RESEARCH ARTICLE

microRNA-140-3p modulates invasiveness, motility, and extracellular matrix adhesion of breast cancer cells by targeting syndecan-4

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Abstract
Syndecan-4, a predicted target of the microRNA miR-140-3p, plays an important role in multiple steps of tumor progression and is the second most abundant heparan sulfate proteoglycan produced by breast carcinoma cell lines. To investigate the potential functional relationship of miR-140-3p and syndecan-4, MDA-MB-231, SKBR3, and MCF-7 breast cancer (BC) cells were transiently transfected with pre-miR-140-3p, syndecan-4 small interfering RNAJ, or control reagents, respectively. Altered cell behavior was monitored by adhesion, migration, and invasion chamber assays. Moreover, the prognostic value of syndecan-4 was assessed by Kaplan–Maier Plotter analysis of gene expression data from tumor samples of 4929 patients. High expression of syndecan-4 was associated with better relapse-free survival in the whole collective of BC patients, but correlated with a worse survival in the subgroup of estrogen receptor negative and estrogen/progesterone-receptor negative patients. miR-140-3p expression was associated with improved survival irrespective of hormone receptor status. miR-140-3p overexpression induced posttranscriptional downregulation of syndecan-4, as demonstrated by quantitative real-time PCR (qPCR), flow cytometry, and luciferase assays, resulting in decreased BC cell migration and matrigel invasiveness. Furthermore, miR-140-3p overexpression and syndecan-4 silencing increased the adhesion of BC to fibronectin and laminin. qPCR analysis demonstrated that syndecan-4 silencing leads to altered gene expression of adhesion-related molecules, such as fibronectin and focal adhesion kinase, as well as in the gene expression of the proinvasive factors matrix metalloproteinase 2 and heparanase (also known as HPSE). We conclude that syndecan-4 is a novel target of miR-140-3p that regulates BC cell invasiveness and cell-matrix interactions in the tumor microenvironment.

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1 | INTRODUCTION

Syndecan-4 is a central mediator of cell adhesion, and also has an important role in cell migration, proliferation, endocytosis, and mechanotransduction. The broad effects of this molecule are exemplified by its unique versatility in extracellular, cell membrane and intracellular interactions.1-3

Several studies have demonstrated that changes in the expression of syndecan-4 contribute to the development and progression of cancers.4 Syndecan-4 expression is reduced in colorectal cancer and osteosarcoma, in contrast syndecan-4 is overexpressed in glioma, melanoma, neuroblastoma, osteosarcoma, papillary thyroid carcinoma, liver, kidney, testicular, and bladder cancer.5 In addition, our previous work demonstrated that acquisition of anoikis resistance is associated with upregulated syndecan-4 expression in endothelial cells.6

According to the literature syndecan-4 inhibits breast carcinoma cell invasion, and its expression in human breast carcinoma was described as being associated with a good prognosis.7 In contrast, another study found that syndecan-4 is overexpressed in an estrogen receptor (ER)-negative, highly proliferative breast carcinoma subtype.8 While these data suggest an important, and possibly subtype specific role of syndecan-4 in breast cancer (BC) pathogenesis, little is known about the mechanisms of its (dys) regulation in BC.

BC is a heterogeneous form of cancer characterized by complex genetic alterations. According to the American Cancer Society, BC is the most common cancer among women, accounting for nearly one in three cancers diagnosed in women. Metastasis formation at different distant sites are the main cause of BC-related death.9 One of the relatively novel and intensively discovered mechanisms of BC development and pathogenesis includes changes in the expression of microRNAs (miRNAs) levels.10,11

Numerous studies have demonstrated that miRNAs acting as tumor promoters or tumor suppressors are involved in the regulation of a variety of malignancies.12-16 Notably, a variety of studies have demonstrated that miRNAs can regulate the expression of several extracellular matrix (ECM) molecules, including heparan sulfate proteoglycans and enzymes mediating the biosynthesis and degradation of their GAG carbohydrate chains.12,17

For example, miR-140-3p upregulated many of the components involved in the synthesis of hyaline ECM and reduced the levels of aggregcanases and syndecan-4 in a model of Interleukin 1β-induced Osteoarthritis. In addition, studies in miR-140-3p knock out mice and miR-140-3p depletion in vitro demonstrated changes such as accelerated aggregcan proteoglycan loss and ECM degradation.18,19 Interestingly, a recent study demonstrated that, miR-140-3p is downregulated in BC tissues and cell lines, and that this downregulation could promote BC cell proliferation and migration.20 Overall, these data mark miR-140 as a plausible candidate for a regulation of syndecan-4 in BC that is worth exploring at the mechanistic level.

In our study, we investigated a possible negative regulation of syndecan-4 by miR-140-3p, and the consequences of syndecan-4 depletion in human BC cell lines. Syndecan-4 is confirmed as a novel regulatory target of miR-140-3p, and both miR-140-3p overexpression and syndecan-4-depletion decrease BC cell motility and invasiveness. At the molecular level, modulation of these processes by syndecan-4 involves altered expression of adhesion molecules.

2 | MATERIALS AND METHODS

2.1 | Cell culture

Human BC cell lines, human umbilical vein endothelial cells (HUVEC), and the monocytic cell line U937 were purchased from ATCC/LGC Promochem. MDA-MB-231, SKBR3 and U937 cells were maintained in Dulbecco’s modified Eagle medium (DMEM) containing 10% fetal calf serum (FCS), 1% glutamine and 1% penicillin/streptomycin in a humidified atmosphere of 7% CO₂ at 37°C. MCF-7 cells were cultured in RPMI-1640 medium containing 10% FCS, 1% glutamine and 1% penicillin/streptomycin in a humidified atmosphere of 5% CO₂ at 37°C. HUVEC cells were cultured in endothelial cell growth Media MV (EGM) (cat. no. C-22020; PromoCell GmbH) at 37°C and 5% CO₂. All reagents were from Sigma-Aldrich Chemie GmbH, except for FCS (PAN-Biotech GmbH).
2.2 | Small interfering RNAJ (siRNA) transfection and miRNA precursor transfection

For siRNA transfection, 2.5 × 10^5 cells/well of a six-well plate were cultured in DMEM medium for 24 h. siRNA transfection was performed on semi-confluent cells (60%–70%) using Dharmafect reagent (cat. no. T-2001-03; Dharmacon™) according to the supplier’s instructions. This reagent contained in a total volume of 1 ml 840 µl Opti-MEM® media (cat. no. 31985-070; Gibco®; Thermo Fisher Scientific), 80 µl 10 µM syndecan-4 siRNA/Opti-MEM® (cat. no. 4392420, ID s12638 and s12640; Ambion® Life Technologies) or negative control siRNA (cat. no. 4390844; Ambion®), and 80 µl 2.5% Dharmafect/Opti-MEM® solution. Cells were left for incubation at 37°C and 7.5% CO₂ for 24 h and then the medium was changed to DMEM or RPMI medium with FCS. Messenger RNA (mRNA) and protein extraction were performed 48 h after the initial transfection. For miRNA transfection, cells were plated in the same way and transfected with miRNA precursor miR-140-3p (5 µM) and pre-miR precursor negative control (ABI). Twenty-four hours after transfection, OPTI-MEM was replaced by Dulbecco’s modified Eagle’s medium as described above. Assays were performed 48–72 h after transfection, as indicated in results.

2.3 | Quantitative real-time PCR (qPCR)

RNA isolation was performed with InnuPREP RNA mini kit (cat. no. 845-KS-2040250; Analytikjena), then reverse-transcribed into complementary DNA (cDNA) using the High-Capacity cDNA Reverse Transcription Kit (cat. no. 4368814; Applied Biosystems) according to the supplier’s protocols. qPCR was performed in duplicates for each target gene using RT2 SYBR Green qPCR Primer Assay (cat. no. 330500; Qiagen) and gene expression levels were measured in an ABI 7300 Real-time PCR detection system (Applied Biosystems). Transcriptional analysis was performed using the ΔΔCT method and samples were normalized to the expression of beta-actin (β-actin) as internal control. Melting curve analysis was performed to confirm specific product amplification. Primer sequences were confirmed by NCBI BLAST analysis and are listed in the Table S1. miRNA expression was determined using Taqman miRNA assays miR-140-3p and RNU6B, detecting the mature RNAs (ABI). qPCR was performed in a 96-well plate format using the ABI PRISM 7300 Sequence Detection System using the default thermal cycling conditions (denaturation at 95°C for 10 min [1X], followed by 40 cycles of denaturation [95°C, 15 s] and combined annealing and extension [60°C, 60 s]). The comparative cycle threshold method was used for relative quantification.

2.4 | Flow cytometry

BC cells were detached with 0.5 mM ethylenediaminetetraacetic acid, centrifuged at 1000 rpm for 4 min, washed three times with washing buffer (phosphate-buffered saline [PBS] containing 0.1% FCS) and counted. Cells were re-suspended in 100 µl PBS containing 0.1% bovine serum albumin (BSA) and incubated with mouse anti-human syndecan-4 antibody (sc-12766; Santa Cruz Biotechnology Inc.) for 1 h in the dark. Negative control was incubated without the primary antibody. Cells were then washed three times with 1 ml each of the above mentioned washing buffer and finally re-suspended in 100 µl PBS/BSA containing incubated as above with a goat anti mouse Alexa 488 secondary antibody (A11029; Invitrogen). Cells were incubated for 1 h in the dark, then three times washed with washing buffer, and analyzed using by a Cytosoft Space flow cytometer (Cytosoft Space; Sysmex/Partec). Fluorescence emission was measured at 527 nm and intensity was calculated as mean fluorescence intensity by setting a region gate using FloMax software (Quantum Analysis).

2.5 | Luciferase assays

pre-miR-140.3p was cotransfected along with 1 µg of plasmid pEZX-MT06 expressing firefly luciferase under the control of the human SDC4 3′-untranslated region (UTR), and renilla luciferase under the control of the Cytomegalovirus promoter (HmiT109023- MT06; GeneCopoeia), and pre-miR-140.3p. Cells cotransfected with the SDC4-Luciferase vector and control miRNA served as negative controls. Luciferase assays were performed in triplicates using the Luc-Pair™ Duo-Luciferase Assay Kit 2.0 (GeneCopoeia) as described by the manufacturer. Cell lysates were assayed in a luminometer 48 h after transfection, normalizing firefly to renilla luciferase activity. Data were expressed as percentage inhibition relative to control miR-transfected cells.

2.6 | Cell viability assay

Forty-eight hours after miRNA/siRNA transfection cells were plated in 96-well plates (5 × 10³ cells/well) and cultured for 24 h with DMEM medium (without phenol red) (cat. No. 31053028; Gibco®) or RPMI medium (without phenol red) with FCS. After the incubation
time, the cells were incubated with 20 µl/well of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) (cat. no. M2128-1G), at 5 mg/ml for 4 h, at 37°C. Subsequently, 100 µl of stopping buffer, pH 4.7 composed of 10% (wt/vol) sodium dodecyl sulfate (SDS) (cat. no. 3599286) and 50% (vol/vol) N,N-dimethylformamide (cat. no. 605365) was added to stop the reaction and dissolve the formazan crystals. The absorbance was measured in a VersaMax Microplate Reader (Molecular Devices) at a wavelength of 595 nm. The proliferation of the control cells was defined as 100%. Results were derived from three independent sets of triplicate experiments.

### 2.7 Cell adhesion assay

In brief, 96-well plates were coated with 20 µg/ml laminin (1 h, 37°C) or 10 µg/ml fibronectin (4°C, 16 h) washed and blocked with DMEM/0.5% BSA. Forty-eight hours after transfection, adhesion of 25,000 cells/well was measured spectrophotometrically after methylene blue staining as described previously.

### 2.8 Matrigel invasion assay

A total of 25,000 MDA-MB-231 cells were seeded per invasion filter (BD Biosciences) in a 24-well plate format 24 h after transfection and incubated for another 24 h. The invasion assay was then performed by addition of serum-free medium to the upper chamber and of 10% FCS-containing media to the bottom well of the invasion chamber for another 24 h, resulting in transmigration of the cells through the ECM along this chemotactic gradient, and cell attachment to the bottom of the filter insert. Cells in the upper chamber were removed with a cotton swab, and cells on the lower surface were fixed and stained with Diff-Quik dye (Medion). Excised and mounted filter membranes were photographed using a Zeiss Axiovert microscope equipped with Axiosvision software (Zeiss) at x100 magnification. Relative invasiveness was expressed as percentage of the siRNA/miRNA-treated cell number per membrane insert compared with control siRNA cell numbers per insert (n > 3).

### 2.9 Cell migration assay

In total, 25,000 MDA-MB-231 cells in 0.5 ml serum-free DMEM/0.1% BSA were added in duplicates to the upper compartments of Transwell migration inserts (pore size: 8 µm; BD Biosciences) 48 h after transfection with control miRNA and control siRNA or premiR-140-3p and siSDC4. The lower compartment contained 0.75 ml DMEM/0.1% BSA/10% FCS. After 24 h, cells on the lower surface were fixed and stained with DiffQuik (Medion). Relative migration was expressed as percentage of cells on compared to control inserts (n > 3).

### 2.10 Kaplan–Meier plot analysis

To assess the prognostic value of syndecan-4 genes, the online tool kmplot.com, which allows a meta-analysis of gene expression in relation to BC patient survival, was employed. Gene expression data were obtained through microarray analysis of widely used arrays of the GEO database and converted into Kaplan–Meier plots. This study includes relapse-free survival (RFS) (n = 4934) and overall survival (OS) (n = 1880). The package “survival” was used in the R programming environment to plot Kaplan–Meier survival curves and compute the number-at-risk. To distinguish between high and low expression, the median was selected as cut-off value to reduce the impact of outliers and produce equal numbers in both categories to only show strong correlations. Also, the JetSet probe set was selected to acquire unambiguous expression estimates and redundant samples were removed to enhance the quality of the sample. Patients were stratified by ER status, PR status. We analyzed the expression of syndecan-4 and visualized the correlation to survival by drawing Kaplan–Meier survival plots. The Affymetrix ID is 202071_at (SYND4). The shown hazard ratios (HR) are not inverted (HR <1 favorable).

To analyze the prognostic value of miR-140-3p, we used the online tool kmplot.com/mirpower. This database uses the GEO, EGA, TCGA, and PubMed repositories to identify datasets with published miRNA expression and clinical data from 2178 samples. To validate the prognosis significance of 41 previously published survival associated miRNAs the database performs a Kaplan–Meier OS analysis. The Affymetrix ID of miR-140-3p is hsa_miR_140_3p.

### 3 RESULTS

#### 3.1 Patients with ER/PR negative status expressing high levels of syndecan-4 have a poor outcome

To establish whether syndecan-4 expression could have an impact on BC prognosis, we investigated its relation to RFS. Gene expression data from tumor samples of 4929 patients without any stratification and patients classified according to different molecular subtypes and receptor status were analyzed using the online tool Kaplan–Meier Plotter. We
observed that high syndecan-4 was a protective factor for patient survival for all BC patients, HR = 0.87; p = .0058 (Figure 1A). However, in patients with ER-negative tumors, high syndecan-4 expression correlated with a worse survival (HR = 1.29; p = .0091) (Figure 1B); the same was found in patients with ER/progesterone receptor (PR)-negative status (HR = 1.41; p = .033) (Figure 1C). In contrast, in patients with ER/PR positive tumors, we did not find a significant correlation of syndecan-4 expression with survival (HR = 1.09; p = .57) (Figure 1D).

### 3.2 Syndecan-4 is a regulatory target of miR-140-3p

Syndecan-4 is expressed in normal human mammary epithelium, and it is the second most abundant HSPG produced by most breast carcinoma cell lines. As both syndecan-4 and miRNAs are dysregulated in BC, we hypothesize that an aberrant, miRNA-dependent regulation of syndecan-4 may have a mechanistic impact on the malignant properties of BC cell lines. In this study, we used three different BC cell lines, MCF-7, SKBR3 and MDA-MB-231, representative for different subtypes of BC.

MDA-MB-231 cells are estrogen- and progesterone-receptor negative mesenchymal-like cells with a highly invasive phenotype. SKBR3 cells also are estrogen- and progesterone-receptor negative but HER2 (human epidermal growth factor-2 receptor)-positive. MCF-7 cells exhibit a luminal epithelial phenotype. They are ER-positive and PR-positive. MCF-7 is a poorly-aggressive and noninvasive cell line. In MCF-7 cells, syndecan-4 expression is downregulated, whereas in the ER negative breast carcinoma subtype, syndecan-4 is overexpressed.

**FIGURE 1** The prognostic value of the expression of syndecan-4 in patients with breast cancer stratified by hormone receptor status. Kaplan–Meier relapse-free survival curves are plotted based on: (A) all breast cancer patients (n = 4929), (B) estrogen receptor (ER) negative status, ER− (n = 1161) (C) Progesterone receptor negative (PR) status and ER negative status, ER−, PR− (n = 501), and (D) Progesterone receptor positive status (PR) and ER positive status, ER+, PR+ (n = 891). Log-rank p values and hazard ratios (HRs; 95% confidence interval in parentheses) are shown. The corresponding Affymetrix IDs is: 202071_at (SYND4)
To identify suitable miRNAs predicted to target syndecan-4, we performed an in silico analysis using TargetScan, a miRNA target prediction algorithm (www.targetscan.org/database). We identified syndecan-4 as a potential target of miR-140-3p,2 (Figure 2A). None of the other members of the syndecan family was predicted to be a target of miR-140-3p, in accordance with recent studies in other experimental systems.18,32 All cell lines expressed miR-140-3p, with MCF-7 cells showing the highest expression level (Figure 2B). As an expression control, we also analyzed whether miR-140-3p is expressed in other cell models representing cell types of the tumor microenvironment such as monocytes (U937 cell line) and endothelial cells (HUVEC cells). We found that miR-140-3p is expressed in both cell types, but it is considerably more highly expressed in the monocytic cell line U937, which showed expression levels in the range of MDA-MB-231 and SKBR3 cells (Figure S1 and Figure 2B).

For functional analyses, we transiently transfected the three cell lines with a miR-140-3p precursor or a control miRNA, and confirmed miR-140-3p overexpression by qPCR (Figure 2C).

After demonstrating that syndecan-4 is targeted by miR-140-3p, we performed a survival analysis to assess the impact of miR-140-3p on the prognosis of the patients with BC. For this purpose, we use the online tool Kaplan–Meyer-plotter/miRpower using the GSE40267 database.26 In a total of 85 patients, we observed that miR-140-3p was associated with better OS (HR = 0.53, p = .014) (Figure 3A). Next, patients were stratified based on the expression of hormonal receptors as we did with the survival analysis of syndecan-4, in which we observed a poor survival correlation in patients with ER- and ER-PR-negative tumors (Figure 3). The analysis of miR-140-3p showed that in the patients with ER+, PR+, and ER-PR-negative tumors, the high expression of this miRNA was associated with better overall survival (HR = 0.49, p = .0091; HR = 0.5, p = .02; HR = 0.47, p = .014, respectively), demonstrating an inverse relation between the impact of miR-140-3p and syndecan-4 on patient survival (Figure 3B–D). We also performed a second analysis using the METABRIC database in Kmoplet/miRpower.26 In this database, a higher number of patients could be analyzed in the general analysis (n = 1262) however, we were only able to stratify the patients into the ER-negative category (n = 266), but not into PR-, ER-PR-negative, due to lack of data. We found a good prognosis correlation in all the patients and we did not find a positive statistical significance in the patients with ER-negative tumors (Figure S2A,B, respectively).

To investigate the regulatory impact of miR-140-3p on syndecan-4, MDA-MB-231 BC cells were cotransfected with dual luciferase plasmid pEZX-MT06-SDC4-3′-UTR and a control pre-miR or pre-miR-140-3p, and assayed for luciferase activity 48 h after transfection. pre-miR-140-3p transfection resulted in a significant 30% decrease in normalized firefly luciferase activity after 48 h, indicating direct regulation of the 3′-UTR of syndecan-4 by miR-140-3p (Figure 2D). qPCR and flow cytometry analysis revealed significant downregulation of syndecan-4 expression in MDA-MB-231 and SKBR3 cells (Figures 2E and 4A,B) at the mRNA and protein level, respectively, upon miR-140-3p overexpression. As MCF-7 cells showed the highest relative expression of miR-140-3p in our cell line panel, we reasoned that the lack of syndecan-4 downregulation upon miR-140-3p expression may be due to these already high endogenous levels. Therefore, we inhibited miR-140-3p by transient antimiR transfection. Consistent with our overexpression results in the ER-negative cell lines, miR-140-3p inhibition resulted in increased expression of syndecan-4 in MCF-7 cells (Figure 2G).

### 3.3 siRNA-mediated knockdown of syndecan-4 and overexpression of miR-140-3p concordantly affect breast cancer cell invasiveness and adhesiveness

A single miRNA is capable of regulating tens to hundreds of targets.12,13 To delineate which miR-140-3p effects were associated with the downregulation of syndecan-4, we employed a parallel siRNA approach, ensuring downregulation of syndecan-4 only. MDA-MB-231 cells were transiently transfected with control and syndecan-4 siRNA. qPCR and flow cytometry analysis confirmed successful silencing of syndecan-4 expression (Figures 2F and 4A,B). Matrigel invasion assays revealed that invasiveness of syndecan-4-deficient MDA-MB-231 cells decreases significantly compared to controls (Figure 4A). Transient transfection of MDA-MB-231 cells with miR-140-3p precursor molecules resulted in a similar significant decrease in invasiveness (Figure 5A). Using MTT assay, no difference in cell viability was detected comparing syndecan-4-depleted and control siRNA-transfected cells (Figure 5B). Likewise, miR-140-3p transfection did not affect cell viability. We next investigated if miR-140-3p-dependent cellular invasiveness was linked to increased cell motility. As shown in Figure 4D, in the pre-miR-140-3p and syndecan-4-siRNA transfected cells, cell migration was significantly decreased compared with that of control cells. Key results shown above were replicated in MDA-MB-231 cells using a second sequence of syndecan-4 siRNA, confirming the absence of potential off-target effects (Figure S3).
Syndecan-4 is a direct target of miR-140-3p. (A) Illustration of the sequence match between miR-140-3p and syndecan-4 mRNA determined using TargetScan. (B) miR-140-3p expression (C) miR-140-3p precursor transfection upregulates miR-140-3p expression relative to controls. Expression was normalized to RNU6B expression. (D) Syndecan-4 is a direct target of posttranscriptional regulation by miR-140-3p. MDA-MB-231 breast cancer cells were cotransfected with dual luciferase plasmid pEZX-MT06-SDC4-3′-UTR and a control pre-miR or pre-miR-140-3p and assayed for luciferase activity 48 h after transfection. miR-140-3p induced a significant, 30% decrease in SDC4-specific normalized firefly luciferase activity (n = 3, ***p < .0001). (E) Syndecan-4 mRNA expression is significantly decreased upon ectopic miR-140-3p expression in MDA-MB-231 and SKBR3 cells (**p < .01; *p < .05; n = 3). (F) Syndecan-4 expression is significantly downregulated following SDC4 siRNA transfection. (G) Syndecan-4 expression is significantly increased in MCF-7 cells after transfection with antimiR-140-3p (*p < .05; n = 3). mRNA, messenger RNA; UTR, untranslated region.
Syndecan-4 is a central regulator of focal adhesions, bridging fibronectin, integrins and intracellular components. Here, we show that syndecan-4 siRNA knockdown and miR-140-3p overexpression significantly enhanced in vitro adhesion to the interstitial matrix constituent fibronectin and the basement membrane constituent laminin compared to controls cells (Figure 5C).

### 3.4 Syndecan-4 silencing leads to alterations in the gene expression of adhesion molecules

To further investigate processes affected by syndecan-4 depletion in the context of cell adhesion and invasion, we analyzed the expression of the ECM-related molecules fibronectin, matrix metalloproteinase 2 (MMP2) and...
FIGURE 4  Flow cytometric analysis of syndecan-4 (SDC4) expression. The percentage of marker-positive cells and the mean fluorescent intensity of events ($n = 3$) are depicted. Negative control: isotype control.
HSPE, known to modulate these processes.35,36 Gene expression was determined using qPCR.

We observed that both syndecan-4 silencing and miR-140-3p overexpression had an impact on the expression of extracellular molecules. Syndecan-4 silencing upregulated the expression of fibronectin in all three cell lines, while miR-140-3p upregulated fibronectin expression in MDA-MB-231 and MCF-7 cells, but downregulated this molecule in SKBR3 cells (Figure 6A). Syndecan-4 silencing increased expression of the metalloproteinase MMP2 in MCF-7 and SKBR3 cells, whereas no effects were observed in MDA-MB-231 cells and upon miR-140-3p upregulation (Figure 6B). We also investigated the expression of focal adhesion kinase (FAK). Syndecan-4 silencing and miR-140-3p overexpression increased FAK expression in MCF-7 and SKBR3, whereas miR-140-3p decreased FAK expression in MDA-MB-231 cells (Figure 6C). Finally, syndecan-4 silencing decreased expression of the proinvasive heparan sulfate degrading enzyme HPSE in MDA-MB-231 and SKBR3 cells, with miR-140-3p overexpression mimicking this effect in MDA-MB-231 cells (Figure 6D). In contrast, HPSE expression was not affected by these treatments in low invasive MCF-7 cells. Overall, our data document a
context-dependent effect of syndecan-4 and miR-140-3p on the expression of ECM-related molecules in BC cells of different subtypes.

4 | DISCUSSION

In our study, we could show that syndecan-4 is a novel and functionally relevant target of the oncomiR miR-140-3p in a panel of BC cells and thus confirm the TargetScan analysis where the seed sequence fits perfectly to the syndecan-4 sequence. A recent study demonstrated that miR-140-3p was revealed to be downregulated in BC tissues and cell lines and low miR-140-3p expression predicted a poor prognosis of patients with BC. Our results are consistent with these reports; we demonstrated that in estrogen- and progesterone-receptor negative and invasive cell lines (SKBR3 and MDA-231 cells), the monocytic cell line U937 and primary endothelial cells (HUVEC), the miR-140-3p
expression was low in comparison to miR-140-3p expression in MCF-7 cells, a poorly-aggressive and non-invasive cell line. In addition, SKBR3 and MDA-MB-231 cells showed high syndecan-4 expression when compared with the syndecan-4 expression in MCF-7. In line with these findings, syndecan-4 was downregulated by overexpression of miR-140-3p in cells lines displaying low expression of this miRNA (MDA-MB-231, SKBR3) and upregulated upon inhibition of this miRNA in cells showing high basal expression levels (MCF-7). These results also corroborate with data found in the literature, as a study demonstrated that in an ER negative breast carcinoma subtype, syndecan-4 is overexpressed and in ER positive MCF-7 cells, syndecan-4 expression is downregulated. Changes in the expression of syndecan-4 contribute to the development and progression of cancer, and have a diagnostic and prognostic value in numerous tumor entities. Interestingly, analyzing the survival data of a collective of over 4000 BC patients, we found that the expression of syndecan-4 was associated with worse RFS in patients with ER- and PR-negative tumors. In addition, the analysis of miR-140-3p showed that in the patients with ER- and PR-negative tumors the high expression of this miRNA was associated with better overall survival, demonstrating an inverse relation between the expression of miR-140-3p and syndecan-4 that corresponds well to our in vitro findings.

ER plays a regulatory role for the expression of proteoglycans. The expression of heparan sulfate proteoglycans, for example, can be mediated through the tyrosine kinase receptors EGFR and IGFR, in coordination with the action of estrogen and its receptors. Recent studies have shown that miRNA expression is regulated depending on the ER status, including an important role of ERβ in triple-negative BC cells. miR-200b for example, restrains epithelial-to-mesenchymal transition and aggressiveness and regulates matrix composition depending on ER status and signaling in mammary cancer, and similar results were observed for estrogen-dependent regulation of miR-10b and miR-145, which were in part due to a regulation of syndecan family members.

Our previous studies have shown that syndecan-4 silencing led to downregulation of proliferative and invasive capacity and angiogenic abilities of anoikis-resistant endothelial cells. Furthermore, compared with the parental cells, syndecan-4 silenced cells exhibited an increase in adhesion to collagen and laminin and also in the apoptosis rate. Here, we show that syndecan-4 siRNA knockdown and miR-140-3p overexpression also resulted in significant changes in BC cell adhesion to both interstitial and basement membrane ECM constituents, as well as cell motility and invasiveness, demonstrating that syndecan-4 acts both in a cancer cell-autonomous manner (this study) and on cells of the tumor microenvironment.

Syndecan-4 has an important role in the hallmarks of cancer, modulating multiple steps of tumor progression, including unlimited cell proliferation, resistance to apoptosis, invasive growth and metastasis, tumor angiogenesis and tumor-associated inflammation. Syndecan-4 mediates these processes as a signaling interface at the cell surface, acting as a classical heparan sulfate coreceptor for soluble ligands such as growth factors and chemokines, but also via interactions of its protein moiety with growth factor receptors, integrins, and fibronectin. We demonstrated that after syndecan-4 silencing and miR-140-3p overexpression, the expression of the interstitial matrix protein fibronectin and the proinvasive metalloproteinase MMP-2 were altered in BC cells. A study showed that syndecan-4 glycosaminoglycan chains can bind a recombinant protein that includes the HepII domain of fibronectin, and that syndecan-4 is incorporated into focal adhesions that form in response to signaling downstream of this interaction. In addition, we previously demonstrated that syndecan-4 is a regulator of MMP-dependent invasive growth in endometriosis, a benign disease characterized by local tissue invasion. In addition, we demonstrated a regulatory impact of syndecan-4 on the expression of the proinvasive heparan sulfate degrading enzyme HPSE in ER-negative BC cells. Previous studies have established a functional link between HPSE and syndecan-4, as syndecan-4 mediates HPSE endocytosis (Higashi et al.), and HPSE is able to induce clustering of syndecan-4, thereby facilitating cell adhesion and spreading (Levy-Adam et al.). Moreover, in melanoma cells, syndecan-4 modulates the interaction of latent HPSE of the integrin VLA4, thereby regulating cancer cell adhesion and metastatic spread (Gerber et al.). Our data expand the range of hHPSE-syndecan-4 interactions to the field of BC pathogenesis.

Our qPCR analysis also shows that syndecan-4 silencing can alter FAK gene expression. As mentioned anteriorly, syndecan-4 is a key mediator in focal adhesion formation. Focal adhesions are composed of transmembrane receptors (primarily syndecan-4 and members of the integrin superfamily), structural molecules (such as actin, talin, tensin, vinculin, and α-actinin), and signaling molecules (i.e., FAK, PKC, and Src). Syndecan-4 modulates FAK phosphorylation. In addition, a recent study demonstrated that the binding of syndecan-4 to PAR-3, involve the Thy-1/CD90-induced FA dynamics in mesenchymal cells by regulating FAK autophosphorylation and Tiam1 activation.
While our study demonstrates a clear regulatory impact of both syndecan-4 and miR-140-3p on ECM-related regulators and mediators of cancer cell adhesion and motility, this regulation partially occurred in a context and cell-type-dependent manner. We ascribe this to the pleiotropic nature of both syndecan and miRNA functions: syndecan-4 acts as a multifunctional coreceptor that impacts on numerous signaling pathways. Changes in syndecan-4 expression will therefore simultaneously affect multiple signaling circuits (e.g., receptor tyrosine kinases, G-protein coupled receptors, morphogen signaling), and the net response of a tumor cell to syndecan-4 (and heparan sulfate) depletion will depend on its individual complement of signaling receptors and their (constitutive) activation status, as previously demonstrated by our group for the heparan sulfate sulfotransferase HS3ST2. Likewise, miRNAs have numerous targets, however, not all targets are expressed to the same degree in a given (tumor) cell, resulting in context-dependent effects. While these effects increase the complexity of proteoglycan and miRNA-dependent regulation, they reflect the pathophysiology of tumors.

The involvement of miRNAs in the initiation and progression of BC holds great potential for new development in current diagnostic and therapeutic strategies in BC management. Several studies have demonstrated that miRNAs can regulate the expression of numerous ECM molecules, including proteoglycans and enzymes mediating the biosynthesis and degradation of their GAG carbohydrate chains. Interestingly, knockout mice deficient in miR-140 showed accelerated aggrecan proteoglycan loss and ECM degradation, while mice overexpressing miR-140 were protected against degradation of aggrecan, demonstrating the impact of miR-140-mediated proteoglycan regulation in vivo. This example demonstrates that the future use of miRNA-based drugs emerges as a viable option for modulating the ECM as an antitumoral strategy.

Although it is well known that miRNAs are capable of synchronous targeting of multiple gene product, this study has revealed a novel role for miR-140-3p in regulating BC cell invasiveness and cell-matrix interactions, which could at least partially be attributed to a targeting of syndecan-4 and associated changes in the expression of ECM constituents. Our findings mark these molecules as potential therapeutic targets in ER-negative BC that are worthy of further evaluation in more complex preclinical experimental systems. These findings are, therefore, an important step towards understanding the mechanism by which miRNAs modulate ECM genes and prevent ECM dysregulation.

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CONFLICT OF INTERESTS
The authors declare that there are no conflict of interests.

AUTHOR CONTRIBUTIONS
Jessica Oyie Sousa Onyeisi conceived the study with Martin Götte, performed the major part of the experiments, analysed data, drafted the majority of the figures and wrote the manuscript draft. Burkhard Greve provided expertise and resources for flow cytometry analysis, analysed data and drafted figures. Nancy Adriana Espinoza-Sánchez performed database analysis, drafted figures, analysed qPCR data and revised the manuscript text, Ludwig Kiesel provided resources and clinical expertise, Carla Cristina Lopes co-supervised Jessica Oyie Sousa Onyeisi and revised the manuscript draft, Martin Götte coconceived, coordinated and supervised the study, revised figures and revised the manuscript draft. All authors reviewed and commented on the manuscript.

DATA AVAILABILITY STATEMENT
All data generated or analyzed during this study are included in this published article.

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