Rheumatology 2009;48:1057–1064
Advance Access publication 14 July 2009

doi:10.1093/rheumatology/kep192

Distinct mesenchymal progenitor cell subsets in the adult human synovium

Alexandra Karystinou[1], Francesco Dell'Accio[2], Tobias B. A. Kurth[1], Henning Wackerhage[1], Ilyas M. Khan[3], Charles W. Archer[3], Elena A. Jones[4], Thimios A. Mitsiadis[5] and Cosimo De Bari[1]

Objective. To analyse the heterogeneity at the single-cell level of human mesenchymal progenitor cells from SM.

Methods. Cell populations were enzymatically released from the knee joint synovium of adult human individuals. Single cell-derived clonal populations were obtained by limiting dilution and serially passaged to determine growth rates. Phenotypic analysis was carried out by flow cytometry. Replicative senescence was assessed by the senescence-associated β-galactosidase staining. Telomere lengths were determined semiquantitatively by Southern blotting. Telomerase activity was measured using a real-time quantitative telomerase repeat amplification procedure. Culture-expanded clonal populations were subjected to *in vitro* differentiation assays to investigate their mesenchymal multipotency.

Results. The 50 clones analysed displayed wide variations in the proliferation rates, even within the same donor sample. The time taken to reach 20 population doublings ranged from 44 to 130 days. The phenotype of the clones tested was compatible with that of mesenchymal stem cells. Mean telomere lengths ranged from 5.2 to 10.9 kb with positive linear trend with telomerase activity, but no correlation with proliferative rates or cell senescence. All clones tested were capable of chondrogenic and osteogenic differentiation, though with large variability in potency. In contrast, only 30% of the clones were adipogenic.

Conclusions. We report for the first time the co-existence, within the synovium, of progenitor cell subsets with distinct mesenchymal differentiation potency. Our findings further emphasize the need for strategies to purify cell populations with the clinically desired tissue formation potentials.

Key words: Mesenchymal stem cells, Synovium, Clones, Chondrogenesis, Osteogenesis, Regenerative medicine.

Introduction

Increasing evidence supports the use of stem cells in the clinic. A major concern is the large structural and clinical variability in outcome, which limits standardization of stem cell-based therapies and impedes comparison of clinical study outcomes [1, 2]. Such variability is at least partly due to donor-related factors and the inconsistency of stem cell preparations [3], as highlighted by the report of contrasting results in clinical trials of cardiac cell therapy [4].

Mesenchymal stem cells (MSCs) are clonogenic undifferentiated cells capable of both self-renewal and differentiation into lineages of mesenchymal tissues, including cartilage, bone, adipose tissue and skeletal muscle. The 'conventional' MSCs are those obtained from bone marrow (BM) [5–7]. However, MSCs have been isolated from several other tissues, including the SM [8] and SF [9], periosteum [10–12], skeletal muscle [13], adipose tissue [14] and articular cartilage [15–17]. In view of their presence in many easily accessible adult tissues and their ability to differentiate into multiple lineages, MSCs are excellent candidates to be used for skeletal tissue repair in regenerative medicine.

We reported the isolation and characterization of multipotent MSCs from adult human SM (SM-MSCs) [8]. These cells were derived from mixed unselected populations of adherent synovial cells following *in vitro* expansion. SM-MSCs could be expanded for up to at least 30 population doublings (PDs) with limited senescence and maintenance of multilineage differentiation capacity towards chondrogenesis, osteogenesis, adipogenesis and skeletal myogenesis [8, 18–20].

The strategy for obtaining cell populations with predictable tissue formation capacity would at least partly depend on the presence of either multiple functionally distinct cell subsets or more primitive stem cells capable of multilineage differentiation. Human SM-MSCs, like the BM counterparts, appear heterogeneous in their growth and differentiation abilities [19, 21]. Mixed cells are likely to produce an unpredictable biological activity when used in the clinic. More functionally homogeneous cell populations are desirable.

Multipotency of SM-MSCs was inherent to the single cell when five clones were investigated as a proof-of-concept [8]. A more systematic characterization of synovial progenitors at the single-cell level is needed to investigate the possible co-existence of cell subsets with distinct tissue formation capacity. Such characterization will help devise novel strategies for purification of cell subsets with a consistent and predictable biological activity.

In the present study, we analysed human synovial progenitor cells at the single-cell level by studying clonal cell populations derived by limiting dilution. We confirm wide variations in growth and differentiation potentials between clones even within the same donor sample and, for the first time, report the co-existence, within the synovium, of progenitor cell subsets with distinct mesenchymal differentiation potency.

Materials and methods

Harvest of synovium and isolation of cells

SM was collected aseptically, after informed consent, from the knee joints of six human donors (Table 1). Three donors had a history of OA and underwent total knee replacement, whereas the remaining three donors had no history of knee joint disorders and were referred to as normal. Ethical approval was obtained from the local ethics committee (St Thomas' Hospital Research Ethics Committee).

[1]Division of Applied Medicine, University of Aberdeen, Aberdeen, [2]Centre for Experimental Medicine and Rheumatology, Queen Mary, University of London, London, [3]Cardiff Institute of Tissue Engineering and Repair, Cardiff University, Cardiff, [4]Leeds Institute of Molecular Medicine, University of Leeds, Leeds, UK and [5]Institute of Oral Biology, University of Zurich, Zurich, Switzerland.

Submitted 4 February 2009; revised version accepted 5 June 2009.

Correspondence to: Cosimo De Bari, Division of Applied Medicine, Institute of Medical Sciences, Foresterhill, Aberdeen AB25 2ZD, UK.
E-mail: c.debari@abdn.ac.uk

TABLE 1. Synovial tissue specimens used in the study

Donor	Age, years	Sex	Clinical history	No. of clones	Passage
A	55	M	N	7	Fresh
B	74	F	OA	6	P6
C	58	F	N	9	P7
D	73	F	OA	8	Fresh
E	90	F	N	10	Fresh
F	74	F	OA	10	P1

M: male; F: female; N: normal (donors had no history of knee joint disorders and did not have active infections or tumours); P: number of passage of parental cells, from which clones were derived by limiting dilution.

SM samples were rinsed twice with Hanks' Balanced Salt Solution (Invitrogen, Paisley, UK) supplemented with 2× antibiotic-antimycotic solution (100 U/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml amphotericin B; Invitrogen), finely minced and digested with 0.2% collagenase (Invitrogen) in growth medium consisting of high-glucose DMEM (Lonza, Slough, UK) supplemented with 10% fetal bovine serum (FBS; Invitrogen) and antibiotics. Following overnight incubation at 37°C, cells were collected by centrifugation, washed, further digested for 5 min at 37°C with trypsin–ethylenediamine tetraacetate (EDTA) (0.25% trypsin, 1 mM EDTA; Invitrogen), washed again and filtered through a 70 µm cell strainer (Marathon Laboratory, London, UK). Red blood cells were lysed. Cells were then counted using trypan blue (Sigma, Poole, UK) to assess cell viability, and were either culture expanded in growth medium or used to generate clonal populations by limiting dilution.

Cell cloning and culture expansion

Cell cloning was performed by limiting dilution. Freshly isolated or cultured cells were suspended in growth medium and plated at a density of 0.3 viable cells per well in 96-well, flat-bottomed culture plates. Four plates were used for each donor sample. At 30 days, aggregates of more than 32 cells were scored as clones. Thirty-two cells were chosen as this represents a population of cells derived from more than five PDs of a single cell, thereby discounting a transit-amplifying cell [22]. Up to 10 clones per donor sample were selected. Cells were culture expanded in monolayer in growth medium at 37°C in a humidified atmosphere of 5% CO_2. The medium was replaced every 3 days. Clonal cell populations were subcultured upon reaching confluence. For each clone, aliquots were cryopreserved.

Kinetics of growth

Cells were plated at a density of 3000 cells/cm^2 in tissue culture plates and were continuously passaged at the same density whenever they reached sub-confluence. The number of viable cells was determined at each passage using a haemocytometer. The growth rate of each clone was expressed as number of cumulative PDs during time. A graph was plotted against duration in culture for a single cell to undergo 20 PDs.

Senescence-associated β-galactosidase staining

Senescence-associated β-galactosidase (SA-β-gal) staining was performed in chamber slides as described [23]. Cells were counterstained with Neutral Red (1% in water). Human Embryonic Kidney (HEK) 293 cells and early passage (P4-P5) SM-MSCs were used as a negative control. Late passage (P18) SM-MSCs cells and senescent human dermal fibroblasts were used as a positive control.

Osteogenesis assay

The *in vitro* osteogenesis assay was performed as described earlier [8]. Each clone was plated in triplicate. Calcium content was determined at 3 weeks of treatment with osteogenic medium and normalized to protein content as described previously [19]. Total calcium was calculated from standard solutions and expressed as micrograms per microgram of protein content. Protein content was determined in parallel wells by the bicinchoninic acid protein assay (Sigma), using bovine serum albumin as standard.

To stain calcium deposits, cells were rinsed twice with PBS, fixed with 4% PFA and covered with alizarin red S solution [2% aqueous solution (pH 4.2); Sigma) for 3 min. Cultures were then washed thoroughly with distilled water.

Chondrogenesis assay

The *in vitro* chondrogenesis assay was performed by combining micromass culture and treatment with 10 ng/ml of TGF-β1 (Biosource, Nivelles, Belgium) in a serum-free medium. Micromass cultures were obtained by pipetting 20-μl droplets of cell suspension at a density of 2×10^7 viable cells/ml into individual wells of 24-well plates. After cells were allowed to attach without medium for 3 h, a serum-free medium consisting of high-glucose DMEM supplemented with 50 μg/ml ascorbic acid, ITS+ and 100 nM dexamethasone (all from Sigma) was added. The day of plating in micromass culture was designated as Day 0. Starting on Day 1, when the culture medium was changed, TGF-β1 was added to the culture medium every other day, at a final concentration of 10 ng/ml. After 6 days, cultures were rinsed twice with PBS, fixed with methanol for 30 min at −20°C, washed and covered with Alcian blue at pH 0.2 [0.5% Alcian blue 8 GS (Carl Roth, Karlsruhe, Germany) in 1 N HCl]. After overnight staining, cultures were washed extensively with distilled water. For quantitative analyses, Alcian blue-stained cultures were extracted with 200 μl of 6 M guanidine HCl for 6 h at room temperature. The absorbance of the extracted dye was measured at 630 nm by spectophotometry.

Adipogenesis assay

The *in vitro* adipogenesis assay was performed as described earlier [24]. One hundred cells were plated in 60-cm^2 cell culture dishes and allowed to grow for 2 weeks. The medium of the treated samples was then replaced with adipogenic induction (AI) medium, consisting of growth medium supplemented with 1 μM dexamethasone, 0.5 mM 3-isobutyl-1-methyl-xanthine, 10 μg/ml insulin and 100 μM indomethacin (all from Sigma). After 72 h, the AI medium was replaced with adipogenic maintenance (AM) medium, consisting of growth medium supplemented with 10 μg/ml insulin. After 24 h, the AM was discarded and cells received two more treatments with alternating AI and AM. By the end of the third treatment, the cells were maintained in AM for a total of 21 days from the beginning of treatment. Cells were then washed with PBS, fixed with 4% PFA, washed with water, rinsed with 60% isopropanol, stained with Oil Red O solution [0.1% Oil Red O (Sigma) in 60% isopropanol], rinsed again with 60% isopropanol and washed extensively with water. The total number of colonies was determined after counterstaining with crystal violet (0.5% in methanol).

Telomere length assay

Genomic DNA was extracted from clonal cell monolayers using one-step lysis and DNA exclusion columns (Nexttec, MP Sciences, Wellingborough, UK) and the amount quantified fluorescently using PicoGreen (Molecular Probes, Eugene, Oregon, USA). Telomere lengths were determined semi-quantitatively by Southern blotting using TeloTAGGG telomere length assay, according to the recommendations of the manufacturer (Roche, Lewes, UK). Mean telomere lengths were determined by densitometric scanning of exposed X-ray film.

Telomerase activity

Comparative analysis of telomerase activity was performed using a real-time quantitative telomerase repeat amplification procedure (RTQ-TRAP) as described [25]. Cell extracts were obtained by adding 200 μl of lysis buffer per 1×10^6 cells (lysis buffer; 10 mM Tris pH 8.3, 1.5 mM $MgCl_2$, 1 mM ethylene glycol tetraacetic acid (EGTA), 10% glycerol, 0.5% 3[(3-Cholamidopropyl)dimethylammonio]-propanesulfonic acid (CHAPS) buffer, 1 mM phenylmethylsulphonyl fluoride (PMSF), 5 mM β-mercaptoethanol and 200 U RNAsin). Each RTQ-TRAP reaction was composed of 1×SYBR Green Mastermix (Eurogentec, Seraing, Belgium), 10 mM EGTA, 0.2 μg T4 gene protein, 0.3 μM primers TS (5′-AATCCGTCGAGCAGAGT T-3′) and ACX (5′-GCGCGG[CTT ACC]$_3$CTAACC-3′) and 25×10^3 cell extract in a final volume of 25 μl. The reaction mixture was incubated at 25°C for 20 min to allow TS primer extension by telomerase, heated to 95°C for 10 min to activate *Taq* polymerase, followed by 40 cycles at 95°C for 20 s, 50°C for 30 s and 72°C for 90 s. Telomerase activity in cell samples was calculated based on the threshold cycle (C_T). All samples were run in triplicate, and lysis buffer was used as a negative control. Immortal human leukaemia cell line 60 (HL60) was used as a positive control for telomerase activity. HL60 cell extracts were serially diluted and assayed by RTQ-TRAP to generate a standard curve that was used to derive telomerase activity based on the HL60 cell equivalent units.

Phenotyping of clonal cell populations using three-colour flow cytometry

Culture-expanded clonal cell populations were used for flow cytometry at 10^5 cells/test. Phycoerythrin (PE)-conjugated antibodies were: CD166 and CD73 (Pharmingen, Oxford, UK), CD105 and CD90 (Serotec, Oxford, UK) and SSEA-4 (R&D Systems, Abingdon, UK). FITC-conjugated antibodies were: CD45 (Dako, High Wycombe, UK), CD13 and CD31 (Serotec), CD81 (Pharmingen) and CD271 (Miltenyi Biotec, Bisley, UK). Isotype-specific negative control antibodies were purchased from Serotec. Data were acquired using BD FACScan and dead cells were gated out based on propidium iodide exclusion (Sigma). All flow cytometry data were analysed with WinMDI v8 (Scripps Research Institute, La Jolla, California, USA).

Statistical analysis

Data are presented as mean ± S.D. apart from flow cytometry data, which are shown as median (range) of percentage positive cells. Each assessment was performed on two or three cultures per clonal cell population. Pearson's product moment correlation coefficient r was calculated in order to assess the linear associations between variables. Means were compared using one- and two-factorial analysis of variance (ANOVA) as *a priori* test and Fisher's least significant difference as *a posteriori* test. For all correlations and comparisons of means, $P < 0.05$ was considered significant.

Results

Growth potential of clonal populations

We first analysed the proliferative capacity of single cell-derived clonal populations. Clonal populations were derived by limiting dilution in 96-well plates. Four plates were set up for each of the six individual donors. A total of 50 clones were randomly selected (see Table 1 for details). Out of these 50 clones, three clones stopped growing after 17–18 PDs, whereas the remaining 47 clones were able to grow beyond 20 PDs. More in detail, seven clones reached 25 PDs, 25 clones grew beyond 30–35 PDs, 11 clones exceeded 40 PDs and four clones grew beyond 50 PDs.

The proliferative capacities of the individual clones were assessed according to the time taken by a single cell to undergo 20 PDs. A wide variation in the proliferation rates was observed between the clones, across the donors and within the same donor. The time taken to reach 20 PDs ranged from 44 to 130 days (Fig. 1). The clones obtained from donor sample B were growing faster and displayed less variation in their proliferative capacities than the clones of the other samples tested ($P < 0.05$). The clones of donor A were significantly faster than the clones of donors C and E ($P < 0.05$).

Phenotype

To evaluate the phenotype of the expanded clonal cells, we performed fluorescence-activated cell sorting analysis, testing five representative clones (A2, A8, D1, D3 and E10) for the expression of a marker set associated with multipotent MSCs [5, 10, 19] (Fig. 2). All clones tested were negative for the haematopoietic cell marker CD45 and the endothelial cell marker CD31, both known to be not expressed by MSCs [5]. CD271/LNGFR was also undetectable, as reported with culture-expanded non-clonal MSC populations [19, 26]. Clones displayed variable expression of the MSC-positive markers CD13, CD105, CD73, CD166, SSEA4, CD81 and CD90. These data indicate that the clonal cells tested display a phenotype compatible with culture-expanded MSCs.

Replicative senescence, telomere length and telomerase activity

MSCs are somatic cells that display a long-term self-renewal capacity *in vitro* that is dependent upon factors such as replicative senescence, telomere length and telomerase activity. We first investigated the occurrence of cell senescence by assessing, during clonal culture expansion, the SA-β-gal, an assay that detects the presence of the β-gal enzyme expressed by senescent cells [23]. For this assay, we selected clones of donor A, which were heterogeneous in their proliferative capacity, and clones of donor B, which instead displayed similar and overlapping growth curves (Fig. 1 and data not shown). After 18 PDs, cells were mostly negative and had a fibroblast-like morphology, whereas after 30–35 PDs cells stained intensely for SA-β-gal and were morphologically flat and bigger in size (Fig. 3A), features consistent with the senescent phenotype. An increase in the proportion of SA-β-gal$^+$ senescent cells during time in culture was observed in most of the clonal populations (Fig. 3B), although with variability across the clones tested. For Donor A, the increase was significant at 25 and 35 PDs compared with 18 PDs ($P < 0.05$), whereas for Donor B it was significant at 35 PDs compared with 18 and 25 PDs ($P < 0.05$).

We then measured telomere lengths and telomerase activity in 11 clonal populations after a number of PDs ranging between 23 and 49. Despite the extensive expansion in culture, all clones displayed relatively long telomeres, ranging from 5.2 (clone C6) to 10.9 kb (clone A5—Fig. 4A). Telomerase activity, determined as the equivalence of each clone to HL60 cell line, was between 0.0734 (B3) and 1.024 (E10). Mean telomere length displayed positive linear trend with telomerase activity ($r = 0.63$, $P < 0.05$; Fig. 4B). Neither telomere length nor telomerase activity correlated with replicative senescence, cumulative number of PDs or PD duration at the time of their measurements.

Differentiation potential of clonal populations

A total of 21 clones were tested for their ability to differentiate towards mesenchymal lineages using well-established *in vitro* assays (Fig. 5). These assays were performed after a cumulative number of PDs ranging between 22 and 28, with the exception of clone A2, tested after 41 PDs, and clones A8, A9 and B2, which were assessed after 48 PDs. There was considerable variation in

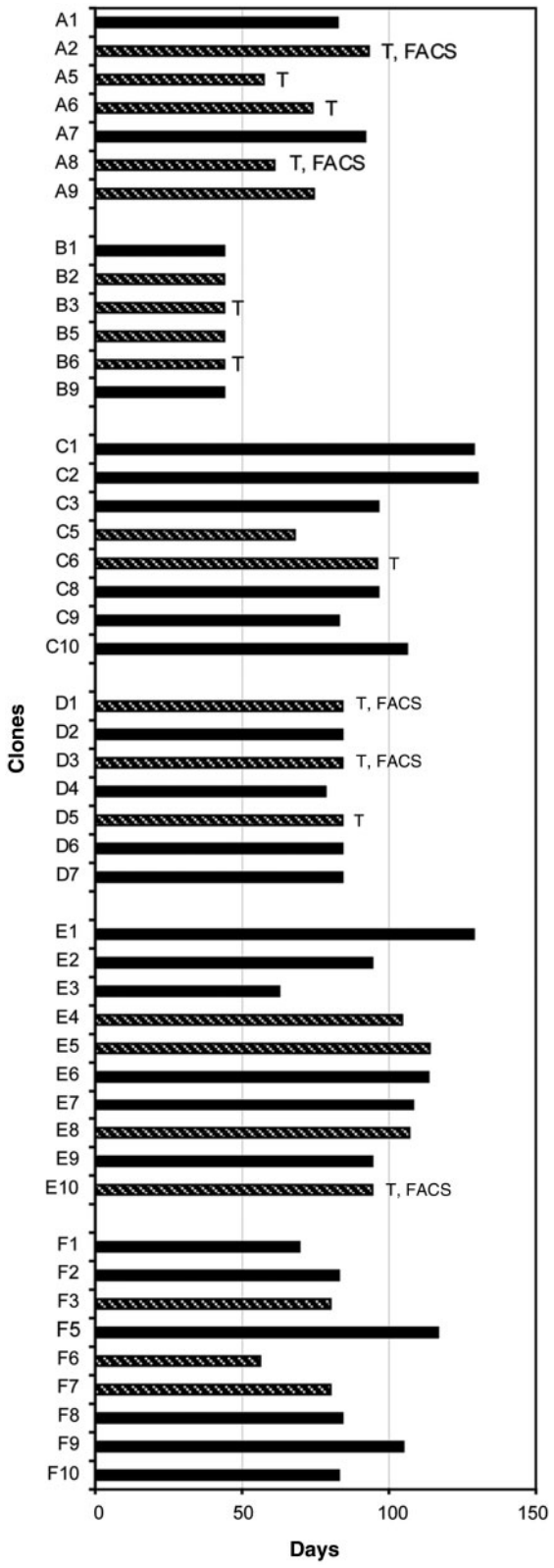

FIG. 1. Proliferation rates of individual clones to undergo 20 PDs. Each bar represents the time needed for each clone to reach 20 PDs. Striped bars indicate the clones that were tested for cell differentiation assays. The clones that were further analysed for telomere lengths and telomerase activity (T) and phenotype by flow cytometry (FACS) are also labelled. On the *y*-axis, capital letters and numbers indicate donors and corresponding clones, respectively.

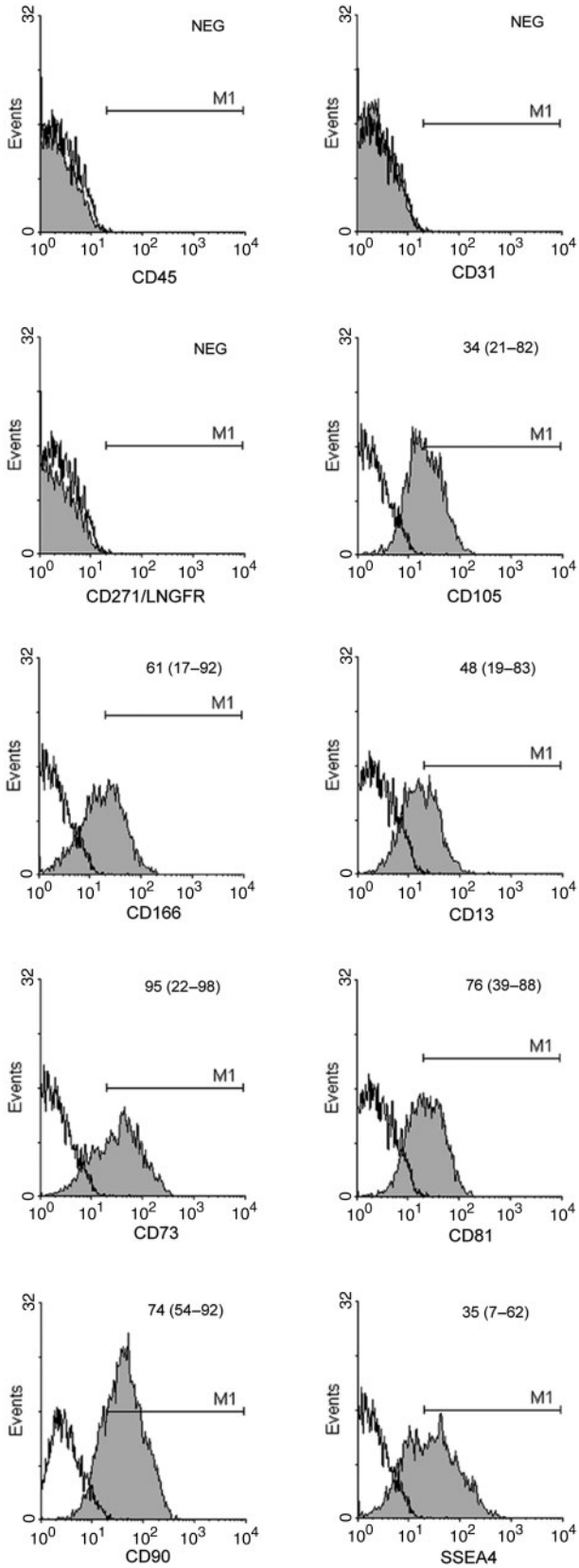

FIG. 2. Surface marker phenotype of synovial clonal cells following culture expansion. Results from a representative clone (A8) are shown. For each marker tested, values are shown as median (range) of the percentage of positive cells for the five clones tested. Solid histograms show marker expression; open histograms show negative isotype controls. Horizontal line shows positive cells.

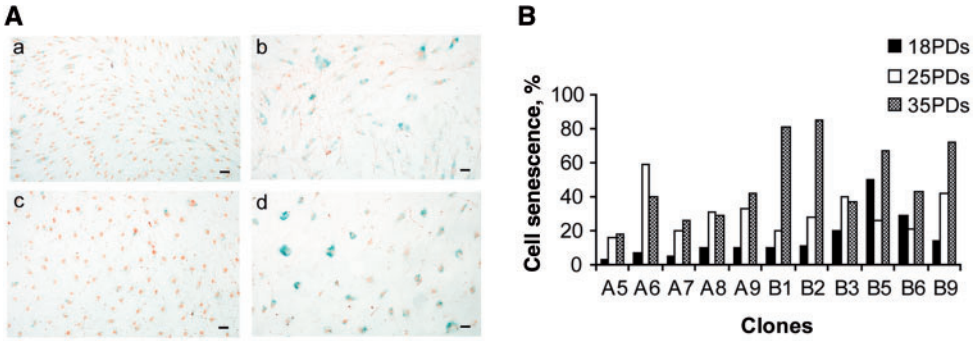

FIG. 3. SA-β-gal activity. (**A**) SA-β-gal staining of a fast-growing clone (A5) and a slow-growing clone (A6) throughout culture expansion. (a) and (b) show clone A5 after 18 PDs (54 days) and 35 PDs (144 days), respectively; (c) and (d) show clone A6 after 19 PDs (54 days) and 30 PDs (171 days), respectively. Senescent cells appear blue. Counterstaining is with neutral red. Scale bar = 50 μm. (**B**) Percentage of SA-β-gal$^+$ (senescent) cells in clones selected from Donors A and B after 18, 25 and 35 PDs. Results indicate a trend in progressive increase in replicative senescence paralleling the increase in PDs.

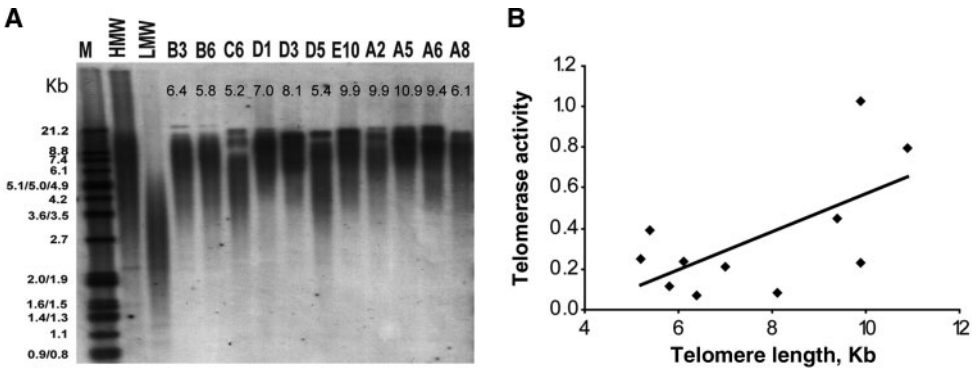

FIG. 4. Telomere length and telomerase activity. (**A**) Southern blotting for telomeres. Eleven culture-expanded clonal populations (as indicated) were analysed by Southern blotting for the length of their telomeres. Note the apparent gaps in the DNA streaks, which confirm the purity of single cell-derived clonal populations. The mean telomere length of each clone is indicated in kilobase pairs. The U937 cell line was used as a positive control (HMW: high-molecular weight; mean telomere length 10.2 kb), and the HL-60 cell line was used as a negative control (LMW: low molecular weight; mean telomere length 3.9 kb). (**B**) Plot of mean telomere length (kb) *vs* telomerase activity, expressed as arbitrary units (ratio of the clonal cell equivalents divided by the HL60 cell equivalent units) ($r = 0.63$, $P < 0.05$).

the extent of differentiation between the clonal populations tested, across donors and within the same donor.

All clones were chondrogenic as determined by alcian blue staining of TGF-β1-treated micromasses, with absorbance ranging from 0.15 to 0.44. One clone (D1) was highly chondrogenic with an absorbance of 0.76. Untreated clones were negative (Fig. 5A and B).

All clones responded to osteogenic treatment in monolayers as assessed by Alizarin red staining for calcium (Fig. 5C). For quantitation, we measured calcium deposition, which we recently reported to be significantly correlated, under specific conditions, with the amount of ectopic bone formation *in vivo* following subcutaneous transplantation of human MSC-seeded scaffolds in nude mice [19]. Under our conditions, untreated clones did not show any calcium deposition (Fig. 5C). In contrast, treated clones responded with deposition of calcium ranging from 0.6 to 4.1 μg per microgram of proteins (Fig. 5D).

Adipogenic differentiation was assessed by Oil Red O staining. While all clones gave rise to colonies as assessed by staining with crystal violet, only seven out of the 21 clones tested generated colonies of adipocytes, which stained with Oil Red O (Fig. 5E). The rate of Oil Red O-positive colonies varied between 1.6 (B6) and 38% (E4—Fig. 5F).

Under our experimental conditions, the differentiation potentials of the tested clonal populations did not correlate with cumulative PDs, markers, replicative senescence, telomere length or telomerase activity. In addition, no correlation was observed between chondrogenic, osteogenic and adipogenic potentials of individual clonal populations.

Discussion

Several studies indicate the therapeutic utility of MSCs in humans [27–32]. However, inconsistency of MSC preparations limits standardization. To develop standard protocols for a cell transplant with the prospect to be used routinely in the clinic, a requirement is the obtainment of a sufficient number of well-defined MSCs, which should possess fundamental characteristics such as proliferative capacity as well as known potency with related ability to deposit the required matrix proteins. We previously reported that human SM-MSCs possess high self-renewal capacity and a multipotency that is inherent to the single cell when using a limited number of clones as a proof-of-concept [8]. Here, we characterized synovial mesenchymal cells more systematically at the single-cell level using clonal populations obtained by limiting dilution, with a goal of discerning multipotent stem cell pools from progenitor cell populations with more restricted potency. Such information is clinically relevant for the standardization of tissue engineering applications employing human MSCs.

It is known from previous studies that single cell-derived clonal MSC populations become heterogeneous during culture expansion [19, 33, 34]. The phenotypic heterogeneity within each clonal population may be due to the different status of the individual cells of the single cell progeny (e.g. different phases of cell cycle, symmetric or asymmetric cell division and different stages of differentiation). Nonetheless, we opted to study, in depth, clonal populations as cells within a single cell-cloned population are genetically and perhaps epigenetically homogeneous,

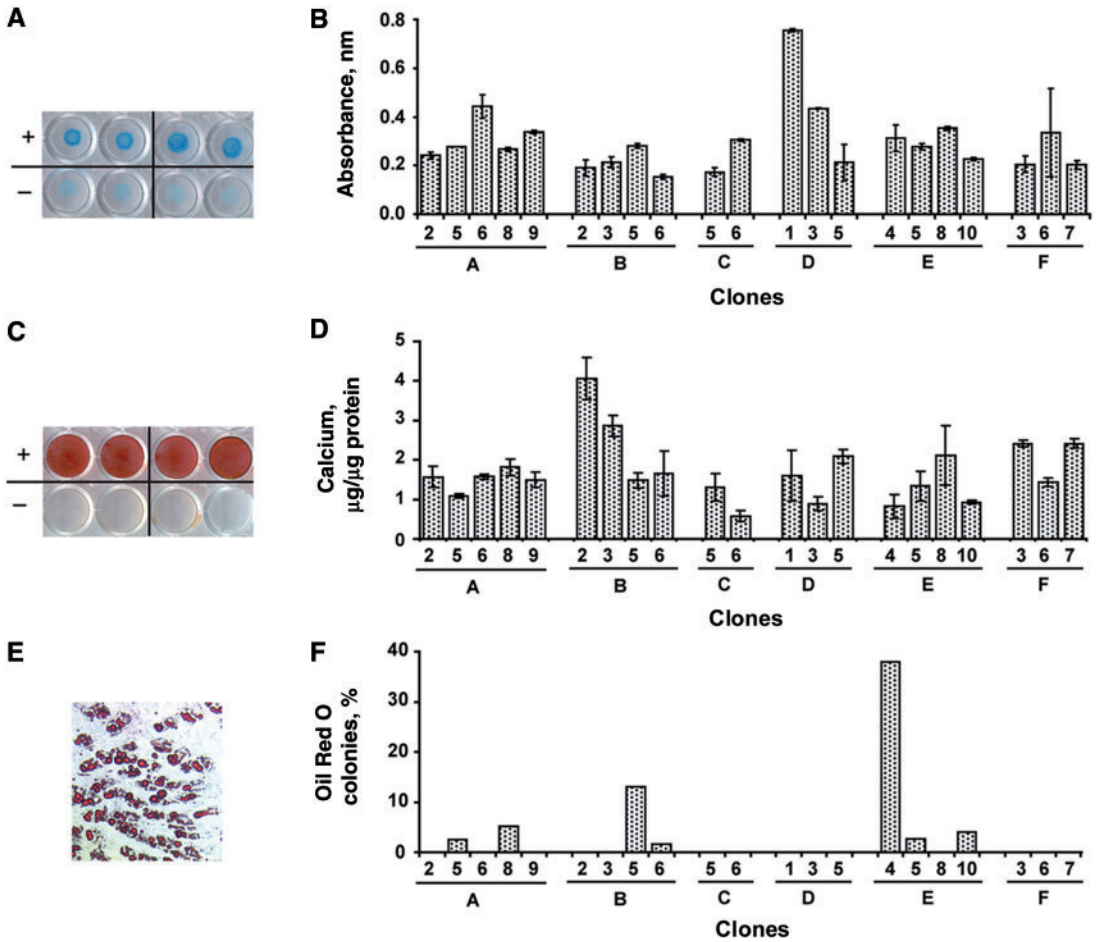

FIG. 5. *In vitro* mesenchymal differentiation assays. Twenty-one culture-expanded clonal populations were subjected to differentiation assays. (**A** and **B**) Chondrogenic differentiation. Cells were plated in micromass culture and were either treated (+) or not treated (–) with TGF-β1 for 6 days. (**A**) Representative picture of Alcian blue stained micromasses (in duplicate). (**B**) Stained micromasses were extracted with 6 M guanidine HCl, and the absorbance of the extracted dye was measured at 630 nm. Data are shown as the mean ± S.D. of duplicates. (**C** and **D**) Osteogenic differentiation. Clonal cell populations in monolayers were either treated (+) or not treated (–) with osteogenic medium for 3 weeks. (**C**) Representative picture of Alizarin red stained cells (in duplicate). (**D**) Quantitation of calcium deposition (expressed as micrograms of calcium per microgram of protein content, determined in parallel wells). Data are shown as mean ± S.D. of triplicates. (**E** and **F**) Adipogenic differentiation. (**E**) Representative picture of Oil Red O-stained cells upon adipogenic treatment. (**F**) Percentage of Oil Red O-positive colonies per each clone.

which would explain why clonal cells maintain in culture a certain degree of functional consistency [19].

In keeping with our previous study [19], wide variations in the proliferation rates were observed between clones, even within clones from the same donor sample. This suggests that plastic adherent non-clonal synovial MSCs do not consist of a uniform population of stem cells. It is worth mentioning that not all of the clones were culture-expandable under our conditions, with three of the 50 clones tested unable to grow beyond 20 PDs.

One factor possibly accounting for the growth heterogeneity of clonal populations is cell senescence, a common phenomenon occurring to various degrees in somatic cells during *in vitro* expansion. As expected, and in keeping with our previous work with non-clonal SM-MSCs [8], we observed a general increase in SA-β-Gal$^+$ cells with increasing PDs. Highly expressing SA-β-gal cells were more enlarged and flatter in morphology, compatible with the senescent phenotype. Interestingly, in some clones the percentage of SA-β-gal$^+$ cells decreased to then increase again over accumulating PDs, rather than increase continuously. This could be due to variability in the assay, or re-activation of telomerase activity [35], or emergence within the clonal progeny of cell subsets having clonogenic advantage due, for instance, to somatic mutations [36]. In addition, there is evidence that replicative senescence may be reversible as some senescent cells can, under specific conditions, recover from growth arrest, re-enter cell cycle and replicate their DNA [37, 38].

Although the phenomenon of senescence is not fully understood, it is believed that rather than being the result of cell ageing, it is more likely to be a protective mechanism, not necessarily irreversible, activated by normal cells in response to various types of stress such as carcinogenesis [39]. Indeed, SM-MSCs do not form tumours when injected into mice [18], a property that would favour their use in cell-based therapies.

Telomere maintenance by telomerase is thought to be necessary for a high number of cell divisions [40]. We observed a positive linear trend between telomerase activity and mean telomere length, supporting the accuracy of our experiments. However, under our experimental conditions, *in vitro* growth rate and cell senescence, known to be related to telomere shortening [41], did not show any correlation with mean telomere length or telomerase activity, and clonal populations were able to grow when their mean telomere lengths were ~5–6 kb. A clear correlation was reported between telomere length and proliferative capacity of human BM non-clonal MSCs, with MSC populations stopping dividing at ~10 kb [42]. These apparent discrepancies could be due to donor-related factors, culture conditions, use of clonal *vs* non-clonal MSC populations or different MSC tissue sources. Together with the high threshold telomere length reported

in BM-MSCs [42], our findings suggest that mechanisms independent of telomere shortening [41], such as accumulated damage, contribute to cell senescence and growth arrest in cultured MSCs.

Variations in the growth potential of clonal populations could be due to the co-existence of multiple cell types selected by plastic adherence and grown under our culture conditions. Indeed, the identity of synovial MSCs in relation to other stromal cells such as fibroblasts is not known. Both synovial MSCs and fibroblasts are derived following enzymatic digestion of synovial tissue and culture expansion of the plastic-adherent fibroblast-like cells. Culture-expanded clonal populations in our study displayed a phenotype compatible with conventional MSCs and expressed SSEA-4, which was not detected in 3T3 fibroblasts [43]. However, markers alone would not be sufficient to rule out the presence of fibroblasts or their progeny in our clones as culture conditions are known to affect cell phenotype. In addition, primary fibroblasts derived from various human tissues including lung and skin were reported to contain cells that were able to differentiate into osteoblasts, chondrocytes and adipocytes [44]. Investigations are therefore needed to elucidate the relationship between MSCs and fibroblasts.

We previously reported that SM-MSCs are multipotential with a single cell-inherent ability to differentiate down the chondrogenic, osteogenic and adipogenic pathways *in vitro*, when using a few clones as a proof-of-concept [8]. In the present study, we analysed 21 clones from six donors and observed that all clonal populations were chondro-osteogenic, whereas seven of the 21 clones were also adipogenic under our experimental conditions. When the same *in vitro* cell differentiation assays were performed using parental cells (i.e. mixed non-clonal cell populations), SM-MSCs were tripotential (data not shown). Altogether, our findings suggest the co-existence in synovium of progenitor cell subsets with distinct differentiation abilities. It has to be noted, however, that in order to characterize single cell clonal populations high numbers of cells are required to perform all the tests described in this study. The extensive culture expansion may have caused, in some clonal populations, loss of adipogenic potential that was originally present in the cells, as has been suggested for BM-MSCs [45]. It is thus possible that some synovial clones retained their multipotentiality through a high number of PDs, whereas other clones lost the ability to form adipocytes whilst retaining chondro-osteogenic capacity.

To our knowledge, this is the first study to report the existence of single cell-derived clonal populations in the synovium that are chondro-osteogenic but unable to differentiate into adipocytes. As reported with BM-MSCs [45], we did not observe clones with a differentiation potential restricted to the osteo-adipogenic or to the chondro-adipogenic phenotype.

In a previous study with BM-derived clonal MSC populations [46], wide variations were observed in clonal cell replication, which were directly related to the clonal differentiation potentials. In our study, we did not observe any correlation between any feature of growth rate and the clonal differentiation potential. All these findings indicate the need of developing protocols for purification of cells that display functional homogeneity and predictable biological behaviour for cell-based therapy. In the present study, as well as in our previous work [19], clones expressed the putative MSC markers, but the markers tested did not correlate with the differentiation potential. This would emphasize the need to search for novel cell surface markers of MSCs that would be linked to specific biological behaviours and could therefore be used as quality controls in the cell manufacturing process.

The generation of consistent MSC-based therapeutic protocols is a pre-requisite to translating stem cell technologies into routine clinical application. We do not advocate the use of clonal populations in the clinic, as this would be impractical and costly. However, studies of clonal populations will help in the identification of marker sets for prospective purification of mesenchymal stem/progenitor cell subpopulations, functionally homogeneous in their clinically preferred differentiation capacities. Such purification will increase the consistency of cell preparations for clinical use.

Rheumatology key messages

- This study reveals heterogeneity of synovial mesenchymal progenitor/stem cells and co-existence of cell subsets with distinct tissue formation abilities.
- This emphasizes the need of purification of progenitor cells displaying functional homogeneity for consistent cell therapy.

Acknowledgements

C.D.B. is a Fellow of the Medical Research Council, UK and F.D'A. is a Fellow of the Arthritis Research Campaign, UK.

Funding: This study was supported by MRC grant G108/620.

Disclosure statement: The authors have declared no conflicts of interest.

References

1 De Bari C, Dell'Accio F. Mesenchymal stem cells in rheumatology: a regenerative approach to joint repair. Clin Sci 2007;113:339–48.
2 De Bari C, Dell'Accio F. Cell therapy: a challenge in modern medicine. Biomed Mater Eng 2008;18(Suppl. 1):S11–17.
3 De Bari C, Pitzalis C, Dell'Accio F. Reparative medicine: from tissue engineering to joint surface regeneration. Regen Med 2006;1:59–69.
4 Rosenzweig A. Cardiac cell therapy—mixed results from mixed cells. N Engl J Med 2006;355:1274–7.
5 Pittenger MF, Mackay AM, Beck SC *et al.* Multilineage potential of adult human mesenchymal stem cells. Science 1999;284:143–7.
6 Caplan AI, Bruder SP. Mesenchymal stem cells: building blocks for molecular medicine in the 21st century. Trends Mol Med 2001;7:259–64.
7 Prockop DJ. Marrow stromal cells as stem cells for nonhematopoietic tissues. Science 1997;276:71–4.
8 De Bari C, Dell'Accio F, Tylzanowski P, Luyten FP. Multipotent mesenchymal stem cells from adult human synovial membrane. Arthritis Rheum 2001;44:1928–42.
9 Jones EA, English A, Henshaw K *et al.* Enumeration and phenotypic characterization of synovial fluid multipotential mesenchymal progenitor cells in inflammatory and degenerative arthritis. Arthritis Rheum 2004;50:817–27.
10 De Bari C, Dell'Accio F, Vanlauwe J *et al.* Mesenchymal multipotency of adult human periosteal cells demonstrated by single-cell lineage analysis. Arthritis Rheum 2006;54:1209–21.
11 Nakahara H, Bruder SP, Haynesworth SE *et al.* Bone and cartilage formation in diffusion chambers by subcultured cells derived from the periosteum. Bone 1990;11:181–8.
12 De Bari C, Dell'Accio F, Luyten FP. Human periosteum-derived cells maintain phenotypic stability and chondrogenic potential throughout expansion regardless of donor age. Arthritis Rheum 2001;44:85–95.
13 Asakura A, Komaki M, Rudnicki M. Muscle satellite cells are multipotential stem cells that exhibit myogenic, osteogenic, and adipogenic differentiation. Differentiation 2001;68:245–53.
14 Zuk PA, Zhu M, Mizuno H *et al.* Multilineage cells from human adipose tissue: implications for cell-based therapies. Tissue Eng 2001;7:211–28.
15 Dell'Accio F, De Bari C, Luyten FP. Microenvironment and phenotypic stability specify tissue formation by human articular cartilage-derived cells in vivo. Exp Cell Res 2003;287:16–27.
16 Alsalameh S, Amin R, Gemba T, Lotz M. Identification of mesenchymal progenitor cells in normal and osteoarthritic human articular cartilage. Arthritis Rheum 2004;50:1522–32.
17 Dowthwaite GP, Bishop JC, Redman SN *et al.* The surface of articular cartilage contains a progenitor cell population. J Cell Sci 2004;117:889–97.
18 De Bari C, Dell'Accio F, Vandenabeele F, Vermeesch JR, Raymackers JM, Luyten FP. Skeletal muscle repair by adult human mesenchymal stem cells from synovial membrane. J Cell Biol 2003;160:909–18.
19 De Bari C, Dell'Accio F, Karystinou A *et al.* A biomarker-based mathematical model to predict bone-forming potency of human synovial and periosteal mesenchymal stem cells. Arthritis Rheum 2008;58:240–50.
20 De Bari C, Dell'Accio F, Luyten FP. Failure of in vitro-differentiated mesenchymal stem cells from the synovial membrane to form ectopic stable cartilage in vivo. Arthritis Rheum 2004;50:142–50.
21 Ho AD, Wagner W, Franke W. Heterogeneity of mesenchymal stromal cell preparations. Cytotherapy 2008;10:320–30.

22 Jones PH, Watt FM. Separation of human epidermal stem cells from transit amplifying cells on the basis of differences in integrin function and expression. Cell 1993;73:713–24.
23 Dimri GP, Lee X, Basile G *et al.* A biomarker that identifies senescent human cells in culture and in aging skin in vivo. Proc Natl Acad Sci USA 1995;92:9363–7.
24 Sakaguchi Y, Sekiya I, Yagishita K, Muneta T. Comparison of human stem cells derived from various mesenchymal tissues: superiority of synovium as a cell source. Arthritis Rheum 2005;52:2521–9.
25 Hou M, Xu D, Bjorkholm M, Gruber A. Real-time quantitative telomeric repeat amplification protocol assay for the detection of telomerase activity. Clin Chem 2001;47:519–24.
26 Jones EA, Kinsey SE, English A *et al.* Isolation and characterization of bone marrow multipotential mesenchymal progenitor cells. Arthritis Rheum 2002;46:3349–60.
27 Quarto R, Mastrogiacomo M, Cancedda R *et al.* Repair of large bone defects with the use of autologous bone marrow stromal cells. N Engl J Med 2001;344:385–6.
28 Vacanti CA, Bonassar LJ, Vacanti MP, Shufflebarger J. Replacement of an avulsed phalanx with tissue-engineered bone. N Engl J Med 2001;344:1511–14.
29 Warnke PH, Springer IN, Wiltfang J *et al.* Growth and transplantation of a custom vascularised bone graft in a man. Lancet 2004;364:766–70.
30 Wakitani S, Imoto K, Yamamoto T, Saito M, Murata N, Yoneda M. Human autologous culture expanded bone marrow mesenchymal cell transplantation for repair of cartilage defects in osteoarthritic knees. Osteoarthritis Cartilage 2002;10:199–206.
31 Horwitz EM, Gordon PL, Koo WK *et al.* Isolated allogeneic bone marrow-derived mesenchymal cells engraft and stimulate growth in children with osteogenesis imperfecta: implications for cell therapy of bone. Proc Natl Acad Sci USA 2002;99:8932–7.
32 Horwitz EM, Prockop DJ, Fitzpatrick LA *et al.* Transplantability and therapeutic effects of bone marrow-derived mesenchymal cells in children with osteogenesis imperfecta. Nat Med 1999;5:309–13.
33 Colter DC, Class R, DiGirolamo CM, Prockop DJ. Rapid expansion of recycling stem cells in cultures of plastic-adherent cells from human bone marrow. Proc Natl Acad Sci USA 2000;97:3213–18.
34 Colter DC, Sekiya I, Prockop DJ. Identification of a subpopulation of rapidly self-renewing and multipotential adult stem cells in colonies of human marrow stromal cells. Proc Natl Acad Sci USA 2001;98:7841–5.
35 Bianchi A, Shore D. How telomerase reaches its end: mechanism of telomerase regulation by the telomeric complex. Mol Cell 2008;31:153–65.
36 Yamanishi Y, Boyle DL, Green DR *et al.* p53 tumor suppressor gene mutations in fibroblast-like synoviocytes from erosion synovium and non-erosion synovium in rheumatoid arthritis. Arthritis Res Ther 2005;7:R12–18.
37 Blank M, Shiloh Y. Programs for cell death: apoptosis is only one way to go. Cell Cycle 2007;6:686–95.
38 Brookes S, Rowe J, Ruas M *et al.* INK4a-deficient human diploid fibroblasts are resistant to RAS-induced senescence. EMBO J 2002;21:2936–45.
39 Ben-Porath I, Weinberg RA. The signals and pathways activating cellular senescence. Int J Biochem Cell Biol 2005;37:961–76.
40 Aubert G, Lansdorp PM. Telomeres and aging. Physiol Rev 2008;88:557–79.
41 Herbig U, Sedivy JM. Regulation of growth arrest in senescence: telomere damage is not the end of the story. Mech Ageing Dev 2006;127:16–24.
42 Baxter MA, Wynn RF, Jowitt SN, Wraith JE, Fairbairn LJ, Bellantuono I. Study of telomere length reveals rapid aging of human marrow stromal cells following in vitro expansion. Stem Cells 2004;22:675–82.
43 Gang EJ, Bosnakovski D, Figueiredo CA, Visser JW, Perlingeiro RC. SSEA-4 identifies mesenchymal stem cells from bone marrow. Blood 2007;109: 1743–51.
44 Sudo K, Kanno M, Miharada K *et al.* Mesenchymal progenitors able to differentiate into osteogenic, chondrogenic, and/or adipogenic cells in vitro are present in most primary fibroblast-like cell populations. Stem Cells 2007;25:1610–17.
45 Muraglia A, Cancedda R, Quarto R. Clonal mesenchymal progenitors from human bone marrow differentiate in vitro according to a hierarchical model. J Cell Sci 2000;113:1161–6.
46 Mareddy S, Crawford R, Brooke G, Xiao Y. Clonal isolation and characterization of bone marrow stromal cells from patients with osteoarthritis. Tissue Eng 2007;13:819–29.