


# MDA-MB-231 breast cancer cell viability, motility and matrix adhesion are regulated by a complex interplay of heparan sulfate, chondroitin–/dermatan sulfate and hyaluronan biosynthesis

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**Abstract** Proteoglycans and glycosaminoglycans modulate numerous cellular processes relevant to tumour progression, including cell proliferation, cell-matrix interactions, cell motility and invasive growth. Among the glycosaminoglycans with a well-documented role in tumour progression are heparan sulphate, chondroitin/dermatan sulphate and hyaluronic acid/hyaluronan. While the mode of biosynthesis differs for sulphated glycosaminoglycans, which are synthesised in the ER and Golgi compartments, and hyaluronan, which is synthesized at the plasma membrane, these polysaccharides partially compete for common substrates. In this study, we employed a siRNA knockdown approach for heparan sulphate (EXT1) and heparan/chondroitin/dermatan sulphate-biosynthetic enzymes ( $\beta$ 4GalT7) in the aggressive human breast cancer cell line MDA-MB-231 to study the impact on cell behaviour and hyaluronan biosynthesis. Knockdown of  $\beta$ 4GalT7 expression resulted in a decrease in cell viability, motility and adhesion to fibronectin, while these

parameters were unchanged in EXT1-silenced cells. Importantly, these changes were associated with a decreased expression of syndecan-1, decreased signalling response to HGF and an increase in the synthesis of hyaluronan, due to an upregulation of the hyaluronan synthases HAS2 and HAS3. Interestingly, EXT1-depleted cells showed a downregulation of the UDP-sugar transporter SLC35D1, whereas SLC35D2 was downregulated in  $\beta$ 4GalT7-depleted cells, indicating an intricate regulatory network that connects all glycosaminoglycans synthesis. The results of our *in vitro* study suggest that a modulation of breast cancer cell behaviour *via* interference with heparan sulphate biosynthesis may result in a compensatory upregulation of hyaluronan biosynthesis. These findings have important implications for the development of glycosaminoglycan-targeted therapeutic approaches for malignant diseases.

**Keywords** Solute carriers · Glycosaminoglycans · Galactosyltransferase I · EXT1 · UDP-sugars ·  $\beta$ 4GalT7

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## Introduction

Glycosaminoglycans (GAGs) are complex polysaccharides composed of a repetitive specific disaccharide units, and on this basis, they are divided into four sub-families: hyaluronan (HA), heparin/heparan sulphate (HE/HS), chondroitin/dermatan sulphate (CS/DS) and keratan sulphate (KS) [1–4]. HA is synthesised by means of three enzymes (hyaluronan synthases, HASs) associated with the plasma membrane and the polymer is immediately extruded into the extracellular matrix (ECM) [2]. All the other GAGs are covalently linked to a protein core thus forming proteoglycans (PGs), and polymerized in the endoplasmic reticulum (ER)

and Golgi compartments [3, 4]. Two classes of GAGs, *i.e.* CS/DS and HS/HE constitute the major part of the PGs in cell membrane and ECM. GAGs synthesis is due to several enzymes that use UDP-sugars concentrated in the lumen of Golgi and ER by members of the transporter family SLC35 [5]. GAGs chains exert several pivotal roles on tissue, mostly due to their interaction with growth factors and morphogens [6–8]. In particular, various PGs (*e.g.* syndecans and glypicans) can act as co-receptors for growth factors [8, 9]. Modification of HS synthesis can alter proper embryonic development, leading to severe malformations or ultimately lethality in both affected model organisms and humans [5–12]. HS/HE and CS/DS are bound to a protein core through a linkage region, the sequence of sugar xylose-galactose-galactose-glucuronic acid, and the same biosynthetic enzymes are active for both families [9, 13, 14]. The enzyme galactosyltransferase I ( $\beta$ 4GalT7), that catalyses the transfer of the first galactose to the xylose residue in the linkage region of CS/DS and HS/HE PGs has been related to the pathogenesis of progeroid Ehlers-Danlos syndrome, characterized by wound repair defects and skeletal malformations [7, 15]. Among the GAGs, the HS/HE chains are composed by the repetitive disaccharide units ( $-\alpha(1,4)\text{-UA-}\beta(1,4)\text{-GlcNAc-}$ )<sub>n</sub>, [1], this chain being polymerized by the exostosin proteins (EXT1 EXT2 and EXTL) [16, 17]. Afterwards, the glucosaminyl *N*-deacetylase/*N*-sulfotransferase (NDST) eliminates the acetyl groups introducing *N*-sulphate into the growing polymer and several sulfotransferases and the epimerase (transforming the glucuronic in iduronic acid) conclude the HS synthesis [9, 13, 16]. The enzyme  $\beta$ 4GalT7, therefore, is of pivotal importance in both the HS/HE and CS/DS synthesis. Seidler *et al.* [15] described a deficiency in the activity of the enzyme, due to a point mutation in the gene leading to an Ehlers-Danlos syndrome-like phenotype. In this disease, CS/DS PGs such as decorin and biglycan showed a defective glycosylation and a modification in the disaccharide composition in HS/HE GAGs [7] even not in the total amount [15] which, in turn, modifies the cell behaviour, wound repair, (*i.e.* migratory ability) and adhesion on fibronectin. The different effect of the  $\beta$ 4GalT7 mutation in the final GAGs structure clearly indicates that the GAGs biosynthetic machineries for CS/DS and HS/HE are separate complexes, the so-called GAGosomes [16, 18]. The GAGs UDP-sugar precursors are UDP-xylose, UDP-glucuronic acid, UDP-*N*-acetyl-glucosamine and UDP-*N*-acetyl-galactosamine; their synthesis involves several cytoplasmic enzymes, while the biosynthesis of GAGs (excluding HA) occurs in Golgi membranes. For this reason, the UDP-sugar precursors need to be imported within the Golgi and ER [19, 20] through specific membrane carriers. The nucleotide-sugar transporter family (SLC35) consists of at least 17 molecular species in humans, localized in the Golgi apparatus and/or ER [21, 22]. Among several carriers, specific UDP-galactose and *N*-acetyl-glucosamine can be important

for HS/HE and CS/DS synthesis, in particular SLC35D1 (Q9NTN3) transports UDP-GlcNAc,  $-\text{GlcA}$ , and  $-\text{Gal}$ ; SLC35D2 (Q76EJ3) UDP-GlcNAc, and SLC35B1 (P78383) carries UDP-Gal,  $-\text{GlcNAc}$ , and CMP-Sia. SLC35D1 controls specifically the CS synthesis by limiting the UDP-sugar precursor inside the Golgi apparatus. Several papers report the immunolocalization of UDP-sugar carriers and have analysed their mechanism and kinetics [23, 24]. HA [25–28] and PGs [29], with their biosynthetic machineries, are reported to be critical in tumour growth and progression, both for their direct effect on cancer cell behaviour and for their role in transducing inflammatory signals in the cancer site. Therefore, we hypothesised that  $\beta$ 4GalT7 or EXT enzymes can be important in cancer cells in the organization of the GAGs proportion and represent useful targets for both unravel the mechanism of GAGs polymerization and control cell proliferation. In fact, HS/HE PGs have various roles in cancer progression, alone or *via* the interaction with integrins [30, 31], even though also CS/DS [32, 33] are reported to be overexpressed, in particular bound to the PG versican and biglycan. Among the HS/HE PGs, the syndecans, a family of 4 members, are the most studied molecules in relation to cancer progression. The invasive breast cancer cell line MDA-MB-231 mainly expresses syndecan-1 and syndecan-4 [31]. Moreover, the interplay between glucose energetic metabolism and GAGs synthesis is critical in tumour cells and may be controlled by the expression of specific UDP-sugar transporters. In this study, we investigate the complex interplay of the HS and HA biosynthetic machinery, and their effect on breast cancer cell behaviour using an *in vitro* siRNA-approach in the aggressive cell line MDA-MB-231. Our data demonstrate that the abrogation of central GAGs synthetic enzymes,  $\beta$ 4GalT7 and EXT1, has effects on PGs expression, (the end products of the synthesis) and on the preceding UDP-sugar carriers, demonstrating that CS/DS and HS/HE have a definite and independent GAGosome. Eventually, we can suggest the UDP-sugar transporters as possible target for therapeutic drugs.

## Materials

Media, fetal calf serum (FCS) and tissue culture supplies were from Gibco BRL (Karlsruhe, Germany). DharmaFECT I® was from Dharmacon, (Inc. Lafayette, CO). siRNAs from Ambion Cambridgeshire, UK and Qiagen, Hilden, Germany. Hyaluronan Enzyme-Linked Immunosorbent Assay Kit (HA – ELISA) (Echelon, Salt Lake City, UT). Unless stated otherwise, all chemicals were from Sigma (Deisenhofen, Germany).

## Methods

**Cell culture and RNA silencing** The breast cancer cell line MDA-MB231 was purchased from LGC Standards (Wesel,

Germany) and cultured at 37 °C under humid atmosphere containing 7.5 % carbon dioxide in Dulbecco's modified Eagle medium (DMEM) high-glucose medium (Invitrogen, Karlsruhe, Germany) supplemented with 10 % FCS, 1 % L-glutamine, 1 % penicillin-streptomycin.

For the silencing of  $\beta$ 4GalT7 or EXT1 genes,  $2 \times 10^5$  cells were plated in a 6-well plate until the 80 % confluency. The silencing was performed using DharmaFECT I® as transfection reagent according to the manufacturer's instruction with the siRNA #111770 (si $\beta$ 4GalT7), #116804 (siEXT1) and #301698 (a negative control siRNA = Mock). Briefly, the cells were incubated with a premixed solution of Dharmafect and siRNA in 1 ml final volume of the transfection medium Opti-MEM®. The final concentration of the reagents was 40 nM siRNA and 2.4 % (v/v) Dharmafect. After 24 h the medium was replaced with the growing medium for 48 h.

After silencing, the cellular RNA was isolated using the basicRNA-OLS (OLS, Hamburg, Germany) and reverse transcribed into complementary DNA applying the First Strand cDNA Synthesis Kit (Fermentas International Inc. Burlington, Canada).

Quantitative RT-PCR was performed in an AbiPrism 7300 instrument (Applied Biosystems) using TaqMan Universal PCR Master Mix (Applied Biosystems) following the manufacturer's instructions. The following TaqMan gene expression assays (probes and primers) were used:  $\beta$ 4GalT7 (Hs00200655), EXT1 (Hs00609162), SLC35D1 (Hs00209446), SLC35D2 (Hs00294687), SLC35B1 (Hs01047744), 18S RNA (Hs99999901), HAS1 (Hs00155410), HAS2 (Hs00193435) and HAS3 (Hs00193436) GAPDH (Hs99999905). Fluorescent signals generated during PCR amplifications were monitored and analysed with AbiPrism 7300 SDS software (Applied Biosystems). Comparison of the amount of each gene transcript among different samples was made using 18S RNA as the reference with the exception of HASs, for which GAPDH was used.

**Western blot analysis** Silenced cells were washed with ice cold PBS and added of reducing lysis buffer (62.5 mM TRIS-HCl, 50 mM dithiothreitol, 10 % (V/V) glycerin, 2 % (m/V) SDS, 0.01 % bromphenol blue, and freshly added protease inhibitor cocktail 1:100 (Sigma, St. Louis, MO), 10 mM NaF, 1 mM NaVO<sub>3</sub>, 10 mM  $\beta$ -glycerolphosphate). Lysates were sonicated for 10 s, heated to 95 °C for 5 min and centrifuged for 5 min at 10,000 g. In the supernatants, proteins were quantified by means of BCA method and 15  $\mu$ g of proteins were loaded to a SDS-polyacrylamide gel (7.5 % acrylamide).

For syndecan-1 (R&D system Antibody, AF2780) Western blot, control cells were lysed as reported above. Mock and si $\beta$ 4GalT7 cells were digested for 40 min at 37 °C with 100 mU Heparinase I in DMEM serum free, washed with PBS and lysed as control cells; 60  $\mu$ g of proteins were loaded

in a SDS PAGE polyacrylamide gel (8 % acrylamide) [34]. Electrophoresis was performed at 25 mA per mini gel (64 cm<sup>2</sup>). Separated proteins were transferred to a nitrocellulose membrane (Hybond; Amersham Pharmacia Biotech, Piscataway, NJ) for 90 min at 16 V. Molecular weights were estimated by comparison with prestained standards. Immunodetection was performed by treating nitrocellulose membranes with blocking buffer, 5 % skim milk in 0.1 % TBS-Tween (TBST) for 1 h at room temperature, followed by incubation with the primary antibody over night at 4 °C. Rabbit antihuman polyclonal antibodies (1:1000 in 5 % BSA in TBST) were used for the detection of phosphorylated p42/44 protein (Cell Signaling, #9102) and total p42/44 (Cell Signaling #9101). After washing in TBST three times, the membranes were incubated for 1 h with horseradish peroxidase-conjugated anti-rabbit IgG diluted 1:2000 (Cell Signaling) in blocking buffer. The membranes were washed and treated with enhanced chemiluminescence detection reagents (Amersham Pharmacia Biotech) for 1 min and exposed to CL-XPosure™ film (Thermo Fisher Scientific, Waltham, MA). Chemiluminescence signal intensity on developed and scanned films was analysed using ImageJ software for PC. For the detection of tubulin as endogenous control protein, membranes were stripped with 0.87 % NaCl and 0.75 % glycine at pH 2.5 three times, washed, and re-incubated with mouse monoclonal anti-tubulin (Sigma) followed by the detection procedure as described previously.

**Cell proliferation assay (MTT)** After 24 h of transfection, the cells were trypsinized, counted and seeded in a 96-well plate at the concentration range of 2500–5000 cells/well in 100  $\mu$ l of complete medium for 24 h. In the wells were then added 10  $\mu$ l/well MTT (0.5 % in PBS) and incubated for 4 h. The reaction was stopped adding 100  $\mu$ l/well of 10 % SDS, 50 % *N,N*-Dimethylformamide pH 4.7, incubated overnight at RT in dark and the plate read at 570/650 nm wavelengths.

**Wound scratch assay** Silenced cells monolayers were grown on 6-well plate and to assess migration ability, a scratch was made with a pipette tip and new complete medium was added. Closing of the scratch wound was monitored by Nomarski contrast light microscopy with a Zeiss Axiovert 100 microscope. The images were taken with a Zeiss AxioCam MRC camera at various time since 24 h. The mobility rate was calculated with respect to time 0 for each treatment as percentage of repopulated area with respect to the starting scratched area, using ImageJ software.

**Cell adhesion assay** 96-well-plate were coated with 10  $\mu$ g/ml FN at 37 °C for 1 h, washed twice with, blocked with 0.5 % BSA in DMEM without FCS at 37 °C in CO<sub>2</sub> incubator for 45 min followed with washing with 0.1 % BSA in DMEM without FCS and chilling the plates on ice. 25.000 cells

washed once and removed with 2 mM EDTA in PBS were added in each well re-suspended in blocking buffer. The plate was incubated in a CO<sub>2</sub> incubator at 37 °C for 30 min. The plate was then shaken at 500 rpm for 1 min, washed with PBS for 3 times, fixed with 3.7 % PBS-buffered paraformaldehyde at RT for 30 min and stained with 1 % methylene blue (Sigma) in 0.01 M borate buffer (pH 8.5) for 30 min. Wells were washed 4 times with borate and cells lysed in ethanol/0.1 M HCl (mixed 1:1). The absorption of extracted methylene blue dye at 620 nm after 10 min and overnight lysis.

**Glycosaminoglycans (GAGs) purification** GAGs/PGs in the medium were precipitated with 1 % CPC (1-hexadecyl pyridinium chloride) for 24 h at 25 °C. Media were centrifuged to obtain GAGs/PGs-CPC complexes at 2500×g for 20 min at RT. Pellets were re-solubilized with 1 ml of 2 M NaCl/ethanol 100:15 (v/v) [35] centrifuged and pellets added of 1.5 ml absolute ethanol to have dissociation of PG/CPC complexes with incubation for 24 h at 25 °C. After centrifugation at 2500×g 20' at RT pellets were washed twice with 1.5 ml and 0.5 ml of absolute ethanol followed by pellet dry in air. Samples were suspended in 150 µl of water and used for uronic acid and hyaluronan quantification.

**Uronic acid assay** In 96 wells microtiter plates 200 µl concentrated sulfuric acid (97 %) were added to 40 µl of precipitated GAGs and incubated for 60 min at 80 °C. Samples were allowed to cool at room temperature, and the plates were read at 540 nm in the spectrophotometer. Afterwards, 40 µl *m*-hydroxyphenyl solution (100 µl di 100 mg/ml hydroxyphenyl in DMSO mixed with 4.9 ml of 80 % H<sub>2</sub>SO<sub>4</sub>), were added to the samples, let at RT and read at 540 nm after 16 h [36].

**Hyaluronan quantification** The HA was measured using a commercial HA-ELISA kit (Echelon). The protocol is a standard competitive ELISA format in which the colorimetric signal is inversely proportional to the amount of HA present in the sample. 20 µl of GAGs precipitated to be assayed were first mixed with the HA Detector, and then added to the HA ELISA plate for competitive binding. An enzyme-linked antibody and colorimetric detection was used to detect the HA Detector bound to the plate. The enzyme / substrate system is a colorimetric assay comprised of alkaline phosphatase / pNPP phosphatase substrate. It was read at 405 nm.

**Time lapse microscopy** 48 h after transfection, 60,000 cells were seeded in 12.5 cm<sup>2</sup> culture flasks (Falcon) and allowed to adapt for 24 h prior to recording. The culture flasks were put into heated chambers on stages of inverted microscopes (Axiovert25, Carl Zeiss, Inc. Göttingen, Germany). Cell migration was recorded in 10 min intervals for 12 h at 37 °C using XC-ST70CE and XC-77 CE video cameras (Hamamatsu/Sony, Japan)

and PC-vision frame grabber boards (Hamamatsu, Herrsching, Germany). Image acquisition was controlled by HiPic and WASABI softwares (Hamamatsu). The circumferences of the cells were labelled applying the AMIRA software (TGS, San Diego, USA). The cell contours served as the basis for further analysis. Parameters such as structural index (SI), migratory velocity (µm min<sup>-1</sup>) and translocation (µm) were analysed using self-made JAVA programs and the NIH ImageJ software. Migration was determined as the movement of the cell centre per time unit, the velocity was estimated from the 10 min time intervals applying a three-point difference quotient and the cell area was measured as the number of pixels.

**Apoptosis analysis** Following transfection cells were stained for apoptosis as well as apoptosis/necrosis using the annexin V test kit from Becton Dickinson (San José, USA). Flow cytometric cell analysis and quantification of cell death took place on a flow cytometer (CyFlow Space, Partec, Germany).

**Statistical analysis** GraphPad Software were used for statistical analysis. The number of independent replicate experiments is indicated in each figure legend. Data are presented as means of independent experiments ± S.E. Statistical significance was tested with unpaired Student's t test and significant values of p are reported in the figure legends.

## Results

### Impact of β4GalT7 and EXT1 silencing on cell survival

Due to the pivotal role of HSPGs of the syndecan family in cancer progression and inflammation, this work has focused on the role of HS/HE GAGs chains. The investigation was performed using the triple negative breast cancer cell line MDA-MB231, a highly invasive and tumorigenic cell line with mesenchymal morphology [37].

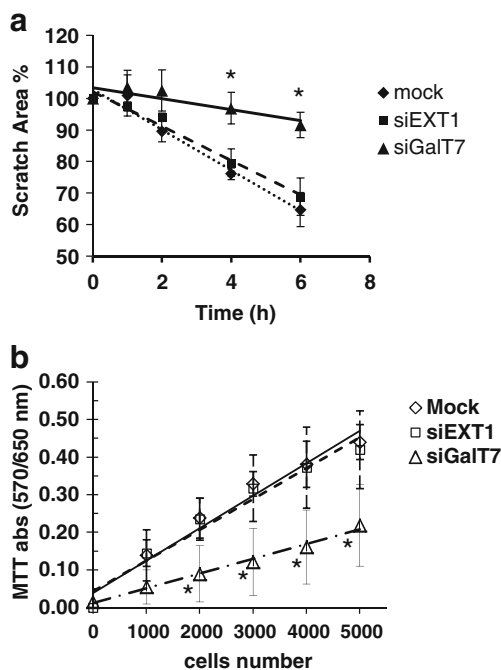
The genes encoding the enzymes β4GalT7 and EXT1 were transiently knocked down using the siRNA system. After control of successful silencing by quantitative real-time PCR, the cells were evaluated for their proliferation and migration ability. When the apoptotic events in the silenced cells for β4GalT7 (siβ4GalT7) and EXT1 (siEXT1) were evaluated by flow cytometry, the results showed a slight, but not significant increase of apoptosis in siβ4GalT7 cells within the 48 h of the experiments, while the apoptotic cells were up to 3fold increased after 72 h (data not shown). The silencing resulted in a decreased expression for β4GalT7 of 93.45 ± 2.35 % and 93.87 ± 4.63 %, for EXT1 of 92.16 ± 3.43 % and 95.18 ± 1.31 % at 24 and 48 h respectively. Moreover,

the silencing of either  $\beta$ 4GalT7 or EXT1 did not alter each other's expression.

### Effect of GAG biosynthetic enzyme downregulation on cell behavior

The functional effect of the gene silencing was evaluated using two GAGs-dependent parameters: cell migration and proliferation. The cell migration was evaluated by means of a scratch assay and as reported in Fig. 1a, the abrogation of  $\beta$ 4GalT7 was heavily affecting the mobility of cells that were not able to close the wound at the same time rate as the control and the siEXT1 cells.

Nevertheless, in time-lapse assays we could not prove significant differences in the cells speed between mock and si $\beta$ 4GalT7 ( $0.383 \pm 0.252$  and  $0.683 \pm 0.317$   $\mu$ m/min respectively following 8 and 10 cells) (data not shown). This inconsistency might be due to the small population we analyzed or to a different behavior of the subpopulation of the cell line [38, 39]. The wound closure was not affected by cell proliferation since the assay was monitored only up to



**Fig. 1** Effect of enzyme abrogation on cell behavior. **a** Migration ability. Mock (control with scrambled siRNA) 1, siEXT1, si $\beta$ 4GalT7 cells monolayers were subjected to a scratch with a pipette tip and the migration with monitored with microscope pictures every 30 min for 6 h. The mobility rate was calculated with respect to time 0 for each treatment as percentage of repopulated area with respect to the starting scratched area using ImageJ software. **b** Cell proliferation. Control, siEXT1, si $\beta$ 4GalT7 were seeded at different density and the proliferation was measured with an MTT assay after 24 h. The plot reports the absorbance after cell lysis at 570/650 nm. Results are expressed as mean  $\pm$  s.d. of triplicate; \* $p < 0.05$

6 h, nevertheless displaying a significant difference between mock/siEXT1 and si $\beta$ 4GalT7.

si $\beta$ 4GalT7 cells were also less proliferative with respect to control (Mock) and siEXT1 cells (Fig. 1b) in a 24 h MTT assays with different seeding density start points.

The results clearly indicate that the GAGs synthesis abrogated by the  $\beta$ 4GalT7 silencing is pivotal for the proliferation and migration, possibly by interfering with signalling pathways of those breast cancer cells. The silenced cells are not able to restore any cell-cell contact signal in order to proliferate.

### Cell response to extracellular stimuli

Further investigations focused on the ability of the silenced cells to correctly transduce growth factor effects. Among the various growth factor receptors which need the presence of a HS/HE co-receptor, we choose Met, the HGF receptor. Met stimulation leads to the intracellular activation, *i.e.* phosphorylation, of the MAP kinases p42/44 [40, 41].

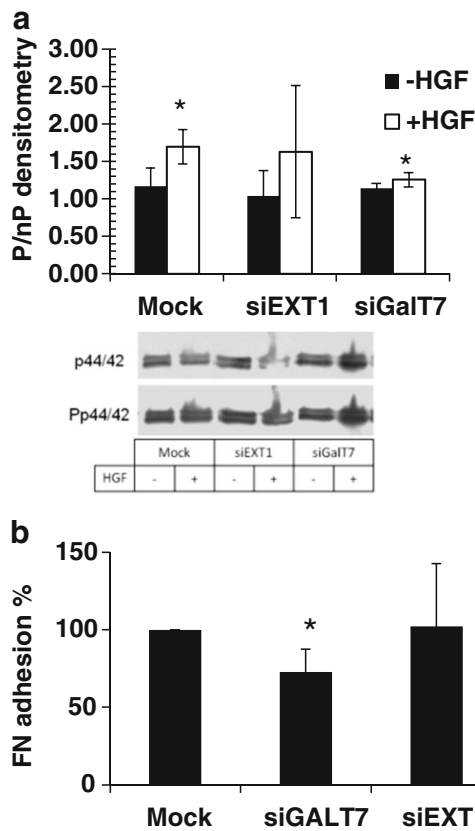
As reported in Fig. 2a, the activation of the intracellular pathway starting from c-Met (+HGF) leads to an increment of phosphorylation of p44/42 (also known as Erk1/2) in Mock cells and, with less extent, in si $\beta$ 4GalT7 cells. The siEXT1 cells showed a highly variable degree of activation, evident in the plot as a trend in the phosphorylation, but not significant.

The data therefore suggest that both silencing of EXT1 and  $\beta$ 4GalT7 could cause a defective synthesis in the cell membrane GAGs, in a different fashion, in fact si $\beta$ 4GalT7 cells maintain probably a functional array of HS/HE GAGs, although in minor concentration, while siEXT1 cells probably display a different arrangement in GAGs relative concentration.

Adhesion to fibronectin was performed as described by Götte *et al.* [7] and the si $\beta$ 4GalT7 showed a decrease in FN adhesion (Fig. 2b). siEXT1 did not display any differences with respect to mock, even though, again, they showed a high degree of variability, as indicated by the standard deviation.

### Analysis of GAGs, and GAGs-related enzymes

The silencing of either  $\beta$ 4GalT7 or EXT1 affect the production of GAGs chains of different families because of their role in the polysaccharides synthesis. We therefore quantified the total uronic acid (UA) content of the conditioned medium, which we consider the hallmark of the silencing of both  $\beta$ 4GalT7 and EXT1. In fact uronic acid is one of the two sugars composing HA, CS/DS and HE/HS. As reported in Table 1, the total UA is highly increased in the si $\beta$ 4GalT7 cells, due to the increment in HA deposition. The HS/HE component, which is of a 20 % in control cells, was in parallel decreased.



**Fig. 2** Cell responses to extracellular stimuli. **a** Cell growth factor sensitivity. Mock, siEXT1, si $\beta$ 4GalT7 were stimulated or not with HGF. Cells lysates were tested with appropriate antibodies and western blot picture analyzed with ImageJ software to obtain the phosphorylated/not phosphorylated ratio of the bands corresponding to p44/42 protein. In the figure an example image of the western blot. **b** Adhesion to fibronectin ECM. Mock, siEXT1, si $\beta$ 4GalT7 were grown on fibronectin-coated wells and their adhesiveness was tested as reported in material and methods, and the adhesion percentage calculated for each treatment with respect to the number of cells attached in the mock. Results are expressed as mean  $\pm$  s.d. of triplicate; \* $p < 0.05$

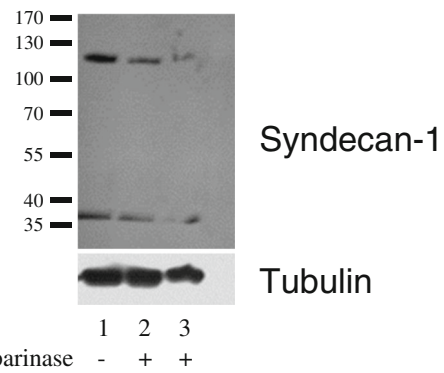
The decrease in the HS/HE can be also related to the loss of proteoglycans at the cell membrane. Since syndecan-1 plays an important role regulating cell motility and invasiveness in MDA-MB-231 cells [39], as well as in contributing to tumour metastasis [42], we investigated the content of syndecan-1 in mock and si $\beta$ 4GalT7 cells. As shown by Western blotting (Fig. 3), silencing of  $\beta$ 4GalT7 resulted in a decrease of syndecan-1 expression in the cells (lane 3 compared to lanes 1 and 2).

**Table 1** UA and HA content normalized to Mock in medium/ $10^5$  cells

	UA content %	HA content %	HS/HE content %	% UA HS/HE $\mu$ g
Mock	100 $\pm$ 18.35	100 $\pm$ 39.2	100 $\pm$ 10.3	20.5 $\pm$ 2.11
si $\beta$ 4GalT7	171.27 $\pm$ 27.13 *	155.45 $\pm$ 23.0 **	67.6 $\pm$ 1.1 *	13.86 $\pm$ 0.15*
siEXT1	87.16 $\pm$ 13.48	90.69 $\pm$ 56.6	76.0 $\pm$ 50	15.58 $\pm$ 7.79

Results are mean and standard deviation (s.d.) of three experiments

\*  $p$  value  $\leq 0.01$ ; \*\*  $p$  value  $\leq 0.005$

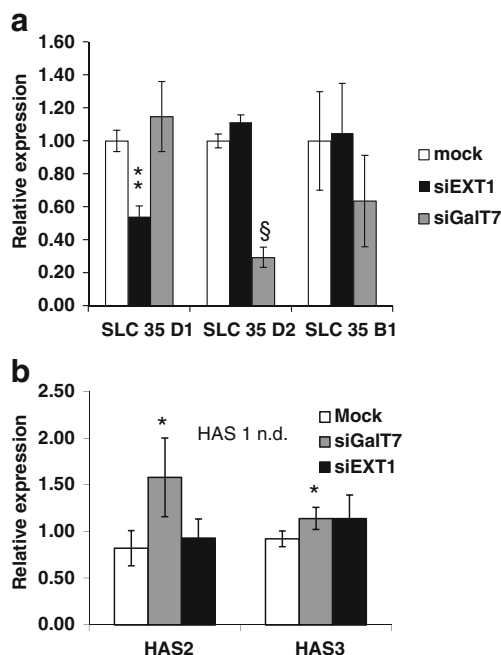


**Fig. 3** Syndecan-1 western blot. Mock and si $\beta$ 4GalT7 analysis of syndecan-1; lane 1: mock cells lysed with RIPA buffer; lane 2: Mock, and lane 3: si $\beta$ 4GalT7 cells digested with Heparinase I and lysed with RIPA buffer. 60  $\mu$ g of protein were separated in a 8 % PAGE electrophoresis, subjected to Western blot and tested with syndecan-1 antibody

Concerning the silencing of EXT1, our data are not as clear. In fact, the silencing of EXT1, that should be relevant only for HS/HE, displayed a decrement in UA content as well as in HS/HE but only as a trend, since the sample variability did not permit to obtain statistically significant data.

The association of several proteins/enzymes in the synthesis of GAGs chains includes also the UDP-sugar transporters, which mediate substrate availability *via* transport through the Golgi and ER membrane. In particular, we investigated the role of three UDP-GlcNAc transporters, SLC35D1 reported to specifically control CS GAGs, SLC35D2 that may take part in HS synthesis and SLC35B1 whose role is unknown in GAGs metabolism [5, 21, 22]. The results of the gene expression analysis of those carriers are reported in Fig. 4a. The silencing of  $\beta$ 4GalT7, affects the expression of the transporter SLC35D2, and this can probably account for the loss of syndecans-1 and for the lower HGF effect on p44/42 activation (see Fig. 2). In addition, SLC35B1 showed a lower expression, even though this difference was not significant. In case of EXT1 silencing, we demonstrated a diminished expression of the SLC35D1 carrier, which controls CS GAGs synthesis by limiting the UDP-sugar availability within the Golgi [22].

In the  $\beta$ 4GalT7 silenced cells, the decreased HS/HE GAGs content (Table 1) reduced the cells ability to completely



**Fig. 4** Quantitative RT-PCR analysis of GAGs-related enzymes. RNA from Mock, siEXT1 and si $\beta$ 4GalT7 cells was analyzed for gene expression; the results are expressed as relative expression with respect to the control treated with siRNA scrambled (Mock). **a** Relative quantification of UDP-sugar transporters *SLC35D1*, *SLC35D2* and *SLC35B1* genes. **b** Relative quantification of Hyaluronan synthases *HAS2* and *HAS3* genes. *HAS1* was not detectable. The Results are expressed as mean  $\pm$  s.d. of triplicate; \* $p < 0.05$ ; \*\* $p < 0.01$ ; § $p < 0.0001$

respond to a HGF stimulus, thus resulting in a less invasive and proliferative behaviour. Moreover, the diminished expression of the UDP-sugar transporter *SLC35D2*, which can be responsible for the described features, can also lead to an increase in UDP-sugars in the cytoplasm that was reported to be associated to the overexpression of the HA synthases and the deposition of HA [43]. Similarly, in si $\beta$ 4GalT7 cells, we observed the overexpression of both *HAS2* and *HAS3* enzymes (Fig. 4b), while the siEXT1 did not show any differences compared to the control.

## Discussion

In the present study, we demonstrate for the first time that GAG biosynthesis in breast cancer cells is a highly controlled mechanism, which includes the UDP-sugar carriers together with the so-called GAGosome, *i.e.* the chain elongating and modifying enzymes [16, 18]. Various glycosyltransferases firstly arrange for a tetrasaccharide named “linkage region” and then elongate the different GAG chains [3]. The exception to this metabolism is represented by HA; this long unsulphated chain is formed by HA synthases located on the plasma membrane which use UDP-sugars from the cytosol [1–4, 44]. The HA synthesis is controlled in several different

ways, first of all by the availability of UDP-sugars in the cells; in fact, Vigetti *et al.* demonstrated that increasing or decreasing the enzyme UDP-glucose dehydrogenase that produces UDP-glucuronic acid (UDP-GlcA), lead to higher or lower production of HA respectively [43]. All other GAGs are only slightly modified by the change in UDP-GlcA availability, probably as the results of specific membrane carrier with high affinity, the soluble ligand carriers (SLC35) [21, 22]. Nevertheless, the loss of activity of the enzyme galactosyltransferase I ( $\beta$ 4GalT7), that catalyses the transfer of the first galactose to the xylose residue in the linkage region, has been reported to induce impairment in the HS/HE and CS/DS synthesis [7, 15]. A point mutation that causes loss of activity in  $\beta$ 4GalT7 was, in fact, demonstrated to be responsible for alterations in cell proliferation, migration, adhesion and response to outer stimuli, due to changes in the structure of GAGs HS/HE, which were shown to be less sulphated [7]. Skin fibroblasts with mutant  $\beta$ 4GalT7 displayed normal large CS/DS and HS/HE PGs but about 50 % of the decorin was secreted without GAGs chain and 70 % of biglycan carried only one GAGs chain. [15]. Although the amount of HS/HE was not altered, the sulphation degree was lower. Moreover, in the  $\beta$ 4GalT7 mutant it was reported that “the authors could not find any evidence for a differential transcriptional regulation of HS biosynthetic enzyme expression” with respect to control fibroblasts [7]. For this reason, we compared the silencing of  $\beta$ 4GalT7 with EXT1, one of the polymeric enzymes of HS/HE chains. Therefore, we would be able to compare two systems, one affecting CS/DS and HS/HE and the other referred only to HS/HE. The siEXT1 results nevertheless, are peculiar to cancer cells; in fact, even though the silencing of either  $\beta$ 4GalT7 or EXT1 did not alter the each other expression, siEXT1 cells showed migration and proliferation rates similar to control and adhesion to fibronectin was unaffected. Since one of the main role of HS/HE GAGs in cells is to act as co-receptor, we investigated the effects of gene silencing on the HGF signalling. HGF, the hepatocyte growth factor, binds to the membrane tyrosine kinase receptor, c-Met, whose prognostic value has been established in several clinical studies on breast cancer. Moreover, c-Met was connected to syndecan-1 expression both in multiple myeloma and aggressive breast cancer cell lines [45] [40]. The signal transduction from c-Met to the MAP kinase p42/44 was still functioning in si $\beta$ 4GalT7 cells, but with less intensity, indicating that the remaining GAGs on the cell membrane were correctly polymerized and sulphated, as the sulphation pattern is fundamental in growth factor recognition [9, 13]. The effect of EXT1 abrogation was unclear: even if the trend was evident, the effects on different experiments were very variable. This results can be the due to the formation of shorter HS chains [17], but since the EXTs enzymes maturation through the ER/Golgi depends also on the relative concentration of EXT2 and NDST1, the sulphation pattern of the HS

synthesized can or cannot be able to bind HGF due to the final composition of the GAGosome complex [16]. Since cell adhesion to the ECM substrate fibronectin is also influenced by HS sulphation [7], the chain length may not be the key point regarding cell adhesion. Apparently, the final GAG disaccharide composition could be the motif for a lack of effect in the siEXT1 cells adhesion assay. The HS/HE chains in the breast carcinoma cell line MDA-MB-231 are mainly associated with syndecan-1 [31, 39, 40, 42], a PG that is important in various steps of cancer progression, both as membrane-bound PG and, after shedding, in soluble form [46]. The syndecan-1 extracted from cell lysates is visible in two main forms, at about 100 kDa and 37 kDa. The bigger form represents the PG with short GAG chains, since normally the protein part of the PG is protected from the interaction with antibodies by the GAG moiety [47]. In highly aggressive breast cancer cells as MDA-MB-231, the presence of the clearly visible high molecular weight band correlates with the expression of heparanase. [48]. Heparanase is an ECM enzyme that degrades HS/HE chains to oligosaccharides and causes an imbalance in growth factor retention, leaving them available for different cell activation and for neoangiogenesis [48]. It is noteworthy that the si $\beta$ 4GalT7 treatment resulted in a syndecan-1 downregulation evident in the decrease of both the protein core and the whole HSPG. We can suppose that the lack in the formation of the linkage region can control the core protein expression.

Since the polymerization of a GAGs chain needs UDP-sugars availability inside the Golgi and ER, we investigated whether the silencing of the enzymes  $\beta$ 4GalT7 and EXT1 could also control the amount of carriers. The nucleotide-sugars transporter family (SLC35) consists of at least 17 molecular species in humans, localized in the Golgi apparatus and/or ER [21, 22]. In particular, we selected SLC35D1 that transports UDP-GlcNAc, -GlcA, and -Gal; SLC35D2 for UDP-GlcNAc, and SLC35B1 which carries UDP-Gal, -GlcNAc, and CMP-Sia. SLC35D1 is reported to control specifically the CS synthesis by limiting the UDP-sugar precursor inside the Golgi apparatus. [22]. SLC35D2 (homologous of *C.elegans* SQV-7), is reported to colocalize with the UDP-glucuronic acid decarboxylase (that produces the link sugar UDP-xylose) and after their silencing, the effect is the same found with the absence of one of the GAGs polymerizing enzymes: the failure of embryonic development, probably due to the absence of HS and/or CS chains [49]. The last transporter, SLC35B1, is reported to transport UDP-GlcA in a variety of species, but nevertheless, it did not correlate to GAGs synthesis; developmental studies in *C. elegans* demonstrated that this protein is required for maintenance of ER structure and homeostasis [50]. Silencing of  $\beta$ 4GalT7 determined a decrease in the only SLC35D2 transporter that was associated with embryonic development, probably due to the absence of HS and/or CS chains. The co-expression of this

enzyme with UDP-GlcA decarboxylase [23, 51] that converts UDP-GlcA to UDP-xylose, seems to indicate a spatial and temporal regulation of the enzymes and transporters implicated in the construction of the tetrasaccharide. In fact, the EXT1 down regulation controls a different UDP-sugar transporter, the SLC35D1, that is mainly localized in the ER [21], where the GAGosome is established in order to prepare the correct complex that will be transported to the Golgi network determining the sulphation pattern and chain length of the HS/HE GAGs [16, 17]. The GAGosome model would therefore include the solute carriers, but further studies are needed to identify the three complexes specialized in linkage region, CS/DS and HS/HE chains. The signals backward from Golgi or ER to the nucleus regulating carrier expression is a remarkable piece of information. We can hypothesize that the UDP-sugars amount plays an important role as demonstrated for the HA synthases. UDP-GlcNAc has a central role in control metabolism, *via* both UDP-sugar availability and the post-translational *O*-GlcNAcylation; as recently published by our group; the increase in UDP-GlcNAc leads to HA synthesis and HAS2 overexpression [20, 52, 53]. We observed the same effect in si $\beta$ 4GalT7 treated cells. In fact, when we silenced  $\beta$ 4GalT7, we also diminished the transportation of UDP-GlcNAc within the ER/Golgi, which accumulated in the cytoplasm. It is noteworthy that, even if the si $\beta$ 4GalT7 cells are not proliferating or migratory, the accumulation of HA in the ECM is usually of poor prognosis in breast cancer [25]. Moreover, at least in multiple myeloma, a high expression of  $\beta$ 4GalT7 is associated with a better prognosis [54].

In the study by Vigetti *et al.* [43] also the increment of UDP-GlcA in the cytoplasm, induced an overexpression of HAS2 and -3, which are able to use those UDP-sugars to make HA. On the other hand, the loss of UDP-GlcA did not modify enzyme expression reducing total cell GAGs. In our cells, the silencing of EXT1 decreased the expression of UDP-GlcNAc and UDP-GlcA transporter (SLC35D1), but even if the UDP-GlcA should be increased in the cytoplasm no effect on HAS gene expression or HA synthesis was detectable. Probably it happens because the two sugars are balanced in the cytoplasm and some metabolic control mechanism occurs.

Concluding, our data outline a control mechanism for GAGs polymerization that clearly separates the formation of the linkage region from the chain elongation, and includes in the complexes the UDP-sugars transporters. Moreover, the abrogation of either  $\beta$ 4GalT7 or EXT1 enzymes can have different effect on the behaviour of cancer cells, both of them helpful but not conclusive in decreasing cell aggressiveness, once again highlighting the need of a multi targeted approach.

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### Compliance with ethical standards

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

**Ethical approval** This article does not contain any studies with human participants or animals performed by any of the authors.

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