

# Altered Gene Expression in Human Adipose Stem Cells Cultured with Fetal Bovine Serum Compared to Human Supplements

Karen Bieback, Ph.D.,<sup>1,\*</sup> Viet Anh-Thu Ha, M.D. (cand.),<sup>1,\*</sup> Andrea Hecker,<sup>1</sup> Melanie Grassl, Dipl. Ing. FH,<sup>1,2</sup> Sven Kinzebach, Dipl. Ing. FH, Dr. Sc. hum. (cand.),<sup>1</sup> Hermann Solz, M.D.,<sup>3</sup> Carsten Sticht, Ph.D.,<sup>4</sup> Harald Klüter, M.D.,<sup>1</sup> and Peter Bugert, Ph.D.<sup>1</sup>

Mesenchymal stromal cells (MSCs) are promising candidates for innovative cell therapeutic applications. For clinical scale manufacturing regulatory agencies recommend to replace fetal bovine serum (FBS) commonly used in MSC expansion media as soon as equivalent alternative supplements are available. We already demonstrated that pooled blood group AB human serum (HS) and thrombin-activated platelet releasate plasma (tPRP) support the expansion of multipotent adipose tissue-derived MSCs (ASCs). Slight differences in size, growth pattern and adhesion prompted us to investigate the level of equivalence by compiling the transcriptional profiles of ASCs cultivated in these supplements. A whole genome gene expression analysis was performed and data verified by polymerase chain reaction and protein analyses. Microarray-based screening of 34,039 genes revealed 102 genes differentially expressed in ASCs cultured with FBS compared to HS or tPRP supplements. A significantly higher expression in FBS cultures was found for 90 genes (fold change  $\geq 2$ ). Only 12 of the 102 genes showed a lower expression in FBS compared to HS or tPRP cultures (fold change  $\leq 0.5$ ). Differences between cells cultivated in HS and tPRP were hardly evident. Supporting previous observations of reduced adhesion of cells cultivated in the human alternatives we detected a number of adhesion and extracellular matrix-associated molecules expressed at lower levels in ASCs cultivated with human supplements. Confirmative assays analyzing transcript or protein expression with selected genes supported these results. Likewise a number of mesodermal differentiation-associated genes were higher expressed in cells grown in FBS. Quantifying adipogenic and osteogenic differentiation lacked to demonstrate a clear correlation to the supplement due to donor-specific variances. Our results emphasize the necessity of comparability studies as they indicate that FBS induces a culture adaptation exceeding that of *ex vivo* culture in human supplements and thus may contribute to the therapeutic potential.

## Introduction

**T**HE CAPACITY OF mesenchymal stromal cells (MSCs) for extensive self-renewal, differentiation and immune modulation renders them interesting for cell-based therapies.<sup>1</sup> Thus MSCs are already in different phases of clinical trials ranging from regenerative medicine to immunological or hematopoietic support. Translating MSCs into clinical settings raises issues on how to expand these rare cells in large-scale good manufacturing practice (GMP)-compliant protocols. The type of supplement to be used for clinical-scale expansion of MSCs is a critical issue as until now a

completely chemically defined medium devoid of xenogenic proteins has not been identified to efficiently promote both MSC isolation and expansion.<sup>2,3</sup> Fetal bovine serum (FBS) used in most expansion protocols provides vital nutrients, attachment factors, toxin scavengers and growth factors. However, due to the ill-defined nature, high lot-to-lot variable composition, the risk of transmitting infectious agents and immunizing effects, serum is regarded unsuitable for therapeutic purposes.<sup>4-6</sup> Especially the latter, in line with guidelines of the regulatory authorities to minimize the use of FBS, has initiated an intense research for possible alternatives.<sup>7</sup> A variety of human supplements have been

<sup>1</sup>Medical Faculty Mannheim, Institute of Transfusion Medicine and Immunology, German Red Cross Blood Service of Baden-Württemberg-Hessen, Heidelberg University, Mannheim, Germany.

<sup>2</sup>Mannheimer Core Facility for Flow Cytometry and Cell Sorting, Medical Faculty Mannheim, Heidelberg University, Mannheim, Germany.

<sup>3</sup>Mannheimer Clinic for Plastic Surgery, Mannheim, Germany.

<sup>4</sup>Medical Faculty Mannheim, Medical Research Center, Heidelberg University, Mannheim, Germany.

\*These two authors contributed equally to the work.

postulated as alternatives to replace FBS and some already applied in clinical trials with human cells: autologous or allogeneic human serum (HS), human plasma, cord blood serum, human platelet derivatives including platelet lysate and platelet released factors.<sup>8–14</sup>

We have recently tested various preparations of pooled human blood-derived components producible in GMP-grade as possible alternative supplements to FBS, namely HS, human platelet lysate and thrombin-activated platelet releasate plasma (tPRP).<sup>15,16</sup> Comparing various methods to release platelet growth factors, thrombin activation yielded a supplement comparably potent as HS to expand adipose tissue-derived MSCs (ASCs). Interestingly, MSCs from human adipose tissue (ASCs) and human bone marrow (BM-MSCs) differed in their demand for human blood-derived supplements. Pooled human platelet lysate was found to be the most suitable FBS substitute in clinical-scale BM-MSC expansion.<sup>16</sup> MSC quality and functionality including cell surface marker expression, adipogenic and osteogenic differentiation and immunosuppressive action were similar in MSCs from all culture conditions. In contrast both HS and tPRP, but not pooled human platelet lysate, were efficient in expanding ASCs. However, differences in size, growth pattern and adhesion were evident.<sup>15</sup>

Despite the fact that no major changes in the quality of ASCs could be demonstrated after isolation and culture in alternative human supplements, the observable differences in the growth and adhesion could potentially modify adhesive and migratory capacities. MSCs/ASCs are attractive cellular therapeutics because they efficiently migrate to sites of tissue remodeling due to injury, inflammation or tumor.<sup>17</sup> Thus to check that the use of HS and tPRP is therapeutically competitive or superior to FBS at first we screened for regulated genes using a whole-genome microarray approach. We verified regulation of selected genes applying reverse transcription quantitative polymerase chain reaction (RT-qPCR), and protein expression by appropriate techniques.

## Materials and Methods

### Isolation and culture of ASCs

ASCs from six donors ranging from 25 to 64 years were obtained by elective tumescence liposuction of the abdomen and hip/thigh region in accordance with the ethical standards of the local ethical committee. Lipoaspirates were processed as described<sup>15</sup> using FBS, HS or tPRP supplemented medium. ASCs were maintained under the three culture conditions with twice-weekly media changes. At a confluence of 70%, cells were passaged using trypsin-ethylenediaminetetraacetic acid (EDTA) (PAA) and either replated at a density of 200 cells/cm<sup>2</sup> for all passages or served for further investigations. Cells were counted using a Neubauer chamber (Marienfeld GmbH). Aliquots of primary and passage 1 cells were cryopreserved in 10% dimethyl sulfoxide in either FBS or HS, respectively, and thawed for subsequent experiments.

### Preparation of supplements and media

ASCs were cultured under three different culture conditions. Dulbecco's modified Eagle's medium-low glucose (Lonza) with 4 mM L-glutamine (PAA) and 0.05 U penicillin/

0.05 µg streptomycin (PAA) served as the basal medium, which was supplemented with (1) 10% FBS (preselected lot; mesenchymal stem cell growth supplement; Lonza), (2) 10% HS, or (3) 10% tPRP.

### Human supplements

HS and tPRP were prepared as described.<sup>15</sup> In brief, for HS whole-blood donations of 10 AB-blood-group-typed donors were used to derive HS by centrifugation at 2000 g for 15 min. The serum was then aliquoted and frozen at –30°C. After thawing, HS of at least five donors was pooled and sterile filtered through 0.2 µm pores (Nalgene filtration device; Nunc International).

tPRP was prepared from two pooled platelet concentrates out of buffy coats derived from whole-blood donations of four AB-blood-group-typed donors, thus representing a pool of eight donors. To derive platelet releasates, platelets were activated by 1 U/mL of human thrombin (Sigma-Aldrich) for 45 min at room temperature with gentle agitation. After centrifugation and sterile filtration, tPRP was aliquoted and frozen at –80°C. Freshly prepared before use, tPRP was thawed and centrifuged for 5 min at 1500 g. To prevent *in vitro* gel formation, 2 U/mL of heparin (Liquemin 5000; Roche) were added to the basal medium before adding the platelet factors.

### Primary dermal fibroblasts, osteoblasts and human umbilical vein endothelial cells as controls

Human primary normal dermal fibroblasts (PromoCell), human primary osteoblasts (PromoCell) and human umbilical vein endothelial cells (HUVECs, isolated according to Ref.<sup>18</sup>) served as positive controls for the protein expression analysis. Fibroblasts and osteoblasts were cultured in Dulbecco's modified Eagle's medium supplemented with 10% FBS (Sigma-Aldrich). For culturing HUVECs gelatin-coated culture flasks and EGM-2 Endothelial Cell Growth Medium (Lonza) were used.

### Trypsin kinetic

To investigate adhesive characteristics, passage 2 ASCs under the three culture conditions in FBS, HS, and tPRP were cultured until a confluence of 70%–80% was reached. Medium was removed, cells washed with phosphate-buffered saline (PBS) (Biochrom AG, www.biochrom.de) and prewarmed trypsin-EDTA (PAA, www.paa.com) added. Under time measurement, a serial picture recording was performed using an Axiovert 100 microscope (Carl Zeiss MicroImaging GmbH, www.zeiss.com).

### RNA extraction

ASCs from six donors, and for internal validation as dye swap one donor tested twice independently, were harvested at 70% confluency. At passage 2, extraction of RNA was performed for each sample using the RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. RNA quantity was determined photometrically. RNA integrity was determined using the RNA 6000 Nano LabChip Kit and Agilent 2100 Bioanalyzer (Agilent Technologies). All samples yielded RNA integrity numbers  $\geq 9.6$  indicating a high quality of intact RNA.

### Microarray procedure and data analysis

Three hundred nanograms total RNA was used in each microarray experiment. RNA amplification and labeling were done using Low RNA Input Fluorescent Linear Amplification Kit (Agilent). The amount of cRNA was determined photometrically. Five micrograms each of a Cy3-labeled and a Cy5-labeled cRNA sample was mixed and hybridized to Human 44K oligonucleotide microarrays. Hybridization was performed in a hybridization chamber for 16 h at 65°C. After stringent washing of the arrays, the hybridization signals of the Cy3 and Cy5 dyes were scanned using a microarray laser scanner (GMS418; Affymetrix). The raw data images (16 bit TIF format) were quantified using ArrayVision 7.0 software (Imaging Research). The raw fluorescence intensity values were normalized applying Loess normalization procedure to adjust the Cy3 and Cy5 channels. Differential gene expression analysis was performed using the one-way analysis of variance (ANOVA) with the commercial software package SAS JMP7 Genomics 3.1 (SAS Institute). A false-positive rate of  $\alpha = 0.05$  with false discovery rate (FDR) correction was taken as the level of significance.

### Reverse transcription quantitative polymerase chain reaction

To verify differential gene expressions independent RT-qPCR analysis was performed in three ASC samples at passage 2 also used for microarray analyses. Twenty-five target genes and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) as reference gene were selected based on high fold change and potential functional impact. One hundred nanograms total RNA from each sample was reverse transcribed using the Transcriptor First Strand cDNA Synthesis Kit (Roche). Quantitative assessment of DNA amplification was detected through TaqMan hydrolysis probes of the human Universal ProbeLibrary (Roche) or through a SYBR Green Assay. Primer sequences are provided in Table 1. Multiplex reactions were carried out in a final volume of 25  $\mu$ L containing 2.5  $\mu$ L cDNA, 12.5  $\mu$ L Fast Start TaqMan Probe Master Mix (Roche Applied Science) or Universal SYBR Green Master Mix (Roche), 2.5  $\mu$ L Primer/Probe and 7.5  $\mu$ L of RNase-free water (Qiagen). No template control reactions, where the cDNA is substituted with RNase-free water and a human reference RNA pool (Clontech) were included in each RT-qPCR run. Using the ABI PRISM 7000 Sequence Detector (Applied Biosystems), thermal cycling was performed with the following conditions: 95°C for 10 min followed by 40 cycles consisting of 15 s at 95°C and 60 s at 60°C. Analyses of the RT-qPCR products were performed using SDS 1.2.3 Software (Applied Biosystems). Relative gene expression was calculated by normalization to *GAPDH* using following formula:

$$\text{relative gene expression} = (CT_{RG}/CT_{TG}) - (CT_{RG}/40.1)$$

where  $CT_{RG}$  represents the threshold cycle (CT) of the reference gene *GAPDH*,  $CT_{TG}$  the of the target gene, and 40.1 the negative CT beyond the detection limit.

### Protein expression analysis

**Antibodies.** Protein expression levels not necessarily equate with transcript levels.<sup>19</sup> To validate differential ex-

pressions on protein level, immunofluorescence, subsequent also flow cytometry and Western blot analysis were performed on ASCs from three to five different donors and at passage 2 or 3 and reproduced in cells up to passage 5. The antibodies listed in Table 2 were chosen based on high fold change, probable functional impact and yielding robust results in validation studies. Antibodies were purchased from Abcam, Progen, Becton Dickinson, Santa Cruz Biotechnology, Molecular Probes, Jackson ImmunoResearch Laboratories, Inc., R&D Systems, and DAKO.

**Indirect immunofluorescence.** Cells cultivated on glass coverslips were rinsed with PBS, fixed with 2% formaldehyde (Sigma-Aldrich) for 20 min, then permeabilized with 0.2% Triton X100 for 1 min and blocked with a solution containing 5% goat serum and 3% albumin in PBS for 15 min. Cells were then incubated with the primary antibodies in a humidified chamber for 60 min. After washing three times with PBS, cells were incubated with a fluorochrome-labeled secondary antibody for 30 min and cell nuclei were subsequently stained with 4',6-diamidino-2-phenylindole (Sigma-Aldrich). Final dilutions for all antibodies are specified in Table 2. Finally, cells were rinsed three times with PBS and once in 96% ethanol and in aqua dest. After being embedded in mounting medium (Fluoromount-G; Southern Biotech) cells were analyzed the following day. Images were captured by a fluorescence microscope (Axio Imager.Z1; Carl Zeiss) and AxioVision 4.7. software (Carl Zeiss).

**Flow cytometry analysis.** Flow cytometric analysis of ASCs was performed to assess cell purity.<sup>15</sup> For intracellular staining harvested cells from at least three to six donors were washed with PBS and fixed for 15 min in 2% formaldehyde. Cells were preincubated, washed, and resuspended in 100  $\mu$ L 0.1% saponin buffer. Two microliters FcR Blocking Reagent (Miltenyi Biotec GmbH) were incubated for 10 min. The primary antibody was incubated on ice for 30 min and then washed twice with saponin buffer. A fluorochrome-conjugated secondary antibody was incubated for 30 min on ice. Cells were washed with saponin buffer and finally CellWash. For extracellular staining harvested cells were washed with CellWash and incubated with 2  $\mu$ L FcR Blocking Reagent for 10 min at 4°C. The respective fluorochrome-conjugated antibody was added and incubated 20 min at 4°C. Cells were washed twice and resuspended in 300  $\mu$ L CellWash. Acquisition and analysis were performed with FACS CANTO II and FLOWJO software (TreeStar Inc.).

**Western blot analysis.** Cells from at least three donors were harvested in RIPA buffer (Santa Cruz Biotechnology). Protein concentration was measured according to Bradford (Bio-Rad Laboratories). Ten micrograms of protein was separated on 12% Bis-Tris gels and transferred onto a polyvinylidene difluoride (PVDF) membrane (GE Healthcare). All antibodies were dissolved in TRIS-buffered saline (TBS) (Carl Roth), 5% skimmed milk powder (Roth), 0.05% Tween-20 (Roth). Band detection was performed using enhanced chemiluminescence reagent (ECL Western Blotting Detection Reagents; GE Healthcare). Equal loading was controlled using antibodies against extracellular-signal-related kinase 2, *GAPDH*, and b-Actin.

TABLE 1. LIST OF POLYMERASE CHAIN REACTION PRIMERS USED TO VALIDATE MICROARRAY RESULTS

| <i>Gene symbol</i> | <i>Gene name</i>                                                           | <i>F-primer (5'–3')</i>           | <i>R-primer (5'–3')</i>           | <i>Method</i> |
|--------------------|----------------------------------------------------------------------------|-----------------------------------|-----------------------------------|---------------|
| NOV                | Nephroblastoma overexpressed gene                                          | CCC AGC TCC AAG AAA AGT TG        | CTC CTA GGG TGG CTT CTG G         | TaqMan        |
| TNFRSF11B (OPG)    | Tumor necrosis factor receptor superfamily, member 11b (osteoprotegerin)   | GAA GGG CGC TAC CTT GAG AT        | GCA AAC TGT ATT TCG CTC TGG       | TaqMan        |
| TRH                | Thyrotropin-releasing hormone                                              | GGG CCT GGA TGA CTT CCT           | GAG AGC CAG TCA GAT TGA AAG AT    | TaqMan        |
| STMN2              | Stathmin-like 2                                                            | CCC TGG AGG AGA TCC AGA A         | CTG GGC CTC CTG AGA CTT T         | TaqMan        |
| A2M                | Alpha-2-macroglobulin                                                      | CCA GCT TCC AAA TCT CCC TA        | AAG CCA GAG ACC ATC TTC ACA       | TaqMan        |
| FMOD               | Fibromodulin                                                               | ACC GTC CCC GAT AGC TAC TT        | CAT CCT GGA CCT TCC AGC AAA       | SYBR Green    |
| ELN                | Elastin (supravalvular aortic stenosis, Williams-Beuren syndrome)          | CAG CTA AAT ACG GTG CTG CTG       | AAT CCG AAG CCA GGT CTT G         | TaqMan        |
| GPNMB              | Glycoprotein (transmembrane) nmb                                           | CAA AAC CCA CCC CTT CTT TAG       | TGG CAG TTT TCA TCA GGA ATC       | TaqMan        |
| EMILIN2            | Elastin microfibril interfacer 2                                           | CGT GAA CAA GAA TGT GAG CTG       | CTA GGT CTG AAG TTC ACT CGA TAC A | TaqMan        |
| COMP               | Cartilage oligomeric matrix protein                                        | CCA AGT GGG CTA CAT CAG G         | GTC CAA GAC CAC GTT GCT G         | TaqMan        |
| ITGA6              | Integrin, alpha 6                                                          | TTT GCT GAA AGA AAA TAC CAG ACT C | CTC GAG CGC AAA ATA AGA GAC       | TaqMan        |
| MYH2               | Myosin, heavy chain 2, skeletal muscle, adult                              | AAC TTC AGG CAA AAG TGA AAT CTT   | GCT AGA TTG GTG TTG GAT TGT TC    | TaqMan        |
| ADAMTS5            | ADAM metalloproteinase with thrombospondin type 1 motif, 5 (aggrecanase-2) | CGG ATG TGT GCA AGC TGA           | GGC CTA CAT TCA GTG CCA TC        | TaqMan        |
| SHROOM3            | Shroom family member 3                                                     | AGG ATG TGA AGG CAA GAG GA        | GCA GGG CAG TTA ACC AAC AT        | TaqMan        |
| EFS                | Embryonal Fyn-associated substrate                                         | TCC TGA ACT GCC CGA GAG           | GCA TTG CCC AGC ATA GAA GT        | TaqMan        |
| COL8A2             | Collagen, type VIII, alpha 2                                               | AGG CCA GTA CCT GGA AAT GC        | CTG GCC CAC TCC ATT ATC CC        | SYBR Green    |
| SOC2               | Suppressor of cytokine signaling 2                                         | GGA GCT CGG TCA GAC AGG           | GTT CCT TCT GGT GCC TCT TTT       | TaqMan        |
| GDF15              | Growth differentiation factor 15                                           | CCG GAT ACT CAC GCC AGA           | AGA GAT ACG CAG GTG CAG GT        | TaqMan        |
| FMN2               | Formin 2                                                                   | ACA TGC TGT TGT GAA CTT GGA       | CGA GTT CGT CTG ACT GTG CT        | TaqMan        |
| PSG1               | Pregnancy-specific beta-1-glycoprotein 1                                   | GTG AAA TAC GGA ACC CAG TGA       | TGA TGG TGA TGT AGG GCT TG        | TaqMan        |
| IGF2R              | Insulin-like growth factor 2 receptor                                      | TGG CCC TGT TGC TCT ACA A         | CAG CAA GTG GTC AGC TTA CTT ATC   | TaqMan        |
| CXCL5              | Chemokine (C-X-C motif) ligand 5                                           | AAG TGG TAG CCT CCC TGA AG        | TGT CTT CCC TGG GTT CAG AG        | SYBR Green    |
| ILF3               | Interleukin enhancer binding factor 3, 90 kDa                              | GCC AAC CAT GGA GGC TAC           | GCC GTA GCA GTC TGT GAA AA        | TaqMan        |
| WWTR1              | WW domain containing transcription regulator 1                             | AGC TCA GAT CCT TTC CTC AAT G     | GGG GAC ACT GAT GCA CCC TA        | TaqMan        |
| NES                | Nestin                                                                     | CTG GAG GCT GAG AAC TCC CG        | GCA TCC TGG GCT CTG ATC TC        | SYBR Green    |

TABLE 2. LIST OF ANTIBODIES USED TO VALIDATE MICROARRAY RESULTS

| (a) Primary Antibodies |                                               |            |         |                 |              |              |              |                          |
|------------------------|-----------------------------------------------|------------|---------|-----------------|--------------|--------------|--------------|--------------------------|
| Abbreviation           | Name                                          | Source     | Clone   | Isotype         | IF           | FC           | WB           | Company                  |
| COMP <sup>a</sup>      | Cartilage oligomeric matrix protein           | Rat        | MA37C94 | IgG1            | 1:10         | 1:50         | 1:10         | Abcam                    |
| b-Actin-HRP            | Beta-actin                                    | Mouse      | C4      | IgG1            | <sup>b</sup> | <sup>b</sup> | 1:18,000     | Santa Cruz Biotechnology |
| ERK2                   | Extracellular-signal-related kinase 2         | Rabbit     | C-14    | IgG             | <sup>b</sup> | <sup>b</sup> | 1:5000       | Santa Cruz Biotechnology |
| FMOD <sup>a</sup>      | Fibromodulin                                  | Rabbit     | H-50    | IgG             | 1:50         | 1:50         | 1:400        | Santa Cruz Biotechnology |
| GAPDH                  | Glyceraldehyde-3-phosphate dehydrogenase      | Mouse      | 0411    | IgG1            | <sup>b</sup> | <sup>b</sup> | 1:30,000     | Santa Cruz Biotechnology |
| ITGA6                  | Integrin alpha 6; CD49f                       | Rat        | GoH3    | IgG2a           | 1:5          | <sup>b</sup> | <sup>b</sup> | Progen                   |
| ITGA6                  | Integrin alpha 6; CD49f                       | Rat        | GoH3    | IgG2a           | <sup>b</sup> | 1:20         | <sup>b</sup> | Becton Dickinson         |
| MYH2 <sup>a</sup>      | Myosin, heavy chain 2, skeletal muscle, adult | Mouse      | 414     | IgM             | 1:10         | 1:50         | <sup>b</sup> | Progen                   |
| OPG                    | Osteoprotegerin                               | Rat        | bony-1  | IgG2a           | 1:10         | <sup>b</sup> | <sup>b</sup> | Abcam                    |
| PSG1                   | Pregnancy-specific beta-1-glycoprotein 1      | Mouse      | BAP3    | IgG1            | 1:10         | <sup>b</sup> | <sup>b</sup> | Abcam                    |
| VIM                    | Vimentin                                      | Guinea pig | GP53    | Whole antiserum | 1:200        | <sup>b</sup> | <sup>b</sup> | Progen                   |

<sup>a</sup>Intracellular staining in flow cytometry performed.<sup>b</sup>Not performed.

IF, immunofluorescence; FC, flow cytometry; WB, Western blot.

## (b) Secondary Antibodies

|                                          | Conjugate       | Dilution | Company                                   |
|------------------------------------------|-----------------|----------|-------------------------------------------|
| Goat anti-guinea pig IgG                 | Alexa Fluor 568 | 1:200    | Molecular Probes                          |
| Goat anti-mouse IgG + IgM                | Cy3             | 1:500    | Jackson ImmunoResearch Laboratories, Inc. |
| Goat anti-rabbit IgG                     | Cy3             | 1:500    | Jackson ImmunoResearch Laboratories, Inc. |
| Goat anti-rat IgG                        | Cy3             | 1:500    | Jackson ImmunoResearch Laboratories, Inc. |
| Goat F(ab') <sub>2</sub> anti-rat IgG    | PE              | 1:10     | R&D Systems                               |
| Goat anti-rabbit IgG                     | PE              | 1:20     | R&D Systems                               |
| Rabbit F(ab') <sub>2</sub> anti-mouse Ig | FITC            | 1:20     | DAKO                                      |
| Goat anti-mouse Ig                       | HRP             | 1:1000   | DAKO                                      |
| Goat anti-rabbit IgG                     | HRP             | 1:2000   | DAKO                                      |
| Goat anti-rat IgG                        | HRP             | 1:5000   | Jackson ImmunoResearch Laboratories, Inc. |

Cy3, cyanine 3; PE, phycoerythrin; FITC, fluorescein isothiocyanate; HRP, horseradish peroxidase.

*Adipogenic and osteogenic differentiation assays*

Adipogenic and osteogenic differentiation assays were performed in ASCs of three donors at passage 2 for 21 days as described in detail in Ref.<sup>15</sup> Differentiation was evaluated qualitatively and quantitatively by different assays: (1) adipogenesis qualitatively by Oil red O; (2) osteogenesis qualitatively by von Kossa staining; (3) adipogenesis quantitatively by triglyceride determination (TG GPO-PAP kit; Analyticon® Biotechnologies) according to Ref.<sup>20</sup>; (4) osteogenesis quantitatively by using the Fluitest Ca-CPC kit (Analyticon). In addition, mRNA expression of adipogenic marker genes (adipophilin, peroxisome proliferator-activated receptor gamma [PPARγ], adiponectin, and perilipin [PERI]) and osteogenic marker genes (osteopontin [OPON] and osteocalcin [OCAL]) was assessed by RT-qPCR.<sup>21</sup> Therefore, RNA was isolated from differentiated cell using RNeasy Mini Kit (Qiagen), reverse transcribed (Transcriptor High Fidelity cDNA Synthesis Kit; Roche) and investigated on a

LightCycler 480 system (Roche) with the use of gene-specific primers and universal probes (Roche). For normalization according to GeNorm<sup>22,23</sup> at least two different house keeping genes selected after validating *SFRS4*, *GAPDH*, *β2-microglobulin*, *HPRT*, *TBP* served as references (Table 3).

*Statistical analysis*

Statistical tests were performed using SPSS 12.0 (SPSS, Inc.) or SigmaPlot 11.0 (Systat Software) statistical software. Data are represented as arithmetic mean ± standard deviation. Data were tested for normality and equal variance before analysis. Statistical differences were calculated using ANOVA (or ANOVA on ranks if equal variance testing failed) and *t*-test (paired *t*-test where applicable). Differences were considered significant at \**p* < 0.05.

Differential gene expression analysis was performed using the one-way ANOVA with the commercial software package SAS JMP7 Genomics 3.1 (SAS Institute).

TABLE 3. LIST OF POLYMERASE CHAIN REACTION PRIMERS USED TO ANALYZE ADIPOGENIC AND OSTEOGENIC GENES

| Gene symbol   | Gene name                                        | F-primer (5'-3')               | R-primer (5'-3')               | Method |
|---------------|--------------------------------------------------|--------------------------------|--------------------------------|--------|
| ADFP          | Adipophilin                                      | tca gct cca ttc tac tgt tca cc | cct gaa ttt tct gat tgg cac t  | TaqMan |
| ADPOQ         | Adiponectin, C1Q and collagen domain containing  | ggg gag aag ggt gag aaa gga    | ttt cac cga tgt ctc cct tag    | TaqMan |
| B2M           | Beta-2-microglobulin                             | ttc tgg cct gga ggc tat c      | tca gga aat ttg act ttc cat tc | TaqMan |
| GAPDH         | Glyceraldehyde-3-phosphate dehydrogenase         | tcc act ggc gtc ttc acc        | ggc aga gat gat gac cct ttt    | TaqMan |
| OCAL          | Osteocalcin                                      | ggc gct acc tgt atc aat gg     | tca gcc aac tcg tca cag tc     | TaqMan |
| OPON          | Osteopontin                                      | cgc aga cct gac atc cag t      | ggc tgt ccc aat cag aag g      | TaqMan |
| PERI          | Perilipin 1                                      | gga cac agt ggt gca tta cg     | gtc ccg gaa ttc gct ctc        | TaqMan |
| PPAR $\gamma$ | Peroxisome proliferator-activated receptor gamma | cag gaa aga caa cag aca aat ca | ggg gtg atg tgt ttg aac ttg    | TaqMan |
| SFRS4         | Splicing factor, arginine/serine-rich 4          | agc tgg caa gac cta aag ga     | ccc ttg tga gca tct gcat       | TaqMan |
| TBP           | TATA box binding protein                         | ccc atg act ccc atg acc        | ttt aca acc aag att cac tgt gg | TaqMan |

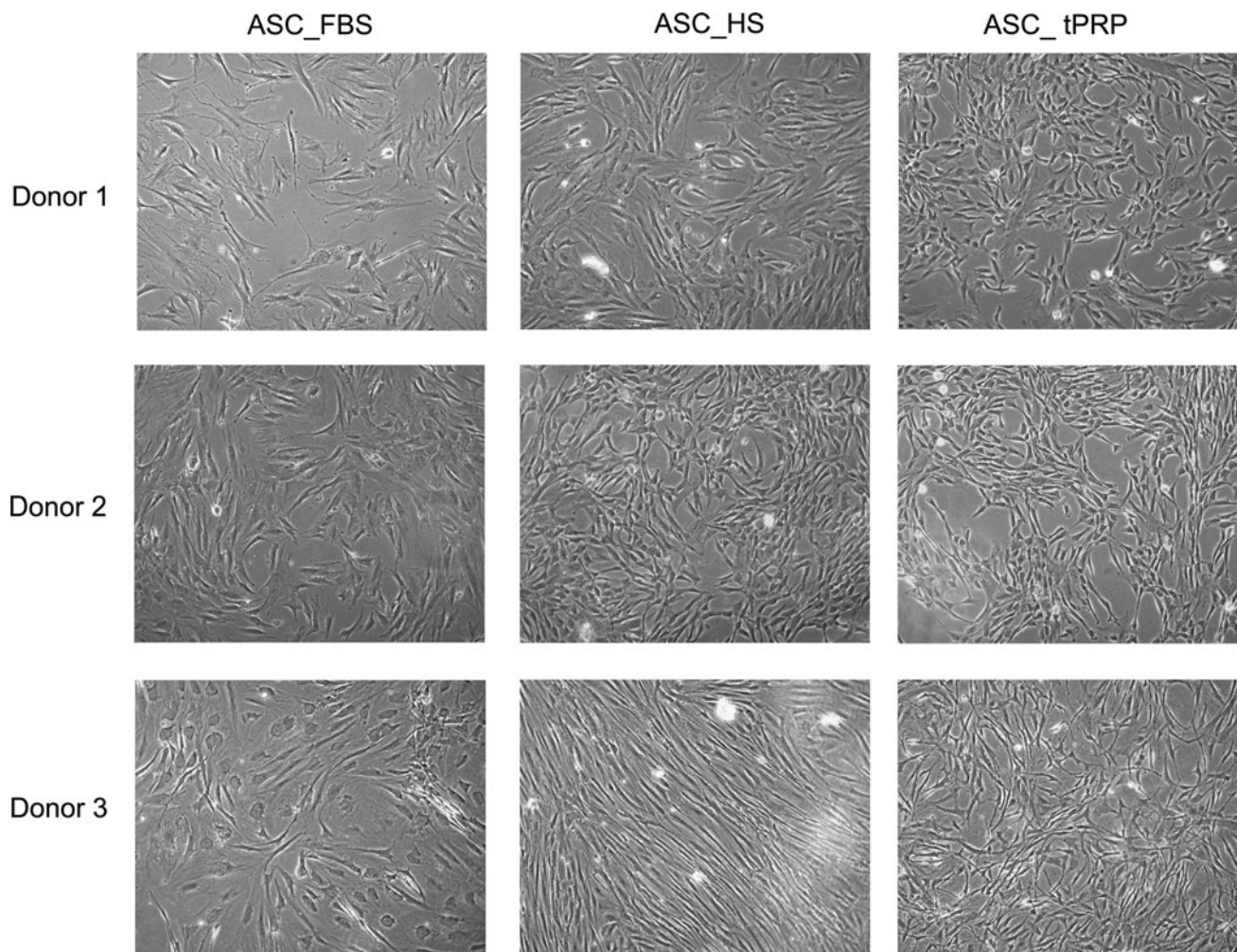


**FIG. 1.** Cell morphology of ASCs cultured under different conditions. ASCs from three representative donors are depicted each isolated and cultivated in either 10% FBS, 10% HS, or 10% tPRP. ASCs, adipose tissue-derived mesenchymal stromal cells; FBS, ASCs cultivated in 10% fetal bovine serum; HS, ASCs in 10% human serum; tPRP, ASCs in 10% thrombin-activated platelet releasate plasma.

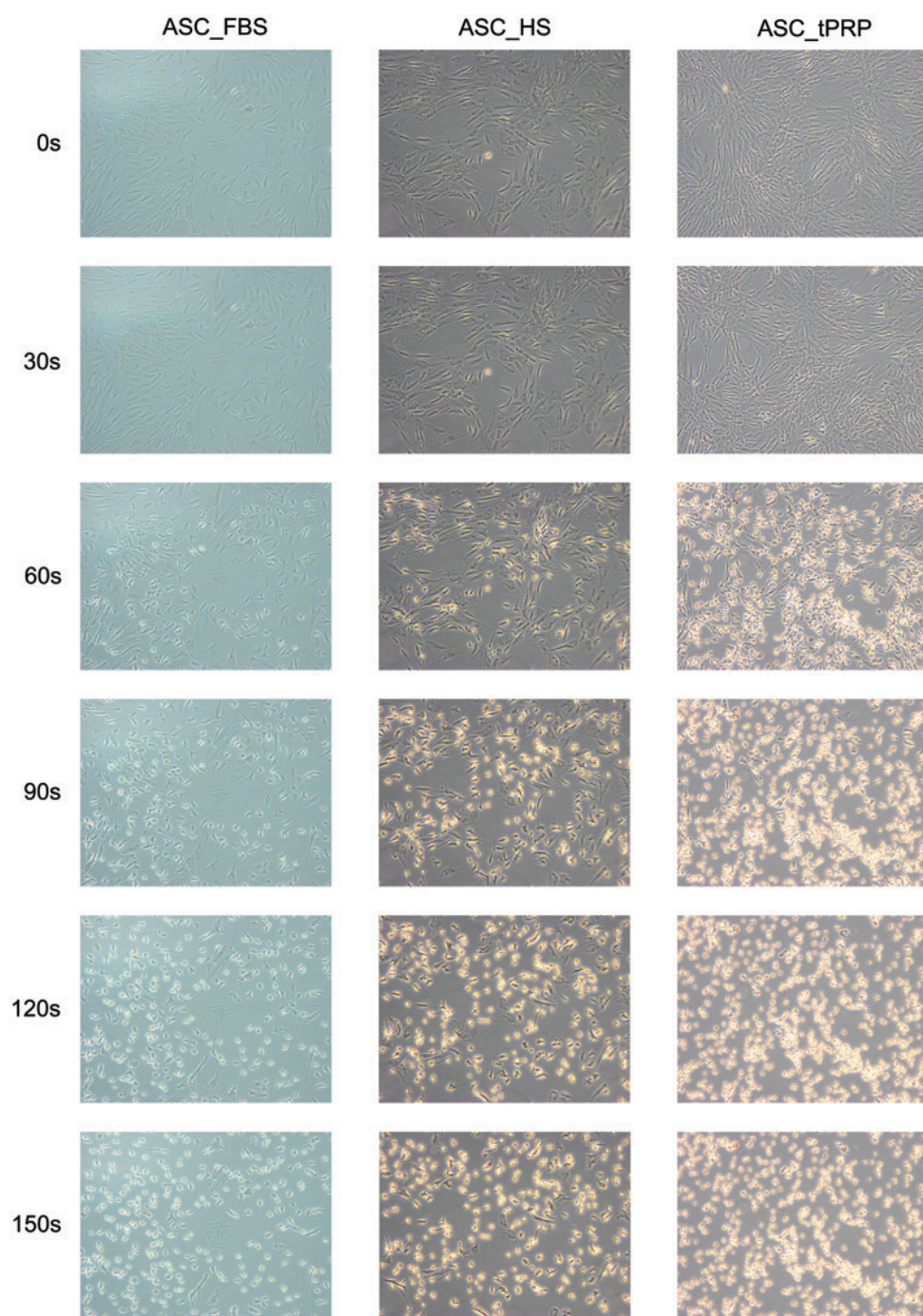


**FIG. 2.** Kinetic response of ASCs to trypsinization. ASCs cultivated in the three different conditions were washed and trypsin-EDTA added. Photomicrographs were taken every 30 s to observe the time course of detachment. ASCs in HS and tPRP responded within 60 s, whereas ASC\_FBS needed 90–120 s. One representative experiment is depicted. EDTA, ethylenediamine-tetraacetic acid. Color images available online at [www.liebertonline.com/ten](http://www.liebertonline.com/ten).

## Results

### MSC growth pattern

As described previously,<sup>15</sup> all adipose tissue-derived cells displayed fibroblast morphology. ASCs isolated and expanded in both human supplements, HS and tPRP expanded at higher rates and appeared smaller, more spindle-shaped than cells cultivated in FBS (Fig. 1).

### Response to trypsin

While passaging the cells, differences in the timely response to trypsin-EDTA were observed as depicted in Figure 2. Cells

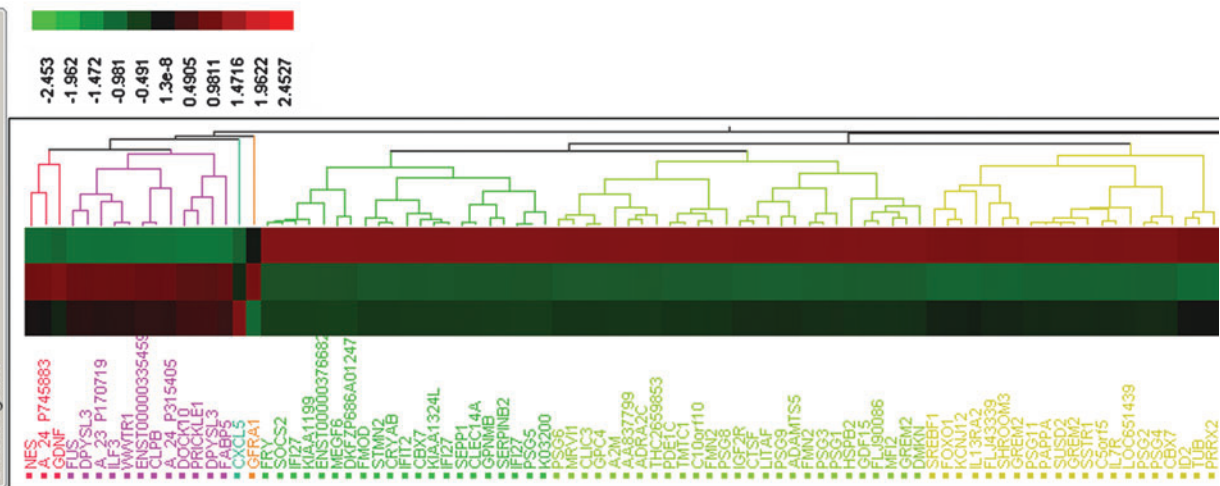
grown in either HS or tPRP responded within 60 s to the enzymatic and chelating activity, whereas ASCs grown in FBS needed 1.5–2 times longer.

### RNA analysis

**Microarray analysis.** To eliminate most of the cell cycle progression and cell proliferation-associated genes,<sup>24</sup> we harvested cells in early expansion culture at a confluency of about 70% where MSCs normally get trypsinized for further passaging. Cells were harvested at passage 2 to minimize the impact of different population doublings (cumulative doublings from passage 1 and 2: FBS  $9.1 \pm 0.3$ , HS  $12.5 \pm 0.8$ , and tPRP  $11.9 \pm 0.8$ ).

## Hierarchical Clustering

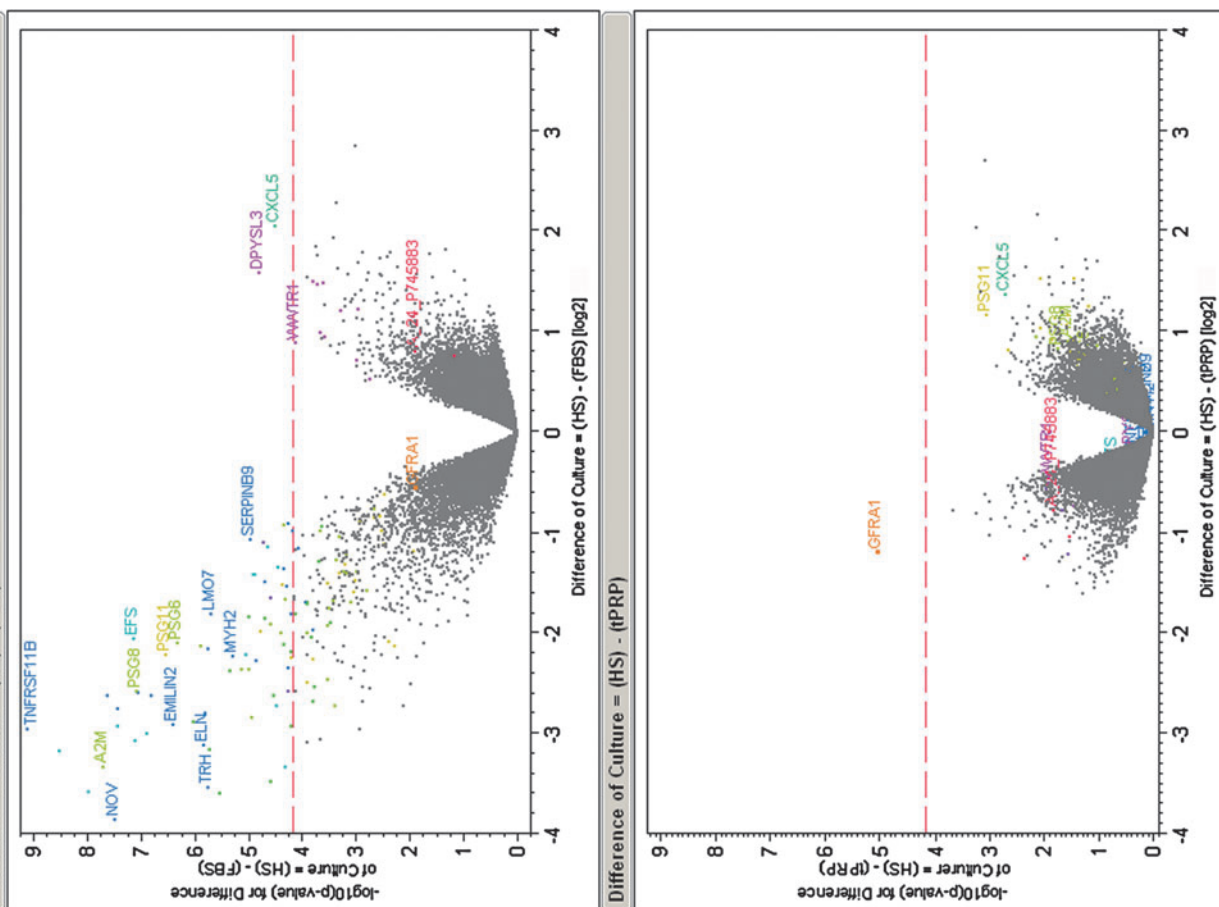
### Dendrogram



Horizontal reference line drawn at  $-\log_{10}(p) = 4.18$

Horizontal reference line drawn at  $-\log_{10}(p) = 4.18$

Difference of Culture = (HS) - (FBS)



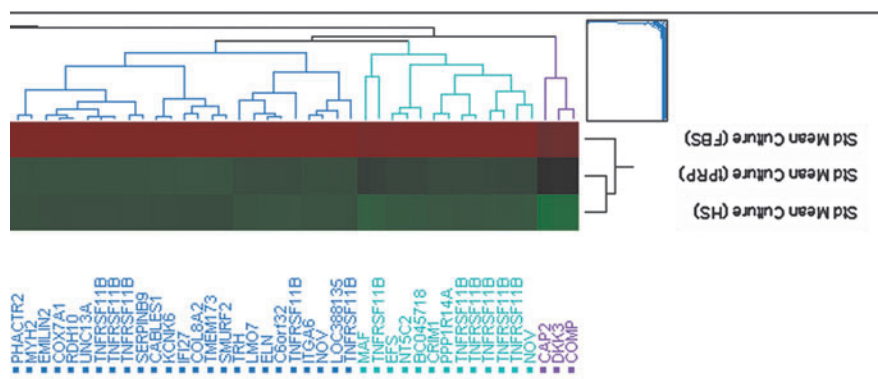


**FIG. 3.** Microarray-based comparative analysis of global ASCs gene expression affected by the culture conditions. RNA from seven samples were subjected to whole genome oligonucleotide microarrays. Statistical analysis is based on mean values of Loess normalized data. The dendrogram (**A**) produced by hierarchical clustering indicates a close relationship between ASC\_HS and ASC\_tPRP, but differences to ASC\_FBS. It further depicts those genes differentially expressed. In the volcano plots (**B**) genes are arranged along dimensions of biological and statistical significance. On a log scale on the *x*-axis the fold change between the groups is depicted, on the *y*-axis on a negative log-scale the *p*-value. Those genes plotted above the multiple testing cut-off at  $-\log(p)$  of 4.18 (dashed line) are genes significantly changed due to the culture conditions. It is obvious that HS compared to tPRP show nearly no differences, whereas FBS induces changes.

TABLE 4. GENES UPREGULATED IN FETAL BOVINE SERUM VERSUS HUMAN SUPPLEMENT CULTURES

| Gene symbol                                  | Gene name                                                                                                       | Entrez gene ID | Fold change |          |
|----------------------------------------------|-----------------------------------------------------------------------------------------------------------------|----------------|-------------|----------|
|                                              |                                                                                                                 |                | FBS/HS      | FBS/tPRP |
| Development and differentiation              |                                                                                                                 |                |             |          |
| TNFRSF11B                                    | Tumor necrosis factor receptor superfamily, member 11b (osteoprotegerin) (osteoclastogenesis inhibitory factor) | 4982           | 11.94       | 8.68     |
| STMN2                                        | Stathmin-like 2                                                                                                 | 11075          | 11.08       | 19.39    |
| GPNMB                                        | Glycoprotein (transmembrane) nmb (osteoactivin)                                                                 | 10457          | 7.57        | 11.03    |
| C6orf32                                      | Chromosome 6 open reading frame 32                                                                              | 9750           | 6.96        | 6.20     |
| PPP1R14A                                     | Protein phosphatase 1, regulatory (inhibitor) subunit 14A                                                       | 94274          | 6.56        | 5.32     |
| DMKN                                         | Dermokine                                                                                                       | 93099          | 5.52        | 10.60    |
| HSPB2                                        | Heat shock 27 kDa protein 2                                                                                     | 3316           | 5.14        | 9.01     |
| RDH10                                        | Retinol dehydrogenase 10 (all-trans)                                                                            | 157506         | 5.07        | 5.34     |
| MYH2                                         | Myosin, heavy chain 2, skeletal muscle, adult                                                                   | 4620           | 4.66        | 4.77     |
| MEGF6                                        | Multiple EGF-like-domains 6                                                                                     | 1953           | 4.53        | 5.36     |
| GREM2                                        | Gremlin 2, cysteine knot superfamily, homolog ( <i>Xenopus laevis</i> )                                         | 64388          | 3.92        | 8.01     |
| CRYAB                                        | Crystallin, alpha B                                                                                             | 1410           | 3.70        | 5.02     |
| COX7A1                                       | Cytochrome c oxidase subunit VIIa polypeptide 1 (muscle)                                                        | 1346           | 3.50        | 3.64     |
| DKK3                                         | Dickkopf homolog 3 ( <i>Xenopus laevis</i> )                                                                    | 27122          | 3.14        | 1.85     |
| FMN2                                         | Formin 2                                                                                                        | 56776          | 2.96        | 4.57     |
| MAF                                          | v-maf musculoaponeurotic fibrosarcoma oncogene homolog (avian)                                                  | 4094           | 2.67        | 2.01     |
| PDE1C                                        | Phosphodiesterase 1C, calmodulin-dependent 70 kDa                                                               | 5137           | 2.64        | 3.57     |
| CRIM1                                        | Cysteine-rich transmembrane BMP regulator 1 (chordin-like)                                                      | 51232          | 2.53        | 2.29     |
| FOXO1                                        | Forkhead box O1                                                                                                 | 2308           | 2.15        | 3.57     |
| FRY                                          | Furry homolog ( <i>Drosophila</i> )                                                                             | 10129          | 1.90        | 2.08     |
| ID2                                          | Inhibitor of DNA binding 2, dominant negative helix-loop-helix protein                                          | 3398           | 1.84        | 3.24     |
| SREBF1                                       | Sterol regulatory element binding transcription factor 1                                                        | 6720           | 1.79        | 2.60     |
| LOC651439                                    | Similar to Keratin, type I cytoskeletal 18 (cytokeratin-18) (CK-18) (keratin-18) (K18)                          | 651439         | 1.78        | 2.39     |
| TUB                                          | Tubby homolog (mouse)                                                                                           | 7275           | 1.61        | 2.51     |
| Extracellular matrix, adhesion and migration |                                                                                                                 |                |             |          |
| NOV                                          | Nephroblastoma overexpressed gene (CC3)                                                                         | 4856           | 14.50       | 13.63    |
| FMOD                                         | Fibromodulin                                                                                                    | 2331           | 8.93        | 14.67    |
| ELN                                          | Elastin (supravalvular aortic stenosis, Williams-Beuren syndrome)                                               | 2006           | 8.64        | 7.59     |
| EMILIN2                                      | Elastin microfibril interfacer 2                                                                                | 84034          | 7.48        | 8.00     |
| COMP                                         | Cartilage oligomeric matrix protein                                                                             | 1311           | 5.93        | 2.58     |
| ITGA6                                        | Integrin, alpha 6                                                                                               | 3655           | 4.83        | 4.67     |
| ADAMTS5                                      | ADAM metalloproteinase with thrombospondin type 1 Motif, 5 (aggrecanase-2)                                      | 11096          | 4.34        | 8.34     |
| SHROOM3                                      | Shroom family member 3                                                                                          | 57619          | 4.21        | 10.05    |
| EFS                                          | Embryonal Fyn-associated substrate                                                                              | 10278          | 4.13        | 3.24     |
| COL8A2                                       | Collagen, type VIII, alpha 2                                                                                    | 1296           | 3.88        | 4.33     |
| LMO7                                         | LIM domain 7                                                                                                    | 4008           | 3.50        | 3.26     |
| Signal transduction, cell-cell signaling     |                                                                                                                 |                |             |          |
| TRH                                          | Thyrotropin-releasing hormone                                                                                   | 7200           | 11.61       | 10.33    |
| ADRA2C                                       | Adrenergic, alpha-2C-, receptor                                                                                 | 152            | 6.61        | 10.75    |
| SSTR1                                        | Somatostatin receptor 1                                                                                         | 6751           | 4.78        | 10.72    |
| CLIC3                                        | Chloride intracellular channel 3                                                                                | 9022           | 3.21        | 4.49     |
| UNC13A                                       | unc-13 homolog A ( <i>C. elegans</i> )                                                                          | 23025          | 2.89        | 3.01     |
| IL7R                                         | Interleukin 7 receptor                                                                                          | 3575           | 2.81        | 4.89     |
| KCNJ12                                       | Potassium inwardly rectifying channel, subfamily J, member 12                                                   | 3768           | 2.63        | 4.95     |
| SMURF2                                       | SMAD-specific E3 ubiquitin protein ligase 2                                                                     | 64750          | 2.21        | 2.35     |
| CAP2                                         | CAP, adenylate cyclase-associated protein, 2 (yeast)                                                            | 10486          | 2.13        | 1.45     |
| KCNK6                                        | Potassium channel, subfamily K, member 6                                                                        | 9424           | 1.97        | 2.06     |
| IGF2R                                        | Insulin-like growth factor 2 receptor                                                                           | 3482           | 1.69        | 2.11     |

(continued)

TABLE 4. (CONTINUED)

| Gene symbol                                 | Gene name                                                                            | Entrez gene ID | Fold change |          |
|---------------------------------------------|--------------------------------------------------------------------------------------|----------------|-------------|----------|
|                                             |                                                                                      |                | FBS/HS      | FBS/tPRP |
| Membrane associated                         |                                                                                      |                |             |          |
| CLEC14A                                     | C-type lectin domain family 14, member A                                             | 161198         | 5.62        | 7.90     |
| MRV11                                       | Murine retrovirus integration site 1 homolog                                         | 10335          | 3.75        | 5.45     |
| IFI27                                       | Interferon, alpha-inducible protein 27                                               | 3429           | 3.56        | 4.38     |
| MFI2                                        | Antigen p97 (melanoma associated) identified by monoclonal antibodies 133.2 and 96.5 | 4241           | 3.16        | 4.76     |
| TMTC1                                       | Transmembrane and tetratricopeptide repeat containing 1                              | 83857          | 3.18        | 4.57     |
| SUSD2                                       | Sushi domain containing 2                                                            | 56241          | 2.77        | 4.71     |
| TMEM173                                     | Transmembrane protein 173                                                            | 340061         | 2.54        | 2.72     |
| GPC4                                        | Glypican 4                                                                           | 2239           | 1.96        | 2.37     |
| Regulation of cell growth and proliferation |                                                                                      |                |             |          |
| CABLES1                                     | Cdk5 and Abl enzyme substrate 1                                                      | 91768          | 4.46        | 4.93     |
| SOCS2                                       | Suppressor of cytokine signaling 2                                                   | 8835           | 3.51        | 4.19     |
| GDF15                                       | Growth differentiation factor 15                                                     | 9518           | 3.34        | 5.22     |
| CBX7                                        | Chromobox homolog 7                                                                  | 23492          | 2.47        | 3.82     |
| PRRX2                                       | Paired-related homeobox 2                                                            | 51450          | 2.27        | 4.97     |
| Immune response                             |                                                                                      |                |             |          |
| IFIT2                                       | Interferon-induced protein with tetratricopeptide repeats 2                          | 3433           | 5.77        | 8.34     |
| IL13RA2                                     | Interleukin 13 receptor, alpha 2                                                     | 3598           | 4.36        | 12.63    |
| LITAF                                       | Lipopolysaccharide-induced TNF factor                                                | 9516           | 1.88        | 2.40     |
| Protease inhibition                         |                                                                                      |                |             |          |
| A2M                                         | Alpha-2-macroglobulin                                                                | 2              | 10.11       | 18.869   |
| SERPINB2                                    | Serpin peptidase inhibitor, clade B (ovalbumin), member 2                            | 5055           | 4.11        | 5.31     |
| SERPINB9                                    | Serpin peptidase inhibitor, clade B (ovalbumin), member 9                            | 5272           | 2.09        | 2.15     |
| Pregnancy-associated proteins               |                                                                                      |                |             |          |
| PSG8                                        | Pregnancy-specific beta-1-glycoprotein 8                                             | 440533         | 5.94        | 10.52    |
| PSG3                                        | Pregnancy-specific beta-1-glycoprotein 3                                             | 5671           | 5.11        | 9.78     |
| PSG4                                        | Pregnancy-specific beta-1-glycoprotein 4                                             | 5672           | 4.73        | 9.93     |
| PSG11                                       | Pregnancy-specific beta-1-glycoprotein 11                                            | 5680           | 4.64        | 10.36    |
| PSG6                                        | Pregnancy-specific beta-1-glycoprotein 6                                             | 5675           | 4.29        | 6.43     |
| PSG9                                        | Pregnancy-specific beta-1-glycoprotein 9                                             | 5678           | 3.99        | 7.50     |
| PSG5                                        | Pregnancy-specific beta-1-glycoprotein 5                                             | 5673           | 3.59        | 4.41     |
| PSG2                                        | Pregnancy-specific beta-1-glycoprotein 2                                             | 5670           | 2.85        | 4.69     |
| PSG1                                        | Pregnancy-specific beta-1-glycoprotein 1                                             | 5669           | 2.40        | 3.44     |
| PAPPA                                       | Pregnancy-associated plasma protein A, pappalysin 1 Endo- and metallopeptidase       | 5069           | 1.96        | 2.79     |
| Metabolic process                           |                                                                                      |                |             |          |
| C10orf10                                    | Chromosome 10 open reading frame 10                                                  | 11067          | 7.14        | 12.99    |
| SEPP1                                       | Selenoprotein P, plasma, 1                                                           | 6414           | 5.19        | 7.16     |
| CTSF                                        | Cathepsin F                                                                          | 8722           | 2.62        | 3.96     |
| NT5C2                                       | 5'-nucleotidase, cytosolic II                                                        | 22978          | 2.20        | 1.92     |
| Others                                      |                                                                                      |                |             |          |
| KIAA1199                                    | KIAA1199                                                                             | 57214          | 12.08       | 17.01    |
| K03200                                      |                                                                                      | —              | 7.38        | 10.38    |
| ENST00000376682*                            | Probable G-protein coupled receptor 133 precursor (G-protein coupled receptor PGR25) | —              | 6.38        | 8.04     |
| DKFZP686A01247                              | Hypothetical protein                                                                 | 22998          | 6.14        | 7.43     |
| KIAA1324L                                   | KIAA1324-like                                                                        | 222223         | 4.32        | 5.94     |
| THC2659853                                  |                                                                                      | —              | 3.79        | 5.30     |
| FLJ43339                                    | FLJ43339 protein                                                                     | 388115         | 2.99        | 5.66     |
| LOC388135                                   | Similar to RIKEN cDNA 6030419C18 gene                                                | 388135         | 2.79        | 2.71     |
| BC045718                                    |                                                                                      | —              | 2.65        | 2.26     |
| AA837799                                    |                                                                                      | —              | 2.33        | 2.91     |
| FLJ90086                                    | Similar to AI661453 protein                                                          | 389389         | 2.04        | 2.66     |

FBS, ASCs cultivated in 10% fetal bovine serum; HS, ASCs cultivated in 10% human serum; tPRP, ASCs cultivated in 10% thrombin thrombin-activated platelet releasate plasma.

TABLE 5. GENES DOWNREGULATED IN FETAL BOVINE SERUM VERSUS HUMAN SUPPLEMENT CULTURES

| Gene symbol      | Gene name                                                         | Entrez gene ID | Fold change |          |
|------------------|-------------------------------------------------------------------|----------------|-------------|----------|
|                  |                                                                   |                | FBS/HS      | FBS/tPRP |
| CXCL5            | Chemokine (C-X-C motif) ligand 5                                  | 6374           | 0.24        | 0.63     |
| DPYSL3           | Dihydropyrimidinase-like 3                                        | 1809           | 0.33        | 0.28     |
| PRICKLE1         | Prickle homolog 1 ( <i>Drosophila</i> )                           | 144165         | 0.35        | 0.29     |
| FABP5            | Fatty acid binding protein 5 (psoriasis associated)               | 2171           | 0.36        | 0.27     |
| DOCK10           | Dedicator of cytokinesis 10                                       | 55619          | 0.36        | 0.30     |
| ILF3             | Interleukin enhancer binding factor 3, 90 kDa                     | 3609           | 0.66        | 0.52     |
| WWTR1            | WW domain containing transcription regulator 1                    | 25937          | 0.54        | 0.38     |
| ENST00000335459* |                                                                   | —              | 0.51        | 0.39     |
| CLPB             | ClpB caseinolytic peptidase B homolog ( <i>Escherichia coli</i> ) | 81570          | 0.52        | 0.40     |
| FUS              | Fusion [involved in t(12;16) in malignant liposarcoma]            | 2521           | 0.61        | 0.47     |
| NES              | Nestin                                                            | 10763          | 0.44        | 0.22     |
| GDNF             | Glial cell-derived neurotrophic factor                            | 2668           | 0.59        | 0.25     |

From a total of 34,039 individual genes analyzed on the 44K whole-genome microarray system, we identified only 102 genes to be significantly and at least twofold differentially expressed between the three groups FBS and HS or tPRP, disregarding donor-specific variances. Analyzing the microarray data, gene expression was considered as significantly changed, if exceeding the multiple testing cut-off, computed in this case with  $-\log_{10}(p)$  of 4.18. A false-positive rate of  $\alpha = 0.05$  with FDR correction was taken as the level of significance. The volcano plots in Figure 3 demonstrate that 90 of 102 identified genes showed a higher expression in FBS compared to HS or tPRP (fold change  $\geq 2$ ; Table 4). Depicted is the  $\log_2$  fold change on the x-axis and the  $-\log_{10}(p\text{-value})$  on the y-axis. In contrast, only 12 of the 102 genes showed a lower expression in FBS compared to HS or tPRP (fold change  $\leq 0.5$ ; Table 5).

To define the functional role of differentially expressed genes in ASCs, gene ontology databases, PubMed and Web of Science literature researches were performed. Accordingly, genes were subjected to functional categories. Among the genes with highest fold change values were genes grouped to “development and differentiation,” “extracellular matrix, adhesion and migration,” and “signal transduction, cell-cell signalling” (Table 4). In line with the observed different response to trypsin, some genes upregulated in FBS

cultures group to the category “protease inhibition.” A large fraction of 10 out of 90 genes grouped to “Pregnancy associated proteins,” including various isoforms of pregnancy-specific beta-1-glycoproteins (PSGs).

Interestingly, differences between cells cultivated in HS and tPRP were hardly evident, only glial cell-derived neurotrophic factor family receptor alpha 1 displayed a fold change of 0.43 comparing HS versus tPRP. Thus further supporting or previous conclusions stated in Ref.<sup>15</sup>, the ease of preparation favors the use of HS as human supplement. Underlying the observations of reduced adhesion of cells cultivated in the human alternatives, we detected a number of adhesion or extracellular matrix-associated genes expressed at lower levels in ASCs cultivated in these supplements. Likewise a number of differentiation especially osteo- and chondrogenesis-associated proteins were higher expressed in cells grown in FBS.

Reverse transcription quantitative polymerase chain reaction. From the list of differentially expressed genes, we selected 25 genes to be validated by RT-qPCR. Among those selected were genes displaying high fold change in gene expression and candidate genes known to be associated with proliferation, adhesion/motility, and morphogenesis. Normalized to the reference gene, all genes analyzed by

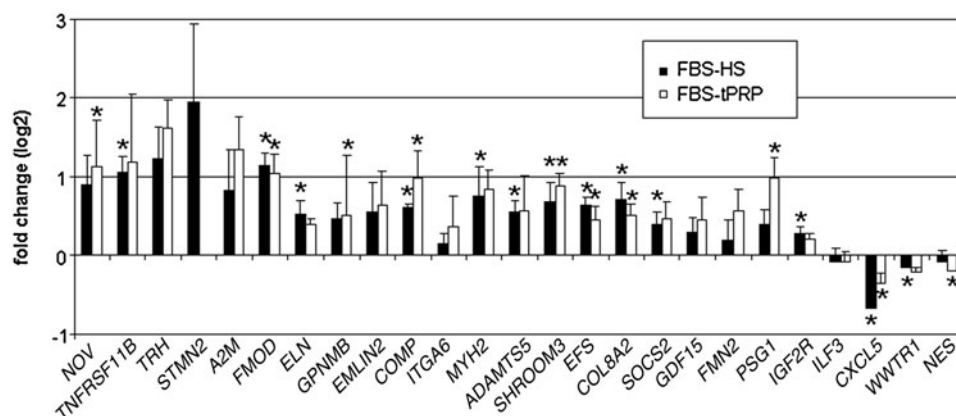


FIG. 4. Validation of microarray results by RT-qPCR. Twenty-five target genes of the 90 gene products identified in microarray analysis were selected for RT-qPCR analysis performed on three ASC donors. Fold change ( $\log_2$ ) of normalized values is depicted with FBS to HS in black and FBS to tPRP in white bars. The fold change calculated within the RT-qPCR analysis showed in all genes analyzed the same tendency of up- or downregulation, respectively. \* $p < 0.05$  calculated by paired  $t$ -test. RT-qPCR, reverse transcription quantitative polymerase chain reaction.

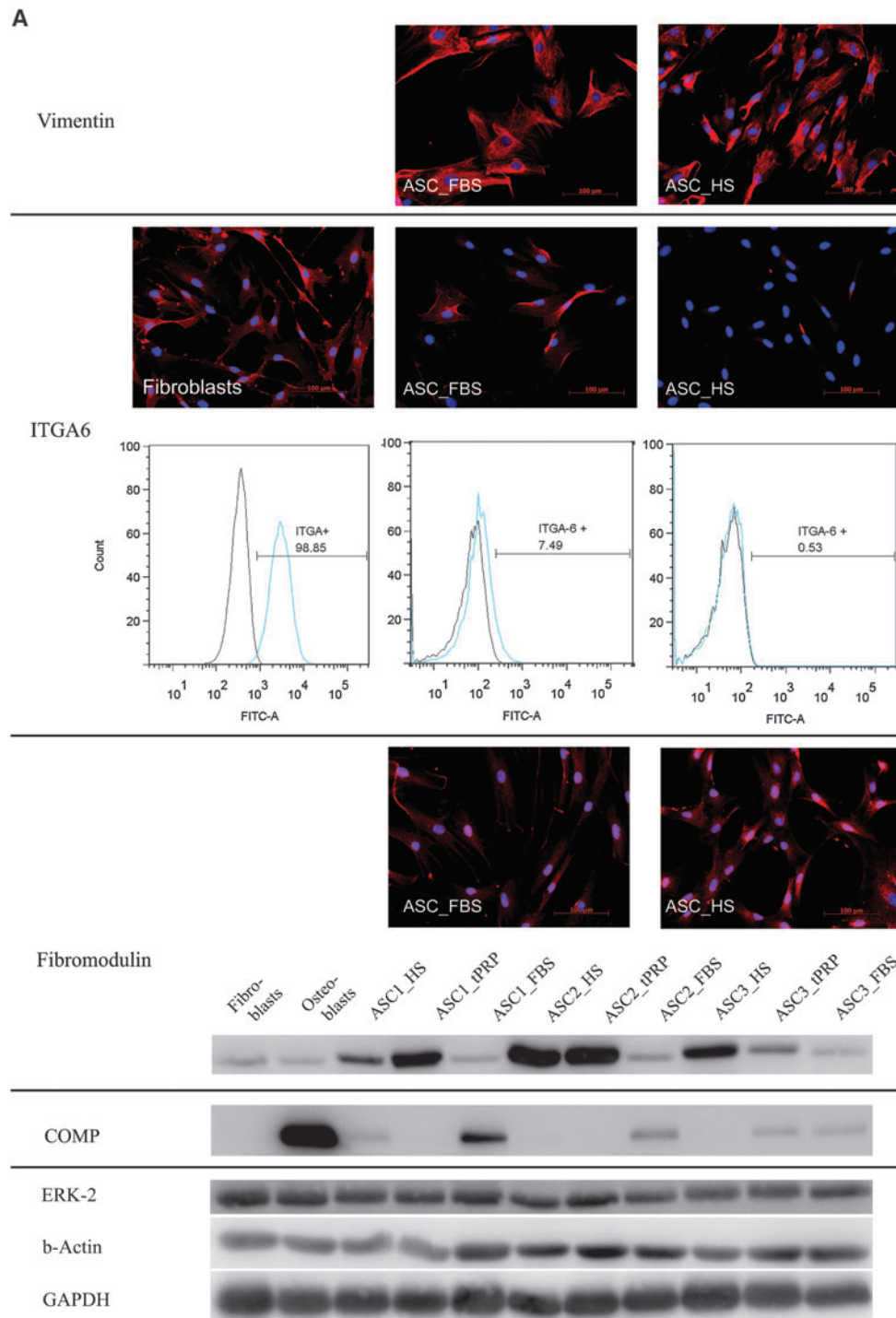


**FIG. 5.** Validation of microarray results by protein analysis. Three different analytical methods were applied to analyze protein expression: immunofluorescence staining, flow cytometric, and Western blot analysis. Fibroblasts, human umbilical vein endothelial cells, and osteoblasts served as positive controls, but are only depicted where relevant. At least three different ASC donors and at different passages from 2 to 5 were analyzed but only representative figures shown. (A) Vimentin staining underlines the differences in size and morphology of ASC\_FBS and ASC\_HS. Integrin alpha 6 is highly expressed in fibroblasts, but only a few ASC\_FBS and single ASC\_HS express it. FMOD analysis by all three methods revealed stronger expression in ASCs cultivated in the human supplements (flow cytometry see text). COMP could be detected by Western blot analysis with donor-dependent intensity in ASC\_FBS and only faint bands detectable for ASC\_HS and ASC\_tPRP. ERK2, GAPDH, and b-Actin served as loading controls for FMOD and COMP Western blots. Comparing three proteins to serve as loading controls indicated that amounts of b-Actin varied, whereas ERK2 and GAPDH supported equal protein loading. (B) Microarray and flow cytometric analysis of MFI in ASCs. Marker expression intensity determined by flow cytometric analysis is comparable to microarray analysis. Flow cytometric analysis was performed on all ASC samples by means of quality control as described in Ref.<sup>15</sup> COMP, cartilage oligomeric matrix protein; ERK2, extracellular-signal-related kinase 2; FMOD, fibromodulin; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; MFI, mean fluorescent signal intensity.

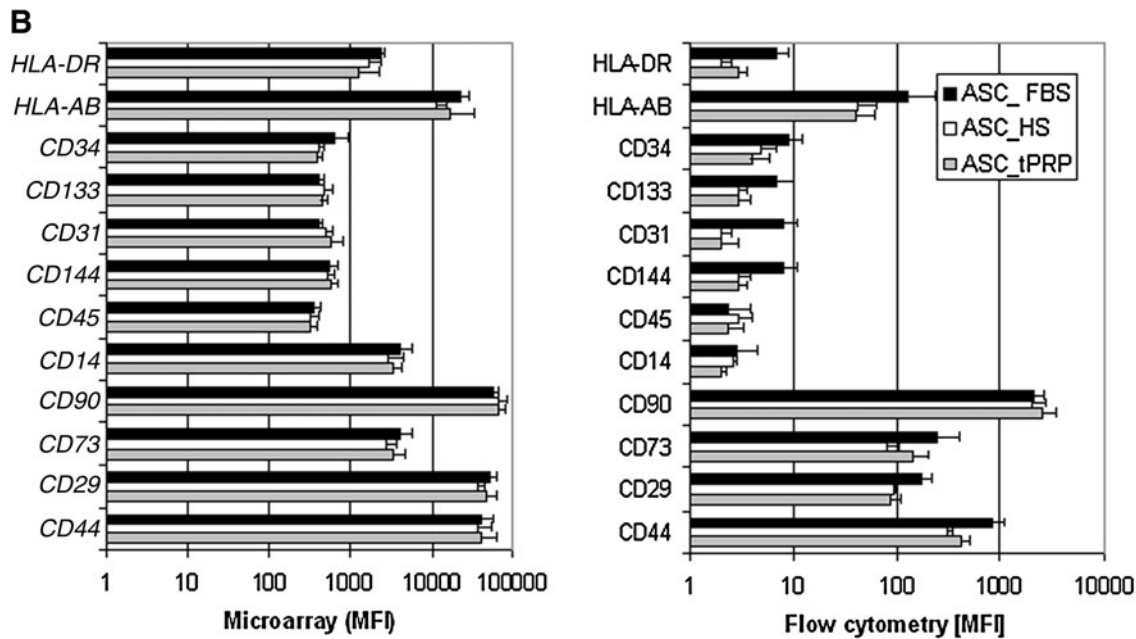


FIG. 5. (Continued).

RT-qPCR displayed similar expression patterns as already assessed in the microarray study and most showed significant regulation under the different culture conditions (Fig. 4). Stathmin-like 2 transcripts were not detectable in any samples of ASCs\_tPRP. A logarithmic transformation to calculate the fold change was not possible. At least on the transcript level 25% of differentially expressed genes identified by microarray analysis were verified to be differentially expressed within an independent experiment by an independent experimental method.

#### Protein expression analysis

It is important to validate microarray analyses by expression of the corresponding protein products. Proteins with at least twofold change plus putative importance in ASC functions were chosen (listed in Table 2) and first tested by immunofluorescence. In some cases immunofluorescence staining did not work with fibroblasts, HUVECs and osteoblasts chosen as positive controls, thus analysis could not be validated. As ASCs grown in tPRP did not adhere to the cover-slips used comparative results were only obtained from ASCs cultivated in FBS and HS. Thus, flow cytometric and Western blot analysis were performed with the same antibody clones. MYH2, OPG and PSG1 were neither in the control cells nor in the ASCs reproducibly detectable, thus an evaluation of these markers was not possible. Protein analysis was performed in cells from at least three donors and reproduced at different passages. Vimentin was chosen as positive control and its expression clearly demonstrates differences in the size and morphology of cells grown in either FBS or HS (Fig. 5A).

Compared to fibroblasts, integrin alpha 6 (ITGA6) expression was lower in all ASCs. Fewer ASC\_HS than ASC\_FBS stained positive for ITGA6 (Fig. 5A). In flow cytometry strong variations were observed, but median values

of % positivity supported gene expression data (ASC\_FBS: 7.49%, range 0.46%–16.1%, ASC\_HS: 0.82%, range 0.46%–23.3%, and ASC\_tPRP: 1.19%, range 0.11%–2.74%). Contradicting microarray and RT-qPCR analyses, protein levels indicated an enhanced expression of fibromodulin (FMOD) in ASCs cultivated in HS and tPRP (Fig. 5A), supported by flow cytometry analyses: median values: ASC\_FBS 25.12%, range 24.01%–26.22%; ASC\_HS 61.02%, range 53.38%–80.77%, and ASC\_tPRP 79.75%, range 78.69%–80.8%;  $p < 0.01$  for HS and tPRP compared to FBS.

Western blot analysis of cartilage oligomeric matrix protein (COMP), however, clearly revealed strong signals in ASCs cultivated in FBS and lower but variable expression in ASCs cultivated in HS or tPRP (Fig. 5A). We also compared the expression of typical MSC markers previously used for flow cytometric ASC characterization.<sup>15</sup> Similar expression patterns in microarray and flow cytometric staining were obvious with both transcript and protein expression for CD44, CD29, CD90, and HLA-AB and low expression of CD14, CD45, CD144, CD31, CD133, CD34, and HLA-DR (Fig. 5B).

#### Adipogenic and osteogenic differentiation potential

ASCs derived from all conditions demonstrated differentiation toward the osteogenic and adipogenic lineage as assessed by von Kossa and Oil red O staining after 21 days of induction. These assessments revealed donor-specific influences of the culture supplement (Fig. 6). However, no statistical significant impact was calculable when averaging the three donors ( $p = 0.08$  for triglycerides;  $p = 0.46$  PPAR $\gamma$ ,  $p = 0.26$  perilipin (PERI) and  $p = 0.44$  for calcium;  $p = 0.07$  OCAL,  $p = 0.54$  OPON). Data for adipophilin and adiponectin are not shown as they showed results comparable to perilipin. For both adiponectin and perilipin mRNA ex-

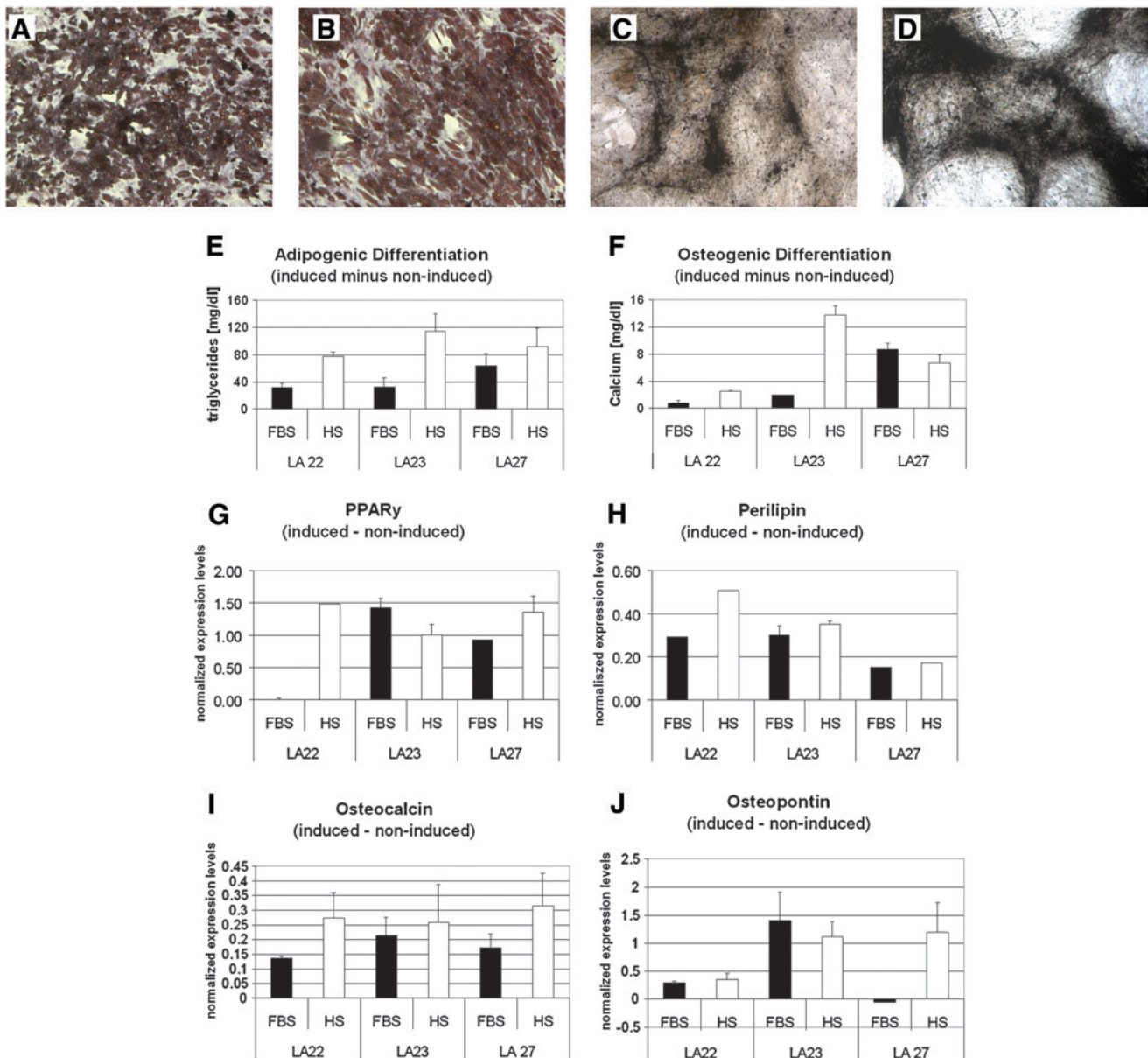


**FIG. 6.** Adipogenic and osteogenic differentiation potential of ASCs at passage 2 cultured in FBS or HS. Differentiation was assessed qualitatively by Oil red O (A, B) or von Kossa staining (C, D), quantitatively by triglyceride (E) and calcium (F) determination and RT-qPCR (G, PPAR $\gamma$ ; H, perilipin; I, osteocalcin; J, osteopontin). Photos depict results from donor LA23. No spontaneous induction of differentiation was observed in any condition. Differentiation in ASCs from three donors has been assessed in replicates and RT-qPCR performed in addition in technical duplicates. Values from noninduced were subtracted from induced samples. Averaging the three donors no statistical significant impact of the supplement was calculable. PPAR $\gamma$ , peroxisome proliferator-activated receptor gamma. Color images available online at [www.liebertonline.com/ten](http://www.liebertonline.com/ten).

pression was only detectable in adipogenic-induced cultures, whereas baseline expression levels detectable for adipophilin, PPAR $\gamma$ , OCAL, and OPON were markedly upregulated in the induced condition, with the exception of OPON in LA27\_FBS.

## Discussion

Changes in the manufacturing process by introducing alternate supplements to FBS might have an impact on the final cellular product. Accordingly regulatory authorities ask to perform comparability studies to ensure that modified

manufacturing protocols yield cells with equivalent qualities.<sup>25</sup> We now provide a basis of comparative microarray analyses of nonclonal ASCs cultivated in FBS or two human alternative supplements, HS or tPRP.

Of note, nonclonal ASC preparations have to be regarded heterogeneous and highly variable according to the isolation and culture procedure.<sup>26</sup> By purpose, our studies have been performed on clinically relevant ASC cultures to see global differences in the transcriptome. Our data indicate that at least at a stage in culture where cells usually get harvested for clinical use, the switch from the human environment to

FBS induces changes in gene expression which might be associated with gain or loss of function in ASCs. By comparing two human supplements to FBS better allowed assessing differences and to attribute them to FBS.

Supporting our notion of altered morphology, expansion capacity and trypsin sensitivity, we detected a variety of genes categorized in "development and differentiation," "extracellular matrix, adhesion and migration," and "signal transduction, cell signalling" highly upregulated in FBS-propagated cultures. Similar observations have been published very recently by Lindroos *et al.*<sup>27</sup> Their study performed at the same time as ours also compared the effects of 10% FBS and 15% allogeneic HS culture on ASCs by microarray and confirmatory RT-qPCR. In their study all genes with a statistically significant change irrespective of the fold change were examined, whereas we focused on those changed at least twofold. Genes overexpressed in HS clustered to cell cycle. Since HS in our hands significantly accelerate cell proliferation, we avoided the gene expression of cell cycle-associated genes by harvesting ASCs at late logarithmic phase of expansion culture. Comparable to us, Lindroos *et al.*<sup>27</sup> detected an excess of differentiation-associated genes expressed in FBS\_ASCs, especially of the transforming growth factor beta signaling pathway: *THBS1*, *COMP*, *Smurf2* and *ID2* appeared also in our study upregulated in FBS. Both ours and Lindroos *et al.*<sup>27</sup> findings are congruent with data by Lange *et al.*<sup>24</sup> and Shahdadfar *et al.*<sup>28</sup> Lange *et al.* defined genes clustered in "differentiation/development" and "cell adhesion/extracellular matrix-receptor interaction" upregulated in BM-MSCs in FBS compared to human platelet lysate.<sup>24</sup> Shahdadfar *et al.* comparing BM-MSCs cultivated in FBS or autologous HS described similar observations with a variety of genes encoding constituents of the extracellular matrix upregulated in FBS.<sup>28</sup> Based on this, the authors suggested that cells cultivated in HS reflect a more premature stage and less predifferentiated cells. This is further supported by Dahl *et al.*, who demonstrated that BM-MSCs cultivated in HS maintained a higher number of genes in unmethylated stages.<sup>29</sup>

The extracellular matrix of cartilage contains large amounts of aggrecan, decorin, biglycan, FMOD, collagen type II, chondroadherin as well as COMP.<sup>30</sup> Our data indicated significantly upregulated gene expression of both *COMP* and *FMOD* in ASCs cultivated in FBS, with similar findings for *COMP* protein. Our own group reported that *FMOD* gene is expressed in undifferentiated BM-MSCs, downregulated at an early phase of chondrogenic differentiation to be upregulated at a late time point of chondrogenesis.<sup>31</sup> Current protein analysis, however, contradicted gene expression data and revealed higher protein expression of FMOD in ASCs in human supplements. Currently we cannot explain the molecular basis for this discrepancy, but an inverse correlation of mRNA and protein levels can relate to differential regulation at the transcriptional or translational level.<sup>19</sup> Tormin *et al.* observed *FMOD* transcripts enriched in the nondividing fraction of MSCs and a time-dependent increase of the *FMOD* antigen-positive subpopulation.<sup>32</sup> Although we have checked ASCs at different passages, we were not able to detect an increase in FMOD expression, but we are now investigating whether the expression correlates to proliferation kinetics and populations doublings.

Of course, the sum of genes clustering to "differentiation" gives a good hint to differential processes modulated by growth supplements or the isolation of different subtypes of cells. Qualitative differentiation assays yet have not indicated any influence on the differentiation potential of ASCs.<sup>33</sup> Our quantitative results analyzing late stages of osteogenesis and adipogenesis demonstrate donor-specific differences on the differentiation potential in response to the supplement. Averaging the three donors analyzed demonstrated no significant impact of the supplement used for both adipogenic and osteogenic differentiation. Lindroos *et al.*<sup>27</sup> demonstrated that early osteogenesis after 14 days of induction is significantly impaired in HS-expanded ASCs whereas adipogenesis appeared to occur spontaneously and further enhanced by the HS supplement. We currently investigate whether differentiation kinetics are different in ASCs correlating to the enhanced proliferation potential. This might explain that we and others assessing BM-MSCs in human alternatives obtained different results. Studies on BM-MSCs cultivated in platelet lysate indicated despite similar differentiation pattern *in vitro* enhanced bone formation *in vivo*.<sup>13,34</sup>

ITGA6 or CD49f either in combination with the beta 1 or the beta 4 subunit is known as laminin receptor promoting cellular adhesion to the extracellular matrix. Our own previous data revealed no *ITGA6* gene expression in ASC\_FBS.<sup>35</sup> Here we validated enhanced *ITGA6* expression. This controversy may be related to the fact that previous protocols cultivated ASCs at 10-fold higher densities than the recent protocols seeding 200 cells/cm<sup>2</sup>. This notion is supported by Lee *et al.* who recently observed that BM-MSCs cultivated for 5 days expressed higher levels of *ITGA6* than cells expanded prolonged for 9 days.<sup>36</sup> In their study, BM-MSCs coexpressing podocalyxin-like protein and *ITGA6* showed enhanced clonogenicity and differentiation potential and importantly showed better homing with few cells trapped in the lung after intravenous infusion.<sup>36</sup> Translating these results to our observations, reduced *ITGA6* expression in ASCs cultivated in human alternatives may potentially negatively affect homing and engraftment. To check whether *ITGA6* expression relates to enhanced adhesion to laminin, preliminary experiments revealed no differences correlating to constant beta 4 and beta 1 expression. Continuation studies characterizing the adhesive and migratory conditions have already been initiated, but interim data show no distinction.

Our notion of reduced sensitivity to trypsin-EDTA in FBS-cultivated ASCs may relate to either enforced adhesion or to the presence of trypsin inhibitors. Our microarray data indicated upregulation of a few genes clustered to "protease inhibition" in FBS cultures. Alpha-2-macroglobulin may function as trypsin inhibitor.<sup>37</sup> SERPINs, a class of serine protease inhibitors, originally identified as plasma proteins capable of inhibiting serine proteases of the chymotrypsin/trypsin family, could also contribute.<sup>38</sup> Despite the obvious differences in *ITGA6* expression, other adhesion molecules like VLA4, 5 or collagens were not differentially expressed. Of course adhesion factors, like, for example, fibronectin may be differentially concentrated in the supplements.<sup>39</sup>

Interestingly and a matter of further studies is the cluster of neurogenic genes upregulated in the human supplements,

suggesting a neurogenic predisposition and the cluster of PSGs highly expressed in FBS. The latter might be attributable to the fetal origin of the bovine serum.

A serum-free chemically defined medium is highly demanded for GMP requirements. Our current data indicate that modifications of the cell culture supplement induce slight, but possibly important changes in the gene and protein expression. We obtained hints on a transcriptome level that switching the supplements may potentially affect ASC functions beyond those typically assessed. Both human supplements did not yield significant distinctions, thus we postulate that primarily the xenogenic supplement FBS induces changes exceeding that of *ex vivo* culture in human supplements. These transcriptome changes may determine ASC characteristics. Accordingly, we recommend validating any significant change in the stem cells culture condition according to the attempted therapeutic setting to ensure equivalency.

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### Disclosure Statement

No competing financial interest exists.

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Address correspondence to:

Karen Bieback, Ph.D.

Faculty of Clinical Medicine Mannheim

Institute of Transfusion Medicine and Immunology,

German Red Cross Blood Service of Baden-Württemberg-Hessen

University of Heidelberg

Ludolf-Krehl-Str. 13-17

Mannheim 68167

Germany

E-mail: karen.bieback@medma.uni-heidelberg.de

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