Towards treatment of liver fibrosis: Cells, targets and models

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CHAPTER 3

3

VCAM-positive mesenchymal stromal cells are most instrumental in ameliorating experimental liver fibrogenesis

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Abstract

Background

Liver fibrogenesis starts with apoptotic hepatocytes that induce proliferation of stellate cells and their subsequent differentiation into myofibroblasts. Myofibroblasts are the main source of extracellular matrix in fibrogenesis. Mesenchymal stromal cells (MSCs) are known to possess pro-regenerative and anti-inflammatory properties, but in relation to the reversal of fibrogenesis contradictory findings have been reported. The reported differences might partly be explained by the use of different subpopulations of MSCs. In the present study we compared the pro-regenerative and anti-fibrotic effects of four different subpopulations of MSCs, categorised on Endoglin (CD105) and VCAM (CD106) membrane expression.

Methods and Results

Proliferation, wound healing and trans-well migration experiments using damaged HepG2 cells showed that VCAM-positive MSC subpopulations have more pro-regenerative capacities compared to the VCAM-negative subpopulations. VCAM-positive MSC populations also expressed higher levels of migratory (SDF-1 and CXCR4) and anti-fibrotic (TGF-β1, VEGF, HGF and IGF) genes. Furthermore, only VCAM-positive MSCs, independent of Endoglin expression, were able to reverse fibrogenesis in a mouse model for liver fibrosis.

Conclusion

To conclude, VCAM-positive subpopulations of MSCs are superior compared to VCAM-negative subpopulations in relation to their anti-fibrotic and pro-regenerative properties. Endoglin expression of MSCs does not have major functional implications regarding their antifibrogenic activity. These observations indicate that differences in subpopulations of MSCs have considerable functional impact that should be implicated in their functional assessment analyses.

Introduction

Liver fibrogenesis is becoming a serious health problem, since therapies specifically targeting this process and thereby preventing progression of fibrosis to cirrhosis are not yet available[1-4]. Fibrogenesis in the liver is caused by injuring stimuli such as excessive alcohol intake, viral hepatitis, non-alcoholic steatohepatitis, autoimmune hepatitis or metabolic syndromes[5-7]. These injuries may lead to apoptosis of hepatocytes which leads to increased proliferation, activation and myofibroblast-differentiation of the stellate cells. These myofibroblasts are responsible for the excessive extracellular matrix deposition observed in fibrosis[5,7].

While removal of the injuring stimulus in some cases may reverse liver fibrogenesis, no medication directly targeting fibrogenesis is available. For example, In the case of hepatitis C infection, a sustained response to anti-viral treatment can lead to a regression of fibrosis[8]. For end-stage cirrhosis, an orthotopic liver transplantation (OLT) is the only curative treatment[2,3]. OLT is a major intervention with associated risks and feasibility for its use depends on donor availability and patient condition[9-11]. Therefore, new therapeutics or interventions specifically targeting the process of hepatic fibrogenesis are needed.

Recently, the use of mesenchymal stromal cells (MSCs) has been explored as a possible treatment for liver fibrosis[12,13]. MSCs are pluripotent cells that can be isolated from various tissues, such as bone-marrow, umbilical cord and adipose tissue. Furthermore, MSCs are not rejected by the immune system upon transplantation and are known to be immuno-suppressive and able to stimulate the repair and regeneration of damaged tissue[14-17]. Several *in vivo* studies, including our own, have shown the potency of MSCs to inhibit the induction and to promote the reversal of fibrogenesis[12,18-21]. MSCs have been used to successfully reverse liver fibrosis in patients with alcohol-related or viral-induced liver injury[18,19]. Different working mechanisms of MSCs in the reversal of liver fibrosis have been proposed. These mechanisms include the capacity of MSCs to inhibit stellate cell proliferation and their subsequent activation and differentiation into myofibroblasts, but also the ability of MSCs to silence the myofibroblasts, and thereby directly target fibrogenesis[22-24]. Furthermore, it is suggested that MSCs can stimulate the proliferation and survival of hepatocytes[22-26]. Other proposed mechanisms include the immunomodulatory abilities of MSCs by -for example- inhibition of T-cell activation and stimulation of pro-inflammatory macrophages to an immunosuppressive phenotype[22,27].

Besides the positive results obtained from *in vivo* studies and clinical trials with MSC therapy for liver fibrosis, other studies have shown different and even contradictory results[18,19,21,28]. Disease stage, timing of MSC administration, source of MSCs, dosage of MSCs and administration routes differ between the studies and may therefore account for the observed contradictory results[21,28]. Another, less studied explanation is the use of different subpopulations of MSCs[28-30]. The current isolation methods for MSCs lead to a rather heterogeneous group of

cells[31-33]. Most of the studies describe MSCs as cells that are able to differentiate *in vitro* into osteoblasts and adipocytes, express CD29, SCA-1 and CD44 on their membranes, and adhere to plastic[14,28,31,33]. However, most studies are less consistent about the Endoglin (CD105) and vascular cell adhesion protein (VCAM, CD106) membrane expression of MSCs[29,31,33-35]. VCAM-negative subpopulations are thought to have less regenerative and immunosuppressive properties as compared to VCAM-positive MSC subpopulations[31,34,35]. Studies describing Endoglin-negative subpopulations reveal a more immunosuppressive phenotype compared to Endoglin-positive MSC subpopulations[29]. Thus, in order to find the optimal treatment and to get reproducible results, it might be highly relevant to characterise the different MSC subpopulations and assess the functional implications. However, there are no studies focussing on the use of different subpopulations of MSCs in relation to the treatment of liver fibrosis. Therefore, in the present study we compared the pro-regenerative and anti-fibrotic abilities of four different subpopulations of MSCs, selected to be double-positive, double-negative, or single-positive for either Endoglin and VCAM. We hypothesized that different subpopulations of MSCs will lead to different experimental outcomes, which may explain the contradictory results in different studies.

Material and Methods

MSC isolation and culturing

Tg(s100a4-cre)1Egn mice (Jackson laboratory, Bar Harbor, USA) were crossed with Bl6-ROSA-LacZ reporter mice (LUMC breeding population) and their offspring was used for the isolation of MSCs following standard protocol[36]. In short, mice were sacrificed by cervical dislocation and femur, tibia and humerus were collected and cleared from surrounding tissues. Bones were flushed with RPMI culture medium supplemented with, L-glutamine, penicillin/streptomycin (P/S; Invitrogen Corp., Paisley, UK), fetal calf serum (FCS; Gibco, Paisley, UK) and Heparin (Pharmacy AZL, Leiden, The Netherlands). Flushed bone-marrow was filtered and subsequently cultured in complete culture medium consisting of αMEM culture medium (Lonza, BE12-169F) supplemented with L-glutamine, P/S and FCS. Floating cells were removed by daily medium refreshment and growing MSC populations were obtained after a few weeks. Cells were used in passage 3-5 and monthly tested for mycoplasma contamination.

Identification and characterisation of MSC subpopulations

MSC subpopulations were identified and characterised by FACS analysis. MSCs were stained for CD29-PE-Cy5, SCA-1-APC, CD45-PE, CD31-APC (eBioscience, Vienna, Austria), CD44-APC, Endoglin-PE and VCAM-PE (BD Pharmingen, San Diego, CA, USA) and fluorescence was measured with LSR II flow cytometer (BD Biosciences, San Diego, CA, USA) with FACS diva software (version 8.7.1., Tree Star Inc. Ashland, OR, USA). Results were analysed using FlowJow analysis software (version 8.7.1., Tree Star Inc. Ashland, OR, USA). FACS analysis identified four

different subpopulations of MSCs: double-positive ($V^{pos}E^{pos}$-MSC), double-negative ($V^{neg}E^{neg}$-MSC), or single-positive for Endoglin or VCAM ($V^{neg}E^{pos}$-MSC or $V^{pos}E^{neg}$-MSC).

To test the ability of the identified MSC subpopulations to differentiate into osteoblasts and adipocytes, the MSCs were cultured for three weeks with adipocyte or osteoblast differentiation medium as previously described by our group[36]. In short, adipogenic differentiation medium consists of complete culture medium supplemented with 1 µM dexamethason, 5 µM insulin, 100 µM indomethacin and 0.5 mM 3-isobutyl-1-methylxanthine (all from Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands). Osteoblast differentiation medium consists of complete culture medium supplemented with 10 nM dexamethason, 50 µg/ml ascorbic acid and 10 mM β-glycerophosphate (all from Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands). After three weeks, fast blue staining for alkaline phosphatase expression and alizarin red staining for calcium deposition were used to verify osteogenic differentiation (Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands). Adipogenic differentiation was confirmed by oil-red-o stained lipid droplets (Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands).

Proliferation, trans-well migration and wound healing assays

Cell proliferation was measured with Promega MTS assay following manufactures' protocol (Promega, Madison, Wisconsin, USA). MSCs were plated in a 96 well plate and the next day (day 0) and at day 2, MTS was added and after 1 h of incubation the colour development was measured. To evaluate the ability of MSCs to influence HepG2 proliferation, 2 days conditioned MSC medium (FCS free) was added to HepG2 cells or wounded HepG2 cells (cross-sectional scratch injuries) and a MTS assay was performed at day 0 and 2.

For wound healing assays 500.000 HepG2 cells were plated on a coverslip in a 24 well plate. Next day, a wound was made, and medium replaced by MSC conditioned medium (FCS free) or 150.000 cells of the different MSC subpopulations. Images (10x magnification) were made at 0 h and 48 h and used to calculate the wound size.

Trans-well migration assays were used to study the migration capacity of the subpopulations of MSCs (8 µm, Thincert TM Greiner Bio-One 12 well, 665638). In all experiments 10.000 MSCs in FCS free αMEM culture medium were added to the upper compartment. Thereafter, migration to 1% FCS medium with or without HepG2 or wounded HepG2 cells (cross-sectional scratches) in the lower compartment was evaluated. After 24 h, migrated cells were visualized by crystal violet staining and counted subsequently.

RNA isolation, cDNA synthesis and quantitative Polymerase Chain Reaction (qPCR)

NucleoSpin RNA kit (Machery-Nagel GmbH, Düren, Germany) was used to isolate mRNA following manufactures' protocol. Next, cDNA was synthesized according to Promega standard

protocol (Promega, Madison, Wisconsin, USA). For qPCR a mix containing 1 nM primers, 5 µl iQ SYBR Green supermix reagent and 4 µl cDNA was used (Bio-Rad Laboratories, Berkeley, California, USA). CXCR4, stromal derived factor-1 (SDF-1), hepatocyte growth factor (HGF), vascular endothelial growth factor (VEGF), insulin-like growth factor (IGF), VCAM, Endoglin and transforming growth factor-β1 (TGF-β1) expression levels were measured and normalised to Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), (Supplemental Table 1: Primer sequences).

Mouse model for liver fibrosis

All experiments were approved by the animal ethics committee of the Leiden University Medical Center. Mice were housed under 12 h day/night cycle and received food and water *ad libitum*. For the induction of liver fibrosis 6 week old male C57Bl/6Jico mice were used (Charles River Laboratories, The Netherlands). For a period of 6 weeks, mice received 3 intraperitoneal injections with carbon tetrachloride (CCL4, 0.5 ml/kg body weight) in mineral oil per week (Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands). After these 6 weeks, mice underwent a partial hepatectomy where the three frontal lobes were removed [37]. During surgery, one of the four different MSC subpopulations (2x10^6 cells) or vehicle control (NaCl) were locally injected in one of the two intact lateral lobs (N=10 mice per group). After 8 days, mice were sacrificed by cervical dislocation and livers were collected, weighed and stored in paraformaldehyde for paraffin embedding.

Histological examination of extracellular matrix

To evaluate the severity of fibrosis a Sirius-red staining was performed to visualize and subsequently quantify the amount of extracellular matrix (ECM). Fixed cell cultures and hydrated paraffin tissue sections were stained for 90 min with 1 g/L Sirius-red F3B in saturated picric acid (both Klinipath, Guildford, UK). Next, the cells or tissue sections were incubated for 10 min with 0.01 M HCL, dehydrated and mounted with Entellan (Merck KGaA, Darmstadt, Germany).

To quantify the amount of ECM in liver tissue, 5-8 random pictures (10x magnifications) with fixed microscopy settings were captured and thereafter analysed with ImageJ (ImageJ 1.47v, National Institutes of Health, USA). Subsequently, the reduction of collagen content in the regenerated liver tissue, relative to the resected pHx tissue was calculated. In addition, lobuli closure was used as a second score for the severity of fibrosis. More lobuli closure indicated a more severe degree of fibrosis.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism software and P values lower than 0.05 were considered to be statistically significant (GraphPad Software, version 5.01, San

Diego, CA). To compare two or multiple groups Student's t-test or One-Way ANOVA test was used respectively. Results are presented as the means ± standard error of the mean (SEM).

Results

Identification and characterisation of VCAM/Endoglin subpopulations of MSCs

$V^{pos}E^{pos}$-MSC, $V^{pos}E^{neg}$-MSC, $V^{neg}E^{pos}$-MSC, $V^{neg}E^{neg}$-MSC subpopulations were identified and characterised by FACS analysis. All four subpopulations revealed to be positive for CD44, CD29 and SCA-1 expression (Figure 1A). Endothelial marker CD31 and haematopoietic marker CD45 were absent in all subpopulations (Figure 1A). Endoglin and VCAM expression as such were independent of each other (Figure 1A). QPCR measurements of mRNA expression of

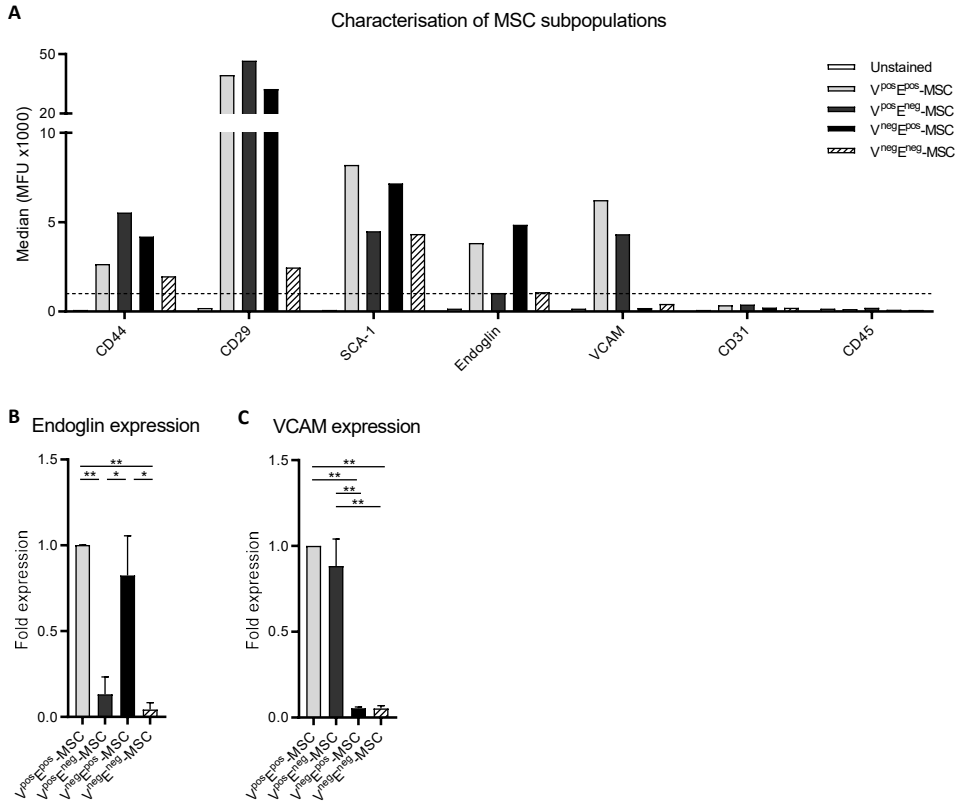

Figure 1. Identification and characterisation of VCAM/Endoglin subpopulations of MSCs. Identification and characterization of the different subpopulations of MSCs by membrane marker expression. (A) CD44, CD29, SCA-1, Endoglin, VCAM, CD31 and CD45 membrane expression measured by flow cytometry. RNA expression levels of (B) Endoglin and (C) VCAM were measured by qPCR and normalized to GAPDH. The qPCR data is represented as mean ± SEM of three independent experiments. *p≤0.05 **p≤0.01

Endoglin and VCAM confirmed the protein results obtained from the FACS experiments (Figure 1B and C). To confirm that the subpopulations are indeed MSCs, osteoblast and adipocyte differentiation assays were performed. All four populations showed to be able to differentiate into adipocytes and osteoblasts (Figure 2). Some small differences in the extent of differentiation were observed. The $V^{neg}E^{neg}$-MSC population showed less differentiation into osteoblasts, while the adipocyte differentiation was more pronounced in both Endoglin-negative subpopulations. These results indicate that the four identified subpopulations of cells can all be classified as classical MSCs.

Conditioned medium of the VCAM-positive MSC subpopulation enhances the survival and proliferation of damaged HepG2 cells

Proliferation and survival of endogenous liver cells are two proposed mechanisms of MSC treatment for liver fibrosis. The results of an *in vitro* assay using HepG2 cells as a model for

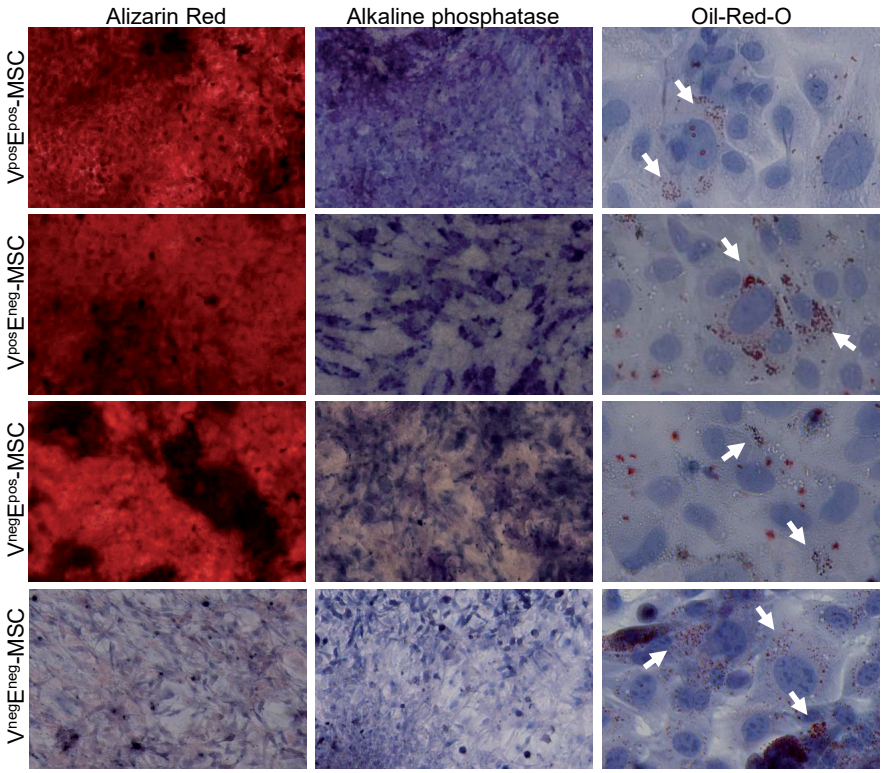

Figure 2. Osteoblast and adipocyte differentiation of MSC subpopulations. Characterisation of the isolated MSC subpopulations by osteoblast and adipocyte differentiation. Osteoblast differentiation was visualized by calcium deposit (Alizarin red staining), and alkaline phosphatase production (fast blue staining, 10x magnifications). Adipocyte differentiation was visualized by Oil-red-o stained cytoplasmic lipid droplets (indicated by the white arrows, 40x magnifications).

endogenous hepatocytes showed that incubation with conditioned medium of the four different MSC subpopulations did not affect basal proliferation of HepG2 cells (Figure 3A). When the HepG2 cells were challenged with injuring scratches, increased proliferation was observed when incubated with conditioned medium obtained from the VCAM positive populations compared to control (non-conditioned medium) and conditioned medium obtained from the VCAM-negative populations (Figure 3B). Next, the ability of MSCs to sense tissue damage and actively migrate to these damaged regions was evaluated. In a trans-well migration assay, the basal migration of the different populations from medium without FCS to medium with 1% FCS was tested. VCAM-positive MSCs showed significantly more migration compared to the VCAM-negative subpopulations (Figure 3C). Similar migration patterns were observed when

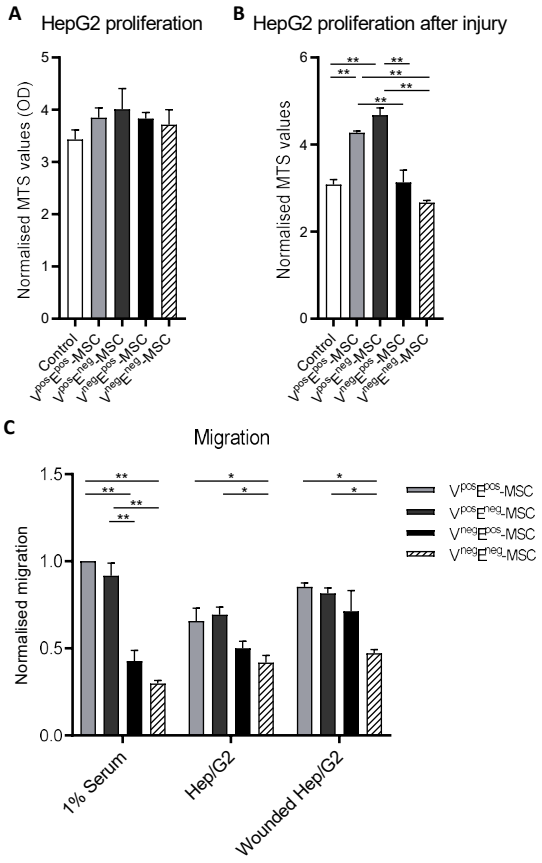

Figure 3. Conditioned medium of the VCAM-positive MSC subpopulation enhances the survival and proliferation of damaged HepG2 cells. The ability of the MSC subpopulations to affect HepG2 cell proliferation was measured by MTS proliferation assays. Proliferation of (A) HepG2 or (B) scratched HepG2 monolayers after 48 h of stimulation with conditioned medium of the different MSC subpopulations, normalized to baseline measurement. (C) In trans-well migration assays the migration to 1% serum, HepG2 or wounded HepG2 cells in 24 h was evaluated. Migrated cells were visualized with crystal violet staining, counted, and normalized to 1% serum initiated migration of the $V^{pos}E^{pos}$-MSC subpopulation. Graphs represented the mean ±SEM of three independent experiments. *p≤0.05 **p≤0.01

the MSC subpopulations migrated to HepG2 or wounded HepG2 cells (Figure 3C). Altogether these data indicate that VCAM-positive MSC subpopulations are more migratory and more able to stimulate proliferation upon injuring stimuli compared to the VCAM-negative MSC subpopulations.

VCAM-positive and VCAM-negative MSC subpopulations equally enhance HepG2 wound closure and form 2D lobuli-like structures

Wound closure assays were performed to study whether the four subpopulations of MSCs differently affect tissue regeneration. The results showed faster wound closure of the HepG2 cells after adding MSCs or MSC conditioned medium but no differences between the subpopulations were observed (Figure 4A and B). Proliferation assays showed that the $V^{neg}E^{neg}$-MSC population proliferate faster compared to the other subtypes which showed equal proliferation rates (Figure 4C). Since the other subpopulations have a similar proliferation rate, this could not affect the results of the wound closure experiments. At the end of the wound closure experiments, hexagonal/lobuli-like structures were observed. Sirius-red staining of these cocultures co-localised with the observed hexagonal structures (Figure 4D). These observed structures are similar to those observed in *in vivo* livers. To study the exact location of the MSCs in these experiments GFP-$V^{pos}E^{pos}$-MSCs were used. Results showed that GFP-$V^{pos}E^{pos}$-MSCs co-localised with the hexagonal structures (Figure 4E, white arrow) and the wound opening (Figure 4E, area surrounded by the dashed line). Furthermore, the results showed that, although MSCs adhere in the wound area, the borders of HepG2 cells did grow towards each other and that the MSCs were excluded from the wounds. The finding that the MSC subpopulations lead to faster wound closure and the formation of liver-like structures implies that MSCs affect tissue regeneration.

VCAM-positive MSC subpopulations express a better pro-regenerative and migratory gene profile compared to VCAM-negative subpopulations

QPCRs were performed to assess whether differences in migration could be explained by different expression levels of genes involved in MSC migration. Results showed that VCAM-positive MSC subpopulations express higher levels of CXCR4 and SDF-1 compared to the VCAM-negative subpopulations (Figure 5A and B). Furthermore, expression levels of known anti-fibrotic and pro-regenerative genes (VEGF, TGF-β1, IGF and HGF) were measured. VEGF expression was less affected by the VCAM profile (Figure 5C). TGF-β1 and IGF were higher expressed in $V^{pos}E^{neg}$-MSC population compared to the other three subpopulations (Figure 5D and E). $V^{pos}E^{pos}$-MSCs showed the highest expression level of HGF (Figure 5F). Altogether these data indicate that VCAM-positive MSC subpopulations have a more pro-regenerative and migratory gene profile compared to the VCAM-negative MSC subpopulations.

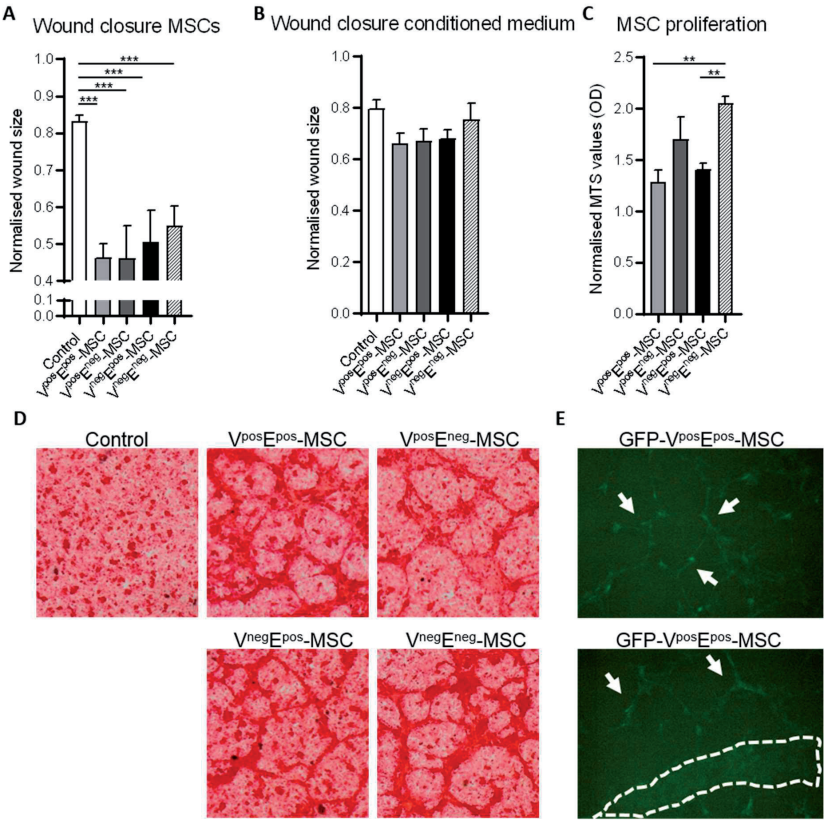

Figure 4. VCAM-positive and VCAM-negative MSC subpopulations equally enhance HepG2 wound closure and form 2D lobuli-like structures. HepG2 wound healing experiments were performed with (A) cells or (B) conditioned medium of the 4 different MSC subpopulations. The graphs are presenting wound closure after 48 h normalised to baseline. (C) Basal proliferation of the 4 different MSC subpopulations measured by a MTS assay. (D) Pictures of Sirius-red stained HepG2–MSC cocultures at the end of the wound healing experiments (10x magnifications). (E) Pictures of wound closure experiments with GFP expressing MSCs. MSCs colocalising with the lobuli-like structures are indicated by white arrows and the wound area is surrounded by a white dashed line. The data is represented as the mean ±SEM (n=3). **p≤0.01, ***p≤0.001

VCAM-positive but not VCAM-negative MSC subpopulations reverse fibrogenesis in regenerating mouse livers

To study the ability of the different MSC subpopulations to reverse fibrogenesis, an *in vivo* model for liver fibrosis was used. After 6 weeks of fibrosis induction with CCL4, mice underwent a partial hepatectomy as regeneration stimulus, and locally received one of the four subsets of MSCs or vehicle as control. During 8 days of regeneration no differences in body weights were observed, except for the last two days where the mice treated with the VCAM-negative MSCs had relative lower body weights (Figure 6A). Eight days after cell treatment, mice were sacrificed and livers collected and weighted. No differences in total liver weights were observed (Figure 6B). Liver lobes which were locally treated with the VCAM-negative subpopulations

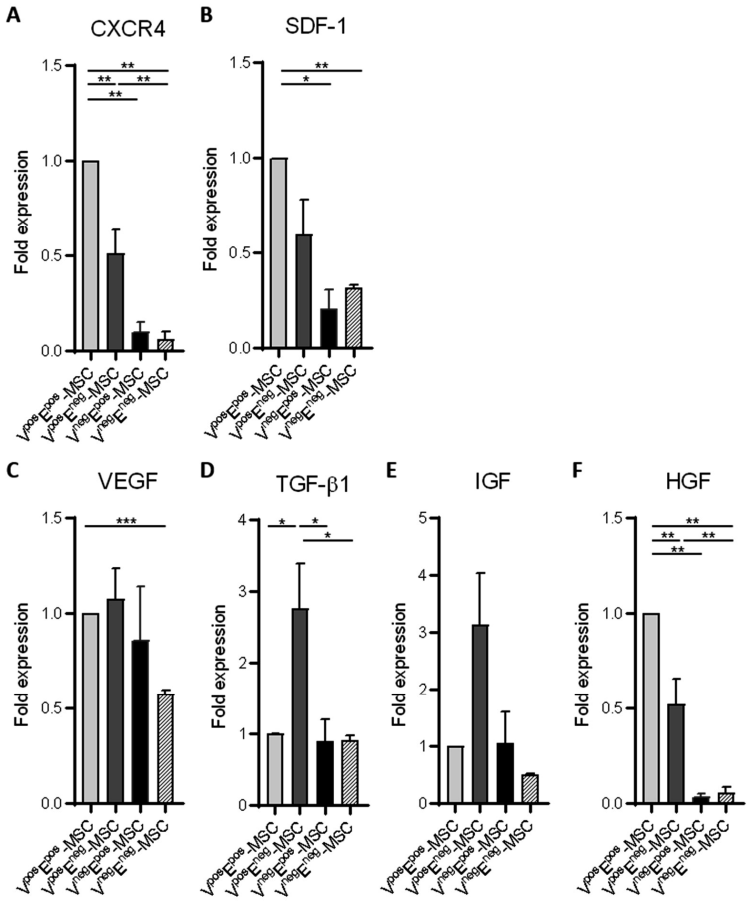

Figure 5. Basal pro-migratory and anti-fibrotic gene expression levels. QPCR analysis of pro-migratory and anti-fibrotic gene expression levels of the different MSC subpopulations. Expression levels of (A) CXCR4, (B) SDF-1, (C) VEGF, (D) TGF-β1, (E) IGF and (F) HGF were measured and normalized to GAPDH. The graphs represent the mean of three independent experiments ±SEM. *p≤0.05, **p≤0.01, ***p≤0.001

were significantly smaller compared to controls. The weights of the locally treated lobes of the groups receiving the VCAM-positive populations were not different compared to control (Figure 6C). Paraffin embedded liver sections were stained for Sirius-red to assess the degree of liver fibrosis. Results showed that local $V^{pos}E^{pos}$-MSC treatment lead to more reduction in collagen content compared to mice treated with vehicle control or the VCAM-negative MSC populations (Figure 6D and E). Next, the tissues were also scored for lobuli closure, in which more closure indicates a more severe fibrosis. More closure was observed in the mice treated with the VCAM-negative subpopulations compared to control, while the mice treated with the VCAM-positive populations did not differ from controls (Figure 6F). $V^{pos}E^{pos}$-MSCs showed, although not significantly, a trend towards less closure (Figure 6F). No significant differences in the weights, reduction of collagen or lobuli closure in the untreated counterpart liver lobes

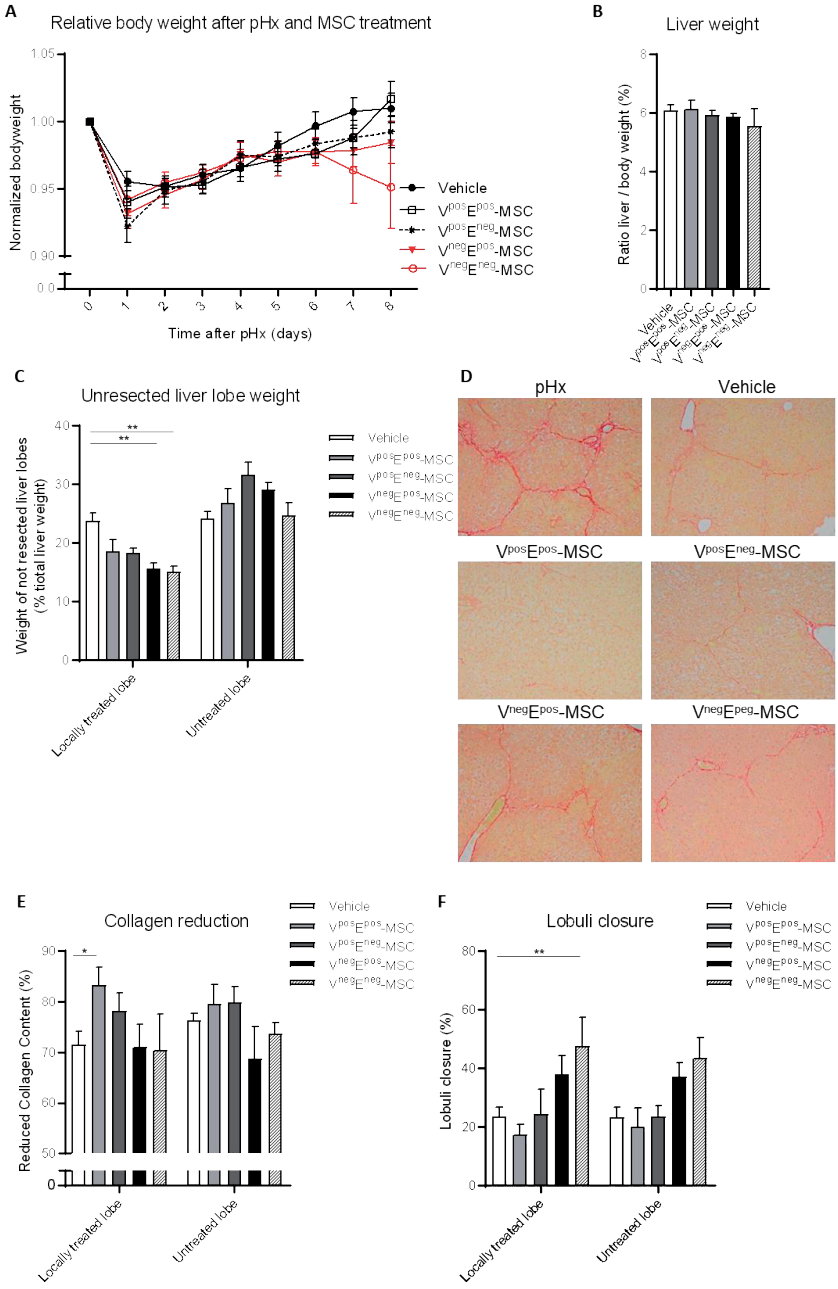

Figure 6. VCAM-positive MSC populations ameliorate fibrosis in regenerating mouse livers. After CCL4 induced fibrosis, mice underwent partial hepatectomy and received local treatment of vehicle, or one the MSC subpopulations in one of two remaining liver lobes (N=10 mice/group). (A) Relative body weight during regeneration. (B) Liver weight normalised to total body weight and (C) treated and untreated lobe weight as percentage liver after regeneration. (D) Representative pictures of Sirius-red stained sections of resected and locally treated liver lobes (10x magnifications). (E) Reduction of Sirius-red staining relative to resected tissue. (F) Estimated lobuli closure of Sirius-red stained sections. Data are expressed as mean ± SEM. *p≤0.05, **p≤0.01

were observed, which indicates a local effect of the MSC treatment (Figure 6C, E and F). These results showed that VCAM-positive subpopulations, independent of Endoglin expression, have the ability to locally ameliorate liver fibrosis in regenerating livers.

Discussion

The incidence and the progression of liver fibrosis to cirrhosis is an increasing health problem for which new interventions or therapeutics are needed[4,38]. Extensive research is ongoing in order to find new treatments specifically targeting fibrogenesis. Currently, several studies have tested the application of MSC therapy as a new treatment strategy, and while some results were promising, other studies had negative outcomes[12,18,19]. Explanations for these opposing effects could be variation in the study design, source of MSCs, dosage, route of administration and possibly the existence and use of different subpopulations of MSCs[12,21,28]. So far, functional assessments of different subpopulations of MSCs in the reversal of liver fibrosis has not been studied. Therefore, in the present study, we compared the pro-regenerative and anti-fibrotic capacities of four different subpopulations of MSCs, selected for Endoglin and VCAM membrane marker expression. Results showed that VCAM-positive MSC subpopulations are more migratory and lead to more proliferation of damaged HepG2 cells compared to the VCAM-negative subpopulations. Furthermore, in a mouse model for liver fibrosis we showed that local MSC treatment with the VCAM-positive subpopulations in combination with a partial hepatectomy as regeneration stimulus, ameliorates fibrosis. The VCAM-negative subpopulations were not able to ameliorate fibrosis in this model. The contribution of Endoglin expression was found to be less relevant.

Studies of Li and Huang et al. have shown that MSCs could protect hepatocytes from apoptosis and are able to promote hepatocyte proliferation[24,26]. The results of the present study were in line with these observations, and showed that conditioned medium of the VCAM-positive MSC populations led to more proliferation of the damaged HepG2 cells compared to control. However, the previous studies did not compare their results to a VCAM-negative subpopulation. Therefore, it is interesting that the current study showed that the conditioned medium of the VCAM-negative MSC populations lacked this property needed for enhanced liver regeneration.

The migration assays showed increased mobility of all tested subpopulations of MSCs to damaged HepG2 cells compared to healthy HepG2 cells. In all experiments, the VCAM-positive MSC subpopulations migrate more compared to VCAM-negative subpopulations. These results are in line with previous research of Gao et al. who also showed less migration of MSCs with altered VCAM expression[39]. In contrast to the present study, they focused on Glioma cell- instead of hepatocyte- induced migration and used VCAM blocking antibodies instead of using different MSC populations. The SDF-1-CXCR4 gradient is a well-known pathway for

directed migration of MSCs[26]. Several studies described that MSCs recognise tissue damage by higher SDF-1 concentrations in damaged tissue. The qPCR results of the present study showed higher expression of these genes in VCAM-positive compared to the VCAM-negative MSC subpopulations which might explain the faster migration of these cells as observed in our *in vitro* trans-well assays. Altogether, the current results indicate that properties of VCAM-positive MSCs are better suitable for the treatment of liver fibrosis as compared to the VCAM-negative MSC subpopulations.

Wound healing experiments with HepG2 cells showed faster wound closure upon MSC treatment, but surprisingly did not show differences between the different MSC subpopulations. Unexpectedly, the traced MSCs in these experiments formed hexagonal structures mimicking the structures observed in *in vivo* livers[24]. We hypothesise that MSCs sense the old liver architecture and try to rebuild this structure. This 2D phenomenon has not been described before and further research is needed to study this architectural aspect in more detail.

Previous studies, including our own, showed that MSCs express proteins involved in tissue-regeneration (HGF, VEGF, IGF, and TGF-β1)[20,22,23,25,27,35,40]. HGF and IGF are thought to stimulate the survival and proliferation of liver-resident cells[22,25,26,41]. Furthermore, HGF is known to inhibit stellate cell activation and is also able to silence activated myofibroblasts and thereby directly inhibiting the fibrogenic process[19,23,25]. The present study showed higher expression levels of these genes in VCAM-positive subpopulations compared to the VCAM-negative subpopulations. These results are in line with earlier studies showing lower basal expression level of HGF, VEGF, and IGF in VCAM-negative MSC populations[31,35]. However, these previous studies did not focus on fibrogenesis and therefore do not explain how their results might affect the potency of VCAM-positive or VCAM-negative MSCs to reverse fibrogenesis. Altogether these gene expression profiles might explain the results as observed in our current *in vitro* studies which showed that VCAM-positive populations protect and stimulate cell proliferation of damaged HepG2 cells. Furthermore, it could also explain the reduced collagen content observed in the mice treated with VCAM-positive MSC subpopulations.

The observed faster proliferation of wounded HepG2 cells *in vitro* was not observed *in vivo* where the weights of the regenerating livers were not affected by local MSC treatment. This might be explained by the time the livers were weighted (on day 8), since other studies observed differences in liver weight at an earlier stage[41]. Weighing on day 8 might have been too late to find differences in liver weight as all livers are already fully regenerated.

In the present study, no major functional differences between the $V^{pos}E^{pos}$-MSC and the $V^{pos}E^{neg}$-MSC populations were observed. Like Anderson et al., we found that the Endoglin-negative subpopulation was more prone to adipogenic differentiation compared to the Endoglin-positive subpopulations[29]. Our results also showed that the $V^{pos}E^{neg}$-MSC population expresses higher

IGF and TGF-β1 RNA levels. Fiore et al. described that IGF produced by MSCs could stimulate macrophage differentiation to an anti-inflammatory and anti-fibrotic phenotype[25]. In our *in vivo* experiment we observed that $V^{pos}E^{neg}$-MSCs led to an intermediate reduction of collagen content compared to the control- or the $V^{pos}E^{pos}$-MSC-treated groups. One could speculate that the working mechanism of $V^{pos}E^{pos}$-MSCs is more HGF pathway related, directly targeting the stellate cells and myofibroblasts leading to a direct effect. On the other hand, as Anderson et al. suggested, it could be that $V^{pos}E^{neg}$-MSCs are working anti-inflammatory leading to an delayed, indirect, effect that might explain the observed intermediate result *in vivo*. Since the immunosuppressive capacity of MSCs was not in the scope of the present study we did not further evaluate this hypothesis.

Several studies have described the use of MSC treatment in relation to liver fibrosis in humans, rodents and zebrafish embryos[12,18-20]. These studies showed contradictive results about the efficacy of MSC treatment on liver fibrosis. In the present study, we hypothesised that these observed differences might very well be due to the use of different subpopulations of MSCs. This hypothesis is strengthened by the present study, as we showed that mice treated with VCAM-positive MSC subpopulations showed a reduction in collagen content and less lobuli closure compared to mice treated with the VCAM-negative MSC subpopulations. Altogether, the present study showed that VCAM-positive MSCs subpopulations have advantageous properties for therapeutic interaction with regenerating fibrotic livers compared to VCAM-negative subpopulations indicating that patients with liver cirrhosis might benefit more from the treatment with VCAM-positive MSC subpopulations. Therefore, it is highly recommendable to include VCAM as a marker in the characterization panel of MSCs before use.

To conclude, VCAM-positive MSC subpopulations are more able to migrate and stimulate survival and proliferation of endogenous liver cells and contain a more pro-regenerative and anti-fibrotic RNA expression profile. Furthermore, the VCAM-positive population showed to be more effective in ameliorating fibrosis in an *in viv*o model for liver fibrosis and regeneration. Endoglin expression of MSCs have less functional implications regarding ameliorating liver fibrosis. These observations lead to the conclusion that the VCAM-positive subpopulation of MSCs is superior compared to the VCAM-negative population regarding their pro-regenerative and anti-fibrotic properties.

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Disclosure of conflicts of interest

The authors confirm that there are no conflicts of interest.

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Supplementary files

Supplemental table 1: Primer sequences

Gene	Abbreviation	Forward	Reverse
Hepatocyte growth factor	HGF	AAGAGTGGCATCAAATGCCAG	CTGGATTGCTTGTGAAACACC
Vascular endothelial growth factor	VEGF	CACAGCAGATGTGAATGCAG	TTTACACGTCTGCGGATCTT
Insulin-like growth factor	IGF	CTACAAAAGCAGCCCGCTCT	CTTCTGAGTCTTGGGCATGTCA
Transforming growth factor-β1	TGF-β1	CAACAATTCCTGGCGTTACC	TGCTGTCACAAGAGCAGTGA
Stromal derived factor 1	SDF-1	GAAAGGAAGGAGGGTGGCAG	TCCCCGTCTTTCTCGAGTGT
CXCR4	CXCR4	TTACCCCGATAGCCTGTGGA	GCAGGACGAGACCCACCAT
Vascular cell adhesion protein	VCAM	TGCCGAGCTAAATTACACATTG	CCTTGTGGAGGGATGTACAGA
Glyceraldehyde 3-phosphate dehydrogenase	GAPDH	AACTTTGGCATTGTGGAAGG	ACACATTGGGGGTAGGAACA