Pleiotropic effects of the steroid hormone 1,25-dihydroxyvitamin D3 on the recruitment of mesenchymal lineage progenitors in fetal rat calvaria cell populations

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Abstract

The steroid hormone 1,25-dihydroxyvitamin D3 (1,25(OH)2D3) inhibits osteogenesis while stimulating adipogenesis in vitro. We hypothesized that 1,25(OH)2D3 redirects the fate of osteoblast/adipocyte bipotential progenitors and other potential progenitors towards adipogenesis, a process possibly underlying the pathogenesis of osteopenic diseases such as osteoporosis. We therefore tested the global effects of 1,25(OH)2D3 on the recruitment of mesenchymal progenitors including osteogenic, chondrogenic, adipogenic and myogenic lineages (colony forming cell (CFC)-osteoblast (CFC-O), CFC-chondrocyte (CFC-C), CFC-adipocyte (CFC-A), and CFC-myoblast (CFC-M) respectively) in rat calvaria (RC) cell populations using gene expression profiling of single cell-derived colonies. Based on expression of lineage specific transcripts, 86% of single cell-derived colonies in untreated cultures simultaneously co-expressed transcripts of two, three, or four of the mesenchymal lineages tested. The distribution of mesenchymal progenitors in 1,25(OH)2D3-treated cultures was significantly changed compared with the control group, i.e. CFC-O were reduced (from 6 to 0%) and CFC-O/A bipotential (0 to 8·2%), CFC-C (4 to 10·2%) and CFC-Fibroblast (CFC-F) (4 to 16%) were increased. 1,25(OH)2D3 did not affect the frequency of tri- or tetra-lineage colonies. Single lineage CFC-A colonies were not detected in either the control or 1,25(OH)2D3 treatment group under the conditions tested. Since the parietal bones used for cell isolation derive from neuroectoderm, we also analyzed for expression of the neural markers nestin and β3 tubulin in these colonies. Surprisingly, 90% (45 of 50) of the colonies in the control group expressed neural markers, a frequency not changed by 1,25(OH)2D3 treatment. The current studies demonstrate the global and developmental stage-specific effects of 1,25(OH)2D3 on mesenchymal lineage progenitors, and suggest that the effects of 1,25(OH)2D3 on osteogenesis and adipogenesis in RC populations are mediated, at least in part, by increased recruitment of CFC-O/A, but not CFC-A type precursors.


Introduction

It is well known that fetal rat calvaria (RC) tissue contains osteoprogenitors that, under appropriate culture conditions, differentiate into mature osteoblasts that form mineralized bone nodules resembling woven bone (Bellows et al. 1986, Bhargava et al. 1988). However, RC cell populations in culture are a heterogeneous mixture of cell types including different differentiation stages of osteoblasts, chondrocytes and adipocytes (Rifas et al. 1982, Bellows et al. 1989, Aubin 1998, Bellows & Heersche 2001). The fact that the clonally-derived RC cell line RCJ 3-1 is capable of differentiating into cartilage, fat, muscle and bone indicates that mesenchymal stem or multipotential progenitor cells are also present in RC cell populations (Grigoriadis et al. 1988, 1990). The reciprocal relationship seen in bone mass and marrow adipose tissue under certain pathological conditions also suggests the presence of a common osteoblast/adipocyte bipotential progenitor in adult marrow (Meunier et al. 1971, Wang et al. 1977, Kawai et al. 1985, Beresford et al. 1992). One study estimated the frequency of bipotential osteo-adipoprogenitors at ~5% in RC cell populations (Bellows & Heersche 2001), but the frequency of various monopotential versus bi- or multipotential cells in RC populations remains unknown.

The steroid hormone 1,25-dihydroxyvitamin D3 (1,25(OH)2D3) has diverse effects on many cell lineages, including osteoblasts where it affects proliferation, apoptosis, specific bone protein expression as well as mineralization (reviewed in van Driel et al. 2004). In the RC cell model, 1,25(OH)2D3 inhibits bone nodule formation while concomitantly increasing the number of
adipocyte foci (Bellows et al. 1994). However, it is not yet clear whether 1,25(OH)2D3 acts separately to inhibit committed osteoprogenitors and stimulate adipocyte precursors and/or whether 1,25(OH)2D3 induces an adipogenic fate in bipotential osteo-adipoprogenitors or both. To address this issue, and dissect the presence and responsiveness of other multi-lineage mesenchymal precursors in RC cell populations, we analyzed gene expression profiles in untreated and 1,25(OH)2D3-treated single cell-derived RC colonies.

Materials and methods

Cell culture and isolation of single cell-derived colonies

All procedures were approved by the University of Toronto Animal Care Committee. Fetal (E20–21) rat calvaria cells were harvested by serial enzymatic digestion as previously described (Bellows et al. 1986). Cells recovered from the last four of five enzymatic digestions were cultured overnight in T-75 flasks containing α-MEM, 10% fetal bovine serum (FBS) and antibiotics (0·1 mg/ml penicillin G, 50 µg/ml gentamycin sulphate, 0·25 µg/ml fungizone), harvested with 0·2% trypsin, re-plated in 10-cm tissue culture dishes at a density of 104/cm2 and incubated at 37 °C for 5 days. Medium was changed every 2 or 3 days. Cells were recovered by trypsinization, washed and re-suspended in Hank’s Balanced Salt Solution containing 2% FBS and 1 mM HEPES.

Single cell-derived RC cell colonies were obtained by sorting single cells into individual wells of 96-well cell culture dishes by fluorescence activated cell sorting (FACS) using a MoFlow equipped with an automatic cell deposition unit (ACDU) (Cytomation, Fort Collins, CO, USA). The cells were cultured in α-MEM containing 10% FBS, antibiotics, ascorbic acid (50 µg/ml), β-glycerophosphate (10 mM) and dexamethasone (10−8 M) at 37 °C in a 5% CO2 atmosphere for 4 weeks. Medium was changed every 2 or 3 days. At the end of 4 weeks, each well was examined by phase contrast microscopy; wells with identifiable colonies were marked and cells were lysed with 350 µl RLT buffer (RNasey, Qiagen) and stored at −80 °C for RNA analysis.

RNA isolation, reverse transcription and real time PCR

Harvested colonies were randomly selected, total RNA was extracted with an RNasey Mini kit (Qiagen) according to the manufacturer’s instructions, and samples were further digested with DNase (Qiagen). First strand cDNA was synthesized with SuperScript II reverse transcriptase and oligo-dT primer according to the manufacturer’s instructions (Invitrogen Inc.), and further purified and precipitated according to the protocol of Liss (2002). Real time PCR was performed on an ABI Prism 7000 Sequence Detection System (Applied Biosystems Inc., Foster City, CA, USA) with SYBR Green PCR Master Mix (Applied Biosystems) as buffer and source of fluorescence. A total of 50 cycles of amplification was used for all transcripts. Primer sequences, concentrations and annealing temperatures are listed in Table 1 for individual genes. An example of real-time PCR amplification of lineage-specific transcripts is shown in Fig. 1.

Lineage-specific markers were used as follows. For osteoblasts: osteocalcin (OCN), bone sialoprotein (BSP); for chondrocytes: collagen type II, Sox9; for adipocytes: adipin, adipocyte lipid binding protein (aP2); for myocytes: myosin heavy chain 6 (Myh6), myogenic regulatory factor (MyoD). Lineage-specific transcripts are designated as present (positive) or absent (negative/undetected) in each colony. The presence or absence of individual markers was determined by amplification and dissociation curves and in comparison with positive control samples (described below). Each colony type was defined by the expression of one or both of the lineage-specific markers used. Colonies with expression of markers of only one of the mesenchymal cell types (i.e. osteoblasts, chondrocytes, adipocytes and myocytes) were designated as CFC-O, CFC-C, CFC-A or CFC-M respectively. Colonies with simultaneous expression of markers of two or more mesenchymal lineages were designated accordingly; for example, colonies with co-expression of osteoblast and adipocyte transcripts were designated as CFC-O/A and so on for all other combinations. Colonies negative for expression of any of the mesenchymal lineage markers but with expression of the housekeeping gene (ribosomal protein L32) comparable to that in all other colonies were designated CFC-F. Positive controls for each lineage were: for osteoblasts/CFC-O, mRNA isolated from microscopically defined mineralized bone nodules present in RC cell cultures under osteogenic conditions (Bellows et al. 1986); for adipocytes/CFC-A, mRNA from colonies with patent lipid droplets after 1,25(OH)2D3 treatment of RC cells (Bellows et al. 1986); for myocytes/CFC-M, mRNA isolated from rat skeletal muscle; for chondrocytes/CFC-C, mRNA isolated from the chondrocyte cell line C5-18 (Grigoriadis et al. 1996). Neural cell mRNA isolated from neurospheres formed from adult and embryonic neural tissue (kindly provided by Dr C M Morshead, University of Toronto; Kim & Morshead 2003) served as a positive control for nestin and β3 tubulin. The sequences of real-time PCR products of nestin and β3 tubulin were also confirmed by direct sequencing.
<table>
<thead>
<tr>
<th>Name of gene</th>
<th>Gene accession no.</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Temperature (°C)</th>
<th>Primer concentration (µM)</th>
</tr>
</thead>
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<tr>
<td>Ribosomal protein L32 CFC-O</td>
<td>NM_013226</td>
<td>5’-AAG CGA AAC TGG CGG AAA C-3'</td>
<td>5’-GGA TCT GGC CCT TGA ATC TTC-3'</td>
<td>59</td>
<td>0.26</td>
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<td>Bone sialoprotein (BSP) Osteocalcin CFC-A Adipsin Adipocyte lipid-binding protein (aP2)</td>
<td>AB001383 NM_013414</td>
<td>5’-CCG GCC ACG CTA CTT TCT T-3'</td>
<td>5’-CCT GGA CTG GAA ACC GTT TC-3'</td>
<td>59</td>
<td>0.26</td>
</tr>
<tr>
<td>Bone sialoprotein (BSP) Osteocalcin CFC-A Adipsin Adipocyte lipid-binding protein (aP2)</td>
<td>NM_053365</td>
<td>5’-CTG ACC TGG CAG GTG CAA A-3'</td>
<td>5’-CTA CTG CCC TCC TGC TGA T-3'</td>
<td>59</td>
<td>0.26</td>
</tr>
<tr>
<td>Bone sialoprotein (BSP) Osteocalcin CFC-A Adipsin Adipocyte lipid-binding protein (aP2)</td>
<td>M92095</td>
<td>5’-TGA TGT GCA AAG TGT AGT GCT TCA-3'</td>
<td>5’-TGG GAG AGC TTA AAG AGC ATG AG-3'</td>
<td>59</td>
<td>0.26</td>
</tr>
<tr>
<td>Bone sialoprotein (BSP) Osteocalcin CFC-A Adipsin Adipocyte lipid-binding protein (aP2)</td>
<td>NM_053365</td>
<td>5’-TCC AGT GAG AAC TTC GAT GAT TAC A-3'</td>
<td>5’-GCC CAT ACC GGC CAC TT-3'</td>
<td>59</td>
<td>0.26</td>
</tr>
<tr>
<td>Bone sialoprotein (BSP) Osteocalcin CFC-A Adipsin Adipocyte lipid-binding protein (aP2)</td>
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<td>5’-CGG CTC CAG CAA GAA CAA G-3'</td>
<td>5’-TGC GCC CAC ACC ATG A-3'</td>
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<tr>
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<td>NM_012929</td>
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<td>5’-TGC GTA CTC GAT GAT GGT CT-3'</td>
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</tr>
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<td>Collagen type II, alpha 1 (col 2a1)</td>
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<td>5’-GAG TGT CAT TTA AGC TTC ATT TGT GG-3'</td>
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<tr>
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<td>NM_017239</td>
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<td>0.26</td>
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<td>5’-CTA TGC CAT GCT GGT CAC TGA T-3'</td>
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<td>0.26</td>
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</table>
Immunocytochemistry

For immunocytochemical analysis of nestin and β3 tubulin, single cells were inoculated and cultured as described above. At times as indicated, cells were fixed in fresh 4% paraformaldehyde, washed with PBS and incubated with antibodies against nestin (1:400, BD Biosciences, San Jose, CA, USA) or β3 tubulin (1:500, Covance Inc., Princeton, NJ, USA), washed, then incubated with FITC-conjugated donkey anti-mouse IgG (1:200, Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA). Secondary antibody alone served as a negative control. After immunostaining, cell nuclei were counterstained with Hoechst 33258.

Statistical analysis

Comparison of the distribution of different types of progenitors in control and 1,25(OH)2D3 treatment groups was performed using a hypergeometric algorithm developed by Adams and Skopek, a generalization of Fisher’s ‘exact’ test for tables with more than two rows and two columns (Adams & Skopek 1987, Cariello et al. 1994). A two-tail Chi-square test for multiple rows was employed to compare individual lineages in different categories (single lineage, bi-lineage etc.) using a GraphPad Instat software package (GraphPad Software Inc., San Diego, CA, USA).

Results

Colony forming efficiency

In each experiment, fifteen 96-well culture dishes were used (total ~ 3000 wells inoculated at 1 cell/well). In the control group, approximately 9% of wells had colonies (4.7 and 13.3% in two independent experiments), while in the 1,25(OH)2D3 treatment group, approximately 8.7% (8.1 and 9.3%) of wells had colonies. There was no statistical difference in the colony forming efficiency between the control and the 1,25(OH)2D3 treatment groups.

The frequency of mesenchymal lineage progenitors in RC cell populations

Amongst 134 and 75 independent colonies collected from the two groups, 60 colonies from each group were randomly selected for analysis by real-time PCR. From
these, a total of 50 control and 49 1,25(OH)2D3-treated colonies were selected for lineage-specific expression profiling based on their similar level of expression of the housekeeping gene (ribosomal protein L32; detected by threshold cycle (Ct) value). The frequency of the different types of colonies from the control and 1,25(OH)2D3 treatment groups is summarized below and shown in Table 2 (the Ct values for expression of particular transcripts in individual colonies are available upon request).

Amongst the 50 colonies in the control group, only five (10%) were monopotential on the basis of expression of markers of a single mesenchymal lineage: three were CFC-O and two were CFC-C. Forty-three colonies (86% of colonies) were bi-, tri- or tetra-potential for the mesenchymal lineages tested (16, 19 and 8 colonies respectively). Two expressed none of the mesenchymal lineage markers tested and were designated CFC-F.

Comparison of the frequency of different types of mesenchymal lineage precursors showed that 1,25(OH)2D3 treatment significantly changed the frequency of some but not all mesenchymal progenitors (\(P=0.014\), Adam–Skopek algorithm). The proportion of tri- and tetra-lineage colonies did not change significantly after 1,25(OH)2D3 treatment (20 and 5 colonies respectively compared with 19 and 8 colonies in the control group) \((P>0.32, \text{two-tail Chi-square test for multiple-row comparisons; the same algorithm was used for all the following statistical analyses})\). However, CFC-F increased significantly from 4 to 16% after 1,25(OH)2D3 treatment \((P=0.04)\). Amongst single lineage colonies, 1,25(OH)2D3 increased CFC-C (from 4 to 10.2%, \(P<0.01\)) apparently at the expense of CFC-O (which decreased from 6 to 0%, \(P=0.03\)). In bi-lineage colonies, 1,25(OH)2D3 increased CFC-O/A (from 0 to 8.2%, \(P<0.01\)), decreased CFC-O/C but had no effect on CFC-A/C \((P=0.03\) for CFC-O/C, one-tail Chi-square test; \(P=0.95\) for CFC-A/C). CFC-A and colonies with myocyte marker expression in mono- and bi-lineage categories were not detected in either the control or 1,25(OH)2D3 treatment group. Tri-lineage colonies CFC-O/A/M decreased from 4 to 0% after 1,25(OH)2D3 treatment; however this change was not statistically significant \((P=0.13)\).

Expression of neural markers in single cell-derived RC cell colonies

Given the high proportion of multi-lineage colonies observed and the fact that the parietal bone used for RC

<table>
<thead>
<tr>
<th>Colony type</th>
<th>Control (50 colonies)</th>
<th>Number of colonies</th>
<th>Percentage</th>
<th>Number of colonies with nestin/(\beta)3 tubulin(^2)</th>
<th>1,25(OH)2D3 treatment (49 colonies)</th>
<th>Number of colonies</th>
<th>Percentage</th>
<th>Number of colonies with nestin/(\beta)3 tubulin(^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Single lineage</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>CFC-O*</td>
<td>3</td>
<td>6%</td>
<td>1 (1)</td>
<td>0</td>
<td>0</td>
<td>0%</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CFC-C*</td>
<td>2</td>
<td>4%</td>
<td>2 (2)</td>
<td>5</td>
<td>10.2%</td>
<td>4 (2)</td>
<td>0</td>
<td>0</td>
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<tr>
<td>CFC-A</td>
<td>0</td>
<td>0%</td>
<td>0</td>
<td>0</td>
<td>0%</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CFC-M</td>
<td>0</td>
<td>0%</td>
<td>0</td>
<td>0</td>
<td>0%</td>
<td>0</td>
<td>0</td>
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<td>Bi-lineage</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>CFC-O/A*</td>
<td>0</td>
<td>0%</td>
<td>0</td>
<td>4</td>
<td>8.2%</td>
<td>3 (3)</td>
<td>0</td>
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<tr>
<td>CFC-O/C*</td>
<td>10</td>
<td>20%</td>
<td>9 (4)</td>
<td>3</td>
<td>6.1%</td>
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<tr>
<td>CFC-O/M</td>
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<td>0%</td>
<td>0</td>
<td>0</td>
<td>0%</td>
<td>0</td>
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<tr>
<td>CFC-A/C</td>
<td>6</td>
<td>12%</td>
<td>6 (3)</td>
<td>4</td>
<td>8.2%</td>
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<tr>
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<td>0</td>
<td>0%</td>
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<td>0</td>
<td>0</td>
<td>0%</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<tr>
<td>Tri-lineage</td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>CFC-O/A/C</td>
<td>16</td>
<td>32%</td>
<td>15 (13)</td>
<td>19</td>
<td>38.8%</td>
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<td>0</td>
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<tr>
<td>CFC-O/C/M</td>
<td>2</td>
<td>4%</td>
<td>2 (2)</td>
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<tr>
<td>CFC-A/C/M</td>
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<td>2%</td>
<td>1 (1)</td>
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<td>2.0%</td>
<td>1 (1)</td>
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<tr>
<td>Tetra-lineage</td>
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<tr>
<td>CFC-O/C/A/M</td>
<td>8</td>
<td>16%</td>
<td>8 (8)</td>
<td>5</td>
<td>10.2%</td>
<td>5 (4)</td>
<td>0</td>
<td>0</td>
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<tr>
<td>CFC-F*</td>
<td>2</td>
<td>4%</td>
<td>1 (0)</td>
<td>8</td>
<td>16.4%</td>
<td>3 (0)</td>
<td>0</td>
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</table>

\(^1\)Comparison of the distribution of different types of mesenchymal lineage progenitors in control and 1,25(OH)2D3 treatment groups by Adam–Skopek algorithm: \(P=0.014\), 95% Confidence Interval=0.010–0.019.

\(^2\)Most mesenchymal progenitors simultaneously express nestin and/or \(\beta\)3 tubulin (numbers within brackets indicate number of colonies with both nestin and \(\beta\)3 tubulin expression).

\(^\ast\)Colony types changed significantly by 1,25(OH)2D3.
cell isolation derives from neuroectoderm, we next asked whether neural markers (nestin and β3 tubulin) were expressed in any of the colonies isolated. Strikingly, 45 (90%) colonies in the control group expressed nestin and/or β3 tubulin (34 expressed both markers; Table 2), and this frequency was not changed by 1,25(OH)2D3 treatment (42 expressed nestin and/or β3 tubulin: 24 expressed both). Neural marker expression was seen across all mesenchymal colony types, and 1,25(OH)2D3 did not change the distribution (P>0.05; Table 2). Amongst colonies designated CFC-F on the basis of the absence of all mesenchymal lineages tested, 1 of 2 in the control and 3 of 8 in the 1,25(OH)2D3 treatment groups had expressed neural markers.

To test whether nestin and β3 tubulin protein expression occurred, 22 colonies from parallel wells as used for mRNA analysis were immunostained; neither nestin nor β3 tubulin was detected in any of the colonies (data not shown).

**Discussion**

1,25(OH)2D3 regulates the development and differentiation of many cell types including monocyte-macrophages (Lindmark & Siegbahn 2002), osteoclasts (Suda et al. 1997), osteoblasts (Ishida et al. 1993, Bellows et al. 1994, van Driel et al. 2004), adipocytes (Kelly & Gimble 1998) and chondrocytes (Grigoriadis et al. 1989). These cell lineages co-exist under physiological and pathological conditions in bone, bone marrow and some other tissue compartments (Caplan 1991, Dorheim et al. 1993, Friedenstein 1995, Aubin 1998), but a comprehensive analysis of the ability of 1,25(OH)2D3 simultaneously to recruit or alter the fate of multiple mesenchymal lineage precursors has not previously been carried out. By using gene expression analysis of single cell-derived colonies, we have achieved new insights into the types of progenitors present in RC cell populations and the ability of 1,25(OH)2D3 to alter their fate choices.

Consistent with our previous estimates by limiting dilution cloning (Aubin et al. 1982, Grigoriadis et al. 1988, 1990) and osteoprogenitor frequency analysis (Bellows & Aubin 1989), ~ 8% of primary RC cells are capable of colony formation, suggesting that the majority of the population comprises relatively mature cells lacking colony forming capacity. Concomitantly, a large proportion (86%) of colonies are multipotential (at least bi- but also tri- and tetra-potential) on the basis of simultaneous expression of markers for multiple mesenchymal lineages. These data support the presence of a mesenchymal cell hierarchy in primary RC cell populations, with a preponderance of multipotent precursors amongst the colony-forming cells. A mesenchymal cell lineage hierarchy was suggested earlier in stromal tissues and clonally derived RC cell populations (Grigoriadis et al. 1988, 1990, Owen & Friedenstein 1988, Friedenstein 1995). Notably, however, we have found that the presence of each type of precursor was not evenly distributed in the RC cell analysis, e.g. about one third of precursors were of the CFC-O/A/C type. This might indicate a ‘default’ lineage selection under the osteogenic and 1,25(OH)2D3 treatment conditions used here, in keeping with the view that extrinsic factors can alter stem cell fate determination (Shah et al. 1994, 1996, Morrison et al. 1995).

In the current studies, RC colonies were defined based on mRNA expression patterns, not on functional criteria such as capacity of a single colony to give rise to more than one differentiated mesenchymal cell type after re-plating under appropriate culture conditions. We showed previously by clonal analyses that such functionally multipotent RC cells exist, but the strategy used to expand cells for testing also caused spontaneous immortalization of the isolated colonies (Grigoriadis et al. 1988, 1990). It remains a challenge to prove directly and without expansion/immortalization that single colonies expressing transcripts of multiple mesenchymal lineages are indeed primed for differentiation along these multiple mesenchymal lineages, since for most of the mesenchymal lineages the only reproducible differentiation culture conditions require cells to achieve high density in colonies (e.g. osteogenesis; Malaval et al. 1999) or require high initial plating cell density (e.g. chondrogenesis; Grigoriadis et al. 1988, Denker et al. 1999). However, evidence from hemopoietic cells suggests a dynamic development process of ‘promiscuous expression’ or ‘lineage primed’ progenitors that may contribute to flexibility and accessibility of multiple fate choices in particular progenitor populations (Miyamoto et al. 2002). Multilineage gene expression has also been detected in human bone marrow mesenchymal stem cells (Seshi et al. 2003). It is also worth noting, given the lineage markers we used, that expression of mature lineage markers in progenitors does not appear to affect their developmental potential; for example, transplanted hemopoietic stem cells expressing the myeloid lysozyme gene repopulate all hemopoietic lineages (Ye et al. 2003). Such data have led to a ‘lineage priming’ model for stem cell differentiation, i.e. genes indicating multi-lineage potential are promiscuously expressed preceding commitment to a single lineage (Hu et al. 1997, Enver & Greaves 1998, Seshi et al. 2000, 2003, Miyamoto et al. 2002, Woodbury et al. 2002). Our data obtained from single RC cell-derived progenitors are consistent with such a model. The hierarchical distribution amongst colony types (tetra-, tri-, bi- and mono-lineage) in RC cell populations suggests that when individual multipotential mesenchymal stem or progenitor cells differentiate to more committed progenitors, the genes responsible for other non-selected lineages are extinguished. Similar to hemopoietic stem cells (Ye et al.
1,25(OH)\(_2\)D\(_3\) increases
tetra-lineage progenitors

\[ \text{MSC (7)} \quad \text{CFC-F} \quad \text{CFC-O/C/A/M} \]

1,25(OH)\(_2\)D\(_3\) no detectable
effect on progeny

\[ \text{Tri-lineage} \times \text{progenitors} \]

1,25(OH)\(_2\)D\(_3\) lineage specific
increases or decreases

\[ \text{Mono-lineage} \times \text{progenitors} \]

\[ \text{Bi-lineage} \times \text{progenitors} \]

\[ \text{Tetra-lineage} \times \text{progenitors} \]

\[ \text{Other mesenchymal} \times \text{lineages} \]

\[ \text{MSC (7)} \quad \text{CFC-F} \quad \text{CFC-O/C/A/M} \]

**Figure 2** Proposed mechanisms of the effects of 1,25(OH)\(_2\)D\(_3\) on the recruitment of mesenchymal lineage precursors. Open up and down arrows indicate respectively a stimulatory or inhibitory effect of 1,25(OH)\(_2\)D\(_3\) on progenitors observed in the current study. The proposed stimulatory effect of 1,25(OH)\(_2\)D\(_3\) on dedifferentiation of CFC-O/C/A/M or other progenitors to CFC-F or redirection to other mesenchymal lineages is based on the evidence of increased CFC-F frequency. CFC-O/A/M, O/M, A/M, C/M, CFC-A and CFC-M were not observed in the current culture conditions and are not shown in the diagram. CFC, colony forming cell; O, osteoblast; C, chondrocyte; A, adipocyte; M, myocyte; F, fibroblast lineage; MSC, mesenchymal stem cell.

In a primed stage, individual RC progenitor cells may express genes encoding lineage exclusive functions (e.g. BSP, adipin, collagen II, etc), but remain in an apparent state of indecision. Interestingly, the proportion of tri- and tetra-lineage colonies was not changed after 1,25(OH)\(_2\)D\(_3\) treatment, suggesting developmental stage-specific effects of 1,25(OH)\(_2\)D\(_3\) on RC mesenchymal progenitors. The developmental stage-dependent recruitment of 1,25(OH)\(_2\)D\(_3\) on different precursors may contribute to the biphasic effects of 1,25(OH)\(_2\)D\(_3\) described previously on adipocyte-osteoblast differentiation (Owen et al. 1991, Ishida et al. 1993, Kelly & Gimble 1998, Atmani et al. 2002). However, the frequency of other progenitor cell types is affected. For example, the frequency of CFC-C was increased after 1,25(OH)\(_2\)D\(_3\) treatment, suggesting a stimulatory effect of 1,25(OH)\(_2\)D\(_3\) on chondrogenesis. 1,25(OH)\(_2\)D\(_3\) also increased the frequency of osteoblast/chondrocyte progenitors (from 20% to 6%; \(P=0.03\) by one-tail Chi-square test). Although the statistical robustness of the observation is relatively low, the increased CFC-C and decreased CFC-O/C frequency suggests that 1,25(OH)\(_2\)D\(_3\) stimulates chondrogenesis by recruitment from the CFC-O/C progenitor pool. However, we cannot rule out an effect of 1,25(OH)\(_2\)D\(_3\) on other pathways that may contribute to the CFC-C pool.

The frequency of osteoblast/adipocyte bipotential progenitors has been predicted to be ~5% in primary RC cell populations (Bellows & Heersche 2001). Such cells are detectable at somewhat higher frequency by histological assessment of single cell-derived colonies in very low density cultures after 1,25(OH)\(_2\)D\(_3\) treatment, where about a quarter of total colonies (25·2 ± 10%) are stained for lipid (Oil red O) and for the presence of the osteoblast marker alkaline phosphatase, whereas only alkaline phosphatase-positive colonies are detectable in the control group (authors’ unpublished data). Consistent with previous studies in high density cultures (Ishida et al. 1993, Bellows et al. 1994, Bellows & Heersche 2001), 1,25(OH)\(_2\)D\(_3\) treatment resulted in loss of CFC-O, indicating an inhibitory effect of 1,25(OH)\(_2\)D\(_3\) on committed osteoprogenitors in RC cell cultures. Data such as these have contributed to the hypothesis that the fates of osteoblast versus adipocyte are reciprocally related in RC and stromal cell populations (reviewed in Nuttall & Gimble 2000, Aubin & Trippitt 2003, Gimble & Nuttall 2004). Notably, however, the loss of CFC-O was not accompanied by an increase in CFC-A frequency in our studies, but instead by a significant increase in frequency of CFC-O/A bipotential progenitors (0% to 8%), suggesting that the reciprocal effects of 1,25(OH)\(_2\)D\(_3\) on osteogenesis and adipogenesis are mediated by the enhanced recruitment of CFC-O/A. Because 1,25(OH)\(_2\)D\(_3\) did not affect the frequencies of tri- and tetrapotential colonies, a dedifferentiation process of CFC-O to CFC-O/A may underlie the decreased CFC-O and increased CFC-O/A frequency observed.

Some progenitors were identified as CFC-Fs in the current study based on absence of expression of any of the mesenchymal lineage markers tested. Such cells may represent a very primitive mesenchymal progenitor or they may belong to lineages for which markers were not tested. In either case, 1,25(OH)\(_2\)D\(_3\) significantly increased the frequency of CFC-Fs (\(P=0.04\)), suggesting that 1,25(OH)\(_2\)D\(_3\) either re-directs some progenitors towards other mesenchymal lineages or dedifferentiates progenitors to a more primitive stem cell stage. Dedifferentiation was suggested to be responsible for the observation that highly differentiated adipocytes revert to a less differentiated, more proliferative fibroblastic precursor and then to an osteogenic phenotype under certain culture conditions (Park et al. 1999). Based on CFC-Fs and the other colony types observed, we propose that 1,25(OH)\(_2\)D\(_3\) alters the fate choice of mesenchymal lineage precursors at several bifurcation points as summarized in Fig. 2.
Given that the parietal bone used for RC cell isolation derives from neuroectoderm, it may not be entirely surprising that, in addition to other mesenchymal markers, a remarkable proportion of colonies analyzed also expressed the neural markers nestin and β3 tubulin, a frequency not changed by 1,25(OH)2D3 treatment. To our knowledge, this is the first study demonstrating expression of neural markers in RC cell populations, although co-expression of neural and mesenchymal markers has been seen in other populations including bone marrow stroma (Woodbury et al. 2000) and multipotent adult progenitor cells (MAPCs) (Jiang et al. 2002). It must be noted that we saw no evidence of nestin or β3 tubulin protein expression and therefore cannot rule out the possibility that nestin and/or β3 tubulin expression represents transcriptional leakiness. However, it is also possible that their expression represents a more global priming state indicative of a more diverse developmental potential of RC cells. Consistent with the latter, when ‘side population’ RC cells were isolated and cultured under various differentiation conditions, enrichment for not only mesenchymal cell fates but also a neurogenic fate was observed (Zhang et al. 2005). Our data support the hypothesis that basal level expression of lineage-specific transcripts is necessary for priming lineage differentiation under appropriate culture conditions (Orkin 2003).

In conclusion, single RC cell-derived colonies define a mesenchymal cell lineage hierarchy in which 1,25(OH)2D3 alters fate choices in a developmental stage-specific manner.

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