

Rapid clinical-scale propagation of mesenchymal stem cells using cultures initiated with immunoselected bone marrow CD105⁺ cells

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Abstract

Current clinical protocols used for isolation and purification of mesenchymal stem cells (MSC) are based on long-term cultures starting with bone marrow (BM) mononuclear cells. Using a commercially available immunoselection kit for enrichment of MSC, we investigated whether culture of enriched BM-CD105⁺ cells could provide an adequate number of pure MSC in a short time for clinical use in the context of graft *versus* host disease and graft failure/rejection. We isolated a mean of $5.4 \times 10^5 \pm 0.9 \times 10^5$ CD105⁺ cells from 10 small volume (10–25 ml) BM samples achieving an enrichment >100-fold in MSC. Seeding 2×10^3 immunoselected cells/cm² we were able to produce $2.5 \times 10^8 \pm 0.7 \times 10^8$ MSC from cultures with autologous serum enriched medium within 3 weeks. Neither haematopoietic nor endothelial cells were detectable even in the primary culture cell product. Expanded cells fulfilled both phenotypic and functional current criteria for MSC; they were CD29⁺, CD90⁺, CD73⁺, CD105⁺, CD45⁻; they suppressed allogeneic T-cell reaction in mixed lymphocyte cultures and retained *in vitro* differentiation potential. Moreover, comparative genomic hybridization analysis revealed chromosomal stability of the cultured MSC. Our data indicate that adequate numbers of pure MSC suitable for clinical applications can be generated within a short time using enriched BM-CD105⁺ cells.

Keywords: clinical scale expansion • mesenchymal stem cells • CD105⁺ cells

Introduction

The ability of mesenchymal stem cells (MSC) to differentiate along multiple pathways, together with their relatively easy isolation from BM and their extensive capacity for *in vitro* expansion, makes MSC potentially the most attractive population among stem cells

for tissue engineering and cell therapy applicable on a variety of congenital and acquired diseases [1–3]. In addition to their role in regenerative therapies, MSC have been consistently shown to possess immunomodulatory properties, which may play a role in the maintenance of peripheral tolerance, the induction of transplantation-related tolerance and control autoimmunity [4, 5]. There are preliminary data supporting their clinical efficacy in controlling steroid resistant graft *versus* host disease (GVHD) and improving engraftment of donor cells in the allogeneic stem cell transplant setting. However, the beneficial role of MSC in GVHD prophylaxis is being questioned by small-scale randomized clinical trials

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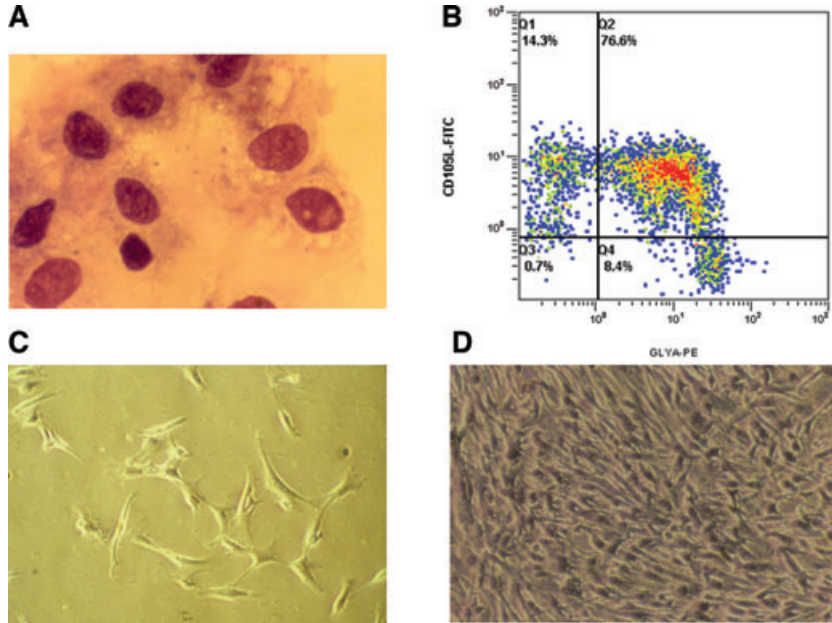


Fig. 1 Characterization of freshly isolated BM-CD105⁺ cells and cultured MSCs. **(A)** Morphology of immunomagnetically isolated BM-CD105⁺ cells after they were cytocentrifuged and counterstained with May-Grünwald solution. **(B)** Three colour flow cytometric analysis of sorted cells demonstrated two distinct CD105⁺ cell populations: CD105⁺Glycophorin-A⁺CD45⁻ and CD105⁺Glycophorin-A⁻CD45⁻. **(C–D)** Fibroblastoid morphology of cells derived from CD105⁺ cells after 3 **(C)** and 10 days **(D)** in culture before first passage (primary cells).

[6, 7]. Further large-scale randomized studies are needed to clarify the benefits and hazards of MSC administration for the prevention and therapy of GVHD.

In all clinical protocols, MSC have been isolated from bone marrow mononuclear cells (BM-MNC) and amplified in culture media supplemented with foetal calf serum [8, 9], or human platelet lysate [10]. Such protocols, however, result in a heterogeneous initial population of adherent BM cells, of which a significant proportion represents adherent monocytic cells. Even when MSC have been harvested after three or more culture passages (>4weeks culture time), the proportion of macrophages identified represented 1–44% of stromal cells [11, 12]. Recent studies have shown that positive selection using several surface markers including stromal (STRO)-1, CD105, CD271 and stage specific embryonic antigen (SSEA)-4 makes it possible to obtain a homogeneous MSC population without contaminating cultures with haematopoietic derived cells [13–16]. However, no such an enrichment method has yet been used in clinical scale *ex vivo* expansion of MSC. Here we describe the isolation and *ex vivo* expansion of MSC from BM-CD105⁺ cells in culture media enriched with autologous human serum for clinical application.

Materials and methods

BM was harvested from the posterior iliac crest of 10 normal donors for a related stem cell transplant, aged 10–30 years old, after informed consent, according to the Ethical Committee of the Aghia Sophia Children's Hospital. Initially, we performed immunomagnetic isolation of BM-CD105⁺ cells using Milteny microbeads according to the manufacturer's instructions (Milteny Biotech, Bergisch Gladbach, Germany). After the isolation,

BM-CD105⁺ cells were suspended in DMEM (Stem Cell Technologies, Vancouver, BC, Canada) enriched with 10% autologous serum, and placed into a 75 cm² flask (Corning Life Sciences, Corning, NY, USA) at a concentration of 2×10^3 cells/cm². The flasks were incubated at 37°C in a humidified environment with 5% CO₂. The medium was changed after 72 hrs and thereafter every 3–4 days. Fibroblastoid cells first became evident 2 to 12 days after inoculating culture flasks with CD105⁺ cells (Fig. 1C). By 9 to 12 days, a homogeneous population of adherent fibroblastoid cells was present (Fig. 1D). At this time-point cells were detached with 0.25% trypsin-ethylenediaminetetraacetic acid (Gibco BRL, Grand Island, NY, USA) for 5 min. at 37°C counted and subsequently re-plated at 4×10^3 cells/cm² in 175 cm² flasks until confluency. Cells derived from primary cultures of CD105⁺ cells were defined as Passage 0 (P0) and each cycle of reseeding of MSC after trypsinization was considered to be one additional passage. After a total time of 3 weeks, the cultures were discontinued. Surface marker expression of MSC derived from both primary (P0) and P1 cultures were analysed by flow cytometry. Cells were stained with monoclonal antibodies against human leukocyte antigen (HLA)-DR, CD14, CD29, CD34, CD45, CD31, CD73, CD105, CD44, CD73, and CD90. In order to investigate the multipotentiality of MSC, MSC derived both from P0 and P1 were directed towards the osteogenic, chondrogenic and adipogenic lineages. For osteogenic differentiation, cells were cultured in complete osteogenic differentiation medium (Stem Cell Technologies). Cells were fed twice a week. After a culture period ranged from 3 to 5 weeks, monolayer cultures were analysed morphologically for the formation of large osteogenic nodules. For adipogenic differentiation, cells were cultured for 2 weeks in complete adipogenic differentiation medium (Stem Cell Technologies). Adipogenic differentiation was confirmed by the formation of neutral lipid vacuoles. Chondrogenic differentiation was performed in high-density pellets containing 2.5×10^5 cells per pellet, cultured for 21 days in StemPro chondrogenesis differentiation medium (Invitrogen, Carlsbad, CA, USA). Total RNA was isolated from differentiated cells using RNeasy MINI Kit (Qiagen GmbH, Hilden, Germany). One microgram of each RNA sample was reverse transcribed with SuperScript III (Invitrogen) with use of

Oligo(dT). The resulting cDNA was then used as a template for PCR amplification with Platinum Taq (Invitrogen); the following genes have been inquired: alkaline phosphatase (ALP, F: TCAGAAGCTCAACACCAACG; R: GTCAGGGACCTGGGCATT) [15] for osteogenesis, lipoprotein lipase (LPL, F: GAGATTCTCTGTATGGCACC; R: CTGCAAATGAGACACTTTCTC) [17] for adipogenesis and collagen 2A1 (COL2A1, F: GGAAACTTTGCTGCCAGATG; R: TCACCAGGTTACCAGGATTGC) [17] for chondrogenesis. To estimate the immune inhibitory function of MSC, we used Ki-67-antigen staining in mixed lymphocyte cultures (MLC) in the presence of MSC as third party [18]. MLC were carried out with human peripheral blood mononuclear cells (hPBMC) from two HLA-DRB mismatched individuals used as effectors and stimulators. Stimulators and MSC were irradiated (30 Gy) before being cultured with effectors. Effectors and stimulators were seeded in triplicate at the concentration of 1×10^5 cells/500 μ l/well in 24-well round-bottom plates (Nunc, Roskilde, Denmark) in the absence (control) or presence of decreasing number of third-party MSCs (ratio MSC/PBMC = 1, 0.5 and 0.1). After an incubation period of 96 hrs, intracellular expression of Ki-67 was determined by immunohistochemistry. An array-based comparative genomic hybridization (array-CGH) analysis was conducted in order to investigate the genomic stability of MSC. Genomic DNA was extracted from four MSC samples derived at the end of expansion culture using the Qiamp DNA Mini kit (Qiagen GmbH, Hilden, Germany). Agilent Human Genome CGH 4×44 K microarrays with an average spatial resolution of 12 kb were used in the study (Agilent Technologies, Santa Clara, CA, USA). Genomic DNA from MSCs and pooled reference DNA of the same gender (Promega Corporation, Madison, WI, USA) were digested with Alul and RsaI (Promega Corporation) and labelled using Agilent Genomic DNA labelling Kit according to manufacturer's instructions. MSCs and reference DNA were labelled with Cy3 and Cy5, respectively, and were co-hybridized to arrays for 40 hrs at 65°C in a rotating oven (Agilent Technologies) at 20 rpm. The arrays were then washed with Agilent wash buffer and scanned by using an Agilent Microarray Scanner. Data were extracted using Feature Extraction 9.1 software (Agilent Technologies) and analysed using CGH Analytics 3.4 software (Agilent Technologies). Genomic copy number changes were identified with the assistance of the Aberration Detection Method 1 algorithm with a threshold of 6. Centralization and fuzzy zero corrections were applied to remove putative variant intervals with small average \log_2 ratios. For the location of genes in the deleted/duplicated genomic segments the UCSC (<http://genome.ucsc.edu/>) and the Database of Genomic Variants (<http://projects.tcag.ca/variation/>) (human genome build 18) were used.

Results

From BM samples of 10–25 ml (16 ml average) we were able to immunomagnetically isolate $5.4 \times 10^5 \pm 0.9 \times 10^5$ cells. We detected two populations of CD105⁺CD45⁻ cells (Fig. 1B): CD105⁺glycophorin⁺CD45⁻ (erythroid cells) and CD105⁺glycophorin⁻CD45⁻ cells (mesenchymal cells) comprising $78.6 \pm 16.2\%$ and $18.6 \pm 4.8\%$ of the total isolated CD105⁺ cells, respectively, whereas the proportion of the latter in the initial BM mononuclear cell population was $0.16\% \pm 0.12\%$. Light microscopic examination of cytopspins prepared with freshly sorted CD105⁺ cells disclosed, apart from typical erythroblasts, median size agranular cells with heterochromatic nuclei and numerous projections of the cell membrane resembling reticular cells

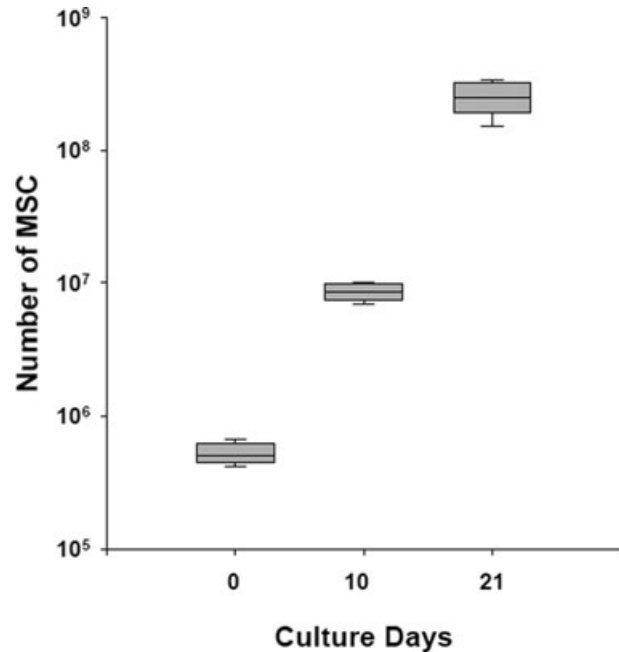


Fig. 2 Expansion potential of MSC derived from selected BM-CD105⁺ cells. Growth profile of CD105⁺ cells sorted on day 0 and plated at 2×10^3 cells/cm² culture surface. On day 10, adherent cells were detached with trypsin and replated at 4×10^3 cells/cm² in culture for an additional period of 11 days. Boxes represent the interquartile range; lines inside boxes represent the median value; whiskers represent the lowest and highest observations, respectively.

(Fig. 1A). Primary culture yielded $8.6 \times 10^6 \pm 1.2 \times 10^6$ MSC within a median time of 10 days. After replating at 4×10^3 cells/cm², P0 MSC were further cultured for an additional period of 11 days, and resulted to a total production of $2.5 \times 10^8 \pm 0.7 \times 10^8$ MSC (Fig. 2). Flow cytometry revealed that P0 MSC did not express certain haematopoietic and endothelial markers such as CD45, CD31, CD14, CD34 and CD62L. On the other hand, cultured cells were found positive for CD105, CD90, CD73, CD44, CD29 and class I HLA (Fig. 3). No difference in marker expression was found for P0 or P1 MSC.

MSC from both primary and passage 1 (P0 and P1) cultures treated with osteogenic medium underwent a change in their morphology from spindle shaped to cuboidal, and formed large nodules after 18 days of induction. MSC cultured in adipocytic differentiation medium formed lipid vacuoles 14 days after induction. Chondrogenic, adipogenic and osteogenic differentiation were confirmed by COL2A1, LPL and ALP expression, respectively (Fig. 4).

We next assessed the ability of MSC to inhibit the alloreaction observed in MLC. As expected, MLC without MSC led to an increase in proliferation of effector lymphocytes quantified by the percentage of Ki-67⁺ cells. Both P0 and P1 MSC, when added to MLC at MSC/effector ratio of 1:1, efficiently reduced the percentage of Ki-67⁺ cells by approximately 85%. With decreasing MSC/effector ratios the proportion of proliferating effector cells

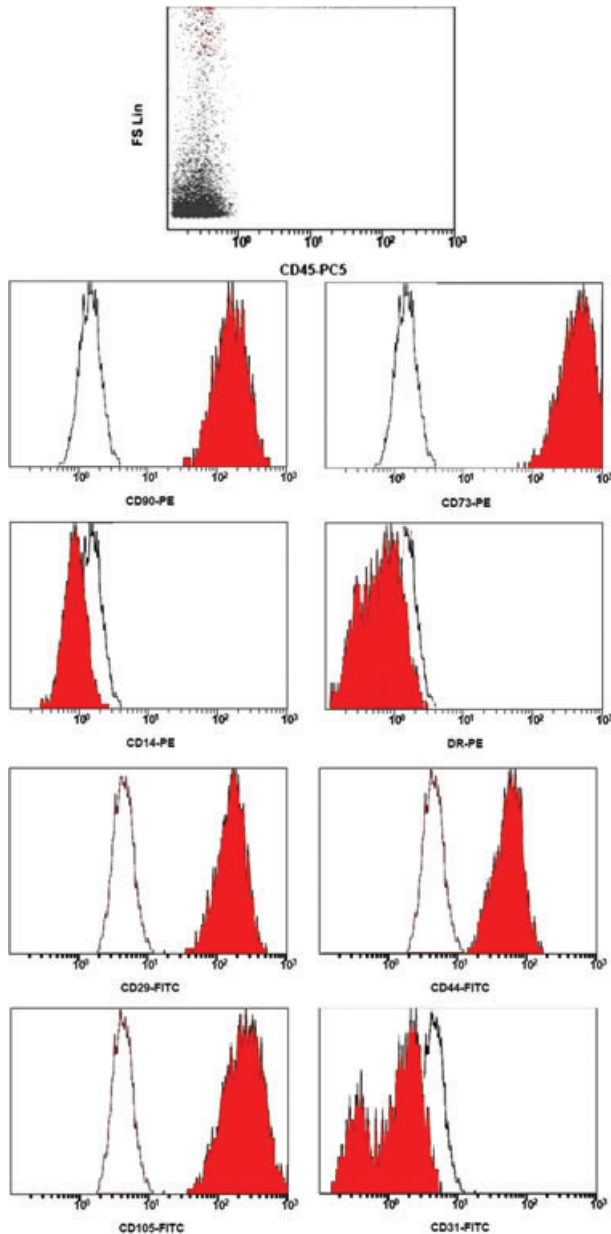


Fig. 3 Immunophenotype of P0-MSCs. Flow cytometric plots showing the immunophenotype of cultured cells at the end of primary culture on day 10. Gray histograms show specific antibody staining profile and open histograms show isotype control IgG staining profile.

was increased. However, even at low MSC to effector ratio of 0.1 there was an inhibition of proliferating cells of about 25–30% (Fig. 5).

We performed a whole-genome microarray-based CGH in order to look for chromosomal rearrangements with multiple loci involvement and for genome-wide aberrations in our MSC samples from normal donors. CGH-plotter software showed balanced profiles for all four MSC samples after culture expansion (Fig. 6).

We detected several small (average size of 15 kb to 1.5 Mb) autosomal copy number variations, both deletions and duplications, which are also observed in normal individuals [19].

Discussion

Our aim was to establish a culture system where sufficient numbers of pure MSC could be generated within reasonable time for 'emergency type' therapeutic strategies in the context of allogeneic stem cell transplant, like treatment of steroid-resistant GVHD and graft failure/rejection. It is important to note that MSC contaminated with a low percentage of CD45⁺ cells and MSC of late passage do not exhibit *in vivo* significant anti-GVHD effect, as recently suggested [20], indicating that early passage and significant immunodepletion are required for MSC-based cell therapy of GVHD. Therefore, we had to develop an *in vitro* approach which would strictly fulfil two requirements: (1) The generation of a 2×10^6 /per kg adult patient weight, that is the upper cell dose used for an adult patient, within a maximum of 3 weeks, capable to suppress the alloreaction *in vitro* and (2) the absence of any contamination of MSC with haematopoietic or/and endothelial cells. Based on our previous experience with chimerism studies of MSC derived from immunoselected CD105⁺ BM cells [21] and the reduced seeding density approach established by Prockop's group [22], we combined these methods aiming to obtain adequate numbers of MSC with immunosuppressive properties within 3 weeks. Starting with CD105⁺ cells isolated from small volume BM samples, we achieved MSC propagation which corresponded to a cell dose of $>2 \times 10^6$ /kg for an adult patient within 3 weeks. More importantly, even MSC from primary culture could be given to patients safely, because they were completely devoid of haematopoietic cells. We also investigated the maintenance of genomic stability using a whole-genome microarray based CGH at the end of the expansion procedure. Although we analysed MSC after a high *in vitro* proliferation rate, they did not show genomic alterations. By comparing our results with published data from clinical studies, similar numbers of MSC were obtained after culturing BM-MNC isolated from >40 ml BM samples for 4–6 weeks [23, 24]. According to our own experience, when cultures started from BM-MNC population, a larger volume of BM is needed (>30 mL) and the median time of expansion is much longer, almost 35 days to obtain adequate numbers for MSC infusion. Two recent, independent studies, however, described the successful production of a large MSC number ($>2 \times 10^8$) within fewer than 4 weeks derived from small BM aspiration samples [25, 26]. Both research groups were based on the fact that reduced MSC seeding density results in marked increase of MSC proliferation. Nevertheless, the fact that the cultures were initiated with unmanipulated BM [25] or BM-MNC [26] and MSC for clinical use were derived from primary culture or P1, respectively, makes the contamination with haematopoietic cells a matter of concern. At the time of writing this report, Poloni *et al.* [27] were able to produce 1×10^9 MSC using BM-CD271⁺ cells

Fig. 4 Chondrogenic, adipogenic and osteogenic differentiation of MSC. Chondrogenic, adipocytic and osteogenic differentiation was demonstrated by the expression of specific genes COL2A1, LPL and ALP, respectively.

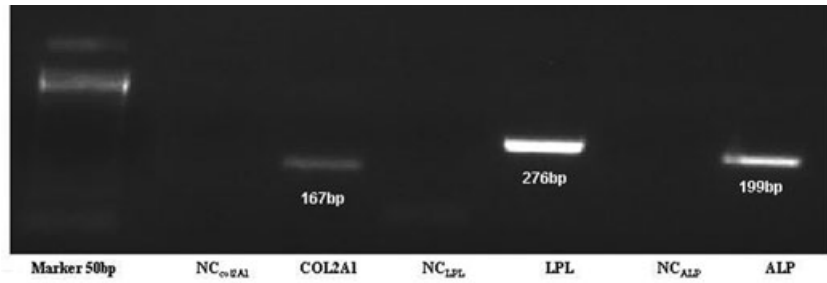


Fig. 5 Inhibitory effect of MSC on lymphocyte proliferation in MLC. hPBMC from healthy donors were co-cultured in one-way MLC with irradiated allogeneic PBMC in the absence (control) or presence of decreasing number of unrelated MSC. At 96 hrs of culture, cells were stained for intracellular Ki-67 antigen. Ki-67 antigen positivity indicates proliferating responder cells. Bars represent the percentage of Ki-67⁺ cells in three independent experiments at 3 MSC to effector ratios of 1, 0.5 and 0.1.

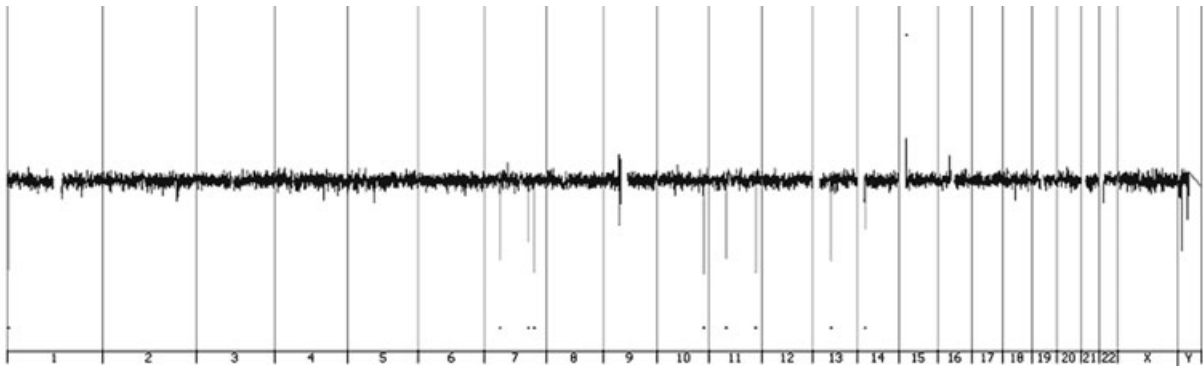
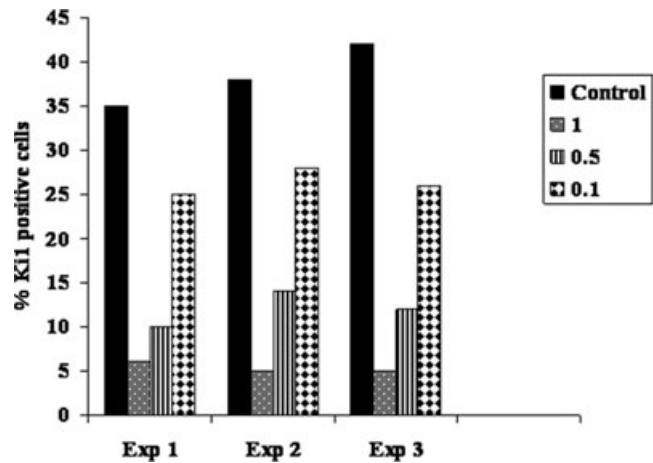


Fig. 6 Whole-genome array CGH profile of MSC. The X-axis represents the 22 autosomes, the X and Y chromosomes, and the Y-axis shows the log₂ of the fluorescence intensity ratio of all spots of the chromosome. Every coloured vertical peak represents a commonly found copy number variations; duplications are represented above the baseline with log₂ ratio >0.25 and deletions below the baseline with log₂ ratio <-0.25.

immunoselected from 5 ml BM in 30 days. However, they did not mention whether the produced cells possessed *in vitro* immunomodulatory abilities. In our hands, expansion cultures of CD271⁺ cells using foetal bovine serum (FBS) or human serum did not produce MSC solely, but also a percentage of neurosphere-like structures indicating a spontaneous differentiation to neural cell lineage. That was the reason for choosing the CD105⁺ cells.

Several criteria are required for clinical application of MSC in human beings. It is of major importance to use a rapid method to

produce adequate numbers of uncontaminated MSC with proven immunosuppressive potential. Especially, in the clinical setting of resistant GVHD or graft failure, alternative treatments are required urgently, timing is crucial and shortening the culture period even by a few days can prove beneficial. The use of purified CD105⁺ BM cells makes the method more expensive, but in terms of cost-effectiveness, the benefit from the time saved can prove valuable. We have shown that our method of deriving MSC from enriched CD105⁺-BM cells fulfils the criteria for a clinically applicable cell therapy protocol.

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Conflict of interest

The authors confirm that there are no conflicts of interest.

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