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Multi-lineage potential of human mesenchymal stem cells following clonal expansion

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Abstract

Bone marrow contains mesenchymal cells that can be isolated and grown *in vitro*. Using appropriate treatment protocols such cultures can be induced to differentiate to yield osteoblasts, adipocytes, and chondrocytes. However, previous experiments had not addressed the question whether single pluripotent stem cells exist and can give rise to these different cell lineages or whether bone marrow mesenchymal cell preparations represent a mixture of committed precursors. We have used human adult bone marrow-derived mesenchymal cells obtained from iliac crest biopsies to demonstrate clonal outgrowth after limiting dilution and we show that some clones can be expanded over more than 20 cumulative population doublings and differentiated to osteoblasts, adipocytes, and chondrocytes. Our data provide direct experimental evidence that cultures of bone marrow-derived mesenchymal cells contain individual cells that fulfil two essential stem cell criteria: (i) extensive self-renewal capacity and (ii) multi-lineage potential.

Keywords: Cell Differentiation, Cell Aging, Osteogenesis, Adipogenesis, Chondrogenesis

Introduction

Multicellular organisms originate from a single totipotent cell and develop through a process of cell proliferation and progressive differentiation. Somatic cells isolated from an adult organism are usually terminally differentiated or restricted with respect to the phenotypes they can adopt under specific culture conditions *in vitro* or upon re-implantation *in vivo*. However, adult organisms also contain a relatively low number of cells that are less restricted and can give rise to several different terminally differentiated cell types. These "pluripotent stem cells" are required for normal tissue remodeling and for appropriate responses to injury. Hematopoietic precursors represent a particularly well studied example of pluripotent stem cells[1].

Mesenchymal stem cells (MSCs) are defined as cells that can give rise to a variety of mesenchyme-derived cell types, notably fibroblasts, chondrocytes, osteoblasts, myoblasts, and adipocytes[2-5]. When implanted in the brain, such cells may also differentiate to become astrocytes, which indicates a surprising degree of "plasticity"[6]. Therefore, MSCs are considered to have a significant therapeutic potential for the treatment of disorders of tissue turnover and repair and for reconstructive surgery[3-7].

It has been reported that MSCs can be isolated in relatively high numbers from cultures of bone marrow by selecting cells that adhere to tissue culture plastic and proliferate rapidly[2-5]. Although it has been clearly demonstrated that cell cultures established this way can give rise to several cell types, there are no data available clearly demonstrating that these different phenotypes can originate from one single genuine stem cell.

To demonstrate the existence of multipotential mesenchymal stem cells both Pittenger et al.[5] and Muraglia et al.[8] isolated and expanded colonies grown in primary marrow cultures and subjected the expanded cell populations to differentiation assays. In both cases, however, the clonal origin of mesenchymal cells can not be clearly demonstrated because of the large number of cells present initially. In addition, it remains unclear whether single pluripotent stem cells are indeed present in populations of bone marrow-derived cells that were expanded *in vitro* to yield several million cells. Such cultures are routinely used by many investigators as "stem cell cultures"[2-5].

We therefore set out to determine whether bone marrow mesenchymal cells can be cloned by limiting dilution and expanded *in vitro* without losing the capacity to differentiate to osteoblasts, chondrocytes, and adipocytes. We show that such clonal expansion is possible and that a subset of clones retains the possibility to undergo multi-lineage differentiation.

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Materials and methods

Chemicals

If not otherwise indicated, fine chemicals were supplied by Sigma (Buchs, Switzerland).

Cells and culture conditions

Preparations of human bone marrow mesenchymal cells (named "human mesenchymal stem cells", MSCs, by Pittenger et al.[5]) from three different donors (D222 [male, 27 years], D290 [male, 22 years], D294 [female, 26 years]) were provided by Osiris Therapeutics, Baltimore, MD, as frozen stocks at passage 1. Cells were thawed and put in culture using DMEM low glucose medium (Seromed, Berlin, Germany) supplemented with 10% fetal calf serum (FCS; Hyclone, Logan, UT). This serum was recommended by Osiris Therapeutics for MSC culture. Clonal growth experiments were initiated with cells collected from these cultures after 3-4 days, before confluence was reached.

As basal growth media we used either DMEM as indicated above or a 1:1 mixture of MEMα (Bioconcept, Allschwil, Switzerland) and Ham's F12 (Seromed, Berlin, Germany) medium supplemented with 10% FCS and antibiotics (100 IU/ml penicillin, 100 mg/ml streptomycin; Bioconcept, Allschwil, Switzerland). These media were further supplemented or not with basic fibroblast growth factor (bFGF or FGF-2; Bachem, Bubendorf, Switzerland) at a final concentration of 10 ng/ml. Supplementation with bFGF has been reported to increase the proliferative and osteogenic capacity of human bone marrow mesenchymal cells[9,10]. To prepare conditioned medium, DMEM-based standard medium was put on early confluent cultures of MSCs (D222) and collected after 2 or 3 days. Batches of conditioned medium were pooled, filtered (0.22 μm), and stored (no longer than 6 weeks) at -20°C until use in clonal growth experiments. Conditioned medium was also supplemented with fresh bFGF before use.

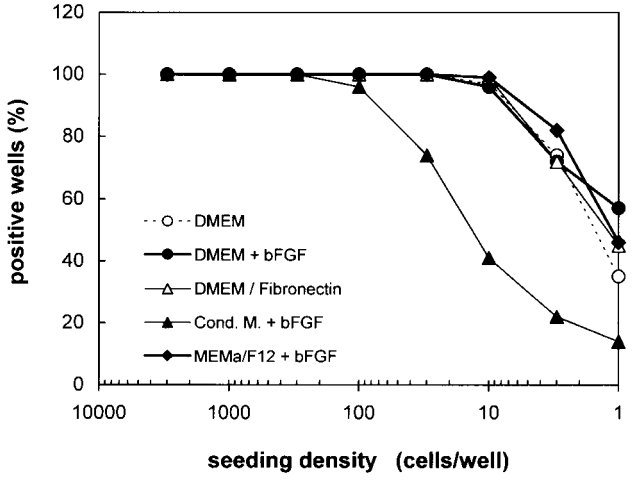

Figure 1. Growth of cells following seeding at different densities and under different growth conditions. Percentage of wells containing more than 5000 cells at the end of the observation period (2 weeks or 3 weeks for seeding densities >100 cells per well or <100 cells per well, respectively) is plotted against seeding density.

Cells were seeded either on standard 96-well tissue culture plates (Costar, Cambridge, MA) or on fibronectin-coated plates (Biocoat®; Beckton-Dickinson, Bedford, MA). The medium was changed twice weekly.

Fluorimetric determination of cell number

Cell numbers were determined using the CyQuant cell proliferation assay kit from Molecular Probes (Leiden, The Netherlands). This method is based on fluorescence quantification of cellular nucleic acids.

Reversible DNA stain and fluorescence microscopy

Cells were incubated with the membrane-permeable DNA-binding dye Hoechst 33342[11] diluted to 0.1 μM in Hepes-buffered saline (130 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.9 mM NaH_2PO_4, 0.8 mM $MgSO_4$, 25 mM Glucose, 20 mM Hepes, pH 7.4). Plates were inspected at low magnification using a Leitz DM10 microscope equipped for fluorescence imaging (excitation: 360 nm, emission: 450 nm). Individual nuclei of living cells could be detected clearly under our experimental conditions. After counting, plates were washed once with culture medium and put back into the incubator with fresh medium.

Osteogenic differentiation

Cells were seeded into 96-well plates at a density of 2000 cells per well and grown to confluence. To stimulate osteogenic differentiation, cells were incubated in "mineralization medium" (MEMα/Ham's F12 + 10% FCS + 0.75 mM $CaCl_2$ [to compensate for the low calcium content of Ham's F12 medium]) and treated with ascorbic acid phosphate (50 μM), β-glycerophosphate (10 mM), and dexamethasone (0.1 μM). Control cells did not receive the latter supplements. Cultures were maintained for >15 days. The medium was changed twice weekly. Every second or third day, groups of 4 wells were washed three times with isotonic saline to remove the culture medium and left dry until the end of the experiment. Matrix-associated calcium deposited in wells was determined using the MPR2 calcium assay kit from Roche Molecular Biochemicals (Basel, Switzerland).

Adipogenic differentiation

Cells were seeded into 6-well plates at a density of 8×10^4 cells per well and grown to confluence. Cultures were then treated in DMEM high glucose medium (Seromed, Berlin, Germany) + 10% FCS supplemented with insulin (10 μg/ml), BRL 49653 (1 μM, Novartis), isobutylmethylxanthine (0.5 mM) and dexamethasone (1 μM) for 15-21 days. The medium was changed twice weekly. To assay adipogenic conversion

quantitatively, cells were collected at the end of the treatment period by trypsinization and adipocytes were quantified using cytofluorimetry with Nile red as a lipophilic dye essentially as described by Gimble et al.[12]. To visualize adipocytes, cultures were fixed in 4% paraformaldehyde on ice for 15 minutes, then washed and stained with Oil Red O (3 mg/ml in H_2O) for 10 minutes.

Chondrogenic differentiation

These experiments were carried out following essentially the protocol described by Johnstone et al.[13]. Cell suspensions were prepared at a density of 5 x 10^4 cells / ml in DMEM high glucose medium supplemented with Hepes (10 mM, pH 7.4), glutamine (2 mM), "ITS" (bovine insulin, transferrin, selenous acid, final concentration 6.25 μg/ml each), linoleic acid (5.3 μg/ml), BSA (1.25 mg/ml), dexamethasone (0.1 μM), ascorbic acid phosphate (50 μg/ml), Na-pyruvate (1 mM) and proline (40 μg/ml). Of such suspensions, 5 ml were pipetted into conical 15 ml polypropylene tubes and cells were sedimented by centrifugation at 150 x g (8 min, room temperature). Cell pellets were washed once more and resuspended in 0.5 ml of the same medium supplemented with TGFβ3 (10 ng/ml, Novartis). To initiate chondrogenic pellet condensation, cells were sedimented once more at 150 x g (2 min). The medium was changed twice weekly and cultures were maintained for >20 days.

At the end of the experiment, the pellets were fixed in 4% paraformaldehyde in PBS (4°C, 24h). The material was then subjected to dehydration (ethanol dilution series) and embedded in Technovit 7100 blocks (Leica, Glattbrugg, Switzerland). Blocks were sectioned and stained with Safranin O. Tissue showing chondrogenic differentiation develops a characteristic purple stain, non-chondrogenic tissue remains blue.

Results

To assess the proliferation potential of bone marrow mesenchymal cells in low density culture, we seeded serially diluted cell suspensions in 96-well plates and measured cell numbers in individual wells after two or three weeks. Subconfluent cultures were used to prepare cell suspensions for seeding in order to minimize formation of cell clumps. Microscopic inspection of diluted suspensions confirmed that cells were well individualized. As basal culture media we compared DMEM and a 1:1 mixture of MEMα and Ham's F12 medium supplemented with FCS and bFGF as indicated. In addition, we prepared DMEM medium conditioned by confluent cultures as described in the methods section. We expected conditioned medium to increase cell survival and proliferation at low cell density. Finally, fibronectin-coated plates were used in some experiments as a culture substrate.

Figure 1 shows the results of a serial dilution experiment carried out with D222 cells grown under different conditions. Unexpectedly, a significant percentage of wells grew confluent within the observation period even down to the lowest seeding density employed (1 cell per well). As human "MSCs" were described to undergo apoptosis at low cell density[14], we expected the number of wells growing confluent to decline sharply already at seeding densities of around 100 cells per well and below. Note that the standard seeding density employed in our laboratory for human bone marrow mesenchymal cells is 6000 cells per cm^2 or about 2000 cells per well on a standard 96-well plate. In contrast to our expectations, use of conditioned medium did not constitute an advantage. Rather, this medium significantly inhibited cell proliferation compared to all other media and conditions tested, which seemed to support outgrowth of cells about equally well.

Cell numbers in individual wells were determined and it became apparent that MEMa/F12 medium supplemented with bFGF supported cell proliferation best, followed by DMEM supplemented with bFGF (Table 1). Normal medium without bFGF supplementation performed moderately. Only low cell numbers were reached in conditioned DMEM. Similar data as in Table 1 were obtained with seeding densities of 3 and 10 cells per well (data not shown).

Average cell number in wells (x100) (± s.e.m.)						
	DMEM conditioned +bFGF	**DMEM fibronectin**	**DMEM**	**DMEM +bFGF**	**MEMα/F12**	**MEMα/F12 +bFGF**
donor D222	7.1 ±1.1 N=21 (26.2%)	10.60 ±0.5 N=37 (46.2%)	10.4 ±1.0 N=31 (38.8%)	12.7 ±0.9 N=52 (65%)	n.d.	18.4 ±0.9 N=39 (48.8%)
donor D290	n.d.	n.d.	8.6 ±0.6 N=36 (45%)	15.3 ±1.1 N=30 (37.5%)	8.9 ±0.6 N=36 (45%)	18.6 ±1.2 N=31 (38.8%)
donor D294	n.d.	n.d.	7.1 ±1.5 N=5 (6.2%)	6.4 ±1.8 N=3 (3.8%)	12.1 ±0.7 N=44 (55%)	15.6 ±1.1 N=43 (54%)

Table 1. Growth of bone marrow mesenchymal cells from different donors following seeding in 96-well plates at a nominal density of 1 cell per well. Effect of different media and growth conditions. Average cell numbers were calculated from all wells that contained >2000 cells. The percentage of wells containing at least this cell number is given in parenthesis. The total number of wells seeded was 80. n.d.: not determined.

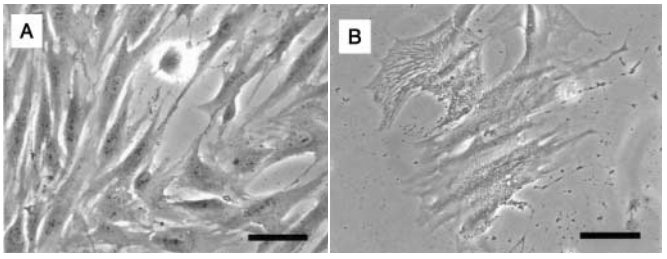

Figure 2. Photomicrographs of growing early passage bone marrow mesenchymal cells (A) and senescent cells (B). Bars: 10 μm.

Having demonstrated that growth of bone marrow mesenchymal cells seeded at very low density is possible, we set out to investigate whether individual cells could be clonally expanded to yield sufficient cell numbers to perform osteogenic, adipogenic, and chondrogenic differentiation assays. Whereas only a few cells are required to carry out osteogenic and adipogenic differentiation assays, chondrogenic differentiation requires larger cell numbers to successfully initiate a pellet culture (>10^5 cells). Clonal expansion from 1 cell to >500,000 requires 20 cumulative population doublings and is therefore not trivial given the limited *in vitro* life span of primary cells.

In a first experiment, a diluted cell suspension of D222 cells was prepared and seeded at a nominal density of 1 cell per well into eight 96-well plates. For two of the plates, the number of cells in individual wells was determined 18h after seeding by fluorescence microscopy following cell labelling with the membrane-permeable and reversibly DNA-binding fluorescent dye H33342[11]. We found that 52% of the wells inspected did not contain any cells, 36% contained 1 cell, 10% contained 2 cells, 2% contained 3 or 4 cells. A similar distribution of cells per well is indeed expected following Poisson statistics at an average seeding density of 1 cell per well. Half of the plates received DMEM and MEMα/F12 medium, respectively, both supplemented with FCS and bFGF. Starting after two weeks of culture, individual clones were transferred to 12-well plates (total number: 101). Of these clones, 61 grew confluent within two to three weeks and could be transferred to T25 culture flasks. Only 27 of these cultures could finally be expanded to confluence and used to initiate cell differentiation assays. Cells in other cultures ceased to proliferate and adopted a very extended and flat appearance, often containing a large number of vacuoles (Fig. 2B). These cells obviously reached the end of their *in vitro* life span[15,16].

Osteogenic, adipogenic, and chondrogenic differentiation assays were carried out according to standard procedures. The results are summarized in Table 2. All clones showed clear mineral deposition *in vitro*, demonstrating osteogenic differentiation.

However, only a subset of clones gave rise to adipocytes and/or produced active chondrocytes in pellet culture.

We could finally show that 12 of 24 clones tested successfully underwent differentiation into all three lineages. Among these clones, 5 could be traced back to a single cell detected initially by fluorescence microscopy.

A second clonal expansion and differentiation experiment

			Differentiation		
			Osteogenic	**Adipogenic**	**Chondrogenic**
clone designation	**medium used for expansion**	**initial number of cells in well**	calcium deposition (ug/well)	adipocyte number (%) or presence of Oil Red O-positive cells	Safranin O-positive pellet
1A-A7	DMEM	1	1.02	-	+
1A-B6	DMEM	1	1.63	2.63	+
1A-D5	DMEM	1	1.80	0.51	+
1A-F4	DMEM	2	1.48	0.3	+
1A-F7	DMEM	1	1.64	+	++
1A-G7	DMEM	1	1.15	6.68	no pellet
1A-G10	DMEM	1	0.92	1.55	+
1B-A3	DMEM	1	1.84	0.47	++
1B-C4	DMEM	1	1.77	0.82	no pellet
1B-C7	DMEM	2	1.05	3.91	no pellet
2A-B7	DMEM	n.d.	1.11	-	+
2A-E7	DMEM	n.d.	1.35	0.07	+
3A-F5	MEMα/F12	n.d.	0.92	+	+
3A-G9	MEMα/F12	n.d.	1.13	+	+
3A-H5	MEMα/F12	n.d.	0.96	+	+
3A-H9	MEMα/F12	n.d.	1.48	+	+
3A-H10	MEMα/F12	n.d.	1.22	+	+
3B-A8	MEMα/F12	n.d.	0.93	+	+
3B-H4	MEMα/F12	n.d.	0.91	-	-
4A-C3	MEMα/F12	n.d.	0.76	+	no pellet
4A-C6	MEMα/F12	n.d.	0.99	-	-
4A-F2	MEMα/F12	n.d.	1.40	+	+
4A-F8	MEMα/F12	n.d.	1.04	-	no pellet
4A-F9	MEMα/F12	n.d.	1.02	-	+

Table 2. Results of cell differentiation assays carried out with expanded clones of human MSCs (donor D222). For 24 clones all assays could be completed correctly and 12 clones scored positive with respect to osteogenesis, adipogenesis, and chondrogenesis. Criteria were as follows: osteogenic differentiation: >0.5 ug/well calcium deposition; adipogenic differentiation: microscopic detection of Oil Red O-positive cells or >0.3% nile red-positive cells in flow cytometry (absence of quantitative data indicates that flow cytometry was not carried out); chondrogenic differentiation: formation of a safranin O-positive pellet culture. n.d.: not determined.

was carried out for confirmation with cells from two additional donors (D290, D294). Cells were seeded in DMEM-based medium supplemented with FCS and bFGF at a nominal seeding density of 0.3 cells per well on eight 96-well plates, respectively. No fluorescence microscopy with H33324 was carried out in this experiment. Only very few clones derived from D294 cells could be expanded to the T25 flask stage and these cells failed to undergo adipogenic or chondrogenic differentiation (not shown). Clones from donor 290, however, could be expanded very well and differentiated along the osteogenic, adipogenic, and chondrogenic lineage. It must be noted that in this experiment only a limited number of rapidly growing clones were analyzed, no effort was made to collect and expand developing clones quantitatively. Results are summarized in Table 3. Of 24 clones initially transferred, 15 could be grown to the T25 flask stage. For 10 clones all three differentiation assays could be terminated correctly, and 7 clones gave positive results in the three tests. Notably, clones from donor 290 gave rise to much higher numbers of adipocytes than clones isolated from donor 222 (Table 2). Figure 3 shows photomicrographs taken of clone D290 a-A6 following osteogenic, adipogenic and chondrogenic differentiation.

Discussion

The data reported here show that secondary cultures of human adult bone marrow mesenchymal cells contain cells that can be grown as clones after limiting dilution and that some clones can be expanded over 20 cumulative population doublings and maintain the capacity to differentiate into osteoblasts, adipocytes, and chondrocytes. This establishes that a significant fraction of bone marrow mesenchymal cells indeed exhibit the two essential stem cell characteristics of extensive self-renewal capacity and multi-lineage potential.

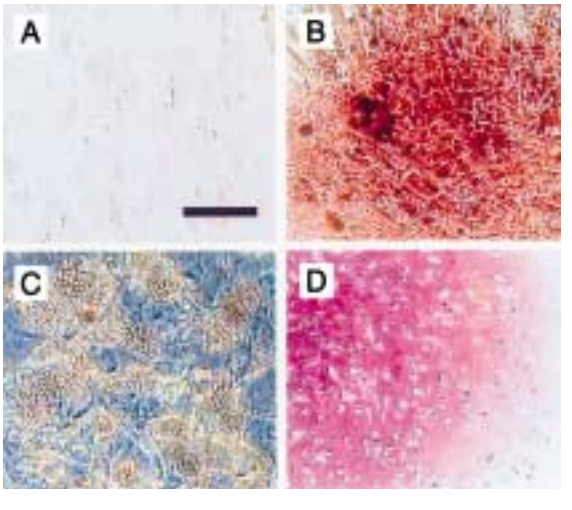

Figure 3. Clone D290 a-A6. Photomicrographs of control cells (A) and cells following osteogenic (B), adipogenic (C) and chondrogenic (D) differentiation. A,B: Alizarin red stain; C: phase contrast; D: Safranin O stain. Bar: 100μm.

It is noteworthy that all clones expanded to the T25 flask stage were able to form a mineralized matrix, indicating that the capacity to undergo osteogenic differentiation is not lost readily. This is in line with the data presented by Muraglia et al.[8] and earlier observations in our laboratory obtained with rat bone marrow mesenchymal cells (unpublished results) which suggested that osteogenic differentiation represents a default pathway. Among the clones that had lost multi-lineage potential, we detected in our experiments no particular preference to maintain either adipogenic or chondrogenic differentiation capacity.

Adipogenic differentiation, assessed here using a quantitative assay, was very variable. Many clones clearly produced mature adipocytes, however, only in low numbers (<1% of the culture). Other clones gave rise to >30% nile red-positive cells (donor 290). We set a low threshold and considered clones producing >0.3% adipocytes as competent for adipogenic differentiation. It is important to note that we encountered also very variable adipogenicity with uncloned marrow mesenchymal cells (range of observations 0-40% nile red-positive cells, depending on donor and passage number (data not shown)).

In an earlier study Kuznetsov et al.[17] had transplanted single colony-derived human marrow stromal cells into nude mice and observed that only 59% of the transplants were able to form bone *in vivo*. On the other hand the osteogenic potential was 100% when cells originating from mixed colonies were used. These results, suggesting heterogeneity in osteogenic potential among individual colonies, appear to be in contrast to the results that we describe. However, it may be assumed that osteogenic differentiation *in vitro* is more readily achieved than bone formation *in vivo*. Factors not relevant in the *in vitro* context may be required for "penetrance" of the osteogenic phenotype in the animal model.

The high cloning efficiency observed in our study was

	Differentiation		
	Osteogenic	**Adipogenic**	**Chondrogenic**
Clone Designation	calcium deposition (ug/well)	adipocyte number (%)	Safranin O-positive pellet
a-A3	1.33	1.26	-
a-A6	2.42	32.18	++
b-D2	2.18	-	-
b-H10	2.86	2.97	+
b-H2	3.02	7.23	+
b-H7	2.62	0.39	++
d-C2	3.64	45.06	++
d-E3	2.75	0.42	++
d-G10	2.14	-	-
d-H12	2.53	-	-

Table 3. Results of cell differentiation assays carried out with expanded clones of human MSCs (donor D290). See Table 2 legend.

unexpected as MSCs from human bone marrow have been described to undergo apoptosis in low density culture. When seeded below 2000 cells/cm^2, proliferation ceased and >60% of the cells were apoptotic[14]. We have no straightforward explanation for these apparently differing results. One reason for a better performance of the cells in our hands could be the fact that we used subconfluent, proliferating cells for low density seeding. Cells from confluent cultures had been used by van den Bos et al.[14].

Our experiments further confirm the positive effects of bFGF on MSC proliferation[9,10] and indicate that inclusion of this growth factor during prolonged *in vitro* culture is compatible with the maintenance of multi-lineage potential. Using FGF as a medium supplement may reduce serum requirements and may also reduce the dependence on specific serum lots to allow MSC isolation and culture[7]. In contrast, the use of medium conditioned by confluent cell cultures, which we expected to benefit clonal growth, turned out to have the opposite effect. Confluent bone marrow mesenchymal cells obviously secrete factors that are inhibitory for cell proliferation. Such factors may act as negative feedback regulators in the bone marrow context *in vivo* and their identification and/or isolation might constitute an interesting research project.

Finally, our data indicate that secondary cultures of human adult bone marrow mesenchymal cells prepared according to standard procedures represent a mixture of cells with respect to proliferation and differentiation potential. Although these cells appear homogenous phenotypically and when analyzed for a number of cell surface markers by flow cytometry[5] such cultures clearly contain cells that lack MSC characteristics. It remains a challenge to find cell surface markers that alone or in combination specifically define pluripotent MSCs.

Acknowledgements

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