Development of a human tissue engineered hypertrophic scar model

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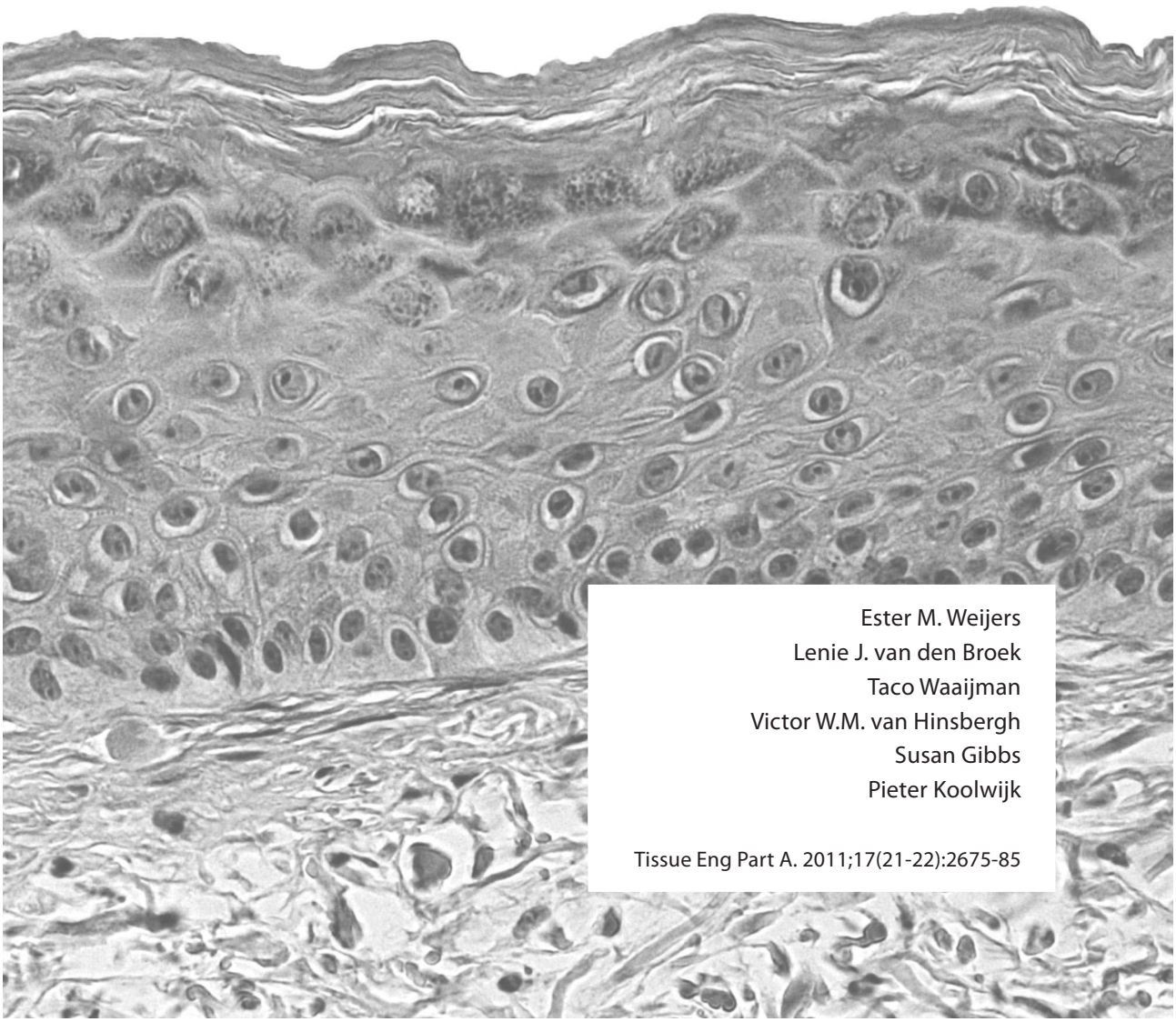
Chapter 5

The influence of hypoxia and fibrinogen variants on the expansion and differentiation of adipose tissue-derived mesenchymal stem cells



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ABSTRACT

Upon implantation of tissue-engineered scaffolds, hypoxia will occur until neovascularization takes place. *In vivo*, the temporary fibrin matrix forms a suitable matrix for this process and fibrin variants can influence the extent of neovascularization. In this study, the influence of oxygen tension and naturally occurring fibrinogen variants on adipose tissue-derived mesenchymal stem cell (ASC) expansion and differentiation were determined. ASC proliferated 1.7-fold faster in 1% oxygen, showed reduced cell aging and their stemness was preserved. The stem cell surface marker expression was similar in 1% and 20% oxygen. The various fibrinogen coatings did not influence ASC expansion and differentiation. Differentiation of ASC towards adipogenic and osteogenic lineages was improved in 20% oxygen, whereas 1% oxygen improved chondrogenic differentiation. In conclusion, optimal oxygen concentrations vary for the intended ASC application, and fibrinogen variants, which can be used to influence neovascularization, do not alter ASC behavior. These data emphasize the importance of oxygen concentrations during stem cell growth and differentiation.

INTRODUCTION

Mesenchymal stem cells can be isolated from bone marrow (BM-MSC), but their retrieval is invasive and low numbers of stem cells are obtained[1,2]. In contrast, adipose tissue is a more easily accessible source for stem cells with high proliferative capacity and multilineage potential. In adipose tissue, mesenchymal stem cells (ASC) are abundantly present in the stromal vascular fraction (SVF), as first described by Zuk et al. in 2001[3,4]. The SVF contains >85% ASC-like cells that adhere to culture plastic. Contaminating cells are low numbers of leukocytes, endothelial cells, vascular smooth muscle cells and pericytes[2]. Relatively homogeneous ASC populations can be obtained via adherence to culture plastic and *in vitro* expansion[5,6]. Freshly isolated ASC were shown to be positive for the surface markers CD34, CD54, CD73, CD90, CD105 and CD166 and negative for CD31, CD45, CD106 and CD146[2,6-8]. *In vitro* culture in conventional conditions changes the surface marker expression on the cells; ASC lose CD34, CD117 and HLA-DR expression, whereas the expression of CD90, CD105 and CD166 increases[2,6,8]. Cultured ASC were shown to maintain their capacity to differentiate into osteogenic, chondrogenic, myogenic, neuronal and cardiomyocytic lineages[3,7,9]. Due to the availability and wide range of applications, ASC have great potential within the field of tissue engineering.

Routinely, ASC are cultured in ambient atmospheric conditions at ~20% oxygen, which is not a physiological environment for cells. The oxygen levels in vascularized tissues ranges up to 5%[10] and presumably mesenchymal stem cells reside *in vivo* in a locally hypoxic niche[11,12]. Increasing evidence favors hypoxic conditions during stem cell expansion[13]. Embryonic stem cells maintain full pluripotency in hypoxic culture conditions[14]. The growth rate of human ASC increases in hypoxic (1-5% O_2) conditions, while preserving stemness[15,16]. Pre-adipocytes showed a differentiation arrest in hypoxic conditions without altering the adipogenic potential of the progenitor cells, as demonstrated by Lin et al[17]. Literature suggests that the expansion of human ASC is supported by hypoxic oxygen levels, whereas their differentiation is likely to be reduced in hypoxic culture conditions[10,18], but at this moment no consensus has been reached[15,19-22]. Particularly the effects regarding hypoxia on ASC differentiation towards chondrogenic lineages are inconclusive[19,22]. The ability of murine ASC to differentiate towards chondrogenic and osteogenic lineages decreases when cultured in 2% oxygen[19]. While expansion of murine ASC in 2% oxygen also was described to enhance chondrogenesis[22]. It is important for tissue engineering that this is clarified, since upon implantation the scaffold will soon become a hypoxic environment until neovascularization occurs.

In vivo, fibrin forms a suitable temporary extracellular matrix for neovascularization[23]. Previously, we showed that naturally occurring fibrinogen variants alter functional and molecular mechanisms of endothelial cells[24]. High molecular weight (HMW) fibrin increases neovascularization *in vitro* and *in vivo*, whereas low molecular weight (LMW)

fibrin diminishes this process[25]. The question arises whether the naturally occurring fibrinogen variants alter mesenchymal stem cell expansion and differentiation. In the present study, the influence of oxygen tension and fibrinogen variants on the expansion and differentiation of human ASC was investigated. Conventional oxygen conditions (20%) were compared with 1% hypoxic oxygen conditions since 1% oxygen represents the environment of the stem cell niche and in particular the severe hypoxic state of non-vascularized implanted scaffolds.

MATERIALS AND METHODS

Isolation and culture of ASC

Human adult skin was obtained from healthy female donors, age 30-60 years (with written informed consent) undergoing abdominal dermolipectomy and was used directly after surgery. The study was conducted according to the Declaration of Helsinki Principles. Adipose tissue-derived stem cells were isolated as described by Kroeze et al[8]. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) medium, containing, 1% UltroserG, 2 mM L-glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin (all Gibco, Invitrogen, Breda, The Netherlands) and maintained at 20% O_2, 5% CO_2, 37°C or at hypoxic culture conditions at 1% O_2, 5% CO_2, 37°C in humidified incubators. The culture medium was replaced twice a week. When 90% confluence was reached, cells were detached using 0.05% trypsin and seeded in 1:5 ratios.

Hypoxic cell culture

Hypoxic cell culture conditions were maintained inside a custom designed hypoxic workstation (T.C.P.S., Rotselaar, Belgium), with a CO_2 and O_2 controlled, humidified incubator (Sanyo, Etten-leur, The Netherlands), placed inside a T4 glovebox (Jacomex, Dagneux, France) equipped with an O2X1 oxygen transmitter (GE Panametrics, Billerica, USA). The oxygen concentration inside the incubator was continuously monitored with an internal zirconia sensor and periodically checked with O_2 test tubes (Drager Safety, Zoetermeer, The Netherlands). To prevent re-oxygenation during hypoxic culture, all media and buffers were pre-incubated for 4h before use.

Fibrinogen coating

Human plasma fibrinogen (Calbiochem, La Jolla, USA) was prepared by dissolving fibrinogen in 0.05 M Tris/HCl pH7.5 and dialysis to phosphate buffered saline (PBS). Fibrinogen isolated from human plasma is defined as unfractionated-fibrinogen and contains HMW- and LMW-fractions. The HMW- and LMW-fractions of plasma fibrinogen were purified using $(NH_4)_2SO_4$ precipitation, as previously described[24]. Fibrinogen

coatings were made on polystyrene culture plates (Costar, Corning, Amsterdam, The Netherlands) using 100 µl/cm^2 with a concentration of 0.2 µM fibrinogen in PBS. Molar concentrations were used to correct for molecular weight differences of the fibrinogen fractions. Fibrinogen coating was performed 1h at 37°C followed by one wash with PBS, resulting in fibrinogen immobilization on plastic.

Proliferation

The growth rate of ASC was determined after seeding 3x10^4 cells/cm^2 on non-coated and unfractionated-, HMW-, and LMW-fibrinogen coated culture plates in 20% and 1% oxygen conditions. Freshly isolated ASC from three different donors were cultured separately in normal culture medium with triplicate wells for each donor. After 2, 5, 8 and 9 days phase contrast pictures were taken with a Qimaging camera on a Zeiss microscope connected to a computer with Optimas image analysis software (Media Cybernetics, Bethesda, USA) and the cell number (>500 cells per condition) in a total area of 25 mm^2 was counted manually after blinded randomization by two independent scientists. In the hypoxic workstation, the camera was attached to the phase contrast microscope, and a similar procedure was followed to determine the ASC proliferation.

Telomere length

The telomere length of ASC from three different donors after 21 days of culture (from passage 0 to passage 3) in 20% and 1% oxygen conditions was determined by Quantitative Real-Time Polymerase Chain Reaction (QPCR), as previously described by Cawthon et al.[26]. DNA was isolated using DNeasy kit (Qiagen, Venlo, The Netherlands). Oligonucleotides for single copy gene (36B4) and telomeres, as specified in literature[26], were synthesized by Invitrogen. The sequences are given in Table 1. QPCR was performed in duplicate wells using SYBR Green in an ABI 7500 sequence detection system (Applied Biosystems, Foster City, USA). The Ct-values for the telomeres and 36B4 in DNA from cultured ASC were by using four different DNA concentrations (0.8, 1.5, 2.7 and 5.0 ng/µl). The relative telomere length was determined using the T/S ratio with the calculation $[2^{Ct\ (telomeres)}/2^{Ct\ (single\ copy\ gene)}]^{-1}=2^{\Delta Ct}$.

Flow cytometry

Freshly isolated and separately cultured ASC from four different donors were used to determine the surface marker expression by flow cytometry. The surface marker expression was measured on FACScan (Becton Dickinson Immunocytometry Systems, Mountain View, USA). Antibodies and corresponding IgG1 isotype controls were from BD Pharming (San Diego, USA) unless stated otherwise; CD31 (clone WM59), CD34 (clone 581), CD54 (clone HA58), CD90 (clone 5E10), CD105 (clone SN6, Caltag, Buckingham, UK) and CD166 (clone 3A6), as described by Kroeze et al[8]. The MFI represents the

Table 1. Oligonucleotide sequences of primers used for Quantitative Real-Time Polymerase Chain Reaction Analyses.

Marker	NCBI	Forward primer	Reverse primer
Single copy gene[26]	–	CAGCAAGTGGGAAGGTGTAATCC	CCCATTCTATCATCAACGGGTACAA
Telomere[26]	–	GGTTTTTGAGGGTGAGGGTGAGG GTGAGGGTGAGGGT	TCCCGACTATCCCTATCCCTATCC CTATCCCTATCCCTA
GUSB	NM_000181	ATCCACCTCTGATGTTCACTGAAG	GCACTCTCGTCGGTGACTGTT
LPL[28]	NM_000237	GAGATTTCTCTGTATGGCACC	CTGCAAATGAGACACTTTCTC
ADPN	NM_004797	CCTGTCCACAACAAACTCTTAATGC	CCACCCCAACCAGATGTGA
PPARγ2	NM_015869	CGACCAGCTGAATCCAGAGT	GATGCGGATGGCCACCTCTT
OPN	NM_001040058	CAGCCTTCTCAGCCAAACG	TAACTGGTATGGCACAGGTGATG
ALP[28]	NM_000487	TCAGAAGCTCAACACCAACG	GTCAGGGACCTGGGCATT
SOX9[27]	NM_000346	CCCAACGCCATCTTCAAGG	CTGCTCAGCTCGCCGATGT
COL2A1[27]	NM_001844	GGATGGGCAGAGGTATAATG	GGTCCTTTGGGTCCTACAA

ADPN, adiponectin; *ALP*, alkaline phosphatase; *COL2A1*, collagen 2A1; *GUSB*, β-glucuronidase; *LPL*, lipoprotein lipase; *NBCI*, National Center for Biotechnology Information; *OPN*, osteopontin; *PPAR*, peroxisome proliferator-activated receptor

mean fluorescent intensity that is calculated by: MFI marker - MFI IgG control. The data were gated for viable cells using forward and side scattering during FACS analysis. Cells smaller than 150 FSC-H were considered as non-viable cells or debris and were excluded. No additional gates were applied in the figures and 10,000 events were counted in total. For the analysis of the surface marker expression the positive cells were gated in R2 (indicated in Figure 4).

Differentiation capacity

Osteogenic, chondrogenic and adipogenic lineage differentiation of cultured ASC was studied both in 20% and 1% oxygen on various coatings. Four different donors were used and all experimental conditions were donor matched. Prior to the differentiation, the cells were expanded in corresponding culture conditions for 21 days with respect to coating and oxygen concentrations. This resulted in confluent ASC in passage 3. Differentiation was induced during 21 days in specific differentiation media.

For adipogenic differentiation ASC passage 4 were seeded in a density of $2x10^4$ cells/cm^2 in adipogenic medium, consisting of normal culture medium, supplemented with 0.5 mM 3-isobutyl-1-methylxanthine (IBMX), 1 µM dexamethasone and 200 µM indomethacin. The adipogenic differentiation potential was evaluated by histochemistry using Oil Red O staining of lipid vacuoles and mRNA expression of adiponectin (ADPN) and lipoprotein lipase (LPL). Quantification of the adipogenic differentiation was performed by measuring the intensity of the red color of ASC stained with Oil Red O, using Nikon NIS Elements Software version AR3.1 (Melville, NY, USA).

For osteogenic differentiation ASC passage 4 were seeded in a density of $5x10^3$ cells/cm^2 in osteogenic medium, consisting of normal culture medium, supplemented with 10 mM β-glycerol phosphate, 50 µg/ml ascorbic acid and 100 ng/ml bone morphogenetic protein-2 (Peprotech EC LTD, London, UK). The osteogenic differentiation capacity was evaluated by histochemistry using Von Kossa staining of calcified matrix and mRNA expression of alkaline phosphatase (ALP) and osteopontin (OPN). Quantification of the osteogenic differentiation was performed by manual counting of the calcified areas of ASC stained with Von Kossa.

The chondrogenic differentiation was induced in ASC passage 4 using chondrogenic medium and the micromass culture method[19] consisting of $4x10^5$ cells. Chondrogenic medium consisted of DMEM, supplemented with penicillin-streptomycin, 0.2% UltroserG, ITS+ premix (BD, USA), 20 ng/ml transforming growth factor β1 (TGF-β1, Biovision) and 25 µM ascorbate-2-phosphate (Sigma). The chondrogenic differentiation potential was evaluated by histochemistry using Alcian blue staining of proteoglycans and mRNA expression of SOX9 and Collagen 2A1 (COL2A1). Quantification of the chondrogenic differentiation was performed by measuring the intensity of the blue color of ASC stained with Alcian blue, using Nikon NIS Elements Software version AR3.1.

Quantitative Real-time PCR

RNA isolation of ASC from four different donors was performed using the RNeasy Mini kit (Qiagen, Venlo, The Netherlands). Copy DNA was synthesized using the Cloned AMV First Strand cDNA Synthesis Kit from Invitrogen with poly(T)primers. Oligonucleotides were designed or specified in literature[27,28] and synthesized by Invitrogen, the sequences are given in Table 1. Primer specificity was tested by homology search with the human genome (BLAST) and confirmed by dissociation curve analysis. QPCR was performed in duplicate wells using SYBR Green in an ABI 7500 sequence detection system (Applied Biosystems, Foster City, USA). β-glucuronidase (GUSB) and β-2-microglobulin were used as endogenous reference genes based on previous studies[29]. Relative expression levels of target genes were calculated with the housekeeping gene GUSB with the comparative Ct method, as previously described[30].

Statistical analysis

Statistical analyses were performed using paired Student t-test or two-way ANOVA with Bonferroni post-hoc test. Numbers of replicates and significant P-values are indicated in the text of figures, $p<0.05$ was considered significant. The results are given as mean ± SEM.

RESULTS

ASC expansion

The optimal expansion condition of three ASC donor isolations was determined in conventional (20% O_2) and hypoxic (1% O_2) conditions on non-coated culture plastic and various fibrinogen coatings. ASC proliferation was shown to be 1.7-fold higher in 1% oxygen compared to cultures grown in 20% oxygen ($P<0.05$, Figure 1a). The number of population doublings during passage 1 was 6.3±0.9 in 1% oxygen compared to 4.1±0.9 in 20% oxygen. The cell morphology of ASC was similar in both culture conditions (Figure 1b). ASC cultured on unfractionated-, HMW- or LMW-fibrinogen coatings and non-coated plastic, showed no significant differences in the growth rate neither in 20% nor in 1% oxygen culture conditions (Figure 2a,b).

Expansion of ASC for 21 days in 20% oxygen led to a significant decrease in T/S ratio compared to 1% oxygen (Figure 3a). The T/S ratio represents the relative length of the telomeres. On average the telomeres were 1.5-fold shorter in ASC cultured in 20% oxygen compared to 1% oxygen. Moreover, the autofluorescence of ASC significantly increased during the three culture passages in 20% oxygen (from 2.5±0.2 to 20.7±4.8, $P<0.05$, Figure 3b,c). ASC expansion in 1% oxygen largely prevented the increase in autofluorescence of the cells (from 2.5±0.2 to 3.8±0.3, not significant). Taken together, these results show that, despite the higher population doublings, culture in 1% oxygen suppresses ASC aging.

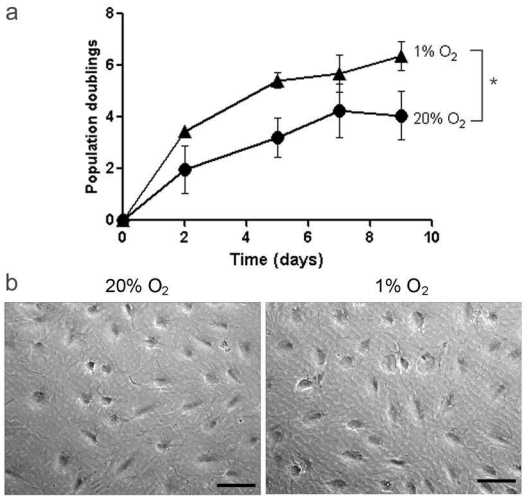

Figure 1. Expansion of ASC in 20% and 1% oxygen. (**a**) The ASC proliferation in 20% oxygen (circles) and 1% oxygen (triangles) differs significantly (n=3, ANOVA: *$P<0.01$). (**b**) Representative pictures of ASC morphology on phase contrast images of ASC in passage 0 on day 7 during expansion in 20% and 1% oxygen. The bars indicated in the figures represent 100 µm.

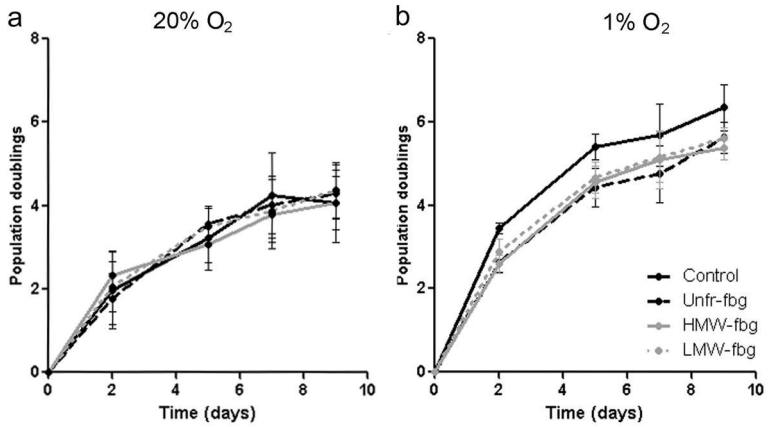

Figure 2. Expansion of ASC on non-coated and fibrinogen-coated plastic. The ASC proliferation in 20% oxygen (**a**) and 1% oxygen (**b**) are given. The black solid line represents non-coated plastic, black striped line represents unfractionated-fbg, grey solid line represents HMW-fbg and the grey dotted line represents LMW-fbg (n=3, ANOVA: NS).

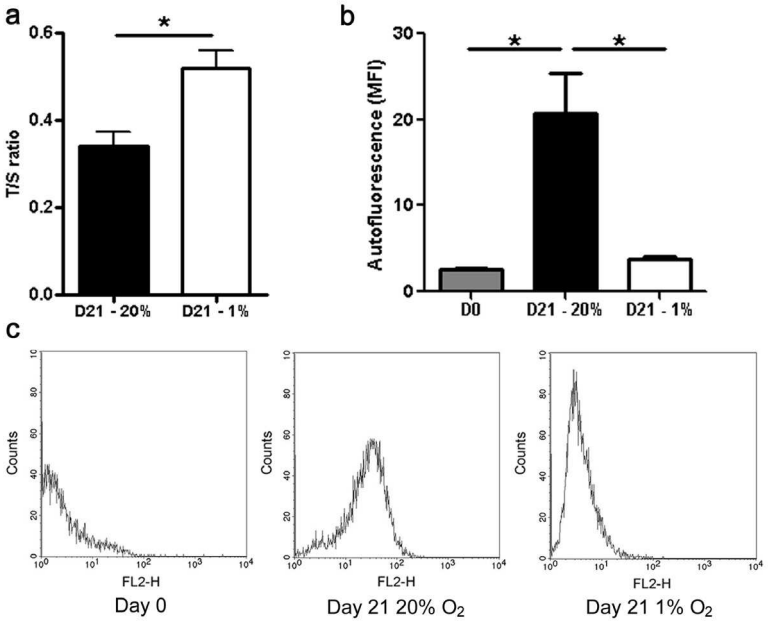

Figure 3. ASC aging during expansion in 20% and 1% oxygen conditions. (**a**) The relative telomere length (T/S ratio) was determined after 21 days expansion in 20% and 1% oxygen conditions (n=3, T-test: P<0.05). (**b**) Mean fluorescent intensity and (**c**) histoplots showing the autofluorescence of non-stained ASC was determined by FACS analysis, directly after isolation and after expansion. Mean fluorescent intensity (MFI) = MFI marker – MGI IgG control of experiments with 4 independent donors (ANOVA: *P<0.05). Grey bars represent ASC directly after isolation (D0), filled bars represent ASC cultured for 21 days in 20% oxygen (D21 – 20%) and open bars represent ASC cultured for 21 days in 1% oxygen (D21 – 1%). Note: Throughout the manuscript the same fill pattern of the bars is used.

Table 2. Percentage of stem cell surface-marker positive cells after expansion in 20% and 1% oxygen.

Culture condition		% of cells expressing				
Day	**Oxygen**	**CD34**	**CD54**	**CD90**	**CD105**	**CD166**
0	NA	65.2±4.7	80.4±6.0	83.8±3.5	62.5±7.9	18.6±2.4
21	20%	90.4±2.2	94.1±2.7	99.9±0.04	99.9±0.02	93.1±3.0
21	1%	98.2±0.8	99.7±0.1	99.6±0.2	99.9±0.04	97.6±1.1

The results are given as percentage of cells with fluorescence above background Values were calculated as indicated for CD34 in Figure 4 for 4 independent donors. NA, not applicable

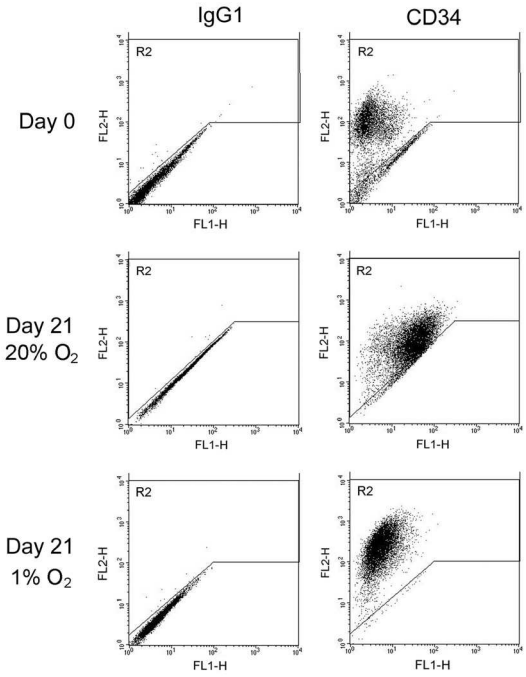

Figure 4. FACS dot-plots of ASC stained with IgG1-isotype control and CD34. FACS dot-plots of one representative donor are shown with control and CD34 staining. Similar FACS settings were used for all analyses. The data are representative for 4 independent experiments with different donors, the mean and SEM are shown in Table 2.

The maintenance of the stem cell phenotype during expansion was determined using standard stem cell markers in flow cytometry analyses. Directly after ASC isolation, the stem cell and hematopoietic lineage marker CD34 was present on a subset of cells (65%, Table 2 and Figure 4). During 21 days culture in 20% and 1% oxygen, the percentage CD34 positive cells increased to 90 and 98%, respectively. However, it should be noted that whereas the high fluorescent intensity of CD34 was maintained for ASC cultured in 1% oxygen, the fluorescent intensity of CD34 on ASC cultured in 20% oxygen clearly decreased (Figure 4 and 5). In addition to CD34, the subsets of cells expressing the sur-

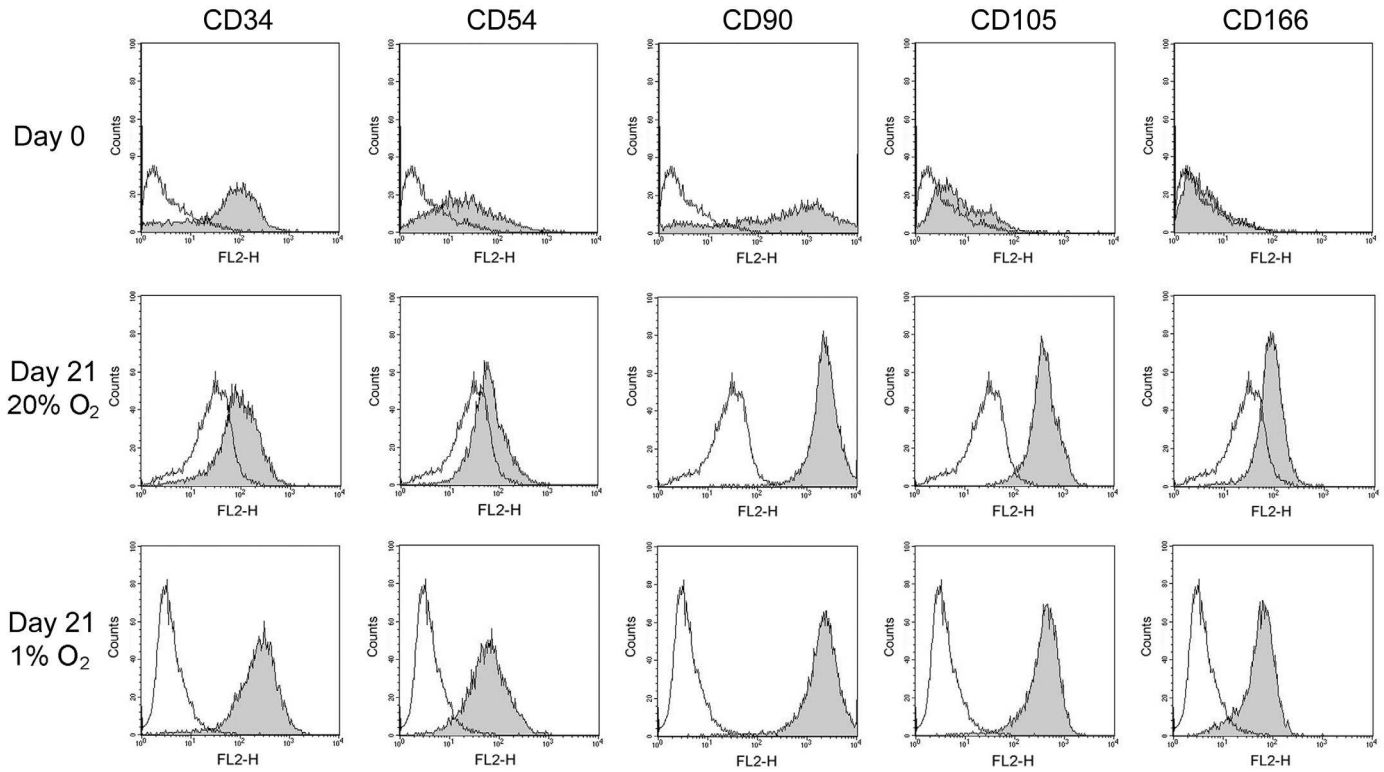

Figure 5. ASC surface-marker expression during expansion in 20% and 1% oxygen conditions. The stem-cell marker CD34, CD54, CD90, CD105 and CD166 expression of ASC were determined with FACS analyses after 21 days of expansion. Histoplots of one donor are shown, and are representative for 4 independent donors. *In vitro* culture of ASC enhances the surface expression of CD105 and CD166[8]. Surface marker (grey) and its IgG1-isotype control (open) are shown (n=4).

face markers CD54 (ICAM-1), CD90 (Thy-1), CD105 (endoglyn) and CD166 (ALCAM) also increased during 21 days culture to >90% positive cells in 20% and 1% oxygen (Table 2). Similar to CD34, the fluorescent intensity of CD54 increased slightly after culture in 1% oxygen. However, the fluorescent intensity of CD90, CD105 and CD166 did not differ on ASC in 20% and 1% oxygen, for these surface markers the MFI increased to similar extents (Figure 5). All cultured ASC were negative for the endothelial marker CD31 on day 21 (data not shown).

In addition to hypoxia, the influence of the matrix on the maintenance of the stem cell phenotype was investigated. As described above, the proliferation and aging of ASC was similar on the different fibrinogen coatings. In addition, isolated ASC were cultured for 21 days on non-coated and unfractionated-, HMW-, and LMW-fibrinogen coated wells. None of the fibrinogen forms influenced the expression of the stem cell surface markers of ASC as shown for CD34, CD90 and CD166 (Figure 6), neither in 20% nor in 1% oxygen. The stemness of ASC remained unchanged by culture on various fibrinogen coatings.

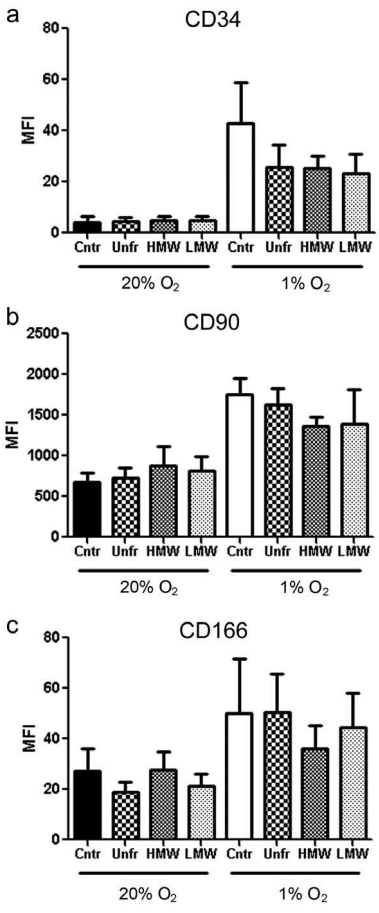

Figure 6. ASC surface-marker expression during expansion on non-coated plastic and fibrinogen coatings in 20% and 1% oxygen conditions. The expression was determined by FACS analysis directly after cell isolation (before attachment on day 0) and after 21 days of expansion. The mean fluorescence intensity (MFI) compared to isotype-control are given of CD34 (**a**), CD90 (**b**) and CD166 (**c**) (n=4). Mean fluorescent intensity (MFI) = MFI marker – MFI IgG control of 4 independent experiments with different donors.

ASC differentiation

Following the ASC expansion in 20% or 1% oxygen, the differentiation capacity towards adipogenic, osteogenic and chondrogenic lineages during 21 days was determined. After adipogenic differentiation, histochemical analysis using Oil Red O staining showed slightly more ASC differentiation in cells expanded and differentiated in 20% oxygen, when compared to their 1% counterparts (Figure 7a). Quantification of the staining intensity supported this observation (20% oxygen; 96.8 A.U. compared to 1% oxygen: 95.5 A.U., $p<0.05$). The increased adipogenic differentiation in 20% oxygen was confirmed using QPCR analysis of the adipogenic lineage-specific genes adiponectin (ADPN) and lipoprotein lipase (LPL). A 3600-fold higher ADPN expression and 330-fold higher LPL expression was observed when compared to undifferentiated cells (Figure 8a,b). The cells differentiated in 1% oxygen displayed less pronounced but increased gene expression of ADPN (468-fold) and LPL (25-fold), respectively.

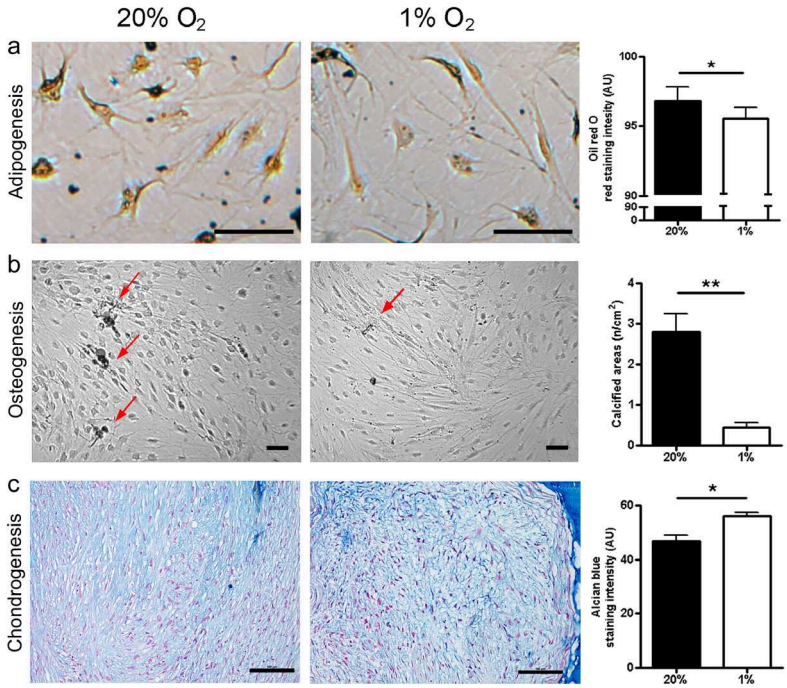

Figure 7. Immunohistochemical staining after 21 days ASC differentiation. (**a**) Lipid vacuoles specific for adipogenic differentiation were stained with Oil Red O. (**b**) Calcification specific for osteogenic differentiation was stained with Von Kossa (see arrows). (**c**) For chondrogenic differentiation the cells were cultured in micromass[19], proteoglycans were stained with alcian blue. Bars indicated in the figures represent 100 µm. Quantification of the stainings specific for differentiation towards adipogenic, osteogenic and chondrogenic lineages are shown in the graphs (n=4, T-test: *P<0.05, **P<0.001). One should consider that the stoichiometry of the immunohistochemical stainings is not always linear. Parallel cultures of non-differentiated cells did not show any signs of differentiation (data not shown).

The von Kossa staining of calcified matrix was used to determine osteogenic differentiation. After stimulation towards the osteogenic lineage, more calcification areas were observed in 20% oxygen than in 1% oxygen (Figure 7b). Quantification of the staining showed a 6.5-fold increase in number of calcification areas in 20% oxygen compared to 1% oxygen (P<0.001). The increased osteogenic differentiation in 20% oxygen was partially confirmed by QPCR of the osteogenic lineage-specific genes alkaline phospha-

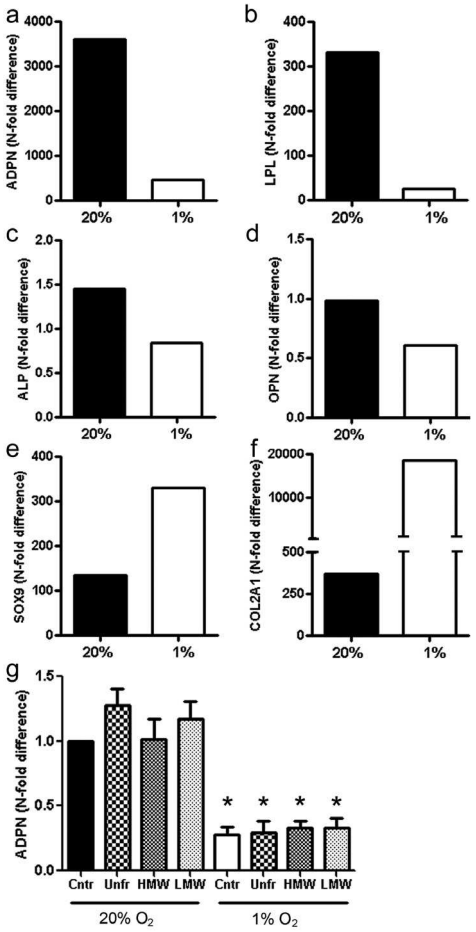

Figure 8. Gene expression data after 21 days ASC differentiation. The adipogenic differentiation (**a**; ADPN and **b**; LPL), osteogenic differentiation (**c**; ALP and **d**; OPN) and chondrogenic differentiation (**e**; SOX9 and **f**; COL2A1) as determined by gene expression (n=4). The graphs show the data of one representative ASC donor from 4 donors. The gene expression levels (**a-f**) were compared relative to those of non-differentiated ASC. (**g**) The adipogenic differentiation of ASC on non-coated plastic and fibrinogen coatings in 20% and 1% oxygen as determined by ADPN gene expression (n=4, T-test: *P<0.05 compared to the corresponding coating in 20% oxygen). The gene expression levels (**g**) were compared relative to those of differentiated ASC towards adipogenic lineage on non-coated plastic 20% oxygen to correct for inter-donor variability (1.0 = 3374±223 N-fold difference as compared to non-differentiated ASC (**a**)).

tase (ALP) and osteopontin (OPN). After osteogenic differentiation, 1.5-fold increased ALP expression, but no increased OPN expression was observed, when compared to undifferentiated cells (Figure 8c,d). ASC differentiated in 1% oxygen displayed a slightly decreased gene expression of ALP (0.8-fold) and OPN (0.6-fold) when compared to undifferentiated cells.

In contrast, proteoglycans produced by ASC during chondrogenic differentiation were more abundant in ASC exposed to 1% oxygen (Figure 7c). Quantification of the staining showed 9.3% more proteoglycan staining in the micromass cultured in 1% oxygen, as compared to 20% oxygen ($P<0.05$). Moreover, after differentiation the expression of the chondrogenic genes SOX9 and Collagen 2A1 (COL2A1) was clearly increased in 1% oxygen compared to 20% oxygen. The gene expression of SOX9 and COL2A1 in 1% oxygen was 331-fold and 18,560-fold higher than undifferentiated cells (Figure 8e,f). The ASC differentiated in 20% oxygen displayed less increased gene expressions of SOX9 (133-fold) and COL2A1 (370-fold).

Comparing the ASC differentiation potential on fibrinogen coatings to non-coated plastic, no differences were found with histology (data not shown). These data were confirmed by gene expression analysis of differentiation lineage-specific genes. Neither in 20% nor in 1% oxygen conditions significant differences were observed, as shown for adipogenic lineage-specific expression of adiponectin (Figure 8g). However, the oxygen induced effect on adipogenic ASC differentiation capacity was significant on all coatings ($P<0.05$, Figure 8g). The ASC differentiation on all three fibrinogen coatings proceeded to a similar extent as on the conventional non-coated conditions. This shows that unfractionated-, HMW- and LMW-fibrinogen coatings do not influence ASC differentiation towards the adipogenic, osteogenic and chondrogenic lineages.

DISCUSSION

In the present study, we provide evidence that the oxygen concentration during expansion and differentiation of adipose tissue-derived mesenchymal stem cells is important for maintenance of stem cell phenotype and commitment to differentiate. ASC proliferate faster in 1% oxygen compared to 20% oxygen, show reduced cell aging, and their stemness is preserved. Differentiation of ASC towards adipogenic and osteogenic lineages was favored in 20% oxygen, whereas chondrogenic differentiation was favored in 1% oxygen. Herewith, the knowledge on the effects of hypoxia on human ASC differentiation into three widely used lineages[6] is expanded and effects of hypoxia on chondrogenic differentiation are elucidated. Notably, the fibrinogen variants applied as coating did not influence ASC expansion and differentiation. These results represent the first findings regarding stem cell characteristics on different naturally occurring fi-

brinogen variants. The present study focuses on comparing ASC culture in conventional oxygen concentrations (20%) and severe hypoxic conditions (1%). This is of interest for two reasons; (I) the stem cell niche *in vivo* is described as a hypoxic area and (II) severe hypoxia will occur upon scaffold implantation.

Upon implantation in the body a tissue-engineered scaffold will soon become hypoxic. Since it is an aim within regenerative medicine to transplant stem cells within a scaffold, it is important to evaluate the influence of hypoxia on stem cell growth and differentiation. Whereas the effects of hypoxia on bone marrow-derived mesenchymal stem cells (BM-MSC) are convincingly demonstrated[11,31,32], ASC characteristics in hypoxia are not fully elucidated[15,19-22]. This is mainly due to the variability in ASC culture conditions, oxygen concentrations, duration of exposure and various differentiation lineages[9,33]. In our study, the oxygen concentration was chronically maintained at 20% or 1% oxygen, without re-oxygenation during media replacements and differentiation. In 1% oxygen, ASC showed increased growth rates, in line with findings of Carrancio et al., who found that BM-MSC expansion is faster in 5% oxygen[15]. Besides the lower ASC proliferation in 20% oxygen, two independent determinants of cell aging demonstrated an increased ASC aging in 20% oxygen, when compared to ASC in 1% oxygen. The telomere length (T/S ratio) was shown to be 1.5-fold shorter in ASC cultured in 20% oxygen. Moreover, the autofluorescence of ASC was enhanced during culture in 20% oxygen. Together, these findings demonstrate increased ASC aging during *in vitro* culture in conventional 20% oxygen conditions. Importantly, this increased aging during expansion was prevented by culturing ASC in hypoxic conditions. Our findings are in line with those of other investigators. Previously, enhanced proliferation and delayed cell aging in 1-3% oxygen was shown for BM-MSC and fibroblasts[31,32]. Aging of fibroblasts caused an intracellular accumulation of the aging pigment lipofuscin, that resulted in an increased autofluorescence of aged fibroblasts[34]. Moreover, ASC isolated from old mice show impaired proliferation and 3-fold shortening of the telomeres, when compared to their counterparts from young mice[18].

Optimal oxygen concentrations during stem cell differentiation are lineage dependent[10,17,22,35]. In the present study, three ASC differentiation lineages were investigated; osteogenic, adipogenic and chondrogenic, respectively. Differentiation towards adipogenic lineages and to a lesser degree towards osteogenic lineages was increased in 20% oxygen, when compared to 1% oxygen. This is in line with findings of Lin et al., who showed a differentiation arrest of pre-adipocytes towards the adipogenic lineage in 1% oxygen, potentially via the HIF-1 pathway[17]. In contrast to the adipogenic and osteogenic differentiation, we observed enhanced chondrogenic differentiation in 1% oxygen compared to 20% oxygen. Henderson et al. reported faster cartilage matrix formation by mature chondrocytes in 5% oxygen[35]. Moreover, Wang et al. showed increased cartilage matrix formation in 5% oxygen with ASC[21]. On the contrary, a markedly decreased chon-

drogenesis of ASC was shown by Malladi et al. employing 2% oxygen concentrations[19]. In the present study, positive effects of hypoxia on chondrogenic differentiation of ASC are demonstrated, this is in concordance with the native situation in cartilage which is a relatively avascular environment with low oxygen levels[21,35].

ASC isolated from subcutaneous fat show higher proliferation rates, but lower differentiation capacities than BM-MSC. Especially differentiation towards the osteogenic lineage is reduced[7]. In our study, indeed a relatively low osteogenic differentiation of ASC was found, both with Von Kossa staining and QPCR. Although the differentiation was not abundant, increased calcification and gene expression of ALP and OPN in 20% and 1% oxygen were demonstrated.

To avoid hypoxia in the implanted scaffold, neovascularization is important. We have previously shown that the HMW-fibrin variant increases neovascularization *in vitro* and *in vivo*, whereas LMW-fibrin inhibited this process[24,25]. Endothelial cells proliferate 52% faster on HMW-fibrinogen in comparison to cells cultured on LMW-fibrinogen coatings[24]. Importantly, our findings indicate that ASC can be combined with different fibrin scaffolds and that fibrin does not influence the potential of ASC to proliferate or differentiate, either in 20% or 1% oxygen. For well-vascularized tissues like bone, ASC in combination with HMW-fibrin scaffolds appear as a good option. When more avascular tissues, like cartilage are engineered, LMW-fibrin might be a good optional scaffold for ASC.

In conclusion, expansion in 1% oxygen conditions stimulates ASC growth, delays cell aging and preserves stemness. Moreover, the differentiation towards adipogenic and osteogenic lineages is improved in the hyperoxic environment (20% O_2), whereas chondrogenic differentiation proceeds better in hypoxic (1% O_2) conditions. The naturally occurring fibrinogen variants do not alter the ASC growth and differentiation. These findings explain disagreements found for ASC in hypoxic conditions and emphasize the importance of oxygen concentrations during stem cell expansion and differentiation.

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