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High-density Lipoproteins Induce the Migration Capacity of Mesenchymal Stromal Cells

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Abstract

Background: Based on the regenerative capacity of HDL and their ability to induce migration of endothelial cells, we aimed to investigate whether HDL can influence the migration of mesenchymal stromal cells (MSC) and to analyze the underlying mechanisms.

Methods and results: MSC express the SR-BI receptor as shown by flow cytometry. Supplementation of HDL or their main apolipoprotein (apo), apo A-I, induces the phosphorylation state of Akt and NO production in MSC. This is associated with an increase in lamellipodia formation as demonstrated via phallotoxin staining and further leads to an induction in migration capacity as indicated by a 1.4-fold ($p<0.05$) and 1.4-fold ($p<0.05$) higher presence of MSC in the lower chamber of a modified Boyden chamber supplemented with HDL or apo A-I, respectively, compared to basal medium. In addition, the migration capacity of MSC in a wound healing assay 24 h after scratching was 1.7-fold ($p<0.05$) and 1.2-fold ($p<0.05$) higher in HDL-and apo A-I-supplemented hydroxyurea-treated MSC compared to basal hydroxyurea-treated MSC. In both assays, the HDL or apo A-I stimulated migration of MSC was reduced in the presence of the phosphatidylinositol-3-kinase (PI3K) inhibitor Ly294002.

Conclusion: HDL induces the migration of MSC in a PI3K-dependent manner.

Keywords: Mesenchymal stromal cells; HDL; Migration

Abbreviations: Apo A: I Apolipoprotein A-I; HDL: High-Density Lipoproteins; MSC: Mesenchymal Stromal Cells; PI3K: phosphatidylinositol-3-Kinase

Introduction

Epidemiological studies [1,2] and studies in experimental animal models [3-5] consistently demonstrate that low high-density lipoprotein (HDL) cholesterol levels are a cardiovascular risk factor. The cardiovascular-protective effects of HDL have mainly been attributed to the role of HDL in "reverse cholesterol transport", the transport of excess of cholesterol from the periphery towards the liver. Though, meanwhile also the pleiotropic effects, including the anti-oxidative [6], anti-inflammatory [4,7,8], anti-apoptotic [9,10], and pro-angiogenic [11] features of HDL are well recognized. These effects are particularly attributed to apolipoprotein (apo) A-I, the main apolipoprotein of HDL, which constitutes 70% of the protein content of HDL. Consequently, a strong correlation between apo A-I plasma concentrations and HDL cholesterol levels exists [12]. Besides the above mentioned pleiotropic effects, HDL also have a regenerative potential: they induce the mobilization of endothelial progenitor cells (EPC)/ circulating angiogenic cells from the bone marrow [5] and promote their incorporation at the site of endothelial damage [13,14].

Mesenchymal stromal cells (MSC) are attractive candidates for cell therapy given their immunomodulatory [15-17], anti-oxidative [15], anti-apoptotic [15], anti-fibrotic [15,16,18], pro-angiogenic [19-21] features and their capacity to migrate towards the site of injury [16,22-24]. Their ability to preferentially engraft into inflamed or ischemic injury follows from experimental animal studies [16,23] as well as from studies showing that endogenous MSC can be mobilized from the bone marrow and recruited into the inflamed [24] or ischemic [22] heart. This characteristic empowers their therapeutic efficacy and favors the intravenous application of MSC as administration route [25]. In fact, approximately half of the clinical trials with MSC involve the systemic administration of MSC into the vasculature [26]. The migratory capacity of MSC towards chemokines and growth factors has already been intensively studied [27]. Though, the impact of HDL on the migration of MSC has not been explored so far.

Based on the capacity of HDL to induce migration of endothelial cells and their regenerative potential, we aimed to investigate whether HDL can influence the migration of MSC.

Materials and Methods

Mesenchymal stromal cell isolation and cell culture

Human adult MSC were isolated from iliac crest bone marrow aspirates of normal male donors after their written approval. The aspiration of iliac crest bone marrow was approved by the ethical committee of the Charité-Universitätsmedizin Berlin (EA1/131/07). Aspirates (3-5 ml) were washed twice with phosphate buffered saline (PBS) (Biochrom, Berlin, Germany), and resuspended in Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen) supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, 1% glutamine, 2% HEPES and 2 ng/ml of basic fibroblast growth factor

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(Tebu-bio, Offenbach, Germany). Cells were purified using a percoll gradient at a density of 1.073 g/ml (Biochrom). Next, cells were washed with PBS and then resuspended in complete DMEM. Cells were plated at a density of 3×10^5 cells/cm^2 and cultured under standard cell culture conditions. Medium was exchanged after 72 hours (h) and every 3 days thereafter. Reaching 90% confluence, cells were trypsinized and replated at a density of 5×10^3 cells/cm^2.

MSC were stimulated with 5 µg of HDL protein/ml or 3.5 µg/ml of apo A-I. Since previous *in vitro* experiments with HDL showed protective effects at 5 µg/ml [4] and 50 µg/ml [6,8,28] of HDL, we first compared the impact of 5 and 50 µg/ml of HDL on MSC migration. This experiment illustrated a more pronounced effect by 5 µg/ml (data not shown). We chose for 3.5 µg/ml of apo A-I, since apo A-I comprises 70% of HDL protein content.

Human aortic endothelial cells (HAEC) (Lonza Walkersville, Walkersville, MD, USA) were cultured in EBM-2 basal medium supplemented with EGM-2 Single Quots (Lonza, USA).

Characterization of mesenchymal stromal cells

MSC were characterized by flow cytometry analysis according to Binger et al. [29] with PE-labeled monoclonal mouse anti-human CD14, CD34, CD73, CD166 and FITC-labeled mouse anti-human CD44, CD45, CD90 and CD105 antibodies (Figure 1). Cells were washed with PBS-BSA 0.5%, resuspended in 100 µl of PBS-BSA 0.5% and incubated with titrated concentrations of antibodies at 4°C for 15 min. Prior to flow cytometry analysis, cells were washed with PBS-BSA 0.5%.

Scavenger receptor-BI flow cytometry

MSC and HAEC, both in passage 5, were cultured in a 6-well plate until 90% confluence, and then trypsinized, collected, and resuspended in cold FACS buffer. Flow cytometry analysis was performed on a *MACSQuant* Miltenyi Biotec after cell labeling with rabbit polyclonal anti-SR-BI primary antibody (Novus Biologicals) and goat anti-rabbit IgG secondary antibody (Invitrogen) at 4°C for 60 minutes. SR-BI positive cells were analyzed with FlowJo 8.7. software (Tree Star).

Western blot

Three minutes (min) after stimulation with 5 µg/ml of HDL (MP Biomedicals, Solon, Ohio, USA) or 3.5 µg/ml of apo A-I (Sigma), cells were lysed in lysis buffer (Invitrogen) containing proteinase inhibitors (Roche). An equal amount of protein was loaded into a SDS-polyacrylamide gel. p-Akt and Akt antibodies (Cell signaling), and β tubulin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were detected with the respective antibody, followed by incubation with an IR dye secondary antibody (LI-COR Biosciences, Lincoln/Nebraska, USA). All blots were visualized with Odyssey (LI-COR Biosciences). Quantitative analysis of the intensity of the bands was performed with Odyssey V3.0 software.

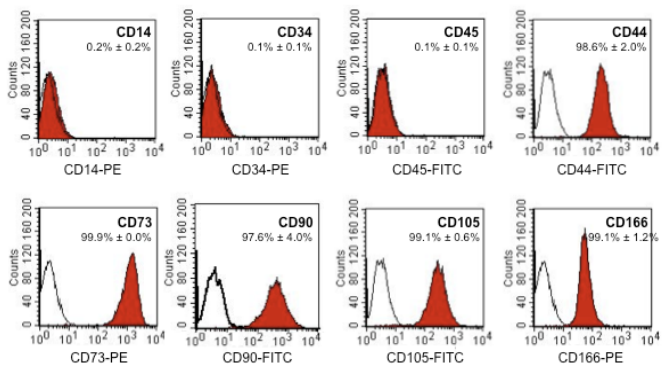

Figure 1: Representative flow cytometry analysis of mesenchymal stromal cells. Flow cytometry histograms indicate that MSC are CD14-, CD34-, CD45- and CD44+, CD73+, CD90+, CD105+ and CD166+. Numbers above the red histograms indicate the mean ± SEM of the percentage (%) of gated cells which are positive of n=3 different donors.

Nitric oxide measurement

Intracellular NO was measured with DAF-FM diacetate (4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate, Invitrogen) as described previously [30]. Following a pretreatment of 2h with 1 µM of the PI3K-inhibitor Ly 294002 (Calbiochem, EMD Chemicals,Gibbstown, NJ, USA) for all conditions with Ly 294002, MSC were supplemented with 5 µg/ml of HDL or 3.5 µg/ml of apo A-I for 5 min in the presence or absence of Ly 294002. Next, MSC were incubated at 37°C for 30 min in PBS containing 1 µM of DAF-FM diacetate. After loading, cells were rinsed two times with PBS and incubated with fresh PBS at 37°C for 30 min. NO fluorescence intensity was read in a Berthold Mithras LB 940 reader at 495 nm excitation and 515 nm emission wavelength.

Lamellipodia formation

MSC (7,500 cells/well) were plated in a CellCarrier black 96-well plate (PerkinElmer, Waltham, Massachusetts, USA). After 24h, cells were treated with 3.5 µg/ml apoA-I, or 5 µg/ml HDL for 5 min, 10 min, 15 min and 30 min, respectively, and fixed with 4% paraformaldehyde. Twenty-four hours after fixation, MSC were stained with 3.6 µM DAPI (Sigma) and 6.9 µM phallotoxin (Alexa Fluor 546). Fluorescent images were taken by Operetta® high content screening system (PerkinElmer, Inc. USA) using the 20x LWD objective. Cells with lamellipodia were evaluated from 3 to 4 fields with approximately 40-50 cells/field.

Modified boyden chamber migration assay

MSC migration was evaluated by using a modified Boyden chamber (Chemo TX® Neuroprobe, Gaitherburg). After reaching confluency, MSC were seeded in the upper chamber at a density of 3×10^4 cells per well in 40 µl of basal medium per well. A volume of 37.5 µl of medium supplemented with 3.5 µg/ml apo A-I or 5 µg/ml HDL, with or without 1 µM of Ly 294002 (Calbiochem) or 10% FBS (positive control) with n=4 per group was placed in the lower chamber. MSC were allowed to migrate for 24h at 37°C and were subsequently stained by crystal violet. The migration of MSC was quantified by absorbance measurement at 595 nm with a spectrophotometer (Spectramax 340 PC®, Molecular devices, USA).

Wound healing assay

MSC were seeded in 6-well plates at a density of 2×10^5 cells per well. After reaching confluency, the medium was subsequently replaced with serum starvation medium (DMEM medium containing 0.01% FBS, 1% penicillin/streptomycin), supplemented with 3.5 µg/ml apoA-I or 5 µg/ml HDL, in the presence or absence of 1 µM of Ly294002. In all the conditions the anti-proliferative agent, hydroxyurea (5 mM, Sigma), was added. MSC were allowed to migrate for 24h at 37°C and the number of migrated MSC was quantified per microscopy field for n=10-12. Migrated MSC were depicted as percentage (%) with the amount of migrated MSC versus basal medium set as 100%.

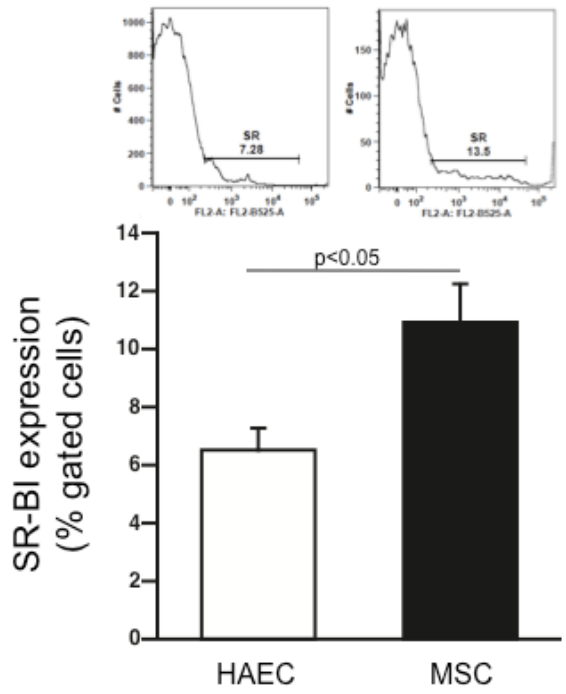

Figure 2: Mesenchymal stromal cells express the SR-BI receptor. Bar graph represents the mean ± SEM of the percentage (%) of SR-BI-positive human aortic endothelial cells (HAEC; open bars) and mesenchymal stromal cells (MSC; black bars), n=5/group.

Statistical analysis

Data are presented as mean ± SEM. Paired and unpaired Student's t tests or Mann Whitney tests were used for statistical analysis. Differences were considered to be significant at $p<0.05$.

Results

HDL-mediated signalling in mesenchymal stromal cells

Flow cytometry analysis demonstrated that MSC express the SR-BI receptor (Figure 2). Supplementation of HDL and apo A-I induced phosphorylation of the downstream target Akt by 1.3-fold ($p<0.05$) and 1.3-fold ($p<0.005$), respectively (Figure 3A). NO production was 1.7-fold ($p<0.001$) and 1.5-fold ($p<0.01$) induced upon HDL and apo A-I supplementation, respectively. This effect was abrogated by the PI3K inhibitor Ly294002, leading to NO levels not significantly different from basal levels (Figure 3B).

HDL induce the migration capacity of mesenchymal stromal cells

Supplementation of MSC with 5 µg/ml of HDL or 3.5 µg/ml of apo A-I induced lamellipodia formation in MSC by 2.0-fold ($p<0.01$) and 1.8-fold ($p<0.05$), respectively, 5min after stimulation as indicated by phallotoxin staining (Figures 4A and 4C). This effect gradually decreased over time with no significant induction after 15 min (Figures 4B and 4D). The HDL- and apo A-I-mediated induction in lamellipodia was reflected in the raised migratory potential of MSC as shown by Boyden chamber and wound healing assays.

The amount of migrated MSC was 1.4-fold ($p<0.05$), 1.4-fold ($p<0.05$), and 2.0-fold ($p<0.05$) higher in the lower chamber of the Boyden chamber containing 5 µg/ml HDL, 3.5 µg/ml apo A-I (Figure 5A) or 10% FBS (positive control; data not shown) compared to basal medium, respectively. Twenty-four hours after scratching and HDL or apo A-I stimulation in the presence of the anti-proliferative agent hydroxyurea, the migration capacity of MSC was 1.7-fold ($p<0.0001$) and 1.2-fold ($p<0.05$) higher in HDL and apo A-I-supplemented MSC compared to hydroxyurea-treated MSC, respectively (Figures 5B and 5C). In both assays, the HDL or apo A-I stimulated migration of MSC was reduced in the presence of the PI3K inhibitor Ly294002.

Discussion

The salient finding of this study is that HDL and their main apolipoprotein, apo A-I, induce the migration of MSC in a PI3K-dependent manner.

MSC are an attractive cell source for cell therapy given their

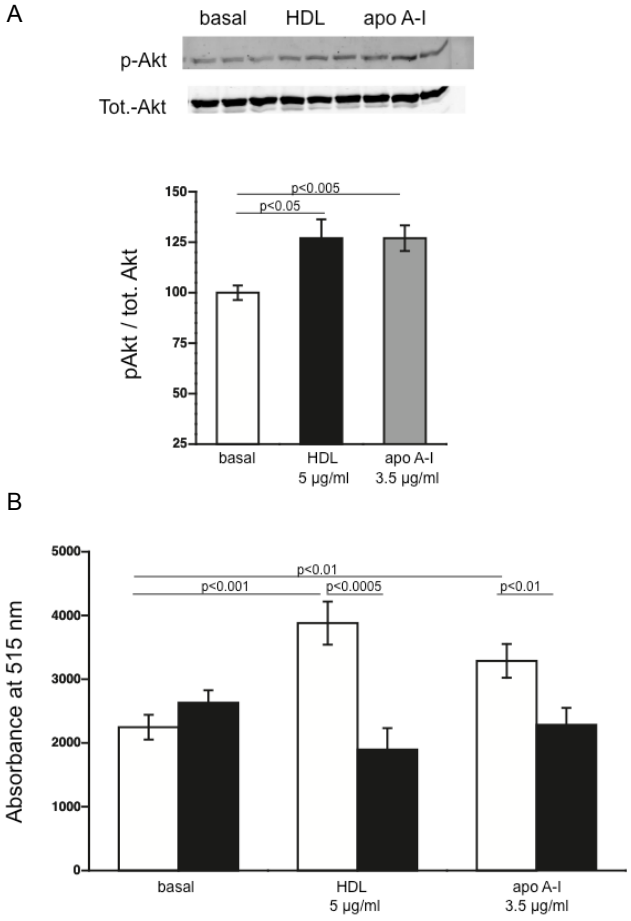

Figure 3: HDL and apo A-I supplementation induce the phosphorylation state of Akt and increase nitric oxide in mesenchymal stromal cells in a phosphatidylinositol-3-kinase-dependent manner. (A) Representative Western blots of p-Akt and Akt in MSC 3 min after HDL (black bar) or apo A-I (grey bar) supplementation. Bar graph represents the mean ± SEM of the p-Akt/Akt ratio expressed as the percentage of the non-stimulated basal group (n=10/group for basal, HDL, and n=7 for apo A-I). (B) Bar graphs representing the mean ± SEM of intracellular NO production depicted as absorbance at 515 nm in untreated (open bars) MSC and MSC pre- and co-treated with the phosphatidyl-inositol-3-kinase inhibitor Ly 294002 (black bars) and supplemented with 5 µg/ml of HDL or 3.5 µg/ml of apo A-I for 5 min (n=10/group).

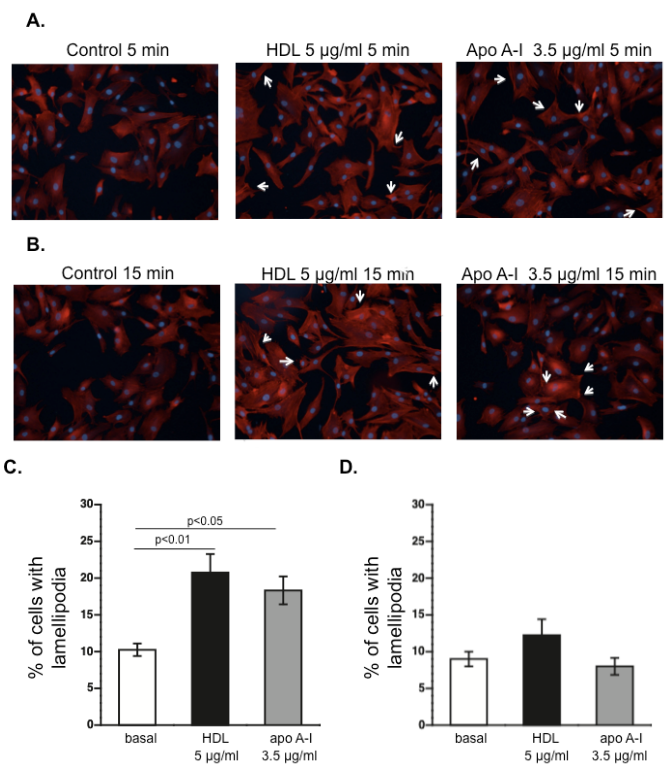

Figure 4: HDL and apo A-I induce lamellipodia formation in mesenchymal stromal cells. Representative images of MSC stimulated with HDL or apo A-I for (A) 5 or (B) 15 min. Arrows indicate lamellipodia. Red staining (Alexa Fluor 546) reveals the actin filaments and lamellipodia, whereas the blue staining (DAPI) indicates the cell nuclei. Bar graphs representing the mean ± SEM of the % of cells with lamellipodia (C) 5 min or D. 15 min post HDL or apo A-I supplementation.

pleiotropic effects and their ability to migrate towards the site of tissue damage. This migration process involves the interaction between released chemokines/growth factors at the site of injury and chemokine receptors on the MSC, and depends on the viability and functionality of the MSC. Comorbidities like diabetes mellitus [31,32] and age [33] decrease the migration capacity of MSC, whereas cell culture conditions, including hypoxia [34] induce their migration potential. The impaired migration of MSC under diabetes mellitus is triggered by hyperglycemia-induced oxidative stress [31]. Though, under type 2 diabetes mellitus also dyslipidemia including high LDL cholesterol and low HDL cholesterol levels might underlie the reduced migration efficacy of MSC.

Several approaches directed at increasing the survival and functionality of MSC have been associated with activation of the PI3K/Akt signaling pathway [35]. HDL has been shown to protect MSC from oxidative stress-induced apoptosis involving PI3K/Akt [36]. However, the impact of HDL on MSC migration has not been investigated so far.

We demonstrated that MSC express the SR-BI receptor and that 3 min post HDL or apo A-I supplementation, the phosphorylation state of Akt was upregulated. In addition, HDL-and apo A-I induced NO in MSC in a PI3K-dependent manner. The involvement of NO in downstream PI3K signaling and cell migration has been documented in several reports [37,38]. In agreement with the HDL-mediated migration of endothelial cells [11] and EPC [14,38], we next illustrated via modified Boyden chamber migration and wound healing assays that HDL and apo A-I induce the migration of MSC in a PI3K-dependent manner. PI3K/Akt signaling regulates multiple biological processes including cell growth, cell division, survival [4], cell migration and invasion [39]. Particularly, Akt enhances actin remodeling and generates membrane protrusions through downstream activation of Rac1 and Cdc42 [40]. Via phallotoxin staining, we further demonstrated that both HDL and apo A-I induce cytoskeleton rearrangement, actin filament deposition in the proximity of the plasma membrane and pronounced lamellipodia

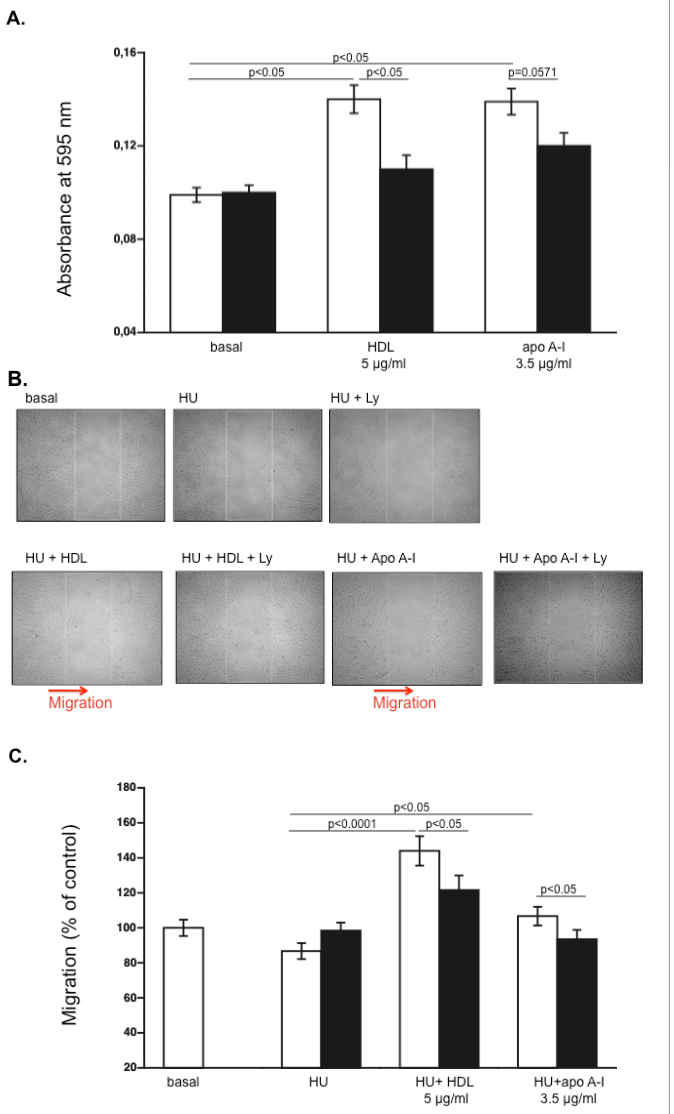

Figure 5: HDL and apo A-I induce migration of mesenchymal stromal cells in a phosphatidyl-inositol-3-kinase-dependent manner. (A) Bar graph represents the mean ± SEM of the absorbance at 595 nm depicting the migration potential of MSC versus basal medium, HDL or apo A-I in the absence (open bars) or presence (black bars) of the phosphatidyl-inositol-3-kinase inhibitor Ly 294002 with n=4/group. (B) Representative pictures of a wound healing assay showing MSC 24 h post-scratching and supplementation of basal medium, medium containing hydroxyurea (HU) with or without HDL or apo A-I in the absence or presence of Ly 294002 (Ly) as indicated. (C) Bar graph represents the mean ± SEM of the percentage of migrating MSC 24 h after scratching and supplementation of basal medium, medium containing HU with or without HDL or apo A-I in the absence (open bars) or presence (black bars) of Ly 294002, expressed towards the percentage of the non-stimulated basal group set as 100. n=10-12 fields/group.

formation, supporting the HDL/apo A-I mediated induction of MSC migration.

In summary, HDL and apo A-I enhance the migratory capacity of MSC involving PI3K - Akt. Therefore, we suggest that the HDL- and apo A-I-induced improvement in MSC functionality facilitates circulating MSC to migrate towards the site of injury and to contribute to endothelial repair via their ability to render endothelial cells support as pericyte-like cell [41] and their capacity to induce angiogenesis in a paracrine manner [19]. We conclude that the regenerative capacity of HDL is broaden by their ability to improve the migration of MSC in a PI3K-dependent manner. Furthermore, this study supports the culture of MSC in the presence of HDL to improve their potency for clinical use.

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Disclosure

There is no conflict of interest.

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