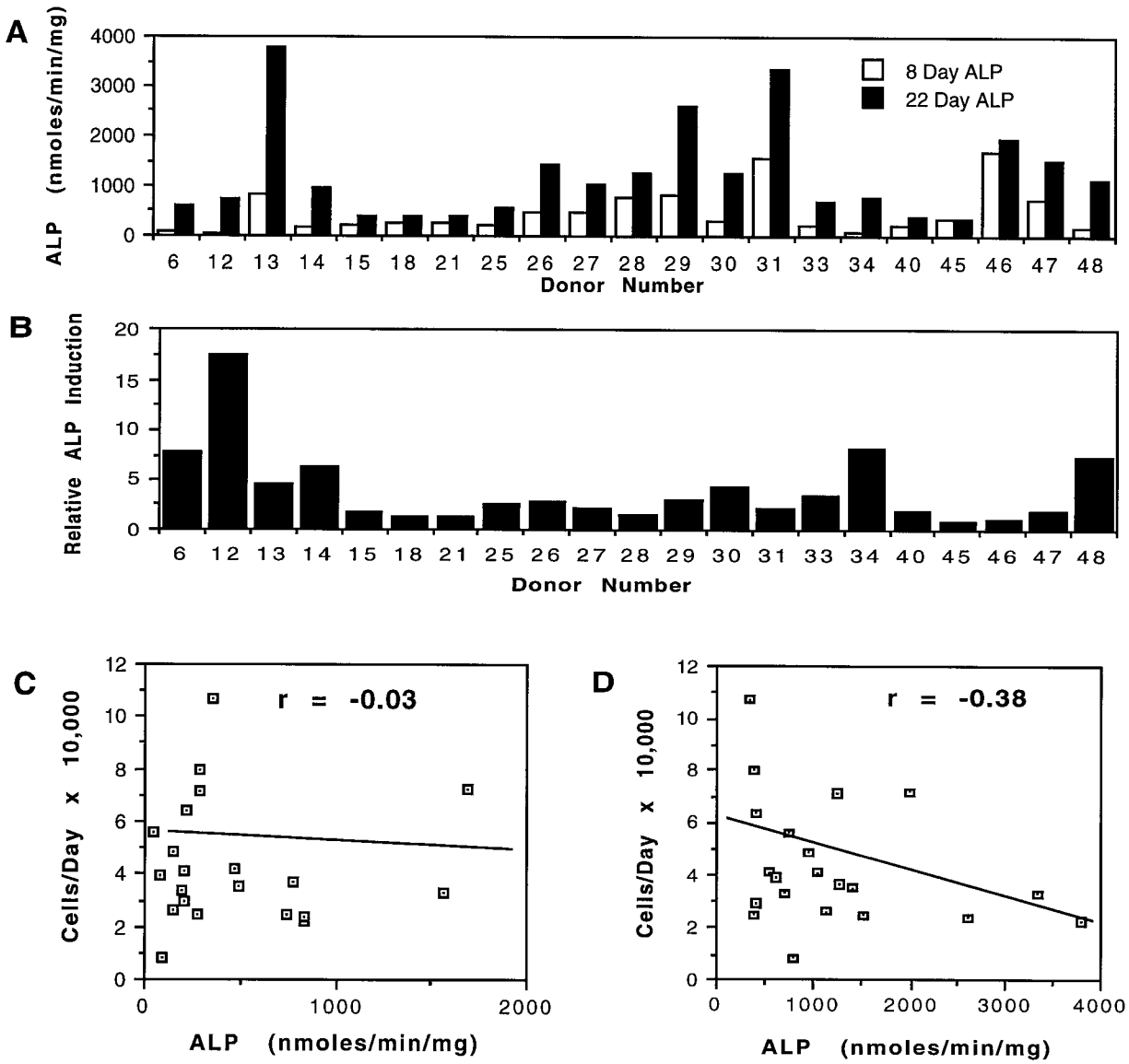


**Fig. 5.** ALP enzyme activity in subconfluent and confluent hMSC populations from different donors. **A**: First passage hMSC cultures from the indicated donors where seeded at 12,000 cells/9.5cm$^2$ and cultured without passage for 8 days (□) or 22 days (■) in basal media. After this time period cell extracts were prepared and ALP enzyme activity measured. Each assay was performed only a single time due to a limited number of available cells. **B:** The relative level of ALP induction between 8 and 21 days was also calculated. **C:** No correlation between the growth rate and ALP activity at 8 days was evident ($r = -0.03$, $P \leq 0.00007$), but (**D**) growth rate was inversely correlated with ALP activity measured at 22 days ($r = -0.38$, $P \leq 0.05$).

supported by the fact that levels of ALP induction between subconfluent (8 days) and confluent (22 days) cultures is strongly correlated with the ability of a given population to upregulate the expression of bone specific genes in response to osteogenic agonists and deposit a mineralized extracellular matrix. Collectively, these data indicate that hMSC cultures are not a uniform population of mesenchymal progenitors. This conclusion is consistent with a recent report by Kuznetsov et al. [1997] who demonstrated that only 58% of clonally derived hMSC populations from a total of four donors demonstrated a capacity to form bone when implanted in vivo. Apparently, ALP induction levels between confluent and subconfluent cultures provides a simple means to assess the osteogenic potential of a given population.

Protocols designed to utilize human MSCs for cell or gene therapy must rely on the ability

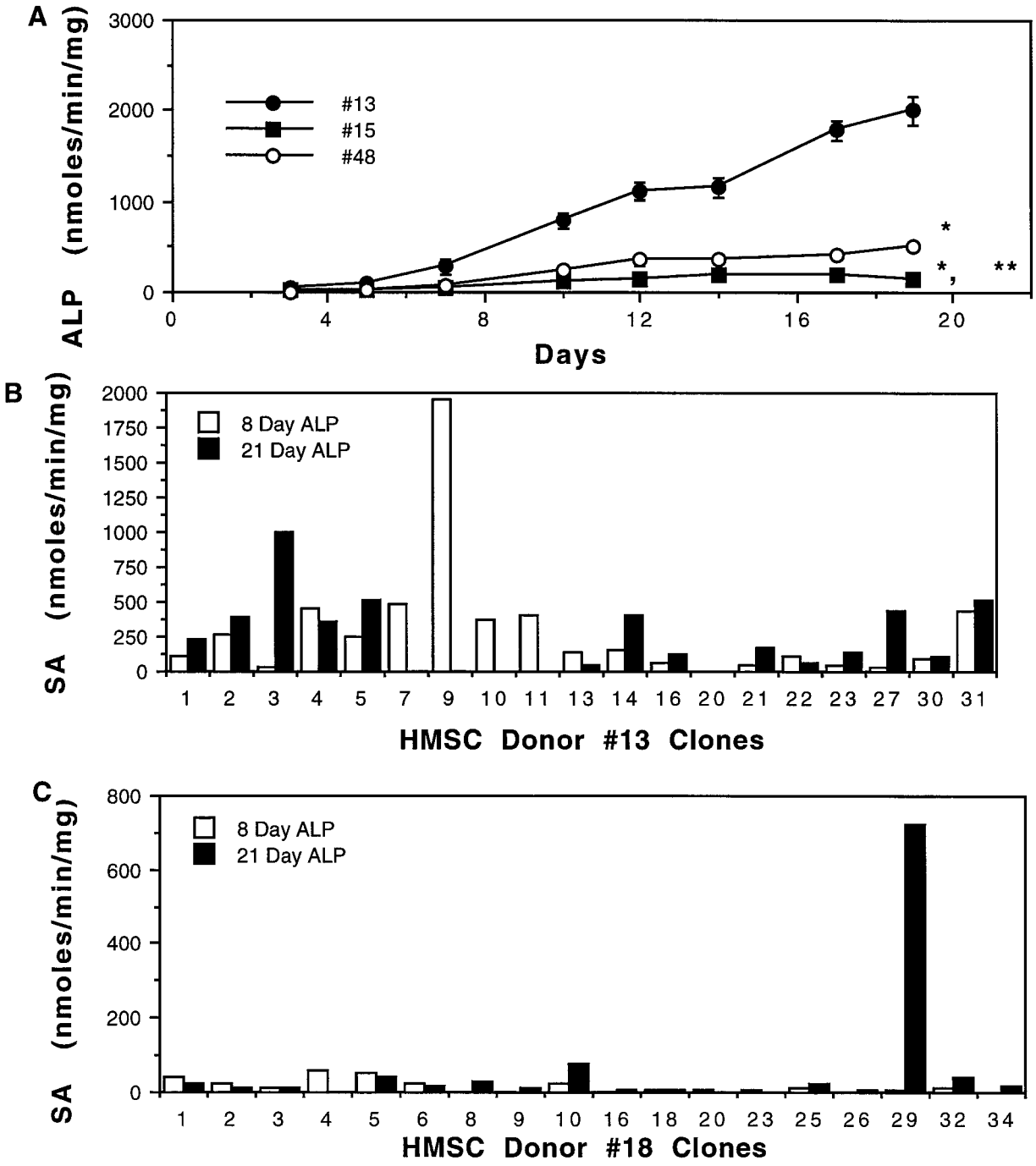


**Fig. 6.** ALP activity in subconfluent and confluent cultures of clonal hMSC populations. **A:** ALP activity in first passage hMSC populations derived from the indicated donors was continuously monitored over a period of approximately three weeks. Samples were done in duplicate and error bars represent the variance in the data ($*P \leq 0.001$, $**P \leq 0.007$). **B,C:** ALP specific activity (SA) in clonal hMSC populations derived from donor 13 (B) or donor 18 (C) after 8 days or 21 days of continuous culture in basal media. Assays were done only once due to limitations in the number of available cells.

to culture-expand these cell populations in vitro. Large sample variations in the growth of rates of MSC populations may make it impossible to obtain sufficient cells from specific donor samples. Moreover, the composition of the donor population with regard to the number of mesenchymal progenitors and committed osteoblasts may limit its utility depending upon its proposed use. For example, populations comprised predominantly of committed osteopro-

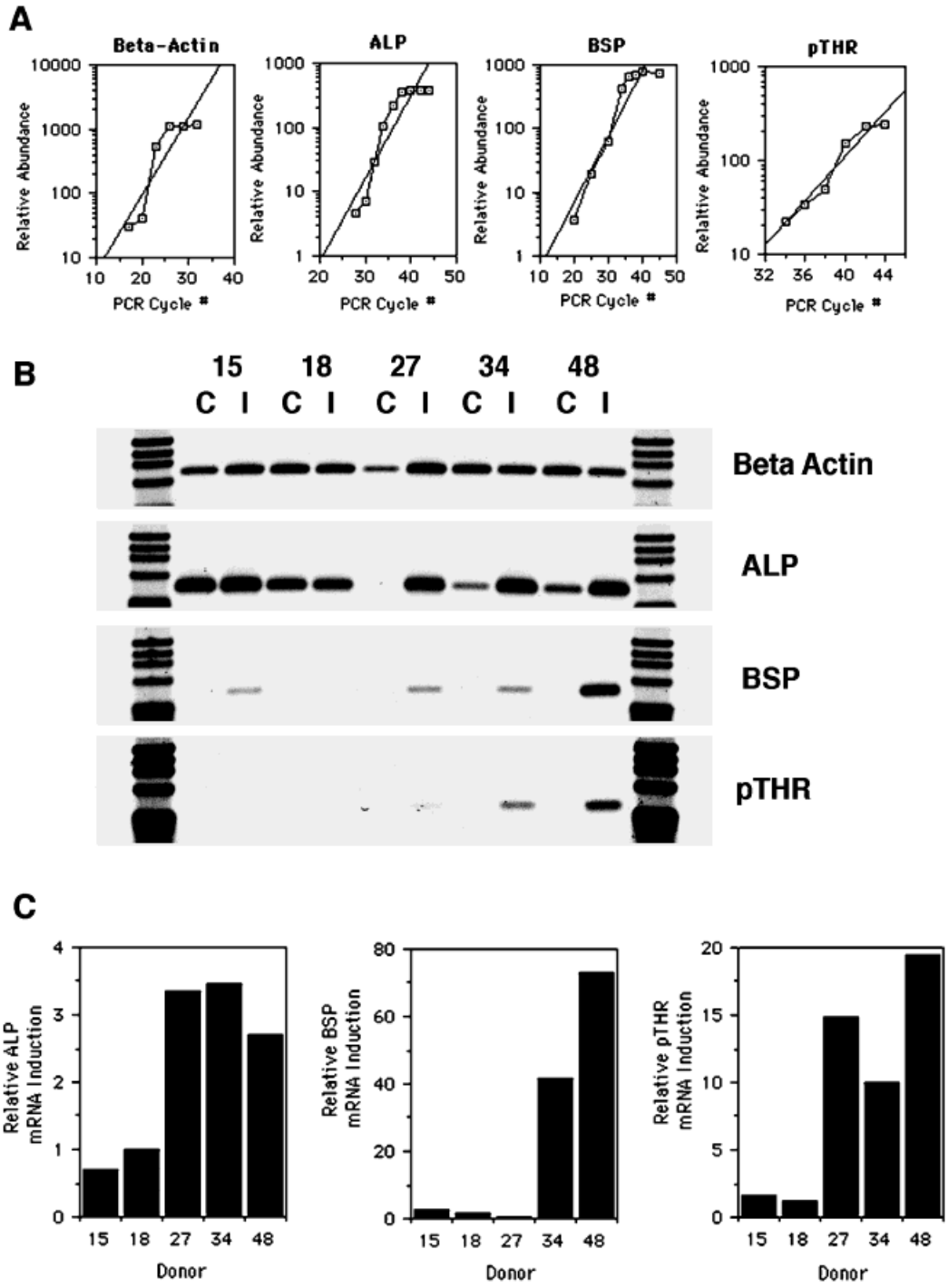


**Fig. 7.** RT-PCR analysis of bone-specific gene expression in hMSC populations exposed to osteo-inductive media. **A:** The number of PCR cycles wherein product accumulation for each gene of interest remained exponential was empirically determined. **B:** Levels of β-actin, alkaline phosphatase (ALP), bone sialoprotein (BSP), and parathryoid hormone receptor (pTHR) mRNA in the indicated hMSC donor populations were measured by semi-quantitative RT-PCR. **C:** Levels of bone-specific mRNA in each sample were normalized to that of β-actin and then plotted as a relative level of expression.

genitors may prove ineffective at effecting repair of damaged cartilage or muscle, assuming that cells expressing high ALP levels cannot be redirected along other mesenchymal cell lineages. As a result, multiple samples of MSCs may need to be harvested from an individual and carefully analyzed in vitro before cells can be implemented in cell or gene therapy regimens.

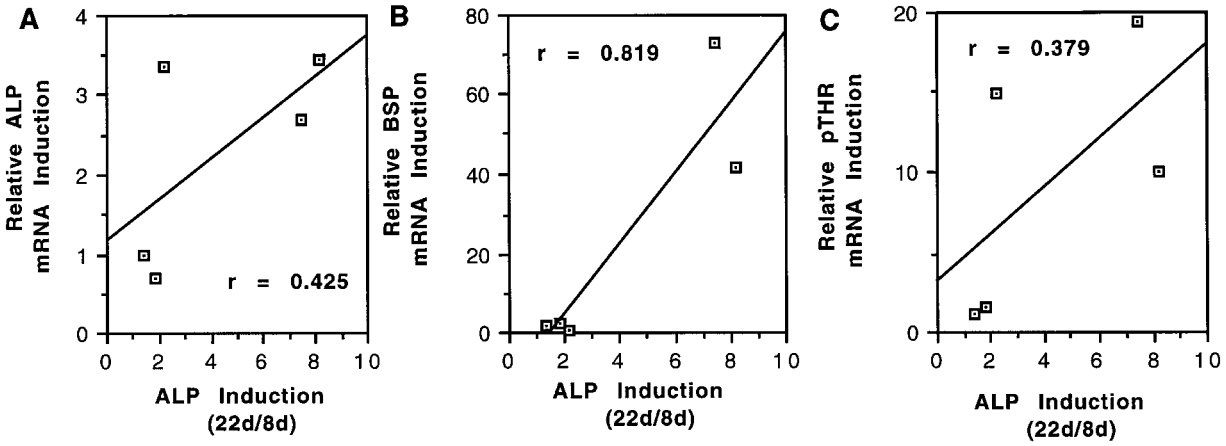


**Fig. 8.** Correlation of basal ALP levels with bone-specific gene induction after exposure to OI media. Scatter plots depict the correlation of ALP induction levels between confluent and subconfluent cultures with relative levels of (**A**) ALP ($P \leq 0.25$), (**B**) BSP ($P \leq 0.05$), and (**C**) pTHR ($P \leq 0.025$) mRNA levels following exposure of cells to OI media.

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