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CCL5/CCR1 axis regulates multipotency of human adipose tissue derived stromal cells

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Abstract Several potential clinical applications of stem cells rely on their capacity to migrate into sites of inflammation where they contribute to tissue regeneration processes. Inflammatory signals are partially mediated by chemokines acting via their receptors expressed on the target cells. Data concerning the repertoire and biological activities of chemokine receptors in human adipose tissue derived stromal cells (ADSCs) are limited. Here we show that CCR1 is one of the few chemokine receptors expressed in ADSCs at a high level. *CCR1* expression varies in ADSCs derived from different donors. It sharply decreases in the early phase of ADSCs in vitro propagation, but further demonstrates relative stability. Expression of *CCR1* positively correlates with expression of *SOX2*, *OCT4* and *NANOG*, transcription factors responsible for maintenance of the stemness properties of the cells. We demonstrate that signaling via CCL5/CCR1 axis triggers migration of ADSCs, activates ERK and AKT kinases, stimulates NF- κ B transcriptional activity and culminates in increased proliferation of CCR1⁺ cells accompanied with up-regulation of *SOX2*, *OCT4* and *NANOG* expression. Our data suggest that chemokine signaling via CCR1 may be involved in regulation of stemness of ADSCs.

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Introduction

Adherent, fibroblastic cell populations have been isolated from many connective tissues, and some have been found to contain a subset of local stem/progenitor cells. Some of

these cell populations are suggested to be nonimmunogenic, and may have the ability to migrate to injured or inflamed sites. Those features support their potential clinical applications including treatment of immune, cardiovascular and degenerative diseases (osteoarthritis and osteoporosis), and their use as drug delivery vehicles (Caplan, 2007; Gimble et al., 2007).

It has been suggested that similarly to embryonic stem cells (ESCs), stemness-related properties of some stromal stem/progenitor cells are supported by regulatory networks of transcription factors OCT4, SOX2 and NANOG (Greco et al., 2007; Ng and Surani, 2011; Park et al., 2012; Riekstina et al., 2009). When over-expressed, these factors reprogram differentiated cells to an embryonic-like state designated as

Abbreviations: ADSCs, adipose tissue derived stromal cells; BCs, peripheral blood mononuclear cells; BMSCs, bone marrow derived stromal cells; ESCs, embryonic stem cells; GPCR, G-protein coupled receptor; p, passage.

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pluripotent stem cells. These reprogrammed cells exhibit the morphology and growth characteristics of ESCs and express ESC marker genes (Takahashi et al., 2007).

Stromal cells with stem/progenitor-like properties can be found in a diverse range of tissues and organs (Parekkadan and Milwid, 2010). White adipose tissue is considered as a rich source of stromal cells due to its simple harvesting methods and high numbers of isolated cells (Gimble et al., 2007). Growth kinetics, immunogenic characteristics, in vitro differentiation potential, senescence ratio and angiogenic activity of adipose tissue derived stromal cells (ADSCs) (also known as adipose-derived "mesenchymal stem cells") are comparable with stromal cells obtained from other sources; however, several differences have been reported (De Ugarte et al., 2003; Izadpanah et al., 2006; Noel et al., 2008; Wagner et al., 2005; Winter et al., 2003).

Purification of homogenous functionally relevant stem/progenitor cells from adipose tissue is still a challenge. Widely used methods of ADSC isolation include separation of adipose tissue stromal vascular fraction, followed by sorting according to the immunophenotype guidelines suggested by the International Society for Cellular Therapy, and selection of a plastic adherent population (Dominici et al., 2006). It has become clear that this method yields highly heterogeneous populations with only a fraction of cells with stem/progenitor characteristics (Gimble et al., 2010; Madonna et al., 2009; Tallone et al., 2011). Heterogeneity of initial populations may result in unpredictable effects and non-reproducible results, and therefore it remains one of the key problems in the field.

Numerous clinical applications of stromal cell populations isolated from different tissues rely on the migration and homing of therapeutic cells in the site of injury or inflammation (Chamberlain et al., 2007). Also, it has been suggested that inflammatory conditions result in activation and directional movement of endogenous cells to the sites of injury where they participate in the processes of tissue regeneration. Signals of inflammation are partly mediated by chemokines. These signaling molecules act via binding to their seven-transmembrane receptors belonging to the G-protein coupled receptor (GPCR) family. Chemokines and their receptors play essential roles in the immune system by regulating mobilization and migration of several cell types including neutrophils and B- and T-lymphocytes (Bendall, 2005). Also, chemokines are associated with a variety of other cellular functions including proliferation, differentiation and establishment of cellular polarity. Several data also indicate that chemokines have cell type and concentration dependent anti- and pro-apoptotic effects (Vlahakis et al., 2002). Twenty chemokine receptors and approximately 50 chemokines are identified in humans.

Bone marrow derived stromal cells (BMSCs) express several chemokine receptors (CCR1, CCR4, CCR6, CCR7, CCR9, CCR10, CXCR4, CXCR5, CXCR6, CX3CR1), and respective chemokines induce migration of BMSCs in vitro (Fox et al., 2007; Honczarenko et al., 2006; Ruster et al., 2006; Sordi et al., 2005; Von Luttichau et al., 2005; Wynn et al., 2004). Data concerning chemokine system in ADSCs are very limited. It has been demonstrated that ADSCs are chemotactic in vitro, but, compared to BMSCs, ADSCs express a smaller subset of chemokine receptors (CCR1, CCR7, CXCR4, CXCR5 and CXCR6) (Baek et al., 2011).

Here we analyze the entire repertoire of chemokine receptors in human ADSCs, peripheral blood mononuclear cells and dermal fibroblasts, and show that *CCR1* is one of the few chemokine receptors highly expressed in ADSCs. Interestingly, expression of *CCR1* in different pools of ADSCs positively correlates with the expression levels of the stem cell marker genes *SOX2*, *OCT4* and *NANOG*, whereas exposure of ADSCs to CCL5, a ligand for CCR1, stimulates proliferation of CCR1⁺ cells accompanied with increased expression of *SOX2*, *OCT4* and *NANOG*. Our results also show that CCR1 is an active receptor in ADSCs, and signaling via the CCL5/CCR1 axis triggers not only migration of cells but also activation of ERK and AKT kinases as well as NF κ B signaling pathway.

Materials and methods

Cell culture

ADSCs were isolated from human subcutaneous adipose tissue as described (Lin et al., 2007) with some modifications. Minced adipose tissue was digested with 0.1% collagenase (Gibco, Invitrogen, Carlsbad, CA, USA) in serum-free low glucose Dulbecco's modified Eagle's medium (DMEM-LG) (Gibco, Invitrogen) at 37 °C for 1.5 h, followed by neutralization with normal growth medium (DMEM-LG) supplemented with 10% fetal bovine serum (HI-FBS) (PAA, Pasching, Austria) heat-inactivated at 56 °C for 30 min and 1% penicillin-streptomycin (PEST) (Invitrogen). After centrifugation at 1000 rpm for 5 min, the cell pellet was incubated for 15 min at room temperature (RT) in NH₄Cl, passed through a 100- μ m nylon mesh (BD Biosciences Pharmingen, San Jose, CA, USA), re-suspended in normal growth medium and plated at density of 10,000 cells/cm² (passage 0). Cells were propagated at 37 °C and 5% CO₂ for 24 h, and non-adherent cells were removed by changing the medium. Each splitting of the confluent cells was considered as the next passage. Immunophenotype of the isolated ADSCs was assessed as CD73⁺/CD90⁺/CD105⁺/CD45⁻/CD34⁻ (Supplementary Fig. 1). Human peripheral blood mononuclear cells were isolated using a Histopaque-1077 (Sigma-Aldrich, Steinheim, Germany) density gradient centrifuge. Primary fibroblast cultures were established by migration from skin explants placed onto Primaria dish containing normal growth medium (Takashima, 2001). The donors of adipose tissue, skin fibroblasts and blood mononuclear cells are described in the Supplementary Table 1.

For kinase activation assay, ADSCs were pre-incubated overnight in serum-free DMEM-LG, treated with recombinant CCL5 (PeproTech, Rock Hill, NJ, USA) for 10, 15 or 60 min, washed with phosphate buffered saline (PBS) and lysed directly in Laemmli sample buffer (60 mM Tris-Cl, pH 6.8, 2% SDS, 10% glycerol, 5% β -mercaptoethanol, 0.01% bromophenol blue). Prolonged treatments of ADSCs were carried out in Light medium (DMEM-LG containing 3% HI-FBS and 1% PEST) supplemented with 50 ng/ml of CCL5 for 8, 24, 48 or 72 h. Treatments were stopped by washing the cells with PBS, trypsinization (trypsin-EDTA) (PAA), fractionation for total RNA and protein isolations and immediate lysis in the respective lysis buffer. Viability of ADSCs was tested using Vialight™ plus kit (Lonza, Basel, Switzerland) according to the manufacturer's instructions.

Total RNA isolation, cDNA synthesis, RT-PCR analyses

Total RNA was isolated using RNeasy micro kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Complementary DNA was synthesized from 1 μ g of total RNA using SuperScript™ III Reverse Transcriptase (Invitrogen) according to the manufacturer's protocol using oligo(dT)₂₀. We used 0.5 μ l of cDNA per one PCR.

RT-PCR was performed using FIREPol® Master Mix (Solis BioDyne, Tartu, Estonia) according to the manufacturer's protocol. PCR products were separated on 1.5% agarose gel and visualized by ethidium bromide staining. Quantitative RT-PCR (qRT-PCR) was performed using Platinum® SYBR® Green qRT-PCR SuperMix-UDG (Invitrogen). The levels of target gene mRNAs and mRNA of *glyceraldehyde 3-phosphate dehydrogenase (GAPDH)* used for normalization were detected in triplicates with LightCycler® 480 Real-Time PCR System (Roche Applied Science, Basel, Switzerland). Data were analyzed using Lightcycler 480 software (Roche) and calculations were carried out using the comparative C_T method. Primers used in the current study are presented in Supplementary Table 2. Statistical analysis was carried out using t-test (two sample assuming unequal variances) (MS Excel). P values and coefficients of determination of a linear regression were calculated using the GraphPad Prism software.

Cell transfection and luciferase assay

ADSCs were electroporated using human MSC Nucleofector® kit and Nucleofector™ II Device (Lonza) with C-17 program according to the manufacturer's protocol. We used 1500 ng of a DNA construct encoding firefly luciferase reporter under control of NF κ B dependent promoter, and 500 ng of a plasmid encoding Renilla luciferase under control of SV40 promoter. After electroporation, the cells were propagated in normal growth medium on a 48-well cell culture dish for 24 h following treatment with CCL5 at different concentrations (1, 10 or 50 ng/ml) in Light medium for 24 h. Transfected but not treated cells were used as controls. Cells were washed with PBS and lysed in 40 μ l/well of passive lysis buffer (Promega, Madison, WI, USA). Firefly and Renilla luciferase activities were measured using Dual-Luciferase® Reporter Assay System (Promega) and Ascent FL Fluoroscan (Thermo Electron Corporation, Waltham, MA, USA) according to the manufacturers' instructions. The obtained firefly luciferase activity data were normalized with Renilla luciferase activity values. The normalized firefly luciferase activity in the control sample was set as 1 and the other samples were calculated accordingly.

Western blot (WB)

Cells were re-suspended in RIPA buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 2 mM EDTA, 1% NP-40, 0.1% sodium dodecyl sulfate) containing ProteoBlock™ protease inhibitor cocktail (Fermentas Inc., Burlington, Ontario, Canada) and incubated on ice for 30 min. Total protein concentrations were measured using Pierce BCA Protein Assay kit; 20 μ g of total protein was separated on 10% polyacrylamide gel and transferred to PVDF membrane (Millipore, Billerica, MA, USA). The membrane was incubated in 5% non-fat dry milk

(AppliChem, Darmstadt, Germany) in Tris buffered saline (TBS)+0.05% Tween20 (Sigma) at RT for 1 h. Antibodies were diluted in TBS-T containing 2.5% non-fat dry milk. The antibodies used in the current study were: CCR1 (CKR-1 (H-52), sc-7934, 1:1500) (Santa Cruz Biotechnology, CA, USA), AKT (9272, 1:1000) (Cell Signaling Technology, Beverly, MA, USA), phospho-AKT (Ser473) (9271, 1:1000) (Cell Signaling Technology), ERK (sc-154-G, 1:1500) (Santa Cruz Biotechnology), phospho-ERK (sc-7383, 1:1500) (Santa Cruz Biotechnology), SOX2 (1:750) (CeMines, Evergreen, CO, USA), and GAPDH (G8795, 1:8000) (Sigma). Secondary HRP-conjugated anti-rabbit, -goat or -mouse IgG antibodies were purchased from Abcam (Cambridge, UK) (ab6721; ab6741; ab6728, respectively, dilution 1:15,000). The proteins were visualized using SuperSignal West Pico (ERK, phospho-ERK and AKT) or Femto (phospho-AKT, CCR1 and SOX2) chemiluminescence substrate kits (both Pierce Biotechnology Inc., Rockford, IL, USA).

Flow cytometry

Approximately 10⁵ cells were harvested by trypsinization, washed with PBS and blocked in 2% bovine serum albumin (BSA) (PAA Laboratories GmbH, Pasching, Austria) in PBS. Primary antibodies against CD34 (H-140) (Santa Cruz Biotechnology), CD45 (CALTAG Laboratories), CD73 and CD105 (both BD Biosciences, Erembodegem-Aalst, Belgium) were added directly into blocking solution at the dilution 1:500. Antibodies against CD90 (Chemicon) and CCR1 (CKR-1 (C-20), Santa Cruz Biotechnology, sc-6125) were diluted 1:100 in the blocking solution. The cells were incubated with primary antibodies for 1 h on ice, washed for 10 min with PBS containing 0.5% BSA and incubated with secondary antibodies Alexa Fluor 488 (A11017 or A31628, Molecular Probes, Eugene, OR, USA) diluted in PBS+4% BSA at 1:400 for 45 min on ice. ADSCs stained only with secondary antibody were used as a nonreactive control. Cells were analyzed on Accuri™ C6 or FACS Calibur flow cytometers (both BD Biosciences). Data acquisition and analysis were performed using BD Accuri C6 or CellQuest software, respectively (BD Biosciences).

Immunofluorescence (IF)

ADSCs were plated on slides (BD Biosciences) at a density of approximately 3000 cells/cm² and treated with 50 ng/ml of CCL5 for 3 or 48 h. For bromodeoxyuridine (BrdU) staining, cells were treated with CCL5 and 10 μ M BrdU for 18 h. Cells were washed 3 times with TBS and fixed in 4% paraformaldehyde in TBS for 15 min at RT and 15 min at +4 °C. For CCR1 immunostaining, the cells were treated with 0.1% Tween20 in blocking solution (TBS supplemented with 2% bovine serum albumin (BSA)) for 10 min at RT. For BrdU and CCL5 labeling, the cells were permeabilized with 0.3% Tween20 in blocking solution for 30 min. BrdU stained cells were additionally treated with 2 N HCl for 8 min at RT with subsequent washing with TBS containing 0.5% BSA. All cells were incubated in blocking solution at +4 °C overnight. Cells were exposed to CCR1 antibody CKR-1 (C-20), mouse monoclonal CCL5 capture antibody from CCL5/RANTES DuoSet ELISA Development System (R&D System, Wiesbaden, Germany), or BrdU antibody mAbG3G4 (Developmental Studies Hybridoma Bank, University of Iowa, IA, USA) at dilutions of 1:300, 1:500 and 1:200,

respectively, for 2 h at RT. The cells were washed 3 times for 10 min with TBS containing 0.5% BSA and incubated with Alexa Fluor 488 or Alexa Fluor 568 secondary antibodies (Molecular Probes, A11017 or A11079) (dilutions 1:1000) for 1 h at RT. CCR1, CCL5 and secondary antibodies were diluted in blocking solution. BrdU antibody was diluted in blocking solution supplemented with 0.3% Triton X-100 (Sigma). Cells were mounted with Prolong Gold antifade reagent containing 4',6-diamidino-2-phenylindole (DAPI) (Invitrogen). The cells were visualized under the fluorescence microscope Olympus BX61 with UPLan SApo 40× or 20× objectives.

Cell migration assay

Cell migration assay was performed using a 24-well colorimetric QCM Chemotaxis Cell Migration Assay kit (Millipore) with 8 μm pore size polycarbonate membrane inserts. ADSCs were subjected to overnight serum-starvation in DMEM-LG with subsequent trypsinization and seeding of approximately 80,000 cells per insert in DMEM-LG. DMEM-LG supplemented with 10 ng/ml of CCL5 was added to the lower chamber. Migration assay was performed at 37 °C and 5% CO₂ for 4 h. Base medium without any supplement was used as a negative control. Migratory cells on the membranes were stained and visualized using a Zeiss Axiovert microscope with 20× objective.

Enzyme-linked immunosorbent assay (ELISA)

The levels of CCL5 secreted into the growth medium were measured using CCL5/RANTES DuoSet ELISA Development System (R&D System). The cells were cultured for 3 days in the normal growth medium. The media were collected, centrifuged at 13,000 rpm for 5 min and immediately used for analysis. ELISA was performed using high binding ELISA plates (Greiner BioOne) at RT according to the manufacturer's instructions. Optical density was measured using the photospectrometer Spectramax 340 PC at the wavelength 450 nm.

Results

ADSCs express a set of chemokine receptors

Expression of chemokine receptor transcripts in ADSCs, human skin primary fibroblasts (FBs) and human peripheral blood mononuclear cells (BCs) was analyzed using RT-PCR with 2 independent pairs of primers. All 20 receptors were expressed in BCs. Analysis of four different pools of ADSCs showed that 13 chemokine receptors (*CCR1*, *CCR2*, *CCR3*, *CCR5*, *CCR10*, *CCR11*, *CXCR1*, *CXCR4*, *CXCR5*, *CXCR6*, *CXCR7*, *CX3CR1* and *XCR1*) were expressed in ADSCs, whereas their expression levels varied significantly (Fig. 1). Taking our data from four independent experiments together, we conclude that 6 chemokine receptors (*CCR1*, *CCR10*, *CCR11*, *CXCR4*, *CXCR6* and *CXCR7*) were expressed in all ADSC isolates at levels comparable with that in BCs, whereas expression of *CCR2*, *CCR3*, *CCR5*, *CXCR1*, *CXCR5*, *CX3CR1* and *XCR1* was very low in most of the ADSCs analyzed. Obtained data are mainly in line with recently published results showing the expression of

CCR1, *CCR2*, *CCR7*, *CXCR4*, *CXCR5* and *CXCR6* in ADSCs (Baek et al., 2011). *CCR10*, *CCR11*, *CXCR6*, *CXCR7* and *XCR1* were expressed also in FBs. *CCR1* was one of the few receptors highly expressed in ADSCs, but not in FBs. It has been shown that human BMSCs possess functional *CCR1* (Honczarenko et al., 2006), and the expression of *CCR1* in ADSCs has been also confirmed (Baek et al., 2011). Therefore, out of 6 chemokine receptors found to be expressed in ADSCs at high levels, *CCR1* has been chosen for further investigations.

The expression levels of *CCR1* vary in ADSCs isolated from different donors

It has been reported that the expression of several chemokine receptors decreases significantly in BMSCs as a result of in vitro expansion of cells (Honczarenko et al., 2006). We analyzed the expression of *CCR1* in individual pools of ADSCs isolated from different donors in the course of prolonged in vitro propagation. Results of *CCR1* mRNA expression analyses in ADSCs derived from 9 individuals (samples I–IX) and cultured two (p2) to twelve (p12) passages are shown in Fig. 2A. *CCR1* expression levels were normalized using *GAPDH* expression levels and set as 1 for ADSC test sample I (I p9). Data from other samples were calculated relative to sample I p9. *CCR1* expression was detected in all samples. However, the levels of *CCR1* expression varied notably between ADSCs isolated from different donors. In general, *CCR1* expression was the highest in freshly isolated cells at passage p2 and decreased significantly (more than 5 times) in the next passage (p3). Also, expression of other analyzed chemokine receptors except *CXCR6* and *CXCR7* decreased significantly by passage p4 (Supplementary Fig. 2A). Further propagation of ADSCs in vitro did not result in significant changes of *CCR1* expression in analyzed ADSC samples except sample IX that showed more than 50 and 10 times reduction in the expression of *CCR1* in p4 compared to p2 and p3, respectively.

Flow cytometry analyses were exploited to estimate the fraction of *CCR1*⁺ cells in ADSC populations using *CCR1* antibody (Fig. 2B). Cells from samples III (p10) and VII (p2) were used for these analyses. We also assessed *CCR1*⁺ cell population in isolated BCs. The obtained results showed that ADSC sample VII contained approximately 25% and sample III approximately 4% of *CCR1*⁺ cells (Fig. 2B). These results correlate well with the results of qRT-PCR analyses (Fig. 2A). Two populations of cells expressing different levels of *CCR1* were detected in BC samples, whereas approximately 69% of cells were *CCR1* positive. Analyses of expression of hematopoietic cell marker CD45 using flow cytometry showed that all BCs were CD45⁺, whereas ADSC samples VII p2 and III p10 contained 4.5% and 1.7% of CD45⁺ cells, respectively (Supplementary Figs. 1 and 2B). These data indicate that hematopoietic CD45⁺ cells may only partly contribute to a subset of *CCR1*⁺ cells in ADSCs.

IF analysis was used to estimate levels of *CCR1* protein in individual cells and to confirm flow cytometry analysis results. Analysis of three different lineages of ADSCs (I p7, V p8 and VI p12) using *CCR1* antibody revealed that levels of *CCR1* protein varied significantly among individual cells (Fig. 2C). The obtained results demonstrate high heterogeneity of ADSCs with respect to *CCR1* expression between

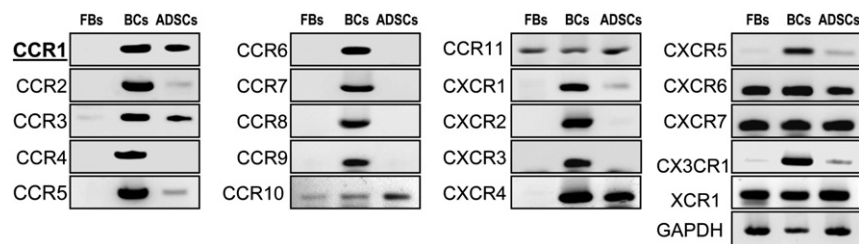


Figure 1 ADSCs express mRNA for several chemokine receptors. The expression of 20 chemokine receptors was analyzed in ADSCs, fibroblasts (FBs) and peripheral blood mononuclear cells (BCs) by RT-PCR. *GAPDH* and chemokine receptor cDNAs were amplified for 22 and 40 cycles, respectively.

pools of ADSCs isolated from different donors and also at the level of individual cells.

Expression of *CCL5* and *CCL3* in ADSCs

Chemokine receptor CCR1 is known to interact with several chemokines including *CCL3*, *CCL5*, *CCL7*, *CCL15* and *CCL23*, whereas *CCL3* and *CCL5* have the highest binding affinity (Neote et al., 1993). Results of analysis of expression of *CCL5*

and *CCL3* mRNA in BCs, 6 independent pools of ADSCs and FBs by RT-PCR are presented in Fig. 3A. High levels of expression of both ligands were detected in BCs. Expression of *CCL3* was not detected in analyzed FBs, and a half of analyzed FB cultures expressed very low levels of *CCL5* mRNA. In contrast, all tested ADSC cultures expressed low levels of *CCL5* mRNA. Expression of *CCL3* was observed in 3 out of 6 pools of ADSCs.

Since *CCL5* mRNA expression was detected in all ADSC samples, we analyzed it also by qRT-PCR in several ADSC samples that were previously tested for *CCR1* expression

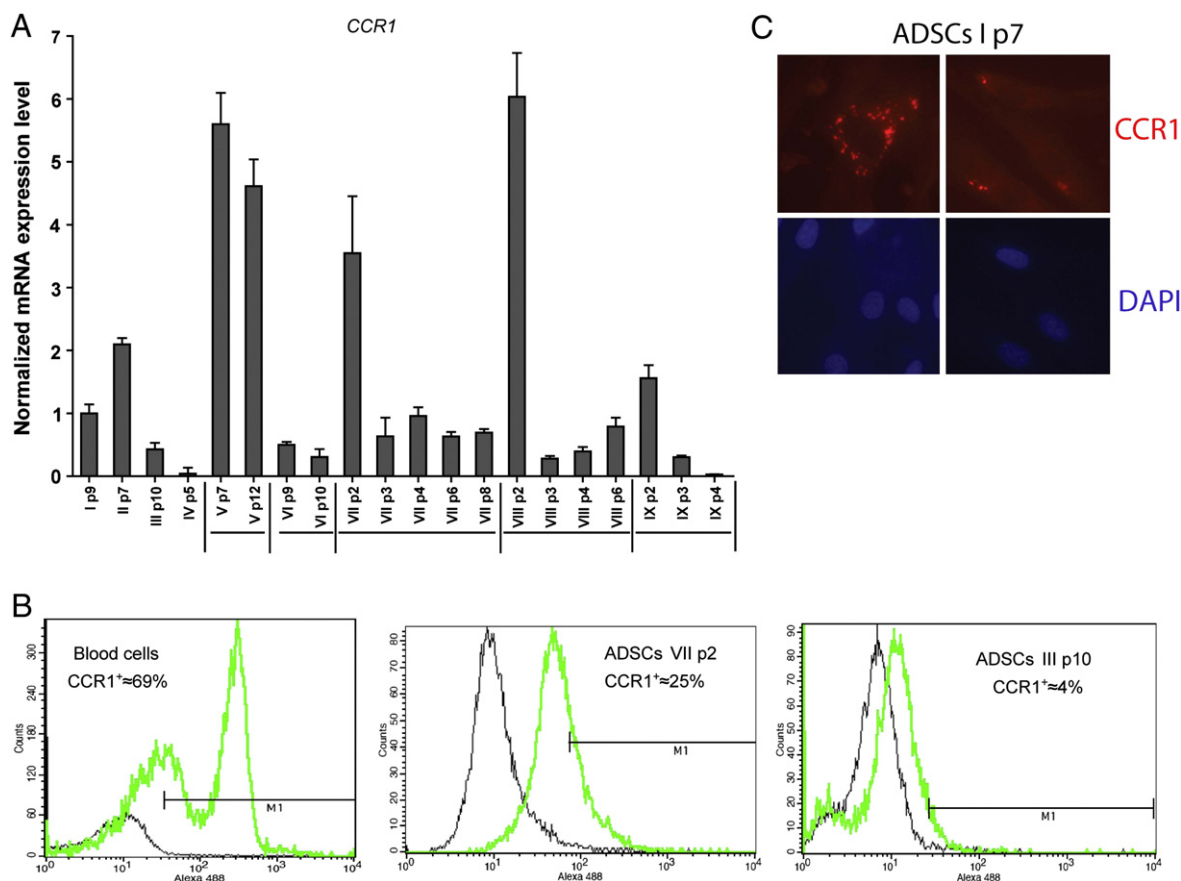


Figure 2 Levels of *CCR1* expression are individually variable. (A) Levels of *CCR1* mRNA expression in ADSCs from nine individuals (samples I–IX) cultured for different periods of time (p2–p12 indicating the passage numbers) were analyzed by qRT-PCR. *CCR1* mRNA expression levels were measured in triplicates, normalized with *GAPDH* mRNA expression level and set as 1 in sample I (I p9). Data from other samples were calculated relative to sample I p9. (B) The portions of *CCR1*⁺ cells within peripheral blood mononuclear cells and ADSC samples III and VII were analyzed using flow cytometry. (C) Immunostaining of ADSCs using *CCR1* antibody (red) and DAPI (blue).

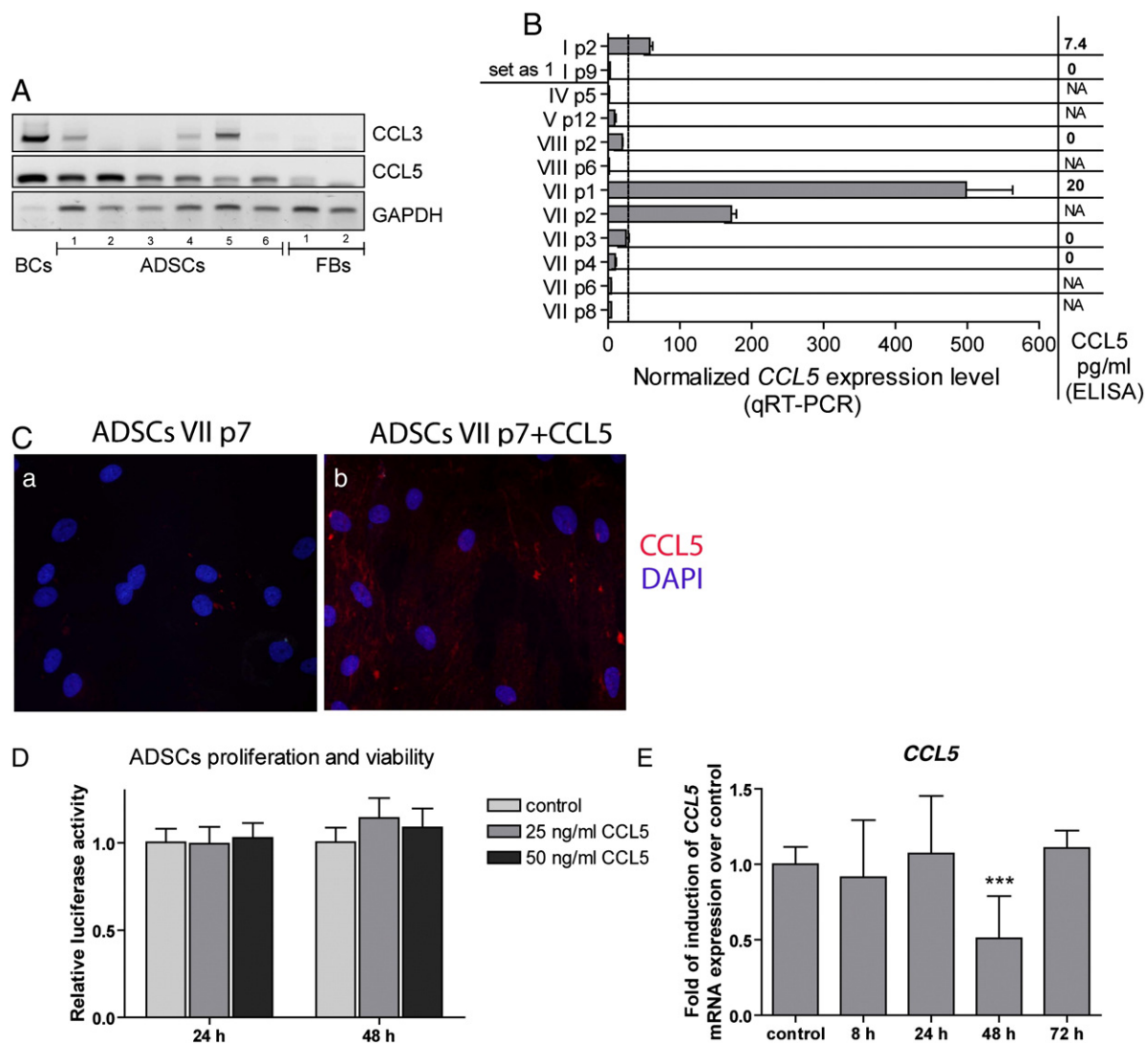


Figure 3 ADSCs may be affected by endogenous CCL5 signaling. (A) Expression of CCR1 ligands *CCL3* and *CCL5* in peripheral blood mononuclear cells (BCs), 6 samples of ADSCs (ADSCs 1–6) and skin primary fibroblasts (FBs 1–2) was examined by RT-PCR (*CCL3/CCL5* and *GAPDH* targets were amplified for 40 and 20 cycles, respectively). (B) The level of *CCL5* mRNA expression in different samples of ADSCs (I, IV, V, VII, VIII) cultivated for different periods of time (passages p1–p12) was measured using qRT-PCR, normalized with *GAPDH* expression level and set as 1 in the sample I p9. The other samples were calculated accordingly. The data are presented as an average mean of one measurement performed in triplicates \pm SD. Concurrently, concentrations of secreted CCL5 protein (pg/ml) in the conditioning media of the samples I p2, I p9, VIII p2, VII p1, VII p3 and VII p4 were measured by ELISA; NA – not analyzed. (C) CCL5 protein in permeabilized naive or treated with 50 ng/ml of recombinant CCL5 ADSCs (left and right panels, respectively) was examined using CCL5 antibody (red). Nuclear staining was performed using DAPI (blue) (40 \times magnification). (D) Proliferation and viability of ADSCs cultured in the presence of 25 and 50 ng/ml of CCL5 for 24 and 48 h were tested using ViaLight™ plus kit. (E) Three independent lineages of ADSCs were treated with 50 ng/ml of CCL5 for 8, 24, 48 and 72 h, and the level of *CCL5* mRNA expression was measured in triplicates, normalized with *GAPDH* expression level and set as 1 in untreated samples. The data are presented as fold of induction of *CCL5* expression in CCL5 treated samples over untreated controls \pm SD; ** $p < 0.01$.

(Fig. 2A, donors I p2 and p9, IV p5, V p12, VIII p2 and p7, VII p1, p2, p3, p4, p6 and p8). Cells were cultured for 3 days in normal growth medium, and lysed for total RNA isolation. *CCL5* mRNA expression levels were normalized using *GAPDH* expression levels and set as 1 in the sample I p9. Expression of *CCL5* in other samples was calculated based on the value of sample I p9. Before lysis of cells, media of samples I p2 and I p9, VI p2, VII p1, VII p3 and VII p4 were collected and analyzed for secreted CCL5 protein using ELISA. Data of both analyses are presented in Fig. 3B. Expression of *CCL5*

decreased dramatically during the propagation of ADSCs. Secreted CCL5 was detected only in the media of the samples with the highest expression of *CCL5* mRNA (I p2 and VII p1). Secretion of CCL5 was not detectable (less than 1 pg/ml) in ADSCs analyzed at later passages and used in the functional assays in the present study. Linear regression analysis revealed no correlation between expression levels of *CCR1* and *CCL5* (coefficient of determination $R^2 = 0.0685$).

IF analysis showed that a small number of ADSCs expressed CCL5 protein and expression of CCL5 varied significantly

between cells (Fig. 3C and Supplementary Fig. 3). Cells treated with recombinant CCL5 protein for 18 h were used as a positive control. Regardless of low levels of CCL5 protein, our data suggest that CCR1 may be affected by endogenous CCL5 signaling in ADSCs. Nevertheless, co-localization of CCR1 and CCL5 was detected only on some non-permeabilized ADSCs treated with recombinant CCL5 for 30 min (Supplementary Fig. 3C).

Proliferation and viability of ADSCs incubated in the presence of 25 or 50 ng/ml CCL5 for 24 or 48 h were not inhibited (Fig. 3D). In order to test whether CCL5 signaling influences *CCL5* expression in ADSCs, the cells were treated with CCL5 for 8, 24, 48, and 72 h and the levels of *CCL5* expression were measured by qRT-PCR in the control and treated samples. The data are presented as an average mean of three independent experiments \pm SD (Fig. 3E). Treatment with CCL5 for 8, 24 or 72 h had no significant effect on endogenous *CCL5* expression. However, expression of *CCL5* decreased approximately 2 times within 48 h in response to exogenous CCL5.

ADSCs respond to CCL5

CCR1 belongs to the GPCR family and is activated by several ligands including CCL5. Upon ligand binding, GPCR may be rapidly internalized (Drake et al., 2006). It has also been reported that CCL5 as some other chemokines may exert its biological effects in a GPCR independent manner via interacting with specific cell surface glycosaminoglycan chains and heparin sulfate proteoglycans (Martin et al., 2001; Roscic-Mrkic et al., 2003; Slimani et al., 2003; Wu and Yoder, 2009). To analyze effect of CCL5 on CCR1 subcellular localization in ADSCs, the cells were treated with 50 ng/ml of CCL5 for 3 h and subjected to immunostaining using CCR1 antibody. Localization of CCR1 protein was assessed in at least 500 cells treated or untreated with CCL5 using fluorescent microscopy, and representative images are shown in Fig. 4A (right and left panels, respectively). Approximately 10% of native ADSCs were positive for CCR1. In response to CCL5 stimulation, the number of CCR1⁺ cells decreased notably. The loss of CCR1 from the surface of

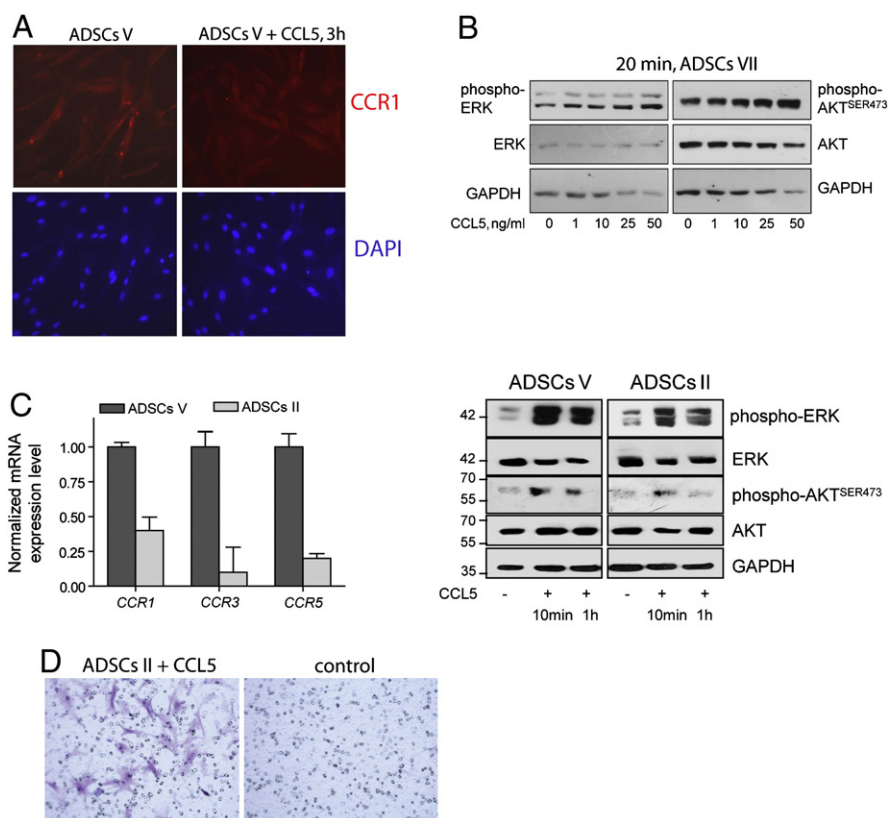


Figure 4 ADSCs respond to CCL5. (A) ADSCs were stimulated with 50 ng/ml of CCL5 for 3 h (right panel) and subjected to immunostaining using CCR1 antibody (red) and DAPI (blue). Non-stimulated cells are shown in the left panel. (B) ADSCs were treated with different concentrations of CCL5 for 20 min. The levels of phospho-AKT, phospho-ERK, AKT, ERK and GAPDH proteins were detected by WB. (C) *CCR1*, *CCR3* and *CCR5* expression levels in two ADSC samples were measured in triplicates by qRT-PCR, normalized with *GAPDH* mRNA expression levels and set as 1 in sample V (left panel). ADSCs were stimulated with 50 ng/ml of CCL5 for 10 min or 1 h, the cells were lysed and subjected to WB analysis using phospho-ERK, phospho-AKT, ERK, AKT and GAPDH antibodies. (D) Migration capacity of ADSCs towards 50 ng/ml of CCL5 (left panel) was examined using a modified Boyden chamber. As a control, CCL5-free media were used (right panel). The migratory cells were stained and observed under a light microscope using 20 \times objective.

ADSCs upon CCL5 stimulation might be a result of its internalization suggesting functional interaction between CCL5 and CCR1.

Several chemokines are known to mediate their effects through mitogen activated protein kinases (MAPK or ERK1/2) that are activated upon phosphorylation of their specific tyrosine and threonine residues (Garrington and Johnson, 1999; Pearson et al., 2001). Also, stimulation of cells with chemokines leads to the activation of PI3K that induces its downstream effectors including AKT kinase activated via phosphorylation of its serine residue 473 (Alessio et al., 2010). It has been shown that stimulation of BMSCs with several chemokines triggers activation of ERK and AKT kinases (Honczarenko et al., 2006). To assess, whether CCL5 leads to the activation of the same signaling cascades in ADSCs, the cells were treated with CCL5 at different concentrations (1, 10, 25 and 50 ng/ml corresponding to 0.13, 1.3, 3.2 and 6.4 nM) for 20 min following WB analysis using antibodies specific for total and phosphorylated forms of ERK and AKT kinases (Fig. 4B). ERK and AKT kinases were activated in a CCL5 concentration dependent manner. Activation of the kinases was achieved at the CCL5 concentration of 1 ng/ml; however, higher concentrations of CCL5 resulted in stronger effects.

To identify the correlation between the kinase activation and *CCR1* expression levels, two lineages of ADSCs expressing different levels of *CCR1* were used (Fig. 4C, left panel). These cells also expressed different levels of other CCL5 receptors, such as *CCR3* and *CCR5*. The cells were treated with 50 ng/ml of CCL5 for 10 min and 1 h and analyzed with WB (Fig. 4C, right panel). Phosphorylation of ERK and AKT kinases occurred already within 10 min upon CCL5 stimulation. The intensity of ERK and AKT phosphorylation correlated with *CCR1* expression levels. ERK remained activated and AKT phosphorylation decreased to the control level following 1 h of treatment.

It has been shown that BMSCs are capable of migration towards chemokines in vitro (Fox et al., 2007; Honczarenko et al., 2006). Recent study suggests that ADSCs also migrate in response to chemotactic stimuli (Baek et al., 2011). In the current study the Boyden chamber test was used to assess the ability of ADSCs to migrate in the presence of CCL5. Analysis showed that ADSCs were able to migrate towards CCL5 (Fig. 4D).

CCL5 signaling activates NF κ B and alters the expression of NF κ B target genes

It has been reported that stimulation of CCR1 by CCL5 drives the activation of nuclear factor-kappa-B (NF κ B) in human osteosarcoma cells (Jang et al., 2007). Also, some inflammatory cytokines of CXC family are known to stimulate the NF κ B signaling pathway in cancer and immune cells (Ye, 2001). To analyze the effect of CCL5 signaling on activation of NF κ B pathway in ADSCs, cells were co-transfected with a construct encoding firefly luciferase under the control of NF κ B dependent promoter together with a plasmid encoding Renilla luciferase under SV40 promoter following stimulation of the cells with 50 ng/ml of CCL5. The average values of firefly luciferase activity normalized with Renilla luciferase activity obtained from three independent experiments

measured in three replicates \pm SD are presented in Fig. 5A. Upon CCL5 treatment, NF κ B transcriptional activity increased in a dose dependent manner.

Analyses of the expression levels of several NF κ B target genes upon stimulation of ADSCs with 50 ng/ml of CCL5 using qRT-PCR showed that expression of analyzed target genes was up-regulated. The average means of induction of gene expression levels over non-stimulated controls \pm SD obtained from the analysis of three independent lineages of ADSCs are shown in Fig. 5B. Treatment of ADSCs with CCL5 for 8 h led to an increased expression of *interleukin-6* (*IL-6*), and

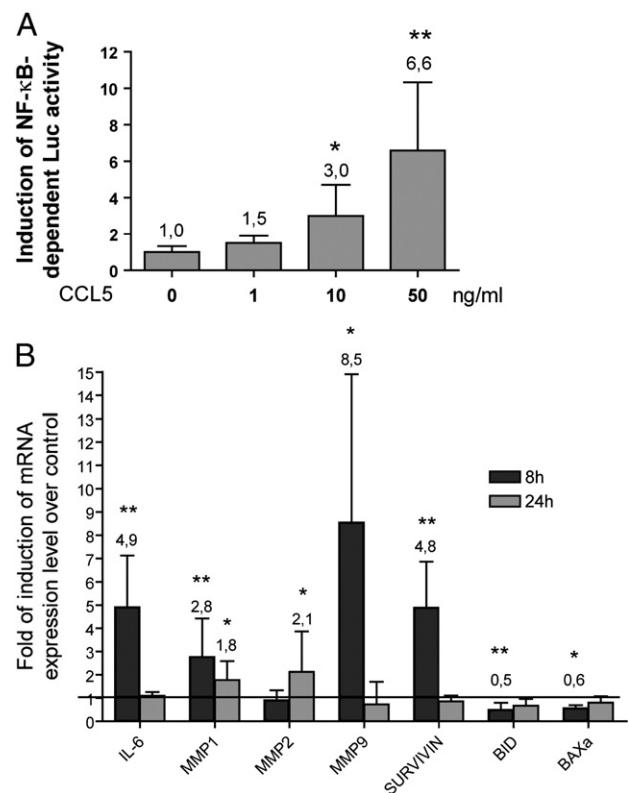
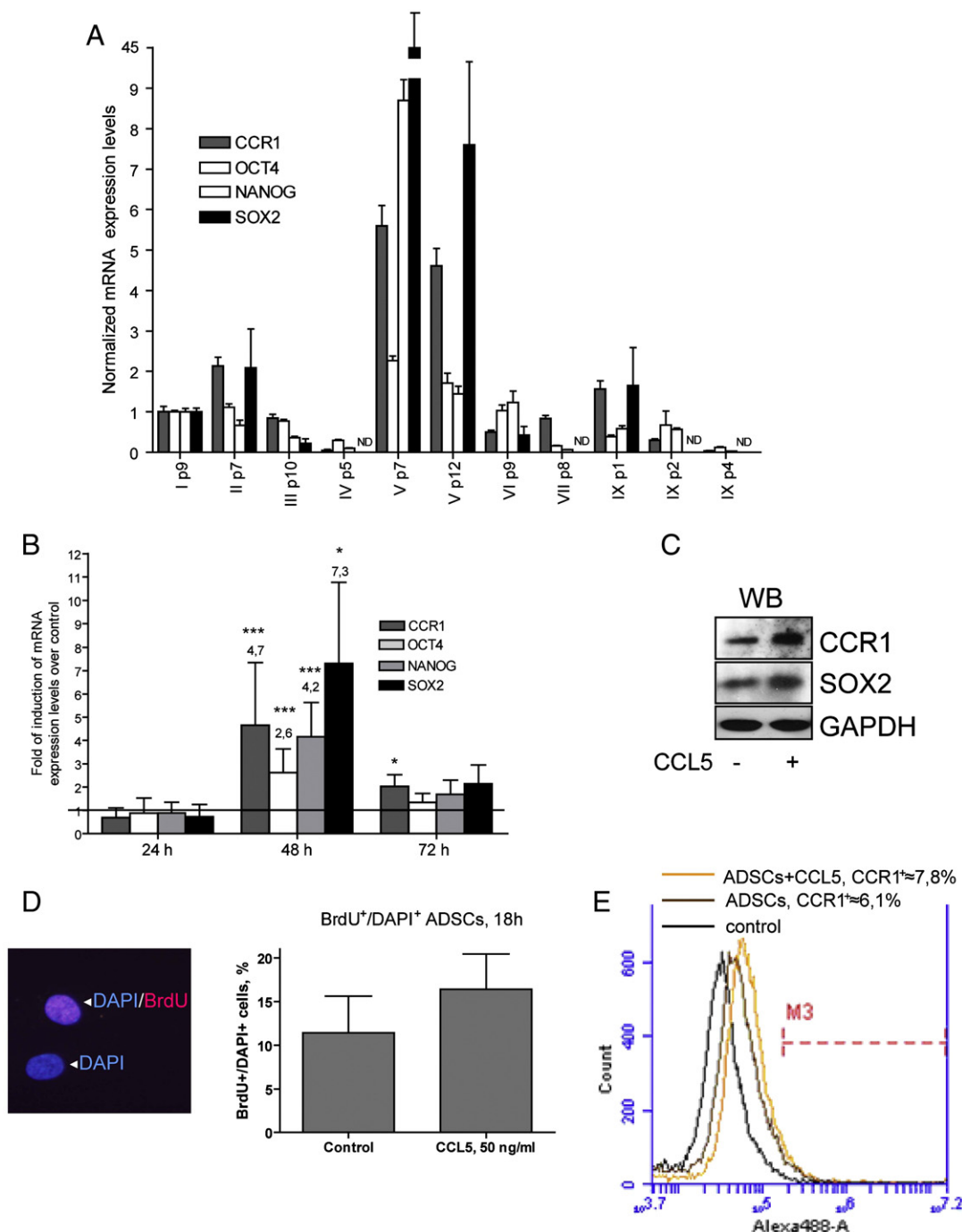


Figure 5 CCL5 induces NF κ B transcriptional activity. (A) ADSCs were co-transfected with plasmids encoding firefly luciferase reporter under the control of NF κ B dependent promoter and Renilla luciferase reporter under SV40 promoter. The cells were stimulated with CCL5 at indicated concentrations for 24 h. Average firefly luciferase activity obtained from three independent experiments measured in three replicates were normalized to Renilla luciferase values and presented as a fold of induction \pm SD over luciferase activity in a non-stimulated ADSC sample (set as 1). (B) ADSCs were stimulated with 50 ng/ml of CCL5 for indicated periods of time and several NF κ B target gene mRNA expression levels were analyzed by qRT-PCR. Target gene mRNA levels were measured in triplicates and normalized with *GAPDH* mRNA expression level. The level of the particular gene expression in the untreated control cells at the same time point was set as 1 (indicated by line). Data are represented as an average mean of fold of induction of the indicated gene expression over non-stimulated control \pm SD obtained from three independent experiments. ((A, B) * p <0.05, ** p <0.01.)

matrix-metalloproteinase (MMP) 1 and 9. Expression of MMP2 was up-regulated within 24 h. Expression levels of tissue inhibitor of metalloproteinases (TIMP) 1 and 2, inhibitors of MMPs, did not change (data not shown). These data suggest that total activity of MMPs involved in ECM remodeling processes required for initiation of migration may be up-regulated in response to CCL5. Also, increased expression of anti-apoptotic and pro-mitotic gene *SURVIVIN* was detected. In contrast, expression of pro-apoptotic genes *BID* and *BAX α* decreased. After 24 h of induction, the expression levels of all tested genes except *MMP1* and 2 decreased approximately to the control level.

Expression of *OCT4*, *SOX2* and *NANOG* positively correlates with the expression of *CCR1*

Transcription factors regulating maintenance of the pluripotent state in embryonic stem cells, such as *SOX2*, *OCT4* and *NANOG*, have been suggested to play similar roles also in adult stem cells. Expression of *OCT4*, *NANOG* and *SOX2* was analyzed in ADSCs derived from 8 different individuals (I–VII and IX) and cultured for different number of passages (p2–p12) by qRT-PCR (Fig. 6A). Comparison of the expression of *OCT4*, *NANOG* and *SOX2* with the expression of *CCR1* showed statistically significant correlation between the gene



expression levels. Linear regression analysis of gene expression levels revealed the following determination coefficients and p values: $R^2(\text{CCR1};\text{OCT4})=0.78$, $p=0.0003$; $R^2(\text{CCR1};\text{NANOG})=0.62$, $p=0.004$; $R^2(\text{CCR1};\text{SOX2})=0.67$, $p=0.0021$; $R^2(\text{NANOG};\text{OCT4})=0.65$, $p=0.0027$; $R^2(\text{SOX2};\text{OCT4})=0.60$, $p=0.0047$; $R^2(\text{SOX2};\text{NANOG})=0.98$, $p<0.0001$ (Supplementary Fig. 4).

To explore whether CCL5 signaling induces alterations in the expression levels of *CCR1* and multipotency genes *OCT4*, *SOX2*, *NANOG*, ADSCs were treated with 50 ng/ml of CCL5 for indicated periods of time. *CCR1*, *OCT4*, *SOX2* and *NANOG* mRNA expression levels in induced and non-induced cells were measured by qRT-PCR. The average induction of the gene expression levels over non-treated controls \pm SD obtained from three independent experiments are shown in Fig. 6B. Expression of receptor *CCR1* and multipotency genes *OCT4*, *NANOG* and *SOX2* increased after 48 h of treatment and remained slightly elevated for 72 h following induction with CCL5. To confirm the qRT-PCR results, ADSCs were treated with CCL5 for 48 h, and cell lysates were subjected to WB analysis using *CCR1* and *SOX2* antibodies (Fig. 6C). Compared with non-stimulated cells, the levels of *CCR1* and *SOX2* proteins were increased within 48 h in response to CCL5.

CCL5 signaling up-regulated *SURVIVIN* expression within 8 h. Increase of *CCR1*, *OCT4*, *NANOG* and *SOX2* expression was detected after 48 h of treatment initiation. These data suggest that CCL5 may stimulate proliferation of ADSCs and, in particular, *CCR1*⁺ cells. To test this hypothesis, the cells were treated with 50 ng/ml of CCL5 and 10 μ M BrdU for 18 h, and assessed for a portion of BrdU⁺ cells by IF. ADSCs exposed only to BrdU were used as a control. Cells considered as DAPI⁺ and BrdU⁺/DAPI⁺ are depicted in the left panel of Fig. 6D. BrdU incorporation was assessed in at least 200 DAPI⁺ ADSCs isolated from three donors, and the data of cell counting are presented as an average mean of BrdU⁺/DAPI⁺ cells \pm SD in the right panel of Fig. 6D. Cell counting revealed that approximately 11.4 \pm 4.2% of ADSCs were positive for BrdU. Treatment with CCL5 led to increase of BrdU⁺ ADSCs to 16.4 \pm 4%. Since double staining of the cells for *CCR1* and BrdU was inefficient for technical reasons, the portion of *CCR1*⁺ cells in ADSCs treated with CCL5 for 48 h was assessed using flow cytometry. The data of a representative experiment performed using ADSCs VIII p7 are shown in Fig. 6E. Amount of *CCR1*⁺ cells

was increased approximately 28 \pm 14% in response to CCL5 signaling.

Discussion

Stromal cells derived from different tissues represent a promising clinical tool for cell therapy targeting a variety of diseases. Efficiency of the cell therapy depends highly on the number of delivered cells and appropriate homing of the cells in the recipient. Therefore, comprehensive understanding of the molecular mechanisms that regulate cell migration and homing enhances safety and efficacy of cell therapies.

The mechanisms of ADSC migration are still largely unknown. Migration of ADSCs in vitro is shown to be induced by several chemokines, VEGF, PDGF, LPA and TxA2 through activation of their biologically active receptors (Amos et al., 2008; Baek et al., 2011; Kang et al., 2005; Lee et al., 2008; Yun et al., 2009). Besides, ex vivo expanded ADSCs are capable of in vitro and in vivo migration towards tumor cells that are known to secrete chemokines and cytokines (Lamfers et al., 2009). However, the expression and biological activity of chemokine receptors in ADSCs are poorly investigated.

Recently, Baek and co-authors have published a paper describing expression of the six chemokine receptors *CCR1*, *CCR2*, *CCR7*, *CXCR4*, *CXCR5* and *CXCR6* in ADSCs (Baek et al., 2011). Here we show that ADSCs express transcripts for 13 out of 20 chemokine receptors (*CCR1*, *CCR2*, *CCR3*, *CCR5*, *CCR10*, *CCR11*, *CXCR1*, *CXCR4*, *CXCR5*, *CXCR6*, *CXCR7*, *XCR1* and *CX3CR1*). Most of the chemokine receptors are expressed at low levels in ADSCs. *CCR1* is one of the receptors showing relatively high expression in ADSCs. Its expression also has high individual variability with more than 50 fold differences in the mRNA levels in ADSCs isolated from different donors. Moreover, variations of *CCR1* expression have been observed also at the single cell level. These differences reflect the heterogeneity of ADSCs, which is a common feature of these cells.

It has been shown that freshly isolated BMSCs contain approximately 70% of *CCR1*⁺ cells, and their ratio decreases to 25–5% during culturing to passages 12–16, respectively (Honczarenko et al., 2006). Our flow cytometry analyses of different ADSC samples suggest that less than 25% of cells are

Figure 6 Expression of *OCT4*, *NANOG* and *SOX2* correlates with the expression of *CCR1*. (A) Expression levels of *OCT4*, *NANOG*, *SOX2* and *CCR1* were analyzed in 9 pools of ADSCs (I–IX) cultivated for different periods of time (passages p2–p10) by qRT-PCR. The expression levels were measured in triplicates, normalized with *GAPDH* mRNA expression level and set as 1 in sample I (I p9). Gene expression levels in other samples were calculated relatively to the sample I p9. (B) ADSCs were treated with 50 ng/ml of CCL5 during indicated periods of time. *OCT4*, *NANOG*, *SOX2* and *CCR1* mRNA expression levels were analyzed by qRT-PCR and normalized with *GAPDH* expression level. The level of the particular gene expression in the untreated control cells at the same time point was set as 1 (indicated by line). The data are represented as an average mean of fold of induction of the particular gene mRNA expression over non-stimulated control \pm SD obtained from three independent experiments; * $p<0.05$, *** $p<0.001$. (C) ADSCs were induced with 50 ng/ml of CCL5 for 48 h, if indicated. The cell lysates were subjected to WB analysis using *CCR1*, *SOX2* and *GAPDH* antibodies. (D) ADSCs were treated with 10 μ M BrdU and, if indicated, 50 ng/ml of CCL5 during 18 h and subjected to immunostaining using BrdU primary and Alexa Fluor 568 secondary antibodies and DAPI (blue). Pink nuclei were considered as BrdU⁺/DAPI⁺, and blue nuclei were considered as DAPI⁺ (left panel). BrdU staining was assessed in at least 200 DAPI⁺ cells, and a percentage of BrdU⁺ cells were calculated. Cell counting data are presented as an average mean of three independent experiments \pm SD (right panel). BrdU staining was detected in 11.4 \pm 4.2% and 16.4 \pm 4% of DAPI⁺ cells in the control and CCL5 stimulated samples, respectively. (E) ADSCs were stimulated with 50 ng/ml of CCL5, if indicated, and the number of *CCR1*⁺ cells was measured by flow cytometry using the *CCR1* antibody. Brown and orange lines indicate *CCR1*⁺ cells that are untreated and treated with CCL5 ADSCs, respectively. ADSCs stained with the secondary antibody were used as a negative control (black).

positive for CCR1, and pools of BCs contained approximately 70% of CCR1⁺ cells. Our data show that *CCR1* expression as well as expression of several other chemokine receptors (*CCR5*, *CCR10*, *CXCR4* and *CX3CR1*) is highest in the freshly isolated cells analyzed at passage p2. Expansion of ADSCs to the next passage p3 causes dramatic reduction of expression of most of the chemokine receptors except *CXCR6* and *CXCR7*. Down-regulation of chemokine receptors in isolated ADSCs within first passages may partly be accounted for by the possible presence of CCR1⁺ plastic adherent blood monocytes (Weber et al., 2001; Yona and Jung, 2010). However, monocytes are not dividing cells under regular in vitro culturing conditions; if they contaminate a culture of dividing cells, their portion decreases during in vitro culturing. Nevertheless, our flow cytometry analyses have shown that the portion of CCR1⁺ cells exceeds several times the portion of CD45⁺ hematopoietic cells in ADSCs even at p2, suggesting the existence of non-hematopoietic CCR1⁺ cells in ADSCs. The observed down-regulation of *CCR1* expression is most likely a result of the apoptosis of CCR1⁺ cells of non-hematopoietic origin. Further culturing of ADSCs from passages p3 to p12 (approximately 6 weeks of in vitro cultivation) did not significantly affect expression of *CCR1* in majority of the samples.

BMSCs and ADSCs also express detectable levels of the CCR1 ligand *CCL5* mRNA (Honczarenko et al., 2006). Expression of *CCL5*, similarly to expression of *CCR1*, is the highest in the freshly isolated ADSCs, but it decreases within each subsequent passage in all ADSCs analyzed. Interestingly, proliferation of CCR1⁺ cells and the resulting increase of *CCR1* expression in response to exogenous *CCL5* signaling are accompanied with a decrease in *CCL5* expression. This may be a result of a decrease in a subset of *CCL5*⁺ cells in ADSC populations or transcriptional down-regulation of *CCL5* expression in CCR1⁺ cells. Although expression of *CCL5* has been detected in some single permeabilized ADSCs by IF, levels of the secreted *CCL5* protein in the media of most ADSC cultures analyzed were lower than 1 pg/ml (detection threshold of the used ELISA kit). These data can be explained by postulating that secreted *CCL5* binds to its receptors on CCR1, CCR3 or CCR5-positive ADSCs or *CCL5* becomes sequestered by heparan sulfate proteoglycans or glycosaminoglycan chains of some cell surface proteins, e.g. CD44 (Roscic-Mrkic et al., 2003; Slimani et al., 2003). Taken together, our data suggest that CCR1⁺ ADSCs may be a subject of paracrine or autocrine regulation by *CCL5*.

It has been demonstrated that CCR1 is biologically functional in BMSCs (Honczarenko et al., 2006). Here we have shown that *CCL5* induces biological responses via CCR1, indicating that the *CCL5*/CCR1 axis is functional also in ADSCs. *CCL5* may also bind to the CCR3 and CCR5 receptors; however, according to our data, expression of these receptors is very low or not detectable in ADSCs. We show that *CCL5* signaling leads to activation of the NF κ B pathway, which is known to be involved in the regulation of cell proliferation, survival, migration and invasion processes (Hayden and Ghosh, 2012; Wu and Zhou, 2010). NF κ B transcriptional activity as well as expression of several NF κ B target genes is altered in ADSCs in response to *CCL5* treatment. We demonstrate rapid up-regulation of genes encoding endopeptidases (*MMP1*, *MMP2* and *MMP9*) that hydrolyze the components of ECM (e.g. collagen, fibronectin, laminin), thus contributing to migratory and wound healing processes (John and Tuszyński, 2001). Up-regulation of *MMP* points to the ability

of ADSCs to re-modulate ECM in response to *CCL5* stimulation. Furthermore, it is an indication of activation of migratory mechanisms in ADSCs demonstrated by cell migration assay here and by others (Baek et al., 2011). Nevertheless, further analyses are needed to elucidate, whether CCR1⁺ ADSCs are also capable of chemotaxis in vivo and whether these cells are able to differentiate and contribute to processes of tissue regeneration.

The NF κ B pathway has been proposed to trigger pro- and anti-apoptotic signals in different cells (Chu et al., 1997; Ryan et al., 2007). Here we show that the NF κ B pathway activated upon *CCL5* stimulation rather inhibits cell death in ADSCs. *CCL5* signaling suppresses the expression of pro-apoptotic genes *BID* and *BAX α* , whereas expression of *SURVIVIN*, an inhibitor of apoptosis and important positive regulator of cell cycle activated in G2/M phases, increases (Chandele et al., 2004). Moreover, we show that a number of BrdU⁺ and CCR1⁺ cells increases in response to *CCL5* signaling after 18 and 48 h of treatment initiation, respectively. Taken together, these data indicate that *CCL5* induces proliferation of CCR1⁺ ADSCs.

It has been suggested that molecular mechanisms regulating the undifferentiated state of stromal stem/progenitor cells from different tissues are related to the expression of distinct transcription factors, such as OCT4, SOX2 and NANOG (Greco et al., 2007; Park et al., 2011; Saulnier et al., 2011). Down-regulation of these factors is associated with loss of multipotency and self-renewal, and is proposed to state the beginning of subsequent differentiation steps (Meshorer and Misteli, 2006; Ng and Surani, 2011; Pan et al., 2006). Here we show that *OCT4*, *SOX2* and *NANOG* are expressed in ADSCs; however, expression of *SOX2* has not been detected in some pools of ADSCs, distinguished by the lower expression of other multipotency genes and *CCR1*. Interestingly, the expression levels of *OCT4*, *NANOG* and *SOX2* demonstrated statistically significant correlation with the expression levels of *CCR1* in ADSC samples. Linear regression analysis has revealed that the coefficients of determination (R^2) obtained for the levels of *CCR1* and multipotency gene expression are comparable with that of multipotency genes, with *SOX2*/*NANOG* demonstrating the highest $R^2=0.98$. Moreover, the levels of multipotency gene expression were elevated in concert with up-regulation of *CCR1* expression upon stimulation of ADSCs with *CCL5*. There are at least two possible explanations of this phenomenon. First, the transcriptional up-regulation of multipotency genes via factors unidentified in the present study may occur at a single cell level. Second, since the increase in the gene expression levels is detected following 48 h of treatment initiation, it is more likely that higher mRNA and protein levels occur due to the increased amount of CCR1⁺ cells demonstrated here. These data suggest that one of the features of "real" adipose tissue derived stem/progenitor cells expressing OCT4, NANOG and SOX2 may be the expression of CCR1 receptor. The presented data suggest that the *CCL5*/CCR1 axis participates in the regulation of ADSC stemness and multipotency, and consequently may have an impact on differentiation of ADSCs. Also, data presented here suggest a possible method for separating populations of ADSCs possessing higher stemness-related properties by exploiting the expression of CCR1 receptor.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.scr.2012.11.004>.

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