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Human cytosolic sialidase NEU2-low general tissue expression but involvement in PC-3 prostate cancer cell survival

Koichi Koseki [a], Tadashi Wada [b], Masahiro Hosono [c], Keiko Hata [b], Kazunori Yamaguchi [a], Kazuo Nitta [c], Taeko Miyagi [a,b,*]

[a] Division of Biochemistry, Miyagi Cancer Center Research Institute, Natori 981-1293, Japan
[b] Division of Cancer Glycosylation Research, Institute of Molecular Biomembrane and Glycobiology, Tohoku Pharmaceutical University, Sendai 981-8558, Japan
[c] Division of Cell Recognition Study, Institute of Molecular Biomembrane and Glycobiology, Tohoku Pharmaceutical University, Sendai 981-8558, Japan

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ABSTRACT

Human cytosolic sialidase (NEU2) has been identified and characterized using a *NEU2* cDNA constructed from a genomic library of human skeletal muscle. However, the tissue distribution of *NEU2* mRNA and the physiological functions of the enzyme remain unclear. In the present study, unlike other human sialidases, *NEU2* expression as assessed by quantitative real-time PCR was found to be extremely low or undetectable in many human tissues and cells, with notable exceptions like the placenta and testis. The gene forms obtained by PCR with cDNAs synthesized from poly $(A)^+$ RNA of human brain and colon were verified to encode cytosolic sialidase with appropriate activity, regardless of the brain gene feature of SNPs. Among a series of human cancer cell lines examined, only prostate cancer PC-3 cells exhibited relatively high expression and *NEU2*-silencing with an siRNA resulted in decreased cell survival and motility. To gain insights into the significance of the high levels, transcription factors in the promoter region of the *NEU2* gene were surveyed for involvement. PC-3 cells were characterized by high expression of *Runx2* and *Sp3*, and their silencing reduced NEU2, suggesting regulatory roles.

1. Introduction

Sialidases (EC 3.2.1.18) are glycohydrolases catalyzing the removal of sialic acids from the non-reducing termini of carbohydrates in glycoconjugates. The catalytic reaction is an initial step in degradation of glycoproteins or glycolipids, leading to modulation of physiological processes like cell division, differentiation, adhesion, invasion and apoptosis. In mammals, there are four types of sialidases classified by subcellular localization and enzymatic properties, designated as NEU1, NEU2, NEU3 and NEU4 [1,2]. Among them, NEU2 was the first example of a mammalian sialidase successfully gene cloned from rat skeletal muscle [3,4], after which further cloning from Chinese hamster ovary cells [5], murine thymus [6], and a genomic library of human skeletal muscle [7,8] have been documented. Previous reports demonstrated that NEU2 involvement in muscle cell differentiation of rat L6 [9] and murine C2C12 myoblasts [10], as well as actions in NGF-dependent neuronal differentiation in PC12 cells [11], suppression of pulmonary metastasis in murine B16 melanoma cells and colon adenocarcinoma 26 cells [12,13], and myoblast hypertrophy and atrophy [14,15]. In the case of human NEU2, enzymatic properties have been characterized using a recombinant protein expressed in *Escherichia coli* with a cDNA constructed from a genomic library of human skeletal muscle [16], and the enzyme three-dimensional structure has been determined by X-ray crystallography [17]. Overexpression of NEU2 in leukemic K562 cells, in which endogenous NEU2 was not detectable, caused suppression of cell proliferation and acceleration of apoptosis [18]. However, physiological roles of human NEU2 are still obscure, even the actual *NEU2* expression profile in human tissues not being available. Herein we report very low *NEU2* expression in human tissues and cells assessed by quantitative real-time PCR, and the unexpected finding of high levels in prostate cancer PC-3 cells with evidence of possible functional significance.

2. Materials and methods

2.1. Materials

Poly $(A)^+$ RNA from human brain, colon, fetal liver, and skeletal muscle were purchased from Clontech. MTC Multi Tissue cDNA panels were also from Clontech.

Abbreviations: 4MU-NeuAC, 4-methylumbelliferyl N-acetyl neuraminic acid; siRNA, small interfering RNA; RT-PCR, Reverse transcription polymerase chain reaction; SNP, single nucleotide polymorphism.

* Corresponding author at: Division of Cancer Glycosylation Research, Institute of Molecular Biomembrane and Glycobiology, Tohoku Pharmaceutical University, Sendai 981-8558, Japan. Fax: +81 22 275 2013.
E-mail address: tmiyagi@tohoku-pharm.ac.jp (T. Miyagi).

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2.2. Cell culture

The human prostate cancer cell lines, PC-3 and DU145, and cervical carcinoma HeLa cells and breast adenocarcinoma MCF-7 cells were obtained from Cell Resource Center for Biomedical Research, Tohoku University (Sendai, Japan). Human colon cancer cell lines, DLD-1 and HCT-116, were obtained from HSRRB (Osaka, Japan) and ATCC, respectively. HEK-293T cells were generously donated by M. Sugai, Kyoto University School of Medicine (Kyoto, Japan). PC-3 cells were routinely maintained in F-12 HAM (Sigma) supplemented with 7% fetal bovine serum (FBS), and DU145 cells in RPMI-1640 (Wako) supplemented with 10% FBS. All other cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Wako) supplemented with 10% FBS. Cells were cultured at 37 °C in a humidified atmosphere of 5% CO_2.

2.3. Assessment of human sialidase NEU2 expression and cDNA cloning and construction of an NEU2 expression vector

NEU2 mRNA levels were assessed by RT-PCR. First-strand cDNAs were synthesized from human brain, colon, and skeletal muscle poly $(A)^+$ RNAs using oligo $(dT)_{12-18}$ primers (Invitrogen) and reverse transcriptase (SuperScriptII RT, Invitrogen) as previously described [19]. The PCR reaction mixture was a total volume of 20 μl with final concentrations of the following reagents: 1 × *LA* PCR Buffer II (Mg^{2+}-free), 2.5 mM $MgCl_2$, 0.4 mM of each dNTP, 0.3 μM of each primers, and 1 unit *LA Taq* DNA polymerase (Takara). PCR was performed at 95 °C for 10 min, followed by amplification using 40 cycles of 94 °C for 30 s, 60 °C for 1 min, and 72 °C for 2 min. Products were sized in 1% agarose gels with ethidium bromide staining.

A PCR fragment coding for the ORF of NEU2 (Gene bank Accession No. NM_005383) was amplified using two sets of primer pairs with an *Eco*RI site (forward-reverse1), or with an *Eco*RI site and a FLAG epitope sequence in reverse primers (forward-reverse2) as follows: 5′-GTGAATTCCACCATGGCGTCCCTTCCTGT-3′ (forward), 5′-CTGAATTCTCACTGAGGCAGGTACTCAG-3′ (reverse1), 5′-CTGAATTCTCACTTGTCATCGTCGTCCTTGTAATCCTGAGGCAGGTACTCAGC-3′ (reverse2). PCR products were digested with *Eco*RI and subcloned into the *Eco*RI site of pBluescript (Stratagene). The cloned cDNAs sequences were confirmed by sequencing. To construct NEU2 expression vectors, NEU2 ORF fragments digested with *Eco*RI were inserted into the *Eco*RI site of expression vector pCAGGS (a generous gift from Dr. J. Miyazaki, Osaka University School of Medicine) under control of the β-actin promoter.

2.4. Expression of human NEU2 sialidase

The expression vector, pCAGGS-NEU2, was transfected into HEK-293T cells using Effectene Reagent (Qiagen) as described elsewhere [20]. After transfection for 48 h, cells were harvested, and homogenized by sonication for 10 s in ice-cold PBS (−) containing 1 mM EDTA, 10 μg/ml leupeptin, 0.5 μg/ml pepstatin A, 0.2 mM PMSF and a protease inhibitor cocktail (Roche Diagnostics). Supernatants were obtained from the homogenates after centrifugation at 1000*g* for 10 min, followed by 100,000*g* for 1 h at 4 °C, and used as the enzyme source. Kinetic analysis was performed with 4-methyumbelliferyl *N*-acetyl neuraminic acid (4MU-NeuAc) as the substrate at pH 5.5–6.0 [20]. For estimation of endogenous human NEU2 activity, PC-3 and DU145 cells were harvested at semi-confluence, homogenized by sonication for 10 s in the same buffer, and cytosolic fractions were prepared as described above.

2.5. Quantitative real-time PCR analysis

Quantitative analysis of *NEU2* mRNA expression levels in several human tissues and cell lines were evaluated with a Lightcycler rapid thermal cycler system (Roche Diagnostics). Total RNAs from several human cell lines were extracted, followed by generation of first-strand cDNAs as described previously [19]. MTC Multi Tissue cDNA Panels were used as a template for analysis of *NEU2* expression for human tissues. Expression of *NEU1, NEU3* and *NEU4* was also determined using the same cDNAs. PCR amplification was carried out using their respective primers with standard curves for each cDNA generated by serial dilution of the pBluescript vector containing the gene encoding ORF as described previously [19]. For expression of *Runx2*, *Sp1* and *Sp3*, the primers were as follows: Runx2 (forward: 5'-ACCATGGTGGAGATCATCGC-3', reverse: 5'-CATCAAGCTTCTGTCTGTGC-3'), Sp1 (forward: 5'-CTTGGTATCATCACAAGCCAGTT-3', reverse: 5'-TCCCTGATGATC CACTGGTAGTA-3'), Sp3 (forward: 5'-TTGACTACATCTAGTGGGCAGGT-3', reverse: 5'-TACAACAGGCTGTGCTGTAGAAA-3').Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control for normalization of the mRNA expression level.

2.6. Cell proliferation, DNA synthesis and apoptosis assays

Transfection of the expression vector pCAGGS-NEU2 or NEU2-siRNA (Dharmacon) into PC-3 cells was performed by nucleofection using the Nucleofector II system (Amaxa Biosystems) according to the manufacturer's instructions. At 36 h after transfection cells were harvested and seeded in 96-well tissue culture plates at a density of 1×10^3 cells/well. Cell proliferation was measured using the WST-1 Cell Proliferation assay System (Takara). After 0 to 3 days incubation, WST-1 reagent was added, followed by incubation at 37 °C for 1 h, and absorbance was measured at 450 nm with a reference at 630 nm. DNA synthesis was determined by BrdU (bromodeoxyuridine) incorporation using a BrdU Labeling and Detection kit III (Roche Diagnostics). After 0–3 days incubation, BrdU was added, and after incubation for a further 4 h incorporated BrdU was measured according to the manufacturer's protocol. Cell apoptosis was analyzed by flowcytometry with annexin V staining (Roche Diagnostics) after treatment with 5 mM sodium butyrate (NaBT).

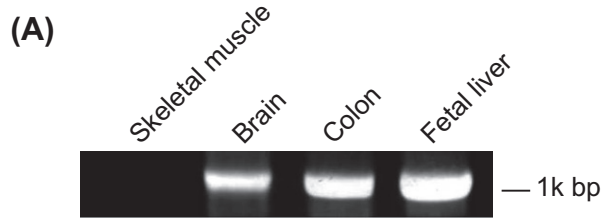
2.7. Cell motility

Transfected PC-3 cells were prepared as described above, and 5×10^4 aliquots were suspended in 300 μl of serum-free medium containing 0.1% bovine serum albumin (BSA, Sigma) and transferred to cell culture inserts (8 μm, Falcon). Cells were incubated in bottom chamber filled with 400 μl of serum-free or conditioned medium at 37 °C. After 5 h the cells migrating through membranes were fixed with 4% glutaraldehyde, stained with Giemsa, and counted under a light microscope.

3. Results

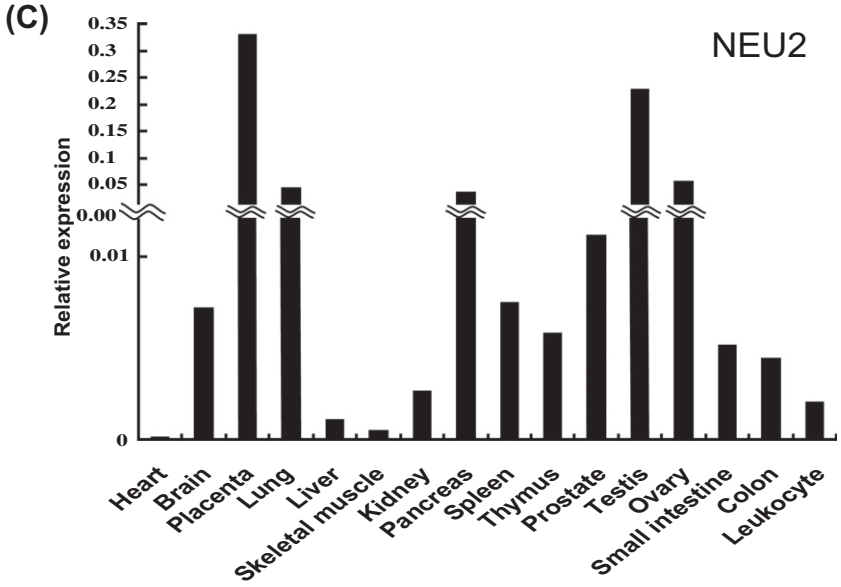
3.1. Cloning of the NEU2 gene from poly $(A)^+$ RNA

Since *NEU2* expression levels in human tissues had hitherto not been clearly evaluated, firstly we examined whether *NEU2* mRNA might be detectable by RT-PCR using primers specific to the 5′ and 3′ termini of *NEU2* gene. As shown in Fig. 1A, bands corresponding to *NEU2* gene (~1.1 kbp) were amplified using cDNA synthesized from poly $(A)^+$ RNA of human brain, colon, and fetal liver, but not from that of skeletal muscle, even after increasing PCR cycles up to 40. When total tissue RNA is used, the band was not clearly amplified. To confirm if the amplified PCR products encoded the *NEU2* gene, cDNAs amplified from brain and colon were subcloned into Bluescript vector and expression vector pCAGGS. Sequence analyses revealed that cDNA from colon was identical



(B)

SNP position	Allele: Colon / NM_005383		Allele: Brain	Amino acid residue	refSNP ID
502	A	→	C	Asn168His	rs2233391
753	C	→	T	Asp251	rs2233394



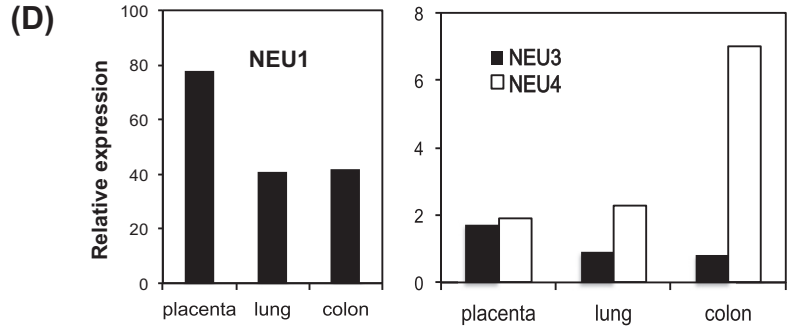


Fig. 1. Determination and quantification of *NEU2* expression in human tissues. (A) RT-PCR analyses of *NEU2* mRNA in human skeletal muscle, brain, colon, and fetal liver. Results are representative of three independent experiments. (B) SNP positions on the ORF of the *NEU2* gene obtained by amplification of sialidase cDNAs from poly $(A)^+$ RNA of human brain and colon. The SNP positions are denoted from the first ATG site. (C) Quantitative RT-PCR analyses of the *NEU2* mRNA in human various tissues. Values are means from two separate analyses. (D) The levels of *NEU1*, *NEU3*, and *NEU4* were also evaluated similarly in placenta, lung and colon. Expression levels were normalized by that of *GAPDH* as an internal control.

with the *NEU2* gene in the GeneBank database (NM_005383). On the other hand, the cDNA from brain contained two SNPs at A502C and C753T, which have been registered as refSNP ID in the dbSNP the database at NCBI: rs2233391 and rs2233394, respectively. These SNPs, A502C and C753T, correspond to amino acids, N168H (nonsynonymous SNP) and D251 (synonymous SNP), respectively (Fig. 1B). To assess NEU2 activity, expression vectors were constructed and transfected into HEK293T cells, and cytosolic fractions were prepared and reacted with 4MU-NeuAc, a good synthetic substrate for cytososlic sialidase [3]. Most of the activity was yielded in the cytosolic fractions of the homogenates. Kinetic analyses showed K_m and V_{max} values to be 1.94 ± 0.16 mM and 642.07 ± 17.60 μmol/h for the brain cDNA, and 1.92 ± 0.14 mM and 634.53 ± 41.22 μmol/h for the colon cDNA, demonstrating that these clones encode sialidase and the N168H SNP in the brain hardly affects the enzyme activity. A previous report demonstrated

that R41Q SNP, an amino acid situated in the catalytic binding site of NEU2, influenced the activity [21]. On the basis of the NEU2 crystal structure [17], N168H SNP was suggested to be located outside of the catalytic cavity, consistent with our present result. In addition, both clones also showed sialidase activity towards GM3, GD1a, sialyllactose, and fetuin as substrates, with no significant differences between them (data not shown).

3.2. NEU2 expression profiling in human tissues and cell lines

As described above, *NEU2* mRNA was detectable in human brain and colon tissues. To extend the evaluation to other tissues, quantitative real-time PCR was performed using MTC panels as cDNA templates. *NEU2* expression was detected in various human tissues, being relatively high in placenta and testis, and then lung, pancreas, ovary and others in that order (Fig. 1C), still extremely low as compared to those of other human sialidases. For example, *NEU2* expression was at most only 300th to 8000th of that of *NEU1* in placenta, lung and colon (Fig. 1D). *NEU2* mRNA was also hardly detectable in almost all the human cancer cell lines tested. However, unexpectedly, as shown in Fig. 2A, *NEU2* expression was significant in prostate cancer PC-3 cells. In comparison with other sialidases, quantitative RT-PCR analyses exhibited *NEU2* in these cell lines to be the lowest among the sialidases (Fig. 2B), whereas interestingly, *NEU3* but not *NEU1* was the highest, consistent with our previous results demonstrating its up-regulation in various cancers [22]. To verify high *NEU2* expression in PC-3 cells, endogenous NEU2 activity in the cytosolic fraction was measured. The sialidase activity at pH 6.0 was much higher than in DU145 prostate cancer cells featuring only slight expression (Fig. 2C). *NEU2* expression in PC-3 cells, however, was low compared to those of the other three sialidases.

3.3. Involvement of NEU2 in cell survival and cell motility

Assuming that high NEU2 expression in PC-3 cells may play roles in physiological functions, the *NEU2* gene was transiently transfected into the cells for overexpression or silenced with an siRNA (Fig. 3A). NEU2 overexpression did not influence cell proliferation, but *NEU2* silencing resulted in the suppression (Fig. 3B). To examine relative contribution of down-regulation of DNA synthesis and acceleration of apoptosis, BrdU and NaBT-induced

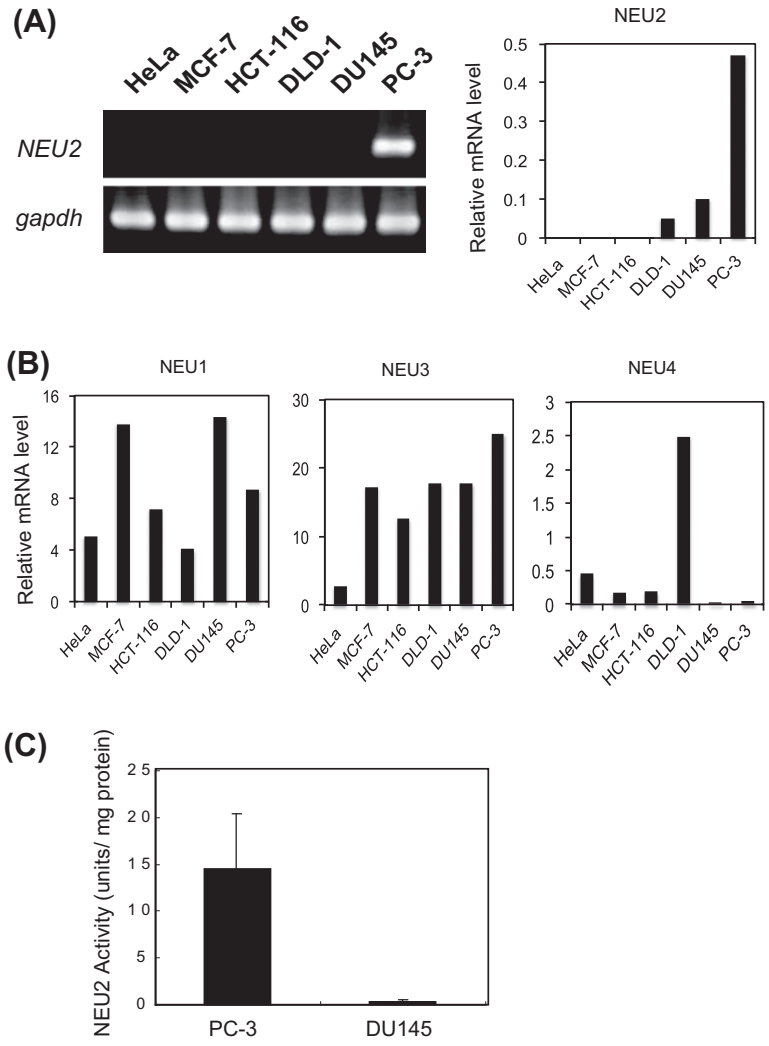


Fig. 2. Determination and quantification of *NEU2* expression in human cancer cell lines. (A) RT-PCR analyses of *NEU2* mRNA in human cancer cells. Results are representative of three independent experiments. The quantification was shown in the right panel. (B) Quantitative RT-PCR analyses of the *NEU1*, *NEU3* and *NEU4* mRNA in human cancer cells. Values are means from two separate analyses. (C) Endogenous NEU2 activity in prostate cancer cell lines, PC-3 and DU145. One unit of activity was defined as the amount of enzyme cleaving 1 nmol of sialic acid in 1 h. The data given are mean values from three experiments ± SD.

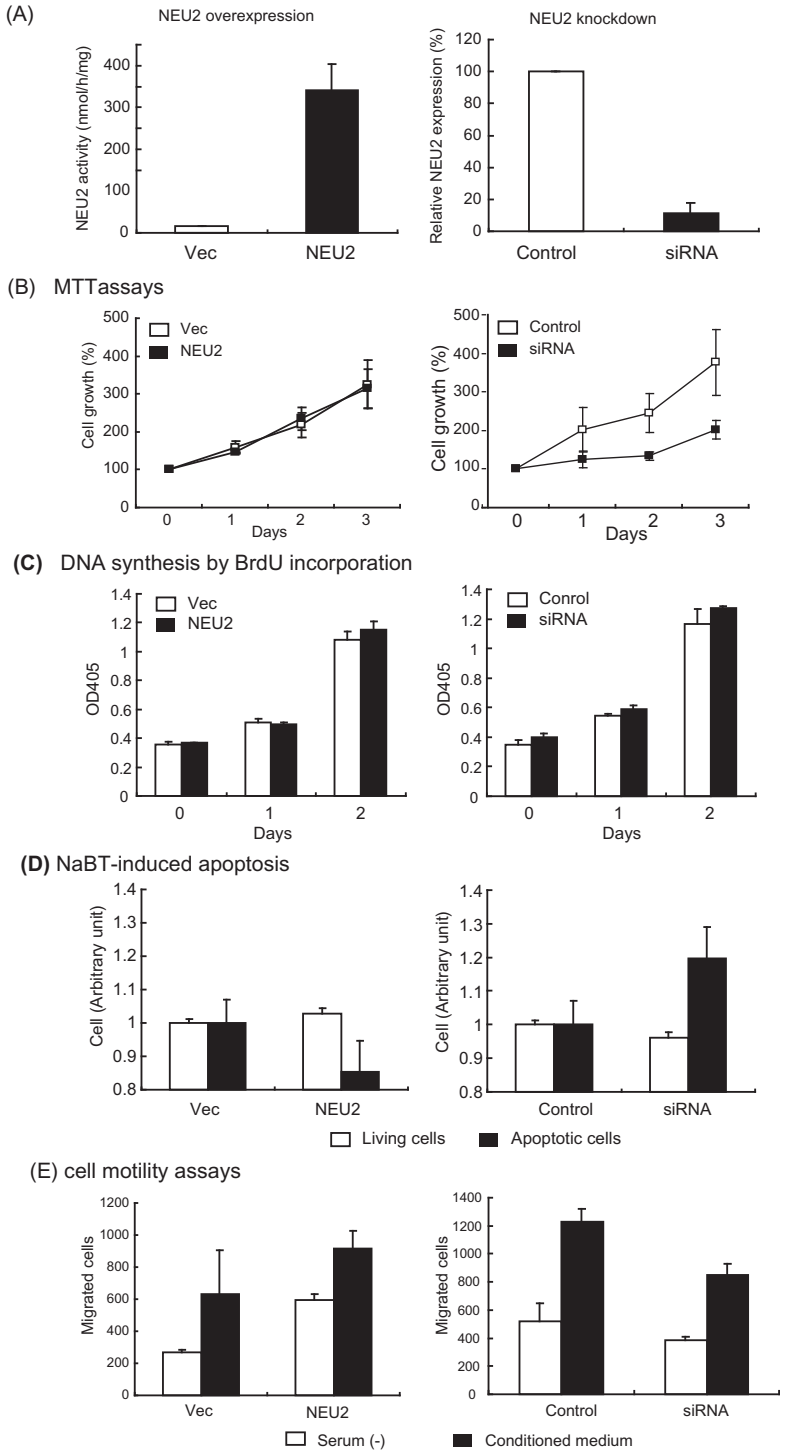


Fig. 3. Effects of *NEU2* expression on cell proliferation, apoptosis and cell motility of PC-3 cells. (A) Increase of sialidase activity in cells with *NEU2* overexpression (left) and decreased *NEU2* mRNA levels by *NEU2* silencing with the siRNA (right). Cell growth by MTT assay (B) and DNA synthesis by BrdU assay (C), NaBT-induced apoptosis (D) and cell motility (E) were determined, as described in Section 2 in NEU2-overexpressing (left) and in *NEU2*-silenced (right) PC-3 cells. The data given are mean values from three experiments ± SD.

apoptosis assays were performed. In the BrdU assays, there were no differences in incorporation of BrdU in PC-3 cells with either *NEU2* overexpression or silencing (Fig. 3C). On the other hand, as shown in Fig. 3D, *NEU2* overexpression suppressed whereas its silencing enhanced NaBT-induced apoptosis. Furthermore, cell motility was enhanced by overexpression, and reduced by silencing in the cells (Fig. 3E). These results suggest that NEU2 may regulate both apoptosis and cell motility in PC-3 cells.

3.4. Reduced NEU2 expression by silencing of the transcription factors, Runx2 and Sp3

To understand why and how *NEU2* expression might be exceptionally expressed in PC-3 cells, we studied possible involvement of some transcription factors. Prediction of putative transcription factor binding sites in the *NEU2* gene promoter region was performed using a web-based prediction program, TFSEARCH [23].

Five putative binding sites for Runx2, thought to be involved in osteodifferentiation, and four GC-box motifs, binding sites for Sp1/Sp3, were found up to 2000 bp 5′-upstream from the ATG start codon of the *NEU2* gene (Fig. 4A). PCR analyses revealed the expression level of *runx2* in PC-3 cells to be much higher than in DU145, whereas both Sp1 and Sp3 were expressed at similar levels in the two cell lines (Fig. 4B). It is interesting because PC-3 cells have higher metastatic potential. To assess whether Runx2, Sp1

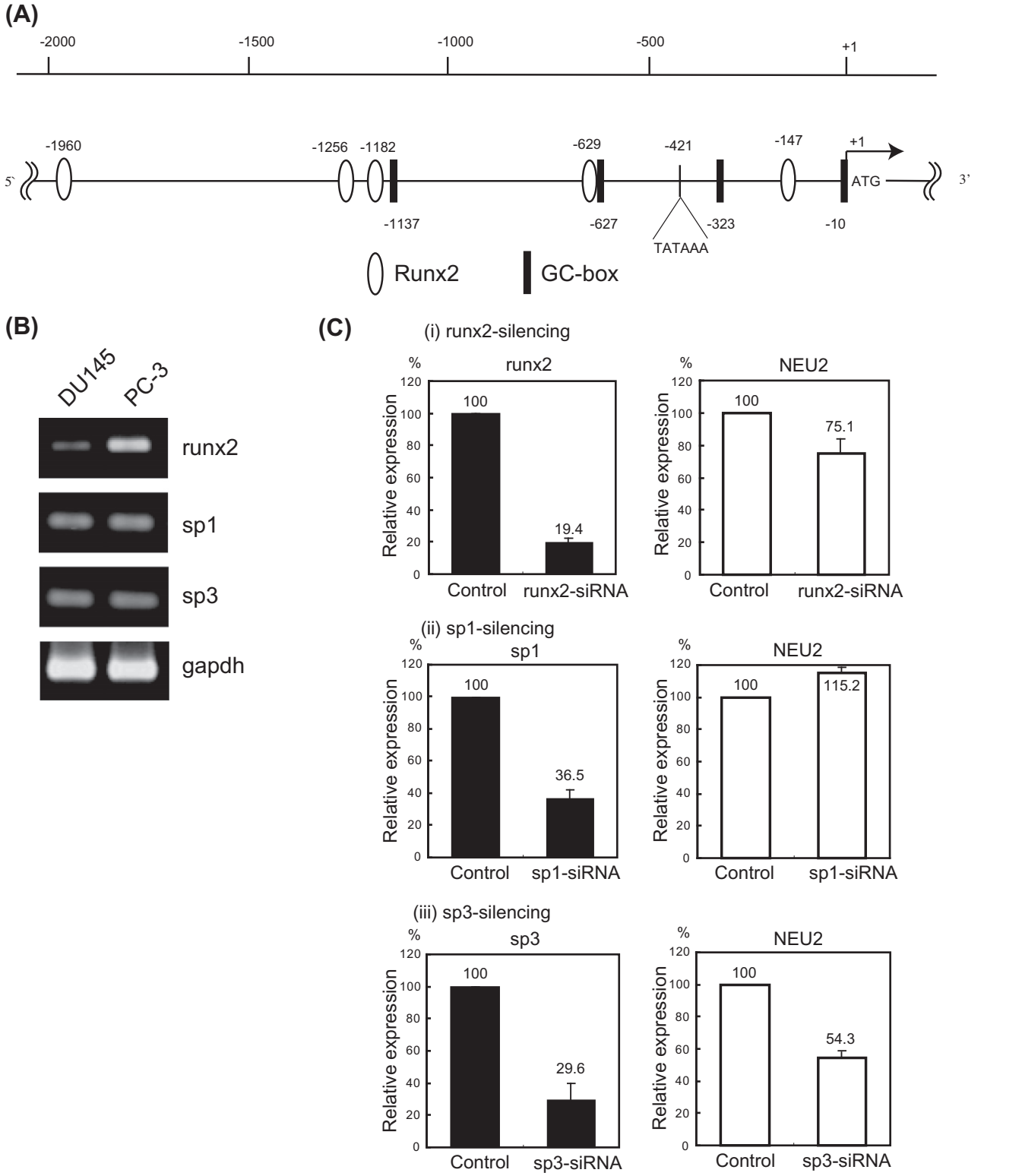


Fig. 4. Effects of transcription factors on *NEU2* expression in PC-3 cells. (A) A schematic representation of transcription factor binding sites, Runx2 and GC boxes. (B) *runx2*, *sp1*, and *sp3* expression in prostate cancer cell lines, DU145 and PC-3, as assessed by RT-PCR analyses. Results are representative of three independent experiments. (C) Down-regulation of *NEU2* by silencing of *runx2*, *sp1* and *sp3* with their respective siRNAs (Dharmacon). Quantitative data present as values relative to those with control siRNAs from three experiments ± SD.

and Sp3 are involved in *NEU2* expression, siRNAs for each were introduced into the cells. As shown in Fig. 4C, when *runx2* was silenced approximately 80% in PC-3 cells, decrease in *NEU2* level by 25% was observed as compared with a negative control. Seventy percent *Sp3*-knockdown led to 46% decrease of *NEU2* level, but *Sp1*-knockdown did not reduce and even caused a slight increase in *NEU2* expression. These results suggest that Runx2 and Sp3 are possibly involved in the regulation of *NEU2* expression in PC-3 cells.

4. Discussion

The present evaluation of endogenous *NEU2* mRNA levels in various human tissues and cells revealed expression to be extremely low compared to those of other sialidases by quantitative RT-PCR, with the exception of considerable expression in PC-3 cells. It could further be confirmed that the cDNAs actually encoded the enzyme, cytosolic fractions demonstrating sialidase activity at pH6.0 when the genes cloned from cDNAs synthesized with poly $(A)^+$ RNA of human colon and brain tissues were transfected in HK-293T cells. The clones showed additional activity toward gangliosides, fetuin as well as 4MU-NeuAc, even in the presence of SNPs in the brain cDNA, indicating actual encoding of the cytosolic sialidase. Although human sialidases, NEU1, NEU3, and NEU4 have been implicated in various biological events [1,2], and previous reports suggested murine NEU2 to be involved in muscle cell differentiation [4,9,10] and in neural differentiation [11], little is known about the physiological functions of the human NEU2. The relatively high NEU2 expression in placenta, testis and ovary (Fig. 1C) may imply a contribution to cell growth and genital differentiation and development, since gangliosides, substrates for NEU2, are present in human testis [24], placenta [25], and ovary [26]. In addition, unexpectedly, we found *NEU2* to be relatively highly expressed in PC-3 cells. We therefore took the opportunity to access its functional significance and obtained evidence that NEU2 may contribute to suppression of apoptosis and stimulation of cell motility (Fig. 3). In clear contrast to our results, Tringali et al. earlier demonstrated overexpression of *NEU2* in K562 cells to lead to induction of apoptosis [18]. We do not have a precise explanation for the inconsistency, but can suggest the difference was probably caused by variation in the status of *NEU2* expression in the cells; PC-3 cells feature the expression whereas it is lacking in K562 cells. Furthermore, human NEU2 has been suggested to participate in degradation of complex-type N-glycans in the cytosol of MKN7 and MKN45 stomach cancer cells, in which *NEU2* expression was undetectable and accumulation of free complex-type N-glycans occurs specifically but not in other several cancer cells [27]. To understand physiological functions of NEU2, including its general influence on apoptosis, further studies are still needed.

We previously demonstrated that abundant rat *Neu2* in skeletal muscle and many E-box (CANNTG) motifs responsible for muscle differentiation exist in the 5′-upstream promoter region of the gene, *Neu2* expression, in fact being increased during differentiation of myoblast L6 cells [4,9]. In contrast, human *NEU2* expression was hardly detectable in the skeletal muscle, despite a similar existence of many E-box motifs in the promoter region [7]. Thus the molecular mechanisms underlying the considerable *NEU2* expression in PC-3 cells are of great interest. Prediction of putative transcription factor binding sites and siRNA experiments revealed that Runx2 and Sp3, but not Sp1, might be involved in *NEU2* expression in PC-3 cells. Runx2 (Cbfa1/AML3) is known as a member of the runt family of transcription factors which act as key regulators of bone development [28,29]. Sp3, as well as Sp1, belong to the Sp transcription factor family recognizing consensus sequences referred to GC boxes present in the promoters of many mammalian genes [30,31]. $Sp3^{-/-}$ mice have been shown to feature impaired endochondral and intramembranous ossification, indicating involvement in bone development [32]. Here, PC-3 cells exhibited much higher *Runx2* expression than another prostate cancer cell line DU145, whereas *Sp1* and *Sp3* levels were similar (Fig. 4B). Furthermore, in this context it should be stressed that PC-3 cells were established from a prostate cancer patient with bone metastasis. Thus it is conceivable that *NEU2* expression in PC-3 cells may be linked to phenomena such as bone metastasis, or controlled by osteogenic factors expressed in the bone environment. The decreased expression level by silencing of *Runx2* or *Sp3* (Fig. 4C) certainly indicates that the NEU2 may be regulated by bone-related factors in PC-3 cells.

In conclusion, the present study demonstrated *NEU2* expression to be detectable but very low in many human tissues and cells, and suggested possible functional roles in PC-3 cells. The molecular basis of the biologically and phylogenically significant relative absence of expression of this sialidase in humans warrants elucidation.

Acknowledgments

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