Mol Cell Biochem (2013) 373:63–72
DOI 10.1007/s11010-012-1475-8

Involvement of Ext1 and heparanase in migration of mouse FBJ osteosarcoma cells

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Received: 12 June 2012 / Accepted: 26 September 2012 / Published online: 10 October 2012

Abstract To know the involvement of glycosaminoglycans (GAGs) in the metastasis of mouse FBJ osteosarcoma cells, N^{α}-lauroyl-*O*-(β-D-xylopyranosyl)-L-serinamide (Xyl-Ser-C12), which initiates elongation of GAG chains using the glycan biosynthesis system in cells, was administered to FBJ cells with different metastatic capacities. Production of glycosylated products derived from Xyl-Ser-C12, especially heparan sulfate (HS) GAG-type oligosaccharides such as GalNAc-GlcA-GlcNAc-GlcA-Gal-Gal-Xyl-Ser-C12, was indicated in poorly metastatic FBJ-S1 cells more than in highly metastatic FBJ-LL cells by LC–MS. The results of RT-PCR revealed that HS synthases, *Ext1* and *Ext2,* were expressed in FBJ-S1 cells more than in FBJ-LL cells. Furthermore, siRNA against *Ext1* suppressed the expression of HS and enhanced the motility of FBJ-S1 cells. In addition, the expression of heparanase (HPSE) was enhanced in Ext-1-knockdown FBJ-S1 cells, and responsible for the increase in cell motility caused by the down-regulation of Ext1 expression. Our data provide the first evidence that Ext1 regulates the expression of HPSE and also indicated that levels of Ext1 and HPSE influenced the motility of FBJ cells.

Keywords Glycosaminoglycan · Cell motility · Ext1 · Saccharide primer · Heparan sulfate · FBJ osteosarcoma · Heparanase

Abbreviations

Hex	Hexose
HexNAc	*N*-Acetylhexosamine
HexA	Hexenuronic acid
Ser	Serine
Xyl	Xylose
RT-PCR	Reverse transcription-polymerase chain reaction

Introduction

Heparan sulfate (HS) is covalently attached to core proteins and known to be involved in tumor growth and metastasis [1, 2]. Tumor suppressors, Exostosin1 (Ext1) and Exostosin2 (Ext2), are type II transmembrane glycoproteins having glycosyltransferase activities for HS biosynthesis, and are located in the endoplasmic reticulum [3, 4]. In the biosynthesis of HS, after the attachment of a GlcNAc residue to GlcA-Gal-Gal-Xyl, Ext1 and Ext2 catalyze the subsequent elongation of glycosaminoglycans (GAGs) by alternately adding GlcA and GlcNAc to the end of the growing chain [4, 5]. Mutations of human *EXT1* and *EXT2* have been reported as an important pathogenic cause of hereditary multiple exostoses (HME), which is an autosomal dominant hereditary disorder characterized by multiple cartilage-capped tumors (exostoses) with a risk of malignancy [6, 7]. Many cases of HME are caused by frameshift or missense mutations in either *EXT1* or *EXT2*.

Heparanase (HPSE), an endoglycosidase that degrades heparan sulfate (HS) [8, 9], has also been shown to be

Electronic supplementary material The online version of this article (doi:10.1007/s11010-012-1475-8) contains supplementary material, which is available to authorized users.

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associated with tumor metastasis [10–14]. The degradation of HS induced by HPSE can affect cell micro-environments, and induce the release of HS-binding growth factors and cytokines involved in tumor metastasis [10–14].

Besides HS, other glycans of glycoproteins, glycolipids, and proteoglycans are also known to play essential roles in cancer metastasis [2, 15, 16]. Thus, detecting the expression of glycans in cancer cells is of great interest for clarification of the mechanism of cancer metastasis and the development of cancer diagnosis. Current methods for detection generally involve the chemical or enzymatic isolation of endogenous glycans and the analysis of glycan structure by enzymatic digestion, immunostaining, liquid chromatography, mass spectrometry, and nuclear magnetic resonance spectroscopy [17, 18]. The saccharide primer method is a new methodology, whereby oligosaccharides are obtained by administering a glycan analog as a precursor, a so-called saccharide primer, for glycan biosynthesis, and has been used to monitor glycan expression in cells [19, 20]. The glycosylation of he saccharide primers is dependent on the cell line, as different cell lines have different intrinsic glycan biosynthesis pathways [19–21]. Saccharide primers such as dodecyl β-lactoside (Lac-C12) [21–23], 12-azidododecyl β-lactoside (Lac-C12N3) [24], dodecyl 2-acetamido-2-deoxy-β-D-glucopyranoside (GlcNAc-C12) [25], and dodecyl β-D-galactopyranosyl-(1 → 4)-2-acetamido-2-deoxy-β-D-glucopyranoside (LacNAc-C12) [25] have been developed to produce ganglio-, globo-, and neolacto-series glycans. A novel saccharide primer, N^{α}-lauroyl-*O*-(β-D-xylopyranosyl)-L-serinamide (Xyl-Ser-C12), mimicking the region in the GAG where xylose attached to the core protein [26], has been synthesized, and it has been indicated that the elongation of GAG chains occurred in CHO and FBJ-S1 cells [27]. These results suggested that Xyl-Ser-C12 is useful in investigating GAG expression as a new methodology for comparative glycomics.

Yamagata et al. [28] have established two cell lines, FBJ-S1 and FBJ-LL, derived from an FBJ virus-induced mouse osteosarcoma. They found that the poorly metastatic FBJ-S1 cells highly expressed the ganglioside GD1a, while the FBJ-LL cells with high metastatic capacity were deficient in GD1a. GD1a regulated cell motility, cell adhesiveness to vitronectin, phosphorylation of c-Met and metastatic capacity in the FBJ cells [16, 29–32]. However, tumor metastasis is a complicated process dependent on many biomolecules. Therefore, to know the involvement of other glycans such as GAGs in the metastasis of FBJ cells, a comparative analysis of glycans between FBJ-LL and FBJ-S1 cells using the saccharide primer method was conducted in this study. As expected, the expression of ganglio-a series was shown to differ between FBJ-LL and FBJ-S1 cells using the saccharide primer Lac-C12 (unpublished result).

In this study, Xyl-Ser-C12 was administered to both FBJ-S1 and FBJ-LL cells to carry out comparative glycomics for the expression of GAG. Based on a comparison of glycosylated products and glycosyltransferases between FBJ-S1 and LL cells, the expression of HS was found to decrease in the highly metastatic FBJ-LL cells. Suppression of Ext1 by siRNA in FBJ-S1 cells resulted in the decreased expression of HS and enhanced motility. The results indicated that HS is involved in the migration of FBJ cells.

Materials and methods

Cell lines and cell culture

The highly metastatic mouse osteosarcoma FBJ-LL cells and the poorly metastatic FBJ-S1 cells were produced from a FBJ virus-induced osteosarcoma of the BALB/c mouse in a previous study [28, 31]. The cells were maintained in RPMI-1640 medium (Nacalai, Japan) supplemented with 10 % FBS (GIBCO, USA) as described previously [33].

Chemicals and antibodies

The saccharide primer Xyl-Ser-C12 was synthesized according to the literature [27]. Goat anti-Ext1, mouse anti-β-actin, rabbit anti-HPSE, and FITC-conjugated anti-mouse IgM antibodies were purchased from Santa Cruz Biotechnology (USA). The mouse anti-HS IgM antibody HepSS-1 was from Seikagaku Biobusiness Corporation (Japan). Peroxidase-conjugated anti-goat IgG and anti-mouse IgG antibodies were purchased from Sigma-Aldrich (USA).

Saccharide primer method

FBJ cells (2×10^6) seeded into 100-mm culture dishes were incubated with serum-free and phenol red-free RPMI-1640 medium (Gibco) containing 50 μM Xyl-Ser-C12, 5 mg/L of transferrin, 5 mg/L of insulin, and 30 nM selene dioxide for 48 h. The glycosylated products were collected from the culture medium using a Sep-Pak C18 cartridge (Waters, Ireland). The products were eluted with methanol. For the analysis with LC–MS, the glycosylated products were dissolved in 1 mL of chloroform/methanol (C/M) (19/1, v/v) and adsorbed to a discovery DSC-NH_2 cartridge (SUPELCO, USA). After washing of the cartridge with chloroform, the acidic products were eluted with 3 % acetic acid/4 % triethylamine in methanol. The eluates were filtrated with a minisart RC4 filter (pore size; 0.2 μm) (Satorius Stedim Biotech, Germany), evaporated to remove the solvent, and dissolved in C/M (9:1, v/v) for LC–MS.

The amount of protein in cells was measured using a Bio-Rad DC protein assay kit (USA).

LC–MS

The glycosylated products dissolved in C/M (9:1, v/v) were subjected to LC–MS as described previously [20]. Peak areas of extracted ion chromatograms (EICs; Online Resource 3) were measured and normalized with the corresponding amount of protein in cells treated with Xyl-Ser-C12. The relative amount of each product was estimated based on the area of the peak corresponding to that product in the chromatogram.

RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted using an RNeasy Mini kit (Qiagen, Germany) following the manufacturer's instructions. The RNA (1 μg) was reverse-transcribed into cDNA, and semi-quantitative PCR was performed with a Takara thermal cycler dice (Takara, Japan) using a ReverTra-Ace RT kit (Toyobo, Japan) and Takara Ex *Taq* according to the instructions provided. PCR products were electrophoresed on a 2 % agarose gel, and quantified using the Bio-rad molecular Imager FX system (Bio-Rad, USA) and ImageJ (National Institute of Mental Health, USA). Ext1, Hpse, and Gapdh expression were also quantified by real-time RT-PCR. Real-time PCR was performed in a 20-μL mixture of SYBER Premix Ex *Taq* II (TAKARA, Japan) and cDNA using a LightCycler (Roche Diagnostics, Switzerland) as per the manufacturer's instructions. The primer sequences used for PCR are listed in Table 1. Data were analyzed according to the comparative CT method [34] using internal control (GAPDH) transcript levels to normalize differences in sample loading and preparation.

siRNA transfection

The distinct predesigned siRNA against mouse *Ext1* (sequence #1 5′-UUCCGUAGUUGAAAGCACAGUGUCC-3′ and sequence #2 5′-UGUCACAGCAGACACCAGGAAAUUC-3′), siRNA against mouse Hpse: (5′-UAUCAUGGUUGACUUGAGAUUUCCA-3′), and RNAi negative universal control were purchased from Invitrogen (USA). FBJ-S1 cells were transfected with siRNA or negative control siRNA (10 nM) using Lipofectamine RNAi MAX according to the manufacturer's instructions (Invitrogen, USA). At 48 h after the transfection, RNA extraction, Western blotting, flow cytometry, and migration assay were carried out.

Western blotting

Western blotting was performed as described previously [33] with a minor modification. In brief, an aliquot of the lysate (25 μg of protein) was fractionated on SDS-polyacrylamide gel, and transferred to Immobilon-P PVDF membranes (Millipore, USA). Membranes were incubated with an anti-β-actin (1:2,000) antibody and an anti-Ext1 antibody (1:2,000) followed by the appropriate peroxidase-conjugated secondary antibodies (1:5,000). Immunoblots were visualized by enhanced chemiluminescence (ECL detection reagent, Amersham, UK). The bands corresponding to β-actin and Ext1 were quantified using the Molecular Imager FX System and Quantity One software (Bio-Rad, USA).

Flow cytometry

The FBJ cell suspension was incubated for 30 min with the anti-HS antibody on ice, washed, further incubated for 30 min on ice with the fluorescein isothiocyanate-conjugated anti-mouse IgM antibody, and then analyzed with a

Table 1 Primers used for amplifying the indicated genes

Gene	Product size (bp)	Sequence (5′–3′)	
		Forward	Reverse
β-Actin	517	ATATCGCTGCGCTGGTCGTC	AGGATGGCGTGAGGGAGAGC
Ext1	530	TCCTGGAGGATTGTTCGTC	TAGCAGCTCCTGTCAACAC
Ext2	228	CCTACAGATCATCAATGACAGG	AGCAGCTTGGACAGACTGG
Chsy1	340	TTGCTCTTCTTCTGTGATGT	CCTGGCTCCTGAATGTCT
Chpf	425	CTGCTGCTGCTGTATGAG	GCCACATAGTCGGAGTTG
Chpf2	415	CTCGGTTACTCTACTTCACA	CAGGTCTTGGTTGATGCTA
Gapdh	205	CATCTGAGGGCCCACTG	GAGGCCATGTAGGCCATGA
Ext1	153	GGAGTTGCCATTCTCCGA	TAAGCCTCCCACAAGAACTG
Hpse	72	GTTCCTGTCCATCACCATCGA	CTTGGAGAGCCCAGGAAGGT

flow cytometer (Epics Altra, Beckman Coulter, USA). All of the incubations and washes were performed with PBS containing 5 % BSA.

Wound healing assay

FBJ-S1 cells were seeded in 6-well plates at a density of 2×10^5 cells/well, and transfected with 10 nM siRNA as described above. At 48 h after transfection, a wound was carefully created by scratching the cell monolayer with a sterile 200-μL pipette tip. After the incubation of cells for 24 h with RPMI-1640 medium (5 % FBS), three or more fields of the wound were photographed, and the cells that had migrated into the wound were counted with ImageJ. The final values were expressed as the fold increase compared to control cells.

Transwell assay

FBJ-S1 cells were seeded in 6-well plates at a density of 2×10^5 cells/well, and transfected with 10 nM siRNA as described above. At 48 h after the transfection, the cells were trypsinized and resuspended in 5 % FBS RPMI-1640 medium. Then 1×10^4 cells in 200 μL were plated in the top chamber with a non-coated PET membrane (24-well format insert, 8.0-μm pore size, BD Falcon, USA) for a migration assay. RPMI-1640 medium with 10 % FBS was added in the lower chamber as a chemo-attractant. After incubation for 24 h, the cells that had migrated to the lower surface of the membrane were fixed and stained with 0.1 % crystal violet in 20 % ethanol for 30 min. The numbers of cells were counted using ImageJ. The final values were expressed as the fold increase compared to control cells.

Statistical analysis

All values are given as the mean ± SD. Statistical analyses were performed using the Student's *t* test available in Microsoft Excel, and the level of significance is indicated in figures when more than three independent experiments were performed.

Results

Analyses of glycosylated products derived from Xyl-Ser-C12 in FBJ-S1 cells and FBJ-LL cells

The Xyl-Ser-C12 primer was administered to poorly metastatic FBJ-S1 cells and highly metastatic FBJ-LL cells. Glycosylated products were extracted from the culture medium and subjected to LC–MS/MS. As shown in Table 2, two neutral products (XN1 and XN2) and six acidic products (XA1-6) were detected in the glycosylated products from FBJ-S1 cells. According to our previous study [27], XN1, XN2, XA1, and XA4-6 were identified as the intermediates of GAG biosynthesis by LC–MS and glycosidase digestion. Two neutral products (XN1-2) and five acidic products (XA1-5) were detected in the glycosylated products derived from FBJ-LL cells (Online Resources 1, 2), and the amount of each of the glycosylated products derived from FBJ-LL cells was less than that from FBJ-S1 cells. Notably, XA6 was not detected in the products derived from FBJ-LL cells. XA6 was deduced to be HexNAc-HexA-HexNAc-HexA-Hex-Hex-Xyl-Ser-C12 by LC–MS, and was considered to be a GAG-type oligosaccharide as it was digested by heparitinase. It was found that the glycosylation initiated by Xyl-Ser-C12 was more active in FBJ-S1 cells than in FBJ-LL cells and the biosynthetic capacity of GAG chains of FBJ-S1 cells was greater than that of FBJ-LL cells.

The expression of Ext1 and HS in FBJ cells

In our previous paper, the tetrasaccharide portion was determined as GlcA-Gal-Gal-Xyl as shown in Table 2. Based on the tetrasaccharide sequence, the elongation of the Xyl-Ser-C12 primer was considered to be mediated by the GAG biosynthetic pathway including HS and chondroitin sulfate. Ext1 and Ext2 catalyze the addition of alternating GlcA and GlcNAc units to the tetrasaccharide linker of GAGs, and are responsible for the elongation of HS [4, 5].

Table 2 The glycosylated products derived from Xyl-Ser-C12-treated FBJ cells

	Predicted structure[a] (X: Xyl-Ser-C12)	Theoretical mass		Relative amount[b]	
				S1	LL
XN1	Hex-X	615.3	$[M+Cl]^-$	1	0.45
XN2	Hex-Hex-X	777.3	$[M+Cl]^-$	1	0.1
XA1	HSO_3-Hex-X	659.3	$[M-H]^-$	1	0.15
XA2	NeuAc-Hex-X	870.8	$[M-H]^-$	1	0.54
XA3	NeuAc-Hex-Hex-X	1032.8	$[M-H]^-$	1	0.03
XA4	HexA-Hex-Hex-X	917.6	$[M-H]^-$	1	0.25
XA5	HexNAc-HexA-Hex-Hex-X	1120.5	$[M-H]^-$	1	0.1
XA6	HexNAc-(HexA-HexNAc)-HexA-Hex-Hex-X	749.3	$[M-2H]^{2-}$	1	N.D.

N.D. not detected

[a] Structural predictions were based on MS/MS spectral assignments of the products and GAG biosynthesis pathway

[b] The relative amounts of product were calculated according to the peak area in chromatograms corresponding to each product. The ratio of the amount of product in LL to that in S1 (set at 1) cells was calculated. Data are the mean for three independent experiments

Chondroitin sulfate synthase 1 (Chsy1), chondroitin polymerizing factor (Chpf), and chondroitin polymerizing factor 2 (Chpf2) mediate the elongation of chondroitin sulfate by addition of GlcA and GalNAc [35]. The composition of glycosylated products derived from Xyl-Ser-C12 in FBJ-S1 and FBJ-LL cells was considered to be directly influenced by these glycosyltransferases. Thus, the gene expression of these glycosyltransferases in FBJ cells was examined by semi-quantitative RT-PCR. The mRNA expression of both *Ext*1 and *Ext2* was higher in FBJ-S1 cells than in FBJ-LL cells (Fig. 1a), whereas there was no significant difference in the expression of chondroitin sulfate glycosyltransferases (Chsy1, Chpf, and Chpf2) between the two cell lines. Based on these results, we focused on Ext1. The difference in the expression of Ext1 between these cell lines was also confirmed by Western blotting. The expression of Ext1 was well consistent with the detection by real-time RT-PCR at the mRNA level (Fig. 1b).

The results of real-time RT-PCR and western blotting revealed a down-regulation of *Ext1* expression in the highly metastatic FBJ-LL cells. These results suggested that the extension of HS in FBJ-LL cells was suppressed compared to that in FBJ-S1 cells. Therefore, the detection of cell surface HS using the HepSS-1 antibody that recognizes the repeating disaccharides in HS was carried out by flow cytometry [36]. The relative amount of bound HepSS-1 was greater for FBJ-S1 cells than FBJ-LL cells. Figure 1c indicates that the expression of HS was significantly higher for FBJ-S1 than FBJ-LL cells. This phenomenon was possibly related to the expression levels of two glycosyltransferases, Ext1 and Ext2, in the cell lines.

Effect of knockdown of Ext1 on cell surface HS

The Ext1/Ext2 complex is known to possess higher glycosyltransferase activity than Ext1 or Ext2 alone, and a reduction in either Ext1 or Ext2 could cause a reduction in HS biosynthesis [4, 5]. Therefore, the suppression of Ext1 by siRNA was carried out. *Ext1* siRNAs with two distinct sequences were separately transfected into Ext1-rich FBJ-S1 cells, and total RNA and protein were extracted. Both real-time RT-PCR and western blotting showed that the expression of the mRNAs and proteins of Ext1 in *Ext1* siRNA-transfected cells (either sequence #1 or #2) was reduced to less than 50 % of levels in the control siRNA-transfected S1 cells as shown in Fig. 2a, b. The binding of the HS antibody to S1 cells was remarkably suppressed by *Ext1*-siRNA transfection (either #1 or #2) compared to control siRNA transfection (Fig. 2c). These results indicated that the reduction of *Ext1* affected the elongation of HS chains.

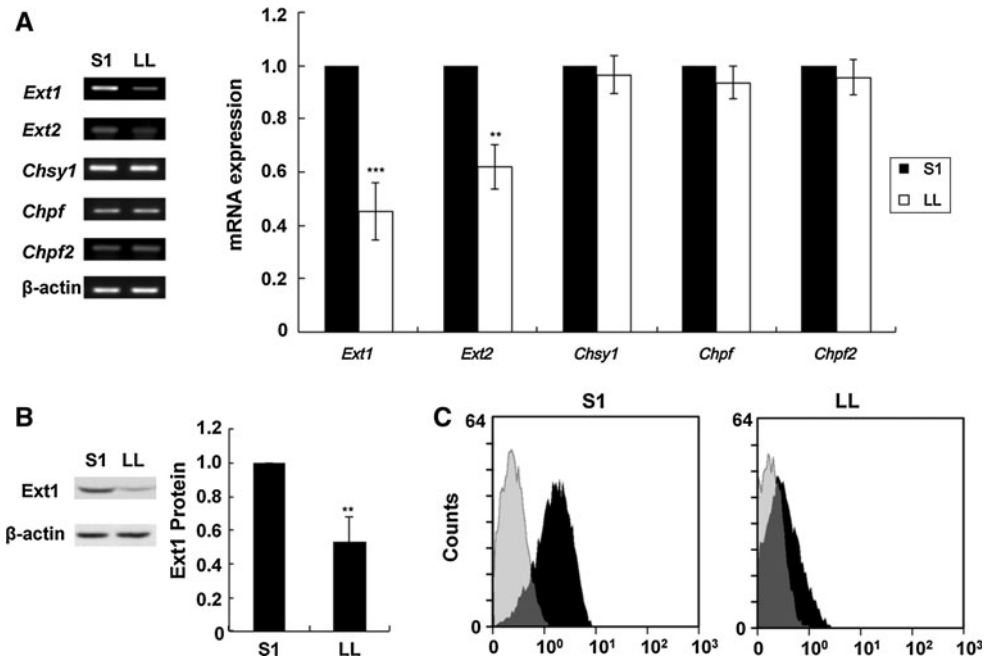

Fig. 1 Comparison of Ext1 and HS expressed in FBJ-S1 and FBJ-LL cells. **a** RT-PCR for the detection of glycosyltransferases related to the biosynthesis of GAGs (*Ext1*, *Ext2*, *Chsy1*, *Chpf*, and *Chpf2*) in FBJ-LL cells compared to FBJ-S1 cells (normalized to *β*-actin). **b** Western blotting for the amounts of Ext1 in FBJ-S1 (set at 1) and LL cells using *β*-actin as an internal control. The data shown in (**a**, **b**) represent the mean for three independent experiments. *Error bars* represent SD. Representative images are shown. $^{**}P < 0.01$, $^{***}P < 0.001$. **c** Flow cytometric analysis of HS expressed in FBJ-S1 and LL cells. These cells were stained with an anti-HS monoclonal antibody. The *abscissas* represent fluorescence intensity, and the *ordinates* represent relative cell number. Representative results of three independent experiments are shown

Effect of knockdown of Ext1 on cell migration

To clarify the contribution of Ext1 to the migration of FBJ cells, a transwell assay (Fig. 3a) and a wound healing assay (Fig. 3b; Online Resource 3) were carried out. The motility of FBJ-S1 cells transfected with *Ext1* siRNA was significantly enhanced. The proliferation and viability of S1 cells cultured in 1 % FBS medium were not affected by the transfection with *Ext1* siRNA (data not shown).

Elevation of the expression of HPSE in Ext1-knockdown S1 cells

Reduced levels of HS synthases in FBJ-LL cells were considered to be responsible for the low expression of HS as described above. Alternatively, the lower levels of HS in FBJ-LL cells may also be attributable to the expression of the HS-degrading enzyme, HPSE, known as a significant pro-metastasis factor.

Hpse expression was significantly lower in FBJ-S1 cells than in FBJ-LL cells (Fig. 4a). Unexpectedly, Hpse expression was enhanced in Ext1-knockdown FBJ-S1 cells (Fig. 4b). To verify the role of Hpse in motility, FBJ-S1 cells were transfected with siRNA against Ext1 and siRNA against Hpse either alone or in combination. Suppression of Hpse by siRNA abrogated the increase in cell migration induced by the down-regulation of Ext1 expression (Fig. 5; Online Resource 5). Furthermore, the motility of FBJ-LL cells was suppressed by knockdown of Hpse, indicating that the expression of Hpse contributed to the migration of

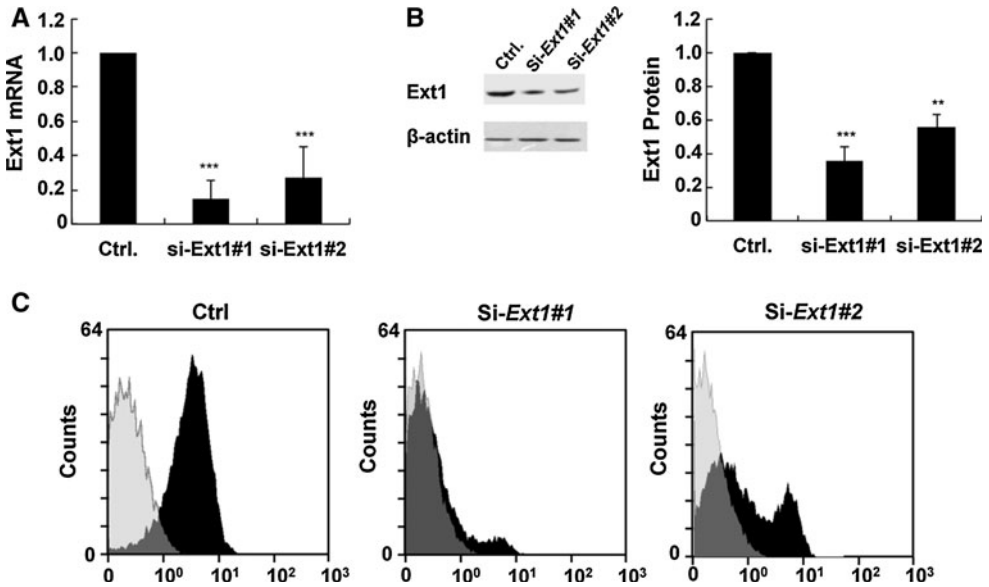

Fig. 2 Effect of *Ext1* siRNA on the expression of *Ext1* mRNA (**a**), Ext1 protein (**b**) and cell surface HS (**c**) in FBJ-S1 cells. FBJ-S1 cells were transfected with either *Ext1* siRNA (*#1* or *#2*, two distinct siRNA sequences against *Ext1*) or control siRNA, and 48 h later, subjected to real-time RT-PCR using GAPDH as an internal control, and western blotting (normalized to β-actin). The expression of HS in FBJ-S1 cells transfected with *Ext1* siRNA (*#1* or *#2*) or control (*ctrl.*) siRNA was investigated by a flow cytometer. *Black line* indicates control (using secondary antibody only) and *filled black profile* indicates HS. Data represent the mean ± SD for three independent experiments. $^{**}P < 0.01$, $^{***}P < 0.001$

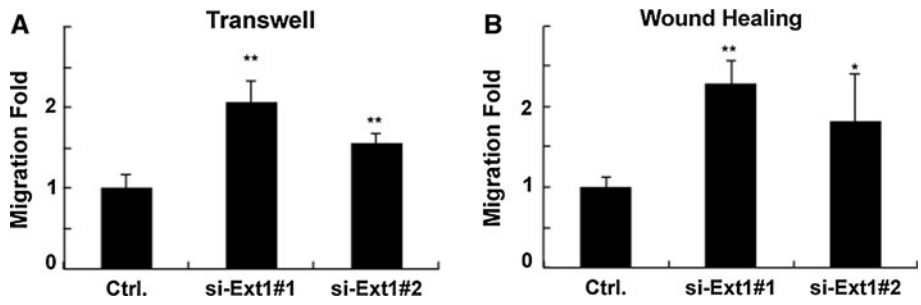

Fig. 3 Motility of FBJ-S1 cells transfected with *Ext1* siRNA. FBJ-S1 cells transfected with *Ext1* siRNA or control siRNA were subjected to transwell (**a**) and wound healing (**b**) assays as described in the "Materials and methods" section. Experiments were carried out three times. *Error bars* represent SD

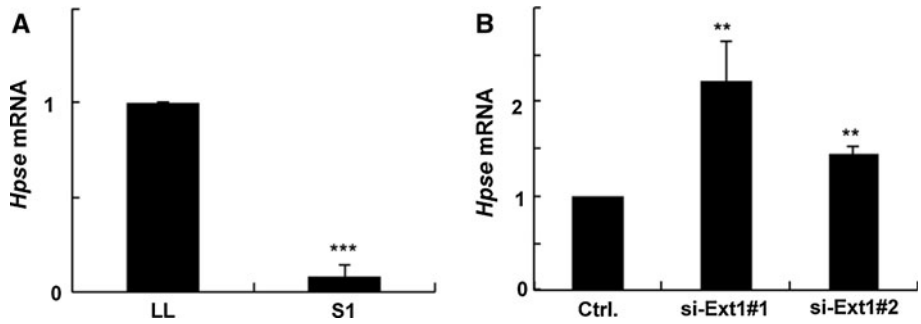

Fig. 4 Real-time RT-PCR for the expression of Hpse mRNA in FBJ-LL cells and FBJ-S1 cells transfected with Ext-1 siRNA. **a** Hpse mRNA expression in FBJ-LL and FBJ-S1 cells. **b** Hpse mRNA expression in FBJ-S1 cells transfected with either *Ext1* siRNA (*#1* or *#2*) or control siRNA. The amounts of Hpse in control siRNA-transfected FBJ-S1 cells (set at 1) were analyzed using GAPDH as an internal control. The data represent the mean for three independent experiments. *Error bars* represent SD. $^{**}P < 0.01$, $^{***}P < 0.001$

FBJ-LL cells (Online Resource 4). On the other hand, no significant change in the migration of FBJ-S1 cells transfected with Hpse siRNA alone was observed. This may be due to the relatively low basal level of Hpse. Based on these results, we conclude that Hpse is involved in the regulation of FBJ-S1 cell motility which is controlled by Ext1.

Discussion

GD1a has been previously shown to be responsible for regulating cell motility, cellular adhesiveness to vitronectin, phosphorylation of c-Met and metastatic ability, in mouse FBJ osteosarcoma cells [16, 29, 30]. Although GD1a plays a major role in controlling the metastatic capacity of FBJ cells, the contribution of other glycans to the complicated process of metastasis could not be excluded. Thus, to determine the involvement of other glycans in the metastasis of FBJ cells, the saccharide primer method was employed to assess the expression profile. Based on the difference in glycan expression between highly metastatic FBJ-LL cells and poorly metastatic FBJ-S1 cells, we carried out the identification of candidate glycans, which may be associated with metastasis. The saccharide primer method is useful for knowing the glycans expressed in cells. Previously, Lac-C12 [19] and GlcNAc-C12 [25] were developed as saccharide primers to identify the biosynthesis of glycans such as the ganglio-, globo-, and neolacto-series. The expression of glycans in FBJ cells was investigated using a saccharide primer, Lac-C12, and FBJ-S1 cells exhibited higher levels of the GD1a type product than LL cells did (unpublished data). This result is in good agreement with previous studies on endogenous gangliosides in FBJ-LL and S1 cells [16]. In this study, a saccharide primer, Xyl-Ser-C12 [27] which is an initiator for the biosynthesis of GAG, was employed to investigate the GAG biosynthetic pathway in FBJ cells. Monitoring GAG biosynthesis using Xyl-Ser-C12 is considered to be useful for knowing the differences in the character of tumor cells. The elongation of GAG-type oligosaccharides from Xyl-Ser-C12 occurred cells more efficiently in FBJ-S1 than FBJ-LL cells. Based on an analysis by LC–MS and enzyme digestion, the products from Xyl-Ser-C12 were deduced to be HS-type oligosaccharides. Therefore, we focused on Ext1 and Ext2 as candidates that cause changes in the expression of GAGs and the difference in metastatic ability between FBJ-LL and FBJ-S1 cells. This notion was also supported by the expression of HS on the surface of FBJ-LL and S1 cells (Fig. 1c). Moreover, the expression of *Chsy1*, *Chpf*, and *Chpf2* showed no significant differences between S1 and LL cells (Fig. 1a). Therefore, in this study, we focused on HS synthesized by Ext1 and Ext2.

HS is ubiquitous, existing on the cell surface or the extracellular matrix, and involved in tumor growth, angiogenesis, and metastasis. HS proteoglycans can promote or inhibit tumor progression depending on the fine structure of GAGs, the core protein, the tumor subtype, and other growth factors [1]. Furthermore, it has been reported that cell surface HS acted as an inhibitor of metastasis by forming a physical barrier along with other ECM components to tumor cell migration or metastasis. Low levels of HS production correlate with high metastatic activity of many tumors [2, 37].

In this study, both Ext1 and Ext2 were expressed more in poorly metastatic FBJ-S1 cells than in highly metastatic FBJ-LL cells. The low expression of cell surface HS would attenuate cell adhesion [37] and would remove the barrier for metastasis of FBJ cells to a certain extent. Wound healing and transwell assays indicated that *Ext1*-siRNA-transfected S1 cells had enhanced motility. These results suggested that Ext1 is involved in the migration of FBJ cells. It has been reported that Ext1 acts as a putative tumor suppressor, and the decreased expression of human EXT1 is associated with HME resulting in bony outgrowth and the formation of tumors [3]. A recent study also showed that the expression of *Ext1* was altered in malignant canine

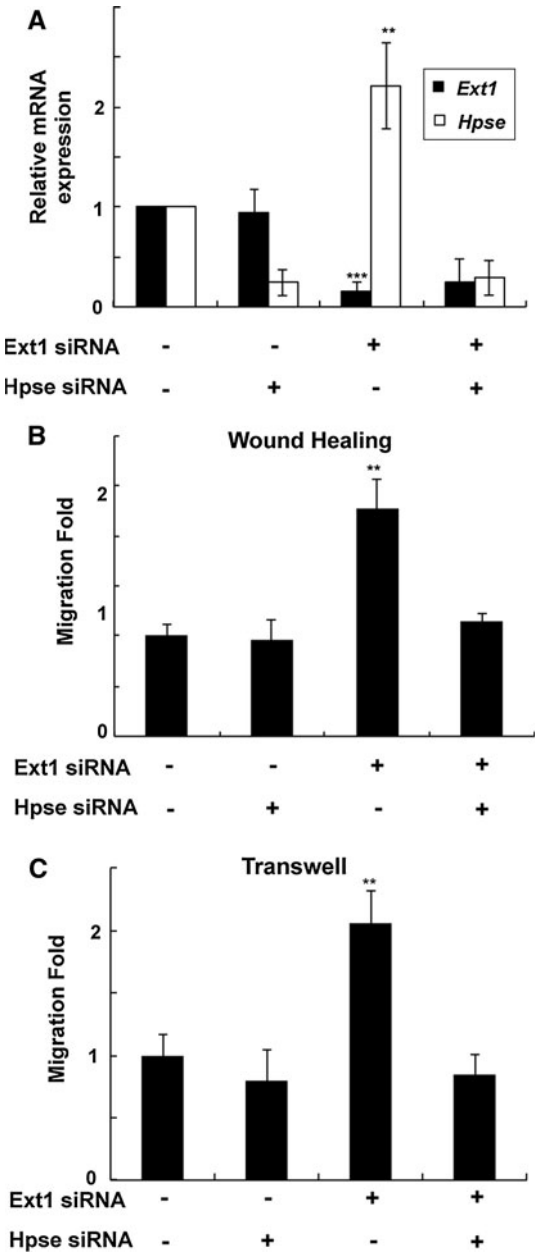

Fig. 5 Effect of siRNA for Hpse and/or Ext1 on the motility of FBJ-S1 cells. FBJ-S1 cells were transfected with *Ext1* and/or *Hpse* siRNA. The mRNA expression of Ext1 and Hpse was detected by real-time RT-PCR (**a**). Cell motility was evaluated by transwell assay (**b**) and wound healing assay (**c**). The data represent the mean for three independent experiments. *Error bars* represent SD. $^{**}P < 0.01$, $^{***}P < 0.001$

osteosarcomas [38]. In addition, both a catalytic function of Ext1 in the biosynthesis of HS [4] and an inhibitory effect of HS on cancer cell metastasis [37] have been reported. However, findings on the inhibition by Ext1 of the migration of osteosarcoma cells have not been reported. Here, we showed the first evidence that Ext1 contributes to the migration of osteosarcoma FBJ cells.

HS chains are capable of binding to growth factors and chemokines including fibroblast growth factors (FGF1 and FGF2), transforming growth factor, and hepatocyte growth factor. Growth factors and chemokines are also known to play an important role in tumor progression [37]. The degradation of HS by HPSEs could affect the extracellular matrix and release HS-binding molecules, thereby promoting tumor metastasis [2, 37, 39]. Elevated levels of HPSE have been detected in a variety of malignancies, suggesting that the expression of HPSE is associated with tumor malignancy and metastasis [10, 12]. Thus, we examined the expression of Hpse in FBJ cells. The expression was greater in the highly metastatic FBJ-LL cells than the poorly metastatic FBJ-S1 cells. In addition, the expression of Hpse was elevated in Ext-1 knockdown FBJ-S1 cells and knockdown of Hpse inhibited the stimulation of cell motility induced by knockdown of Ext1. It was indicated that Ext1 regulates cell motility via Hpse. The molecular mechanisms of increased HPSE expression or activity in malignancies are not well understood. Recent studies showed hypomethylation of the HPSE promoter and transcription factor early growth response-1 to be involved in the overexpression of HPSE in several types of tumor cells [40, 41]. The expression of p53, RAS, and BRAF has also been reported to be responsible for the regulation of HPSE expression [42, 43]. Cathepsin L has been shown to be essential for the activation of HPSE [44]. However, the involvement of Ext1 in the regulation of Hpse has not been reported so far. We hypothesize that the suppression of Ext1 altered the metabolism of HS and further influenced Hpse. The finding that the knockdown of Ext1 stimulated Hpse expression in FBJ cells could be useful for elucidating the mechanism of FBJ cell metastasis.

Acknowledgments This study was partly supported by the Global COE Project "Global COE for Human Metabolomic Systems Biology'' (YW) from the Ministry of Education, Culture, Sports, Science and Technology and a Grant-in-aid for Challenging and Exploratory Research (2265108, TS) from the Japan Society for the Promotion of Science.

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