Lipids (2020)
DOI 10.1002/lipd.12282

ORIGINAL ARTICLE

S1P Lyase siRNA Dampens Malignancy of DLD-1 Colorectal Cancer Cells

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Received: 18 December 2019 / Revised: 30 July 2020 / Accepted: 19 August 2020

Abstract Sphingosine-1-phosphate lyase 1 (S1P lyase or SGPL1) is an essential sphingosine-1-phosphate-degrading enzyme. Its manipulation favors onset and progression of colorectal cancer and others *in vivo*. Thus, SGPL1 is an important modulator of cancer initiation. However, in established cancer, the impact of retrospective SGPL1 modulation is elusive. Herein, we analyzed how SGPL1 siRNA affects malignancy of the human colorectal cancer cells DLD-1 and found that in parallel to the reduction of SGPL1 expression levels, migration, invasion, and differentiation status changed. Diminished SGPL1 expression was accompanied with reduced cell migration and cell invasion in scratch assays and transwell assays, whereas metabolic activity and proliferation was not altered. Decreased migration was attended by increased cell–cell-adhesion through upregulation of E-cadherin and formation of cadherin-actin complexes. Spreading cell islets showed lower vimentin abundance in border cells. Furthermore, SGPL1 siRNA treatment induced expression of epithelial cell differentiation markers, such as intestinal alkaline phosphatase and cytokeratin 20. Hence, interference with SGPL1 expression augmented a partial redifferentiation of colorectal cancer cells toward normal colon epithelial cells. Our investigation showed that SGPL1 siRNA influenced tumorigenic activity of established colorectal cancer cells. We therefore suggest SGPL1 as a target for lowering malignant potential of already existing cancer.

Keywords S1P lyase · migration · invasion · epithelial-mesenchymal transition · differentiation

Lipids (2020).

Abbreviations

ALPi	intestinal alkaline phosphatase
CK 20	cytokeratin 20
S1P	sphingosine-1-phosphate
S1PR1/2/3/4/5	sphingosine-1-phosphate receptor 1/2/3/4/5
SGPL1	sphingosine-1-phosphate lyase 1
SGPP1/2	sphingosine-1-phosphate phosphatase 1/2
siRNA	small interfering RNA
SPHK1/2	sphingosine kinase 1/2

Introduction

Endogenous sphingolipids and their metabolizing enzymes are known to play an important role in progression, growth, and resistance to therapeutic treatment of human colorectal cancer (Camp et al., 2017). Furthermore, sphingolipids are involved in inflammatory processes and, thus, several drugs targeting the metabolic and signaling pathways of sphingolipids have been developed (Sanllehi et al., 2016). Studies have also reported that dietary sphingolipids operate in cancer development *e.g.* by suppressing the Wnt

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signaling and modulation of intracellular ß-catenin distribution in colon carcinogenesis (Kumar et al., 2012; Schmelz et al., 2001; Schmelz and Merrill Jr., 1998). One of the most studied bioactive sphingolipid is sphingosine-1-phosphate (S1P), which regulates several key biological functions in colorectal cancer like cell proliferation, migration, and apoptosis (Garcia-Barros et al., 2014). S1P is enzymatically catabolized by SGPL1 and the S1P phosphatases 1/2 (SGPP 1/2). Only SGPL1 irreversibly degrades S1P. Therefore, SGPL1 has a strong influence on intracellular and tissue S1P levels. Previous research and databases demonstrate that SGPL1 is upregulated in human colorectal cancer tissues (Goldman et al., 2020) (Uranbileg et al., 2018). Survival analyses have shown that the 5-year survival rate of patients with low SGPL1 mRNA expression were higher in comparison to patients with high SGPL1 mRNA expression (Uhlen et al., 2017). Although, a decrease in SGPL1 expression has been shown to reduce cell proliferation (Uranbileg et al., 2018), studies have demonstrated that loss of SGPL1 results in resistance to apoptotic effects induced by chemotherapeutic drugs and promotes oncogenesis (Colie et al., 2009). These data suggest that SGPL1 expression is strongly associated with the carcinogenic activation of colon epithelial cells and may distinctively affect cancer initiation *versus* progression in opposite directions.

Typical characteristics of cancer include cell proliferation, growth signaling autonomy, escape from antiproliferative signals and apoptosis, induction of angiogenesis, and enhancement of migratory activity (Hanahan and Weinberg, 2000, 2011). SGPL1 downregulation has been shown to stimulate migration of breast cancer cell lines, while another study in mesangial cells demonstrated an inhibition of cell migration upon SGPL1 knockdown (Engel et al., 2018; Lovric et al., 2017). Again, the data suggest that SGPL1 may be an ambivalent regulator dependent on the cell type.

One of the pathways to induce cell migration includes the decrease of cell–cell adhesion by enhanced expression of adhesion molecules. E-cadherin, an epithelial marker and Ca^{2+}-dependent cell–cell adhesion molecule, is localized at the junction between neighboring epithelial cells and plays an important role in cancer suppression (van Roy and Berx, 2008). *Via* forming complexes with *e.g.* β-catenin in the plasma membrane, E-cadherin is linked to the actin cytoskeleton and can induce morphological changes in cells (van Roy and Berx, 2008). The loss of E-cadherin is a key feature of epithelial cancer cells and is required for the induction of epithelial-mesenchymal transition (EMT). Contrarily involved in the process of EMT is vimentin, a type III intermediate filament protein expressed in mesenchymal cells (Dave and Bayless, 2014).

Besides cell migration, dedifferentiation is a further hallmark of cancer cells. Colon epithelial cell transformation toward cancer cells is frequently assessed by levels of intestinal alkaline phosphatase (ALP_i). The ALP_i is a member of the phosphate transferases family and is highly expressed in the brush border membrane of epithelial cells in the intestine (Noda et al., 2016). Its physiological function consists in the hydrolytic removal of phosphomonoesters with release of inorganic phosphate and alcohol (Wu et al., 2017). Another well-known differentiation marker for intestinal epithelial cells is cytokeratin 20 (CK20), a member of the intermediate filament protein family. Although CK20 can be used as a marker for the determination of metastasis origin, a study indicated that colorectal cancer cells in late stages showed decreased expression of CK20 (Cross et al., 1996). Moreover, immunohistochemical staining of human colorectal cancer tissues revealed that tissues with reduced CK20 expression showed low differentiation and large tumor size (Harbaum et al., 2012).

In this present study, we aimed to identify the role of SGPL1 in the human colorectal cancer cell line DLD-1 in the context of migration, proliferation, and differentiation to elucidate its role in the tumorigenic cellular activity of those cells.

Material and Methods

Cultivation of DLD-1, Caco-2, and CCD 841 CoN Cells

DLD-1 and Caco-2 human colorectal cancer cell lines and CCD 841 CoN human non-cancerous epithelial cell line were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). DLD-1 cells were grown in RPMI 1640 Medium (Gibco, Waltham, MA, USA) supplemented with 10% FCS, 100 IU/mL penicillin and 100 µg/mL streptomycin (Sigma-Aldrich, Steinheim, Germany). Caco-2 cells were cultured in Minimum Essential Medium (Gibco) containing 20% FCS, 100 IU/mL penicillin, and 100 µg/mL streptomycin (Sigma-Aldrich). CCD 841 CoN cells were cultured in Minimum Essential Medium (Gibco) containing 10% FCS, 100 IU/mL penicillin, and 100 µg/mL streptomycin (Sigma-Aldrich). Cells have been tested negative for mycoplasm contamination. All cell lines were cultured in an incubator with 5% CO_2 at 37 °C. Cell counting has been performed manually with a Neubauer counting chamber.

Transfection with siRNA

DLD-1 cells were transfected using DharmaFECT 1 Transfection Reagent with the On-TARGETplus human SGPL1 siRNA SMARTpool (L-008747, Dharmacon, CO, USA) or scramble control siRNA (SIC001, Sigma-Aldrich). First, cells were plated in 6-well plates in cell culture medium without antibiotics and incubated at 37 °C with 5% CO_2 overnight. Thereafter, transfection medium with SGPL1 or scramble siRNA was prepared according to the manufacturer's

instructions and added to the cells. After 24 h of incubation, transfection medium was replaced with cell culture medium with antibiotics. Cells were harvested 72 h (for the ALP, XTT assay, Transwell, and Invasion assay) or 96 h (for the Scratch Assay) later to perform experiments. The expression of SGPL1 protein and mRNA was determined by Western Blots and qRT-PCR analysis, respectively (see below).

RNA Extraction and Quantitative Real-Time PCR

Total RNA of pelleted cells was extracted using the peqGOLD Total RNA Kit (peqlab, Erlangen, Germany) as recommended by the manufacturer. RNA concentration was measured using the Nano-Drop 1000 (Thermo Fisher Scientific, Waltham, Massachusetts, USA) analyzer and was adjusted to 1 μg/μL for first-strand cDNA synthesis using the high-capacity cDNA reverse transcription kit (Life Technologies, Carlsbad, CA, USA). TaqMan® gene expression assays (Life Technologies) were applied for SPHK1, SPHK2, SGPL1, SGPP1, SGPP2, S1P1, S1P2, S1P3, S1P4, S1P5, CK20, and for the housekeeping genes GAPDH and RPL13A (Applied Biosystems, Darmstadt, Germany). The Precision FAST Mastermix (Primer Design, Southampton, UK) was used, and quantitative real-time PCR was run at 95 °C for 2 min and 40 times at 95 °C for 5 s and 60 °C for 20 s (7500 Fast Real-Time PCR System, Applied Biosystems). The comparative C_T method was used for analyzing the results using the mean of the two housekeeping genes as a reference.

Liquid Chromatography Tandem Mass Spectrometry

Quantification of S1P was performed by high-performance liquid chromatography tandem mass spectrometry as described before (Schwiebs et al., 2017).

Western Blot

For Western Blot analysis, pelleted cells were lysed in a buffer containing 10 mM HEPES-KOH, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 0.5 M NaF, 100 mM Na_3VO_2, and 1× complete™ protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany) by sonification on ice for 3 × 10 s. Respective protein concentration was determined by BCA assay (Thermo Fisher Scientific), according to the manufacturer's instructions. Whole cell extracts were used for detection with antibodies against SGPL1 (HPA021125, dilution 1:500) (Atlas Antibodies, Bromma, Sweden), E-cadherin (24E10, dilution 1:666) (Cell Signaling, Danvers, MA, USA), CK20 (EPR1622Y, dilution 1:1000) (Abcam, Cambridge, UK), and β-actin (A5441, dilution 1:30.000) (Sigma-Aldrich) after SDS-PAGE. According to the first antibodies, the second antibody anti-rabbit IgG (dilution 1:4000) or anti-mouse IgG (1:2500) (GE Healthcare, Little Chalfont UK) has been used. The protein bands were detected by ECL (Thermo Fisher Scientific) following the manufacturer's protocol. Semi-quantitative evaluation was performed by densitometry using Quantity one (Bio-Rad, Hercules, CA, USA) and normalization to β-actin bands.

Scratch Cell Migration Assay

A scratch assay was used to assess cell migration. DLD-1 cells were seeded (2×10^5 cells/well; 6-well plates), transfected with SGPL1 siRNA or scramble control RNA, cultured under standard conditions for 3 days, and then starved in serum-free medium additionally over night to suppress cell proliferation. The cell monolayer was wounded by scratching with a 10 μL pipet tip within a grid that was marked before at the bottom of the dish. The cell layer was then washed and fresh serum-free medium was added to the cells. Additionally, the medium was spiked with 1 μg/mL anti-S1P antibody (ab140592, Abcam) to avoid an S1P signaling effect by S1P that possibly was transported out of the cell (Visentin et al., 2006). At this time point, the first contrast-phase microscopic picture (time point 0 h) was made (Axiovert 200 Microscope, Zeiss, Jena) directly next to the very left or right gridline to enable recognition of the same spot for later analysis. To quantify cell migration, another picture was made 24 h later of the very same area and the non-covered cell gap was measured with ImageJ software (open source). The dimension of each scratch was measured 10 times at different positions and the mean was used for data evaluation. The scratch assay was done in three independent experiments in triplicates.

Transwell Cell Migration Assay

Migration was measured using Transwell inserts with polycarbonate membrane with 8.0 μm poresize (Corning, NY, USA). The upper insert chambers were seeded with 2×10^5 cells in serum-free medium that had been transfected with SGPL1 si-RNA or scramble-RNA 72 h before. Thereafter, the lower chambers were replaced by RPMI medium containing 20% FCS as chemoattractant or serum-free RPMI medium as a control. After incubation for 20 h in an incubator with 5% CO_2 at 37 °C, the cells were fixed with 4% paraformaldehyde and stained with Crystal violet. The cells above the membrane have been removed by a cotton swab. Cells below the membrane have been analyzed under an inverted microscope. Further analysis was performed with Image J. The migration assay was performed in three independent experiments in triplicates.

Cell Invasion Assay

Cell invasion was conducted using pre-coated growth factor-reduced Matrigel inserts with a PET membrane with 8.0 μm poresize (Corning, NY, USA). The lower chambers were filled with RPMI medium containing 20% FCS as chemoattractant or serum-free RPMI medium as a control. The upper insert chambers were seeded with 2×10^5 cells in serum-free RPMI medium. After incubation for 25 h in an incubator with 5% CO_2 at 37 °C, the cells were washed, fixed with 4% paraformaldehyde, and stained with Crystal violet. The cells above the membrane have been removed by a cotton swab. Cells below the membrane have been analyzed under an inverted microscope. Further analysis was performed with Image J.

Metabolic Activity Assay

In different experimental groups, DLD-1 cells were seeded (2.5×10^4 cells/well; 6-well plates) and transfected with either SGPL1 or scramble siRNA for 72 h. According to specific experimental settings mentioned in the text, scramble siRNA samples have been additionally stimulated with 1 μM S1P (Cayman Chemical, Ann Arbor, MI, USA) for 24 h.

Subsequently, the XTT Cell Viability Assay (Thermo Fischer Scientific) was used according to the manufacturer's manual for determination of metabolic activity. In brief, the final XTT solution (2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2H-Tetrazolium-5-Carboxanilide) was added to the cells and to the medium control. Upon 3 h of incubation time at 5% CO_2 and 37 °C, absorbance of supernatants was analyzed in flat 96-well culture plates at 460 nm and normalized to 650 nm. Metabolic activity was calculated and expressed as absorbance at 460 nm.

Cell Proliferation Assay

DLD-1 cells were seeded (2.5×10^4 cells/well; 6-well plates) and cultured for 4 days. In different experimental groups, cells were transfected with either SGPL1 or scramble siRNA for 72 h, or stimulated with 1 μM S1P (Cayman Chemical) or vehicle for 24 h. On day 5 after seeding, the cell proliferation was quantified by determination of the numbers of living cells by trypan blue staining.

Multiple Immunofluorescence Staining and Analysis

Cells were grown on tissue-treated 8-well chambered cover slides (Ibidi, Martinsried, Germany), washed twice with ice-cold PBS, and fixed for 4 min with ice-cold methanol on ice. After washing three times with PBS, the cells were blocked with 2% BSA-PBS for 1 h and have been incubated subsequently with one of the following first antibodies: Anti-E-cadherin (24E10, dilution: 1:200) (Cell Signaling), Anti-vimentin (ab8978, dilution 1:200) (Abcam), or Anti-cytokertain20 (ab76126, dilution 1:300 (Abcam) were incubated overnight at 4 °C. After three washing steps, the second antibody (anti-rabbit, dilution 1:400) (GE Healthcare), DAPI (4′,6-Diamidine-2′-phenylindole dihydrochloride, dilution 1:500) solution (Roche Diagnostics) or for respective experiments phalloidin solution (A12380, Alexa Fluor 568, dilution 1:100 of stock solution according to manufacturer's manual) (Thermo Fisher Scientific) for F-actin staining were applied for 1 h. Finally, cells were washed and kept in the dark at 4 °C until microscopic analysis. Confocal laser scanning microscopy was performed with a Zeiss LSM510 Meta system equipped with an inverted Observer Z1 microscope and a Plan-Apochromat 63× /1.4 or 40× oil immersion or 20× objective (Carl Zeiss MicroImaging GmbH, Göttingen, Germany). Quantification of the staining was performed with Image J.

ALP Activity Assay

DLD-1 cells were seeded (5×10^4 cells/well; 6-well plates), cultured overnight, and then transfected with SGPL1 or scramble siRNA for 72 h.

Subsequently, the fluorometric ALP assay (ab83371, Abcam) was used according to the manufacturer's manual for determination of ALP activity. In brief, cells were resuspended in assay buffer, supernatants were transferred to a flat 96-well culture plate (110 μL/well) and were incubated with a reaction mix containing the substrate 4-Methylumbelliferyl phosphate for 30 min at 25 °C and 5% CO_2. Next, fluorescence intensity was measured at extension/emission of 355/460 nm. For normalization, the protein contents of the respective cell lysates were determined by BCA assay (Thermo Fisher Scientific, Massachusetts, USA). Finally, ALP activity was calculated and expressed as absorbance of 4-Methylumbelliferone (4-MU) in [%].

Statistical Analysis

The software GraphPad Prism 7.0 (La Jolla, CA, USA) was used to enter data and display graphs. For statistical analysis, we used *unpaired* or *paired Student's t-test* as indicated in the figure legends. Data are represented as means ± SD and significant values are symbolized as asterisks (* /** /***/****), which represent p-values of ≤0.05/ ≤0.01/ ≤0.001/ ≤0.0001. The number of experiments performed (n) has been indicated in the figure legends for each graph.

Results

SGPL1 Levels Are High in Colorectal Cancer Cell Lines

First, we analyzed the expression levels of S1P receptors and metabolizing enzymes to identify their potential role in signaling and sphingolipid level balance in colon cells *versus* colorectal cancer cells. In order to elucidate cancer-related differences, we compared the human colorectal cancer cells DLD-1 and Caco-2 with the non-transformed embryonic epithelial cell line CCD 841 CoN.

As shown in Fig. 1a, b, S1P receptor mRNA expression was marginal in all three cell lines except for S1PR2, which was higher expressed compared to all other receptors but with still low absolute expression values. A significant upregulation of S1PR2 was present in the two colorectal cancer cell lines compared to CCD 841 CoN cells. S1P enzyme mRNAs *SPHK2*, *SGPP1*, *SGPP2*, and *SGPL1* were all significantly upregulated in the cancer cells compared to CCD 841 CoN cells. Relative and absolute *SGPL1* mRNA expression was by far highest among all S1P metabolizing enzymes.

To examine the role of SGPL1 for key hallmarks of cancer spreading, we thrived to reduce the SGPL1 abundance in DLD-1 cells with SGPL1 siRNA. As expected, transfection significantly reduced the mRNA expression of SGPL1, with some differences in siRNA efficiency (Fig. 1c). Reduction of *SGPL1* mRNA expression (and complementary SGPL1 enzyme level reduction as presented later on) increased intracellular S1P levels (Fig. 1d). Expression of S1P metabolizing enzymes mRNAs *SPHK1*, *SPHK2*, *SGPP1*, and *SGPP2* remained unchanged upon SGPL1 siRNA treatment (Fig. 1e).

SGPL1 siRNA Treatment Reduces Migration and Invasiveness of Cancer Cells

Since cancer cell migration and invasion is a hallmark of tumor progression, we investigated the role of SGPL1 for the spatial motility of DLD-1 cells using one-dimensional scratch assays and multidimensional transwell and invasion assays.

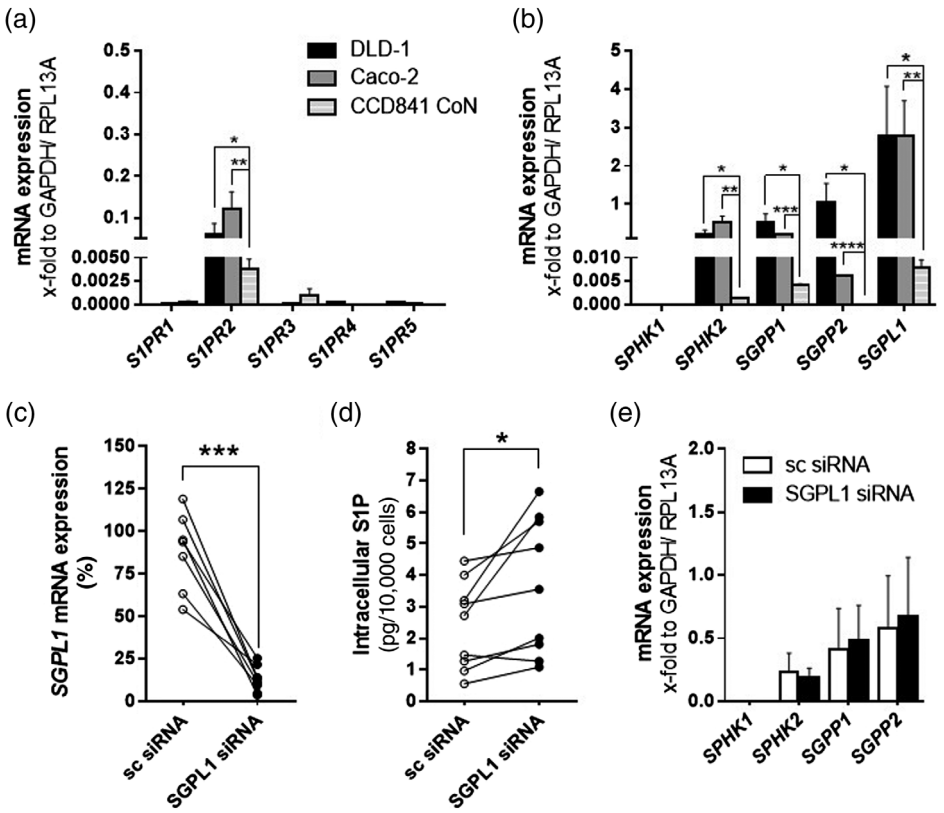

Fig 1 SGPL1 gene expression is upregulated in colon cancer cell lines. Expression of mRNA of (a) S1P receptors (n = 3) and (b) S1P metabolizing enzymes (n = 3) in the colon cancer cell lines DLD-1, Caco-2 and the epithelial cell line CCD841 CoN. Comparison of (c) *SGPL1* mRNA expression (n = 7), (d) intracellular S1P levels (n = 9) and of (e) *SPHK1*, *SPHK2*, *SGPP1*, *SGPP2* mRNA expression (n = 3) between DLD-1 cells that were treated with SGPL1 or scramble siRNA for 96 h. data are shown as mean ± SD. Statistical analysis was performed using *unpaired student's t-test*. Significances: * for $p \leq 0.05$, ** for $p \leq 0.01$, *** for $p \leq 0.001$, **** for $p \leq 0.0001$. (SGPL1, S1P lyase 1; S1PR1/2/3/4/5, S1P receptor 1/2/3/4/5; SPHK1/2, sphingosine kinase 1/2; SGPP1/2, S1P phosphatase 1/2, sc, scrambled)

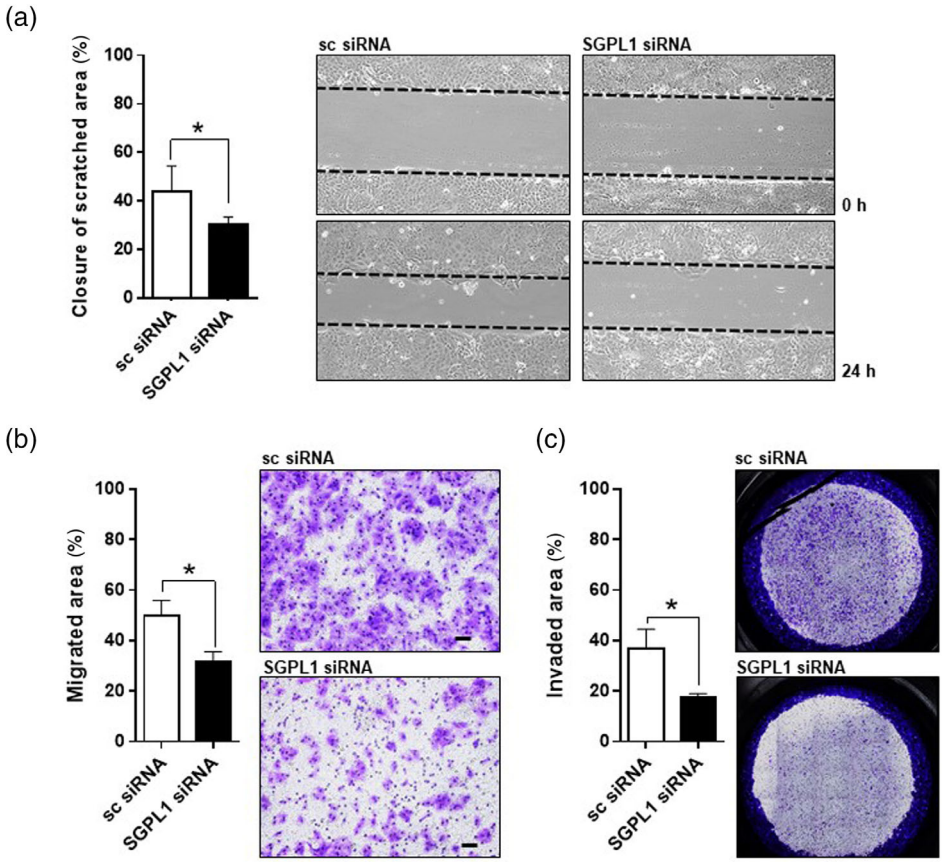

Fig 2 SGPL1 siRNA treatment inhibits migration and invasion of DLD-1 cells. DLD-1 cells were treated with SGPL1 or scramble siRNA for 96 or 72 h before the assays: (a) scratch assay: Closure of wound area 24 h after scratch (n = 5 in triplicates) and exemplary microscopic pictures of wound areas (magnification 10x). (b) Transwell assay: Quantified area of migrated cells 20 h after seeding and exemplary microscopic pictures (bar = 20 μm; n = 3 in duplicates). (c) Invasion assay: Quantified area of invaded cells 25 h after seeding and exemplary microscopic pictures (whole 24-well insert, n = 4). Data are shown as mean ± SD with * for $p \leq 0.05$. Statistical analysis was performed using *unpaired student's t-test*. (sc, scrambled; SGPL1, S1P lyase1)

As depicted in Fig. 2a SGPL1 siRNA transfection resulted in a slight but significant reduction of cell migration during horizontal scratch closure compared to control siRNA transfection. The transwell assay revealed also a reduced vertical chemotactic migration of SGPL siRNA-treated cells (Fig. 2b). Moreover, the ability of cancer cell spreading through an extracellular matrix during the invasion assay was cut in half after SGPL siRNA treatment (Fig. 2c).

SGPL1 siRNA Treatment Is Not Affecting Proliferation and Metabolic Activity of Cancer Cells

As cell growth and activity are other pro-carcinogenic features, we next investigated the role of SGPL1 siRNA treatment in cell viability assays. SGPL1 siRNA transfection did not affect cell proliferation or metabolic activity of the cells (Fig. 3a, b). We additionally compared the results to exogenous S1P stimulation. As shown in former studies too, S1P-treatment led to an increase in cell proliferation (Fig. 3c). Metabolic activity however was not affected (Fig. 3d). After stimulation, S1P was apparently partly internalized and accumulated in the cell (Fig. 3e). This resulted in a complementary upregulation of *SGPL1* mRNA expression, respectively (Fig. 3f).

SGPL1 siRNA Treatment Increased Cell Adhesion of Cancer Cells

Since SGPL1 siRNA transfection showed no significant effect on cell proliferation or metabolic activity, the mechanism by which SGPL1 reduces cell migration and invasion remained unclear. To identify whether SGPL1 possibly modulated cell–cell adhesion as a key feature of cell migration, we first performed Western Blot and analyzed the protein expression level of E-cadherin. We observed that overall E-cadherin protein expression in cell lysates was marginally, however significantly, increased parallel to SGPL1 enzyme reduction in all experiments (Fig. 4a–c).

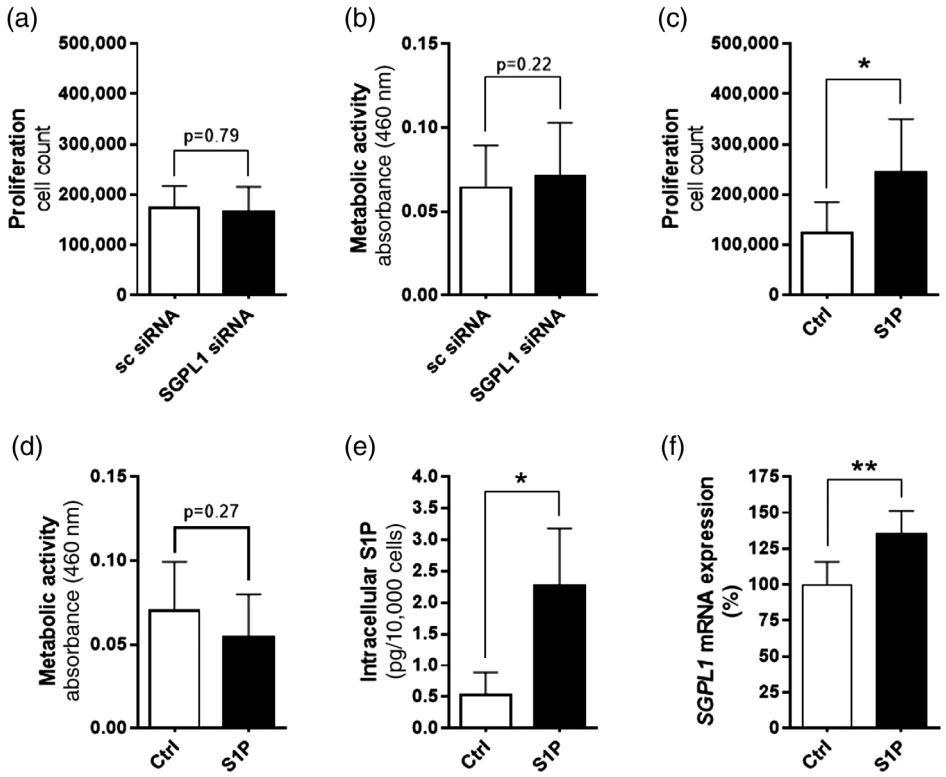

Fig 3 Effects of SGPL1 siRNA and S1P on proliferation and metabolic activity of DLD-1 cells. (a) Proliferation and (b) metabolic activity of DLD-1 cells was determined after cells were transfected with SGPL1 or scramble siRNA for 72 h (n = 9 in triplicates). (c) Proliferation, (d) metabolic activity, (e) intracellular S1P-concentration (n = 3), and (f) expression of SGPL1 mRNA in DLD-1 cells, stimulated with 1 μM S1P or vehicle for 24 h; (n = 7 in triplicates if not indicated otherwise). Data are shown as mean ± SD with * for $p \leq 0.05$, ** for $p \leq 0.01$. Statistical analysis was performed using *unpaired student's t-test*. (sc, scrambled; S1P, sphingosine-1-phosphate; SGPL1, S1P lyase 1; ctrl, control)

To further explore this result, we additionally performed immunofluorescence staining. Anti-E-cadherin staining was especially present at plasma membranes of DLD-1 cells and prominently accumulated at such upon SGPL1 siRNA treatment (Fig. 4d,f). Since E-cadherin is a driver of cell–cell adhesion, subsequently, we investigated the cell contacts between DLD-1 cells in more detail by an additional Anti-F-actin (Phalloidin) staining. Quantification of overall Anti-F-actin staining of the cells revealed a higher abundance of the protein (Fig. 4e). Selectively focusing on Anti-F-actin staining at the basement of the cell layer showed that the cytoskeleton was reorganized upon SGPL1 reduction from regular fibers to a more cross-linked and packed appearance between the cells (Fig. 4g). Double staining showed a co-localization of Anti-E-cadherin and Anti-F-actin patches forming complexes at distinct sites of neighboring cells (Fig. 4h, white arrows).

Additionally, we analyzed the abundance of the anchor protein vimentin, which is typically present at outer islet sites of migrating cells. We therefore cultured DLD-1 cells in a sub confluent cell layer, where expanding cell islets were present. We found that vimentin staining was dominantly present at outer cell islet sites of DLD-1 cells but partly lost in SGPL1 siRNA transfected DLD-1 cells (Fig. 4i). Taken together, these results indicate that SGPL1 siRNA treatment strengthens cell–cell adhesion *via* cytoskeletal remodeling, increased expression of the adhesion molecule E-cadherin, and assembly of cadherin-actin complexes. Furthermore, by regulating vimentin expression, SGPL1 seemed to influence the spreading of cell islets.

SGPL1 siRNA Treatment Partially Induces Redifferentiation of Cancer Cells

As vimentin is a marker of mesenchymal cells rather than epithelial cells and cell islet staining was reduced upon SGPL1 siRNA treatment, we suggested that the differentiation status of DLD-1 cells was altered *per se*. Thus, we finally, explored ALP_i activity and CK20 abundance as marker systems for epithelial cell differentiation.

Investigations revealed that are in parallel to a reduction of SGPL1 expression, ALP_i activity increased (Fig. 5a). *CK20* mRNA levels were raised in SGPL1 siRNA-treated cells (Fig. 5b) and in addition, immunofluorescence staining with Anti-CK20 depicted larger cohesive regions of epithelial cell-like islands upon SGPL1 siRNA treatment, while in ordinary

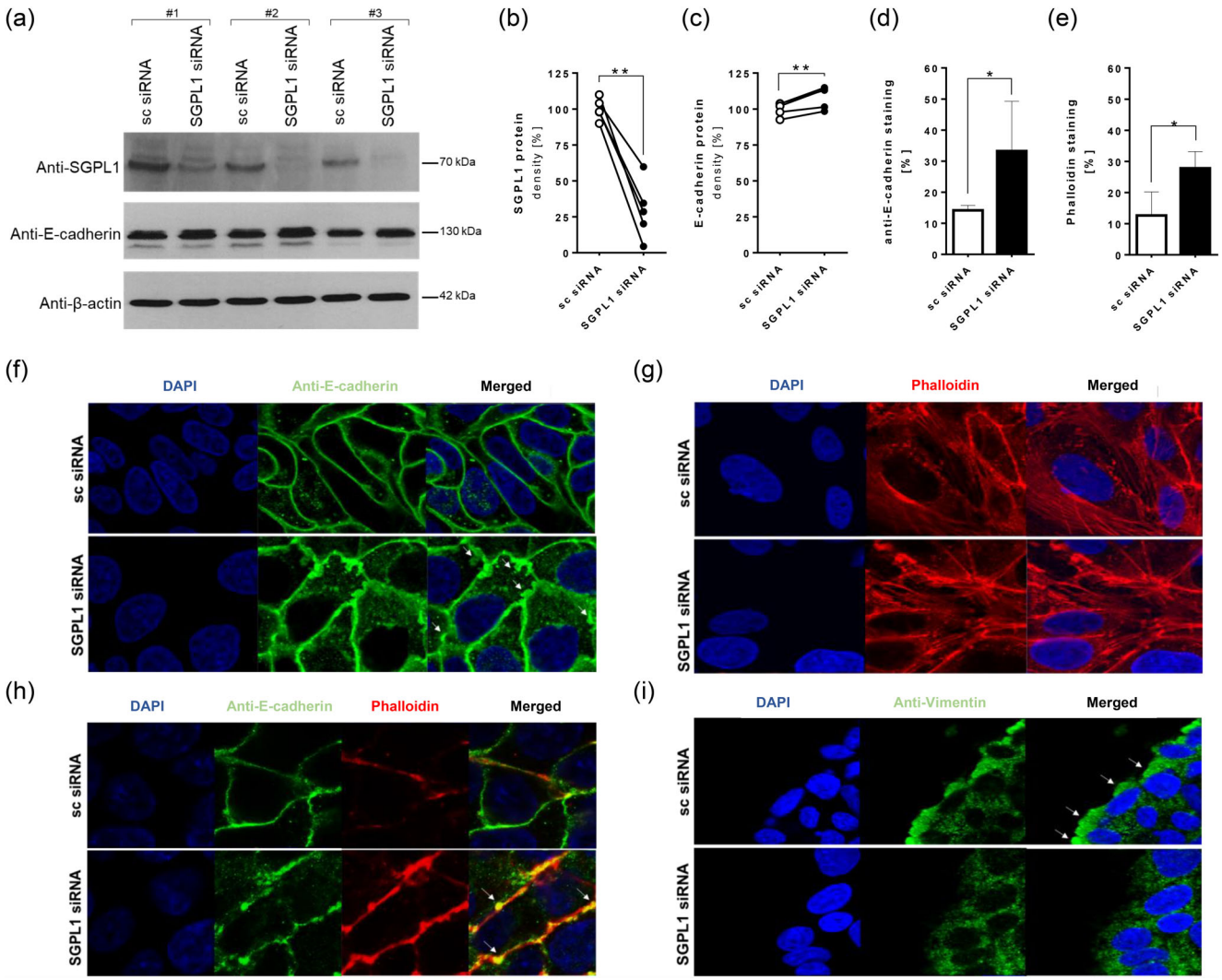

Fig 4 SGPL1 Sirna treatment increases cell adhesion. (a) Representative pictures of Western blot analysis of SGPL1 and E-cadherin protein expression in DLD-1 cells transfected with SGPL1 or scramble siRNA for 96 h (n = 5). Quantification of (b) anti-SGPL1 and (c) anti-E-cadherin staining normalized to anti-ß-Actin staining in Western blot (n = 5). Quantification of (d) anti-E-cadherin and (e) Phalloidin staining of fluorescence imaging (n = 5) (f–h) representative fluorescence images (magnification 63x) of cells stained with antibody against E-cadherin (green (f)) or stained with phalloidin solution for F-Actin (red (g)) or double-stained (h). (i) Representative fluorescence images (magnification 40x) of cell islet border of sub confluent cells stained with antibody against vimentin (green). (f-i) Simultaneous DAPI staining (blue) was used to visualize the nuclei of cells. Data are shown as mean ± SD with * for $p \leq 0.05$, ** for $p \leq 0.005$. Statistical analysis was performed using *paired (b,c) and unpaired students t'test (d,e)*. (sc, scrambled; SGPL1, S1P lyase 1)

DLD-1 cells fewer and more disconnected islands were found (Fig. 5c,d). Quantification revealed slightly higher overall CK20 abundance. These data demonstrate that SGLP1 siRNA treatment partly augments characteristics of epithelial cell differentiation in the colorectal cancer cell line DLD-1.

Discussion

Important physiological processes like cell migration, inflammation, angiogenesis, and carcinogenesis are modulated by S1P (Aguilar and Saba, 2012; Degagne and Saba, 2014; Kumar et al., 2011; Lucke and Levkau, 2010). SGPL1 irreversibly degrades S1P and thus controls S1P pools available for intra- and extracellular signaling. In our study, the relative increase of SGPL1 transcripts in the human colorectal cancer cell lines DLD-1 and Caco-2 compared to non-transformed epithelial cells suggested a functional importance of SGPL1 expression level for their cancer characteristics (Fig. 1b). Several studies have shown that SGPL1 knockout or knockdown in *in vivo* models of colorectal cancer supported transformation of normal colon epithelium into cancer cells (Degagne et al., 2014; Oskouian et al., 2006; Schwiebs et al., 2019; Uranbileg

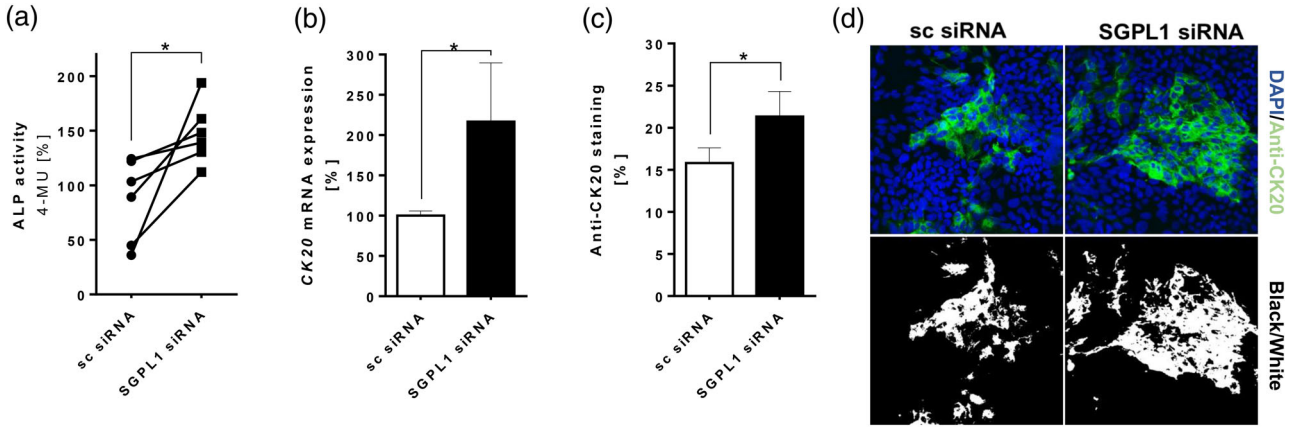

Fig 5 Effects of SGPL1 siRNA treatment on differentiation of DLD-1 cells. (a) Determination of the ALPi activity by fluorometric ALP assay in DLD-1 cells transfected with SGPL1 or sc siRNA for 72 h; (n = 6). (b) *CK20* mRNA analysis in DLD-1 cells transfected with SGPL1 or sc siRNA for 72 h (n = 3), (c) quantification of anti-CK20 immunofluorescence staining after transfection with SGPL1 or sc siRNA for 96 h (n = 3–5) (d) representative pictures of fluorescence stained DLD-1 cells using antibody for cytokeratin 20 (green) as well as DAPI staining (blue). Representative images are displayed in color and in black/white (magnification 20x). Data are shown as mean ± SD with * for $p \leq 0.05$. Statistical analysis was performed using *unpaired student's t-test*. (sc, scrambled; SGPL1, S1P lyase 1; ctrl, control; ALPi, intestinal alkaline phosphatase; CK20, cytokeratin 20)

et al., 2018). However, in human colon cancer tissues, SGPL1 mRNA expression levels seem to be increased (Goldman et al., 2020). Furthermore, high expression of SGPL1 in tumors was shown to worsen survival probability of colorectal cancer patients (Uhlen et al., 2017). Based on this knowledge, it seems that reduction of SGPL1 levels initiate colorectal cancer on the one hand and at the same time, high levels of SGPL1 seem to worsen colorectal cancer outcome in established cancer. Thus, the consequences of SGPL1 modulation during cancer development are presumably different from the consequences for established colon cancer cells. However, little is known about the impact of modulation of the high SGPL1 levels in colorectal cancer cells for their persisting malignancy. In this study, we analyzed the role of SGPL1 in migration, invasion, proliferation, and differentiation of the human colorectal cancer cell line DLD-1.

SGPL1 siRNA treatment significantly reduced SGPL1 mRNA and protein expression and subsequently S1P levels raised, albeit this increase was only one fourth compared to a 70% reduction in SGPL protein levels (Figs. 1d and 4a, b). Although SGPL1 is a key enzyme in S1P degradation, the intracellular S1P pool results also from the existence and modulation of S1P-phosphatases, S1P producing enzymes and S1P transporters that presumably co-regulated S1P levels in DLD-1 cells. The intracellular accumulation of S1P can have direct effects by either binding or indirectly affecting intracellular targets. On the other hand, 'inside out signaling' can occur in an autocrine manner when S1P is exported and binds to its receptors. In our study, we aimed to block this inside out signaling of S1P by the addition of an S1P antibody as shown before (Visentin et al., 2006; Zhang et al., 2015). We thus assume that effects we see are primarily effects of intracellular S1P or even of reduced SGPL1 expression levels themselves.

S1P is attributed to its role in cell proliferation and according to own studies we know that reduction of intracellular S1P levels reduces proliferation of *e.g.* immune cells (Schwiebs et al., 2016). We have compared the effects of SGPL1 reduction by siRNA transfection as well as S1P-treatment as two different mechanistic pathways resulting in increased intracellular S1P levels. Interestingly, S1P accumulation by SGPL1 siRNA did not affect cell proliferation in DLD-1 cells while exogenous S1P stimulation with subsequent intracellular S1P accumulation increased cell proliferation (Figs. 2a and 3c). *Chen et al* have shown that proliferation of intestinal epithelial cells was regulated *via* S1P2 signaling (Chen et al., 2017). Interestingly, relative S1P2 expression levels were high in DLD-1 cells and exogenous S1P stimulation might have forced cell proliferation *via* S1P2 rather than intracellular S1P or inside out signaling, respectively.

An involvement of SGPL1 modulation in immune cells including their migration ability, is studied widely. However, knowledge about SGPL1 modulation on the migration and thus malignity of established cancer cells is rare. Very recently it was demonstrated that a reduction of DLD-1 cell proliferation was accompanied by a reduction of *SGPL1* mRNA expression by heterogeneous nuclear ribonucleoprotein H1 siRNA treatment (Takahashi et al., 2020). Previous studies have described as well, that low endogenous expression levels of SGPL1 were accompanied with reduced proliferation and invasion on the colon cancer cell lines HCT116 and LoVo (Uranbileg et al., 2018) or with decreased migration of hepatocellular carcinoma cells

(Uranbileg et al., 2016). To understand the reason why SGPL1 siRNA treatment decreased migration and invasion we analyzed cell–cell adhesion in DLD-1 cells, which defines function and structure of tissues and determine cell migration. We found that SGPL1 siRNA treatment led to enhanced accumulation of the adhesion molecule E-cadherin at the cellular membrane (Fig. 4d). Cell adhesion is based on cadherin interaction at cell–cell contacts and integrin interactions at cell-ECM contacts. Whereas integrin acts as receptor for different ECM proteins like fibronectin, E-cadherin binds adapter proteins (*e.g.* ß-catenin) that link to the actin cytoskeleton (Collins and Nelson, 2015). Thus, increased E-cadherin strengthens cell–cell contacts *via* interactions with adapter proteins. For cell migration, dynamic changes in the actin cytoskeleton are necessary (Collins and Nelson, 2015). We have shown that cadherin-actin complexes were upregulated at cell–cell contacts upon SGPL1 siRNA treatment (Fig. 4f) and cell condensation was triggered through changes in the actin cytoskeleton (Fig. 4g). Earlier studies have shown that S1P increases the expression of E-cadherin in intestinal epithelial cells and also enhances E-cadherin accumulation at the cell–cell junction, indicating an increase in barrier integrity (Greenspon et al., 2011). In addition, the distribution of the mesenchymal marker vimentin, was reduced in regions, where cell expansion takes place (Fig. 4i). Vimentin is associated with the metastatic ability of cancer cells and sphingolipid stimulation has been shown to regulate vimentin-dependent cell migration (Hyder et al., 2015). A direct effect of SGPL1 expression on vimentin distribution in cancer cell migration has not been demonstrated before this study. Collectively, our results show that reduction in SGPL1 expression is accompanied by reduced cell migration and invasion in DLD-1 cells and strengthens cell–cell adhesion *via* upregulation of cadherin-actin complexes and vimentin downregulation at outer cell islet regions. Therefore, we hypothesize that SGPL1 controls the migration potential of DLD-1 cells and thus probably plays a role in metastasis of DLD-1 cells.

Meshcheryakova et al. (2016) found a strong upregulation of SPHK1 and SGPP2 as well as moderate upregulation of SGPL1 upon TGFbeta and/or TNFalpha treatment at mesenchmayl/fibroblast-like cell stage in lung cancer cells, triggering EMT process and resulting in accumulation of intracellular S1P (Meshcheryakova et al., 2016). The sphingolipid/EMT-associated gene signature identified for lung cancer showed similarity to the expression patterns of other malignant tumors such as colorectal cancer and thus, same EMT mechanism can be suggested and might contribute to metastatic process in colorectal cancer (Meshcheryakova et al., 2016). Recent studies revealed that S1P induces EMT in hepatocellular carcinoma cells *via* an autocrine loop, involving MMP-7, syndecan-1, and TGF-ß (Zeng et al., 2016). Other studies demonstrated that globosides are essential for E-cadherin-mediated cell–cell adhesion and loss of globosides by depletion of α1-4 Galactosyltransferase, a globoside synthesizing enzyme, induces EMT (Jacob et al., 2018). The exact mechanism by which SGPL1 siRNA treatment increases E-cadherin expression and E-cadherin-F-actin complex formation needs to be elucidated further.

The ALP activity and CK20 expression can be used as markers of differentiated intestinal epithelial cells (Shin et al., 2014; Zhang et al., 2014). SGPL1 siRNA treatment resulted in increased ALP activity and CK20 mRNA expression and enhanced clustering of CK20-positive cells into islets (Fig. 5a–d). Together with the data on E-cadherin restoration and vimentin loss, these results create the hypothesis that SGPL1 siRNA treatment leads to partial induction of redifferentiation of DLD-1 colon cancer cells into epithelial cells.

Overall, our results suggest that SGPL1 is able to modulate the degree of malignancy of DLD-1 colorectal cancer cells by suppressing key hallmarks of cancer such as invasion and dedifferentiation.

Acknowledgements The authors thank Sandra Trautman for lipid extraction and analysis. This study was supported by the Deutsche Forschungsgemeinschaft (grant number: SFB1039) to (WFF, HHR, AS, KGS, DT, JMP). We also acknowledge the support of the Translational Research Innovation Pharma graduate school (KGS) funded by the Else-Kröner-Fresenius Foundation and awarded to (HHR). For helpful discussion and advice, we would like to thank Dr. H. Vienken and Prof. D. Meyer zu Heringdorf.

Author Contributions WFF and AS designed the study, performed experiments, data acquisition, analysis and evaluation, and wrote the manuscript. KGS and HHR contributed to data analysis and were involved in correcting and proofreading the manuscript. DT conducted LC–MS/MS analysis. JMP provided basic lab equipment and discussed data. All authors reviewed the relevant intellectual content and WFF, AS, and HHR discussed and approved the final manuscript.

Conflicts of Interest The authors declare that they have no conflicts of interest.

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