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A Novel Method of Dynamic Culture Surface Expansion Improves Mesenchymal Stem Cell Proliferation and Phenotype

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ABSTRACT

Repeated passaging in conventional cell culture reduces pluripotency and proliferation capacity of human mesenchymal stem cells (MSC). We introduce an innovative cell culture method whereby the culture surface is dynamically enlarged during cell proliferation. This approach maintains constantly high cell density while preventing contact inhibition of growth. A highly elastic culture surface was enlarged in steps of 5% over the course of a 20-day culture period to 800% of the initial surface area. Nine weeks of dynamic expansion culture produced 10-fold more MSC compared with conventional culture, with one-third the number of trypsin passages. After 9 weeks, MSC continued to proliferate under dynamic expansion but ceased to grow in conventional culture. Dynamic expansion culture fully retained the multipotent character of MSC, which could be induced to differentiate into adipogenic, chondrogenic, osteogenic, and myogenic lineages. Development of an undesired fibrogenic myofibroblast phenotype was suppressed. Hence, our novel method can rapidly provide the high number of autologous, multipotent, and nonfibrogenic MSC needed for successful regenerative medicine. STEM CELLS *2009;27:200–209*

Disclosure of potential conflicts of interest is found at the end of article.

INTRODUCTION

Mesenchymal stem cells (MSC) are multipotent cells that can differentiate into different cell types attractive for tissue repair [1]. These include chondrocytes and osteocytes for cartilage and bone reconstitution [2–5], myoblasts for skeletal muscle repair [6], hepatocytes for liver regeneration [7], cardiac and smooth muscle cells to repair the cardiovascular system [8–11], fibroblasts for soft connective tissue repair [12, 13], and neuronal cells to treat neurological disorders [14]. Human mesenchymal stem cells (hMSC) are isolated from bone marrow [2, 10, 15–18], umbilical cord blood [19, 20], adipose tissue [21, 22], pancreas [23], pleural cavity [24], muscle and brain [25], connective tissue of dermis and skeletal muscle [26], exfoliated deciduous teeth, and the eye conjunctiva [27].

Irrespective of origin, it is necessary to multiply MSC in culture to obtain sufficient cell numbers for tissue repair; for example, ~4.0 million hMSC are needed for a single injection aimed at infarcted heart repair [28]. Preceding cell implantation, lineage commitment is generally induced using specific culture media [2]. Alternatively, MSC can be directly seeded to repair a damaged organ, as in cardiovascular cell therapies [16] or in bone-forming graft [29], where the microenvironment is then expected to drive differentiation [30]. In any case, hMSC are desired to be kept in an undifferentiated, highly proliferative state during the culture phase of cell multiplication.

Conventional MSC culture requires weekly passaging. To retain efficient proliferation rates and to preserve stemness [31], hMSC must not be seeded and grown too sparsely; this usually limits the ideal dilution during passaging to 1:2. Furthermore, hMSC must be replated after reaching 70%–90% confluence because contact inhibition of growth reduces the cell proliferation rate. With repeated passaging hMSC lose their pluripotency and proliferation capacity partly because of exposure to enzymes such as trypsin that degrade cell surface proteins [31–33]. Whereas early MSC ($\leq$5 passages) preserve pluripotency, late MSC ($\geq$15 passages) are able to differentiate only into adipocytes [32].

We here describe an innovative method that maintains relatively constant cell densities that are near-optimal for proliferation while preventing contact inhibition of cell growth. Our strategy is to dynamically enlarge and thereby adapt the culture surface to the increasing cell numbers. This is achieved with a

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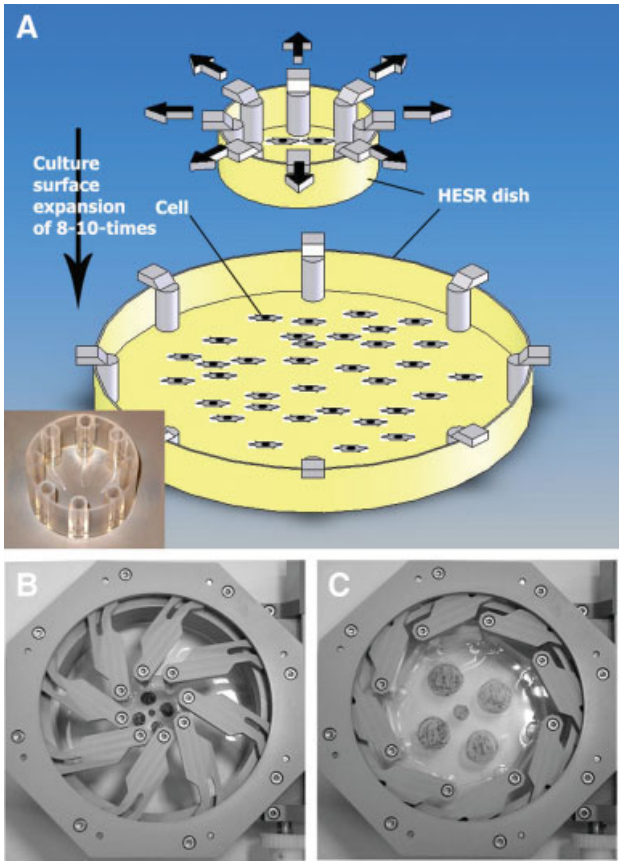

Figure 1. An innovative, highly expandable culture surface. **(A):** Schematically represented is a gradually expanded culture surface that minimizes contact inhibition of cell growth. Surface expansion is achievable with an HESR, shaped as a cell culture dish (inset). **(B, C):** The HESR dish was mounted with its initial area of 10 cm^2 **(B)** and was mechanically expanded by the iris-like device to 80 cm^2 **(C)**. Abbreviation: HESR, high-extension silicone rubber.

novel highly elastic culture dish whose diameter is uniformly expanded using a motorized mechanical device. The innovative culture system is applicable for all adherent cell types that are desired to grow rapidly in large quantities for regenerative medicine. Using our system for hMSC culture, we reduced the number of enzymatic passages by a factor of 3 and achieved significantly higher hMSC yields and continuous growth in contrast to low and ceasing growth rates in conventional culture. On the expanded culture surface, hMSC maintain stem cell characteristics over months in normal culture medium, as assessed by expression of undifferentiated hMSC markers. As final proof of principle, hMSC were still inducible to follow different lineages when placed in specific differentiation media after 9 weeks of culture on the expandable surface. Hence, our method has two major advantages over classic cell culture: (a) dramatic reduction of unwanted effects caused by enzymatic cell passaging, and (b) faster and longer cell proliferation.

MATERIALS AND METHODS

Production, Characterization, and Coating of Highly Extendable Culture Surfaces

High-extension silicone rubber (HESR) (Wacker Silicones, Burghausen, Germany) was injection molded in the shape of a cell culture dish (Fig. 1A, inset), with fittings suitable for mounting within a motorized iris-like device (both produced and distributed by Cytomec GmbH, Spiez, Switzerland, http://www.cytomec.com) (Fig. 1B, 1C; supporting information Video 1). The HESR dish is biocompatible and exhibits excellent optical qualities. This combination allowed for uniform and programmable culture surface expansion up to 1,000%. The Young's modulus of HESR culture surfaces was measured with atomic force microscopy (AFM) (Nanowizard II; JPK Instruments, Berlin, http://www.jpk.com) in the relaxed state and at increasing area expansions, always stabilized against a solid surface [34]. To promote cell adhesion, collagen type I was covalently linked to the fully expanded HESR culture surface and to conventional culture dishes (controls). Briefly, culture surfaces were etched with piranha solution, followed by silanization with 1% (3-aminopropyl)triethoxysilane (Sigma-Aldrich, Buchs, Switzerland, http://www.sigmaaldrich.com), functionalized with 6% (wt/wt) glutaraldehyde, and finally coated with 1 $\mu g/cm^2$ monomeric collagen I (Sigma-Aldrich).

Cell Culture and Quantification of Cell Adhesion and Proliferation

After informed consent was obtained, following protocol approval by the local ethical committee, cell cultures obtained from three donor bone marrow biopsies (passage 0 [P0]) were each initiated at 100,000 cells per cm^2 for one passage (P1) until reaching confluence in complete α-minimal essential medium, supplemented with 10% fetal calf serum (FCS) and 10 ng/ml basic fibroblast growth factor (R&D Systems Inc., Minneapolis, http://www.rndsystems.com). hMSC were then purified and controlled for the absence of hematopoietic cells as described previously [29, 35]. To initiate each generation, 5,000 hMSC per cm^2 were seeded and subcultured in the same medium as above. Culture duration on individual HESR dishes was 20 days, defined as one generation. One generation on HESR corresponded to three conventional 1:2 passages (Fig. 2A); confluence at each conventional passage was ~70%.

The quantities of hMSC on culture surfaces after 12 hours (adhesion) and after 1–7 days (proliferation) were determined using colorimetric living cell quantification methods (Cell Titer 96 AQueous One Solution Cell Proliferation Assay; Promega, Madison, WI, http://www.promega.com). Light absorption was measured at 490 nm in triplicate using a plate reader (Centro LB; Berthold Technologies, Regensdorf, Switzerland, http://www.bertholdtech.com) and corrected for cell-free blanks. Mean values were calculated from three independent experiments and expressed as arbitrary units ± SD.

Reverse Transcription-Polymerase Chain Reaction

Marker genes for undifferentiated hMSC and for the adipogenic, chondrogenic, osteogenic, and myogenic cell lineages were selected from human genes inventoried to customize TaqMan low-density arrays (LDA) (Applied Biosystems, Rotkreuz, Switzerland, http://www.appliedbiosystems.com) for quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis (supporting information Table 1). LDA with 384 wells were customized; each array consisted of 48 wells, each containing TaqMan assays for 48 specific genes. Total RNA was extracted and purified using RNeasy mini kit (Qiagen, Hilden, Germany, http://www1.qiagen.com) following the manufacturer's instructions and quantified using Nanodrop (Witec AG, Littau, Switzerland, http://www.witec.ch/E/index.php). Purity was verified using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, http://www.agilent.com). Expression of target genes was quantified using two-step qRT-PCR analysis. Total RNA (200 ng) was mixed with 0.25 ng of random hexamers (Invitrogen, Carlsbad, CA, http://www.invitrogen.com) and reverse-transcribed with 200 U of Superscript Reverse Transcriptase II (Invitrogen) and RNasin (Promega). The equivalent of 2 ng of total RNA was loaded with TaqMan Universal PCR Master Mix (Applied Biosystems) per 384 wells of the LDA, and qRT-PCR was processed with ABI Prism 7900 (Applied Biosystems). Conditions for qRT-PCR were as follows: 50°C for 2 minutes, initial denaturation at 95°C for 10 minutes, and 45 cycles of 15 seconds at 95°C and 1 minute at 60°C. Results were analyzed with SDS software, version 2.2 (Applied Biosystems), and the cycle threshold (C_T) values were exported into qBase 1.3.5 (Microsoft, Redmond,

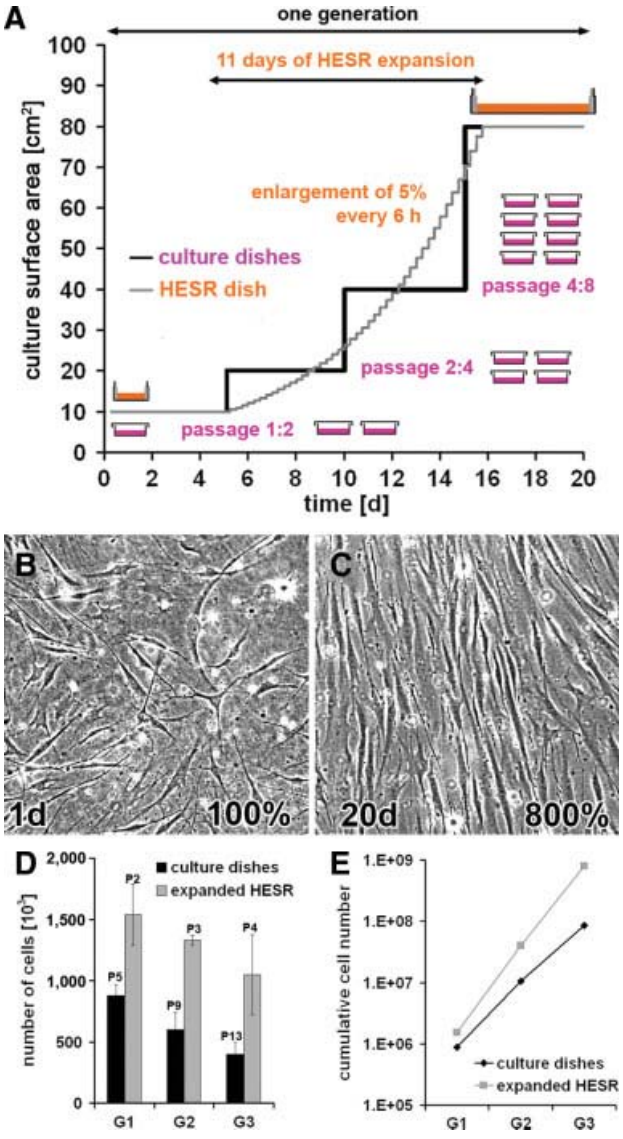

Figure 2. HESR culture boosts human mesenchymal stem cell (hMSC) proliferation compared with conventional culture. **(A):** The optimized culture surface expansion protocol for hMSC growth in the HESR dish device (gray line) is schematized for the course of one G (eight-fold surface expansion) and is compared with the corresponding three Ps in conventional culture (black line). **(B, C):** Mesenchymal stem cell morphology is shown after 1 d of growth on the HESR dish **(B)** and after 20 d of culture **(C)** following eight-fold area expansion. **(D, E):** Proliferation of hMSC on HESR (gray columns) and plastic dishes (black columns) was assessed by cell counting after each G **(D)** and translated into cumulative cell numbers **(E)**. Represented are means from three independent experiments with cells from three different donors ± SD. Abbreviations: d, day; G, generation; h, hour; HESR, high-extension silicone rubber; P, passage.

WA, http://www.microsoft.com), an Excel Visual Basic script for automated analysis of qRT-PCR data [36, 37]. C_T values were transformed to relative quantities and analyzed with geNorm 3.4 software [37]. This application for Microsoft Excel (Microsoft) allows determining the most stable reference genes from a set of candidate normalization genes in a given panel of cDNA samples. To correct for any variation in mRNA content and variation in enzymatic efficiencies, the relative quantities of the genes of interest were normalized with the geometric mean of the two most stable normalization genes, TBP (TATA box binding protein) and TFRC (transferrin receptor, p90, CD71). Gene expression levels were normalized to the values of the most stable housekeeping genes and expressed as the normalized relative quantity (NRQ). Relative values of mRNA were determined by dividing the NRQ value of each gene from the experimental condition by the NRQ value of the same gene obtained from the source hMSC (P1).

Immunofluorescence, Flow Cytometry, and Western Blotting

For immunofluorescence, fluorescence-activated cell sorting (FACS), and Western blot, hMSC were processed as described previously [2, 34] using antibodies against stem cell and lineage marker proteins (Table 1). Confocal images were acquired with a scanning confocal microscope (DM RXA2 with TCS SP2 acousto-optical beam splitter head; Leica, Glattbrugg, Switzerland, http://www.leica.com) using a ×40/numerical aperture 1.25 objective (Leica); all figures were assembled using Adobe Photoshop (Adobe Systems Inc., San Jose, CA, http://www.adobe.com). FACS was performed with four-laser-line FACS (CyanADP; Dako, Glostrup, Denmark, http://www.dako.com).

Multilineage Differentiation and Analysis of hMSC

Human MSC were differentiated in conventional culture dishes according to established protocols [2, 21, 38, 39]. To induce adipogenic lineage we used adipogenic medium (10% FCS, 100 μM indomethacine, 1 μM dexamethasone, 0.5 mM isobutyl-methylxanthine, and 10 μg/ml insulin in Dulbecco's modified Eagle's medium [DMEM]). Osteogenic lineage was induced using osteogenic medium (10% FCS, 100 μM ascorbate-2-phosphate, 10 nM dexamethasone, and 10 mM β-glycerophosphate in DMEM). To induce myogenic lineage we used myogenic medium (10% FCS, 5% horse serum, 10 nM dexamethasone, and 50 μM hydrocortisone in DMEM). To differentiate to the chondrogenic lineage, one aliquot of hMSC was cultured in suspension forming a pelleted micromass in chondrogenic medium (10 ng/ml transforming growth factor β3, 10 μg/ml insulin, 100 μM ascrobate-2-phosphate, and 10 nM dexamethasone in DMEM). To identify adipose cells, oil red O staining was performed. Osteogenic cells were identified by alkaline phosphatase kit (Sigma-Aldrich) and by detecting calcareous salt (mineralization) using von Kossa silver nitrate reduction; collagen fibrils were evidenced using Sirius red. To identify chondrogenic cells, micromasses of MSC pellet culture were fixed in formol buffer, embedded in paraffin, cut with a microtome and stained with Alcian Blue. Myogenic cells were identified by immunostaining for α-smooth muscle actin (α-SMA), smooth muscle myosin heavy chain, and SM22.

RESULTS

An Innovative Culture Device with Highly Expandable Surface

The essence of our approach is to gradually adapt the culture surface to growing cell numbers, which provides constantly high cell density while simultaneously preventing contact inhibition of growth (Fig. 1A). Dynamic expansion of culture surfaces is currently applied to stretch cells on membranes made of polydimethylsiloxane (PDMS); the material properties of PDMS limit surface enlargement to ~130% [40]. To achieve higher culture surface expansions, we identified a transparent and biocompatible HESR that allows isotropic surface expansion up to 1,000%. For use in cell culture HESR was injection molded in the shape of a cell culture dish (Fig. 1A, inset). The Young's modulus of the HESR dish was measured using AFM [34, 39] as a function of surface area expansion; the elastic modulus ranged from 8.5 ± 1.1 kPa when relaxed to 23.4 ± 5.4 kPa when fully expanded. This is ~100 times more compliant than stretchable PDMS membranes and ~1,000 times more compliant than tissue culture plastic.

Table 1. List of antibodies used in FACS, immunofluorescence, and Western blotting

Stem cell markers	Used in	Isotope and conjugation
CD34 (hematopoietic)	FACS	PE-conjugated, mIgG3k (Alexis Biochemical, Lausen, Switzerland, http://www.axxora.com)
CD44 (HA receptor)	FACS	Pacific blue-conjugated rat IgG2b (BioLegend, San Diego, http://www.biolegend.com)
CD73 (SH3 or SH4)	FACS	PE-conjugated, mIgG1 (BD Biosciences, Franklin Lakes, NJ, http://www.bdbiosciences.com)
CD90 (Thy1)	FACS	mIgG2a (Abcam, Cambridge, U.K., http://www.abcam.com)
CD105 (SH2, endoglin)	FACS	mIgG1 (Abcam)
CD166 (ALCAM)	FACS	mIgG2a (Abcam)
Myogenic markers		
α-SMA	IF/WB	mIgG2a, SM-1 (gift of G. Gabbiani, University of Geneva, Geneva, Switzerland)
SM-MHC	IF	rb (BTI, Stoughton, MA, http://www.btiinc.com)
SM22	IF	m (clone 1B8; gift of S. Sartore, University of Padua, Padua, Italy)
Myofibroblast marker		
α-SMA	IF/WB	mIgG2a, SM-1 (gift of G. Gabbiani, University of Geneva, Geneva, Switzerland)
Osteogenic marker		
Collagen type I	IF	mIgG1 (Sigma-Aldrich)
Secondary antibodies/dyes	IF	anti-m-Alexa568 (Molecular Probes, Basel, Switzerland, http://probes.invitrogen.com)
	IF	anti-rb-Alexa488 (Molecular Probes)
	IF	Alexa350-conjugated phalloidin (F-actin) (Molecular Probes)
	IF	DAPI (nuclei) (Sigma-Aldrich)
	FACS	anti-m-IgG1-APC (BD Biosciences)
	FACS	anti-mIgG2a-Alexa647 (Molecular Probes)
	WB	HRP-conjugated goat anti-m (Sigma-Aldrich)

Abbreviations: ALCAM, activated leukocyte cell adhesion molecule, DAPI, 4,6-diamidino-2-phenylindole; FACS, fluorescence-activated cell sorting; HA, hyaluronan; HRP, horseradish peroxidase; IF, immunofluorescence; m, mouse; PE, phycoerythrin; rb, rabbit; SMA, smooth muscle actin; SM-MHC, smooth muscle myosin heavy chain; WB, Western blotting.

To uniformly expand the HESR dish, an iris-like mechanical device was constructed (Fig. 1B, 1C). In our culture experiments HESR dishes were expanded from 10 cm^2 (Fig. 1B) to 80 cm^2 (800%) (Fig. 1C) (supporting information Video 1) and compared with 35-mm culture dishes (controls). To attain hMSC attachment similar to conventional culture dishes, we adapted an existing protocol to covalently coat the HESR with collagen type I [41]. After coating, the static HESR dish promoted moderately lower adhesion and proliferation of MSC compared with equivalently coated conventional culture dishes (supporting information Fig. 1).

Dynamically Expanded HESR Dish Culture Dramatically Enhances hMSC Proliferation

We then established an HESR dish protocol to expand the culture surface by eightfold, surface-equivalent to three passages in conventional culture dishes (schematized in Fig. 2A). Following an initial culture phase of 5 days on the static HESR dish to reach optimum growth density (~70% confluence) we expanded the HESR dish in steps of 5% within 60 seconds every 6 hours over 11 days (Fig. 2A, gray line). Our pilot studies indicated that the delay of 6 hours was largely sufficient for hMSC to recover their original projected surface area after 5% stretch (2 hours appeared to suffice). Once the culture surface reached 80 cm^2 (equivalent to eight 35-mm culture dishes), hMSC were grown to 90% confluence for another 3 days without further enlargement (Fig. 2A). We defined one passage from the HESR dish (here, 20 days) as one "generation" (Fig. 2A, orange), which was surface- and time-equivalent to three sequential 1:2 passages in conventional culture dishes (Fig. 2A, pink, black line).

Each culture was initiated with 5,000 hMSC per cm^2 on 10-cm^2 HESR dishes and 10-cm^2 conventional culture dishes. At the beginning of the 20-day culture period, hMSC grew sparsely on the relaxed HESR dish, with morphology similar to that in conventional culture (Fig. 2B). At the end of one HESR generation after eightfold area expansion and 20 days of culture, hMSC reached confluence and attained an elongated morphology, identical to that on confluent conventional culture dishes (Fig. 2C). To compare hMSC proliferation between expanded HESR dish culture (Fig. 2D, gray columns) and conventional culture dishes (Fig. 2D, black columns), cells were trypsinized and counted at the end of each generation; effective passage numbers at the time of counting are indicated above the columns in Figure 2D. When starting hMSC culture with P1, the cell number obtained after generation (G) 1 (corresponding to P2 of HESR culture and P5 of conventional culture) was ~2 times greater in HESR culture versus conventional culture (Fig. 2D). To further evaluate our method for long-term hMSC growth, we passaged HESR cultures for two more generations, always with identical starting cell numbers. HESR cell number was ~2.5 times greater after G2 and ~3 times greater after G3 versus the respective passages, P9 and P13, of conventional culture (Fig. 2D). Conventionally cultured hMSC became growth-arrested around P15, whereas the respective HESR G4 continued to proliferate (data not shown). On the basis of the measured growth rates for each generation, we calculated the cumulative cell number (Fig. 2E) (i.e., the number theoretically obtained if all cells were used to initiate a new generation). After three generations (9 weeks), starting with 50,000 hMSC, the cumulative cell number from HESR culture was ~800 million, which was 10 times greater than the number obtainable from conventional culture (~80 million) (Fig. 2E). Thus, our novel HESR culture method can provide dramatically higher cell yield compared with classic culture methods.

Dynamic HESR Culture Preserves an Undifferentiated and Nonfibrogenic hMSC Phenotype

We further tested whether dynamic culture surface expansion has unwanted effects on the undifferentiated hMSC phenotype in normal culture medium, since the stretch component of surface expansion can potentially induce mechano-responses [42]. Three parameters were tested as a function of culture generation: (a) preservation of stem cell character, (b) induction of specific mesenchymal lineage without specific induction me-

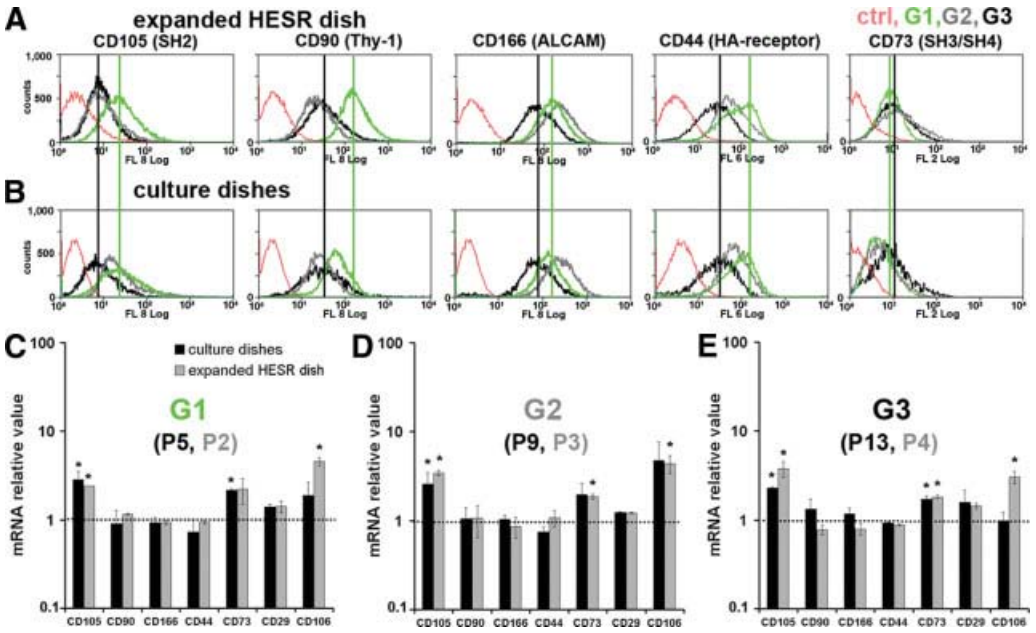

Figure 3. Culture on HESR dishes preserves the stem cell character of human mesenchymal stem cells (hMSC). hMSC were cultured in normal culture medium for three G in HESR **(A)** and in conventional culture dishes **(B)**. hMSC were processed for fluorescence-activated cell sorting analysis of stemness cell surface protein markers (G1, green; G2, gray; G3, black) and compared in histograms together with unstained ctrls (red). **(C–E):** Expression of the same markers was also assessed on the mRNA level using quantitative reverse transcription-polymerase chain reaction. Relative mRNA expression of each gene was first normalized by a stable housekeeping gene and then related to the normalized expression level of the same gene in the source hMSC (P1) (dotted line = 1). All experiments were performed with samples of three different donors, and mean values are expressed ± SD. Asterisks indicate Student's *t* test *p* values <.01, tested against mRNA expression levels of P0 source hMSC (mean = 1). Abbreviations: ctrl, control; G, generation; HESR, high-extension silicone rubber; P, passage.

dium, and (c) development of an unwanted fibrogenic cell phenotype. P1 hMSC were cultured for three generations in expanded HESR dishes (four trypsin passages) and in conventional culture dishes (12 trypsin passages) for a total period of 9 weeks in conventional culture medium.

First, we assessed by FACS the expression of surface protein markers of the hMSC stem cell character CD105, CD90, CD166, CD44, and CD73 (Fig. 3A, 3B), after G1 (green), G2 (gray), and G3 (black). To better compare expression profiles between HESR dishes (Fig. 3A) and normal culture dishes (Fig. 3B), cell count histogram peaks from HESR G1 and G3 are indicated with vertical lines. hMSC cultured on HESR dishes (Fig. 3A) expressed higher or similar amounts of all stem cell surface markers compared with conventional culture (Fig. 3B), which was particular evident after G1. Stemness marker expression was reduced in both culture systems after three generations but still high compared with negative staining controls (red). These results were corroborated on the mRNA level by performing qRT-PCR for the same proteins and CD29 and CD106 (Fig. 3C–3E; supporting information Table 1). Relative mRNA expression of each gene was first normalized by a stable housekeeping gene and then related to the normalized expression level of the same gene in the source hMSC (P1) (Fig. 3C–3E, dotted line = 1). All markers remained either unchanged or were upregulated (Fig. 3C–3E; supporting information Table 1). Hence, the stem cell character of hMSC is preserved over three generations of HESR culture.

Second, we assessed whether expanded HESR culture alone, without any specific induction medium, will drive hMSC into specific adipogenic, chondrogenic, osteogenic, or myogenic lineages (Fig. 4; supporting information Fig. 2). For this we quantified mRNA levels of lineage marker genes by qRT-PCR (supporting information Table 1; supporting information Fig. 2), normalized as indicated above (Fig. 4; supporting information Fig. 2, dotted lines = 1). Neither HESR culture nor conventional culture induced a specific lineage in normal culture medium over three generations (Fig. 4; supporting information Fig. 2). We observed a tendency to osteogenic induction in conventional and HESR culture; however, mRNA osteogenic gene levels did not exceed fourfold upregulation compared with source hMSC P0 (Fig. 4, yellow shaded regions), a value that is generally considered to represent normal mRNA expression variance. For all other lineages, the expression levels of some genes were increased, but this was not restricted to a particular culture method or generation and did not produce a consistent lineage pattern (Fig. 4). However, one common pattern was detected after culture on HESR dishes compared with conventional culture. HESR dish-cultured hMSC exhibited clearly lower levels of extracellular matrix (ECM) protein mRNA specific for chondrogenesis, such as aggrecan (ACAN), collagen type X α1 (COL10A1), and cartilage oligomeric matrix protein (COMP), and characteristic of osteogenesis, including osteopontin (SPP1) and collagen type 1 α1 (supporting information Fig. 2). With the exception of SPP1, all these ECM protein mRNA levels dropped even below the baseline expression in source hMSC (P1). Thus, expanded HESR culture in normal medium preserves the nondifferentiated hMSC phenotype and downregulates ECM protein markers.

Third, we examined whether expanded HESR culture induces spontaneous differentiation of hMSC into fibrogenic so-called myofibroblasts. Like most mesenchymal cell types, hMSC are prone to this mechanically driven transformation that is hallmarked by de novo expression of α-SMA, a key element in fibrosis [43, 44]. In contrast to strong upregulation of α-SMA expression in hMSC after three generations in conventional culture, HESR culture suppressed development of the myofibroblast phenotype (Fig. 5).

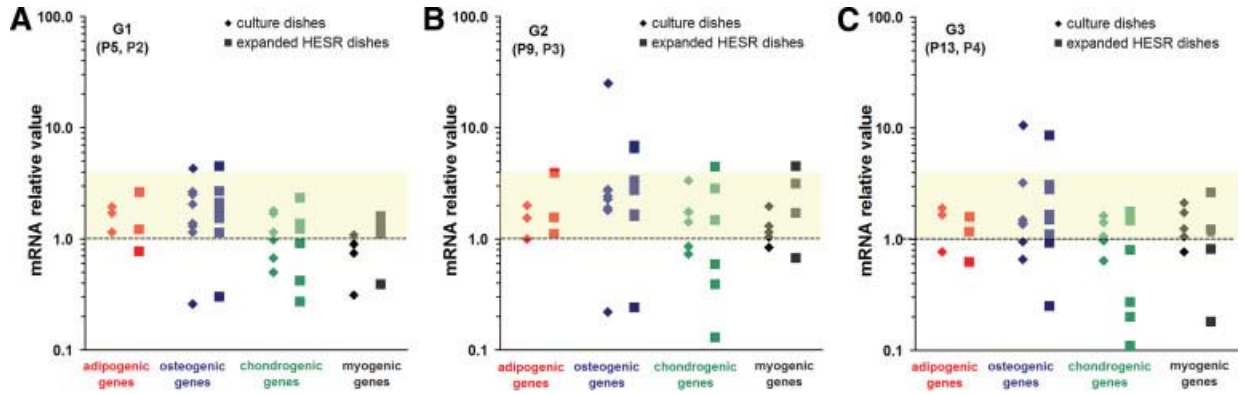

Figure 4. Growth in HESR dish culture preserves the undifferentiated human mesenchymal stem cell (hMSC) phenotype. hMSC were cultured in normal culture medium for three G in HESR and in conventional culture dishes. mRNA samples were prepared after G1 **(A)**, G2 **(B)**, and G3 **(C)** and analyzed by quantitative reverse transcription-polymerase chain reaction using primers for adipogenic, chondrogenic, osteogenic, and myogenic genes. Relative mRNA expression of each gene was first normalized by a stable housekeeping gene and then related to the normalized expression level of the same gene in the source hMSC (P1) (dotted line = 1). Charts are displayed with logarithmic scaling. All experiments were performed with samples of three different donors, and mean values are expressed ± SD. Yellow shaded regions include relative mRNA expression values ≤4 times baseline, which are generally considered in the normal range of variation. Abbreviations: G, generation; HESR, high-extension silicone rubber; P, passage.

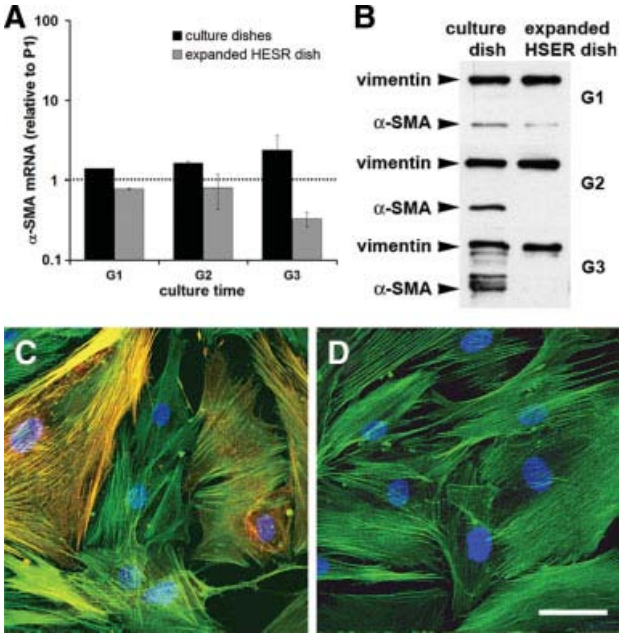

Figure 5. HESR dish culture protocol suppresses development of a fibrotic human mesenchymal stem cell (hMSC) phenotype. hMSC were cultured in normal culture medium for three G in HESR and in conventional culture dishes. Spontaneous development of fibrogenic myofibroblasts was assessed by probing expression of α-SMA by quantitative reverse transcription-polymerase chain reaction **(A)**, Western blotting **(B)**, and immunofluorescence staining **(C, D)**. Western blots were normalized to housekeeping vimentin. G3 hMSC from conventional culture **(C)** and HESR culture **(D)** were stained for α-SMA (red) and actin stress fibers (green, phalloidin). Scale bar = 50 μm. Abbreviations: G, generation; HESR, high-extension silicone rubber; α-SMA, α-smooth muscle actin.

hMSC Obtained from Expanded HESR Culture Are Committable to Mesenchymal Lineages

Finally, we investigated the potential of hMSC from HESR G1–G3 to differentiate into mesenchymal lineages in response to specific induction media. Lineage gene markers were evaluated with qRT-PCR, supported by histological detection of marker gene products (Fig. 6). Induction media and markers were used for the adipogenic (Fig. 6A, 6B), chondrogenic (Fig. 6C, 6D), osteogenic (Fig. 6E, 6F), and myogenic (Fig. 6G, 6H) lineages. Expression levels were always normalized to noninduced source hMSC (P1) (Fig. 6A, 6C, 6E, 6G, dotted lines = 1) and displayed in logarithmic scale. Apart from the myogenic line, no decrease in the potential to being inducible with the respective media was noticeable even after G3 of HESR culture (Fig. 6). Compared with the respective induction of early-passage hMSC (P4) obtained from conventional culture, late (G3) hMSC from HESR culture showed similar or in most circumstances even greater expression of the respective lineage marker genes (supporting information Fig. 3).

The lineage-inducing effect of adipogenic medium was confirmed by the upregulation of peroxisome proliferator-activated receptor γ (~15-fold) and lipoprotein lipase (~40,000-fold) (Fig. 6A); de novo accumulation of cytoplasmic lipid droplets was detected with oil red O staining (Fig. 6B, red lipid droplets). Lineage commitment induced by chondrogenic medium in pellet culture [2] was observed by increased expression of ACAN (~10-fold), COL10A1 (~400-fold), COMP (~100-fold), and biglycan (~10-fold) (Fig. 6C). Collagen types II (COL2A1) and IX (COL9A1) were expressed de novo (data not shown). Because of the missing reference in source hMSC (P1), neo-expressed genes are not displayed in Figure 6. Synthesis of cartilage-specific ECM was further evidenced by Alcian Blue staining for acidic sulfated mucosubstances (blue) accumulating around hMSC (red) in culture pellets (Fig. 6D). Osteogenic differentiation in specific induction medium was demonstrated by increased expression of runt-related transcription factor 2 (approximately fivefold), OB-cadherin (CDH11; approximately fivefold), homeobox (MSX2; approximately eightfold), sparc/osteonectin (approximately threefold), and bone morphogenetic protein 6 (approximately ninefold) (Fig. 6E). Osteogenic-induced hMSC stained positive for alkaline phosphatase (Fig. 6F, red), and mineralization was demonstrated by von Kossa silver stain (Fig. 6F, inset). Although collagen mRNA expression was only moderately higher after osteogenic induction compared with noninduced hMSC of P0 (~twofold of P0 in G3 hMSC), these levels were clearly increased compared with noninduced hMSC obtained from HESR culture G3 (supporting information Fig. 2; ~0.3-fold of P0 hMSC). Collagen upregulation was confirmed by im-

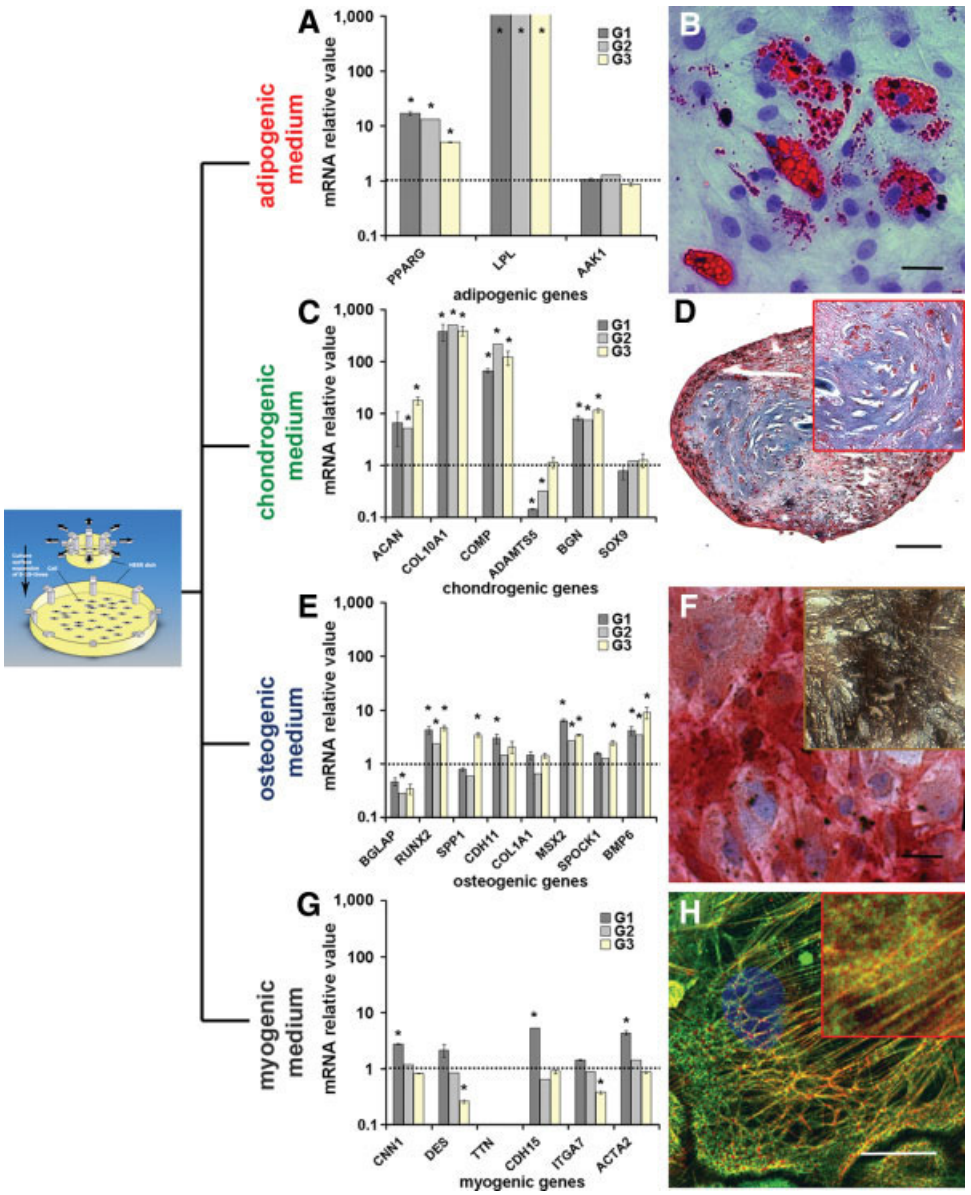

Figure 6. HESR dish culture preserves human mesenchymal stem cell (hMSC) potential to differentiate into mesenchymal cell lineages. Passage 1 (P1) hMSC were cultured for three G in HESR dish culture and harvested from G1, G2, and G3 to be placed for 3 weeks further in the indicated lineage-inducing culture conditions. RNA was extracted from lineage-induced cells and reverse-transcribed for quantitative reverse-transcription-polymerase chain reaction measurements **(A, C, E, G)**. Relative mRNA expression of each gene was first normalized by a stable housekeeping gene and then related to the normalized expression level of the same gene in the source hMSC (P1) (dotted line = 1). All experiments were performed with samples of three different donors, and mean values are expressed ± SD. Asterisks indicate Student's *t* test *p* values $<.01$, tested against mRNA expression levels of P0 source hMSC (mean = 1). **(B):** Adipogenesis was detected with oil red O. **(D):** Chondrogenesis was demonstrated by Alcian Blue. **(F):** Osteogenesis was shown by alkaline phosphatase staining (red) and with von Kossa staining for calcification (inset, black). **(H):** Myogenesis was detected by immunofluorescence staining for smooth muscle myosin heavy chain (green) and α-smooth muscle actin (red) in a striated stress fiber pattern (inset). Scale bars = 20 μm **(B, F)** 100 μm **(D)**, and 10 μm **(H)**. Abbreviations: G, generation; HESR, high-extension silicone rubber; LPL, lipoprotein lipase; PPARG, peroxisome proliferator-activated receptor γ.

munostaining for collagen I protein and positive reaction using Sirius red (data not shown).

After myogenic induction, hMSC from HESR G1 exhibited upregulated mRNA expression of calponin-1 (approximately threefold), desmin (approximately threefold), M-cadherin (CDH15; approximately sixfold), and α-SMA (approximately fivefold) (Fig. 6G, gray bars). With later generations, however, expression dropped to the level of P1 source hMSC or below (Fig. 6G). Myogenic gene expression in hMSC from HESR G3 was also decreased versus P4 hMSC from conventional culture after lineage induction (supporting information Fig. 3). However, we observed strong protein expression and stress fiber organization of the late smooth muscle markers α-SMA (Fig. 6H, green), smooth muscle myosin heavy chain (Fig. 6H, red), and SM22 (data not shown). These proteins were not expressed in myogenic-induced P4 hMSC. In summary, hMSC grown for three generations in expanded HESR culture retain the ability to undergo lineage differentiation after incubation with specific induction media.

DISCUSSION

We have developed a novel culture system that allows dynamic expansion of the culture surface by more than 1,000%. This approach has two major advantages over conventional cell culture: (a) longer preservation of the cell phenotype because cell surface protein degradation by enzymatic passaging is reduced, and (b) higher cell yield because dynamic culture surface expansion eliminates contact inhibition of growth and keeps cell growth in the exponential phase.

Rapid population expansion of desired cell phenotypes is a critical issue in cell therapy and tissue engineering approaches to repair damaged tissues with autologous cells. Grafting of cultured cells is an emerging technology to repair tissues and organs of mesenchymal origin using hMSC [10] and is routinely applied to cover burns with skin cells [45]. Bone marrow is one of the most important sources of hMSC that are relatively easy to purify from other residents, such as hematopoietic cells [2]. However, hMSC make up only approximately 0.01% of nucleated bone marrow cells, and the number of hMSC obtained per 20 ml of aspirate is usually low (~50,000 hMSC) [29]. This low yield is in sharp contrast to what is currently needed for therapy. Repeated injections of at least 1–10 million hMSC per kilogram of body weight can require more than 1 billion hMSC per patient; this is achievable only with allogenic hMSC and at that scale may require immunosuppression [10].

Our culture method can provide 800 million multipotent hMSC from one starting population of 50,000 cells within only 9 weeks; this is 10 times higher than the yield from conventional culture. In addition, because hMSC proliferation ceased after 9 weeks in conventional culture (P15) but continued at a high rate in HESR dishes, one can obtain several orders of magnitude more cells with the new culture system by further prolonging culture time. Another improvement is possible by changing the HESR surface expansion protocol. Here we have used a conservative protocol of 5% expansion every 6 hours. By reducing the delay to 3–4 hours, we are now attempting to reduce the culture time with the same cell yield. Additional growth improvements can be made by increasing the expansion steps to 6%–10% and by reducing the static culture periods before and after applying the expansion protocol; ongoing studies along these lines have delivered promising results. Finally, the ultimate goal is to directly seed P0 hMSC obtained from biopsy onto HESR culture to optimize even the initial and probably most sensitive phase of hMSC growth. However, because P1 culture on tissue culture plastic is used to removed hematopoietic "contaminants" from the hMSC population, we decided to keep this standard procedure in our current study. Future studies will have to evaluate whether the HESR surface similarly selects hMSC over other cell types for survival and growth.

A number of other techniques have been proposed to achieve higher cell yields by providing hMSC with a proliferation-promoting environmental factor. MSC were shown to increase proliferation in response to cyclic stretch [46], low oxygen pressure [47], specific ECM [48], and growth in bioreactors [49, 50]. A frequently encountered problem of these methods and of classic culture is the loss of MSC stemness and pluripotency [32, 51]. Part of this problem arises from the passaging process itself, which includes degradation of cell surface proteins with enzymes such as trypsin [31–33]. This harmful treatment is reduced by a factor of 3 in our culture system. Another reason for the loss of hMSC pluripotency is that the growth-promoting condition often induces a particular cell lineage; for example, application of cyclic stretch increases hMSC proliferation but also upregulates smooth muscle markers [52].

The question arises of why the mechanical stretch applied to hMSC in our HESR culture does not induce unwanted hMSC differentiation despite the known effects of stretch on these cells and other cell types [40, 52]. In fact, culture in our expanded HESR dishes maintains the multipotent stem cell phenotype and does not upregulate markers of any distinct lineage. One explanation is the very low frequency of stretch application (every 6 hours) compared with standard frequencies used in stretch experiments (1 per minute to 1 per second) [40, 52]. Another possibility is the much lower stiffness of our HESR (10–20 kPa) compared with conventional PDMS membranes (~3 MPa). Low substrate stiffness can equally explain the downregulation of ECM protein mRNA during HESR culture. In addition, substrate stiffness has been shown to drive cell differentiation in general [52, 53] and to determine hMSC fate in particular, then even overriding the effect of lineage-committing growth factors [39]. From these findings of Engler et al. [39], one could expect that hMSC differentiation is modulated by the increasing substrate stiffness occurring with HESR dish expansion from ~9 kPa when relaxed to ~23 kPa when fully expanded. However, the naïve stem cell character was maintained in the HESR culture. One explanation for this apparent discrepancy may be the fact that substrate stiffness of the HESR dish increases in a gradual fashion, whereas Engler et al. provided static culture substrates of one defined stiffness [39]. It is thus conceivable that the dynamic nature of our experiments generates more complex mechanical signals that are integrated differently by the cells.

Mechanical influence of the substrate may also underlie the finding that growth on expanded HESR suppresses expression of α-SMA, a cytoskeletal marker and functional protein for the development of fibrogenic myofibroblasts [43]. We have previously achieved a similar suppression of myofibroblast development from fibroblastic cells by growth on similarly compliant polymer substrates [34]. Prevention of myofibroblast development from hMSC has important implications for therapeutic use of cultured hMSC. Cells of myofibroblastic character display augmented collagen synthesis and elevated contractile activity; injecting such fibrogenic cells into damaged tissue or the bloodstream not only may fail to restore tissue function but could contribute to undesired contracture and fibrosis [43]. This view is supported by recent studies showing that MSC turn into fibrogenic myofibroblasts when cultured in a dermal equivalent model [54], after being systemically transplanted into immunodeficient mice with acute liver injury and fibrosis [44], and when engrafting in a mouse model of chronic lung fibrosis [55].

The goals of hMSC culture are not only to produce high cell numbers of undifferentiated cells but also to be able to differentiate them into the desired cells for tissue repair. We examined this capacity by driving hMSC obtained from HESR culture into adipogenic, chondrogenic, osteogenic, and myogenic lineages. To the current standards of available lineage markers [2, 21, 33, 35], hMSC from 9 weeks of HESR culture displayed the same high pluripotency as hMSC taken from 3 weeks of conventional culture. Concerning myogenic differentiation, we were even able to induce the late differentiation marker smooth muscle myosin heavy chain [21] despite the fact that myogenesis was low on the mRNA expression level. It is conceivable that this was due to the advanced smooth muscle differentiation.

CONCLUSION

Our novel culture methods represent an efficient method by which to expand multipotent hMSC for cell therapeutic purposes. Moreover, these methods have potential application for a

wide range of adherent cells relevant for tissue repair. By coating the HESR dish with different ECM molecules and by adapting the expansion protocol, we are currently optimizing our method for fast growth of human dermal fibroblasts and epidermal keratinocytes, pivotal cells for allogenic skin repair [45]. Although we did not explore this potential here, the HESR dish and iris-like expansion device are also applicable for cell mechano-stimulation, with a much wider range of stretch possibilities than achieved with systems based on PDMS culture surfaces. It is also conceivable to combine high-frequency stretch protocols that have been shown to increase cell proliferation [46] with low-frequency surface expansions to further amplify cell growth rates. Hence, these expanded HESR culture methods meet the needs of both clinical and fundamental research applications.

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DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicate no potential conflicts of interest.

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