

CD45-positive cells of haematopoietic origin enhance chondrogenic marker gene expression in rat marrow stromal cells

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Abstract. Adult mesenchymal stem cells (MSCs) can be readily isolated from bone marrow, expanded in culture and subsequently subjected to differentiation into various connective tissue lineages. In general, for animal studies separation of MSCs from other bone marrow-derived cells is achieved by sole adherence to plastic surface of tissue culture flasks; however, this procedure produces a heterogeneous cell population containing CD45-positive haematopoietic cells (HCs) and haematopoietic stem cells (HSCs). It is known, that mixed cell cultures consisting of cocultures of differentiated somatic cells with adult stem cells promote differentiation towards specific cell lineages. For determining the effect of the CD45-positive cell population on the differentiation potential of MSCs, we sorted out the bone marrow-derived adherent cells by immunomagnetic technique (MACS™) to attain a subpopulation of CD45-depleted cells. Herein, we show that the presence of adherent CD45-positive HCs not only promote expression of the chondrogenic marker genes *Col2a1*, *COMP* and *Sox9*, but also of *Col1a1*, *Col10a1* and to a certain degree *Cbfa1* in MSCs when cultured in an appropriate three-dimensional environment. These observations constitute a step towards unravelling the influence of haematopoietic cells on chondrogenic differentiation of MSCs.

Introduction

Adult bone marrow stromal cells regarded as mesenchymal stem cells are progenitors of connective tissue cells, thus are excellent candidates for chondrogenic differentiation studies. The progeny of adult stem cells includes both daughter stem cells and committed progenitors with a more restricted differentiation potential. These progenitors in turn give rise to differentiated cell types. Mesenchymal stem cells (MSCs) can differentiate under specific cultural and physical conditions into multiple mesenchymal lineages namely, osteocytes, chondrocytes, adipocytes, astrocytes and myocytes (1,2). Bone marrow consists of primarily non-adherent haematopoietic cells and haematopoietic stem cells (99%) along with a minor population of MSCs ($\leq 1\%$). Both of the stem cell types are known to co-exist and have been suggested to cooperate in one another's differentiation (3). The role of MSCs in haematopoietic microenvironment formation is beginning to unravel, and it is assumed that the presence of adherent MSCs and their progeny facilitates HSC differentiation into granulocytes and erythrocytes both *in vivo* and *in vitro* (4-6). Conversely, in depth studies are scarce concerning the role of HSCs in the MSC commitment and differentiation towards a distinct lineage.

Adult rat MSCs are routinely isolated from tibio-femoral bone marrow by relying solely on their adherence to the plastic surface of tissue culture flasks (7,8). The isolated cells constitute a heterogeneous population that always contains HCs; therefore, obtaining a pure population of non-haematopoietic cells remains difficult. This haematopoietic cell fraction varies depending on the species; being relatively high in initial cultures of mouse marrow cells (9) and $<30\%$ in human cell cultures. In rat marrow stromal cells this trend has not been studied thoroughly, although the presence of haematopoietic cells in primary (7 days) and secondary (18 days) bone marrow cultures has been indicated (10). Human and murine MSC studies show subsequent loss of haematopoietic cell surface markers when cultures are maintained for 2 or 3 weeks. Prior to differentiation experiments the haematopoietic cell

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population is often separated from MSCs by employing a magnetic associated cell sorting (MACS) system. The system immunologically separates different cell types by labelling cell surface antigens with magnetic beads followed by sorting through a magnetic column. As a unique identifying marker for MSCs is still lacking the negative selection protocol is carried out using CD34 (My10) or CD45 (leukocyte common antigen) for exclusion of undesired positively labelled haematopoietic cells.

One of the major differentiation pathways of MSCs occurs along the chondrogenic lineage which can occur autonomously in three separate mesenchymal cell lines, cranial neural crest, sclerotome cells and lateral plate mesoderm cells (11). The first stage of chondrogenic differentiation is conversion of undifferentiated MSCs to committed osteochondroprogenitor cells leading to cell condensation and growth arrest. The major proteins involved in condensation initiation are either fibronectin or belong to the transforming growth factor β family of proteins (TGF β -1, -2 and -3). Cells present at the centre of the condensation nodules first form pre-chondrocytes and then chondrocytes which start to produce cartilage matrix marked by upregulation of structural protein genes such as *Col2a1*, *Col9a2*, *Col11a2*, *aggrecan* and *COMP*. Expression of chondrocyte marker genes is controlled by members of the Sox-family, in particular Sox9 has been characterized as a master transcription factor with essential direct or indirect regulatory effects exerted along the entire chondrogenic differentiation pathway. It is expressed in all chondroprogenitor cells and also in differentiated chondrocytes (12-15).

In this study, we have evaluated the degree of haematopoietic cell coexistence in primary cultures of rat MSCs and their influence on the chondrogenic differentiation potential of MSCs. We have determined the difference in chondrogenic marker gene expression of heterogeneous MSCs isolated by sole adherence to plastic surfaces compared to that of a more homogeneous subpopulation of MACS-sorted CD45-depleted cells.

Materials and methods

MSC isolation and primary cell culture. A modified version of the MSC isolation procedure from rat bone marrow was optimized (16,17). Briefly, 6-week-old male Sprague-Dawley rats were sacrificed with CO₂ and tibiae and femora were aseptically removed. The bones were cut from the middle and centrifuged in a 1.5 ml tube at 2000 rpm for 3 min (mini centrifuge-Eppendorf). The centrifuged bone marrow cells were collected and homogenized with 18, 21 and 23 g needles and seeded at the rate of 2.5×10^5 cells/cm² in 175 cm² tissue culture flasks in proliferation medium containing 5% glutamate, 1% antibiotics/antimycotics and 10% FBS (Gibco, Invitrogen, UK; Lot No.: 40F7430K) in α -MEM (Sigma Aldrich, Germany). All non-adherent cells were removed on the 3rd day and the adherent cells were proliferated.

Magnetic separation of cells (MACS). When the flasks were ~75% confluent (9-11 days) cells were harvested with trypsin digestion. Separation procedure was carried out according to the manufacturer's instructions; in short, cells were incubated

with 4 μ l of CD45 antibody/10⁶ cells for 5 min at 37°C followed by washing and a second incubation with goat anti-mouse secondary antibody coupled with magnetic beads (Miltenyi Biotec, Germany) for 15 min at 4°C. The suspension was passed through a magnetic column (Miltenyi Biotec's LS-MACS columns); while labelled cells coupled to the magnetic field, the flow through containing unlabelled CD45-negative fraction was collected.

Immunofluorescence. Twenty thousand cells/chamber were seeded on chamber slides (Biocoat slides, BD, Bioscience) and incubated overnight at 37°C and 5% CO₂. Cells were blocked for 1 h at 37°C in Complete Mini 1:5 (Roche, Germany) plus 5% normal goat serum and 1% BSA in PBS. After washing with PBS, cells were stained with monoclonal antibodies directed against CD45 (Cbl 1502, Chemicon, Germany), CD68 and D7Fib (Acris, Germany) in 1:50 dilution ratio overnight at 4°C. The appropriate Alexa568 or Alexa488 conjugated secondary antibodies (goat anti-mouse, 5 μ g/ml; Molecular Probes, USA) were added for 1 h at RT. After washing, slides were permanently mounted with Dako fluorescent mounting medium (Dako, USA) and covered with coverslips. Slides were evaluated with scanning laser microscopy (C1 confocal microscope from Nikon) and photos were taken with a Nikon C4 camera and software.

Flow cytometric analysis. Cells obtained from one animal (~ 8×10^7 cells, as determined after NH₄Cl lysis of the erythrocytes) were seeded in 4x150 cm² flasks (5×10^5 cells/cm²). The cells were left to adhere 1, 2, 3 or 4 days before non-adherent cells were removed. Cells were trypsinized on day 7 after the isolation and resuspended in PBS. The cell suspension was first blocked for 15 min at 4°C with sheep serum followed by incubation with antibodies against rat CD45-PE (mouse IgG1 against rat CD45, Acris Antibodies, Germany), CD29-FITC (Hamster IgM against rat CD29, BD Pharmingen, USA) and with corresponding isotype controls for 30 min at 4°C. Cells were washed with PBS containing sodium azide and bovine serum albumin. Shortly before the FACS, analysis propidium iodide was added to each sample in order to discriminate the dead cells which were excluded from the evaluation. All measurements were performed on a FACS-Calibur® instrument (Becton Dickinson, USA) with CellQuest data acquisition software (Becton Dickinson, USA). Data analysis was preformed with WinMDI 2.8 software (free access on <http://facs.scripps.edu/software.html>). A minimum of 1×10^4 viable cells was acquired per data set.

Chondrogenic differentiation. A batch of adherent cells (undepleted) was subjected directly to chondrogenesis favourable condition; the other batch was depleted of CD45-positive cells by MACS prior to chondrogenic differentiation experiments. For chondrogenic differentiation, high density 3-D cultures were attained by preparing cell-alginate amalgam in 1.2% alginate at a concentration of 10^7 cells/ml. Cell-alginate beads (2-3 mm) containing ~ 10^5 cells/bead, were formed by dropping the suspension into 102 mM CaCl₂ solution. The culture was carried out in 12-well tissue culture plates for 4 weeks; medium was changed every 2-3 days. Ten alginate beads were removed every 7th day for RNA isolation

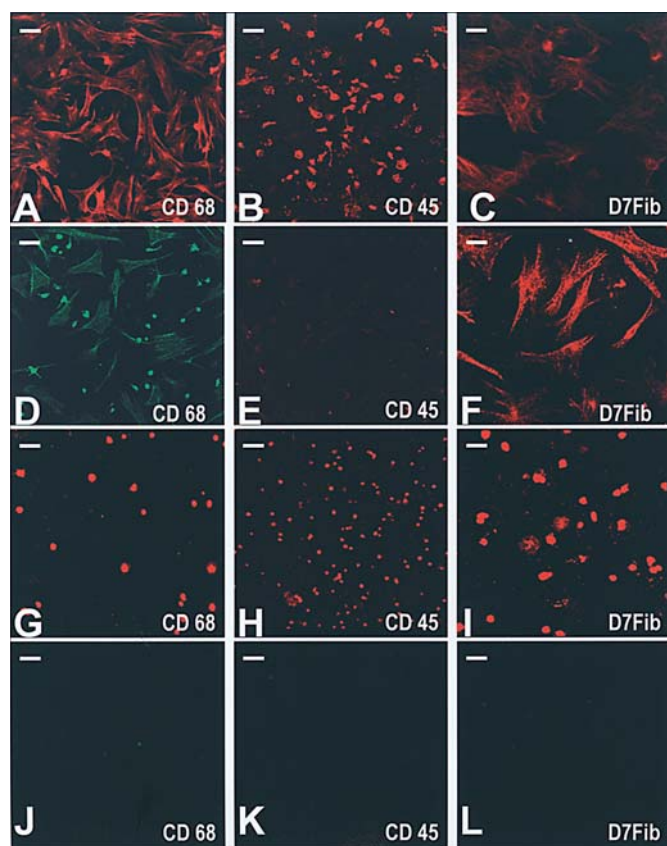


Figure 1. Morphological characterization of MSCs. Immunofluorescence imaging of heterogeneous adherent MSCs (A-C) and CD45-depleted MACS sorted cells (D-F), showing typical fibroblast-like spindle shaped cells, while CD45-positive cells (G-I) are small and rounded in appearance. The negative controls for all three antibodies are unstained (J-L). All three cell types are CD68- and D7fib-positive while the CD45-depleted cell population does not exhibit CD45 harbouring cells (E). Magnification is $\times 200$ for all panels, bars are $10\ \mu\text{m}$.

and gene expression analysis. To release the cells from alginate, the beads were incubated for 30 min at 37°C on a shaker in 55 mM sodium citrate and 0.15 M sodium chloride buffer; cells were recovered after a 3 min spin at $750 \times g$. The chondrogenic medium contained; ITS+ premix (6.25 $\mu\text{g/ml}$ insulin, 6.25 ng/ml selenium acid, 6.25 $\mu\text{g/ml}$ transferrin, 1.25 mg/ml BSA and 5.35 $\mu\text{g/ml}$ linoleic acid; BD Biosciences, USA), 110 $\mu\text{g/ml}$ pyruvate, 40 $\mu\text{g/ml}$ proline, 0.1 μM dexamethasone, 50 $\mu\text{g/ml}$ ascorbic acid and 10 ng/ml TGF β -3 (18) (R&D Systems) in α -MEM high glucose (Gibco, Invitrogen, UK). Alginate sodium salts were acquired from Sigma Aldrich, Germany (Cat. No.: A0682-100G).

RNA isolation and reverse transcription. RNA was isolated by an affinity column chromatography method with Ambion's RNeasy4-PCR kit according to the manufacturer's protocol. For removal of possible DNA contamination, isolated RNA was incubated for 1 h at 37°C in 2 units of DNaseI enzyme (DNA-free, Ambion). RNA integrity was determined on 0.8% agarose-formaldehyde gels. The RiboGreen RNA quantification kit (Molecular Probes, USA) was used for determination of RNA concentration at 585 nm wavelength. For RNA conversion to cDNA, Invitrogen SuperScript II reverse transcriptase kit was used with 0.5-1 μg of RNA in

20 μl of total reaction volume in presence of 40 units/ μl recombinant ribonuclease inhibitor (RNase OUT[®]). Reverse transcription was carried out with 500 $\mu\text{g/ml}$ of Oligo-dT primers, 10 mM dNTPs and 200 units of SuperScript II enzyme in First-Strand buffer and 0.1 M DTT for 50 min at 42°C followed by an extension period of 15 min at 70°C .

Relative quantitative PCR. Relative quantitative PCR was carried out with SYBR Green Dye I on ABI 7000 Prism Sequence detection system (Applied Biosystems, USA) according to the manufacturer's instructions. Briefly, 1 μl of cDNA was amplified in 50 μl final volume of 0.2 μM of each primer suspended in SYBR green master mix (Applied Biosystems). Amplification parameters were the same for all primer pairs and were repeated for 40 cycles, denaturation at 95°C for 0.15 min and annealing at 60°C for 1 min. Mean relative quantification (RQ) value evaluated from three independent experiments was plotted on a semi-logarithmic graph. RQ values were calculated by the software 'RQ study application v1.1' (ABI Prism 7000 SDS software v1.1) according to the $\Delta\Delta\text{Ct}$ method using β -actin as endogenous control and undifferentiated MSCs (day 0) as calibrator. Primers were designed with either Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) or 'Primer Express' software supplied by Applied Biosystems. All primers were manufactured at MWG - Biotech and are listed in Table I. Prior to RQ studies, a CT value (cycle threshold) standard curve with 10-fold diluted cDNA was plotted to determine PCR efficiency of each primer pair, only 90-100% efficient primers were used. The efficiency was determined with $10^{(1/-S)} - 1$, where S is the slope of the curve (19).

Statistical analysis. One way ANOVA and Student's t-test were employed for quantitative PCR experiments performed in triplicate. Results obtained by flow cytometry are the average of 4 independent experiments \pm standard error of mean. The level of significance was determined by Tukey's test with 2 group comparison.

Results

MSC isolation procedure and CD expression profile. To determine the influence of adherence time of rat bone marrow cells on the presence of CD45-positive haematopoietic cells, the cells were left to adhere for 3, 4 and 5 days before non-adherent and weakly attached cells were removed. Upon immunofluorescence analysis, the adherent cells showed a D7fib⁺, CD68⁺ and CD45^{+/low} expression profile regardless of the time of removal of non-adherent cells. Fig. 1 shows representative immunomicrographs of the cells left to adhere for 3 days and compares the staining of non-sorted cells, left for 3 days to adhere before medium removal, are contaminated with CD45-positive cells (Fig. 1A-C). After magnetic sorting, most of the CD45-positive cells were excluded, leaving a CD45-depleted cell fraction (Fig. 1D-F). However, we still observe few rounded cells among the CD68 positively stained fraction (Fig. 1D) of unknown surface antigen characterization. The CD45-positive cell fraction includes cells which stain for CD68 and D7Fib (Fig. 1G-I).

Table I. Primers used for quantitative PCR.

Gene/RefSeq #	Amplicon (bp)	Primer (5'-3')
<i>β-actin</i> NM_031144	104	Forward -GTAGCCATCCAGGCTGTGTT-3' Reverse -CTGTAGGTGAATCGACTGTTGC-3'
<i>Col1a1</i> RGD61817 ^a	59	Forward -TCCAGGGCTCCAACGAGA-3' Reverse -CTGTAGGTGAATCGACTGTTGC-3'
<i>Col2a1</i> NM_012929	60	Forward -CCCCTGCAGTACATGCGG-3' Reverse -CTCGACGTCATGCTGTCTCAAG-3'
<i>Col10a1</i> AJ131848 ^a	247	Forward -CCC TAT TGG ACC ACC AGG TA -3' Reverse -TCT CTG TCC GCT CTT TGT GA -3'
<i>COMP</i> NM_012834	167	Forward -TGACTTCGATGCTGACAAGG-3' Reverse -GAACGATCTCCATTCCCTGA-3'
<i>Sox9</i> XM_343981	140	Forward -CTGAAGGGCTACGACTGGAC-3' Reverse -TACTGGTCTGCCAGCTTCCT-3'
<i>Cbfa1</i> XM_34016	86	Forward -GGCCGGAATGATGAGAACTA-3' Reverse -AGATCGTTGAACCTGGCCACT-3'

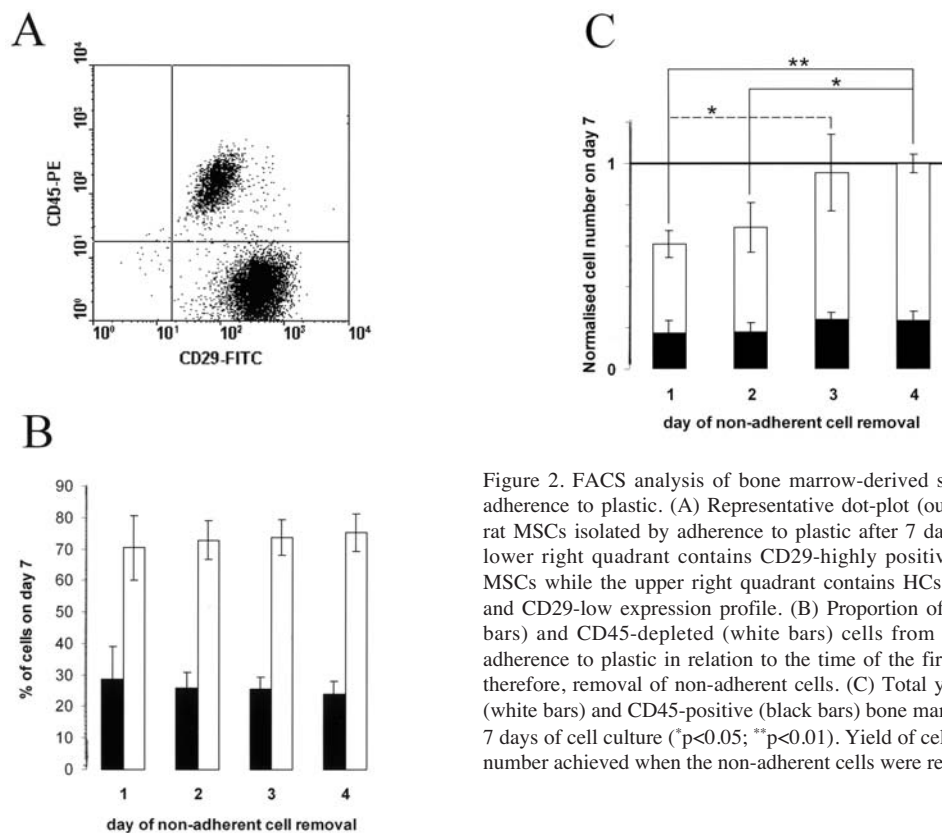
^aLocus ID.

Figure 2. FACS analysis of bone marrow-derived stem cells prepared by adherence to plastic. (A) Representative dot-plot (out of 3 experiments) of rat MSCs isolated by adherence to plastic after 7 days of cell culture. The lower right quadrant contains CD29-highly positive and CD45-depleted MSCs while the upper right quadrant contains HCs with a CD45-positive and CD29-low expression profile. (B) Proportion of CD45-positive (black bars) and CD45-depleted (white bars) cells from rat MSCs isolated by adherence to plastic in relation to the time of the first medium change and therefore, removal of non-adherent cells. (C) Total yield of CD45-depleted (white bars) and CD45-positive (black bars) bone marrow-derived cells after 7 days of cell culture (* $p < 0.05$; ** $p < 0.01$). Yield of cells is normalized to cell number achieved when the non-adherent cells were removed on day 4.

Quantification of CD45-positive cell contamination of primary MSC cultures. In order to quantitate the ratio between haematopoietic CD45-positive cells and MSCs, we employed three-colour flow cytometry (FACS) which allowed distinction between the two different cell types according to their surface antigens (CD45, CD29) and dead cells (Fig. 2A). Fig. 2B

shows that the fraction of CD45-positive haematopoietic cells was not influenced by the adherence time after cell isolation. We mostly found ~20-30% of the attached cells in bone marrow cell cultures to be CD45-positive when selected according to sole adherence to plastic surfaces. Flow cytometric results were concurrent with the observations made after

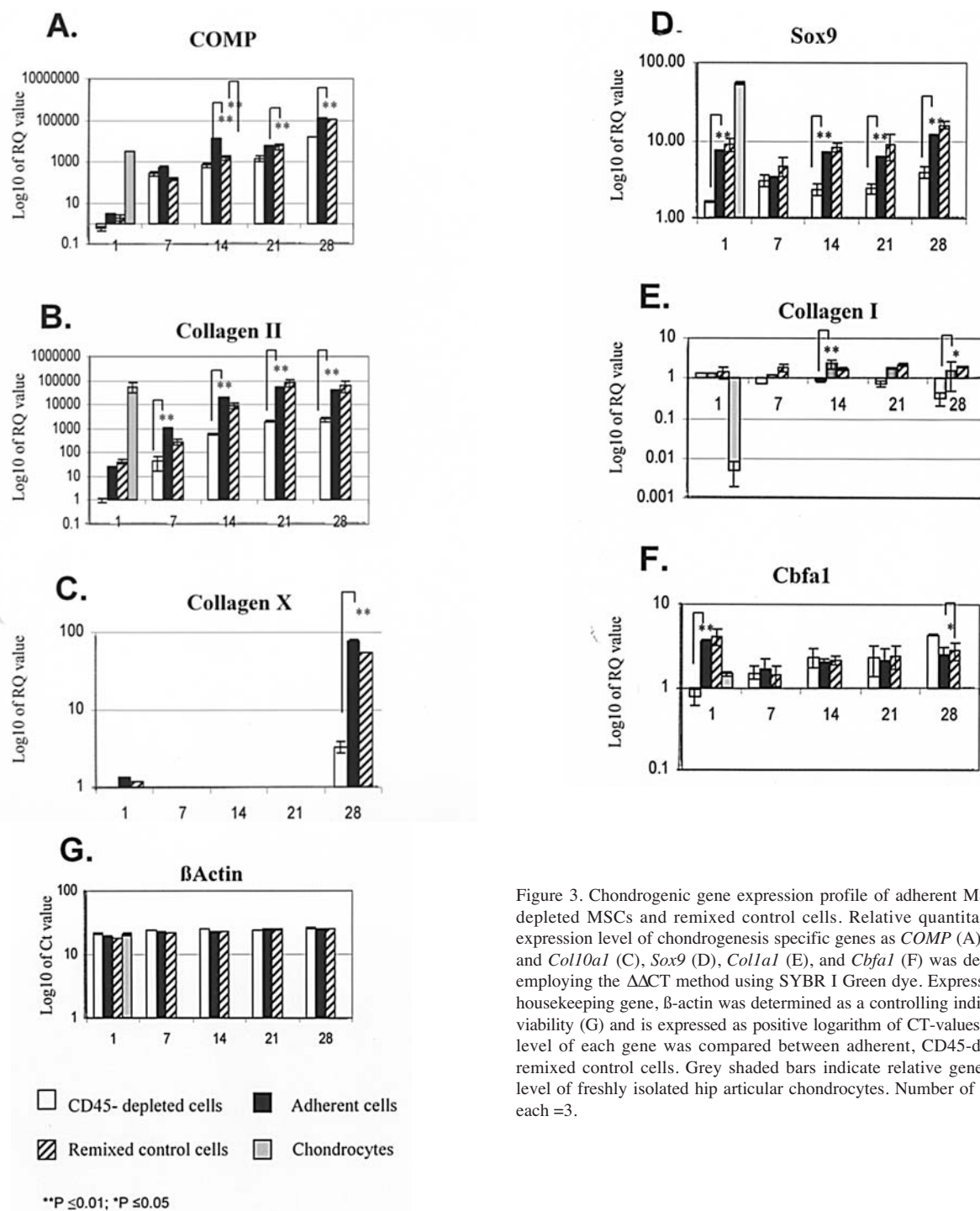


Figure 3. Chondrogenic gene expression profile of adherent MSCs, CD45-depleted MSCs and remixed control cells. Relative quantitative mRNA expression level of chondrogenesis specific genes as *COMP* (A), *Col2a1* (B) and *Col10a1* (C), *Sox9* (D), *Col1a1* (E), and *Cbfa1* (F) was determined by employing the $\Delta\Delta CT$ method using SYBR I Green dye. Expression level of housekeeping gene, β -actin was determined as a controlling indicator of cell viability (G) and is expressed as positive logarithm of CT-values. Expression level of each gene was compared between adherent, CD45-depleted and remixed control cells. Grey shaded bars indicate relative gene expression level of freshly isolated hip articular chondrocytes. Number of experiments each = 3.

immunofluorescent staining. In one out of four experiments, we detected variations between 10-40% in individual flasks.

We observed that CD45-depleted cells expressed CD29 stronger than CD45-positive cells (Fig. 2A) which offers an additional selection parameter. In cultures where non-adherent cells were removed on days 3 and 4, we obtained a significantly increased number of desired CD45-depleted MSCs compared to removal on day 1 and 2 (Fig. 2C). Apparently, 3-4 days were a more appropriate duration for the adherent fraction to get acclimated to the cell culture conditions, hence exhibit a firmer attachment to the cell culture plastics.

*Effect of CD45-positive cells on gene expression levels of *Col1a1*, *Col2a1*, *Col10a1*, *COMP*, *Sox9* and *Cbfa1*.* During the time course of cell culture we observed a strong increase in

relative mRNA levels of COMP and collagen II, while Sox9 mRNA levels exhibited a moderate upregulation (Fig. 3A, B, and D). Collagen X gene expression remained undetectable the first three weeks of cell culture; however, at day 28 expression of this collagen was found to be induced (Fig. 3C). The internal control gene, β -actin, remained unaltered during the complete time course of cell culturing (Fig. 3G). Comparing gene expression of CD45-depleted cells and the non-sorted population, we found collagen I gene expression to be increased in the non-sorted cell population during the culture period, while it became decreased in CD45-depleted MSCs. However, in CD45-depleted cells collagen I was still expressed at a relative higher level as in freshly isolated hip articular chondrocytes (Fig. 3E). Notably, the non-sorted cells displayed

strikingly higher upregulation of collagen II, COMP and Sox9 gene expression compared to the CD45-depleted MSCs throughout the culture period. In the non-sorted population collagen II gene expression is upregulated on average ~25-fold, COMP gene expression ~10-fold, collagen X expression ~23-fold and Sox9 mRNA levels ~3-fold when compared to CD45-depleted MSCs. For collagen I gene expression we observed a significant difference only between the two groups at day 14 and 28, where the adherent MSCs demonstrated an upregulation resulting in a 3-5-fold higher gene expression when compared to CD45-depleted MSCs. Collagen I remained upregulated from day 14 on in adherent cells while CD45-depleted MSCs exhibit an increasingly downregulation during the time course of cell culture. We also observed a striking difference in the collagen X expression pattern between non-sorted and CD45-depleted cells. The collagen X mRNA expression profile of CD45-depleted cells experienced only a moderate increase of ~3.3-fold at the end of the culture period while adherent cells upregulated their mRNA level ~76-fold compared to day 0 (Fig. 3C). Notably, *Cbfa1* gene expression is differentially regulated with respect to the two cell populations (Fig. 3F). On day 1 *Cbfa1* mRNA level is significantly higher in adherent cells (4.7-fold) and also in chondrocytes (2.0-fold) compared to CD45-depleted cells. This pattern became inverted at day 28 with CD45-depleted cells exhibiting an elevated expression (1.7-fold) compared to the adherent MSCs. In between these time points, relative gene expression of *Cbfa1* is similarly regulated in both cell populations.

In remixed control cell populations (30% CD45-positive cells with 70% CD45-depleted cells) collagen II, X, I, Sox9 and *Cbfa1* gene expression profile resembles closely that of adherent cells (Fig. 3B-F) and exhibits no significant differences. Only COMP gene expression at day 14 of the culture period significantly differs from adherent cells but the level at day 21 and 28 shows no difference to non-sorted cells (Fig. 3A). β -actin gene expression profile is similar for all three culture conditions (Fig. 3G).

Discussion

Adult bone marrow is the major source of haematopoietic and mesenchymal stem cells (1). In animal studies, adherence to tissue culture plastic is accepted as a satisfactory parameter for attaining marrow stromal cell population depleted of haematopoietic cells. However, when employing FACS analysis for surface antigen characterization we observed a contamination of these non-sorted MSCs with 20-30% of CD45-positive cells. For additional characterization of sorted and non-sorted cell population we applied immunofluorescence staining for CD68 and D7Fib. The D7Fib antigen constitutes a fibroblast-specific molecule of yet unknown function, supposedly a specific marker for MSCs as suitable as STRO-1 and CD105 (20). We observed expression of the D7Fib antigen on adherent CD45-positive cells; however, these cells displayed a rounded morphology compared to the fibroblast-like CD45-depleted cells. CD68 and its murine analogue macrophage, a member of the lamp family, appear to have a rather limited tissue distribution, being found on macrophages, Langerhans cells and dendritic cells (21). Notably, we

have found all adherent cells CD68-positive suggesting that in non-committed undifferentiated cells, this protein might exhibit a different distribution pattern and possibly exert functions related to cell-cell or cell-ligand interactions (22).

CD45-positive haematopoietic cells were always detected in MSC cultures, regardless of the time of the first change of culture medium. This indicated a persistent presence and attachment of CD45-positive cells in the MSC environment when no further selection based on specific surface antigens is carried out. Adherence to the tissue culture plastic alone is a weak discriminating criterion for a homogeneous MSC population because neural cells, monocytes and macrophages are also known to be isolated according to their adherence to plastic surfaces (6,23,24). The presence of CD45-positive haematopoietic cells and the variation of their proportion in primary cultures of MSCs could explain the occurrence of extensive variations usually seen in differentiation experiments with bone marrow-derived cells.

The fact that CD45-depleted cells expressed CD29 stronger than CD45-positive cells offered us an easier separation of two populations and limited the possible existence of more than two major subpopulations in our cultures. The integrin β 1 subunit CD29 exhibits a broad tissue distribution, including lymphocytes, endothelia, smooth muscle, and epithelia (25) and plays an important role in cellular processes, e.g. embryogenesis and HSC development. Although we did not find the haematopoietic cell fraction to vary according to the time of removal of non-adherent cells, we observed a correlation between adhesion time and yield of CD45-depleted marrow stromal cells. In parallel, cell adhesion plus proliferation could also be promoted by the prolonged presence of the non-adherent fraction via cytokine release and/or cell-cell contacts. A comparable effect of haematopoietic cells on MSC proliferation has been observed for megakaryocytes and platelets (26). Based on our results, we decided to leave the cells to adhere for 3 days before performing differentiation experiments.

Differentiation studies of HSCs have demonstrated that the small number of adherent MSCs present in total bone marrow aspirates provide an important microenvironment for growth of HSCs and their differentiation into several blood borne cell types. In long-term cultures of HSCs, these adherent cells even interact directly with the haematopoietic precursors (5). Herein, we showed that vice versa adherent CD45-positive haematopoietic precursor cells create a microenvironment which may enhance expression of particular marker genes in MSCs. For studying the effects of contaminating CD45-positive cells, we prepared a relatively homogeneous population of CD45-depleted MSCs by immunogenic depletion of CD45-positive cells. Both, the heterogenic adherent cells and the CD45-depleted subpopulation, were subjected to chondrogenic favourable 3-D environment by formation of a cell alginate amalgam. For control purposes in order to assure that difference in gene expression profile between sorted and unsorted MSCs is due to depletion of CD45-positive cells and not because of general depletion of differentiation competent cells or due to cell damage caused by the sorting process, we prepared a third population of remixed cells (30% CD45-positive mixed with 70% CD45-depleted cells) following MACS preparation which was treated

according to the differentiation protocol used for adherent and sorted cells.

We monitored relative gene expression of collagen II (27) and COMP (28,29) as examples for structural extracellular matrix proteins highly specific for hyaline cartilage. Additionally, we have investigated Sox9 as a master transcription factor imperative for chondrogenic differentiation (13) and collagen I as differentiation control for a gene normally not active in healthy hyaline cartilage (30) but occurring together with collagen II in fibrocartilage (31). Gene expression of collagen X, specific for terminal differentiated hypertrophic chondrocytes in the calcifying zone of cartilage and secondary ossification centres within the growth plate (32) indicates maturation of MSCs towards an undesired cartilage condition.

Gene expression of Cbfa1, a transcriptional activator for osteoblastic differentiation during the process of endochondral ossification is analyzed for monitoring differentiation towards osteogenic condition. Cbfa1 is also expressed in the pre-condensation stage during early development in a cell type with the potential to become either a chondrocyte or an osteoblast. In later developmental stages, expression of this transcription factor is restricted to prehypertrophic and hypertrophic chondrocytes where it acts as a hypertrophic differentiation inducer (33,34).

The observed gene expression profile indicates not only a beneficial influence of CD45-positive cells on the differentiation process of MSCs towards the chondrogenic lineage, but also shows that when culture time is extended beyond 28 days chondrogenically differentiating MSCs might enter the prehypertrophic/hypertrophic differentiation cascade and proceed towards terminal differentiation. Usage of chondrogenic culture medium does not prevent upregulation of Cbfa1 and collagen X. This indicates either a not yet defined influence of medium components (i.e. TGF- β or dexamethasone) on intrinsic cell-derived factors (i.e. signalling molecules, proteases, transcription factors) which are involved in regulation of the chondrogenic differentiation cascade or a medium independent process occurring by default through paracrine interaction of MSCs. A variety of growth factors i.e. members of the TGF- β superfamily, IGF-1 and bFGF could account for gene modulating effects in our system (35-38).

In conclusion, we demonstrated that bone marrow-derived fibroblast-like CD45-depleted rat MSCs share a heterogeneous cellular environment with ~20-30% CD45-positive HCs, if no other selection procedure than adherence to plastic is employed. Our differentiation results indicate that adherent CD45-positive haematopoietic cells may create a micro-environment promoting differentiation of MSCs towards the chondrogenic lineage by upregulating specific genes. However, expression of undesired genes such as collagen I and collagen X is likewise promoted by HCs. On the contrary, at early condensation stage HCs strongly increase gene expression of Cbfa1 thus supporting chondrogenic differentiation of MSCs, while at a later differentiation stage when Cbfa1 plays an integral role in hypertrophy the regulation appears to be independent of the presence of HCs.

We speculate that the stromal CD45-positive population *in toto* is responsible for regulation of gene expression. The heterotrophic nature of bone marrow-derived cells suggest that

in vivo interactions of different cell types is very likely and should be addressed accordingly. Characterization of additional subpopulations of CD45-positive cells according to their surface antigens and determination of their potential influence on gene expression may elaborate this point even further. Studies using conditioned media are needed to clarify whether physical contact between cells or paracrine factors, alone or in concurrence, are necessary for promoting gene expression leading to chondrogenic differentiation and maturation of MSCs. Identification and isolation of factors which are capable of modulating genes involved in differentiation and dedifferentiation processes of chondrocytes is vital for *in vitro* engineering of cartilage tissue repair.

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