FGF-2 addition during expansion of human bone marrow-derived stromal cells alters MSC surface marker distribution and chondrogenic differentiation potential

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Received 30 March 2013; revision accepted 10 May 2013

Abstract

Objectives: Although clinical applications using mesenchymal stromal cells (MSCs) are becoming more frequent, procedures for their *in vitro* culture are far from standardized. Growth factors such as FGF-2 are frequently added during expansion to improve population growth and differentiation characteristics. However, up to now its influence on surface marker distribution of MSCs has been close to unknown. The purpose of this study was therefore to analyse effects of FGF-2 supplementation on pre-selection of MSC subpopulations.

Materials and methods: Mesenchymal stromal cells were harvested from bone marrow of six patients and expanded in alpha-MEM or DMEM-LG. Starting in passage 2, 10 ng/ml FGF-2 was administered and non-supplemented media were used as controls. Growth indices were calculated from P0 to P4. After P4, fluorescence cytometry for common MSC surface markers was performed and standard chondrogenic, adipogenic and osteogenic differentiation protocols were applied.

Results: Cell population growth indices were higher for those in FGF-2 supplemented media. Significant differences in surface marker distribution were observed for CD13, CD14, CD49, CD90, CD340 and STRO-1 depending on respective culture conditions. FGF-2 suppressed CD146 expression in both alpha-MEM and DMEM-LG. No

differences in adipogenic and osteogenic differentiation potential could be observed, while FGF-2 significantly improved chondrogenic differentiation in DMEM-LG.

Conclusions: While holding the benefit of improving MSC chondrogenic differentiation potential, FGF-2 pre-selects certain MSC subtypes. Our data clearly show that expansion culture conditions have a significant effect on distribution of a number of MSC surface markers.

Introduction

Mesenchymal stem or stromal cells (MSCs) were first described in the 1960s and 1970s when they were mainly perceived for their remarkable expansion potential (1,2). Since then they have become an important tool in tissue engineering, due to their multilineage differentiation potential (3–7). Moreover, MSCs have been recognized for their immunomodulatory properties (8,9), thus broadening their potential for therapeutic use. MSCs have already successfully entered clinical applications, for example in treatment of patients with severe graft-versus-host disease (10) and have also proven to be promising targets for treatment of myocardial infarction (11) and multiple sclerosis (12); beneficial effects of MSCs have also been demonstrated in organ transplantation (13,14). MSCs currently are the subject of numerous experimental surgical and non-surgical trials (15), but although these applications have become more frequent over the past number of years, there remains uncertainty concerning their precise and specific entity.

It is generally accepted that MSCs are identified by their characteristic adherence to plastic, their mesenchymal descent and their expansion potential, however,

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recent findings suggest that they are a heterogeneous population with respect to most of their properties, including surface marker distribution and differentiation potential (16–18). An attempt to narrow the definition of MSCs has been made by the International Society for Cellular Therapy (19); to qualify as MSCs, they must express CD105, CD73 and CD90 and lack expression of CD45, CD34, CD14 and CD11b. Additionally, clonal cultures must display multilineage potential, at least by differentiating into fibroblasts, osteoblasts, adipocytes and chondroblasts, under specific culture conditions. However, conditions under which MSCs are differentiated and expanded, are far from being standardized. Although some authors have pointed out that altered culture conditions might have a striking impact on differentiation (20–23), this effect is generally underestimated. As culture conditions vary enormously between different laboratories, this may result in culturing different cell populations in different places, although all of them are referred to as MSCs (23).

Growth factors have become an important instrument in the attempt to control MSC differentiation properties. In this context, fibroblast growth factor 2 (FGF-2) has been shown to improve chondrogenic differentiation of MSCs (24–27) and is the growth factor most frequently administered during their expansion (28). It remains unclear though, whether preconditioning of MSCs by growth factors could result in deterioration of other properties. With regard to their ongoing and increasing use in human subjects, we believe that better understanding of how distinct variations of MSC culture conditions may alter their properties is essential. This will contribute to attempts at standardizing their handling, and thus improve safety as well as comparability of scientific results. Thus, the aim of this study was to determine effects of FGF-2 supplementation during expansion of human MSCs, in two widely used cell culture media, on surface marker distribution and cell differentiation properties.

Materials and methods

Bone marrow donors

Human MSCs were collected from ilium, tibia or femur bone marrow, of 6 donors with mean age 43.33 ± 20.26 years (5 male and 1 female patients), within the framework of bone grafting to another body site or total joint arthroplasty. The study protocol was approved by the Ethics Committee of the University of Heidelberg, Germany, and all patients provided informed consent according to the latest version of the Helsinki Declaration.

Mesenchymal stromal cells

Bone marrow was diluted to 1:1 with isotonic saline solution (B. Braun, Melsungen, Germany) containing 5000 I.E. heparine (Ratiopharm, Ulm, Germany). Mononuclear cells (MNCs) were collected by Ficoll Paque plus gradient centrifugation (GE Healthcare, Uppsala, Sweden). MNCs were resuspended at 5×10^5 cells/cm² in T75 cell culture flasks (Nunc, Roskilde, Denmark). They were then cultured under the same conditions (37 °C with 6% CO₂ in a humidified thermostat) in two different culture media compositions:

- 1 Dulbecco's modified Eagle's medium low glucose (DMEM-LG; Invitrogen, Karlsruhe, Germany) with 10% foetal calf serum (FCS; Biochrom, Berlin, Germany) and 1% penicillin/streptomycin (Invitrogen).
- 2 Alpha minimum essential medium (α MEM) with L-glutamine and 10% FCS (Biochrom) and 1% penicillin/streptomycin (Invitrogen).

Non-adherent cells were discarded by medium change after 24 h. MSCs were inspected for confluence daily by polarization microscopy. At 80% confluence (end of P0), cells were detached by incubation with trypsin (Biochrom) and washed in complete medium. After being stained in 0.4% trypan blue solution, MSCs were counted in triplicate (Sigma-Aldrich, Steinheim, Germany) then re-suspended at 5×10^5 cells/cm². At the end of passage 1, cells were cultured under the following four conditions:

Medium 1 (alpha-MEM, see above) with and without addition of 10 ng/ml FGF-2 (Acris, Herford, Germany) and Medium 2 (DMEM-LG, see above) with and without addition of 10 ng/ml FGF-2 (Acris).

Cells were expanded according to protocols detailed above to the end of passage 4 ($n = 6$ per group). Cell population growth index was defined as quotient of cells before and after a passage.

Flow cytometry

Flow cytometry was performed with cells of all six donors after P4. To prepare them for labelling, cells were detached with trypsin, washed in whole medium (see above) and re-suspended in phosphate buffered saline (PBS; Miltenyi Biotec, Bergisch Gladbach, Germany) with 0.5% FCS (Invitrogen), and 2 mM EDTA (Sigma-Aldrich).

Cells were then labelled for the following antigens (all anti-human antibodies): CD10 FITC mouse IgG1 (HI10a), CD13 PE mouse IgG1 (WM15), CD14 FITC mouse IgG2a (M5E2), CD34 PE mouse IgG1 (8G12), CD44 FITC mouse IgG2b (G44-26), CD45 FITC mouse IgG1 (2D1), CD49a PE mouse IgG1 (SR84), CD90 FITC mouse IgG1 (5E10), CD140b PE mouse IgG2a

(28D4), CD146 PE mouse IgG1 (PIH12), CD166 PE mouse IgG1 (3A6), CD271 PE mouse IgG1 (C40-1457) (all BD Biosciences, Heidelberg, Germany), CD105 PE mouse IgG1 (43A4E1), CD133 PE mouse IgG1 (AC133) (Miltenyi Biotec), HLA-ABC PE (W6/32) (Dako, Glostrup, Denmark). CD340 mouse IgG1 (6C2) (Genway, San Diego, CA, USA) and STRO-1 mouse IgM (STRO-1) (R+D Systems, Wiesbaden, Germany) were labelled with secondary goat anti-mouse FITC antibodies (Dako). Assessment of background fluorescence was conducted by corresponding isotype-matched control antibodies (IgG1 FITC and PE, IgG2a FITC and PE, IgG2b FITC, all Dako). One- and two-colour cytometry were performed on a FACS Scan flow cytometry analyser (BD Biosciences, San Jose, CA, USA) using Cellquest Pro Software (BD Biosciences). 10 000 events were assessed for each antibody. Positive fluorescence was defined as any event above 99.5% background isotype antibody fluorescence, defined in a histogram plot.

Chondrogenic differentiation

After P4, MSCs were detached with trypsin as described above and centrifuged into pellets, each containing 5×10^5 cells ($n = 3$ pellets per group). Chondrogenic differentiation was induced using chondrogenic medium containing 286 ml DMEM HG (Invitrogen), 150 μ l transferrin 10 mg/ml (Sigma-Aldrich), 1 μ l sodium selenite 100 μ g/ml (Sigma-Aldrich), 3 ml sodium pyruvate 350 mM (Sigma-Aldrich), 5 ml BSA 7.5% (Invitrogen), 3 ml P/S (Invitrogen), supplemented by 50 μ l dexamethasone/5 ml (Sigma-Aldrich), 5 μ l ascorbic acid/5 ml (Sigma-Aldrich), 5 μ l TGF- β /5 ml (Acris) and 6.9 μ l insulin glargine/5 ml (Sanofi Aventis, Frankfurt, Germany); chondrogenic medium was changed three times a week. Histology and DMMB (1,9-dimethyl-methylene blue) assay were used to evaluate chondrogenic differentiation. Pellets were therefore fixed in 4% paraformaldehyde, mounted and cut into 5 μ m sections. Centre-cut sections were then stained with safraninO/fast green (Sigma-Aldrich). For the DMMB-assay, pellets were digested with pepsin solution overnight and stained for glycosaminoglycans with 1,9-dimethyl-methylene blue (dye content 80 %, Sigma-Aldrich). Absorption was measured at 530 nm and compared to a chondroitin 4-sulphate standard (Sigma-Aldrich). DNA content was measured using Quant iT ds Pico Green DNA Assay Kit (Invitrogen) according to the manufacturer's protocols.

Osteogenic differentiation

After P4, MSCs were harvested as described above and 35 000 cells per well were seeded in osteogenic induc-

tion medium consisting of DMEM high glucose (Invitrogen), 10% FCS (Biochrom), 0.1 mM dexamethasone (Sigma-Aldrich), 0.17 mM ascorbic acid 2-phosphate (Sigma-Aldrich), 10 mM β -glycerophosphate (Sigma-Aldrich) and 1% penicillin/streptomycin (Biochrom). After 21 days, osteogenesis was quantified using the alkaline phosphatase assay. In brief, cells were lysated in 0.5 ml 1% Triton X-100 (Sigma-Aldrich), and 100 μ l lysate was incubated in 100 μ l 1 mg/ml p-nitrophenylphosphate in ALP-buffer (0.1 M glycine, 1 mM $MgCl_2$, 1 mM $ZnCl_2$, pH 10.4). Substrate turnover was measured at 405/490 nm in a MRX ELISA reader (Dynatech Laboratories, Stuttgart, Germany). Lysate protein content was analysed using a Micro BCA Protein Assay Kit (Pierce, Rockford, IL, USA) according to the manufacturer's instructions. ALP levels were standardized to protein content. Calcium deposition was quantified by 0.5% alizarin red S staining (Chroma, Münster, Germany) at 570 nm and standardized to whole protein content as described above.

Adipogenic differentiation

After P4, MSCs harvested as described above were seeded 35 000 cells per well in adipogenic induction medium, consisting of DMEM high glucose (Invitrogen), 10% FCS (Biochrom), 1 mM dexamethasone (Sigma-Aldrich), 0.2 mM indomethacin (Sigma-Aldrich), 0.5 mM isobutyl methylxanthine (Sigma-Aldrich), 0.01 mg/ml insulin glargine (Sanofi Aventis) and 1% penicillin/streptomycin (Biochrom). After 21 days adipogenic induction, cells were fixed in 4% paraformaldehyde and stained in 0.3% oil red O solution (Chroma); re-extraction of dye was conducted with 60% isopropanol and optical density was measured at 490 nm.

Statistical analysis

Normal distribution was assessed by analysing QQ-plots and box plots, performing a ratio analysis and Kolmogorov–Smirnov (with Lilliefors significance correction) as well as Shapiro–Wilk testing. Student's paired *t*-tests were performed for parametric data and comparison of two medium conditions, when applicable, while analyses of variance (ANOVA), followed by Bonferroni correction, was performed to compare parametric data with more than two groups (P0 cell yield, growth indices, passage time, GAG/DNA content, alizarin red analysis, ALP analysis). Friedman tests were performed to compare paired non-parametric data sets for four group comparisons, followed by Wilcoxon tests to determine differences between two conditions (surface marker expression). Differences were considered statistically

significant for P -values less than 0.05. Results presented as means $\pm$ standard deviation. All calculations were performed with SPSS software (SPSS Inc., released 2009, PASW Statistics for Windows, Version 18.0. Chicago, IL, USA).

Results

Cell population growth indices were higher in FGF-2-supplemented media

Mean cell yield after Ficoll separation was $2.56 \pm 1.04 \times 10^6/\text{ml}$ bone marrow aspirate. No differences were observed concerning P0 cell yield per MNCs between alpha-MEM and DMEM-LG (101.35 ± 92.56 versus 6.2 ± 18.08 , $P = 0.44$); calculated growth indices in P1, P2, P3 and P4 are displayed in Fig. 1. Population growth index in P1 did not differ between alpha-MEM and DMEM-LG ($P = 0.89$). Upon addition of FGF-2 in P2, it was significantly higher in both FGF-

supplemented alpha-MEM ($P = 0.034$) and DMEM-LG ($P = 0.038$) than in non-supplemented media. Higher population growth indices in FGF-2-supplemented media were also observed in P3 and P4; however, there were no significant differences between the media ($P = 0.1$ and $P = 0.18$ for alpha-MEM $\pm$ FGF-2; $P = 0.18$ and $P = 0.3$ for DMEM $\pm$ FGF-2).

FGF-2 administration altered surface marker distribution on the cells

Results of surface marker distribution on the MSCs, under different culture conditions are displayed in Table 1. Despite important variation between the donors, surface markers such as CD10, CD14, CD34 and CD45 (so called 'negative MSC markers') had low fluorescence values in all groups. However, CD10 showed greater variation of fluorescence in both DMEM-LG and DMEM-LG+FGF-2. Significantly higher CD14 fluorescence was observed in alpha-MEM

Figure 1. Growth indices in passages 1(a), 2(b), 3(c) and 4(d). Results are displayed as means $\pm$ SD. * $P < 0.05$.

Table 1. Surface marker distribution on human bone marrow-derived mesenchymal stem cells in four different culture conditions

	Alpha-MEM	Alpha-MEM + FGF-2	DMEM-LG	DMEM-LG + FGF-2	Friedman
CD10	0.63 ± 0.23	2.83 ± 1.58	12.62 ± 22.79	9.09 ± 11.66	<i>P</i> = 0.06
CD13	98.52 ± 2.61	99.9 ± 0.55	97.67 ± 2.6	99.02 ± 0.85	<i>P</i> = 0.003
CD14	1.31 ± 1.56	0.29 ± 0.27	0.33 ± 0.19	0.30 ± 0.08	<i>P</i> = 0.031
CD34	0.21 ± 0.2	0.22 ± 0.16	0.34 ± 0.3	0.55 ± 0.41	<i>P</i> = 0.261
CD44	97.83 ± 2.52	98.21 ± 2.08	96.16 ± 3.88	95.53 ± 4.81	<i>P</i> = 0.221
CD45	0.41 ± 0.2	0.23 ± 0.12	0.23 ± 0.22	0.33 ± 0.11	<i>P</i> = 0.284
CD49	56.88 ± 17.67	59.36 ± 25.11	47.89 ± 12.77	22.19 ± 17.76	<i>P</i> = 0.032
CD73	97.31 ± 2.47	99.60 ± 0.29	94.93 ± 5.43	92.76 ± 13.29	<i>P</i> = 0.102
CD90	98.69 ± 2.90	98.41 ± 2.74	96.98 ± 2.81	82.03 ± 23.25	<i>P</i> = 0.007
CD105	93.42 ± 14.85	98.01 ± 1.72	94.15 ± 5.57	80.35 ± 26.30	<i>P</i> = 0.102
CD140b	91.26 ± 13.11	93.78 ± 4.27	87.31 ± 8.15	73.31 ± 33.03	<i>P</i> = 0.172
CD146	40.77 ± 23.59	12.12 ± 10.80	53.2 ± 19.99	3.82 ± 6.04	<i>P</i> = 0.006
CD166	91.52 ± 11.82	96.73 ± 2.58	89.09 ± 14.67	76.62 ± 14.08	<i>P</i> = 0.12
CD271	0.86 ± 0.35	0.86 ± 0.47	0.94 ± 0.68	0.84 ± 1.03	<i>P</i> = 0.706
CD340	69.44 ± 24.52	82.71 ± 19.51	38.80 ± 21.97	70.89 ± 11.27	<i>P</i> = 0.007
STRO-1	78.13 ± 25.55	85.75 ± 14.30	38.27 ± 26.24	26.45 ± 27.14	<i>P</i> = 0.0017
HLA-ABC	96.22 ± 6.97	98.90 ± 0.9	97.35 ± 1.81	93.33 ± 7.74	<i>P</i> = 0.261

Results are displayed as means ± standard deviation.

P-values represent overall results of Friedman tests among the four groups.

Bold values significant differences among the four groups detected.

compared to the other media (Table 1, Fig. 2). Among the surface markers generally taken to be 'positive' markers of MSCs, CD13, CD44, CD73, CD90, CD105, CD140b and CD166 had high fluorescence in all groups, although mean fluorescence was lowest for all of these markers in FGF-2-augmented DMEM-LG. There was significant difference in CD13 expression between alpha-MEM+FGF2 and DMEM (*P* = 0.028) and DMEM+FGF-2 (*P* = 0.028). No MSCs expressed CD271, regardless of culture condition applied.

Significant differences in distribution of CD49, CD90, CD146, CD340 and STRO-1 were observed (Figs 2a–d, 3a–c). CD49 expression was significantly lower in DMEM-LG+FGF-2 compared to DMEM-LG and alpha-MEM (Table 1, Fig. 2c, *P* = 0.028). CD90 expression was significantly lower in DMEM-LG+FGF-2 (Fig. 2d, *P* = 0.028 compared to alpha-MEM and alpha-MEM+FGF-2). CD146 expression was significantly lower in both alpha-MEM and DMEM-LG when FGF-2 was administered (Table 1, Fig. 3a, Wilcoxon tests *P* = 0.046 for AlphaMEM/Alpha-MEM+FGF-2; *P* = 0.028 for DMEM-LG/DMEM-LG+FGF-2). CD340 expression seemed to be upregulated under FGF-2 addition; the difference was, however, only significant between DMEM-LG and DMEM-LG+FGF-2 (*P* = 0.028), while CD340 expression was significantly lower in DMEM-LG compared to alpha-MEM (Fig. 3c, *P* = 0.028). STRO-1 expression was significantly lower in both DMEM-LG+FGF-2 compared to alpha-MEM with and without addition of FGF-2 (*P* = 0.028 for Alpha-MEM/DMEM-LG+FGF-2 and Alpha-MEM+FGF-2) and DMEM-LG

(*P* = 0.046 for DMEM-LG/Alpha-MEM+FGF-2, Table 1, Fig. 3b).

FGF-2 altered MSC chondrogenic differentiation potential

Under all four culture conditions, MSCs could successfully be differentiated into osteogenic (as reflected by light microscopy, calcium deposition and ALP analysis) and adipogenic (as reflected by quantitative and qualitative assessment of oil red O staining) phenotypes after P4 (Fig. 4). No differences were observed concerning osteogenic and adipogenic differentiation (data not shown). Although there was a tendency towards higher GAG/DNA content of FGF-2-treated MSC in both DMEM-LG and alpha-MEM, highest values with significant difference were only observed in DMEM-LG+FGF-2 (Fig. 5, *P* = 0.013).

Discussion

Mesenchymal stromal cells remain one of the most promising tools for use in regenerative medicine due to their multilineage potential (29–33). Although already having entered clinical applications (34,35), conditions under which they are cultured vary widely between different laboratories. Effects of culture conditions, such as oxygen supply (36,37) and culture composition (22,38,39), on expansion and differentiation of MSCs, have been described in a range of studies, but variations in the latter have lately become too numerous to overlook.

Figure 2. Flow cytometry results after P4 in four different culture conditions: Alpha-MEM±FGF-2, DMEM±FGF-2, $n = 6$ measurements per group. * $P < 0.05$. (a) CD13, (b) CD14, (c) CD49 and (d) CD90.

Use of growth factors during expansion and differentiation of MSCs, for example, has become more and more popular. One of the most commonly employed growth factors is basic fibroblast growth factor (bFGF or FGF-2). Its frequent use is explained by its determining influence on MSC proliferation and differentiation characteristics. Several studies have reported enhanced proliferation of MSCs upon FGF-2 administration (22,40–43), although FGF-2 seems to be only one besides other growth factors to promote MSC proliferation, such as PDGF-BB and TGF- β 1 (44). FGF-2 has also been demonstrated to promote chondrogenic differentiation when used during cell population expansion

(26,45). We chose to apply FGF-2 at the beginning of P2 to ensure that cell preparations were composed of homogeneous MSCs. Also, we needed to determine whether administration of FGF-2 had any influence on MSCs when applied to cells that had been cultured without growth factors for two passages. Various concentrations of FGF-2 from 1 to 10 ng/ml have elsewhere been applied during expansion of MSCs [overview in (28)]. We chose to use a high concentration of FGF-2 to clearly surpass any natural occurrence of the growth factor in FCS, but at a concentration proven in previous studies (46,47) to allow safe expansion and chondrogenic differentiation. Our data, based on a 10 ng/ml FGF-2

Figure 3. Flow cytometry results after P4 in four different culture conditions: Alpha-MEM±FGF-2, DMEM±FGF-2, $n = 6$ measurements per group. * $P < 0.05$, ** $P < 0.01$. (a) CD146 (b) STRO-1 and (c) CD340.

addition starting in P2, add to other results reporting the positive effect of FGF-2 on MSC proliferation and chondrogenic differentiation. One recent investigation has focused on the importance of proliferation for chondrogenic differentiation (48). These findings are supported by our own data, clearly indicating acceleration of proliferation from the beginning of FGF-2 administration, and improved chondrogenic outcome for FGF-2 augmented groups.

It has recently been shown that FGF-2 improves proliferation of umbilical cord-derived MSCs (UC-MSCs), but that their 'nature' remains unaffected, as reflected by immunophenotype and immunomodulatory properties (49), although it was also clearly stated that cytokine profiles were significantly altered by FGF administration. However, those authors noted that their cells met all criteria for MSCs defined by the International Society for Cellular Therapy (ISCT) (19), although these data were not shown (49). The main difference between use of our bone marrow MSCs (BM-MSCs) and their UC-MSCs is that our data rely on systematic flow cytometry of more surface antigens than suggested by the ISCT. Indeed, with the exception of CD90 and CD14, markers defined by the ISCT were among the stable ones in our experiments, unaffected by culture composition.

Yet, our study reveals that FGF-2 had a significant influence on other important surface markers, such as expression of CD90, STRO-1, CD340 and CD146. One new investigation has shown that FGF-2 administration during expansion is associated with CD146 downregulation (39), which is in accordance with our findings, as well as that several typical stem cell markers remained unaffected by FGF-2. However, in contrast to this, we were also able to detect that differences in surface marker distribution also applied to other surface markers, such as CD90, which had reduced expression in DMEM-LG+FGF-2 and, less drastically, in DMEM. We cannot explain the mechanisms underlying variation in CD90 expression in DMEM-LG+FGF-2. It can only be speculated that these findings may have applied to other studies as well when applying quantitative expression marker analysis. Mean expression level (82%) in DMEM-LG+FGF-2 would still have been qualified as 'positive' for CD90 in most investigations when qualitatively observed; yet, considering that in this medium condition one-fifth (equalling 10^5 cells in pellet cultures) were negative for CD90, it is a significant finding for tissue engineering. More importantly, it would also imply that this quantity of cells could not be considered to be MSCs when following ISCT recommendations (19), although it has to be noted that the other criteria for MSC (expression of CD105, CD73, absence of CD34 and CD45) are fulfilled. Comparable to findings

Figure 4. Selected slides of Oil Red O staining after adipogenic differentiation, Alizarin Red staining after osteogenic differentiation. Light microscopy, index 200/500 μm .

Figure 5. (a) GAG/DNA content after chondrogenic differentiation in the four groups. $n = 6$ experiments per group. (b) Safranin O/Fast Green staining results (selected slides). Light microscopy, index 500 μm .

of Gharibi and Hughes (39), differences in surface marker distribution did not result in impaired differentiation; on the contrary, we found here that chondrogenic differentiation was higher in DMEM with addition of FGF-2. These findings could add to a current hypothesis that a more heterogeneous population of MSCs could be superior to their regenerative potential; however, more experiments will have to be conducted in this regard.

It has been demonstrated that MSCs derived from patients with haematological disorders have altered surface marker expression such as CD90, CD105, CD184 and HLA-DR (50), clearly indicating that change in surface markers can be a consequence of changes in MSC microenvironment. Our study can contribute to these findings by pointing out additional markers that may depend on conditions of media that MSCs are grown in.

CD49a (alpha-1 integrin) was one of the first surface markers described for MSCs. In our experiments, CD49a was one of the most donor-dependent ones found on our BM-MSCs. Its expression was significantly lower in DMEM-LG+FGF-2 compared to alpha-MEM±FGF-2. One study has shown that CD49a clones generated by fluorescence sorting showed enhanced CD90 and CD105 expression. Although our data do not allow directly correlating these surface markers, our findings are comparable to these data as both DMEM-LG and DMEM-LG+FGF-2 had lower CD49 and CD90 expression compared to alpha-MEM±FGF-2. No correlations between CD49a expression and differentiation outcomes can be made from our data, although it has been reported that differentiation outcomes in CD49a-sorted cells were superior to unsorted cells (35,51). Other attempts to purify MSC cell cultures by using CD49, however, have produced contradictory results (52). STRO-1+ cells have been associated with containing osteogenic precursor cells in bone marrow (53). We were not able to detect any differences in osteogenic differentiation potential between the different media conditions. STRO-1 expression in our experiments did not seem to be influenced by FGF-2, but rather by choice of basal culture media. Although STRO-1 expression was significantly higher in alpha-MEM with or without addition of FGF-2, no correlation with differentiation outcome can be made, as chondrogenesis as reflected by GAG/DNA content was equal in DMEM-LG and alpha-MEM.

CD340, also known as HER-2/neu, was among some novel markers for BM-MSCs described in 2007 (54). To our knowledge, we are the first to report changes in CD340 expression depending on medium composition. CD340 expression not only seemed to be upregulated under the influence of FGF-2 but also seemed to be dependent on basal medium composition, as it was found to be significantly lower in DMEM-LG than in alpha-MEM.

It remains to be proven whether these phenotypic alterations result in changes in functional properties such as other differentiation outcomes and immunomodulatory potential. Our data suggest that observed dramatic differences in surface marker distribution did not correlate with dramatic changes in osteogenic or adipogenic potential, while correlation between single surface markers and chondrogenic differentiation results will have to be assessed in future experiments. However, with regard to differences in surface marker distribution, a central statement of our data must be that ISCT criteria have to be understood in the context of their original publication – *minimal* criteria to define MSCs. The scope of heterogeneity of these cells, how-

ever, is far beyond this definition. We were able to demonstrate that more surface markers deserve attention, being directly influenced by culture conditions applied.

While biological significance of change in surface marker distribution remains to be revealed, the intention of this study was to demonstrate that MSCs remain an entity that is not clearly understood, and that understanding properties of their subpopulations is crucial. The results of our experiments indicate that the culture conditions in which MSCs are expanded preselect certain MSC phenotypes. Given the fact that a wide range of culture conditions are in use in different laboratories, this may have an impact on the quality of MSCs intended for clinical applications. Our data are also in favour of strengthening attempts to standardize MSC culture procedures.

Age range of donors in our study was wide, thus, MSCs may have exerted distinct properties related to age and comorbidities, most importantly osteoarthritis (OA). This may be reflected by the differentiation results, which revealed important variation between donors. Donor dependency regarding regenerative potential of MSCs, however, is most controversially discussed and although some studies have reported reduced chondrogenic capacities of OA patients (55), other important studies have been of the opinion that OA does not affect MSC chondrogenic differentiation potential (56,57), which is in accordance with our findings (58). Thus, we are convinced that variation in chondrogenic differentiation is merely a result of donor dependency itself rather than in relation to OA or age. Variation between MSCs from different donors is a long-known problem (59); however, reasons for this are still not fully understood. Donor dependency may have contributed to the fact that some differences in alpha-MEM were only observed as tendencies, but were not statistically significant.

The main interest of our study was detecting changes in surface marker distribution under different culture conditions and, more importantly, under the influence of FGF-2. All media conditions were tested in all six patients, thus providing information that clearly could be separated from donor-related effects. A further important factor with major influence on differentiation properties of human MSCs is FCS, which was used in all media here. By employing the same FCS lots and FCS concentrations throughout, we believe that effects demonstrated here were determined by the basal media or FGF-2 administration. The study was not intended to attempt to correlate surface marker distribution and differentiation outcome. This, however, must be an imperative goal for future experiments, as understanding of how phenotypic features of MSCs determine functional

properties may lead to improving tissue engineering in all fields of regenerative medicine.

In summary, our study contributes to previous findings that underline the ability of FGF-2 to improve proliferation and chondrogenic differentiation of human BM-MSCs. However, FGF-2, as well as basal culture media, has an important impact on surface marker distribution of the cells. To the best of our knowledge, we are the first to report changes in a distinct set of MSC surface markers depending on media composition and FGF-2 supplementation. While biological significance of these findings in terms of functional properties remains unclear, our study clearly indicates that attention must be paid to surface markers that are not yet part of the MSC definition. In our opinion, understanding these properties is crucial to standardizing MSC culture procedures, which should be imperative due to their growing use in human subjects.

Acknowledgements

We acknowledge Rene Wetzel, Patrick Göthlich, Marc Hoffmann and Elena Tripel for their support. The study was carried out with funding by the state of Baden-Württemberg, Germany. None of the authors received external funding in connection with the study presented in this publication.

Competing interests

The authors declare that they have no competing interests.

Author's contributions

SH, BM, WR and TG conceived of the study. SH drafted the manuscript. SH, TD, TG, BM and SF provided the tissue and blood samples. SH and SF carried out the experiments. SF, PWK and SH performed the statistical analysis. SH, WR, BM, TD, PWK and TG participated in study design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

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