

Characterization of human skin-derived mesenchymal stem cell proliferation rate in different growth conditions

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Abstract This study investigated conditions for optimal in vitro propagation of human skin-derived mesenchymal stem cells (S-MSC). Forty primary skin-derived precursor cell (SKP) cultures were established from both male and female donors (age 29–65 years) and eight of them were randomly selected for in-depth characterization. Effects of basic fibroblast growth factor (FGF-2), epidermal growth factor (EGF), leukemia inhibiting factor (LIF) and dibutyryl-cyclic adenosine monophosphate (db-cAMP) on S-MSC proliferation were investigated. Primary SKP cultures were >95% homogenous for CD90, CD73, and CD105 marker expression enabling to classify these cells as S-MSC. FGF-2 dose-dependent stimulation was observed in low serum medium only, whereas EGF neither stimulated S-MSC proliferation nor potentates the effect of FGF-2. Pronounced donor to donor differences among S-MSC cultures were observed in 3-day proliferation assay. This study demonstrates that homogenous S-MSC populations can be reproducibly isolated from individual donors of different age. Optimal cell culture conditions for in vitro propagation

of S-MSC are B27 supplemented or low serum media with FGF-2 (4 ng/ml). EGF and LIF as well as db-cAMP are dispensable for S-MSC proliferation.

Keywords Skin-derived mesenchymal stem cells (S-MSC) · Proliferation · FGF-2 · EGF · db-cAMP · FBS · FACS

Introduction

Skin is the largest human organ, which is under constant renewal process and contains a number of cell populations that originate from both mesoderm and ectoderm (Tsatsmali et al. 2002; Ancans et al. 2001). More recently skin has attracted attention as the stem cells reservoir for numerous cell lineages (Fernandes et al. 2004; Fuchs et al. 2004; Blanpain and Fuchs 2006). Several studies have confirmed that there are at least three different types of stem cells in the skin: hair follicle, bulge, and dermal precursor cells (Blanpain and Fuchs 2006; Ohyama et al. 2006; Watt et al. 2006). It has been shown, that dermal and hair follicle precursor cells are able to differentiate into neuronal, smooth muscle, melanocyte, chondrocyte and Schwann cells in vitro (Toma et al. 2005). Following studies have shown that skin-derived precursor cells (SKPs) can differentiate into adipogenic, osteogenic and chondrogenic lineages in vitro (Fernandes et al. 2004; Toma et al. 2005; Shih et al. 2005) and their surface marker profile was similar to bone-marrow mesenchymal stem cells (Shih et al.

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2005). SKPs have been obtained from foreskin (Toma et al. 2005), human hair follicles and bulge cells (Ohshima et al. 2006; Raposio et al. 2007), skin punch biopsies from healthy adult volunteers (Joannides et al. 2004; Rittie and Fisher 2005) and scalp tissue (Shih et al. 2005). Several recent studies have confirmed that stem cells derived from adult skin have properties common to all stem cells: capability to proliferate for many passages in culture to maintain relatively unspecialized phenotype and to differentiate into specialized cell types under specific conditions of cultivation (Fernandes et al. 2004; Fuchs et al. 2004; Blanpain and Fuchs 2006).

However, cell culture methods and media composition to obtain and to maintain different SKPs populations for potential clinical applications clearly need further elaboration. Also, the age and gender of the donor could affect response of primary cell cultures to growth conditions (Zhu and Joyce 2004). Proliferation rate under different cell culture conditions, secretion of specific growth factors and surface marker expression needs to be analyzed in detail to develop optimal SKPs propagation procedures.

Stem cells *in vivo* reside in specific microenvironment in the tissue (stem cell niche) wherein they receive the extrinsic signals, which are required to maintain their undifferentiated phenotype (Fuchs et al. 2004). Cultivation media and media supplements are critical factors, which may stimulate stem cell proliferation *in vitro* or may expand cultures whilst maintaining cell plasticity. Thus, experiments to determine optimal growth conditions for specific stem cells populations, e.g., SKPs obtained from adult human tissue are essential.

In this study we investigated factors that regulate the expansion of adult SKPs cultures while preserving their potential to differentiate into various cell types, which may be potentially useful for re-transplantation into the patient's body. We characterized the expression of hemopoietic and mesenchymal cell lineage surface markers in human skin derived stem cell populations and investigated cell proliferation in the presence of different exogenous stimuli, namely, fetal bovine serum (FBS), basic fibroblast growth factor (FGF-2), epidermal growth factor (EGF), leukemia inhibitory factor (LIF), and cell-permeable cAMP analog dibutyryl-cAMP (db-cAMP). Also, SKPs potency to secrete FGF-2, a growth factor essential for stem cell renewal (Liu et al. 2006), was determined.

Materials and methods

Chemicals

The cell cultivation media, reagents and materials were purchased from Invitrogen (UK). Growth factors FGF-2 (Cat. N° 233-FB) and EGF (Cat. N° 236-E.G) were from RnD Systems (UK), LIF (Cat. N° LIF1010) was purchased from Chemicon International (Germany), B27 supplement (Cat. N° 10889-038) was purchased from Invitrogen (UK). Dispase (Cat. N° 10269638001), Liberase Blendzyme 1 (Cat. N° 11988409001) and Bromodeoxyuridine (BrdU) Labeling and Detection kit III (Cat. N° 11444611001) were purchased from Roche Applied Science (Germany). Methyl-[³H]thymidine (Cat. N° TRK120-1MCI, 37 MBq, 1 mCi) was from Amersham GE Healthcare (UK). Protein ready-to-use microtiter plate assay kit for the colorimetric detection of proteins was purchased from BioChemika (Cat. N° 77371, Sweden). Basic fibroblast growth factor (FGF-2) quantitative determination kit was obtained from R&D Systems (Cat. N° DY233, UK).

Primary cell cultures

Human skin samples were obtained from post-surgery materials in accordance with Latvian Central Ethics Committee authorized approval. Patients signed informed consent form. Human skin tissue samples were transported to laboratory in ice-cold transport solution containing Ca²⁺/Mg²⁺ ion free PBS, 2% antibiotic mix (penicillin/streptomycin) and fungizone 2 µg/ml. The specimens were washed with cold PBS buffer, cut into 4–6 mm² pieces and incubated in dispase 0.6 U/ml for 1–3 h at 37°C.

Skin-derived precursor cell cultures were obtained as described earlier (Toma et al. 2005) with some modifications. The epidermis was manually removed from tissue pieces after dispase incubation and dermis was cut into 1 mm³ pieces following enzymatic digestion with Liberase Blendzyme 1 (0.62 Wunsch U/ml) for 30–40 min at 37°C. Afterwards, tissue pieces were dissociated by pipetting into 5 ml pipette, passed through a 70-µm cell strainer (BD Falcon, USA), and centrifuged at 1,500 rpm for 5 min. The pellet was suspended in cell growth media DMEM-F12 (3:1) containing penicillin and streptomycin 100 u/ml and 100 µg/ml respectively, supplemented

with variable concentrations of FBS, FGF-2, EGF, B27, and LIF. Cell suspensions were transferred into 25-cm² tissue culture flasks (T-25) or 24-well plates (Sardsted Inc.) and grown until 80% confluence. Eight primary stem cell culture samples, from both male and female donors (age from 29 to 65 years, passage number ranging from 3 to 5) were selected for in-depth characterization from the total of forty cultures established.

Morphology analysis

Cell morphology was analysed on subconfluent cell monolayers at 100× magnification on phase-contrast microscope (Leica). Photos were taken by Kodak camera. Cells were counted in hemacytometer and cell viability was assessed by Trypan blue staining.

FACS analysis

Adherent skin-derived precursor cells were trypsinized and stained with primary antibodies. Briefly, $1-2 \times 10^5$ cells per sample were incubated with the following primary antibodies: CD90-FITC, CD34 (all from Dako), CD73 (Abcam), CD105 (R&D Systems), CD45-FITC, CD14-APC, HLA-DR-APC (all from BD Biosciences) and isotype controls IgG1-FITC (Dako), IgG1-PE, IgG2A-APC (both from BD Biosciences), and IgG1 (R&D Systems). Secondary goat-antimouse IgG-PE and goat-antimouse IgG-APC (R&D Systems) were used where appropriate. Flow cytometry data were acquired on FACS Calibur and analysed by CellQuest (BD Biosciences) software.

Cell proliferation

Cell proliferation rate was measured in two assays: a 3-day proliferation assay using [³H]thymidine incorporation and a 7-day assay using BrdU incorporation. About 5×10^3 cells per well were seeded on 96-well tissue culture plates (BrdU test) or 5×10^4 cells per well in 24-well plates ([³H]thymidine test). Cell growth was synchronized in medium consisting of 0.5% FBS in DMEM/F12 (3:1) for 48 h. After 48 h, synchronization medium was changed to the cell proliferation media. Proliferation media was prepared as follows: in the BrdU assay FGF-2 in serial dilution (5, 10, 20, and 40 ng/ml), EGF in serial dilutions (5,

10, 20, 40 ng/ml), FGF-2 serial dilutions in combination with EGF at a constant concentration 20 ng/ml; in [³H]thymidine assay FGF-2 was used in serial dilutions 4, 20, and 40 ng/ml. All dilutions of the growth factors were prepared in basal medium DMEM/F12 (3:1) supplemented with antibiotic mix and varying concentration of FBS; 10, 5, and 0.5%, respectively. In addition, similar proliferation experiment was performed using serum-free basal medium DMEM/F12 (3:1), supplemented with antibiotic mix, 2% of B27 supplement (Invitrogen) and growth factors. In some experiments db-cAMP was added at the concentrations of 0.5 and 1 mM. Control wells contained appropriate medium only.

[³H]thymidine (2 µCi/ml) was added to the cell cultures simultaneously with the test compounds for 3 days. After that cell medium was aspirated, cells were washed three times with ice-cold phosphate buffered saline (PBS), precipitated with ice-cold 15% trichloroacetic acid overnight, lysed with 1 N NaOH, and incubated at room temperature for 2 h (Liang et al. 2008). Afterwards, 50 µl of the lysate were transferred to Multiscan 96-well plates in duplicates, and 200 µl of scintillation solution OptiPhase were added. The plate was put on shaker for 4 h and the amount of radioactivity was counted on liquid scintillation and luminescence counter 1450 Microbeta Trilux (Wallac, Finland).

BrdU assay was carried out according to the manufacturer's instructions. Briefly, BrdU was added simultaneously with proliferation media (final concentration 10 µM). Seven days later, cells were fixed and BrdU incorporation was detected with anti-BrdU monoclonal antibody from mouse-mouse hybrid cells (clone BMG 6H8, Fab fragments) conjugated with peroxidase (POD). ABTS substrate was added to the wells and optical density was measured at 405 nm by Elx808 BioTek Instruments microplate reader.

Total protein content was measured as additional index to the cell proliferation. Protein content was determined according to manufacturer's instructions for the ready-to-use microtiter plate assay.

All experiments were repeated three times in duplicates.

FGF-2 measurements

Concentration of FGF-2 in cell culture supernatants was measured on day 7 accordingly to DuoSet ELISA

development kit instructions (R&D Systems, UK). Briefly, 96-well plates were coated with capture antibody and incubated overnight at room temperature. Afterwards, 100 μ l of cell culture supernatants as well as FGF-2 standard dilutions were added to the pre-coated microtiter plate (Nunc, Denmark) and incubated 2 h at room temperature. Next, detection antibody reagent was added to the plates, following by the secondary reagent and substrate. The optical density was measured at 450 nm by microplate reader Elx808 (BioTek). FGF-2 concentrations (pg/ml) were calculated according to the standard curve using Microplate Data Collection & Analysis Software Gen5 (BioTek). All experiments were repeated three times in duplicates.

Statistical analysis

Data are expressed as mean \pm standard error mean (S.E.M.). Statistical significance was assessed using one-way analysis of variance (ANOVA) followed by Bonferroni multiple comparisons test or Student *t*-test. Statistical analysis was performed with the GraphPad Prism package (GraphPad Software). *P* values less than 0.05 were considered statistically significant.

Results

Isolation and culture of primary cells

Isolated dermal SKPs were cultured in medium based on DMEM-F12 (3:1) containing 1% of antibiotic mix, supplemented with variable concentrations of FBS, FGF-2, EGF, B27 and LIF. Formation of

floating spheres was observed in serum-free media supplemented by FGF-2, EGF, B27 and LIF (Fig. 1b, c). Approximately 3.5×10^5 – 4×10^5 viable cells per T-25 flask were found at near 100% confluence.

Cell counting revealed that approximate doubling time for dermal SKPs was 7–8 days. Primary cells were cultured in the defined culture medium almost 1 year and during this period significant changes in the cell doubling time were not observed.

Examination of the cultures revealed pronounced media-dependent morphology changes (Fig. 1). As seen from Fig. 1a in 10% FBS medium cells resembled fibroblast-like morphology, whereas in serum-free medium supplemented by FGF-2 and EGF (Fig. 1b) cells showed changed morphology—dendritic cell bodies and some floating spheres. Floating spheres were observed in LIF supplemented cultures (Fig. 1c). Trypan blue test demonstrated more than 95% cell viability in all cell culture conditions tested.

Flow cytometry analysis

Skin-derived precursor cells (SKPs) were propagated in 10% FBS supplemented medium and characterized for specific surface antigen expression by flow cytometry analysis. SKP cells were analysed for hemopoietic marker expression (CD14, CD45, HLA-DR, and CD34). Double-staining for CD34 and HLA-DR co-expression revealed a small population of SKPs positive for CD34 and not HLA-DR (Fig. 2a). SKP cells were negative for CD45 and CD14 cell surface markers (Fig. 2a). However, more than 95% of SKPs were positive for cell surface markers CD90, CD73 and CD105 (Fig. 2b), typically assigned for human mesenchymal stem cells. Based of these data

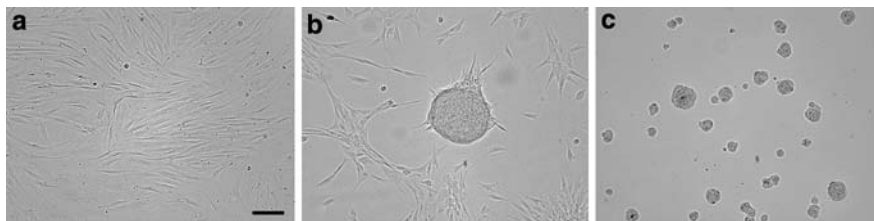
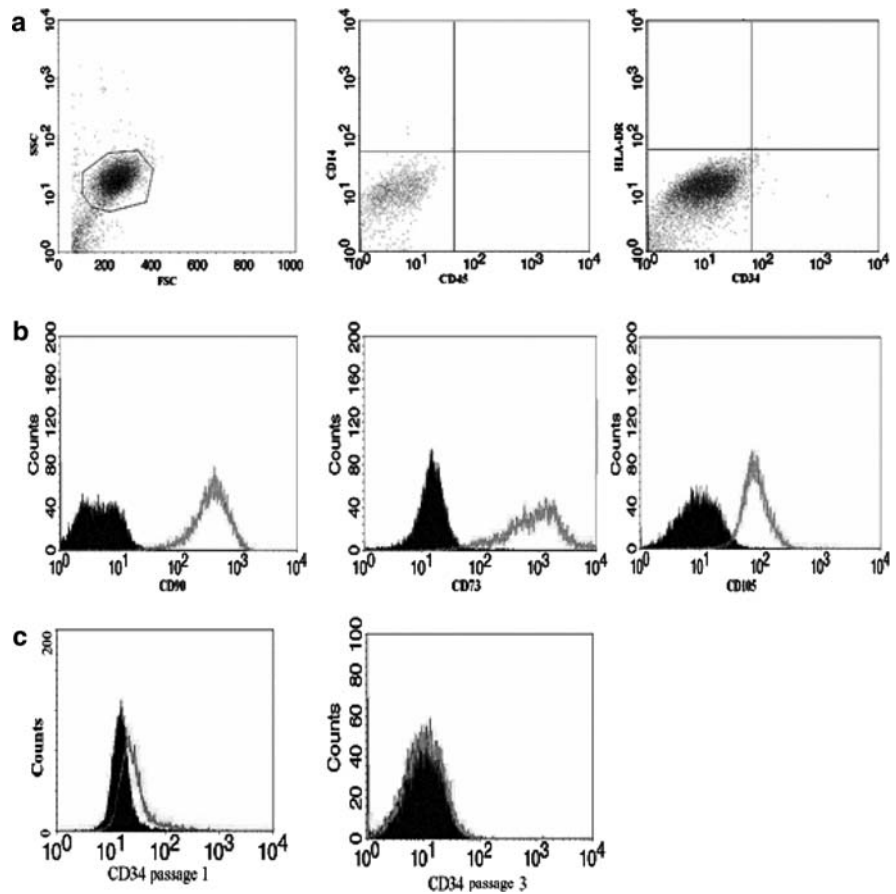


Fig. 1 Skin-derived precursor cell morphology changes in the different culturing media (magnification 100 \times , **a** scale bar indicates 100 μ m). **a** DMEM-F12, 10% FBS medium. **b** DMEM-F12 serum-free medium supplemented by FGF-2 of

40 ng/ml, EGF of 20 ng/ml, and 2% B27. **c** DMEM-F12 serum-free medium supplemented by FGF-2 of 40 ng/ml, EGF of 20 ng/ml, 2% B27 and LIF of 10 ng/ml

Fig. 2 Flow cytometry analysis of skin-derived mesenchymal cell markers. **a** Haematopoietic cell surface marker analysis (co-expression of CD14/CD45 and CD34/HLA-DR). **b** Mesenchymal marker CD105, CD73, CD90 analysis. **c** CD34 analysis in passage 1 and passage 3 skin-derived mesenchymal stem cells



we assumed that SKPs analysed in this study are skin-derived mesenchymal stem cells (S-MSC).

CD34 analysis in the first passage cell culture revealed that S-MSCs contained a small population ($\sim 1.5\%$) of CD34 positive cells (Fig. 2c). However, in passage 3 the amount of CD34 positive cells decreased to 0.27% (Fig. 2c). Confirming flow cytometry data, adherent CD34 positive cells were detected by immunocytochemistry analysis (data not shown).

S-MSC proliferation rate

Cell proliferation rate was measured in two main settings of basal media: FBS-conditioned and serum-free medium, supplemented with serial dilutions of growth factors.

In addition to the changes in cell morphology, pronounced difference in cell proliferation rate was observed in growth factor supplemented cultures (Fig. 3a). After 3-day cultivation of S-MSCs in FGF-2, EGF and B27 supplemented DMEM-F12 media,

[³H]thymidine label was incorporated in twofold higher amount than that in only FBS-based media (Fig. 3a). Addition of LIF at 10 ng/ml completely reversed the effect of FGF-2 and EGF. As seen in Fig. 3b, FGF-2 and EGF increased the content of total protein, whereas addition of LIF blocked the effect of the growth factors.

FBS had a pronounced concentration-dependent effect on the cell proliferation after 7 days in culture of three representative donor samples tested, namely 07D, 10D, and 11D (Fig. 4a). FGF-2 concentration-dependent stimulation of the proliferation was observed only in the medium supplemented by 0.5% of FBS and not in 5%, 10% FBS (Fig. 4b) or B27 supplemented media (data not shown). Addition of EGF to the corresponding control medium and FGF-2 conditioned medium had no synergic effect on cell proliferation in both serum-free (data not shown) and serum supplemented media (Fig. 4c).

There were remarkable differences between individual donor samples in the [³H]thymidine 3-day

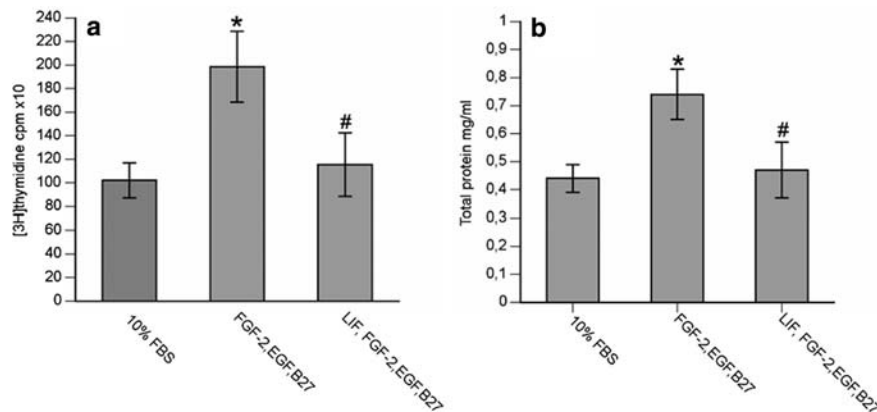


Fig. 3 Effect of growth factors on the cell proliferation and total protein content in the cells. **a** Effect of the growth factors on the skin-derived mesenchymal stem cell proliferation. **b** Effect of the growth factors on total protein content in the skin-derived mesenchymal stem cells. Three independent

experiments were carried out in duplicates. Each point represents average value \pm S.E.M. (eight primary cultures, $n = 48$). * $P \leq 0.05$ versus 10% FBS supplemented DMEM/F12 media. # $P \leq 0.05$ versus FGF-2 of 40 ng/ml, EGF of 20 ng/ml, B27 of 2% supplemented DMEM/F12 media

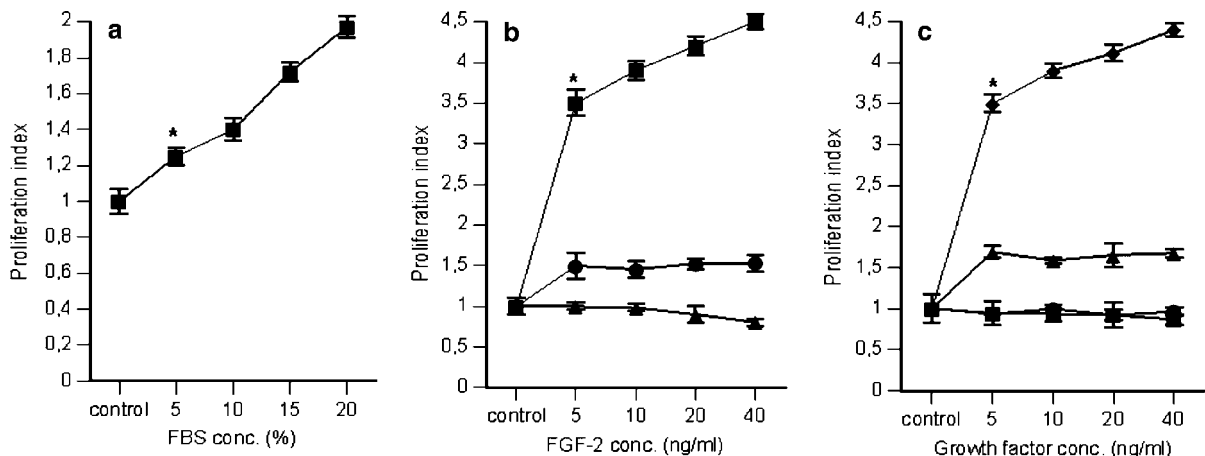


Fig. 4 Effect of cell culture medium supplements on cell proliferation measured by BrdU assay. Proliferation index was calculated as fold increase above serum conditioned basal medium, in each case taken as 1. **a** Effect of FBS concentration on the cell proliferation. Control medium was DMEM-F12 (3:1) supplemented with serum replacement B27. **b** Effect of the FGF-2 concentration on the cell proliferation in 0.5% FBS (■), 5% FBS (●) and 10% FBS (▲) supplemented cell culture media. **c** Effect of EGF on the cell proliferation. EGF in

various concentrations (■), FGF-2 in various concentrations with EGF 20 ng/ml in 0.5% FBS medium (●), FGF-2 in various concentrations with EGF 20 ng/ml in 5% FBS medium (▲), FGF-2 in various concentrations with constant EGF 20 ng/ml in 10% FBS medium (●). Three independent experiments were carried out in duplicates. Each point represents average value \pm S.E.M. (S-MSC cultures 07D, 10D and 11D, $n = 18$). $P \leq 0.05$ versus corresponding control (first point on the curve and following)

proliferation assay demonstrating high donor to donor variability in response to the varying concentration of FGF-2 (Table 1). The highest effect of FGF-2 was reached at the concentration 4 ng/ml, with small tendency to increase cell proliferation at the concentration 20 ng/ml. However, at the concentration

40 ng/ml the proliferation rate was at the same level as with 4 ng/ml of FGF-2. Individual donor cells proliferated with the different rate in 0.5 and 10% FBS conditioned media. Noteworthy, 10D and 11D samples proliferated faster than others and 07D, 26D, and 27D did not respond to FGF-2 stimuli in low

Table 1 Effect of FGF-2 on individual donor S-MSC cell proliferation in [3H]thymidine assay

Sample	Age	0.5% FBS control	FGF-2		
			4 ng/ml	20 ng/ml	40 ng/ml
01D	59	260 ± 45	570 ± 50*	760 ± 24*	532 ± 32*
06D	47	355 ± 40	780 ± 56*	1,030 ± 80*	800 ± 42*
07D	37	230 ± 21	268 ± 33	216 ± 12	290 ± 51
10D	62	692 ± 54	1,294 ± 75*	1,513 ± 87*	1,153 ± 76*
11D	65	570 ± 56	997 ± 75*	1,296 ± 98*	1,173 ± 80*
23D	29	405 ± 32	800 ± 43*	870 ± 72*	800 ± 54*
26D	52	289 ± 22	288 ± 42	297 ± 25	286 ± 20
27D	37	460 ± 30	310 ± 30	549 ± 25	460 ± 22
Sample	Age	10% FBS control	FGF-2		
			4 ng/ml	20 ng/ml	40 ng/ml
01D	59	770 ± 63**	1,300 ± 87*	1,400 ± 98*	1,400 ± 86*
06D	47	990 ± 80**	1,450 ± 96*	1,780 ± 99*	1,534 ± 88*
07D	37	1,107 ± 77**	1,560 ± 89*	1,700 ± 85*	1,540 ± 76*
10D	62	1,350 ± 66**	2,500 ± 97*	2,780 ± 97*	2,400 ± 88*
11D	65	1,270 ± 80**	2,340 ± 98*	2,670 ± 96*	2,450 ± 95*
23D	29	925 ± 56**	1,100 ± 77	2,000 ± 84*	1,200 ± 78*
26D	52	650 ± 48**	1,230 ± 87*	1,500 ± 76*	1,300 ± 80*
27D	37	813 ± 67**	1,300 ± 96*	1,540 ± 82*	1,320 ± 69*

Data are shown in cpm. Three independent experiments were carried out in duplicates. Each point represents average value ± S.E.M ($n = 6$)

* $P \leq 0.05$ versus corresponding FBS control

** $P \leq 0.05$ 10% FBS control versus corresponding cell 0.5% FBS control

serum media. The increase of FBS concentration from 0.5 to 10% resulted in approximately twofold acceleration of the S-MSC proliferation rate.

After 3 days, db-cAMP at concentration of 0.5 and 1 mM decreased cell proliferation rate for 40% in comparison with control (10% FBS medium), whereas after 7 days no inhibitory effect was observed. The inhibitory effect of db-cAMP in the 0.5% FBS medium was statistically insignificant either after 3 or 7 days. Also, db-cAMP did not stimulate cell proliferation in the growth factor supplemented serum-free medium.

Secretion of FGF-2 by S-MSC

Release of FGF-2 was measured in cell-free supernatants obtained from S-MSC cultures grown in 0.5% of FBS and 10% of FBS supplemented media. Adjustment of FGF-2 level to cell numbers revealed no significant differences in FGF-2 secretion by S-MSC grown in 10% FBS media or 0.5% FBS media.

In 0.5% FBS medium FGF-2 was secreted 317 ± 91 pg/ml per 5×10^4 cells, whereas in 10% FBS medium 410 ± 85 pg/ml per 5×10^4 cells, respectively.

Discussion

Primary S-MSC cultures from forty individual donor skin samples were isolated according to the published skin stem cell isolation protocols (Toma et al. 2001, 2005; Shih et al. 2005). Eight S-MSC cultures were freely selected for the in-depth studies in equal experimental set-ups, and effect of commonly used media supplements on the cell proliferation was analysed.

We observed significant changes in cell morphology depending on growth medium. In serum supplemented medium adherent, spindle-shaped cell morphology was observed. In contrast, floating

spheres (embryonic bodies) that are characteristic for early stage stem cells were formed under serum-free culture conditions, which is in line with previous report (Toma et al. 2005). FACS analysis used to characterise phenotype of the skin-derived precursor cells, revealed greater than 95% expression of mesenchymal markers CD90, CD105, and CD73 (Shih et al. 2005; Dominici et al. 2006; Sudo et al. 2007). Also, FACS results showed that a minor population of S-MSC were positive for CD34 marker during early passages (up to number 3). Noteworthy, CD34-positive cells have been found before in the connective tissues of dermis, dermal precursor cells (up to 15 passages) and skeletal muscle of fetal and adult human tissue (Young et al. 2001; Belicchi et al. 2004; Brown et al. 1991). Interestingly, both CD34-positive and CD34-negative bone marrow-derived mesenchymal stem cells possess the ability to differentiate into osteocyte, adipocyte and chondrocyte lineages (Kaiser et al. 2007).

We compared various growth media supplements for their potency to influence stem cell proliferation (passage number 3–5). Our results indicate that FBS had the most pronounced dose-dependent effect on S-MSC proliferation in comparison to FGF-2, EGF, LIF, and db-cAMP. Alternatives to FBS-supplemented medium have been subject of discussion due to the potential hazards that FBS may have for clinical applications (Sotiropoulou et al. 2006; Shahdadfar et al. 2005; Mannello and Tonti 2007; Chachques et al. 2004). However, it has been suggested that risks from the use of FBS are insignificant (Doerr et al. 2003).

FGF-2 stimulates growth of fibroblasts, myoblasts, osteoblasts, neuronal cells, endothelial cells, keratinocytes, chondrocytes, and many other cell types of mesodermal and neuroectodermal origin and tumors derived from these tissues (Okada-Ban et al. 2000). The mitogenic property of FGF-2 has enabled researchers to establish long-term cultures of progenitors from the adult CNS (Shihabuddin et al. 1997). Moreover, poor survival of skin derived precursor cultures was observed in the absence of FGF-2 (Schumm et al. 2002). Although the number of donor samples analyzed was insufficient to draw conclusions about age and gender effect on cell propagation, we have observed high inter-donor sample proliferation variability in response to FGF-2. Differences amongst cultures after 3 days were so

pronounced that generalized estimation of FGF-2 effect on the cell proliferation was not possible. After 7 days in culture, FGF-2 stimulated S-MSC proliferation in dose-dependent manner in 0.5% FBS media only. In accordance with our findings, variable FGF-2 effect has been shown on human neural progenitor cells: low concentrations of FGF-2 (up to 2.5 ng/ml) increased neurogenesis while high levels of FGF-2 (10–100 ng/ml) maintained progenitor cell proliferation (Nelson and Svendsen 2006). We have demonstrated that S-MSC secrete FGF-2, thus supporting self-renewal in an autocrine manner, similarly to that shown in human multipotent adipose-derived stem cells (Zaragosi et al. 2006).

EGF is another growth factor that has proliferative effects on cells of both mesodermal and ectodermal origin, particularly keratinocytes and fibroblasts, and promotes proliferation of mesenchymal, glial and epithelial cells (Oda et al. 2005). However, in our experimental set-up EGF did not influence the proliferation of S-MSC. Interestingly, loss of responsiveness to EGF has been described after cell maintenance in vitro for 1 month in human corneal endothelial cells obtained from 50 years and older donors versus 30-year old donor (Zhu and Joyce, 2004). In our study S-MSC were maintained longer than 1 month in vitro and skin samples were obtained from donors older than 29-years. However, it would be too early to discuss gender and age variations as a reason for the cell non-responsiveness to the EGF stimuli.

Our results demonstrated that addition of LIF had inhibitory effect on cell proliferation. Such effect could be due to the dual LIF role on maintaining balance between the proliferation and anti-proliferation signals that is a key to homeostatic regulation of stem cell self-renewal in their niche (Williams et al. 1988; Metcalf 2003).

Dibutyl-*l*-cyclic adenosine monophosphate, a cell-permeable cAMP analog which preferentially activates cAMP-dependent protein kinase pathway is also known for its dual effects (Tsatsmali et al. 2000). Db-cAMP may act as proliferation inducer or depletory agent. Here, db-cAMP exerted short-term proliferation inhibitory effect on S-MSC cells grown in the FBS-containing medium, but did not affect cell division in serum-free medium.

In summary, our study demonstrates that homogeneous S-MSC populations can be reproducibly isolated

from both male and female donors (age 29–65 years). However, we have obtained results that show donor to donor variability of S-MSC proliferation in response to FGF-2 in short-term culture which should be taken into consideration for adult stem cell therapy. Previously established propagation medium for growth of the skin-derived precursor cells contains mixture of EGF (20 ng/ml), FGF-2 (40 ng/ml), B27 supplement and LIF (10 ng/ml) in DMEM/F12 3:1 (Shih et al. 2005; Toma et al. 2005; Wong et al. 2006). However, in our hands, the cocktail of growth factors could be replaced with FGF-2 alone at concentration 4 ng/ml in basal medium. Additionally, we are first to show that dbcAMP, EGF, and LIF are dispensable for adult human S-MSC proliferation. Moreover, addition of LIF to serum-free medium supplemented with FGF-2 and EGF inhibited the proliferative stimulus of FGF-2. Hence, our findings suggest that optimal cell culture conditions for in vitro expansion of S-MSC are low serum media supplemented with FGF-2 at concentration 4 ng/ml. B27 supplemented medium with 4 ng/ml FGF-2 could be used as alternative to serum conditioned medium for propagation of human S-MSC.

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