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# Functional, molecular and proteomic characterisation of bone marrow mesenchymal stem cells in rheumatoid arthritis

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## ABSTRACT

**Objective:** Bone marrow (BM) mesenchymal stem cells (MSCs) are being considered as potential therapeutic agents in various inflammatory autoimmune diseases for their tissue-repair and anti-inflammatory tissue-protective properties. This study investigates the reserves and function, the molecular and proteomic profile and the differentiation potential of BM MSCs in patients with active rheumatoid arthritis (RA).

**Methods:** We evaluated the frequency of MSCs in the BM mononuclear cell fraction using a limiting dilution assay, the proliferative/clonogenic potential and the capacity of cells to differentiate towards the osteogenic/chondrogenic/adipogenic lineages using appropriate culture conditions. We also assessed the molecular and proteomic characteristics in terms of inflammatory cytokine gene and protein expression, the relative telomere length and the survival characteristics of BM MSCs.

**Results:** MSCs from patients with RA ( $n = 26$ ) and age- and sex-matched healthy individuals ( $n = 21$ ) were similar in frequency, differentiation potential, survival, immunophenotypic characteristics, and protein profile. Patient MSCs, however, had impaired clonogenic and proliferative potential in association with premature telomere length loss. Transcriptome analysis revealed differential expression of genes related to cell adhesion processes and cell cycle progression beyond the G1 phase. Previous treatment with methotrexate, corticosteroids, anti-cytokine and biological agents or other disease-modifying anti-inflammatory drugs did not correlate with the clonogenic and proliferative impairment of BM MSCs.

**Conclusion:** In spite of some restrictions related to the impaired clonogenic and proliferative potential, our findings support the use of autologous BM MSCs in RA and may have important implications for the ongoing efforts to repair tissue injury commonly seen in the course of the disease.

Adult multipotential stem cells isolated from a variety of tissues have been demonstrated to participate in tissue homeostasis and repair under the influence of appropriate signals. Among the most well characterised types of adult stem cells are the bone marrow (BM) derived mesenchymal stem cells (MSCs).<sup>1,2</sup> These cells reside in BM stroma providing the supporting feeder cells necessary for the haematopoietic progenitor cell growth but they may also differentiate into cells of connective tissue such as osteocytes, chondrocytes, tenocytes, adipocytes and smooth muscle cells.<sup>3,4</sup>

There is currently great interest in exploring the potential use of MSCs in regenerative medicine with particular target the bone and cartilage defects.<sup>5-10</sup>

Rheumatoid arthritis (RA) is a systemic autoimmune disease characterised by cartilage and bone destruction associated with local production of inflammatory mediators such as tumour necrosis factor  $\alpha$  (TNF $\alpha$ ) and interleukin (IL)1 $\beta$ . Joint destruction renders RA a candidate disease for cartilage and bone repair using MSCs. There are, however, some specific issues concerning the reserves and function of BM MSCs in RA that need to be addressed before proceeding to clinical application. In addition to the concept that BM MSCs may be primarily involved in joint damage in RA,<sup>11-15</sup> there are studies demonstrating that increased local production of TNF $\alpha$  may injure the BM microenvironment and may affect the reserves of BM haematopoietic progenitor cells.<sup>14</sup> Accordingly, a critical issue is whether BM MSCs are also depleted or functionally altered in RA.

This study evaluates the reserves and function of BM MSCs in patients with RA in terms of their proliferative, clonogenic and differentiation potential. Molecular and proteomic characteristics of patient MSCs with special focus on inflammatory cytokine gene and protein expression were also studied.

## PATIENTS AND METHODS

### Patients

We studied 26 patients with active RA<sup>15,16</sup> and 21 healthy individuals, age- and sex-matched with the patients (table 1). The Institutional ethics committee approved the study and informed consent according to the Helsinki Declaration was obtained from all subjects.

### MSC culture and differentiation

BM mononuclear cells (BMMCs) obtained from posterior iliac crest aspirates were cultured in Dulbecco modified Eagle medium, low glucose (DMEM-LG; Gibco/Invitrogen, Paisley, Scotland)/10% foetal calf serum (FCS; Hyclone, Logan, Utah, USA)/100 IU/ml penicillin-streptomycin (MSC medium) and MSCs were grown as previously described.<sup>17,18</sup> Cell-free supernatants were stored ( $-72^{\circ}\text{C}$ ) for cytokine measurements by ELISA (Quantikine; R&D Systems, Minneapolis,

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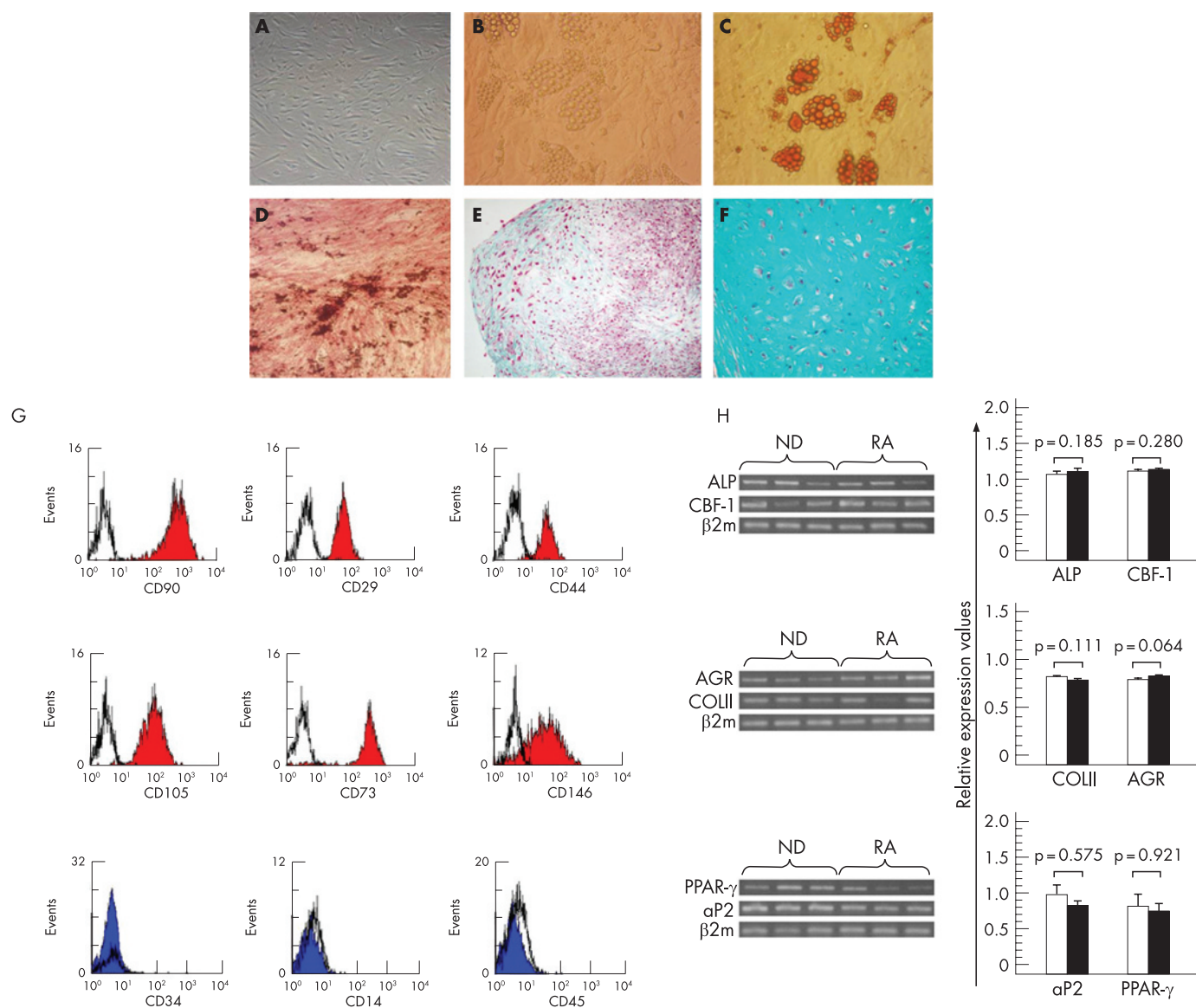
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**Table 1** Clinical and laboratory data of the patients studied

UPN	Age/sex	Duration (months)	DAS	Previous medication			PDM	DMARDs	Biologic	Current medication*
				NSAIDs	MTX	MTX				
1	43/F	6	7.1	No	15 mg/week	No	LEF	Infliximab <sup>†</sup>	MTX, Infliximab	
2	61/M	36	5.3	No	20 mg/week	No	LEF, CSA, SSA	Etanercept <sup>‡</sup> , Adalimumab <sup>§</sup>	MTX	
3	77/F	3	7.6	No	No	No	None	None	None	
4	58/M	72	8.4	No	20 mg/week	No	LEF, SSA	Kineret <sup>¶</sup>	Kineret	
5	71/M	6	4.6	No	20 mg/week	No	None	Infliximab	Infliximab	
6	32/F	3	5.1	No	15 mg/week	No	CSA, SSA, HCQ	None	MTX, HCQ, SSA	
7	77/F	9	5.4	No	15 mg/week	5 mg/day	LEF, CSA, SSA, D-Pen	None	LEF, PDM	
8	58/M	15	3.8	No	20 mg/week	7.5 mg/day	LEF, CSA, SSA, D-pen	Adalimumab	MTX, LEF, PDM	
9	73/F	4	5.6	No	15 mg/week	No	LEF, CSA, SSA, D-pen	Infliximab, Adalimumab	MTX, Adalimumab	
10	56/F	25	3.0	No	15 mg/week	5 mg/day	CSA	None	MTX, PDM	
11	50/F	32	7.1	No	15 mg/week	5 mg/day	LEF, CSA, HCQ	Infliximab, Kineret, Rituximab**	LEF, Rituximab, PDM	
12	66/F	240	6.1	No	15 mg/week	5 mg/day	LEF, CSA	Adalimumab	Adalimumab, PDM	
13	54/F	6	5.8	Yes	15 mg/week	No	HCQ	None	MTX, HHQ	
14	77/M	60	2.6	No	20 mg/week	5 mg/day	CSA	None	MTX, PDM	
15	75/F	15	3.9	No	15 mg/week	No	LEF, CSA, SSA, HCQ, D-pen	Adalimumab	MTX, Adalimumab	
16	62/M	156	5.8	No	20 mg/week	No	LEF, CSA	Infliximab, Rituximab	LEF, Rituximab	
17	53/M	72	6.8	No	20 mg/week	No	LEF, CSA	Infliximab, Rituximab	LEF, MTX, CSA, Infliximab	
18	31/F	12	2.2	No	No	No	None	None	None	
19	58/M	5	7.3	No	No	No	None	None	None	
20	59/F	48	8.4	Yes	15 mg/week	5 mg/day	LEF, CSA, D-pen	None	LEF, CSA, PDM	
21	51/F	240	6.5	Yes	15 mg/week	No	None	None	MTX	
22	77/F	3	8.4	No	No	No	None	None	None	
23	58/F	60	6.8	Yes	15 mg/week	No	HCQ	None	HHQ, PDM	
24	54/M	3	5.8	No	No	No	None	None	None	
25	33/F	6	2.6	No	No	No	None	None	None	
26	45/F	36	5.3	No	20 mg/week	5 mg/day	CSA	None	MTX, HHQ	

\*Patients had discontinued any medication for at least 24 h prior to BMI aspiration. <sup>†</sup>Human/murine chimeric anti-TNF- $\alpha$  monoclonal antibody (3 mg/kg every 4–8 weeks). <sup>‡</sup>Recombinant human soluble p75 TNF receptor fusion protein (25 mg/biweekly). <sup>§</sup>Humanised anti-TNF monoclonal antibody (40 mg every 2 weeks). <sup>¶</sup>Interleukin 1 $\beta$  receptor antagonist (100 mg/day). \*\*Human/murine chimeric anti-CD20 monoclonal antibody (2 doses of 1 g within 2 weeks). CSA, ciclosporine-A (150 mg/day); D-pen, D-penicillamine (200 mg/day); DAS, Disease Activity Score; DMARDs, disease-modifying antirheumatic drugs; HCQ, hydroxychloroquine (400 mg/day); HHQ, hexahydroquinoline; LEF, leflunomide (10–20 mg/day); MTX, methotrexate; NSAIDs, non-steroidal anti-inflammatory drugs; PDM, prednisone; SSA, sulphasalazine-A (2 g/day); TNF, tumour necrosis factor; UPN, unique patient number.



**Figure 1** Differentiation potential and immunophenotypic characteristics of mesenchymal stem cells (MSCs). The upper panel shows undifferentiated MSCs from passage 2 (P2) (A) and differentiated cells towards the adipogenic (B and C), osteogenic (D) and chondrogenic (E and F) lineages from a representative patient with rheumatoid arthritis (RA). Cell differentiation was identified with Oil Red O (C), alkaline phosphatase (ALP)/Von Kossa (D), Masson (E) and Alcian blue (F) staining. G. Immunophenotypic characteristics of patient MSCs at P2. The red and blue histograms depict the positive and negative markers, respectively, compared to the isotype-matched negative controls (open histograms). H. Specific gene mRNA expression (on the left) of P2 BM MSCs upon differentiation towards the osteogenic, chondrogenic and adipogenic lineages in representative RA ( $n = 3$ ) and control ( $n = 3$ ) subjects as well as cumulative data (mean relative values (SEM)) of specific gene mRNA expression from all RA (solid squares) and controls (open squares) studied (on the right). Comparison has been performed by means of the non-parametric Mann–Whitney U test.

Minnesota, USA). Trypsinised MSCs from passage (P)-2 were induced for differentiation (fig 1A–F).

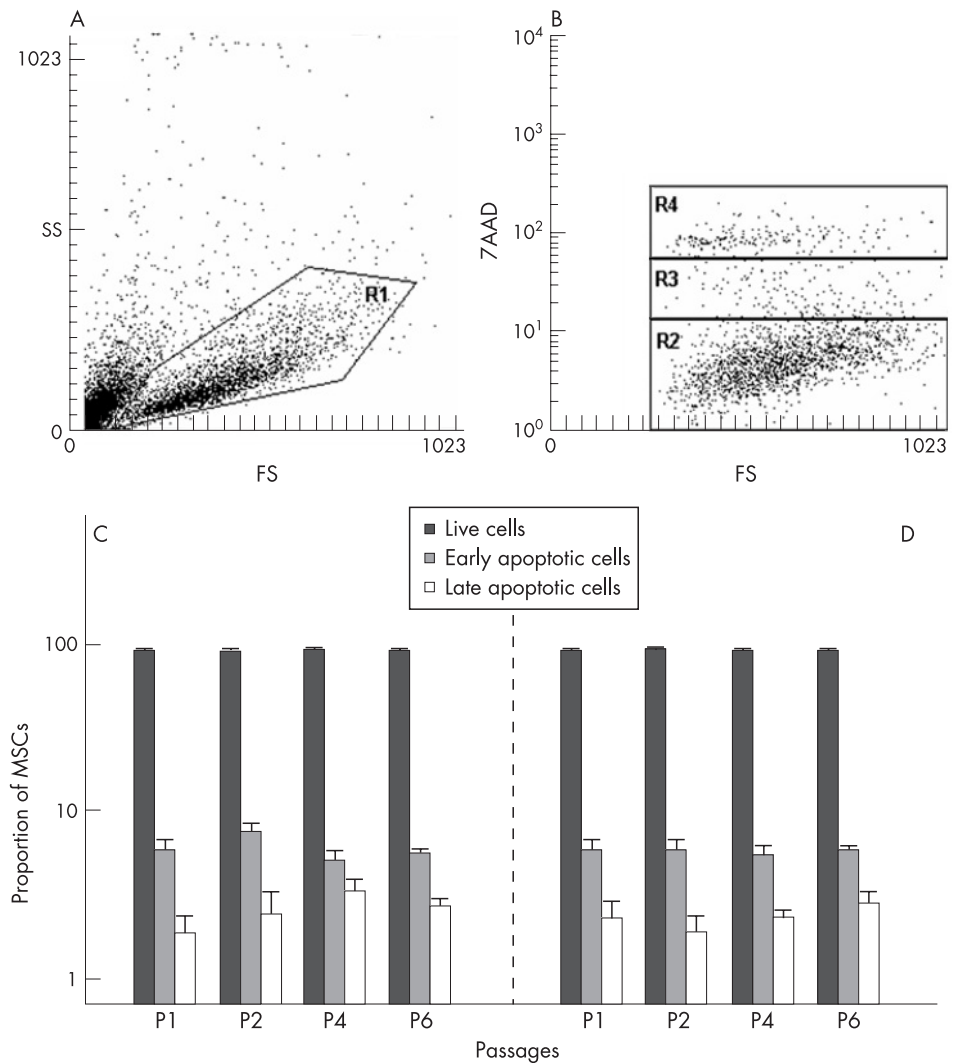
MSC adipogenic and osteogenic differentiation was induced as previously described and assessed by Oil Red O and alkaline phosphatase (ALP)/Von Kossa stains, respectively.<sup>19</sup> For chondrogenic induction, MSCs were pelleted and cultured in DMEM high glucose (DMDM-HG, Gibco), supplemented with 6.25  $\mu\text{g}/\text{ml}$  insulin, 6.25  $\mu\text{g}/\text{ml}$  transferrin, 1.33  $\mu\text{g}/\text{ml}$  linoleic acid, 1.25 mg/ml bovine serum albumin, 1 mM sodium pyruvate, 0.17 mM ascorbate-2-phosphate, 0.1  $\mu\text{M}$  dexamethasone, 0.35 mM L-proline, 6.25 ng/ml selenous acid and 0.01  $\mu\text{g}/\text{ml}$  transforming growth factor (TGF)- $\beta_3$  (R&D Systems).<sup>20</sup> Chondrogenic differentiation was identified with Alcian blue and Masson trichrome stains. Reagents were purchased from Sigma (St Louis, Minnesota, USA) unless otherwise indicated.

### MSC immunophenotypic and survival characteristics

Trypsinised MSCs from passages 1–6 (P1–P6) were immunophenotypically characterised by flow-cytometry, using anti-CD29 (4B4; Cyto-Stat, Beckman-Coulter, Fullerton, California, USA), anti-CD44 (J173; Immunotech/Coulter, Marseille, France), anti-CD73 (AD2; Pharmingen, San Diego, California, USA), anti-CD90 (F15.42; Immunotech/Coulter), anti-CD105 (SN6; Caltag, Burlingame, California, USA), anti-CD146 (P1H12; Pharmingen), anti-CD45 (IMMU19.2; Immunotech/Coulter), anti-CD14 (RMO52; Immunotech/Coulter) and anti-CD34 (QBend10; Beckman-Coulter) monoclonal antibodies.

Apoptosis was studied by flow-cytometry using 7-aminoactinomycin-D (7-AAD; Calbiochem-Novabiochem, La Jolla, California, USA).<sup>14–21</sup> Results were expressed as 7-AAD<sup>neg</sup> (live), 7-AAD<sup>dim</sup> (early apoptotic) and 7-AAD<sup>bright</sup> (late apoptotic) cells (fig 2A,B).

**Figure 2** Survival characteristics of mesenchymal stem cells (MSCs). MSCs from passages 1–6 (P1–P6) were stained with 7-aminoactinomycin-D (7-AAD) and analysed by flow cytometry for the study of apoptosis. Scattergram B shows the proportion of 7-AAD<sup>neg</sup> (R2; live), 7-AAD<sup>dim</sup> (R3; early apoptotic) and 7-AAD<sup>bright</sup> (R4; late apoptotic) cells in the gate of R1 (scattergram A) representing MSCs from P2. Bars in the lower panel represent the mean (SEM) proportion of live, early and late apoptotic cells timecourse in patients (C) and healthy controls (D) analysed as above. No statistically significant differences were documented between patients and controls by means of two-way analysis of variance (ANOVA).



### MSC quantification in the BMBC fraction

A limiting dilution assay (LDA) was used to evaluate MSC frequency within BMBCs following 6-week culture of seven different BMBC concentrations (250–10 000 cells/well) in 96-well plates.<sup>17</sup> Wells with >50 adherent spindle shaped cells were considered positive and MSC frequency corresponded to the dilution resulting in 37% negative wells.<sup>22</sup>

### Clonogenic potential and proliferative potential of MSCs

A colony forming unit fibroblastic assay (CFU-F) was also used to evaluate MSC frequency within BMBCs. Briefly, day-0 BMBCs were seeded at three different concentrations, expanded for 14 days and CFU-F number was estimated using linear regression analysis. The clonogenic potential of trypsinised MSCs from P1–P6 was also evaluated by CFU-F. MSC proliferative potential was evaluated by a methyl triazolyl tetrazolium (MTT) based assay in P2 and also by estimating population doubling time through P1–P6.

### RT-PCR

Total RNA isolated from P2 and differentiated MSCs (RNeasy mini kit; Qiagen GmbH, Hilden, Germany) was reverse transcribed (SUPERScript II; Gibco) and amplified by RT-PCR. Products were normalised according to  $\beta_2$ -microglobulin ( $\beta_2m$ ) using the ImageJ densitometry analysis system ([\[rsb.info.nih.gov/ij/\]\(http://rsb.info.nih.gov/ij/\)\). Primer sequences are listed in supplementary table 1.](http://</a></p>
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### Real time quantitative PCR

#### IL1 $\beta$ and TNF $\alpha$ expression

Platinum<sup>®</sup>qPCR SuperMix-UDG (Invitrogen) and TaqMan<sup>®</sup> Gene Expression Assays (Applied Biosystems; Foster City, California, USA) were used for IL1 $\beta$ , TNF $\alpha$  and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) identification. Measurements were performed using the ABI Prism 7000 System (Applied Biosystems). Results were normalised according to GAPDH quantity.

#### Telomere length measurement

DNA was extracted from P2 MSCs (Qiagen).  $\beta$ -Globin was used as control single copy gene.<sup>23, 24</sup> iTaq SYBR Green Supermix with ROX (Biorad, Hercules, California, USA) was used for the reactions. Primer sequences are listed in supplementary table 1. Telomere length was reflected by the relative telomere/single-copy-gene ratio (T/S):  $T/S = 2^{-\Delta C_t} (\Delta C_t = C_t^{\text{telomere}} - C_t^{\beta\text{-globin}})$ .

#### Two-dimensional gel electrophoresis

Whole cell lysates were obtained in 8 M urea, 2 M thiourea, 4% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate

(CHAPS), 50 mM dithiothreitol (DTT) (Sigma). For the first dimension, 30  $\mu$ g proteins from P2 MSCs were added to rehydration buffer. Immobilised pH gradient (IPG) strips (pH 3–10) were re-hydrated and focalised (70 000 v/h). For the second dimension, strips were equilibrated and proteins were separated by SDS-PAGE. Gels were stained with silver nitrate<sup>25</sup> and scanned using the Labscan-3 software (GE Healthcare; Little Chalfont, Buckinghamshire, UK) after calibration by the Kaleidoscope LaserSoft Imaging software (Kodak ref: R020123; Eastman Kodak, Rochester, New York, USA). Spot detection and quantitation were performed by ImageMaster 2D Platinum software (GE Healthcare).

### Microarray experiments

Hybridisation on HG-U133 Plus 2.0 microarrays was performed according to manufacturer (Affymetrix; Santa Clara, California, USA). Arrays were washed, stained and scanned on a Hewlett Packard Genearray Scanner (Affymetrix). Affymetrix GCOS 1.2 software was used to control washing and scanning, generate DAT/CEL/EXP files and process data. Group comparison and gene retrieval was performed by SiPaGene (BioRetis GmbH; Berlin, Germany). Cluster analysis was calculated with Genesis (A. Sturn; Graz University, Graz, Austria).

### Statistical analysis

Data were analysed by Mann–Whitney U test, one-way and two-way analysis of variance (ANOVA) tests (GraphPad Software; San Diego, California, USA). Two-dimensional gel electrophoresis data were analysed by the Hierarchical Clustering Explorer 3.0 software (<http://www.cs.umd.edu/hcil/multi-cluster/>) and Pearson correlation coefficient.<sup>26</sup> Grouped data are expressed as mean (SD).

## RESULTS

### MSC immunophenotype and differentiation potential

Immunophenotypic analysis at the end of each passage demonstrated that cultures constituted of a homogenous cell population positive for CD73, CD90, CD146, CD105, CD29, CD44 and negative for CD45, CD14, CD34 surface antigens (fig 1G).<sup>19</sup>

Culture-expanded MSCs from patients were able to differentiate into osteogenic/chondrogenic/adipogenic lineages as shown by the respective cytochemical staining (fig 1B–F). Furthermore, the osteogenic/chondrogenic/adipogenic differentiating capacity assessed by the relative expression of ALP and CBF-1 mRNA, COL1I and AGR (aggrecan) mRNA, and aP2 (adipose fatty-acid binding protein 2) and peroxisome proliferator-activated receptor (PPAR)- $\gamma$  mRNA expression, respectively, did not differ between patients and controls, suggesting normal differentiation potential of RA MSCs (fig 1H).

### MSC frequency in the BMMC fraction

The estimated frequency of MSCs in the BMMC fraction did not differ significantly between patients (20.74 (12.41)/10<sup>5</sup> BMMCs) and controls (23.90 (15.65)/10<sup>5</sup> BMMCs) ( $p = 0.8894$ ) by LDA. Quantification of BM MSCs by CFU-F analysis at day 0 was in accordance with LDA data. CFU-F number obtained by day-0 MSCs did not differ significantly between patients (18.42 (13.75)/10<sup>5</sup> BMMCs) and controls (19.85 (18.96)/10<sup>5</sup> BMMCs) ( $p = 0.3464$ ), further suggesting normal numbers of MSCs in RA. No statistically significant difference was documented between the LDA and CFU-F assays in the estimation of MSC frequency in either patients ( $p = 0.210$ ) or healthy controls ( $p = 0.199$ ).

### MSC clonogenic and proliferative potential

The growth potential of BM MSCs in patients with RA was studied by evaluating their clonogenic and proliferative capacity over time. CFU-F recovery by culture-expanded MSCs was significantly lower in patients with RA compared to controls through P1–P6 ( $p < 0.01$ ) (fig 3A). In association to the lower clonogenic potential, patient MSCs displayed also defective proliferative capacity compared to controls as estimated by the population doubling time over P1–P6 ( $p < 0.001$ ) (fig 3B). These findings were further substantiated by the MTT assay; as shown in fig 3C depicting results from a representative passage (P2), the number of live cells remained significantly lower in patients with RA compared to controls ( $p < 0.001$ ).

The majority of patients had previously been treated with cytotoxic and/or immune suppressant or anti-cytokine agents. To exclude the possibility of a drug-related damage of MSCs, a subset analysis was performed in the group of previously treated ( $n = 20$ ) and untreated ( $n = 6$ ) patients. Compared to healthy controls, treated and untreated patient groups displayed lower CFU-F recovery through P1–P6 ( $p < 0.001$  and  $p < 0.05$ , respectively). Similarly, treated and untreated patients displayed increased MSC doubling time ( $p < 0.001$  and  $p < 0.05$ , respectively) and defective proliferative potential in the MTT assay ( $p < 0.001$  and  $p < 0.05$ , respectively) compared to controls. However, no statistically significant differences were found between the two patient groups in the above parameters. Thus drug-induced damage is unlikely to be the cause of the defective clonogenic/proliferative potential of MSCs in RA.

### MSC survival characteristics

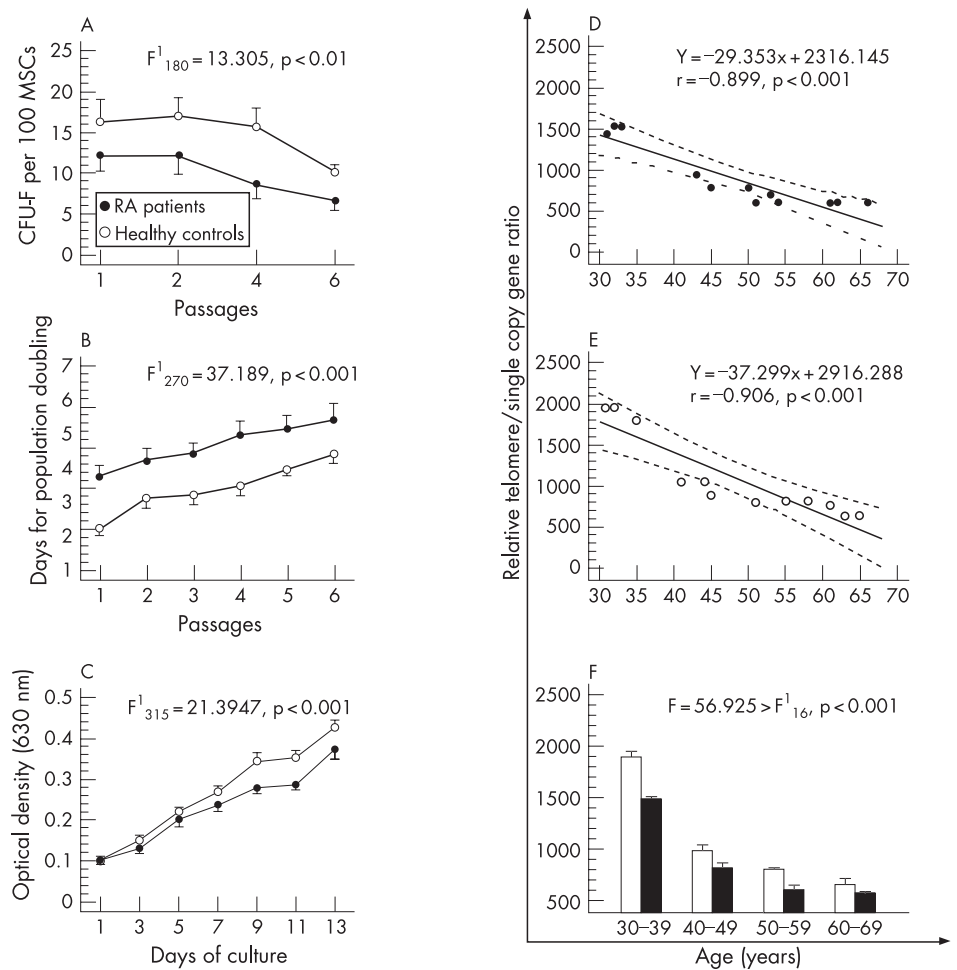
The impaired clonogenic and proliferative ability of MSCs in patients with RA might have resulted from increased cell apoptosis. The total proportion of apoptotic cells ranged from 7.68 (3.70)% (P1) to 8.24 (2.01)% (P6) in the patients and from 8.25 (4.15)% (P1) to 8.24 (2.85)% (P6) in the controls. Overall, no statistically significant differences were found between patients and controls in the proportion of early ( $F = 0.8206 < F_{1,84}^1$ , not significant) or late apoptotic ( $F = 0.9977 < F_{1,84}^1$ , not significant) cells throughout P1–P6 culture period, suggesting that the impaired clonogenic and proliferative potential of MSCs in RA is not due to accelerated cell apoptosis (fig 2C,D).

### Cytokine production by MSCs

To investigate whether BM MSCs from patients with RA display abnormal cytokine production similar to RA synovial fibroblasts<sup>27</sup> we initially assessed IL1 $\beta$  and TNF $\alpha$  production. The relative IL1 $\beta$ /GAPDH and TNF $\alpha$ /GAPDH mRNA levels at P2 did not differ significantly between patients and controls (0.059 (0.029) and 0.040 (0.022), respectively). Similar to the mRNA data, protein levels of IL1 $\beta$  and TNF $\alpha$  determined by ELISA in culture supernatants did not differ significantly between patients and controls through P2–P6 ( $F = 0.1474 < F_{1,48}^1$  and  $F = 1.2373 < F_{1,48}^1$ , respectively).

The levels of the MSC growth-promoting cytokines vascular endothelial growth factor and stromal-derived factor-1 and the values of the inhibitory/inflammatory cytokines IL6, TGF- $\beta_1$ , IL8 and IL15 in culture supernatants through P2–P6 did not differ significantly between patients and controls. These data indicate that abnormal cytokine production by MSC per se seems unlikely to be the cause of the impaired growth potential of MSCs in RA.

**Figure 3** Clonogenic/proliferative potential and relative telomere length of mesenchymal stem cells (MSCs). The left panel depicts the mean (SEM) number from colony forming unit fibroblastic assay (CFU-F) (A) and days for population doubling (B) over passages 1–6 (P1–P6) as well as the mean (SEM) optical density corresponding to the number of live cells over 13-day culture using the methyl triazolyl tetrazolium (MTT) assay in P2 (C). The right panel illustrates the correlation (regression line within 95% confidence limits) between age and relative MSC telomere/single-copy-gene values (diagrams D and E, respectively) and the mean (SEM) relative telomere length per decade of years (F) in RA (solid squares) and normal (open squares) subjects. Analyses have been performed by two-way analysis of variance (ANOVA).



### Proteomic analysis

Two-dimensional electrophoresis was performed using P2 MSCs in patients with RA ( $n = 10$ ) and healthy controls ( $n = 6$ ) (fig 4A,B). By using the Hierarchical Clustering method, we could not define any cluster that might discriminate patient and control cells (fig 4C). The Pearson correlation coefficient was not significantly different between patient and control cells ( $r = 0.933$  (0.022) and  $r = 0.929$  (0.020), respectively). These data corroborate the lack of significant changes in cytokine production between patients and controls.

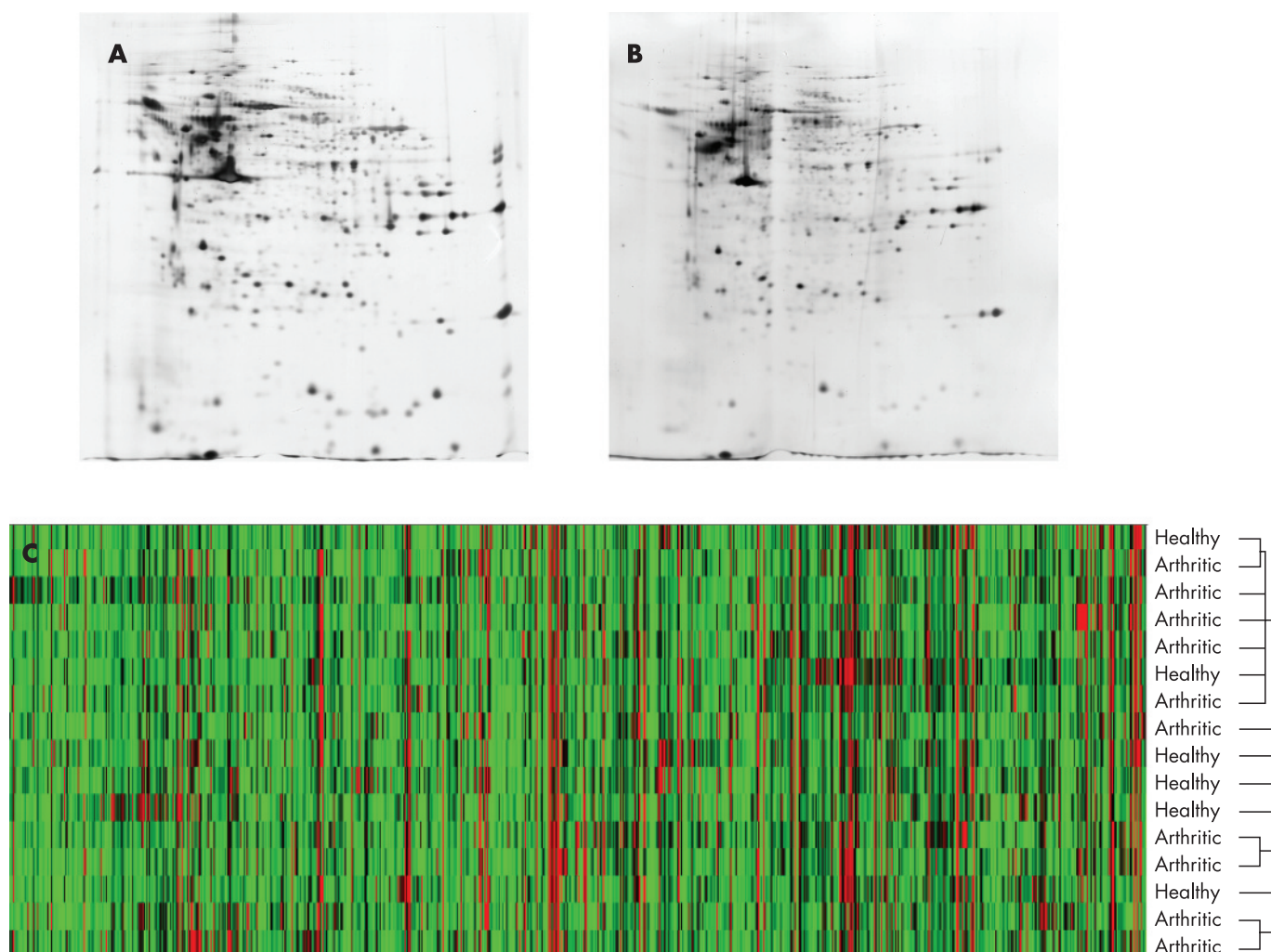
### MSC telomere length

Decreased cellular growth associated with premature telomeric loss has previously been described in periarticular osteoblasts in RA.<sup>28</sup> We therefore evaluated the relative telomere length (RTL) of MSCs from P2 in 12 patients and 12 age- and sex-matched healthy controls. An age-related decrease of the RTL as estimated by the relative T/S ratio was observed in patients ( $r = -0.899, p < 0.001$ ) and controls ( $r = -0.906, p < 0.001$ ) (fig 3D,E). To characterise patient MSC RTLs appropriate or inappropriate for a given age, we defined the observed/predicted RTL ratio (O/P ratio) for each sample, according to the equation derived from the linear regression analysis of the correlation between RTL and age (years) of the controls. We found that the mean O/P ratio of the patients (0.83 (0.20)) was out of the 95% confidence limits of the controls (mean O/P ratio 0.67 (0.17),  $p = 0.0226$ ), suggesting inappropriate MSC telomeric loss by

age. By analysing the mean RTL per decade of years in patients and controls, a highly significant difference was obtained ( $p < 0.001$ ) (fig 3F), indicating further premature telomeric loss of MSCs in RA that might account, at least in part, for the defective clonogenic and proliferative potential of cells in culture.

### Gene expression profiling of MSCs

Genes encoding for cell surface molecules, cytokines and differentiation markers were analysed in particular. Selection for genes differentially expressed in RA compared to normal MSCs allowed separation of the RA from normal samples by hierarchical clustering (fig 5). Affymetrix probe sets that were differentially expressed in more than 80% of the pairwise comparisons between three RA and six normal MSC samples were selected to identify objects in canonical pathways of the Kyoto Encyclopaedia of Genes and Genomes (KEGG) database. The most differentially expressed genes were those encoding for proteins implicated in focal adhesions (supplementary fig 1 and supplementary table 2). Specifically, several genes involved in cytoskeleton regulation and therefore linked to cell motility, proliferation and survival, were found to be differentially expressed between patients and controls. Of particular interest was the downregulation of cyclin-D, which plays an important role in the transition from G1 to S-phase in cell cycle progression, as well as the upregulation of cyclin-D inhibitors Ink4a-d and Kip1,2 and their upstream inductors Smad2/3 and TGF- $\beta$  (supplementary table 3).



**Figure 4** Proteomic analysis of mesenchymal stem cells (MSCs). Two-dimensional gel electrophoresis was performed using whole protein cell extracts from P2 MSC cultures of patients with rheumatoid arthritis (RA) ( $n = 10$ ) (A) and healthy controls ( $n = 6$ ) (B). After scanning, spot detection, quantification and normalisation, gels were compared using Hierarchical Clustering Software and Pearson test (C). No cluster could be detected using these proteomic profiles.

## DISCUSSION

To explore the potential use of MSCs for cartilage and bone repair, we conducted the current study to evaluate the reserves, functional, molecular, proteomic characteristics, and differentiation potential of BM MSCs in patients with RA, a candidate disease characterised by destruction of articular structures. Characterisation of autologous MSCs in patients with RA is important since emerging data suggest that allogeneic MSCs may not be as “immune privileged” as previously considered.<sup>29</sup>

We found that the frequency of MSCs within BMSCs did not differ between patients and controls. Our findings are in accordance with previously reported data on the frequency of MSCs in normal BM suggesting that patients with RA have normal BM MSC reserves.<sup>4,30</sup> Furthermore, culture-expanded MSCs from patients with RA were morphologically and immunophenotypically indistinguishable from the controls.

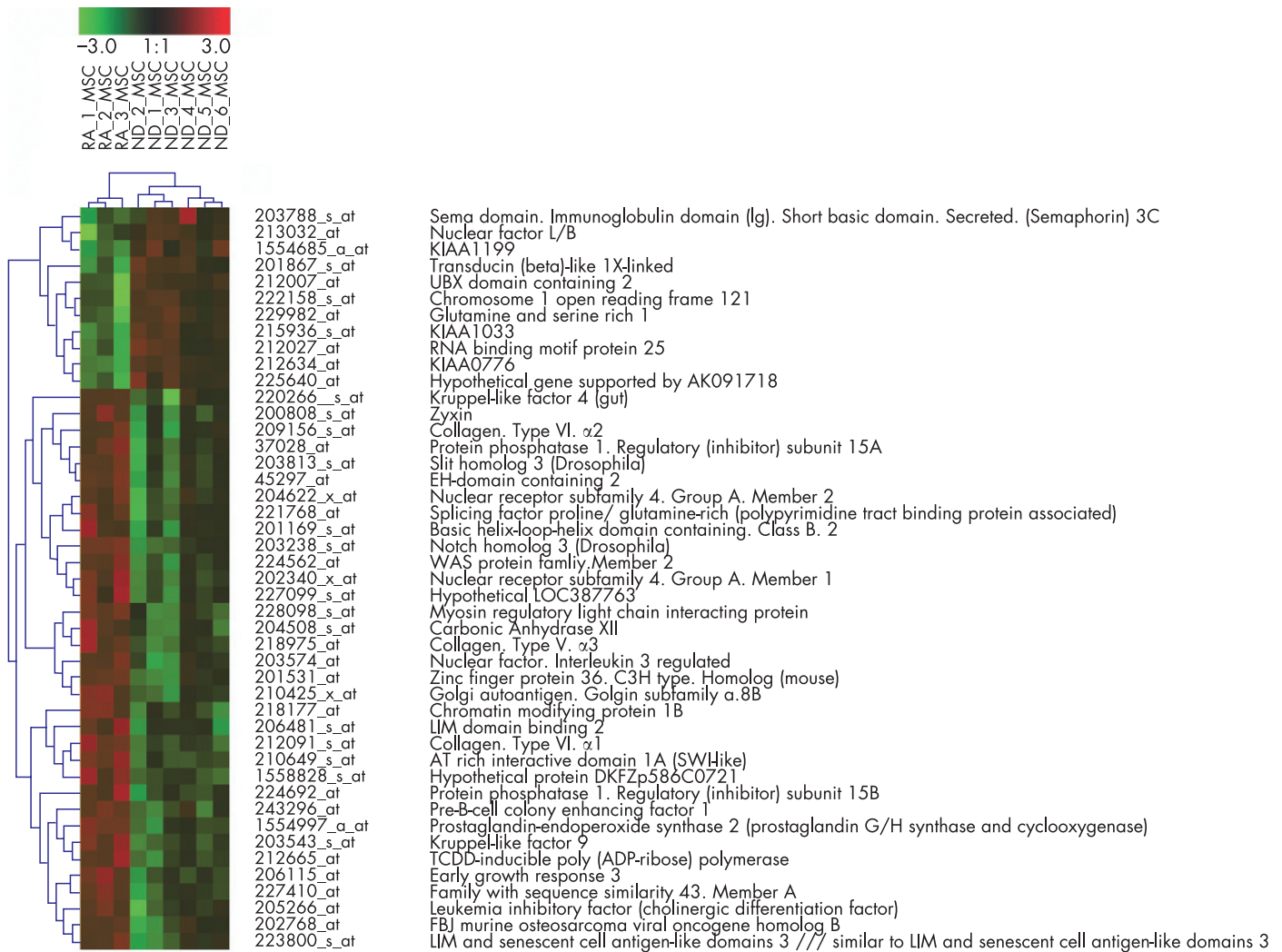
Culture-expanded MSCs from our patients with RA displayed normal osteogenic, chondrogenic and adipogenic differentiation. Interestingly, reduced chondrogenic activity of BM MSCs has been reported for osteoarthritis patients and this defect has been associated with the disturbed homeostatic maintenance of the articular cartilage.<sup>31</sup> Normal differentiation

potential of BM MSCs however, has been described in other autoimmune diseases such as in systemic sclerosis.<sup>32</sup>

MSC expansion through passages, assessed by the population doubling time, was significantly reduced in patients with RA, suggesting defective proliferative capacity. This finding was further substantiated by the MTT assay. The defective proliferative potential was in accordance with the impaired clonogenic capacity of patient MSCs as demonstrated by the low CFU-F recovery timecourse. In addition, the impaired growth of MSCs in RA is not likely to be due to accelerated cell apoptosis, since apoptosis in culture was not found to be significantly different between patients and controls.

The majority of our patients had been treated with methotrexate and/or corticosteroids, anti-cytokine or disease-modifying anti-inflammatory agents. It has been reported that methotrexate does not affect the survival and functional characteristics of MSCs, while corticosteroids may damage their proliferative potential.<sup>33</sup> No available data on the effect of anti-cytokine and anti-inflammatory agents on MSCs are presently known. To exclude the possibility of drug-mediated damage, we have separately analysed MSC clonogenic/proliferative potential in untreated patients. Cellular growth and





**Figure 5** Gene expression profiling of mesenchymal stem cells (MSCs). RNA from P2 bone marrow (BM) MSCs from patients with rheumatoid arthritis (RA) ( $n = 3$ ) and healthy controls ( $n = 6$ ) was hybridised to the GeneChips and arrays were processed for signal calculation and pairwise chip comparison. Selection of genes differentially expressed in RA compared to non-differentiated (ND) bone marrow mononuclear cells (BMMCs) allowed us to distinguish RA MSCs from normal MSCs by hierarchical clustering.

CFU-F recovery were significantly lower even in this group of patients compared to controls, whereas no significant differences were documented between treated and untreated patients with RA. These findings suggest that treatment with antirheumatic agents is not the major factor affecting MSC growth in RA.

It has been suggested that BM derived MSCs with altered properties may repopulate the synovial membrane in RA, whereas abnormal expression of inflammatory cytokines has been reported for RA synovial fibroblasts.<sup>12–13</sup> MSCs from our patients displayed normal cytokine expression in array profiling and ELISA. We have specifically focused on IL1 $\beta$  and TNF $\alpha$  since these cytokines have been found overexpressed in synovial fluid and BM culture supernatants.<sup>14–34–35</sup> No differences were found between patients and controls in the inflammatory cytokine expression or the growth promoting cytokine levels, suggesting that the impaired growth potential found in patient MSCs is not due to altered cytokine expression.

It has been previously shown that aging may affect the proliferative potential of MSCs.<sup>36</sup> Since patients with RA and controls in our study were age-matched, an age-related defect in the clonogenic and proliferative capacity of patient MSCs is

unlikely. Patient MSCs, however, displayed age-inappropriate relative telomere loss. Since there is evidence suggesting that the replicative capacity of cells correlates with the initial cellular telomere length<sup>37–38</sup> we may speculate that the defective growth potential of patient MSCs is due to inappropriate telomere loss. Although telomerase activity was not assessed in our study, we hypothesise that it is under the influence of the inflammatory BM milieu.<sup>14</sup> MSCs in RA undergo accelerated proliferation resulting in premature replicative exhaustion as previously described for RA periarticular osteoblasts.<sup>28</sup> The recently described accelerated telomere shortening under the influence of chronic mild oxidative stress further corroborates this assumption.<sup>24</sup>

In the search for direct molecular mechanisms by array profiling, differentially expressed genes were found to be involved especially in cell adhesion processes and matrix (collagen) synthesis. In particular, downregulation of cyclin-D was identified. Thus, a repressive influence on the transition from G1 to S-phase could provide a direct molecular explanation for the altered proliferative capacity of MSCs from patients with RA. This could be related to the increased local production of TGF- $\beta$  in patients' BM microenvironment (data not shown)

that may result in increase of TGF- $\beta$  signalling in MSCs and consecutive expression of cyclin-dependent kinase inhibitors, as suggested from the "cell cycle" pathway from KEGG. Thus, MSCs from patients with RA may present in vitro with a phenotype of cells that are more adherent, produce more matrix and are less active in proliferation. However, functional data are needed to substantiate this hypothesis.

In conclusion, our data have shown that patients with RA display a normal number, molecular and proteomic profile and differentiation potential of BM MSCs, encouraging, therefore, the use of autologous MSCs for the repair of cartilage and bone damage associated with long-standing RA. The proliferative and clonogenic potential of patient BM MSCs, however, was reduced compared to normal MSCs, and this defect was associated with decreased cellular telomere length and altered expression of genes implicated in focal adhesion and cell cycle pathways. This altered MSC activity may potentially influence the preparation of the cells for therapeutic usage in RA. In summary, this is the first study characterising BM MSCs in patients with RA under defined culture conditions in the context of potential clinical use for the repair of tissue injury associated with RA.

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**Ethics approval:** The Institutional ethics committee approved the study and informed consent according to the Helsinki Declaration was obtained from all subjects.

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