DLK1(PREF1) is a negative regulator of adipogenesis in $CD105^+/CD90^+/CD34^+/CD31^-/FABP4^-$ adipose-derived stromal cells from subcutaneous abdominal fat pats of adult women

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Abstract

The main physiological function of adipose-derived stromal/progenitor cells (ASC) is to differentiate into adipocytes. ASC are most likely localized at perivascular sites in adipose tissues and retain the capacity to differentiate into multiple cell types. Although cell surface markers for ASC have been described, there is no complete consensus on the antigen expression pattern that will precisely define these cells. DLK1(PREF1) is an established marker for mouse adipocyte progenitors which inhibits adipogenesis. This suggests that DLK1(PREF1) could be a useful marker to characterize human ASC. The DLK1(PREF1) status of human ASC is however unknown. In the present study we isolated ASC from the heterogeneous stromal vascular fraction of subcutaneous abdominal fat pats of adult women. These cells were selected by their plastic adherence and expanded to passage 5. The ASC were characterized as relatively homogenous cell population with the capacity to differentiate *in vitro* into adipocytes, chondrocytes, and osteoblasts and the immunophenotype $CD105^{+}/CD90^{+}/CD34^{+}/CD31^{-}/FABP4^{-}$. The ASC were positive for DLK1(PREF1) which was well expressed in proliferating and density arrested cells but downregulated in the course of adipogenic differentiation. To investigate whether DLK1(PREF1) plays a role in the regulation of adipogenesis in these cells RNAi-mediated knockdown experiments were conducted. Knockdown of DLK1(PREF1) in differentiating ASC resulted in a significant increase of the expression of the adipogenic key regulator PPARγ2 and of the terminal adipogenic differentiation marker FABP4. We conclude that DLK1(PREF1) is well expressed in human ASC and acts as a negative regulator of adipogenesis. Moreover, DLK1(PREF1) could be a useful marker contributing to the characterization of human ASC.

Key words

Human adipose-derived stromal cells, Adipogenesis, ASC, CD31, CD34, CD90, CD105, DLK1(PREF1), FABP4, PPARγ2.

1.1 Introduction

White adipose tissues are the primary triglyceride/energy storages of the body acting as metabolic regulator in glucose homeostasis and lipid metabolism [1], and as endocrine organ [2]. The main cell type in fat tissues fulfilling these functions is the mature adipocyte. In adult humans approximately 10 % of fat cells in adipose depots are renewed annually [3], and surgical removal of fat tissue in rodents induces compensatory recovery [4]. This indicates a high demand of progenitor cells for renewal and, hence, maintenance of adipose tissue functions. In fact, the number of adipocyte progenitors in a given fat tissue can vary between 15–40 % of the cell population dependent on the tissue type, localization in the body, physiological conditions, and age [2, 5, 6].

White adipocyte progenitor cells were identified *in vivo* [7], and evidence provided by several studies indicates that these cells are responsible for regenerating adipocyte numbers in fat tissues [7-12]. In addition to adipocyte hypertrophy, an increased proliferation and adipogenic differentiation of ASC also contributes to the adipose tissue growth that is characteristic for obesity [12-14]. ASC reside most likely at perivascular sites in adipose tissues [15-17]. The number of intermediate stages between adipose progenitors and mature adipocytes is uncertain but once committed, ASC can enter an adipocyte differentiation program (adipogenesis) to acquire their specific functions as adipocytes [18-20]. Adipogenesis in human ASC involves growth arrest, early and terminal differentiation, including morphological changes, lipid accumulation and the expression of fat cell specific genes, for example fatty acid binding protein-4 (FABP4) and adipokines, such as leptin and adiponectin. The stages of adipocyte differentiation are orchestrated by a transcriptional cascade involving adipogenic keyfactors, such as the nuclear receptor peroxisome proliferator-activated receptor-γ2 (PPARγ2) and members of the CCAAT/enhancer-binding protein (C/EBP) family [18, 20].

Adipose-derived stromal cells (ASC) are most frequently isolated by collagenase digestion of white adipose tissue, yielding a stromal vascular fraction (SVF) which contains a mixture of heterogeneous cell types that are further purified by differential centrifugation, plating and plastic adherence [10, 11, 21]. It has been shown that a single adherent cell derived from the SVF can be expanded generating a daughter cell population with multipotent differentiation capacity, defined as an adult stem cell [21, 22]. As ASC possess no unique morphological characteristics great efforts are made to characterize this cell type by specific marker proteins. Although in many studies specific pattern of cellular surface markers were assigned to human ASC comprising the combination $CD90^+/CD105^+/CD34^+/CD31^-$ [15, 21, 23-28], there is no complete consensus on the antigen expression pattern that will precisely define human ASC. Moreover, evidence was presented that the immunophenotype of ASC can change during expansion in cell culture, likely influenced by adherence to plastic culture dishes, and proliferation in medium with high concentrations of fetal bovine serum (FBS), that contains variable and undefined factors influencing proliferation, differentiation and survival of adult stem cells [21, 28, 29].

Delta-like protein 1/Preadipocyte factor-1 (DLK1/PREF1), which was originally identified as an inhibitor of adipogenesis in the murine preadipocyte cell line NIH 3T3-L1 [30], is used as preadipocyte marker [7, 17, 30-32]. Similar to Notch, DLK1(PREF1) is an epidermal growth factor repeat containing transmembrane protein that is proteolytically converted at the extracellular domain to generate a physiologically active soluble form that inhibits adipocyte differentiation [33]. Adipocyte progenitors are the sole cell type expressing DLK1(PREF1) in mouse adipose tissues. DLK1(PREF1) knockout mice show accelerated fat deposition, whereas mice overexpressing DLK1(PREF1) in adipose tissue exhibit decreased fat mass [12, 33-36]. DLK1(PREF1) was shown to play a role in the regulation of cell commitment and differentiation in murine multipotent mesenchymal cells, regulating fat mass and bone mass formation in vivo [34, 36-39]. DLK1(PREF1) can also stimulate bone resorption in mice [38]. DLK1(PREF1)

suppresses C/EBPβ and C/EBPδ gene expression in mouse preadipocytes which leads to prevention of adipogenesis [37]. Moreover, DLK1(PREF1) can direct murine multipotent mesenchymal cells to the chondrogenic lineage but it inhibits differentiation into osteoblasts as well as chondrocytes [37, 40, 41]. DLK1(PREF1) was also shown to be expressed in human bone marrow-derived (BM) mesenchymal stem cells (MSC), but at relatively low levels [38, 42-44]. It keeps these cells in an undifferentiated state and regulates their differentiation into osteoblasts or adipocytes, at least in part by influencing their microenvironment composition [38, 42-44]. Low expression of DLK1(PREF1) was also found in human cord blood (CB) MSC correlating with strong adipogenic differentiation [45]. Moreover, DLK1(PREF1) overexpression inhibits adipogenesis as well as osteogenesis in CB MSC [45]. Together these data suggest that DLK1(PREF1) is an important regulator of mesenchymal stem cell differentiation. Thus DLK1(PREF1) could be a useful marker to characterize human ASC, however, the DLK1(PREF1) status of these cells is unknown.

In the present study, we isolated defined populations of ASC from human abdominal subcutaneous fat tissue derived from 4 different women. The immunophenotype and the *in vitro* differentiation capacity of these cells was analysed after expansion in medium with low concentrations of FBS until passage 5. Moreover, the significance of DLK1(PREF1) for adipogenic differentiation of these cells was studied.

1.2 Material and methods

1.2.1 Subjects

Subcutaneous adipose tissue pads (~100 g wet weight) were obtained by incision from four women undergoing elective plastic abdominal surgery. [46, 47] The age of the donors was between 30 and 47 years. The body mass index of the subjects was between 22 – 27 kg/m^2. All participants gave their informed written consent, and the protocol was approved by the ethical committee of Innsbruck Medical University, Austria.

1.2.2 Isolation of human adipose-derived stem cells

The adipose precursor cells were isolated essentially as described [10, 48]. Immediately after surgery adipose tissue biopsies were transferred into sterile serum-free ASC medium (DMEM/F-12 medium (1:1) with HEPES and L-Glutamine (Gibco, Vienna, Austria), supplemented with 33µM biotin, 17µM pantothenate and 12.5µg/ml gentamicin) and kept at 4°C for 1-3 hours before sterile processing in a laminar flow sterile work bench class II. After rinsing with PBS fibrous material and blood vessels were dissected. The tissue was cut into pieces (~ 1-2 mg) and digested in digestion buffer (PBS containing 200U/ml collagenase (CLS Type I, Worthington Biochemical Corp., Lakewood, NJ) and 2% w/v BSA) under stirring for 60 min at 37°C and 450 rpm; 1 mg adipose tissue/3 ml digestion buffer. The dispersed tissue was centrifuged for 10 min at 200 RCF. The floating adipocytes were aspirated and the sedimented stromal-vascular fraction (SVF) was suspended in erythrocyte lysis buffer (0.155 M NH_4CI, 5.7 mM K_2HPO_4, 0.1 mM EDTA, pH 7.3) and incubated for 10 min at room temperature. To remove tissue debris the cell suspension was filtered through a nylon mesh (pore size 100 µm). After another centrifugation step (10 min at 200 RCF) the pelletized SVF was suspended in ASC medium supplemented with 10% FBS (Gibco, Vienna, Austria), and filtered through a 35µm mesh to remove residual cell aggregates. The pelletized SVF was composed of single cells. Aliquots of cells were inoculated at a density of

70,000/cm^2 into 6-well plates. After 16 h of cell attachment, cells were washed with ASC medium to remove non-adhering material, mainly white blood cells and cell debris. Thereafter the almost confluent cells were maintained for 6 days in ASC medium without FBS. The medium was changed every second day. This led to the elimination of all non-adherent cells. The remaining cell population was referred to as the adipose-derived stromal cell (ASC) fraction which was used for further studies. This cell population contains an enriched population of adipose stem/progenitor cells although most likely not all of the cells are stem cells, as defined by rigorous criteria.

1.2.3 Cell culture

The ASC were expanded essentially as described [49]. Briefly the cells were washed with PBS and trypsinized using 0.5% trypsin-EDTA 1x (Gibco, Vienna, Austria). Trypsin was removed by centrifugation at 300 RCF for 5 min and the cells seeded in a density of 5000 cells/cm^2 in ASC medium plus 10% FBS and maintained at 37°C with 5% CO_2. 16h later the medium was replaced by PM4 medium (ASC medium containing 2.5% FBS, 10ng/ml EGF, 1ng/ml bFGF, 500ng/ml Insulin) [49]. ASC were passaged at a ratio of 1:2, medium was changed every third day and the cells were grown to 70% confluence before splitting. Population doublings (PDL) were calculated using the following equation: 1 PDL = $Log_{10}(N/N^0)$ x 3.33 (N = number of cells at the end of a passage, N^0 = number of cells that were seeded at the beginning of a passage). For storage cells were pelleted as described above, diluted in cryomedium (DMEM/F-12 medium (1:1) (1x) with HEPES and L-Glutamine (Gibco, Vienna, Austria), with 20% FBS and 7.5 % DMSO), at a density of 10^6 cells/ml, slowly brought to -80°C and then stored in frozen nitrogen. After thawing cryomedium was immediately removed by centrifugation.

1.2.4 Adipogenic differentiation

For the induction of adipogenesis the ASC were seeded at a density of 10,000 cells/cm^2 and grown till confluence for 6 days in PM4 medium. After a resting period of 48 hours in ASC medium adipogenesis was induced using differentiation medium, composed of 0.2 µM insulin (Roche, Vienna, Austria), 0.5mM IBMX, 0.25µM dexamethasone, 2.5 % FBS, 10µg/ml transferrin (Sigma Vienna, Austria) in ASC medium. After day 3 of differentiation the medium was changed and the cells were cultivated in differentiation medium without IBMX. For visualization of lipid droplets cells were fixed with 4% paraformaldehyde in PBS for 1h and stained with 0,3 % OilRedO (Sigma, Vienna, Austria) in isopropanol/water (60:40) for 1h. Final washing was carried out twice with distilled water.

1.2.5 Osteogenic differentiation

For induction of osteogenesis ASC were seeded at a density of 20,000 cells/cm^2 in ACS medium plus 10 % FBS. After 16h medium was replaced by osteogenic differentiation medium (STEMPRO Osteocyte/Chondrocyte Differentiation Basal Medium combined with STEMPRO Osteogenesis Supplement (Gibco, Vienna, Asutria)) and renewed every second day. After 21 days osteoblasts are fixed with 4% paraformaldehyde in PBS for 30 minutes, washed with PBS and stained with 2% Alizarin-Red-S (Sigma, Vienna Austria) for 20 minutes. Final washing procedure was carried out two times with PBS pH 4.0.

1.2.6 Chondrogenic differentiation

The ASC were seeded in 5 separate 5µl droplets of ASC medium plus 10% FBS, each containing 16,000 cells per 9.6 cm^2 well. After 2 hours each well was carefully filled with 2 ml of STEMPRO Osteocyte/Chondrocyte Differentiation Basal Medium combined with STEMPRO Chondrogenesis Supplement (Gibco, Vienna, Austria). Medium was replaced every second day. After 21 days chondrocytes were fixed with 4% paraformaldehyde (Sigma) in PBS for 30

minutes, washed with PBS and stained with 1% Alcian-Blue 8GX (Molekula, Taufkirchen, Germany) in 0.1N HCl for 30 minutes. Final washing was done three times with 0.1N HCl and two times with distilled water.

1.2.7 Cultivation of human foreskin fibroblasts, HUVEC, and PBMCs

Human foreskin fibroblasts and human umbilical vein endothelial cells (HUVEC) were isolated and cultivated essentially as described [50]. PBMCs were prepared and cultivated as described [51].

1.2.8 Quantitative RT-PCR

Total RNA was isolated with the RNeasy Micro Kit (Qiagen, Hilden, Germany), and cDNA synthesis was performed with the First Strand cDNA Synthesis Kit (Fermentas, St.Leon-Rot, Germany). Quantitative expression analysis was performed using the LightCycler® 480 Real-Time PCR System (Roche, Vienna, Austria). The mRNA quantification was performed using βactin or TATA binding protein (TBP) for normalization. Primers: The sequences, genbank accession numbers, and the base numbers of the primers within the sequence are indicated in table 1.

1.2.9 Immuofluorescence experiments

Immunofluorescence experiments were conducted as described [52]. Briefly, cells were fixed with 4% (w/v) PFA/1×PBS, permeabilized with 0.1% (w/v) Na-Citrate/0.2% (v/v) Triton-X-100, blocked with 1×PBS/1%BSA and incubated for 1 h at 37 °C with primary antibodies in 1× PBS/1%BSA. After washing in 1×PBS/1%BSA and staining with secondary IgGs, (DAKOCytomation, Hamburg), cells were processed for indirect immunofluorescence microscopy and viewed using a confocal laser-scanning system. Antibodies: Goat anti human

endoglin/CD105, C-20, (Santa Cruz, California, # sc-19790), mouse anti human CD31 (BD Pharmingen, Heidelberg, # 555446), mouse anti human CD34 (BD Pharmingen, Heidelberg, #555822), mouse anti human CD90 (BD Pharmingen, Heidelberg, #555596), rat anti mouse/human F4/80 (Biolegend, San Diego, #122615), goat anti human FABP4 C-15 (Santa Cruz, California, # sc-18661), mouse anti human MTS1/FSP1, X9-7 (Santa Cruz, California #sc-100784), mouse anti human DLK1 / PREF1 (R&D, Minneapolis, #MAB1144), mouse anti human VWF [F8/86] (Abcam, Cambridge ab855), mouse anti human calnexin, clone 37 (BD Pharmingen, Heidelberg, #610523), mouse anti rat GM130, clone 35 (BD Pharmingen, Heidelberg, #610822), anti mouse/rabbit/goat IgG FITC labeled (DAKOCytomation, Hamburg), anti mouse/goat IgG Alexa Fluor 555nm/488nm (Invitrogen, Lofer); TO-PRO-3 served as nucleic acid dye (Invitrogen, Lofer).

1.2.10 Westernblot analysis

Cell lysates were prepared in sample buffer (2% SDS, 6% glycerine, 0,1M DTT, 0,5% brom-phenol-blue, 62.5mM Tris-HCl pH 6.8), separated by SDS-PAGE and western blot analysis was performed essentially as described [53]. Antibodies: Mouse anti human βactin, clone AC-15 (Sigma, Missouri, #A5441), goat anti human FABP4, clone C-15, (Santa Cruz, California, # sc-18661), rat anti human DLK1 (PREF1) (AdipoGen, San Diego, # AG-25A-0091), anti mouse IgG HRP conjugate (Promega, Mannheim #W420B), donkey anti goat IgG-HRP (Promega, Mannheim #V805A), rabbit anti-rat IgG HRP (Dako Cytomation, Hamburg, #P0450).

1.2.11 Plasmids/ Lentiviruses

pMD2.G, psPAX2 and pLKO.1 TRC (sh control vector) (D. Trono # 12259, D. Trono #12260, Root #10879, Addgene, Cambridge, USA). The DLK1(PREF1) overexpressing lentivirus, pLentiDLK1(PREF1), was constructed inserting the full length human DLK1(PREF1) cDNA

from pCMV6-AC-DLK1(PREF1), NM_003836 **(**SC320741**)** (Origene, Rockville) into pLenti6/DEST (Invitrogen, Lofer, Austria) using the Gateway Lentiviral Cloning System, Invitrogen, Lofer, Austria. The correct DLK1(PREF1) cDNA sequence in plentiDLK1(PREF1) was verified by sequencing. DLK1(PREF1) shRNA lentiviruses: sh#2 and sh#3 (TRCN0000055979 and TRCN0000055980 of the shRNA set #RHS4533 for NM_003836, Open Biosystems, Vienna).

1.2.12 Retroviral gene expression system

5 different pLKO.1 lentiviral DLK1(PREF1) shRNA vectors for accession NM_003836 (#RHS4533, Open Biosystems, Vienna) were used for lentiviral particle production employing the ViraPowerTM Lentiviral Expression System (Invitrogen, Lofer, Austria) and a second-generation system provided by the Trono lab (Addgene, Cambridge, USA). The same procedure was used to generate virus particles of the DLK1(PREF1) overexpressing lentivirus pLentiDLK1(PREF1). The purified viral particles were stored at -80°C. For viral infection, 900,000 ASC were seeded in a 75-cm^2 bottle, with 8 ml medium, 6 µg/ml polybrene and virus particles at a multiplicity of infection (MOI) of 1:4. After 24 h, the medium was changed, and the cells were grown for another 48 h before they were trypsinized and seeded at 10,000 cells/cm^2 for subsequent differentiation. The efficiency of the DLK1(PREF1) knockdown was measured by qRT-PCR and Western blot analysis 4 days after infection or later. The most efficient knockdown clones for DLK1(PREF1) (sh#2 and sh#3) which were identified by qRT-PCR and Western blotting were used for further experiments.

1.2.13 Flow cytometry analysis

SVF and trypsinized early passage ASC, 250,000 cells per sample, were simultaneously fixed and permeabilized using BD Cytofix/Cytoperm™ solution to prepare the cells for intracellular and

surface marker staining. The cells were subjected to immunofluorescence staining using a panel of directly conjugated mouse monoclonal antibodies: CD31-FITC-A (WM59) #557508, CD34-PE-Cy7A (581) #560710, CD90-PE-A (5E10) #555596, CD105-PerCP-Cy5-5-A (266) #560819; BD Pharmingen, Heidelberg) For DLK1/PREF1 immunofluorescence staining a rat monoclonal anti human DLK1/PREF1 antibody (AG-25A-0091, Adipogen, San Diego) was used in combination with an anti-rat-APC-A antibody (BD Pharmingen, Heidelberg, #551019). The labeled cells were measured using a FACSCanto II (BD Biosciences) and the data were analyzed using the FACSDiva software (BD Biosciences).

1.3 Results

1.3.1 Immunophenotype of human ASC derived from abdominal subcutaneous fat pads

Fresh human abdominal subcutaneous fat pads obtained by surgical resection were processed for the isolation of the stromal-vascular cell fraction (SVF) (Supplemental Fig. 1A), essentially as described [10, 28, 48]. The heterogeneous SVF was maintained in ASC medium with 10% FBS for 16 hours to allow cell attachment on plastic cell culture dishes (passage -1, P-1). Subsequently the cells were kept in serum-free ASC medium for 6 days. Contaminating non adherent cells were removed by changing the medium. The resulting cell fraction was referred to as ASC passage 0 (P0). Under these conditions 230,000 +/- 100,000 SVF cells were isolated per gram fat tissue and 19 % +/- 6 % were recovered from the SVF as adherent ASC at P0. It should be considered that the cell yield per mg fat depends on the donor tissue. The ASC were expanded until passage 5 in PM4 medium [49] containing low concentrations of Insulin, EGF, FGF and 2.5% FBS in a 20% O_2/5% CO_2 atmosphere. Under these conditions the ASC proliferated robustly with a doubling time of ~ 60 hours. To identify potential heterogeneous cells, the adherent cell fraction was stained with antibodies against fibroblast specific protein 1 (FSP1), F4/80(EMR1), a macrophage marker, and von Willebrand factor (VWF), an endothelial cell specific protein (Supplemental Fig. 1B). The analysis of 10,000 cells for each marker by indirect immunofluorescence analysis viewed through a confocal laser-scanning microscope demonstrated that the vast majority of the ASC culture was free of fibroblasts, macrophages and endothelial cells. To underline these results, qRT-PCR was performed to show the mRNA expression of FSP1, F4/80, and VWF. F4/80 mRNA was not detectable in ASC compared to PBMC and monocytes (Supplemental Fig. 1C, upper panel). This was also found for VWF mRNA compared with HUVEC (Supplemental Fig.1C, middle panel). FSP1 mRNA was also relatively low in ASC relative to human foreskin fibroblasts (Supplemental Fig. 1C, lower panel). These data indicate that after isolation from the adipose SVF, 6 days FBS starvation on plastic cell culture dishes, and expansion until passage 5

in PM4 medium, we obtained a homogeneous cell population concerning the examined markers, although it is likely that there are other markers that are not homogeneously expressed. This cell population was referred to as the adipose-derived stromal cell (ASC) fraction which was used for further studies. This cell population contains an enriched population of adipose stem/progenitor cells but most likely not all of the cells are stem cells, as defined by rigorous criteria.

The population of ASC was further characterized in indirect immunofluorescence experiments by antibodies against established marker proteins used as major lineage determinant proteins (Supplemental Fig. 1D). CD90 (Thy1) and CD105 (endoglin) are known as mesenchymal stem cell (MSC) markers [54] which are also expressed in HUVEC cells [55] (Supplemental Fig. 1D). CD34 is not expressed in MSCs [56], but in both hematopoietic [57] and endothelial [58] progenitors (Supplemental Fig. 1D). CD31 (PECAM-1) is a marker for endothelial cells [54, 59] (Supplemental Fig. 1D). Our ASC highly expressed CD90, CD105 and CD34 but not CD31 (Fig. 1A), as shown previously [23]. We found that a considerable proportion of CD105 was not localized on the cell surface but in intracellular structures. To identify these structures we co-stained the ASC with antibodies against CD105 in combination with antibodies against either calnexin, a protein of the endoplasmatic reticulum (ER), or GM130, a protein of the Golgi apparatus. Confocal microscopy analysis demonstrated that considerable proportions of CD105 co-localized with structures of the Golgi apparatus as well as the ER (Fig. 1B). DLK1(PREF1) is an established marker for mouse adipocyte progenitors [33]. The DLK1(PREF1) status of human ASC is unknown. Indirect immunofluorescence experiments demonstrated that all cells in the ASC population at passage 5 stained strongly positive for DLK1(PREF1) (Fig. 1A). As indicated by their specific marker composition, the ASC vary significantly from MSC, HSC, HUVEC, macrophages and fibroblasts. The immunofluorescence microscopy analysis of 10,000 cells for each marker indicated a homogeneity of the DLK1(PREF1)$^{+}$/CD105^{+}/CD90^{+}/CD34^{+}/CD31^{-} ASC fraction of $\geq$ 99.8 %. To corroborate these data and to demonstrate the presence of

DLK1(PREF1), CD105, CD90, and CD34 and the absence of CD31 in the same cell, passage 5 ASC were subjected to multi-parameter detection FACS analysis. We found that the vast majority of the cells was in fact DLK1(PREF1)$^{+}$/CD105^{+}/CD90^{+}/CD34^{+}/CD31^{-} (Fig. 1C).

Since DLK1(PREF1) was identified as novel marker protein for human ASC it was interesting to monitor the purification of DLK1(PREF1) positive cells during enrichment of the ASC from the SVF and sub cultivation to passage 6 by FACS analysis (Fig. 1D, upper panel). In the SVF we detected 41.1 (± 3,6) % DLK1(PREF1) positive cells. After attachment of the ASC on plastic cell culture dishes (passage -1) 82.3 (± 1.6) % of the cell population stained positive for DLK1(PREF1). At passage 0, we measured 98.5 (± 0.3) % DLK1(PREF1) positive cells and at passage 1, 99.1 (± 0.2) % of the cells were DLK1(PREF1) positive. At passage 4 and 6 the number of DLK1(PREF1) positive cells was 100 %. The enrichment of DLK1(PREF1) expressing cells was also monitored by indirect immunofluorescence microscopy analysis (Fig. 1D, lower panel). Similar to the results of the FACS analysis we counted ~ 43 % DLK1(PREF1) positive cells in the SVF; ~ 82 % in passage -1, ~ 98 % in passage 0 and ~ 99 % in passage 1. Thus almost all passage 0 cells of the human ASC population were positive for DLK1(PREF1) and the expression of this protein remained high at later passages. To follow up the enrichment of the ASC population from the SVF we also analysed the expression of lineage determinant genes by qRT-PCR in the cell mixture of the SVF and the isolated ASC. We found a 2.5 fold increase in the CD90 expression (Fig. 1E), suggesting that CD90^{+} stem cells are enriched. In contrary, CD31 mRNA expression was 3 times reduced in the ASC population (Fig. 1E), most likely reflecting the removal of floating endothelial cells.

To determine the amount of adipocyte committed and/or differentiated cells in the ASC fraction the expression levels of the adipogenic differentiation marker FABP4 was measured (Fig. 1F). FABP4 mRNA was not detectable in the ASC fraction, suggesting that adipoyctes were

removed during purification. In summary, the isolated ASC were characterized as DLK1(PREF1)$^+$/CD105$^+$/CD90$^+$/CD34$^+$/CD31$^-$/FABP4$^-$ cells. We obtained similar results with ASC populations isolated from four different women with an age between 30 and 47 years.

1.3.2 DLK1(PREF1)$^+$/CD105$^+$/CD90$^+$/CD34$^+$/CD31$^-$/FABP4$^-$ human ASC possess multipotent differentiation capacity in *in vitro* assays

Tissue-specific stem cells possess the capability to differentiate into various lineages [11, 48, 54, 56, 60]. To get a better insight into the potential of the ASC population to differentiate into adipocytes, adipogenesis was induced with a hormone cocktail containing dexamethasone, IBMX, insulin and 2.5 % FBS (DMI) (Fig. 2A). Confocal immunofluorescence microscopy analysis demonstrated that the ASC marker DLK1(PREF1) was highly expressed in proliferating (Fig. 2A, top panel) and to a similar extent in density-arrested ASC, which stay in the G0/G1 phase of the cell cycle at day 0 of differentiation (Fig. 2A, middle left panel). After 14 days of differentiation the DLK1(PREF1) protein was almost undetectable (Fig. 2A, bottom left panel). In contrast, the adipogenic differentiation marker FABP4 was not detectable at day 0 but strongly induced at day 14 of differentiation (Fig. 2A, right panel). The strong reduction of DLK1(PREF1) and induction of FABP4 during differentiation was corroborated at the mRNA level by qRT-PCR (Fig. 2B) and by western blot analysis (Fig. 2C). qRT-PCR analysis also demonstrated that the expression of the adipogenic keytranscription factors PPARγ2 (Fig. 2D) and C/EBPα (Fig. 2E) was strongly induced during adipogenesis. Moreover, two terminal adipogenic differentiation markers, the adipokines adiponectin and leptin, were well induced in the ASC at day 9 of differentiation (Fig. 2F and 2G). To finally assess the ability of the ASC to differentiate in *in vitro* assays into other mesenchymal lineages, ASC were put under chondrogenic, osteogenic and adipogenic differentiation conditions (Fig. 2H). As expected, based on their tissue of origin, the ASC underwent strong adipogenic differentiation, shown by Oil RedO staining of the formed lipid

droplets. We detected differences in the strength of the *in vitro* adipogenic maturation of single ASC isolated from the same depot, as reflected by the difference in the quantity and size of lipid droplets (Fig. 2H, compare white and yellow arrows). Staining of the glucosaminoglycans in chondrocytes with Alcian Blue and of the osteocyte specific calcification with Alizatin Red S demonstrated that the ASC could differentiate in all three mesenchymal cell types *in vitro*. Although these findings warrant further studies, employing a chondrogenic assay in a pellet culture and an osteogenic assay best done by in vivo transplantation, they suggest that clones isolated from our purified cell population most likely fulfill the criteria of adult tissue-specific multipotent stem cells [11, 48, 54, 56, 60].

1.3.3 DLK1(PREF1) negatively regulates adipogenesis in human ASC

To better understand the function of DLK1(PREF1) in human ASC biology, we studied the effect of DLK1(PREF1) shRNA knockdown on the adipogenic differentiation potential of ASC. DLK1(PREF1) was knocked down using two different shRNA constructs for DLK1(PREF1) mRNA, sh#2 and sh#3 (Fig. 3). A strong reduction of DLK1(PREF1) mRNA was found in sh#2- and sh#3-containing ASC (Fig. 3A) and DLK1(PREF1) protein was significantly reduced by both sh#2 and sh#3 (Fig. 3B). ASC infected with a DLK1(PREF1) overexpressing lentivirus, pLentiDLK1(PREF1), served as positive control (Fig. 3B). The effects of the DLK1(PREF1) knockdown on the induction and progression of adipogenesis were monitored by assessing the expression of the key adipogenic regulator PPARγ2 and of the terminal adipogenic differentiation product FABP4. We found a significant increase in the induction of PPARγ2 and FABP4 expression in the DLK1(PREF1) knockdown cells at day 3 of adipogenesis compared to the control cells (Fig. 3C). We conclude that the knockdown of DLK1(PREF1) enhances adipogenic differentiation in human ASC.

1.4 Discussion

In the present study we isolated ASC populations from subcutaneous abdominal fat pats of different donors and characterized these cells by their plastic adherent properties, their immunophenotype, DLK1(PREF1)$^{+}$/CD105^{+}/CD90^{+}/CD34^{+}/CD31^{-}/FABP4^{-}, and a multipotent *in vitro* differentiation potential. Moreover, we demonstrated that DLK1(PREF1) functions as negative regulator of adipogenic differentiation in these cells.

The ASC could differentiate into adipocytes, osteoblasts and chondrocytes in *in vitro* assays. The absence of the cell surface marker F4/80, VWF, and FSP1 in the ASC population ruled out the presence of macrophages, endothelial cells and fibroblasts. The FABP4^{-} immunophenotype of the ASC demonstrated the absence of cells already committed to the adipogenic lineage. Subcutaneous fat has been established as a source of cultured, relatively homogeneous ASC and ASC fractions are used as defined cell population in clinical studies [27]. Although specific pattern of cellular surface markers were assigned to human ASC [21, 23-26], some debate over the criteria defining a human ASC remains and a consensus on minimal obligatory criteria for ASC has not yet been defined. In keeping with the immunophenotype defined by the International Society for Cell Therapy for mesenchymal stromal cells (MSCs) [61], the vast majority of our ASC robustly expressed the MSC markers CD90 and CD105 [54] and were negative for the endothelial cell marker CD31 [54, 59]. CD34, a transmembrane glycoprotein, is known as a marker for human hematopoietic [57] and endothelial [58] progenitors. In contrast to MSCs [56] our ASC stained strongly positive for CD34. This result is in keeping with previous studies from other research groups [15, 23, 25, 28, 62, 63]. In our ASC CD34 was at peak after 21 days of cultivation at passage 5 and expression remained stable even after passaging the ASC for 82 days until passage 15. The stable expression of CD34 in later passages is in contrast to a previous study showing the loss of CD34 at early ASC passages [21].

However, in that study the ASC were cultivated in 10 % FBS. The employment of growth medium with 2.5 % FBS in our study could account for these differences. It is known that long-term cultivation of cells in high concentration of FBS leads to changes in the proliferation and differentiation phenotype induced by mitogenic growth factors, survival factors and differentiation controlling signals [29, 64]. This leads to changes in cell specific markers as well as in the terminal differentiation capacity [49]. Similar to our results a recent study by Maumas et al., [62] demonstrated the maintenance of the CD34 cell surface marker in ASC cultured in 2 % FBS and the loss of CD34 in 10 % FBS. Thus our and other studies [15, 23, 25, 28, 62] strongly suggest that ASC belong to an adipose tissue-SVF-derived cell type displaying a $CD34^+$ immunophenotpye.

The $CD34^+/CD31^-/CD90^+$ immunophenotype of our ASC population matches with stromal cells showing a high adipogenic potential. These cells are predominantly detected in the outer adventitial ring of vessels and in close proximity to capillaries in adipose tissue *in situ* [15, 62], which may be major localizations of ASC in vivo. Another MSC marker endoglin/CD105 [61], an accessory receptor for TGFβ [65], was not analyzed in the study by Zimmerlin et al., [15]. In accordance with previous studies [24, 66] CD105 was highly expressed in our passage 5 ASC. Some studies found increasing expression of CD105 during early passaging of ASC in growth medium with 10 % FBS [21]. Similarly, human BM MSCs show higher levels of stromal markers, including CD105, in later stages of culture [56]. Conflicting results came from one study using fluorescent activating cell sorter (FACS) analyses showing that the major source of cells in the SVF capable of *in vitro* adipogenesis was the $CD105^-/CD34^+/CD31^-$ fraction [25]. Another group, however, demonstrated that a $CD34^+/CD90^+/CD105^+$ ASC fraction isolated from the SVF by FACS was capable of undergoing endothelial as well as adipose differentiation [63], but the standard "tube" assay (done with Matrigel) was not performed in this study. In addition, the cells were not clonal, and bona fide endothelial cells may have been present. Our indirect

immunofluorescence experiments based on confocal microscopy, allowing visualization of intracellular structures, demonstrated that CD105 was predominantly localized in the transport compartments of the ASC and only in a minor amount in the outer membrane. There might be differences in the detectability of intracellular CD105 between studies dependent on the methods used for cell permeabilizing, fixing and antigen detection. Nevertheless, in the present study we showed that CD105 was highly expressed in passage 5 ASC, as demonstrated by FACS and by indirect immunofluorescence experiments based on confocal microscopy.

We analyzed whether DLK1(PREF1), that was firstly identified as a putative marker for murine preadipocytes [30], could be used as additional marker to characterize human ASC. We found that DLK1(PREF1) is highly expressed in human ASC. The DLK1(PREF1) protein levels were similar in human ASC proliferating in PM4 medium and in density-arrested G0/G1-phase ASC. In the course of adipogenic differentiation the DLK1(PREF1) protein level declined. DLK1(PREF1) was almost undetectable in adipocytes 14 days after the induction of adipocyte differentiation. Thus DLK1(PREF1) is expressed in proliferating ASC, in density-arrested ASC, and most likely at early stages of adipogenic differentiation but completely downregulated in human adipocytes. During adipogenic differentiation of MEFs (mouse embryonic fibroblasts) the DLK1(PREF1) protein levels are relatively low in density arrest before differentiation, transiently increased at day 1 and 2, and then decreased and undetectable at day 5 after conversion into adipocytes [37]. Similar in adipogenesis of the preadipocyte cell line NIH 3T3 L1 the DLK1(PREF1) protein levels are low in density arrest before differentiation and transiently increase until day 2 – 3 [67]. When these cells cease clonal expansion and enter terminal adipogenic differentiation [20], the DLK1(PREF1) protein levels decrease but membranous forms of DLK1(PREF1) are detectable until day 5 [67]. Thus certain forms of DLK1(PREF1) protein are still present at early stages of terminal adipogenic differentiation and most likely contribute to adipocyte differentiation [67]. DLK1(PREF1) has been identified as a negative regulator of

adipocyte differentiation in mouse preadipocytes [30]. Moreover, in its active form, DLK1(PREF1) was shown to function in an autocrine and paracrine manner as soluble factor to inhibit differentiation of murine multipotent MSCs into adipocytes, osteoblasts and chondrocytes [37, 68]. This suggests that DLK1(PREF1) has a general role in keeping mouse precursor cells in an undifferentiated state. In the present study we found that DLK1(PREF1) functions as negative regulator of adipogenesis in human ASC. Previous studies have identified DLK1(PREF1) as regulator of differentiation in human BM MSCs [42, 44] and CB MSCs [45]. DLK1(PREF1) was used as a marker to distinguish human unrestricted somatic stem cells and CB MSC [45]. Moreover, DLK1(PREF1) was employed as surface marker for the isolation and differentiation of chondrogenic cells derived from human embryonic stem cells [41]. DLK1(PREF1) is also involved in additional differentiation processes in rodent cells, including haematopoiesis [69], pancreatic islet cell differentiation [70], Schwann cell differentiation [71], hepatic cell differentiation [72], and differentiation of muscle satellite cells. Moreover, DLK1(PREF1) is expressed in thymocytes and neuroblastoma cells [reviewed in ref. 33]. Thus DLK1(PREF1) should be used in combination with additional markers to distinguish ASC from MSC and other cell types, especially in combination with the $CD34^+$ and $CD31^-$ immunophenotype, as considered earlier [12].

Studies in mice suggest that the age, depot site and sex of the donor can influence the features and functionality of the derived ASC [5, 73, 74]. Clinical studies found a correlation between donor age and differentiation capacitiy of the given ASC [75-77]. In humans also an influence of the BMI [78] and fat depot origin [79, 80] on ASC features was shown. In the present study, we isolated ASC from subcutaneous adipose tissue pads of four different women with an age between 30 – 47 years and a BMI between 22 – 27 kg/m^2. No differences in the analyzed cell surface marker patterns were found between the ASC from the abdominal subcutaneous depots of the different donors. Moreover, all ASC populations had a high capacity to differentiate into

adipocytes *in vitro*, as demonstrated by the formation of high levels of four terminal adipogenic differentiation products, FABP4, adiponectin, leptin and triglycerides. This suggests that the relatively small differences in age and BMI between our four donors did not account for major differences in these ASC features. We detected however differences in the strength of the adipogenic maturation at the level of single ASC isolated from the same depot. This was reflected by the difference in the quantity and size of lipid droplets formed 20 days after the induction of adipogenesis (Fig. 2H, compare white and yellow arrows). These findings are in keeping with the differences in the single cell FABP4 protein level (Fig. 2A, compare white and yellow arrows). These data suggest that there may be heterogeneity regarding the adipogenic capacity of ASC within the same depots. Almost all passage 5 ASC stained strongly positive for the marker composition DLK1(PREF1)$^{+}$/CD105^{+}/CD90^{+}/CD34^{+}/CD31^{-}/FABP4^{-}. However, this marker combination cannot distinguish ASC with high or low adipogenic capacity. Evidence for differences between ASC populations cloned from a single human subcutaneous fat depot regarding the differentiation capacity was previously shown [15, 81, 82]. Common observations in our study were islets of strongly differentiated ASC surrounded by areas of weaker differentiated ASC. This could, for example, result from differences in the autocrine/paracrine micromilieu caused by a different cell density. Such findings could mirror the existence of different subtypes of ASC in subcutaneous fat depots *in vivo*, as discussed previously [12]. More studies are necessary to better understand these observations.

1.5 Acknowledgements

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Tables

Gene	Accession Number	Direction	Sequence	Base Numbers
βActin	NM_001101	forward	AGA AAA TCT GGC ACC ACA CC	332-350
		reverse	AGA GGC GTA CAG GGA TAG CA	500-519
AdipoQ	NM_004797	forward	CCT GGT GAG AAG GGT GAG AA	268-287
		reverse	GTA AAG CGA ATG GGC ATG TT	463-482
C/EBPα	NM_004364	forward	GCG GCG ACT TTG ACT ACC	418-435
		reverse	GCT TGG CTT CAT CCT CCT C	606-524
CD31	NM_000442	forward	GAG TCC TGC TGA CCC TTC TG	254-273
		reverse	ATC TGG TGC TGA GGC TTG AC	412-431
CD90	NM_006288	forward	GAA CCA ACT TCA CCA GCA AA	286-305
		reverse	ATG CCC TCA CAC TTG ACC A	436-454
PU.1 / F4/80	NM_001080547	forward	CAC CCC ATC CCA GTC TTG	531-548
		reverse	TCT TCT TGC TGC CTG TCT CC	712-731
FABP4	NM_001442	forward	CAG TGT GAA TGG GGA TGT GA	176-195
		reverse	CGT GGA AGT GAC GCC TTT	408-425
FSP1 / S100A4	NM_002961	forward	GGG CAA AGA GGG TGA CAA	129-146
		reverse	CTT CCT GGG CTG CTT ATC TG	350-369
Leptin	NM_000230	forward	CAC ACG CAG TCA GTC TCC TC	196-215
		reverse	AGG TTC TCC AGG TCG TTG G	350-368
PPARγ2	NM_015869	forward	ATG GGT GAA ACT CTG GGA GA	92-111
		reverse	TGG AAT GTC TTC GTA ATG TGG A	316-337
PREF1	NM_003836	forward	TGA CCA GTG CGT GAC CTC T	315-333
		reverse	GGC AGT CCT TTC CCG AAGT A	508-526
TBP	NM_003194	forward	ATG ACC CCC ATC ACT CCT G	668-686
		reverse	GCA CAC CAT TTT CCC AGA AC	876-895
VWF	NM_000552	forward	CGC ATC CAG CAT ACA GTG AC	1691-1710
		reverse	GAG GGG GTA AGG AAG TCG TC	1847-1866

Table 1: Primer list: The sequences, genbank accession numbers, and the base numbers of the primers within the sequence are indicated**.**

Legend to the Figures

Fig. 1: Characterization of human adipose-derived stromal cells (ASC). (A) Identification of ASC as DLK1(PREF1)$^{+}$/CD105^{+}/CD90^{+}/CD34^{+}/CD31^{-} cells. Immunofluorescence staining of freshly isolated ASC (passage 5) is shown. Cells were fixed in 4% PFA, stained with primary antibodies against DLK1(PREF1), CD105, CD90, CD34, CD31, and FITC-conjugated secondary antibodies (green) and processed for immunofluorescence microscopy using a confocal laser scanning system. Nuclei were stained with TO-PRO-3 (red) (magnification 400x). **(B)** Co-localization of CD105 with calnexin (ER protein) and GM130 (Golgi apparatus protein) in ASC. Immunofluorescence staining of freshly isolated ASC (passage 5) is shown. Cells were fixed in 4% PFA, stained with a primary antibody against CD105, calnexin, and GM130 and Alexa Fluor 488nm-conjugated (green) or Alexa Fluor 555nm-conjugated secondary antibodies (red) as indicated. Nuclei were stained with TO-PRO-3 (red). Merge (yellow). The stained ASC were processed for immunofluorescence microscopy using a confocal laser scanning system (magnification 1000x). **(C)** Characterization of ASC using multi-parameter detection FACS analysis. 50,000 cells were counted using a BD FACS CantoII™ flow cytometer. FACS histograms of passage 5 ASC simultaneously stained for CD105, CD90, CD34, CD31, and DLK1(PREF1) are shown. Histograms are representative of 3 independent flow cytometry analyses. Red histograms: unstained control; blue histograms: antibody staining. **(D)** Enrichment of DLK1(PREF1) expressing cells during purification of ASC from the SVF and sub cultivation until passage 6. (Upper panel) 50,000 cells from each fraction (SVF, passage P-1, P0, P1, P4 and P6) were analyzed for DLK1(PREF1) staining using a BD FACS CantoII™ flow cytometer. The percentage of DLK1(PREF1) positive cells in each fraction is shown. Three independent flow cytometry analyses were performed for each fraction. (Lower panel) Immunofluorescence staining of DLK1(PREF1) positive cells in the SVF and early passage ASC. Cells were fixed in

4% PFA, stained with a primary antibody against DLK1(PREF1) and FITC-conjugated secondary antibodies (green). Nuclei were stained with TO-PRO-3 (red). Cells were viewed by immunofluorescence microscopy using a confocal laser-scanning system (magnification 400x). For the determination of the percentage of DLK1(PREF1) positive cells a total of 300 cells were analyzed. **(E)** CD90 and CD31 mRNA expression in ASC. RNA samples of SVF and ASC cells were isolated and qRT-PCR analysis performed using specific primers against CD90 and CD31. Expression results were normalized to TATA binding protein (TBP) and induction calculated based on ASC values which were set to 1. The induction was calculated based on SVF values. Three independent qRT-PCR analyses were performed. **(F)** FABP4 mRNA expression in ASC. RNA samples of SVF and ASC cells were isolated and qRT-PCR analysis performed using specific primers against FABP4. Expression results were normalized to TATA binding protein (TBP) and induction calculated based on ASC values which were set to 1. The induction was calculated based on SVF values. Three independent qRT-PCR analyses were performed.

Fig. 2: Differentiation of ASC. ASC were freshly isolated, grown until passage 5 and used for differentiation. Representative results for ASC from four different donors are shown. **(A)** Immunofluorescence analysis of proliferating and density-arrest ASC at day 0 and day 14 of adipogenesis. Indicated proteins were stained with primary antibodies against DLK1(PREF1) and FABP4 and FITC-conjugated secondary antibodies (green). Nuclei were stained with TO-PRO-3 (red) (magnification 400x). **(B)** DLK1(PREF1) and FABP4 mRNA levels at day 0 and day 6 of adipogenesis. RNA samples of ASC and control cells were isolated at the indicated days and qRT-PCR analysis performed using specific primers against DLK1(PREF1) and FABP4. Expression results were normalized to TATA binding protein (TBP) and induction calculated based on ASC values which were set to 1. Three independent qRT-PCR analyses were performed for each parameter. **(C)** DLK1(PREF1) and FABP4 protein levels in ASC at day 0 and day 6 of adipogenesis. ASC lysates were separated by SDS-PAGE and analyzed by western blotting using antibodies against DLK1(PREF1) and FABP4. βactin served as input control. **(D-G)** Induction of PPARγ2 **(D)**, C/EBPα **(E)**, adiponectin **(F)** and leptin (**G**) mRNA expression during adipogenesis. Differentiation was induced in ASC, mRNA isolated at the indicated days of differentiation and relative gene expression was analyzed by qRT-PCR. Three independent qRT-PCR analyses were performed for each parameter. **(H)** ASC were *in vitro* differentiated into three mesenchymal cell types each for 20 days. Adipocytes were stained with Oil RedO, chondrocytes with Alcian Blue and osteoblasts with Alizarin Red-S. All three differentiation conditions changed the morphology of ASC and lineage specific staining was found to be positive for each resulting cell type.

Fig. 3: The knockdown of DLK1(PREF1) enhances adipogenesis in human ASC. ASC were infected with two different lentivirus constructs expressing shRNA against DLK1(PREF1), sh#2 and sh#3, and a mock construct (sh#Control) as a control. **(A)** The DLK1(PREF1) knockdown was analysed by qRT-PCR. Normalization was calculated relative to the mock construct. The data are presented as the mean +/- SD of 3 measurements, (**) $p \leq 0.01$; (***) $p \leq 0.001$. **(B)** The efficiency of the DLK1(PREF1) protein knockdown was measured by Western blot analysis. ASC infected with the DLK1(PREF1) overexpression vector, pLentiDLK1(PREF1), served as positive control. In lane pLentiPREF1 1/10 only 10 % of the input from lane pLentiPREF1 was loaded. βActin served as loading control. **(C)** The knockdown of DLK1(PREF1) enhances adipogenesis in ASC. ASC were infected with lentivirus constructs expressing shRNA against DLK1(PREF1) (sh#2 and sh#3) or a mock construct (sh#Control) as indicated, and adipogenesis was induced by adding hormone cocktail. The mRNA levels of the adipogenic regulator PPARγ2, and of the adipogenic differentiation marker FABP-4 were determined by qRT-PCR analysis at day 3 of adipogenesis. The data are presented as the mean +/- SD of 3 measurements, (*) $p \leq 0.05$; (***) $p \leq 0.001$.

Supplemental Fig. 1: Isolation of human adipose-derived stromal cells (ASC). (A) Approach to isolate ASC from human subcutaneous abdominal fat tissue biopsies. SVF, stromal vascular fraction. ASC, adipose-derived stromal cell. **(B)** Immunofluorescence staining of freshly isolated ASC (passage 5), and, as controls, human foreskin fibroblasts (HFF), peripheral blood mononuclears cells (PBMC), and endothelial cells (HUVEC). Cells were fixed in 4% PFA, stained with primary antibodies against FSP1, F4/80(EMR1) and VWF, and FITC-conjugated secondary antibodies (green) and processed for immunofluorescence microscopy using a confocal laser scanning system. Nuclei were stained with TO-PRO-3 (red) (magnification 400x). **(C)** F4/80, VWF and FSP1 mRNA expression in ASC. RNA samples of ASC and control cells were isolated and qRT-PCR analysis of cell type specific genes performed using specific primers against F4/80, VWF and FSP1. Expression results were normalized to TATA binding protein (TBP) and induction calculated based on ASC values which were set to 1. Three independent qRT-PCR analyses were performed for each parameter. **(D)** Detection of CD105, CD90, CD34, CD31 and DLK1(PREF1) in control cells. Immunofluorescence stainings of HUVEC, U2OS/pLentiPREF1 (U2OS cells transiently transfected with pLentiPREF1), U2OS, CaSki, and Granulocytes which served as positive or negative controls for DLK1(PREF1), CD105, CD90, CD34, and CD31 stainings are shown as indicated. Cells were fixed in 4% PFA, stained with primary antibodies (anti DLK1(PREF1), CD105, CD90, CD34, CD31) and FITC-conjugated secondary antibodies (green) and processed for immunofluorescence microscopy using a confocal laser scanning system. Nuclei were stained with TO-PRO-3 (red). Only merge is shown (magnification 400x).