

**Figure 2.** (A) Effects of interleukin-6 (IL-6) and tocilizumab on adiponectin production in human adipocytes. Cells were differentiated for 12 days, and then treated without (control) or with 10 µg/mL IL-6 antibody, and/or 100 µg/mL, or 10 mg/mL tocilizumab. Data are presented as means ± SEM of at least four independent experiments. \*P < 0.05; \*\*P < 0.01 vs. control. Pio: pioglitazone. (B) Effects of interleukin-6 (IL-6) and tocilizumab on fatty acid-binding protein 4 levels in human adipocytes. Cells were differentiated for 12 days and treated without (control) or with 10 µg/mL IL-6 antibody, and/or 100 µg/mL, 10 mg/ml or tocilizumab. Data are expressed as means ± SEM of at least four independent experiments. \*P < 0.05; \*\*P < 0.01 vs. control. Pio: pioglitazone. (C) A representative western blot of fatty acid-binding protein 4 expression in human adipocytes treated without (control) or with 10 µg/mL interleukin-6 antibody, and/or 100 µg/mL, or 10 mg/mL tocilizumab. Pio: pioglitazone.

study. Treatment with tocilizumab for 3 months significantly suppressed the inflammatory process, demonstrated by a decrease in mean serum CRP levels from  $1.8 \pm 0.4$  to  $0.2 \pm 0.1$  mg/dL ( $P < 0.01$ ). Levels of DAS-28 also improved significantly, from  $3.9 \pm 0.3$  to  $1.8 \pm 0.4$  ( $P < 0.01$ ). The baseline serum adiponectin concentrations were  $13.7 \pm 1.7$  µg/mL, which increased significantly to  $18.0 \pm 2.1$  µg/mL ( $P < 0.01$ , Fig. 1A) after treatment with tocilizumab for 3 months. In contrast, baseline FABP4 concentrations were  $31.3 \pm 9.0$  ng/mL, which decreased to  $27.4 \pm 9.4$  ng/mL after treatment ( $P = 0.05$ , Fig. 1B). Although previous reports suggested that treatment with steroids significantly decreased serum adiponectin levels [17],

oral prednisolone did not affect serum FABP4 or adiponectin levels in our study (data not shown).

Next, the effect of IL-6 and tocilizumab on adiponectin and FABP4 was assessed in human adipocytes. After treatment of differentiated adipocytes with 100 µg/mL tocilizumab, adiponectin secretion was increased approximately  $1.25 \pm 0.04$ -fold ( $P < 0.01$ ) compared with control. Tocilizumab treatment enhances adiponectin secretion by suppressing IL-6 (Fig. 2A). In contrast, FABP4 expression was decreased approximately  $0.85 \pm 0.03$ -fold ( $P < 0.01$ ) in cells treated with tocilizumab compared with control. However, pioglitazone substantially increased FABP4 expression approximately  $1.19 \pm 0.10$ -fold

(Fig. 2B, C).

## Discussion

In this study, we found for the first time that treatment with tocilizumab, an IL-6R Ab, for 3 months reduced serum FABP4 levels in patients with RA. Both adiponectin and FABP4 are downstream targets of PPAR- $\gamma$  in adipocytes. PPAR agonists such as telmisartan and thiazolidines induce the secretion of adiponectin [16] and simultaneously increase serum FABP4 levels [18, 19]. A previous study demonstrated olmesartan, which has little PPAR- $\gamma$  activity, decreased serum FABP4 in patients with hypertension [20], while little is known about the effect of medication on FABP4 levels.

FABP4 promotes atherosclerotic diseases by acting on macrophages [21]. FABP4-deficient macrophages display defects in cholesterol accumulation and decreased pro-inflammatory cytokines TNF $\alpha$ , IL-6, and MCP-1 levels by reducing I $\kappa$ B kinase and NF- $\kappa$ B activity [22]. Although FABP4 is a circulating protein, the mechanism by which it enters the circulation is unknown. In both cross-sectional and prospective studies, serum FABP4 levels were positively correlated with lipid profiles, hyperglycemia, and non-alcoholic fatty liver diseases [23]. In addition, a 12-year community-based cohort study in a Chinese population indicated that plasma FABP4 levels were a strong predictor of CVD [24]. Because elevated FABP4 levels are a risk factor for CVD in patients with end-stage renal disease [8] and are correlated with numerous metabolic syndrome symptoms [9], the up-regulation of FABP4 may induce unfavorable side effects in patients treated with PPAR agonists.

Tocilizumab, a monoclonal antibody that blocks both membrane-bound and circulating IL-6R has anti-inflammatory actions that extend beyond reducing the concentrations of C-reactive protein and fibrinogen [25]. Patients with chronic inflammatory diseases, such as RA, are at increased risk of developing CVD [3], which is in part caused by increased IL-6 levels. Although further studies are required to confirm that these effects are also mediated by factors including macrophages and IL-6 *in vivo*, the present results suggest that changes in adiponectin and FABP4 levels reflect metabolic defects in adipose tissue and thus may be a useful biomarker of CVD in patients with RA.

Tocilizumab was identified as an agent that may help prevent coronary heart disease [26]. Because the suppression of adiponectin gene expression by IL-6 is mediated in part by p44/42 MAP kinase [27], the inhibition of this signaling pathway by tocilizumab may induce adiponectin secretion. The concurrent decrease in FABP4 levels and increase in adiponectin levels induced by tocilizumab may help prevent CVD and metabolic syndrome. However, additional studies are required to define the mechanism behind the differential effects of tocilizumab on FABP4 and adiponectin expression. For example, tocilizumab may act as a selective PPAR gamma modulator (SPPARM) [28] to reduce oxidative stress [29] or improve hypoxia [30]. However, SPPARM exhibited limited effects on FABP4 gene expression in mature 3T3-L1 adipocytes [28] and inhibited the differentiation of human preadipocytes compared

with PPAR- $\gamma$  agonists. SPPARM also displayed a diminished ability to induce FABP4 mRNA expression compared with rosiglitazone [29], whereas in human trophoblasts cultured under hypoxic conditions, the expression of FABP4 was enhanced [30].

In conclusion, tocilizumab treatment decreases FABP4 levels in patients with RA and could provide a novel therapeutic approach to prevent CVD.

## Acknowledgement

We sincerely appreciate the cooperation of Miki Sakaue and Megumi Matsumoto from the Department of Complementary and Alternative Medicine, Osaka University Graduate School of Medicine, Osaka, Japan for accomplishing this research successfully.

## References

1. Dessein PH, Norton GR, Woodiwiss AJ, Joffe BI, Wolfe F. Influence of nonclassical cardiovascular risk factors on the accuracy of predicting subclinical atherosclerosis in rheumatoid arthritis. *J Rheumatol.* 2007;34(5):943-951.
2. Solomon DH, Kremer J, Curtis JR, Hochberg MC, Reed G, Tsao P, Farkouh ME, et al. Explaining the cardiovascular risk associated with rheumatoid arthritis: traditional risk factors versus markers of rheumatoid arthritis severity. *Ann Rheum Dis.* 2010;69(11):1920-1925.
3. Kaplan MJ. Management of cardiovascular disease risk in chronic inflammatory disorders. *Nat Rev Rheumatol.* 2009;5(4):208-217.
4. Neumann E, Frommer KW, Vasile M, Muller-Ladner U. Adipocytokines as driving forces in rheumatoid arthritis and related inflammatory diseases? *Arthritis Rheum.* 2011;63(5):1159-1169.
5. Makowski L, Hotamisligil GS. Fatty acid binding proteins--the evolutionary crossroads of inflammatory and metabolic responses. *J Nutr.* 2004;134(9):2464S-2468S.
6. Iso T, Maeda K, Hanaoka H, Suga T, Goto K, Syamsunarno MR, Hishiki T, et al. Capillary endothelial fatty acid binding proteins 4 and 5 play a critical role in fatty acid uptake in heart and skeletal muscle. *Arterioscler Thromb Vasc Biol.* 2013;33(11):2549-2557.
7. Maeda K. Role of adiponectin and adipocyte fatty acid binding protein in the metabolic syndrome. *Diabetes Res Clin Pract.* 2007;77(Suppl 1):S17-22.
8. Furuhashi M, Ishimura S, Ota H, Hayashi M, Nishitani T, Tanaka M, Yoshida H, et al. Serum fatty acid-binding protein 4 is a predictor of cardiovascular events in end-stage renal disease. *PLoS One.* 2011;6(11):e27356.
9. Hsu BG, Chen YC, Lee RP, Lee CC, Lee CJ, Wang JH. Fasting serum level of fatty-acid-binding protein 4 positively correlates with metabolic syndrome in patients with coronary artery disease. *Circ J.* 2010;74(2):327-331.
10. Matsuzawa Y, Funahashi T, Kihara S, Shimomura I. Adiponectin and metabolic syndrome. *Arterioscler Thromb*

- Vasc Biol. 2004;24(1):29-33.
11. Pischon T, Girman CJ, Hotamisligil GS, Rifai N, Hu FB, Rimm EB. Plasma adiponectin levels and risk of myocardial infarction in men. *JAMA*. 2004;291(14):1730-1737.
  12. Schett G. Rheumatoid arthritis and multiple sclerosis: direful siblings, different strategies. *FEBS Lett*. 2011;585(23):3601-3602.
  13. Schultz O, Oberhauser F, Saech J, Rubbert-Roth A, Hahn M, Krone W, Laudes M. Effects of inhibition of interleukin-6 signalling on insulin sensitivity and lipoprotein (a) levels in human subjects with rheumatoid diseases. *PLoS One*. 2010;5(12):e14328.
  14. Lagathu C, Bastard JP, Auclair M, Maachi M, Capeau J, Caron M. Chronic interleukin-6 (IL-6) treatment increased IL-6 secretion and induced insulin resistance in adipocyte: prevention by rosiglitazone. *Biochem Biophys Res Commun*. 2003;311(2):372-379.
  15. Gustafson B, Jack MM, Cushman SW, Smith U. Adiponectin gene activation by thiazolidinediones requires PPAR gamma 2, but not C/EBP alpha-evidence for differential regulation of the aP2 and adiponectin genes. *Biochem Biophys Res Commun*. 2003;308(4):933-939.
  16. Inomata-Kurashiki Y, Maeda K, Yoshioka E, Fukuhara A, Imagawa A, Otsuki M, Shimomura I. Measurement of adiponectin production from differentiated metabolic stem cells. *Horm Metab Res*. 2010;42(5):318-323.
  17. Harle P, Sarzi-Puttini P, Cutolo M, Straub RH. No change of serum levels of leptin and adiponectin during anti-tumour necrosis factor antibody treatment with adalimumab in patients with rheumatoid arthritis. *Ann Rheum Dis*. 2006;65(7):970-971.
  18. Fujimoto M, Masuzaki H, Tanaka T, Yasue S, Tomita T, Okazawa K, Fujikura J, et al. An angiotensin II AT1 receptor antagonist, telmisartan augments glucose uptake and GLUT4 protein expression in 3T3-L1 adipocytes. *FEBS Lett*. 2004;576(3):492-497.
  19. Kim H, Haluzik M, Gavrilova O, Yakar S, Portas J, Sun H, Pajvani UB, et al. Thiazolidinediones improve insulin sensitivity in adipose tissue and reduce the hyperlipidaemia without affecting the hyperglycaemia in a transgenic model of type 2 diabetes. *Diabetologia*. 2004;47(12):2215-2225.
  20. Miyoshi T, Doi M, Hirohata S, Kamikawa S, Usui S, Ogawa H, Sakane K, et al. Olmesartan reduces arterial stiffness and serum adipocyte fatty acid-binding protein in hypertensive patients. *Heart Vessels*. 2011;26(4):408-413.
  21. Boord JB, Maeda K, Makowski L, Babaev VR, Fazio S, Linton MF, Hotamisligil GS. Combined adipocyte-macrophage fatty acid-binding protein deficiency improves metabolism, atherosclerosis, and survival in apolipoprotein E-deficient mice. *Circulation*. 2004;110(11):1492-1498.
  22. Makowski L, Brittingham KC, Reynolds JM, Suttles J, Hotamisligil GS. The fatty acid-binding protein, aP2, coordinates macrophage cholesterol trafficking and inflammatory activity. Macrophage expression of aP2 impacts peroxisome proliferator-activated receptor gamma and IkappaB kinase activities. *J Biol Chem*. 2005;280(13):12888-12895.
  23. Koh JH, Shin YG, Nam SM, Lee MY, Chung CH, Shin JY. Serum adipocyte fatty acid-binding protein levels are associated with nonalcoholic fatty liver disease in type 2 diabetic patients. *Diabetes Care*. 2009;32(1):147-152.
  24. Chow WS, Tso AW, Xu A, Yuen MM, Fong CH, Lam TH, Lo SV, et al. Elevated circulating adipocyte-fatty acid binding protein levels predict incident cardiovascular events in a community-based cohort: a 12-year prospective study. *J Am Heart Assoc*. 2013;2(1):e004176.
  25. Genovese MC, McKay JD, Nasonov EL, Mysler EF, da Silva NA, Alecock E, Woodworth T, et al. Interleukin-6 receptor inhibition with tocilizumab reduces disease activity in rheumatoid arthritis with inadequate response to disease-modifying antirheumatic drugs: the tocilizumab in combination with traditional disease-modifying antirheumatic drug therapy study. *Arthritis Rheum*. 2008;58(10):2968-2980.
  26. Hingorani AD, Casas JP. The interleukin-6 receptor as a target for prevention of coronary heart disease: a mendelian randomisation analysis. *Lancet*. 2012;379(9822):1214-1224.
  27. Fasshauer M, Kralisch S, Klier M, Lossner U, Bluher M, Klein J, Paschke R. Adiponectin gene expression and secretion is inhibited by interleukin-6 in 3T3-L1 adipocytes. *Biochem Biophys Res Commun*. 2003;301(4):1045-1050.
  28. Balint BL, Nagy L. Selective modulators of PPAR activity as new therapeutic tools in metabolic diseases. *Endocr Metab Immune Disord Drug Targets*. 2006;6(1):33-43.
  29. Einstein M, Akiyama TE, Castriota GA, Wang CF, McKeever B, Mosley RT, Becker JW, et al. The differential interactions of peroxisome proliferator-activated receptor gamma ligands with Tyr473 is a physical basis for their unique biological activities. *Mol Pharmacol*. 2008;73(1):62-74.
  30. Biron-Shental T, Schaiff WT, Ratajczak CK, Bildirici I, Nelson DM, Sadovsky Y. Hypoxia regulates the expression of fatty acid-binding proteins in primary term human trophoblasts. *Am J Obstet Gynecol*. 2007;197(5):516 e511-516.



## Generation and characterization of a bispecific diabody targeting both EPH receptor A10 and CD3



Haruhiko Kamada<sup>a,b,1</sup>, Shintaro Taki<sup>a,c,1</sup>, Kazuya Nagano<sup>a</sup>, Masaki Inoue<sup>a</sup>, Daisuke Ando<sup>a,c</sup>, Yohei Mukai<sup>a,d</sup>, Kazuma Higashisaka<sup>a,e</sup>, Yasuo Yoshioka<sup>a,b,e</sup>, Yasuo Tsutsumi<sup>a,b,d,e</sup>, Shin-ichi Tsunoda<sup>a,b,c,d,\*</sup>

<sup>a</sup> Laboratory of Biopharmaceutical Research, National Institute of Biomedical Innovation, 7-6-8 Saito-Asagi, Ibaraki, Osaka 567-0085, Japan

<sup>b</sup> The Center for Advanced Medical Engineering and Informatics, Osaka University, 1-6 Yamadaoka, Suita, Osaka 565-0871, Japan

<sup>c</sup> Laboratory of Biomedical Innovation, Graduate School of Pharmaceutical Sciences, Osaka University, 1-6 Yamadaoka, Suita, Osaka 565-0871, Japan

<sup>d</sup> Laboratory of Innovative Antibody Engineering and Design (iAED), Center for Drug Innovation and Screening, National Institute of Biomedical Innovation, 7-6-8 Saito-Asagi, Ibaraki, Osaka 567-0085, Japan

<sup>e</sup> Laboratory of Toxicology and Safety Science, Graduate School of Pharmaceutical Sciences, Osaka University, 1-6 Yamadaoka, Suita, Osaka 565-0871, Japan

### ARTICLE INFO

#### Article history:

Received 26 November 2014

Available online 17 December 2014

#### Keywords:

Bispecific antibody

Diabody

EPHA10

CD3

Redirected T cell response

### ABSTRACT

The EPH receptor A10 (EphA10) is up-regulated in breast cancer but is not normally expressed in healthy tissue, thus it has been suggested that EphA10 may be a useful target for cancer therapy. This study reports a diabody, an antibody derivative binding two different target molecules, EphA10 expressed in tumor cells and CD3 expressed in T cells, which showed T cell dependent-cytotoxicity. The diabody, which has His-tagged and FLAG-tagged chains, was expressed in *Escherichia coli* and purified in both heterodimer (Db-1) and homodimer (Db-2) formulations by liquid chromatography. Flow cytometry analysis using EphA10-expressing cells showed that binding activity of heterodimers was stronger than that of homodimers. Addition of diabodies to PBMC cultures resulted in T-cell mediated redirected lysis, and the bioactivity was consistent with the stronger binding activity of heterodimeric diabody formulations. Our results indicate that diabodies recognizing both EphA10 and CD3 could have a range of potential applications in cancer therapy, such as breast cancers that express the EPH receptor A10, especially triple negative breast cancer.

© 2014 Elsevier Inc. All rights reserved.

### 1. Introduction

The EPH receptor A10 (EphA10) [1], known as an ephrin receptor family protein, is known to be involved in cancer progression. The roles of EphA10 in cancer have not yet been fully elucidated [2], although it has been shown to be a contributing factor in tumor progression and invasion and has been associated with enhanced tumorigenic properties and reduced survival times in breast carcinoma. Its expression in normal human tissue seems to be confined to the testis [1] and it is up-regulated in several cancers including prostate cancer [3], ovarian cancer and breast cancer [4,5]. EphA10 transcripts are absent in normal prostate and breast cells but are present in cancer cells of prostate and breast, respectively. Interestingly, high levels of EphA10 are found in the context of triple

negative breast cancers (TNBCs) [5]. Targeting EphA10 by blocking EphA10-dependent activation of the MAPK pathway has resulted in tumor growth inhibition *in vivo*. Therefore, EphA10 has emerged as a promising target for antibody therapies, while the exact functions and mechanism of action of EphA10 in normal physiology or in pathological conditions remain to be determined.

Creating bispecific antibodies (BsAbs), which are capable of simultaneous binding to two different targets, could overcome many defects of monoclonal antibody therapies [6]. Such molecules would be able to retarget not only a large variety of cancer cells but other cell types as well, such as lymphocytes [7–9]. The potential of this approach has been demonstrated by several studies and large amounts of heterogeneous BsAbs have been produced using techniques of molecular biology. In particular, a diabody, which is a kind of BsAb, is constructed from non-covalently associated bivalent molecules, created from scFvs by shortening the polypeptide linker between the VH and VL domains [10–13]. These antibody derivatives may be used as therapeutic drugs to treat cancer and blood coagulation diseases.

\* Corresponding author at: Laboratory of Biopharmaceutical Research, National Institute of Biomedical Innovation, 7-6-8 Saito-Asagi, Ibaraki, Osaka 567-0085, Japan. Fax: +81 72 641 9817.

E-mail address: [tsunoda@nibbio.go.jp](mailto:tsunoda@nibbio.go.jp) (S.-i. Tsunoda).

<sup>1</sup> These authors contributed equally to the work.

Antibodies that react specifically with EphA10 could have diagnostic and therapeutic utility, particularly if they show functional blocking activity. Towards this end, we previously created murine IgG reactive with EphA10 [5]. This anti-EphA10 antibody, in full IgG format, showed anti-tumor activity against breast cancer model mice, however, the effect of BsAb against EphA10-expressing cells was not clear. Here we describe the development of an anti-EphA10 and CD3 BsAb in diabody format. The bivalent nature of diabodies is advantageous for targeting and they provide a flexible platform for development of targeted therapeutics. The anti-EphA10 and CD3 diabody showed cytotoxicity *in vitro* against EphA10-expressing cells.

## 2. Materials and methods

### 2.1. Cell lines and culture

Hybridoma 38.1 (mouse Hybridoma HB-231) and MDA-MB-435 (human breast cancer cell line HTB-129) cells were obtained from American Type Culture Collection (ATCC, Rockville, MD) and cultured under the recommended conditions. Human cells that overexpressed EphA10, MDA-MB-435 (MDA-MB-435<sup>EphA10</sup>), were established in our laboratory. In brief, a lentiviral vector encoding human EphA10 was transfected into MDA-MB-435 cells and stably transfected cells were obtained by Blasticidin (Invitrogen) selection. A hybridoma producing anti-EphA10 IgG was established from splenocytes of a human EphA10-immunized mouse by fusion with a mouse myeloma line.

### 2.2. Cloning of variable (V) immunoglobulin domains

The genes of V light-chain (VL) and V heavy-chain (VH) domains from each hybridoma were subcloned using 5'-Full RACE kits (Takara Bio, Kyoto, Japan). The amplified DNA was directionally subcloned into a plasmid vector using the TOPO TA cloning kit (Invitrogen) and sequenced using a 3130xl Genetic Analyzer (Applied Biosystems, Carlsbad, CA).

### 2.3. Vector construction

The vectors to express the bispecific antibody or single chain Fv (scFv), respectively, were constructed as described previously [14]. The primer sequences are shown in Table 1. To construct the co-expression vector, two additional restriction sites (*Sac*II, *Spe*I) were inserted into the pET20b vector (Invitrogen) and the new vector was named pET20b (SS+). The *E. coli* TOP10 strain (Invitrogen) was used to subclone target genes. To obtain a scFv A (EphA10-VL-Linker-CD3-VH) and a scFv B (CD3-VL-Linker-EphA10-VH), the corresponding VL and VH regions were cloned into separate

vectors as templates for VL- and VH-specific PCR using the primer pairs 5' *Nco*I-VL (hEphA10 or hCD3)/3' VL (hEphA10 or hCD3)-Linker and 5' Linker-VH (hEphA10 and hCD3)/3' VH (hCD3)-*Not*I (scFv A) or 3' VH (hEphA10)-FLAG tag (DYKDDDDKA) *Xho*I (scFv B), respectively. Overlapping complementary sequences were introduced into the PCR products, which combined to form the coding sequence of the 5-amino acid (G<sub>4</sub>S) Linker during the subsequent fusion PCR. This amplification step was performed with the primer pair 5' *Nco*I-VL (hEphA10 or hCD3)/3' VH (hCD3)-*Not*I (scFv A) or 3' VH (hEphA10)-FLAG tag *Xho*I (scFv B), and the resulting fusion product was cleaved with the restriction enzymes *Nco*I and *Not*I (scFv A) or *Xho*I (scFv B), then cloned into the pET20b (SS+) vector (scFv A) and pET20b vector (scFv B). Next, to construct the bispecific antibody (diabody) expression vector, the previously described scFv B vector was used as a template for scFv-specific PCR with the primer pair 5' *Sac*II-peIB/3' FLAG-tag-stop-*Spe*I. The PCR product was cleaved with the restriction enzymes *Sac*II and *Spe*I, then cloned into the pET20b (SS+) scFv A vector (pET20b (SS+) diabody).

### 2.4. Expression and purification of the diabody

In order to express the bispecific diabody, plasmid pET20b (SS+) diabody was transformed into *E. coli* BL21 (DE3) Star (Invitrogen). *Escherichia coli* cells containing the recombinant plasmids were inoculated into 3 ml of 2xYT medium containing 1 mg/ml ampicillin. Overnight cultures were transferred to 300 ml of fresh medium and were grown at 37 °C until they reached an A<sub>600</sub> = 0.8. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.5 mM and the cultures were further grown overnight at 20 °C. *E. coli* cells were collected by centrifugation (8000g for 20 min at 4 °C) and re-suspended in Osmotic Shock buffer (20 mM Tris-HCl, pH 8.0, 0.5 M sucrose, and EDTA added to 0.1 mM final). After 1 h incubation at 4 °C, the cells were shocked by adding ice water and then centrifuged (8000g for 30 min at 4 °C). The diabody-containing supernatant was brought to 60% ammonium sulfate and stirred overnight. The diabody was precipitated by centrifugation (8000g for 30 min at 4 °C). The protein pellet was resuspended in phosphate-buffered saline (PBS) buffer and dialysed exhaustively against PBS at 4 °C.

After dialysis, the diabody was purified by immobilized metal affinity chromatography (IMAC). The diabody was eluted using 150 mM imidazole/PBS (Db-1 Elution) and 300 mM imidazole/PBS (Db-2 Elution) buffers. Each fraction was subjected to gel filtration chromatography with a Superdex200 prep grade column (GE Healthcare, Little Chalfont Bucks, UK) equilibrated in PBS. SDS-PAGE and Western blot analysis with an anti-His or anti-FLAG tag antibody were performed to detect and confirm the size and purity of the diabody-containing fractions. Purified proteins were concentrated in PBS by ultrafiltration with a Centriprep® 30 K or 50 K

**Table 1**  
Oligonucleotide sequences of PCR primers used for construction of diabody (EphA10/CD3) vector.

Primer	Nucleotide sequence (5'-3') <sup>a</sup>
5' <i>Nco</i> I-VL (hEphA10)	NNNCCATGGCCAGTTTTGTGATGACCCAGACTCCC
3' VL (hEphA10)-Linker	CTGGCTACCACCACCAGCCCGTTTGATTCCAGCTTGGT
5' Linker-VH (hEphA10)	GAAAGGTGGTGGTGGTAGCCAGGTTCTGCTGCAGCAGCT
3' VH (hEphA10)-FLAG- <i>Xho</i> I	NNNCTCGAGTCATCAGGCCCTTGTATCGTATCCTTGTAGTCTGAGGAGACGGTGACTGAGGTT
5' <i>Nco</i> I-VL (hCD3)	NNNCCATGGCCCAAATTGTTCTCACCCAGTCTCCAG
3' VL (hCD3)-Linker	CTGGCTACCACCACCACCTTTCAGTCCAGCTTGGTCCC
5' Linker-VH (hCD3))	GCTGGTGGTGGTGGTAGCCAGGTCAGCTGCAGCAGT
3' VH (hCD3)- <i>Not</i> I	NNNCGCGCCGCTGAGGAGACGGTGACTGAGGTT
5' <i>Sac</i> II-peIB	NNNCCGCGGATGAAATACCTGCTGCCGACCC
3' FLAG-tag-stop- <i>Spe</i> I	NNNACTAGTTTCATCAGGCCCTTGTATCGTATC

<sup>a</sup> The restriction enzyme site is underlined.

device (Millipore, Billerica, MA, USA), and protein concentrations were estimated using a Coomassie Plus Protein Assay kit (Thermo Fisher Scientific, Rockford, IL).

### 2.5. Flow cytometric analysis

MDA-MB-435 or MDA-MB-435<sup>EphA10</sup> ( $5 \times 10^5$  cells) were suspended in Suspension buffer (2% FBS containing PBS) and incubated with 20  $\mu$ g diabody or 2  $\mu$ g control IgG (anti-EphA10, anti-CD3) for 1 h on ice, respectively. After washing with Suspension buffer, the cells were incubated with Surelight P3 (614 nm excitation and 662 nm emission) labeled antibodies against the His tag (Columbia Biosciences, Frederick, MD) and Surelight P3 labeled antibodies against the mouse IgG (Columbia Biosciences) for 1 h on ice. The cells were washed again and resuspended in 500  $\mu$ l Suspension buffer and flow cytometric analysis was performed (FACScanto; BD Biosciences, San Jose, CA). All tests were carried out in triplicate.

### 2.6. Cytotoxicity assays

Cytotoxicity assays were performed as described previously with slight modifications [14]. In brief, MDA-MB-435<sup>EphA10</sup> cells and MDA-MB-435 parent cells as target cells ( $10^3$  cells/well) were added to 96-well plates with 10% FBS containing D-MEM at 37 °C in a humidified atmosphere containing 5% CO<sub>2</sub>. After overnight

culture, supernatants were removed and non-stimulated human PBMC from healthy donors as effector cells were added to an effector-to-target (E/T) ratio of 10 with each of the antibodies (0.1–10  $\mu$ g/mL), respectively. After 48 h of incubation, lactate dehydrogenase (LDH) released into the supernatant was measured using a CytoTox 96<sup>®</sup> non-radioactive cytotoxicity assay (Promega, Madison, WI). Percentages of specific lysis were calculated according to the formula: % cytotoxicity = [(experimental release) – (effector spontaneous release) – (target spontaneous release)] / [(target maximum release) – (target spontaneous release)]  $\times$  100. All tests were carried out in triplicate.

### 2.7. Statistical analysis

Differences in cytotoxicity assay results between the control and target groups were compared using the unpaired Student's *t*-test.

## 3. Result and discussion

### 3.1. Formulations of diabody binding to EphA10 and CD3

A BsAb was constructed using two different scFv fragments (scFv A and scFv B) derived from the anti-EphA10 IgG and anti-CD3 IgG. His-tagged and FLAG-tagged VL-VH chain (EphA10-VL-Linker-CD3-VH; scFv A and CD3-VL-Linker- EphA10-VH; scFv

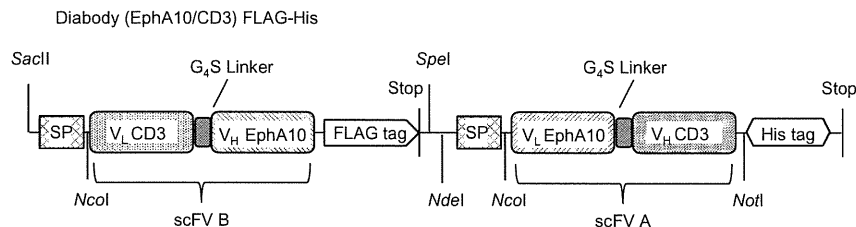


Fig. 1. Construction of the diabody-expressing vector.

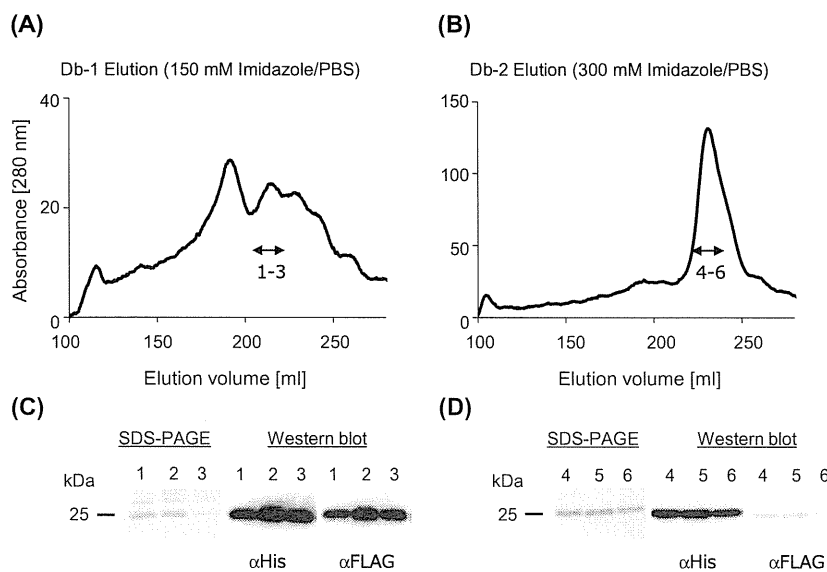
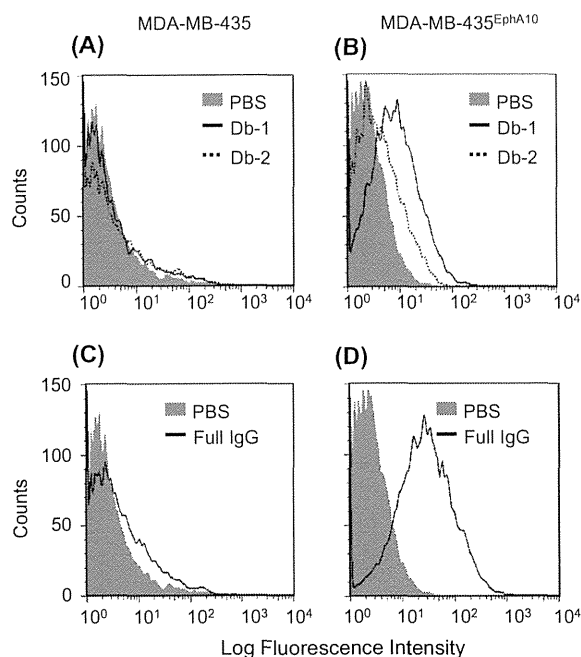


Fig. 2. Characteristics of diabody (Db-1 and Db-2). Gel filtration chromatography profile of diabodies, which were purified by IMAC. (A) 150 mM imidazole elution pattern (Db-1 as heterodimer) and (B) 300 mM imidazole elution pattern (Db-2 as homodimer). SDS-PAGE and Western blot analysis of dimeric Db-1 (C) and Db-2 (D). The line indicates the apparent molecular weight (25 kDa).



**Fig. 3.** Binding activity of diabody against EphA10-transfected cells and the parent cells (MDA-MB-435). The left panels (A, C) show the binding ability of the diabolies (A) and of the full IgG (C) against parental cells (MDA-MB-435) and the right panels (B, D) are against EphA10-transfected cells. Binding activities were measured using 20  $\mu$ g of each diabody sample. Cell-binding proteins were detected using SureLight P3 conjugated anti-His tag or anti-mouse IgG mAb. Filled bars are vehicle control (PBS).

B, respectively) were constructed (Fig. 1). The plasmid vector construct was designed by adding an N-terminal signal peptide to express BsAb in a soluble form and adding a C-terminal hexahistidine (His  $\times$  6) tag or FLAG tag to allow purification by affinity chromatography on a Ni-Sepharose column. This plasmid vector was transfected into BL21 (DE3) Star *E. coli* cells. Pooled supernatants were purified by IMAC using two elution buffers, and fractions containing the diabody further purified by gel-filtration chromatography (Fig. 2A and B). SDS-PAGE under reducing conditions followed by Western blot analysis showed only a single band indicating a  $\sim$ 25 kDa protein (Fig. 2C and D), consistent with the calculated

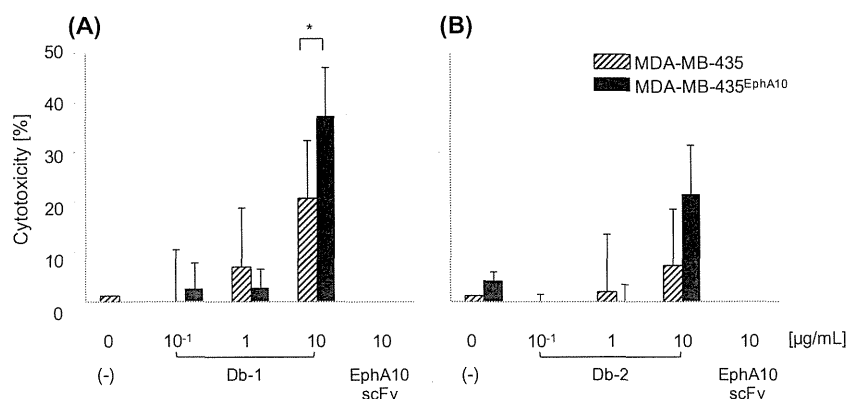
molecular mass of approximately 25 kDa for each scFv. Because these two scFv chains are structured as homodimers, they would be expected to show only low binding activity compared with the heterodimeric form that can fully recognize the target molecules. Therefore, the diabody formulation was checked by western blot against both an anti-His and an anti-FLAG antibody (Fig. 2C and D). These results showed that the diabody existed as heterodimer in the condition of 150 mM imidazole elution, because the amounts of His- and FLAG-tagged scFvs were similar (Fig. 2C). However, the fraction eluted by 300 mM imidazole was primarily composed of homodimers, because the anti-His-tag staining was much stronger than the anti-FLAG tag staining (Fig. 2D). Because His-tagged homodimer antibodies would get trapped strongly by a Ni-Sepharose column, two concentrations of imidazole were used to elute the scFvs (Db-1: 150 mM imidazole/PBS, Db-2: 300 mM imidazole/PBS). The heterodimers indeed were eluted at a lower imidazole concentration than the homodimers.

### 3.2. Binding activity of diabody for human EphA10

Binding activities of these diabolies (Db-1 and Db-2) were examined by flow cytometric analysis using the MDA-MB-435 parental cells, MDA-MB-435<sup>EphA10</sup> cells. Specific binding of EphA10 antigens to both Db-1 and Db-2 was observed (Fig. 3). Interestingly, the binding activity of diabody Db-1 was stronger than that of Db-2. These results indicated that the binding activity of the homodimer was reduced because this formulation would have mismatches between each VL and VH. Furthermore, the structural difference between homodimers and heterodimers had a significant effect on the binding activity.

### 3.3. Redirected target cell lysis of diabody with PBMC

The efficacy of T-cell mediated redirected lysis of MDA-MB-435<sup>EphA10</sup> cells and the parental cells following addition of each diabody was examined using an LDH cytotoxicity assay. Non-stimulated PBMC were used as effector cells at E/T ratios of 10, respectively. As shown in Fig. 4, the Db-1 and Db-2 diabolies showed dose-dependent cytotoxic activity against MDA-MB-435<sup>EphA10</sup> cells compared with the scFv constructs (anti-EphA10 scFv). Furthermore, the cytotoxic efficacy of Db-1 was higher than that of Db-2 at low antibody concentrations, indicating that the heterodimer would increase the cytotoxicity related to binding of the antigen.



**Fig. 4.** *In vitro* cytotoxicity of diabody formulations (Db-1 and Db-2) against MDA-MB-435<sup>EphA10</sup> and parental cells. The left panels are heterodimeric diabody, Db-1 (A) and the right panels are homodimeric diabody, Db-2 (B). MDA-MB-435 parental cells (slashed column) and MDA-MB-435<sup>EphA10</sup> (black column) cells were co-cultured respectively with human PBMC at E/T ratios of 10. Each point represents the mean of triplicate determinations; error bars represent the standard deviations of triplicate determinations. Asterisks label readings that were statistically significant (unpaired Student's *t*-test) from MDA-MB-435 and MDA-MB-435<sup>EphA10</sup> (\**p*  $\leq$  0.05).

The results of this study demonstrate that heterodimeric diabodies can show potent binding activity and specificity against cells that express the target antigen. Purified heterodimeric diabody formulations would lead to higher activity because of their increased affinity against two antigens, compared to homodimers or mixtures of homodimers plus heterodimers. Therefore, it is necessary to optimize purification protocols using HPLC etc. However, diabody formulations consisting of two chains of VL and VH could in principle form several types of mixed species. Thus, the protocols for bispecific antibodies should be optimized to produce a formulation containing a single species, e.g. by using linkers to produce a single chain diabody or tandem scFV. This should improve and standardize the desired binding functions of the BsAbs. The construction of such modified antibodies, e.g. scDb and faFv, shows great potential for the development of novel therapeutic drugs.

### Acknowledgment

This work was supported by JSPS KAKENHI Grant Number 24680093.

### References

- [1] H.C. Aasheim, S. Patzke, H.S. Hjørthaug, E.P. Finne, Characterization of a novel Eph receptor tyrosine kinase, EphA10, expressed in testis, *Biochim. Biophys. Acta* 1723 (2005) 1–7.
- [2] L. Truitt, A. Freywald, Dancing with the dead: Eph receptors and their kinase-null partners, *Biochem. Cell Biol.* 89 (2011) 115–129.
- [3] K. Nagano, T. Yamashita, M. Inoue, K. Higashisaka, Y. Yoshioka, Y. Abe, Y. Mukai, H. Kamada, Y. Tsutsumi, S. Tsunoda, Eph receptor A10 has a potential as a target for a prostate cancer therapy, *Biochem. Biophys. Res. Commun.* 450 (2014) 545–549.
- [4] K. Nagano, S. Kanasaki, T. Yamashita, Y. Maeda, M. Inoue, K. Higashisaka, Y. Yoshioka, Y. Abe, Y. Mukai, H. Kamada, Y. Tsutsumi, S. Tsunoda, Expression of Eph receptor A10 is correlated with lymph node metastasis and stage progression in breast cancer patients, *Cancer Med.* 2 (2013) 972–977.
- [5] K. Nagano, Y. Maeda, S. Kanasaki, T. Watanabe, T. Yamashita, M. Inoue, K. Higashisaka, Y. Yoshioka, Y. Abe, Y. Mukai, H. Kamada, Y. Tsutsumi, S. Tsunoda, Ephrin receptor A10 is a promising drug target potentially useful for breast cancers including triple negative breast cancers, *J. Control. Release* 189 (2014) 72–79.
- [6] D. Schrama, R.A. Reisfeld, J.C. Becker, Antibody targeted drugs as cancer therapeutics, *Nat. Rev. Drug Discovery* 5 (2006) 147–159.
- [7] M.K. Gleason, J.A. Ross, E.D. Warlick, T.C. Lund, M.R. Vermeris, A. Wiernik, S. Spellman, M.D. Haagenson, A.J. Lenvik, M.R. Litzow, P.K. Epling-Burnette, B.R. Blazar, L.M. Weiner, D.J. Weisdorf, D.A. Vallera, J.S. Miller, CD16xCD33 bispecific killer cell engager (BiKE) activates NK cells against primary MDS and MDSC CD33+ targets, *Blood* 123 (2014) 3016–3026.
- [8] C. Somasundaram, R. Arch, S. Matzku, M. Zoller, Development of a bispecific Flab<sup>TM</sup>2 conjugate against the complement receptor CR3 of macrophages and a variant CD44 antigen of rat pancreatic adenocarcinoma for redirecting macrophage-mediated tumor cytotoxicity, *Cancer Immunol. Immunother.* 42 (1996) 343–350.
- [9] S.R. Frankel, P.A. Baeuerle, Targeting T cells to tumor cells using bispecific antibodies, *Curr. Opin. Chem. Biol.* 17 (2013) 385–392.
- [10] I. Shimomura, S. Konno, A. Ito, Y. Masakari, R. Orimo, S. Taki, K. Arai, H. Ogata, M. Okada, S. Furumoto, M. Onitsuka, T. Omasa, H. Hayashi, Y. Katayose, M. Unno, T. Kudo, M. Umetsu, I. Kumagai, R. Asano, Rearranging the domain order of a diabody-based IgG-like bispecific antibody enhances its antitumor activity and improves its degradation resistance and pharmacokinetics, *MAbs* 6 (2014).
- [11] R. Asano, T. Kumagai, K. Nagai, S. Taki, I. Shimomura, K. Arai, H. Ogata, M. Okada, F. Hayasaka, H. Sanada, T. Nakanishi, T. Karvonen, H. Hayashi, Y. Katayose, M. Unno, T. Kudo, M. Umetsu, I. Kumagai, Domain order of a bispecific diabody dramatically enhances its antitumor activity beyond structural format conversion: the case of the hEx3 diabody, *Protein Eng. Des. Sel.* 26 (2013) 359–367.
- [12] R. Asano, K. Ikoma, I. Shimomura, S. Taki, T. Nakanishi, M. Umetsu, I. Kumagai, Cytotoxic enhancement of a bispecific diabody by format conversion to tandem single-chain variable fragment (taFv): the case of the hEx3 diabody, *J. Biol. Chem.* 286 (2011) 1812–1818.
- [13] R. Asano, K. Ikoma, Y. Sone, H. Kawaguchi, S. Taki, H. Hayashi, T. Nakanishi, M. Umetsu, Y. Katayose, M. Unno, T. Kudo, I. Kumagai, Highly enhanced cytotoxicity of a dimeric bispecific diabody, the hEx3 tetrabody, *J. Biol. Chem.* 285 (2010) 20644–20649.
- [14] A. Löffler, P. Kufer, R. Lutterbuse, F. Zettl, P.T. Daniel, J.M. Schwenkenbecher, G. Riethmüller, B. Dorken, R.C. Bargou, A recombinant bispecific single-chain antibody, CD19 × CD3, induces rapid and high lymphoma-directed cytotoxicity by unstimulated T lymphocytes, *Blood* 95 (2000) 2098–2103.





## Eph receptor A10 has a potential as a target for a prostate cancer therapy



Kazuya Nagano<sup>a</sup>, Takuya Yamashita<sup>a,b</sup>, Masaki Inoue<sup>a</sup>, Kazuma Higashisaka<sup>a,b</sup>, Yasuo Yoshioka<sup>a,b,c</sup>, Yasuhiro Abe<sup>d</sup>, Yohei Mukai<sup>e</sup>, Haruhiko Kamada<sup>a,c</sup>, Yasuo Tsutsumi<sup>b,c,e</sup>, Shin-ichi Tsunoda<sup>a,b,c,e,\*</sup>

<sup>a</sup> Laboratory of Biopharmaceutical Research, National Institute of Biomedical Innovation, 7-6-8 Saito-Asagi, Ibaraki, Osaka 567-0085, Japan

<sup>b</sup> Graduate School of Pharmaceutical Sciences, Osaka University, 1-6 Yamadaoka, Suita, Osaka 565-0871, Japan

<sup>c</sup> The Center of Advanced Medical Engineering and Informatics, Osaka University, 1-6 Yamadaoka, Suita, Osaka 565-0871, Japan

<sup>d</sup> Cancer Biology Research Center, Sanford Research/USD, 2301 E. 60th Street N, Sioux Falls, SD 57104, USA

<sup>e</sup> Laboratory of Innovative Antibody Engineering and Design, National Institute of Biomedical Innovation, 7-6-8 Saito-Asagi, Ibaraki, Osaka 567-0085, Japan

### ARTICLE INFO

#### Article history:

Received 28 May 2014

Available online 9 June 2014

#### Keywords:

Eph receptor A10  
Prostate cancer  
Antibody drug

### ABSTRACT

We recently identified Eph receptor A10 (EphA10) as a novel breast cancer-specific protein. Moreover, we also showed that an in-house developed anti-EphA10 monoclonal antibody (mAb) significantly inhibited proliferation of breast cancer cells, suggesting EphA10 as a promising target for breast cancer therapy. However, the only other known report for EphA10 was its expression in the testis at the mRNA level. Therefore, the potency of EphA10 as a drug target against cancers other than the breast is not known. The expression of EphA10 in a wide variety of cancer cells was studied and the potential of EphA10 as a drug target was evaluated. Screening of EphA10 mRNA expression showed that EphA10 was overexpressed in breast cancer cell lines as well as in prostate and colon cancer cell lines. Thus, we focused on prostate cancers in which EphA10 expression was equivalent to that in breast cancers. As a result, EphA10 expression was clearly shown in clinical prostate tumor tissues as well as in cell lines at the mRNA and protein levels. In order to evaluate the potential of EphA10 as a drug target, we analyzed complement-dependent cytotoxicity effects of anti-EphA10 mAb and found that significant cytotoxicity was mediated by the expression of EphA10. Therefore, the idea was conceived that the overexpression of EphA10 in prostate cancers might have a potential as a target for prostate cancer therapy, and formed the basis for the studies reported here.

© 2014 Elsevier Inc. All rights reserved.

### 1. Introduction

The development of antibody engineering has enabled a monoclonal antibody (mAb) to become a safe and effective drug for refractory diseases, such as cancer. Today, more than 30 kinds of antibody drugs are approved all over the world. Continued growth in the market is expected in the future [1]. However, the cases to which antibody drugs are applied are limited. Therefore, the development of new antibody drugs is especially needed in the cases without effective treatments, such as a triple negative breast cancer, a castration-resistant prostate cancer, as well as pancreatic cancers or malignant mesotheliomas.

Several Eph receptor family members such as EphA2 or EphB4 are highly expressed in various tumor cell types found in refractory cancers [2], and with expressions associated with tumorigenesis [3,4], proliferation [5,6], vasculogenesis [7,8] and metastasis [9,10]. Therefore, there is a current focus on the development of therapies targeted on Eph members [11]. In this context, MedImmune LLC is developing an antibody-drug conjugate against EphA2 which inhibits tumor growth *in vitro* and *in vivo* [12,13]. It has been tested in phase I to investigate the safety profile and maximum tolerated dose. However, the most recent report announced the trial was stopped halfway due to adverse events such as bleeding and liver disorders [14]. Some databases such as MOPED or PaxDb have reported that EphA2 is highly expressed in platelets and liver tissues. Therefore, the target protein needs to display specific expression in cancer tissues. However, EphA10 which we identified as a novel breast cancer-related protein is hardly expressed in normal human tissues [15] [16]. Furthermore, we also showed that an in-house developed anti-EphA10 mAb inhibited breast cancer cell proliferation at both *in vitro* and *in vivo* levels [16]. These findings

**Abbreviations:** EphA10, Eph receptor A10; mAb, monoclonal antibody; TMA, tissue microarray; HMEC, human mammary epithelial cell; PrEC, prostate epithelial cell; cDNA, complementary DNA; FCS, fetal calf serum; PBS, phosphate buffered saline; IHC, immunohistochemistry; CDC, complement-dependent cytotoxicity.

\* Corresponding author. Fax: +81 72 641 9817.

E-mail address: [tsunoda@nibio.go.jp](mailto:tsunoda@nibio.go.jp) (S.-i. Tsunoda).

<http://dx.doi.org/10.1016/j.bbrc.2014.06.007>

0006-291X/© 2014 Elsevier Inc. All rights reserved.

suggest that EphA10 is a promising target for breast cancer therapy. However, the only other known report was that EphA10 is expressed in the testis at the mRNA level [17]. Therefore, the potency of EphA10 as a drug target against cancers other than the breast has not been tested. Here, we report EphA10 expression in various kinds of cancer cells and the potential of EphA10 as a target in other cancer therapies.

## 2. Material and methods

### 2.1. Cell lines

The following cancer cell lines were purchased from the American Type Culture Collection (Manassas, VA): HCC70, MDA-MB-157, HCC1599, MDA-MB468, DU4475, 22Rv1, VCaP, colo201, SW620, HCT116, BxPC3, Panc1, AsPC1, H2452, H2052, H28 and Jurkat. The following cancer cell lines were purchased from the Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan): RERF-LC-KJ, RERF-LC-MS, MKN1, MKN45, NEC8, NEC14, A2058, G318, Mewo and K562. PC3 and LNCaP were purchased from the Riken Bioresource Center Cell Bank (Ibaraki, Japan). Normal Human Prostate Epithelial Cells (PrEC) and Normal Human Mammary Epithelial Cells were purchased from Lonza (Basel, Switzerland). All cells were cultured at 37 °C in a humidified atmosphere of 5% CO<sub>2</sub> according to the provider's protocol.

### 2.2. Real-time PCR

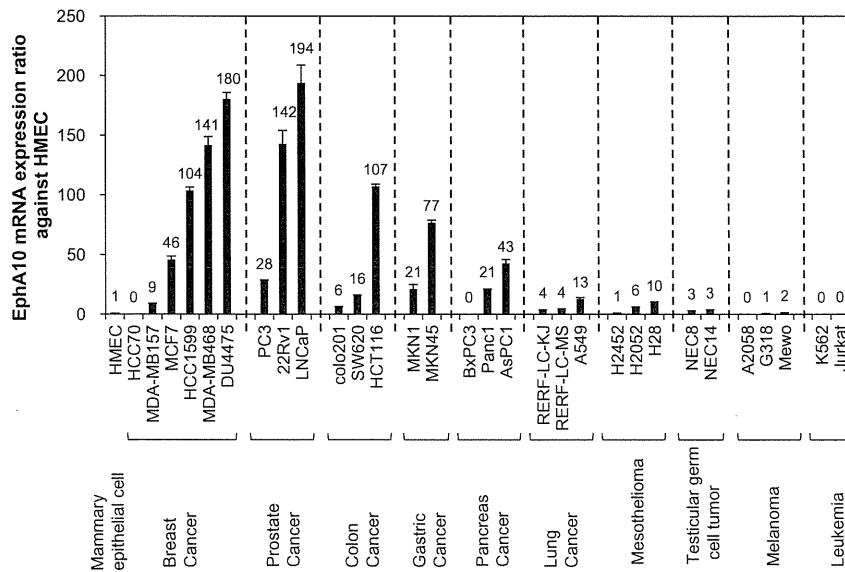
Complementary DNAs (cDNAs) derived from human prostate tumors were purchased from OriGene Technologies (Rockville, MD). The PCR mixture included cDNA template, TaqMan Gene Expression Master Mix and TaqMan probe (EphA10: Hs01017018\_m1 or actin-beta: Hs99999903\_m1) (Life Technologies, Carlsbad, CA) and the reaction was performed according to the manufacturer's instructions. The threshold cycles were determined using the default settings. EphA10 mRNA expression levels were normalized against actin-beta.

### 2.3. Cell immunofluorescent staining

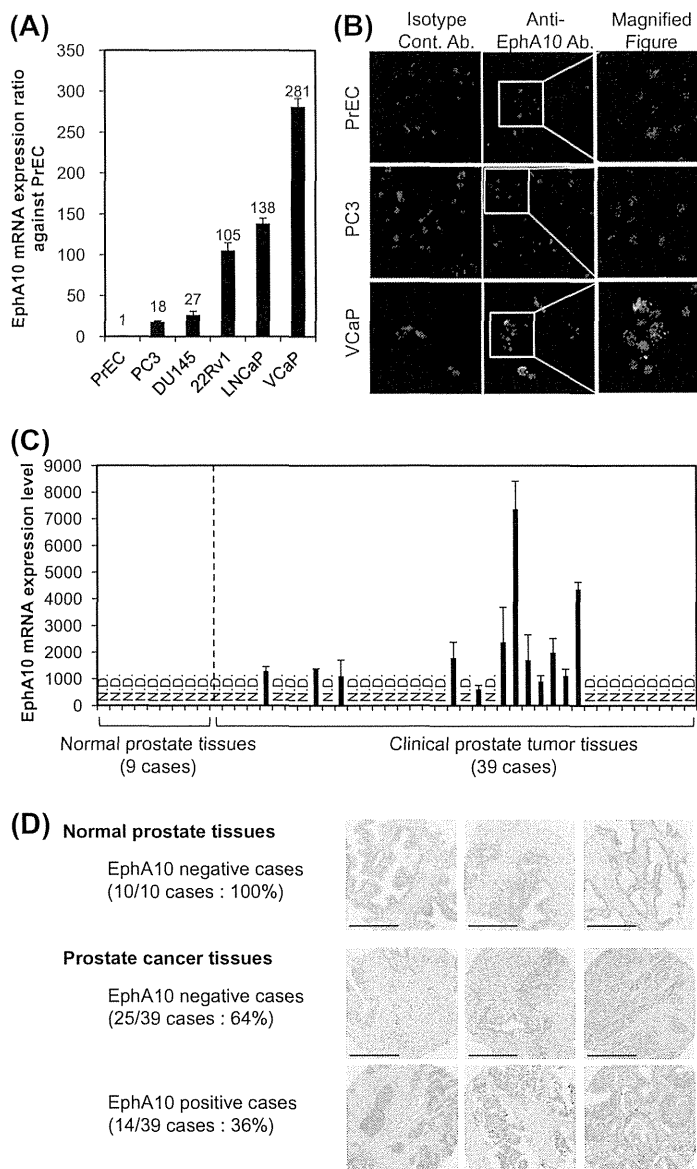
PrEC, PC3 and VCaP cells were seeded at  $1 \times 10^4$  cells/well in Lab-Tek™ 8-well chamber slides (Thermo Fisher Scientific Inc., Waltham, MA). After 24 h, cells were washed twice with PBS, and then fixed with PBS containing 4% paraformaldehyde, pH 8.0 for 10 min. After washing with PBS, fixation was quenched with PBS containing 0.1 M glycine, pH 7.4 for 15 min. Fixed cells were blocked with PBS containing 5% FCS (blocking solution), pH 7.4 for 30 min, and then treated with the anti-EphA10 monoclonal primary antibody and isotype control antibody at 10 µg/ml in blocking solution for 1 h. Donkey anti-mouse IgG conjugated with Alexa Flour 488 (Life Technologies, Carlsbad, CA) was used as the second antibody at 2 µg/ml in the blocking solution for 1 h in the dark. Slides were mounted using a vectashield mounting medium for fluorescence with DAPI (Vector Laboratories Inc., Burlingame, CA) and analyzed with a Leica TCS SP2 confocal laser scanning microscope (Leica Microsystems GmbH, Wetzlar, Germany). Images were further processed using the Adobe Photoshop software.

### 2.4. Immunohistochemical (IHC) staining

TMA with prostate tumor and normal prostate tissues (US Biomax, Rockville, MD) were deparaffinized in xylene and rehydrated in a graded series of ethanol. Heat-induced epitope retrieval was performed by maintaining the Target Retrieval Solution (Dako, Glostrup, Denmark) by following the manufacturer's instructions. After treatment, endogenous peroxidase was blocked with 0.3%. The TMA slides were incubated with rabbit anti-human EphA10 polyclonal antibody (Abgent Inc., San Diego, CA) for 30 min and then with ENVISION+ Dual Link (Dako, Glostrup, Denmark) for 30 min. The reaction products were rinsed three times with 0.05% Tween20/Tris buffer saline and then developed in liquid 3,3'-diaminobenzidine for 3 min. After development, sections were lightly counterstained with Mayer's hematoxylin. All procedures were performed using an AutoStainer (Dako, Glostrup, Denmark). Study samples were divided into high and low expression groups based on the two criteria of distribution and quantity. In terms of distribution, the percentage of positive cells across all tumor cells



**Fig. 1.** Screening of EphA10 expression profile in various kinds of cancer cell lines. EphA10 expression in various kinds of cancer cells were screened by quantitative real time PCR. EphA10 expression level in each cell was normalized by actin-beta expression level and described as a ratio against EphA10 expression level in HMEC, normal human mammary epithelial primary cells.  $n = 3$  in each group. Error bars represent the SD.



**Fig. 2.** EphA10 expression analysis in prostate cancer cell lines and clinical prostate cancer tissues at mRNA and protein level. (A) EphA10 mRNA expression level in prostate cancer cell lines (PC3, DU145, 22Rv1, LNCaP and VCaP) was quantified by real time PCR. It was normalized by actin-beta expression level and described as a ratio against EphA10 expression level in PrEC, normal human prostate epithelial primary cells.  $n = 3$  in each group. Error bars represent the SD. (B) EphA10 protein expression in prostate cancer cell lines was analyzed by cell immunofluorescent staining. PrEC, PC3 (EphA10-mRNA low expressing cells) and VCaP (EphA10 mRNA high-expressing cells) were treated with anti-EphA10 mAb or the isotype control mAb (20  $\mu\text{g}/\text{ml}$ ), and then with Alexa Flour 488 conjugated anti-mouse IgG antibody. EphA10 protein expression was detected by confocal microscopy. Blue and green signals relate to DAPI and EphA10, respectively. (C) EphA10 mRNA expression levels in clinical prostate cancer tissues (39 cases) and the normal prostate tissues (9 cases) were quantified in the same method with (A). N.D. means not detectable. (D) TMAs with clinical prostate cancer tissues (39 cases) and the normal tissues (10 cases) were stained using anti-EphA10 mAb. Representative images of normal breast tissue (positive ratio: 0%), EphA10 negative cancer tissue, and EphA10 positive cancer tissues (positive ratio: 36%) are shown. Scale bar: 200  $\mu\text{m}$ .

was scored as 0 (0%), 1 (1–50%), and 2 (51–100%). In terms of quantity, the signal intensity was scored as 0 (no signal), 1 (weak), 2 (moderate) or 3 (marked). Cases with a total score of more than 3 were classified into the high expression group.

### 2.5. Complement-dependent cytotoxicity (CDC) assay

VCaP cells were seeded at  $2 \times 10^4$  cells/well in a 96 well culture plate (Thermo Fisher Scientific Inc., Waltham, MA) and cultured overnight. After removing the medium, antibodies (anti-EphA10 antibody or the isotype control antibody) and mouse

serum as complement were added and incubated for 24 h. Cytotoxicity was evaluated using the WST-8 assay.

## 3. Results and discussion

### 3.1. EphA10 mRNA was overexpressed not only in breast cancer cell lines but also in prostate and colon cancer cell lines

In order to screen the types of cancer in which EphA10 is expressed, EphA10 mRNA expression was analyzed not only in breast cancer cells in which we had already shown EphA10

expression, but also in cell lines of colon cancer, gastric cancer, leukemia, lung cancer, melanoma, mesothelioma, pancreas cancer, prostate cancer and testicular germ cell tumors by real time PCR. EphA10 mRNA was expressed by normalizing the actin-beta expression level and represented as the ratio against normal human mammary epithelial primary cells (HMEC). Quantitative analysis demonstrated that EphA10 was expressed not only in breast cancer cells (HCC1599: 103x, MDA-MB468: 141x, DU4475: 181x), but also in prostate cancer cells (22Rv1: 142x, LNCaP: 194x) and colon cancer cells (HCT116: 107x) by more than 100 fold over human mammary epithelial primary cells (HMECs). EphA10 mRNA expression level in breast cancer cell lines was equivalent to that in prostate cancer cell lines (Fig. 1). These data suggested that EphA10 could also be associated with prostate cancers. Therefore, we next focused on prostate cancers and analyzed in more detail the expression of EphA10 at the mRNA and protein levels in cancer cell lines and clinical tissues.

### 3.2. EphA10 was overexpressed in prostate cancer cell lines and clinical prostate tumor tissues at mRNA and protein level

In order to examine EphA10 expression in prostate cancers, EphA10 expression at the mRNA and protein levels was evaluated in five prostate cancer cell lines (22Rv1, DU145, LNCaP, PC3 and VCaP) and normal human prostate epithelial primary cells (PrECs). Fig. 2(A) shows EphA10 was highly expressed in all cancer cell lines compared to the normal cells. Furthermore, we also analyzed EphA10 expression at the protein level in these cells. Immunofluorescent staining showed that EphA10 expression could not be detected in both PrEC and PC3 (EphA10 mRNA low-expressing cells). On the other hand, EphA10 protein expression was only observed in anti-EphA10 antibody-treated VCaP cells (EphA10 mRNA high-expressing cells), but not in the isotype control antibody-treated VCaP cells (Fig. 2(B)). These data are consistent with the pattern of EphA10 mRNA expression, further demonstrating that EphA10 was overexpressed in prostate cancer cell lines compared to the normal cells.

In order to pursue the overexpression of EphA10 in prostate cancers, we next analyzed EphA10 expression in clinical prostate cancer tissues and in normal prostate tissues. EphA10 expression at the mRNA level was first evaluated using cDNA derived from clinical prostate tumor tissues and the normal prostate tissues. A real time PCR analysis showed that EphA10 mRNA could not be amplified in all 9 normal prostate cases and 27 prostate tumor cases. In contrast, EphA10 expression was observed in 12 prostate tumor cases (approximately 31% in total cases) (Fig. 2(C)). Furthermore, we analyzed the EphA10 protein expression by IHC-staining TMA with clinical prostate cancer tissues and the normal tissues. TMA data showed that EphA10 expression was observed in 14 prostate cancer cases (approximately 36% in total cases), but not in 10 normal prostate tissues and in 25 prostate cancer cases. These data suggested that EphA10 was definitely overexpressed in prostate cancer cell lines as well as in clinical prostate tumor tissues.

We previously showed that EphA10 expression was positively associated with stage progression and lymph node metastasis in clinical breast cancers [18]. Thus, in order to evaluate the role of EphA10 overexpression in prostate cancers, we tried to analyze the relationship between EphA10 expression in clinical prostate cancer tissues and the clinical information such as the size and spread of primary tumor (pT), regional lymph node metastasis (pN), the distant metastasis (pM), and the cancer progression (pStage). Statistical analysis showed that EphA10 expression was not significantly associated with all of the above factors (Supplementary Table S1). It was reported that some Eph receptor members were overexpressed in various kinds of cancers such as

breast and prostate [2], and activated by hetero-dimerizing between Eph receptors [19,20]. Therefore, in addition to focusing only on EphA10, analysis of other Eph receptors are needed in order to reveal the role of EphA10 in prostate cancers.

### 3.3. Anti-EphA10 mAb significantly caused complement-dependent cytotoxicity (CDC) activity dependent on EphA10 expression

In order to evaluate the potential of EphA10 as a target for prostate cancer therapy, we analyzed CDC effects of anti-EphA10 mAb on VCaP cells in which EphA10 was highly expressed. We added anti-EphA10 mAb and mouse serum as complements into VCaP cells and evaluated cytotoxicity on the next day. Fig. 3 shows that cytotoxicity in VCaP was observed only in the co-culture group of anti-EphA10 mAb and mouse serum, but not in the co-culture group of isotype control mAb and mouse serum as well as in mAb alone group. The data indicated that the cytotoxicity of anti-EphA10 mAb was dependent on EphA10 expression, and suggested that EphA10 targeted therapy might be effective in EphA10 positive prostate cancer cases.

Since molecular targeted drugs such as antibody drugs show therapeutic effects related to affinity and specificity for each antigen, it is important that the target protein display enriched expression in cancer tissues. In this respect, we previously reported that EphA10 expression was not observed in almost all normal human organs, except for testis [16]. In order to develop anti-EphA10 mAb therapy and apply it to male patients, EphA10 function in the testis should be analyzed and consider the effects of anti-EphA10 mAb on dysfunction of the testis. On the other hand, many prostate cancer patients are surgically or medically castrated with the purpose of reducing the amount of androgens which promote the growth of prostate cancer cells. However, almost all of the patients have a recurrence which is known as castration-resistant prostate cancer (CRPC). CRPCs are a bottleneck for prostate cancer therapy, because CRPCs have no effective treatment and poor prognosis. Clinical trials of antibody drugs (such as anti-CTLA4 mAb or anti-PD1 mAb) against CRPCs are currently in progress. However the therapeutic effects have been insufficient [21], emphasizing that a novel drug target is urgently needed. In this respect, EphA10 might be a promising target at least for CRPC patients, although further basic experiments are needed such as EphA10 expression analysis in CRPC cases.

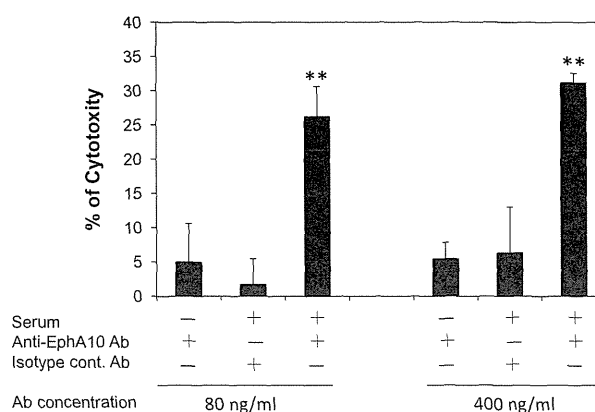


Fig. 3. Complement-dependent cytotoxicity (CDC) effects of anti-EphA10 mAb on VCaP cells. Anti-EphA10 mAb or the isotype control mAb (80 and 400 ng/ml) with/without mouse serum as complements were added to VCaP cells. After 24 h incubation, CDC effects were assessed by WST-8 assay. \*\* $p < 0.01$  vs the isotype control mAb with mouse serum.  $n = 3$  in each group. Error bars represent the SD.

In conclusion, we showed that EphA10 was overexpressed in prostate cancers and suggest that EphA10 is a potential target for prostate cancer therapy.

### Conflict of interest statement

The authors have no conflict of interest.

### Acknowledgments

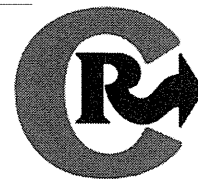
This study was supported in part by Grants-in-Aid for Scientific Research from the Project for Development of Innovative Research on Cancer Therapeutics, the Ministry of Education, Culture, Sports, Science and Technology of Japan, and from the Japan Society for the Promotion of Science. This study was also supported in part by Health Labor Sciences Research Grants from the Ministry of Health, Labor and Welfare of Japan.

### Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.bbrc.2014.06.007>.

### References

- [1] A.L. Nelson, E. Dhimala, J.M. Reichert, Development trends for human monoclonal antibody therapeutics, *Nat. Rev. Drug Discov.* 9 (2010) 767–774.
- [2] H.Q. Xi, X.S. Wu, B. Wei, L. Chen, Eph receptors and ephrins as targets for cancer therapy, *J. Cell. Mol. Med.* 16 (2012) 2894–2909.
- [3] D.M. Brantley-Sieders, G. Zhuang, D. Hicks, W.B. Fang, Y. Hwang, J.M. Cates, K. Coffman, D. Jackson, E. Bruckheimer, K.S. Muraoka-Cook, J. Chen, The receptor tyrosine kinase EphA2 promotes mammary adenocarcinoma tumorigenesis and metastatic progression in mice by amplifying ErbB2 signaling, *J. Clin. Invest.* 118 (2008) 64–78.
- [4] N. Munarini, R. Jager, S. Abderhalden, C. Zuercher, V. Rohrbach, S. Loercher, B. Pfanner-Meyer, A.C. Andres, A. Ziemiecki, Altered mammary epithelial development, pattern formation and involution in transgenic mice expressing the EphB4 receptor tyrosine kinase, *J. Cell. Sci.* 115 (2002) 25–37.
- [5] S.R. Kumar, J. Singh, G. Xia, V. Krasnoperov, L. Hassanieh, E.J. Ley, J. Scheinet, N.G. Kumar, D. Hawes, M.F. Press, F.A. Weaver, P.S. Gill, Receptor tyrosine kinase EphB4 is a survival factor in breast cancer, *Am. J. Pathol.* 169 (2006) 279–293.
- [6] K.A. Mohammed, X. Wang, E.P. Goldberg, V.B. Antony, N. Nasreen, Silencing receptor EphA2 induces apoptosis and attenuates tumor growth in malignant mesothelioma, *Am. J. Cancer Res.* 1 (2011) 419–431.
- [7] N.K. Noren, M. Lu, A.L. Freeman, M. Koolpe, E.B. Pasquale, Interplay between EphB4 on tumor cells and vascular ephrin-B2 regulates tumor growth, *Proc. Natl. Acad. Sci. U.S.A.* 101 (2004) 5583–5588.
- [8] S. Sawamiphak, S. Seidel, C.L. Essmann, G.A. Wilkinson, M.E. Pitulescu, T. Acker, A. Acker-Palmer, Ephrin-B2 regulates VEGFR2 function in developmental and tumour angiogenesis, *Nature* 465 (2010) 487–491.
- [9] D.M. Brantley-Sieders, W.B. Fang, D.J. Hicks, G. Zhuang, Y. Shyr, J. Chen, Impaired tumor microenvironment in EphA2-deficient mice inhibits tumor angiogenesis and metastatic progression, *FASEB J.* 19 (2005) 1884–1886.
- [10] X.D. Ji, G. Li, Y.X. Feng, J.S. Zhao, J.J. Li, Z.J. Sun, S. Shi, Y.Z. Deng, J.F. Xu, Y.Q. Zhu, H.P. Koefler, X.J. Tong, D. Xie, EphB3 is overexpressed in non-small-cell lung cancer and promotes tumor metastasis by enhancing cell survival and migration, *Cancer Res.* 71 (2011) 1156–1166.
- [11] A.W. Boyd, P.F. Bartlett, M. Lackmann, Therapeutic targeting of Eph receptors and their ligands, *Nat. Rev. Drug Discov.* 13 (2014) 39–62.
- [12] D. Jackson, J. Gooya, S. Mao, K. Kinneer, L. Xu, M. Camara, C. Fazenbaker, R. Fleming, S. Swamynathan, D. Meyer, P.D. Senter, C. Gao, H. Wu, M. Kinch, S. Coats, P.A. Kiener, D.A. Tice, A human antibody-drug conjugate targeting EphA2 inhibits tumor growth in vivo, *Cancer Res.* 68 (2008) 9367–9374.
- [13] J.W. Lee, R.L. Stone, S.J. Lee, E.J. Nam, J.W. Roh, A.M. Nick, H.D. Han, M.M. Shahzad, H.S. Kim, L.S. Mangala, N.B. Jennings, S. Mao, J. Gooya, D. Jackson, R.L. Coleman, A.K. Scod, EphA2 targeted chemotherapy using an antibody drug conjugate in endometrial carcinoma, *Clin. Cancer Res.* 16 (2010) 2562–2570.
- [14] C.M. Annunziata, E.C. Kohn, P. LoRusso, N.D. Houston, R.L. Coleman, M. Buzoianu, G. Robbie, R. Lechleider, Phase 1, open-label study of MEDI-547 in patients with relapsed or refractory solid tumors, *Invest. New Drugs* 31 (2013) 77–84.
- [15] S. Imai, K. Nagano, Y. Yoshida, T. Okamura, T. Yamashita, Y. Abe, T. Yoshioka, Y. Yoshioka, H. Kamada, Y. Mukai, S. Nakagawa, Y. Tsutsumi, S. Tsunoda, Development of an antibody proteomics system using a phage antibody library for efficient screening of biomarker proteins, *Biomaterials* 32 (2011) 162–169.
- [16] K. Nagano, Y. Maeda, S. Kanasaki, T. Watanabe, T. Yamashita, M. Inoue, K. Higashisaka, Y. Yoshioka, Y. Abe, Y. Mukai, H. Kamada, Y. Tsutsumi, S. Tsunoda, Ephrin receptor A10 is a promising drug target potentially useful for breast cancers including triple negative breast cancers, *J. Control Release*, in press.
- [17] H.C. Aasheim, S. Patzke, H.S. Hjorthaug, E.F. Finne, Characterization of a novel Eph receptor tyrosine kinase, EphA10, expressed in testis, *Biochim. Biophys. Acta* 1723 (2005) 1–7.
- [18] K. Nagano, S. Kanasaki, T. Yamashita, Y. Maeda, M. Inoue, K. Higashisaka, Y. Yoshioka, Y. Abe, Y. Mukai, H. Kamada, Y. Tsutsumi, S. Tsunoda, Expression of Eph receptor A10 is correlated with lymph node metastasis and stage progression in breast cancer patients, *Cancer Med.* 2 (2013) 972–977.
- [19] B.P. Fox, R.P. Kandpal, A paradigm shift in Eph receptor interaction: biological relevance of EphB6 interaction with EphA2 and EphB2 in breast carcinoma cell lines, *Cancer Genomics Proteomics* 8 (2011) 185–193.
- [20] A. Freywald, N. Sharfe, C.M. Roifman, The kinase-null EphB6 receptor undergoes transphosphorylation in a complex with EphB1, *J. Biol. Chem.* 277 (2002) 3823–3828.
- [21] S.L. Topalian, P.S. Hodi, J.R. Brahmer, S.N. Gettinger, D.C. Smith, D.F. McDermott, J.D. Powderly, R.D. Carvajal, J.A. Sosman, M.B. Atkins, P.D. Leming, D.R. Spigel, S.J. Antonia, L. Horn, C.G. Drake, D.M. Pardoll, L. Chen, W.H. Sharfman, R.A. Anders, J.M. Taube, T.L. McMiller, H. Xu, A.J. Korman, M. Jure-Kunkel, S. Agrawal, D. McDonald, G.D. Kohla, A. Gupta, J.M. Wigginton, M. Sznol, Safety, activity, and immune correlates of anti-PD-1 antibody in cancer, *N. Engl. J. Med.* 366 (2012) 2443–2454.



## Ephrin receptor A10 is a promising drug target potentially useful for breast cancers including triple negative breast cancers



Kazuya Nagano<sup>a</sup>, Yuka Maeda<sup>a,b</sup>, So-ichiro Kanasaki<sup>a,b</sup>, Takanoobu Watanabe<sup>a,b</sup>, Takuya Yamashita<sup>a,b</sup>, Masaki Inoue<sup>a</sup>, Kazuma Higashisaka<sup>a,b</sup>, Yasuo Yoshioka<sup>a,b,c</sup>, Yasuhiro Abe<sup>a</sup>, Yohei Mukai<sup>d</sup>, Haruhiko Kamada<sup>a,c</sup>, Yasuo Tsutsumi<sup>b,c</sup>, Shin-ichi Tsunoda<sup>a,b,c,\*</sup>

<sup>a</sup> Laboratory of Biopharmaceutical Research, National Institute of Biomedical Innovation, 7-6-8 Saito-Asagi, Ibaraki, Osaka 567-0085, Japan

<sup>b</sup> Graduate School of Pharmaceutical Sciences, Osaka University, 1-6 Yamadaoka, Suita, Osaka 565-0871, Japan

<sup>c</sup> The Center of Advanced Medical Engineering and Informatics, Osaka University, 1-6 Yamadaoka, Suita, Osaka 565-0871, Japan

<sup>d</sup> Laboratory of Innovative Antibody Engineering and Design, National Institute of Biomedical Innovation, 7-6-8 Saito-Asagi, Ibaraki, Osaka 567-0085, Japan

### ARTICLE INFO

#### Article history:

Received 31 March 2014

Accepted 9 June 2014

Available online 16 June 2014

#### Keywords:

Ephrin receptor A10

Antibody drug

Triple negative breast cancer

Cell proliferation

### ABSTRACT

Ephrin receptor A10 (EphA10) is a relatively uncharacterized protein which is expressed in many breast cancers but not expressed in normal breast tissues. Here, we examined the potential of EphA10 as a drug target in breast cancer. Immunohistochemical staining of clinical tissue sections revealed that EphA10 was expressed in various breast cancer subtypes, including triple negative breast cancers (TNBCs), with no expression observed in normal tissues apart from testis. Ligand-dependent proliferation was observed in EphA10-transfected MDA-MB-435 cells (MDA-MB-435<sup>EphA10</sup>) and native TNBC cells (MDA-MB-436). However, this phenomenon was not observed in parental MDA-MB-435 cells which express a low level of EphA10. Finally, tumor growth was significantly suppressed by administration of an anti-EphA10 monoclonal antibody in a xenograft mouse model. These results suggest that inhibition of EphA10 signaling may be a novel therapeutic option for management of breast cancer, including TNBCs which are currently not treated with molecularly targeted agents.

© 2014 Elsevier B.V. All rights reserved.

### 1. Introduction

Expression of the estrogen receptor (ER), progesterone receptor (PR) and Her2 in breast cancer tissues is an important indicator in determining treatment options against the disease [1,2]. Anti-hormone therapies and anti-Her2 therapies such as tamoxifen and trastuzumab are chosen against ER/PR positive cases and Her2 positive cases, respectively [1,2]. However, triple negative breast cancer (TNBC), which lacks expression of ER, PR and Her2, is known to be refractory due to absence of molecularly targeted drugs [3–5]. Therefore, there is considerable interest worldwide in respect to the development of therapeutics against TNBC.

Ephrin receptor superfamily members are subdivided into nine type A molecules (EphA1–A8, A10) and five type B molecules (EphB1–B4, B6) in mammals [6,7]. Several Eph members such as EphA2 or EphB4 are overexpressed in various tumor cell types [8,9], with expression also related to tumorigenesis [10–12], proliferation [13,14], vasculogenesis [15–17] and metastasis [18–20]. There is a current focus on the development of Eph member-targeted therapies. In this context, an antibody-

drug conjugate against EphA2 is being developed by MedImmune LLC which inhibits tumor growth *in vitro* and *in vivo* [21–23] and has been tested in phase I to investigate the safety profile and maximum tolerated dose. However, the most recently reported phase I results show that the trial study was stopped halfway due to adverse events such as bleeding and liver disorders [24]. Certainly, it is reported in some databases such as MOPED or PaxDb that EphA2 is highly expressed in platelet and liver tissues. As antibody-based drugs show therapeutic effects related to affinity and specificity for the respective antigen, the target protein needs to display enriched expression in cancer tissues.

Against this background, we had identified EphA10 as being expressed in many breast cancer tissues, but not in normal breast tissues, using an antibody proteomics system [25]. EphA10 is a relatively uncharacterized protein. The only finding known before our report was that EphA10 is expressed in the testis at mRNA level [26]. Therefore, EphA10 is a highly novel breast cancer-related protein. However, the expression profile of EphA10 protein in normal and cancer tissues, as well as function, must be clarified in order to determine its potential as a drug target.

Here, we firstly showed EphA10 expression in various breast cancer cases, including TNBC, with no expression observed in normal tissues apart from testis. Furthermore, we investigated the potency of EphA10 targeted therapy through the use of an anti-EphA10 monoclonal antibody (mAb).

\* Corresponding author at: Laboratory of Biopharmaceutical Research, National Institute of Biomedical Innovation, 7-6-8 Saito-Asagi, Ibaraki, Osaka 567-0085, Japan. Tel.: +81 72 641 9814; fax: +81 72 641 9817.

E-mail address: [tsunoda@nibio.go.jp](mailto:tsunoda@nibio.go.jp) (S. Tsunoda).

## 2. Material and methods

### 2.1. Cell lines and culture

MDA-MB-435 and MDA-MB-436 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA). Both cells were cultured in Dulbecco's modified eagle medium (DMEM) with 10% fetal calf serum (FCS). Stably expressing EphA10 transfectant cells (MDA-MB-435<sup>EphA10</sup>) were established in our laboratory. In brief, lentiviral particles encoding human EphA10 were prepared using 293T cells (ATCC, Manassas, VA) and infected into MDA-MB-435 cells at a multiplicity of infection of 100. Stable EphA10 transfectants were selected and maintained for growth in 10% FCS/DMEM medium containing 8 µg/ml blasticidin. All cells were cultured at 37 °C in a humidified atmosphere of 5% CO<sub>2</sub>.

### 2.2. BrdU incorporation assay

MDA-MB-435, MDA-MB-435<sup>EphA10</sup> and MDA-MB-436 cells were seeded at  $5 \times 10^3$  cells/well in a 96-well cell culture plate and incubated overnight. After 24 h of starvation in medium without FCS, EphrinA3-Fc, EphrinA4-Fc and EphrinA5-Fc chimera recombinant proteins (R&D Systems Inc., Minneapolis, MN) or anti-EphA10 mAb were added and incubated for 12 h. Cell proliferation was evaluated using a dedicated ELISA kit which measures BrdU incorporation (Roche Applied Science, Penzberg, Germany). For this assay, BrdU solution was added to those cells being stimulated with EphA10 ligand and incubated for 2 h. After cell fixation, POD-labeled anti-BrdU antibody was added and incubated at RT for 90 min. After three washes, the level of BrdU incorporation was determined by adding the substrate.

### 2.3. Development of anti-EphA10 mAb

BALB/c mice were immunized with EphA10-Fc chimera recombinant protein (R&D Systems Inc., Minneapolis, MN) four times. After the antibody titers for EphA10 were shown to be optimal by ELISA, hybridoma cells were obtained by fusion of myeloma cells with immunized spleen cells in the usual manner. Positive clones for EphA10 binding were selected by flow cytometry and ELISA methods.

### 2.4. Affinity evaluation as determined by surface plasmon resonance (SPR)

Protein interactions were evaluated using a BIAcore3000 system (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). Anti-EphA10 mAb or each EphA10 ligand (EphrinA3-Fc, EphrinA4-Fc and EphrinA5-Fc) was mixed in series of combinations with EphA10-Fc chimera recombinant protein immobilized on a sensor chip CM5. The kinetic parameters of these interactions were calculated using a single- or multi-cycle kinetic analysis method.

### 2.5. Tumor accumulation analysis of anti-EphA10 mAb

Alexa Fluor 647-labeled Anti-EphA10 mAb or IgG2b isotype control mAb was intravenously administered into xenograft mouse models bearing MDA-MB-435<sup>EphA10</sup> or MDA-MB-435 at 200 µg/mouse. The mice were then imaged daily (24–96 h) and associated fluorescent

intensity was measured using the OV110 *in vivo* observation system (Olympus Corp., Tokyo, Japan). Tumor accumulation was quantified as the ratio of mean fluorescent intensity in tumors compared to that observed on the contralateral side skin. After 96 h of administration, tumor tissues were isolated from dissected mice, and their mean fluorescent intensity was measured.

### 2.6. Anti-EphA10 mAb treatment in a mouse xenograft model

A xenograft mouse model was constructed by orthotopic transplantation of MDA-MB-435<sup>EphA10</sup>. When the tumor size reached approximately 100 mm<sup>3</sup>, saline, anti-EphA10 mAb and control mAb were intraperitoneally administered twice a week (1 mg/mouse or 0.5 mg/mouse) or intravenously administered once a week (0.4 mg/mouse or 0.2 mg/mouse). Tumor volume was assessed over time using the following formula – tumor volume =  $LW^2/2$ , where L is long diameter and W is the short diameter. At day 47 (intraperitoneal administration) or day 53 (intravenous administration), tumor tissues were isolated from dissected mice, and their weights measured.

### 2.7. Statistical methods

All analyses were performed using GraphPad Prism 5 version (GraphPad Software Inc., La Jolla, CA). Student *t* test or one-way ANOVA test was used to compare the two or multiple groups, respectively. All hypothesis testing were two-tailed with a significance level of <0.05.

## 3. Results

### 3.1. EphA10 was expressed in various subtypes of breast tumors, but not in many types of normal tissues

We previously showed that EphA10 is preferentially expressed in breast cancer compared to normal breast tissues. However, the expression profile of EphA10 has not been examined in relation to the various subtypes of breast cancer as yet. Breast cancer patients are divided into the following four therapeutically-relevant subtypes on the basis of Her2, ER and PR expressions in tumor cells: luminal A (Her2<sup>-</sup>, ER<sup>+</sup> and/or PR<sup>+</sup>), luminal B (Her2<sup>+</sup>, ER<sup>+</sup> and/or PR<sup>+</sup>), Her2-enriched (Her2<sup>+</sup>, ER<sup>-</sup>, PR<sup>-</sup>) and TNBC (Her2<sup>-</sup>, ER<sup>-</sup>, PR<sup>-</sup>) [1,27]. In order to comprehensively evaluate the utility of EphA10 as a drug target in breast cancer, we firstly analyzed its expression profile within the above-mentioned subtypes using tissue microarray (TMA) technology. As determined by immunohistochemical (IHC) staining, EphA10 was seen to be specifically expressed in cancer cells from each subtype (Fig. S1 (A)). Moreover, assessment of staining images indicated that the proportion of EphA10-positive tumors per subtype was as follows: luminal A (54%), luminal B (68%), Her2-enriched (64%) and TNBC (67%) (Table 1). These data suggest EphA10 as an attractive target in breast cancer, particularly in relation to patients with TNBC which is under-served by current molecularly targeted drugs.

In order to evaluate the specificity of EphA10 expression, we next analyzed EphA10 protein levels in 36 types of normal tissues. IHC analysis of a normal human organ TMA showed EphA10 to be expressed in testis tissues (Fig. S1 (B)). However, EphA10 expression was not

**Table 1**  
EphA10 expression profile in four subtypes of breast cancer.

	Positive cases(ratio)		Negative cases (ratio)		Total cases
Luminal A	22	(54%)	19	(46%)	41
Luminal B	25	(68%)	12	(32%)	37
Her2-enriched	16	(64%)	11	(36%)	27
TNBC	10	(67%)	5	(33%)	15
Total cases	73	(61%)	47	(39%)	120

**Table 2**  
Expression profile of EphA10 in various types of normal tissues.

Tissue	Positive cases (ratio)		Tissue	Positive cases (ratio)	
Adrenal gland	0/3	(0%)	Nerve	0/3	(0%)
Bladder	0/3	(0%)	Ovary	0/3	(0%)
Bone marrow	0/3	(0%)	Pancreas	0/3	(0%)
Breast	0/3	(0%)	Parathyroid gland	0/3	(0%)
Cerebellum	0/3	(0%)	Pituitary	0/3	(0%)
Cerebral gray matter	0/3	(0%)	Prostate	0/3	(0%)
Cerebral white matter	0/3	(0%)	Salivary gland	0/3	(0%)
Colon	0/3	(0%)	Skeletal muscle	0/3	(0%)
Esophagus	0/3	(0%)	Skin	0/3	(0%)
Eye	0/3	(0%)	Small intestine	0/3	(0%)
Head and neck	0/3	(0%)	Spleen	0/3	(0%)
Heart	0/3	(0%)	Stomach	0/3	(0%)
Kidney	0/3	(0%)	Testis	3/3	(100%)
Larynx	0/3	(0%)	Thymus gland	0/3	(0%)
Liver	0/3	(0%)	Thyroid	0/3	(0%)
Lung	0/3	(0%)	Tonsil	0/3	(0%)
Lymph node	0/3	(0%)	Uterine cervix	0/3	(0%)
Mesothelium	0/3	(0%)	Uterus	0/3	(0%)

observed in the other 35 kinds of normal tissues tested (Table 2). Moreover, we also confirmed the testis-specific expression profile of EphA10 at the mRNA level *via* real-time quantitative PCR methods (Fig. S2). Although a modest degree of EphA10 mRNA expression was detected in colon, kidney and small intestine tissues, this was not observed at the protein level by IHC staining (Table 2 and Fig. S1 (B)). These data suggested that EphA10 is a highly specific tumor antigen. Therefore, EphA10 is a potentially useful target for breast cancer including TNBC on the basis of particular expression profiles.

### 3.2. Cell proliferation was promoted by EphA10 ligand stimulation and inhibited by co-addition of anti-EphA10 mAb *in vitro*

Clarifying the role of EphA10 in cancer is essential for the development of safe and effective drugs targeting this protein. In order to analyze its function in breast cancer, we generated cell lines ectopically overexpressing EphA10 and compared the phenotype of these cells *versus* the corresponding parental line. For this, we infected EphA10-encoding lentiviral particles into MDA-MB-435 cells (which express a low level of EphA10), generating transduced cells stably overexpressing EphA10 (MDA-MB-435<sup>EphA10</sup>) (Fig. S3 (A)). The expression level of EphA10 observed in certain clinical breast tumor tissues was similar to that seen in MDA-MB-435<sup>EphA10</sup> cells (Fig. S3 (B)), suggesting that the transduced cells were suitable for functional analysis of EphA10 *in vitro*. In order to analyze the effects of EphA10 signaling on tumor cell proliferation, a key hallmark of malignancy, we compared the rates of BrdU incorporation (a measure of DNA synthesis) between MDA-MB-435<sup>EphA10</sup> and parental cells following stimulation with the EphA10 ligands, EphrinA3, EphrinA4 and EphrinA5. Addition of the ligands to both cell lines significantly induced BrdU incorporation in a dose-dependent manner in MDA-MB-435<sup>EphA10</sup> cells as compared to MDA-MB-435 cells (Fig. 1(A) and (B)). Next, we evaluated the proliferative activity of breast cancer cells that endogenously express EphA10. We examined EphA10 expression levels in three different breast cancer cell lines by quantitative RT-PCR assay and found that MDA-MB-436, TNBC cells, contained a moderate level of endogenous EphA10 (Fig. S4). Thus, we also performed a BrdU incorporation assay with EphrinA3, A4 and A5. As a result, Ephrin-dependent proliferation was also observed in MDA-MB-436 cells (Fig. 1(C)). Furthermore, we also confirmed the positive effects on proliferation mediated by EphA10 signaling in MDA-MB-436 cells *via* WST-8 assay (data not shown). These data suggested that EphA10 signaling may promote proliferation of breast cancer cells.

In order to analyze the phenomenon in detail, we generated a first-in-kind inhibitory anti-EphA10 mAb. Binding affinity studies showed

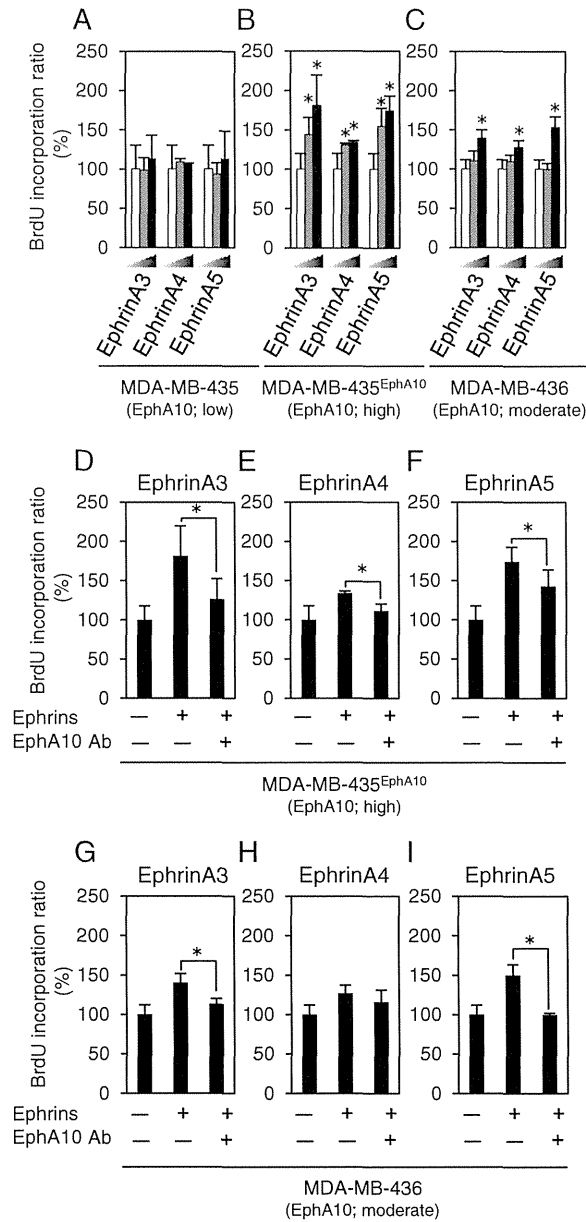
that mAb specifically bound to EphA10, but not to eight other EphA family proteins (Fig. S5 (A)). Moreover, SPR analysis indicated that the mAb displayed an affinity against EphA10 in the nanomolar range (KD = 1.9 nM) (Fig. S5 (B)). Furthermore, flow cytometric analysis showed that the mAb reacted with MDA-MB-435<sup>EphA10</sup> but not with parental cells (Fig. S5 (C)). These data suggested that the anti-EphA10 mAb had high specificity and affinity towards its target, equivalent to that of existing antibody-based drugs such as trastuzumab (KD = 1–5 nM) and bevacizumab (KD = 1.1 nM).

In order to evaluate the potential inhibitory effect on EphA10 signaling, we added the anti-EphA10 mAb to the BrdU incorporation assay. As a result, the rate of BrdU incorporation was significantly inhibited in a group co-treated with EphA10 ligands and anti-EphA10 mAb in EphA10 transfectant cells (MDA-MB-435<sup>EphA10</sup>) (Fig. 1(D)–(F)) and native TNBC cells (MDA-MB-436) (Fig. 1(G)–(I)). Consequently, inhibition of EphA10 signaling *via* this antibody suppressed ligand-dependent stimulation of tumor cell proliferation. These findings suggested that EphA10 targeted therapy might have significant potency in a breast cancer context.

### 3.3. Proliferation induced by EphA10 signaling may be mediated by p38 phosphorylation

In order to analyze the molecular mechanism underlying EphA10-mediated proliferation, we focused on the mitogen-activated protein kinase (MAPK) pathway, which is centrally involved in regulating cell proliferation. In the case of many growth factor receptors, such as epidermal growth factor receptor or vascular endothelial growth factor receptor, cell proliferation is caused by promoting the phosphorylation of Erk1/2 [28–30]. Moreover, in some receptor classes, such as transforming growth factor receptor or fibroblast growth factor receptor, cell proliferation is caused by promoting the phosphorylation of p38 [31,32]. Therefore, we compared the phosphorylation of Erk1/2 or p38 in MDA-MB-435<sup>EphA10</sup> and parental cells following stimulation with EphA10 ligands. Western blotting analysis showed that the degree of phosphorylated Erk1/2 in both cell types essentially unchanged following exposure to any of the EphA10 ligands (Fig. S6 (A)). Next, we compared the extent of phosphorylated p38 between EphA10-overexpressing and parental MDA-MB-435 cells. In this context, the level of phosphorylated p38 in parental MDA-MB-435 cells was practically unchanged in response to any of the EphA10 ligands. On the other hand, phosphorylated p38 levels in MDA-MB-435<sup>EphA10</sup> cells tended to be elevated following stimulation with EphrinA3, A4 and A5 (Fig. S6 (B)). In order to reveal the relationship of p38 phosphorylation to Ephrin-mediated effects on cell proliferation, we analyzed BrdU





**Fig. 1.** BrdU incorporation analysis in relation to EphA10 signaling. After incubation of EphrinA3-Fc, EphrinA4-Fc or EphrinA5-Fc at a series of concentrations (white, gray or black bars indicate 0, 0.25, 4 μg/ml, respectively) with (A) MDA-MB-435, (B) MDA-MB-435<sup>EphA10</sup> or (C) MDA-MB-436 cells for 12 h, cell proliferation mediated by EphA10 was evaluated by measuring the rate of BrdU incorporation (\*:  $p < 0.05$  vs non-treatment group, one-way ANOVA). Moreover, (D)–(F) EphrinA3-Fc, (E)–(H) EphrinA4-Fc and (F)–(I) EphrinA5-Fc (4 μg/ml) and/or anti-EphA10 mAb (20 μg/ml) were incubated with (D)–(F) MDA-MB-435<sup>EphA10</sup> or (G)–(I) MDA-MB-436 cells for 12 h. Cell proliferation and inhibition effects were analyzed as before (\*:  $p < 0.05$  vs EphA10 ligand only group, one-way ANOVA). Error bars indicate the mean + S.D. ( $n = 4–6$ ).

**Table 3**  
Kinetic parameters of the interaction between EphA10 and its ligands.

	$k_a$ ( $M^{-1} s^{-1}$ )	$k_d$ ( $s^{-1}$ )	KD (M)
EphrinA3	$1.3 \times 10^6$	$1.9 \times 10^{-3}$	$1.4 \times 10^{-9}$
EphrinA4	$3.7 \times 10^5$	$1.4 \times 10^{-3}$	$3.8 \times 10^{-9}$
EphrinA5	$1.0 \times 10^6$	$9.2 \times 10^{-4}$	$8.9 \times 10^{-10}$

Indication of each kinetic parameter is as follows.

$k_a$ : association rate constant.

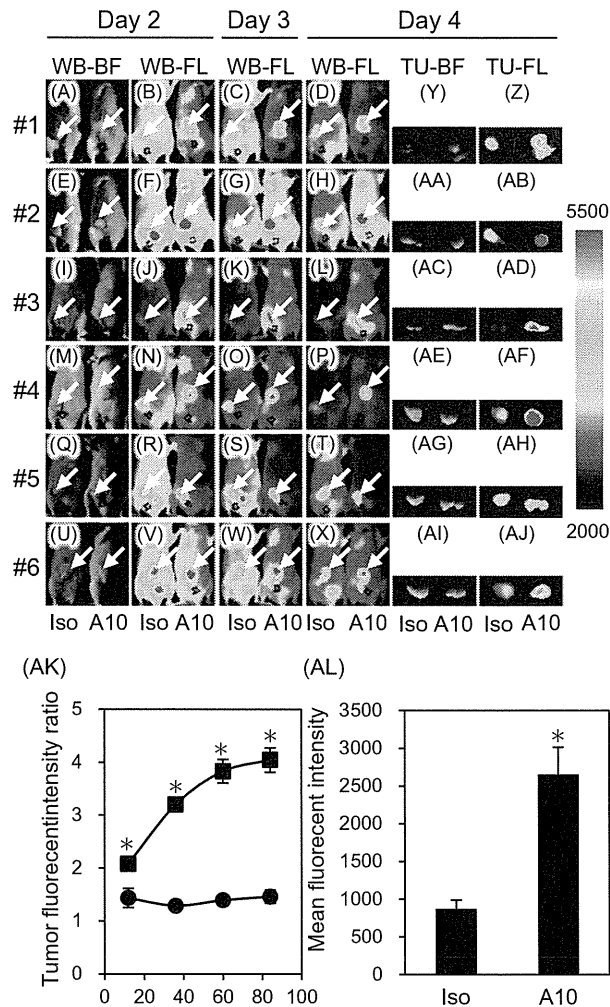
$k_d$ : dissociation rate constant.

KD: equilibrium dissociation constant.

incorporation following Ephrin exposure plus/minus a p38 inhibitor (SB203580). The results showed that the rate of BrdU incorporation was significantly inhibited in cells co-treated with the p38 inhibitor (Fig. S6 (C)). These data suggested that cell proliferation by EphA10 signaling may be mediated via p38 phosphorylation.

### 3.4. Affinity of EphA10 ligands, EphrinA3, A4 and A5 against EphA10

It has previously been reported that EphrinA3, A4 and A5 bind as ligands to EphA10 [26]. However, the affinities of these ligands for EphA10 have not been clarified as yet. In order to evaluate the potential difference in signal transduction activities caused by stimulation of



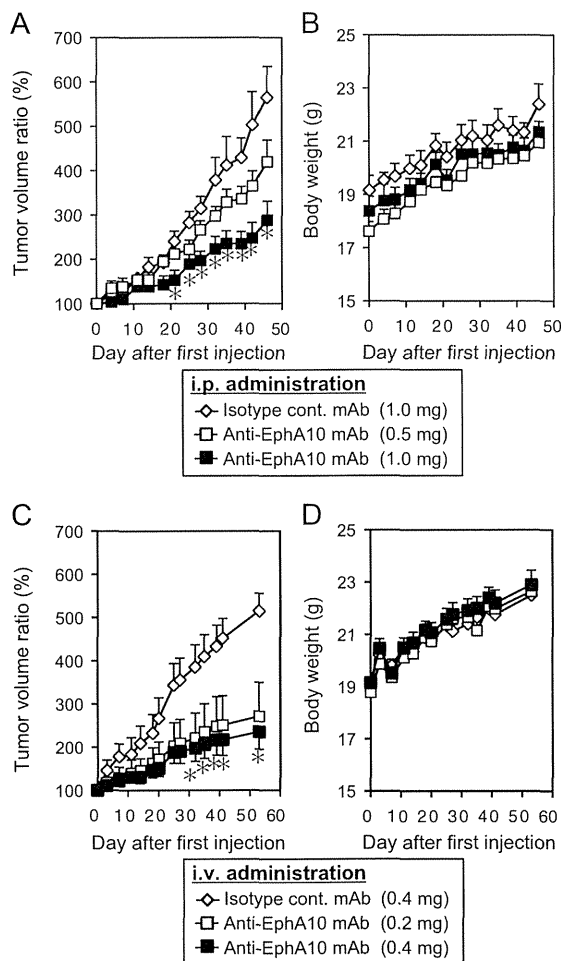
**Fig. 2.** *In vivo* biodistribution of anti-EphA10 mAb in a xenograft mouse model. Alexa Fluor 647-labeled anti-EphA10 mAb or an isotype control mAb was intravenously administered (200  $\mu$ g/mouse) into a xenograft mouse model carrying EphA10-expressing tumors ( $n = 6$ ). (A)–(X) Fluorescence was observed on a daily basis using a whole animal imaging system. The panel on the extreme left and the adjacent panels are brightfield (day 2) and fluorescent (day 2, 3 and 4) images, respectively. The left- and right-hand mice in each image were treated with Alexa Fluor 647-labeled control mAb and anti-EphA10 mAb, respectively. Arrows in each image indicate location of tumor tissues. WB, BF, FL and TU in the upper panel indicate whole body, brightfield, fluorescence and tumor tissue, respectively. Iso and A10 indicate Alexa Fluor 647-labeled isotype control mAb treated mice and Alexa Fluor 647-labeled anti-EphA10 mAb treated mice, respectively. (Y)–(AJ) At day 4, tumor tissues were isolated from dissected mice, with associated fluorescence measured. The left- and right-hand panels are brightfield and fluorescent images of the tumor tissues, respectively. The right-hand bar chart indicates fluorescent intensity range. (AK) Tumor accumulation of anti-EphA10 mAb (■) and the control mAb (●) was quantified as the ratio of fluorescence intensity observed at the tumor over that seen at the contralateral site skin. \* $p < 0.05$  ( $t$ -test). (AL) Mean fluorescent intensity of each tumor tissue was quantified. \* $p < 0.05$  ( $t$ -test). Error bars indicate the mean  $\pm$  S.E.

various ligands, we calculated the kinetic parameters of particular interactions. SPR analysis showed that all ligands bound to EphA10 with a high affinity (KD value of the interaction between EphA10 and EphrinA3; 1.4 nM, EphA10 and EphrinA4; 3.8 nM, EphA10 and EphrinA5; 0.89 nM) (Table 3). Their affinities (EphrinA5  $\geq$  EphrinA3 > EphrinA4) tend to be correlated with the rate of BrdU incorporation. These findings suggest that variation in EphA10 signaling activity and associated cellular responses is due to the affinity of particular ligands against EphA10.

### 3.5. Anti-EphA10 mAb accumulated in EphA10-expressing tumor tissues *in vivo*

In order to evaluate the behavior of anti-EphA10 mAb *in vivo*, we assessed its biodistribution in xenograft mice bearing EphA10-expressing tumors. Alexa Fluor 647-labeled anti-EphA10 mAb or control mAb was intravenously injected into the mice, with fluorescence

assessed on a daily basis thereafter. *In vivo* images showed that fluorescence was non-specifically detected across the whole body of mice treated with either the anti-EphA10 mAb or the control mAb at day 2. At day 4, fluorescence accumulated more in the tumor tissues of anti-EphA10 mAb-treated mice compared to the control mAb-treated mice (Fig. 2 (A)–(X)). The ratio of fluorescent intensity of tumor tissues against that seen in the contralateral site was practically unchanged in the control antibody-treated group, while the ratio was increased within the anti-EphA10 mAb-treated group at the same timepoint (Fig. 2 (AK)). Indeed, the mean fluorescent intensity of tumor tissues isolated from dissected mice at day 4 was significantly stronger within the anti-EphA10 mAb-treated group than in the control mAb-treated group (Fig. 2 (Y)–(AJ) and (AL)). In order to validate the specificity of anti-EphA10 mAb *in vivo*, we compared the accumulation of anti-EphA10 mAb in MDA-MB-435 or MDA-MB-435<sup>EphA10</sup> xenograft model mice. As a result, the mAb significantly accumulated in MDA-MB-435<sup>EphA10</sup> tumor tissues compared to MDA-MB-435



**Fig. 3.** *In vivo* anti-tumor effects of anti-EphA10 mAb in a xenograft mouse model. A xenograft mouse model was generated by orthotopic transplantation of MDA-MB-435<sup>EphA10</sup> cells. When tumors reached approximately 100 mm<sup>3</sup> in size, (A) (B) an isotype control mAb (1.0 mg/mouse) (◇) and anti-EphA10 mAb (0.5 (□) or 1.0 (■) mg/mouse) were intraperitoneally administered twice a week. (C) (D) An isotype control mAb (0.4 mg/mouse) (◇) and anti-EphA10 mAb (0.2 (□) or 0.4 (■) mg/mouse) were intravenously administered once a week. (A) (C) Tumor volumes were measured over time and their growth ratio against the tumor volume at day 0 was compared with the control mAb group (\**p* < 0.05, one-way ANOVA). (B) (D) Body weight change in mice treated with the control mAb or anti-EphA10 mAb. Error bars indicate the mean + S.E. (n = 5–6).

tumor tissues (Fig. S7). Therefore, the anti-EphA10 mAb accumulated in EphA10-expressing tumor tissues, indicating its potential as an anti-tumor therapeutic tool.

### 3.6. Anti-EphA10 mAb caused tumor growth suppression *in vivo*

In order to evaluate the biological effects of the anti-EphA10 mAb accumulated in tumor tissues, we intraperitoneally administered saline, the anti-EphA10 mAb or the control mAb (1 mg/mouse) twice a week into the same xenograft mouse model. Tumor volumes increased in the saline- and the control mAb-treated groups. On the other hand, tumor growth was significantly suppressed within the anti-EphA10 mAb treated group as compared to the other groups (Fig. S8 (A)). Next, we administered the anti-EphA10 mAb (1 mg or 0.5 mg/mouse) or the control mAb (1 mg/mouse). On the basis of volume measurements, tumor growth was significantly suppressed in the case of the anti-EphA10 mAb-treated mice, with this effect occurring in a dose-

dependent manner (Fig. 3 (A)). We obtained tumor tissues from dissected mice at day 47 and measured the corresponding weights. In agreement with volumetric analysis over time, tumor tissue weights were less for the anti-EphA10 mAb-treated groups, with this occurring again in a dose-dependent manner (Fig. S8 (B)). As the body weight of the mice within the different groups was unchanged during the administration (Fig. 3 (B)), indirect effects of mAb administration on body weight loss were ruled out. Furthermore, we observed anti-tumor effects *via* the same administration route (intravenous injection) as used for the biodistribution studies, in a similar vein to that obtained *via* intraperitoneal injection (Fig. 3 (C)–(D) and Fig. S8 (C)). Consequently, the mAb accumulated in EphA10-expressing tumors and significantly suppressed growth without any obvious adverse effects. These findings suggest that EphA10 is a promising drug target potentially useful for breast cancers including TNBC.

## 4. Discussion

EphA10, the most recent addition to the Eph family, is a relatively uncharacterized protein. Thus, its potential as a molecular target has been somewhat unclear. In this manuscript, we showed that EphA10 was specifically expressed in all subtypes of breast tumor tissues, including TNBC, but is absent in normal tissues apart from testis. The proportion of breast tumors expressing EphA10 is around 60% across all subtypes. Therefore, EphA10 expression is independent of ER, PR and Her2 status, suggesting that EphA10 targeted therapy could be utilized as a monotherapy for TNBC cases or as a combination therapy with anti-hormone and anti-Her2 drugs for luminal A, luminal B and Her2-enriched cases, as relevant. Furthermore, a key clinical problem with anti-hormone and anti-Her2 treatments is frequently tolerance to therapy over time [33–36]. Therefore, EphA10 targeted therapy may also be potentially useful in such resistant cases.

We next found that EphA10 signaling promoted DNA synthesis, which was dependent on stimulation by EphA10 ligands. However, clarification of the mechanism involved is needed. In order to elucidate the mechanistic basis underlying the interaction between EphA10 and its corresponding ligands, we examined the expression of particular ligands in tumor tissues in the context of proximity to EphA10. For this, breast cancer tissue derived from the same patient was immunostained with the mAbs against EphrinA3, A4 or A5. IHC staining revealed that all of the ligand molecules were expressed by the breast cancer cells in a similar fashion to that observed with EphA10 (Fig. S9 (A)). Moreover, their ligands were also expressed in MDA-MB-435<sup>EphA10</sup> cells (Fig. S9 (B)). Therefore, one possibility is that EphA10 may be activated *via* its ligands by direct tumor cell-tumor cell contacts in breast cancer patients or MDA-MB-435<sup>EphA10</sup> xenograft model mice. In future studies, it will be of interest to investigate whether anti-EphA10 can suppress signal transduction by intact Ephrins which exist on the cell membrane, as opposed to free form.

Finally, we developed a novel anti-EphA10 mAb as a therapeutic tool, showing it to accumulate in EphA10-positive tumor tissues and capable of mediating significant tumor growth suppression both *in vitro* and *in vivo*. As mentioned previously, the target protein for antibody-based drugs commonly needs to be specifically expressed in cancer tissues, so as not to cause severe adverse events in off-target tissues. We confirmed that while the anti-EphA10 mAb is also able to cross-react with mouse EphA10 (Fig. S4 (D)), the mAb tended to be restricted to tumor compared to normal tissues (Fig. 2). These data are consistent with the pattern of EphA10 protein expression observed in normal human organs (Table 2), further suggesting that the anti-EphA10 mAb could have potential as a safe drug.

Moreover, the mechanism by which the anti-EphA10 mAb inhibits ligand stimulated-proliferation is also interesting. Fig. S4 (E) showed that mAb recognized the fibronectin III (FNIII) domain within the extracellular region of EphA10, an area which consists of the ligand binding (LB) domain and the FNIII domain [6]. It has been suggested that the

FNIII domain plays an important role in the dimerization and activation of receptors [37–39]. Therefore, the inhibitory effects on proliferation may be due to reduced receptor dimerization, as opposed to competitive inhibition in respect to ligand binding. Recently, pertuzumab, a new class of anti-Her2 mAb, has demonstrated powerful and extensive anti-tumor effects in breast cancer patients by inhibiting not only homo-dimerization but also hetero-dimerization such as Her2–Her3 interactions [40,41]. It is also reported that certain Eph family members, such as EphA2 and EphB6, dimerize with other receptors, activate cancer cells and promote malignancy [10,42]. Taken together, our first-in-class anti-EphA10 mAb might have the same potency. On the other hand, it is known that Eph receptor/Ephrin signaling is associated with cancer metastasis [18–20]. We also reported that expression of EphA10 is related to lymph node metastasis in breast cancer patients [43]. It may, thus, be of interest in future work to elucidate the role of EphA10 in tumor metastasis, as well as possible therapeutic targeting of same via anti-EphA10 mAb.

## 5. Conclusions

EphA10 was specifically expressed in various subtypes of breast cancer tissues, but not within most normal tissues. Moreover, EphA10 promoted cell proliferation following ligand stimulation. Conversely, an in-house developed anti-EphA10 mAb inhibited tumor proliferation significantly at both *in vitro* and *in vivo* levels. These data should contribute to the development of novel drugs against refractory breast cancers, including TNBC.

## Acknowledgments

This study was supported in part by Grants-in-Aid for Scientific Research from the Project for Development of Innovative Research on Cancer Therapeutics and the Scientific Research (C) (#26430180), the Ministry of Education, Culture, Sports, Science and Technology of Japan, and from the Japan Society for the Promotion of Science. This study was also supported in part by Health Labor Sciences Research Grants from the Ministry of Health, Labor and Welfare of Japan.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.jconrel.2014.06.010>.

## References

- [1] S.X. Lin, J. Chen, M. Mazumdar, D. Poirier, C. Wang, A. Azzi, M. Zhou, Molecular therapy of breast cancer: progress and future directions, *Nat. Rev. Endocrinol.* 6 (2010) 485–493.
- [2] A. Ocana, J.J. Cruz, A. Pandiella, Trastuzumab and antiestrogen therapy: focus on mechanisms of action and resistance, *Am. J. Clin. Oncol.* 29 (2006) 90–95.
- [3] L. Carey, E. Winer, G. Viale, D. Cameron, L. Gianni, Triple-negative breast cancer: disease entity or title of convenience? *Nat. Rev. Clin. Oncol.* 7 (2010) 683–692.
- [4] S.K. Pal, B.H. Childs, M. Pegram, Triple negative breast cancer: unmet medical needs, *Breast Cancer Res. Treat.* 125 (2011) 627–636.
- [5] F. Podo, L.M. Buydens, H. Degani, R. Hilhorst, E. Klipp, I.S. Gribbestad, S. Van Huffel, H. W. van Laarhoven, J. Luts, D. Monleon, G.J. Postma, N. Schneiderhan-Marra, F. Santoro, H. Wouters, H.G. Russnes, T. Sorlie, E. Tagliabue, A.L. Borresen-Dale, Triple-negative breast cancer: present challenges and new perspectives, *Mol. Oncol.* 4 (2010) 209–229.
- [6] E.B. Pasquale, Eph receptor signalling casts a wide net on cell behaviour, *Nat. Rev. Mol. Cell Biol.* 6 (2005) 462–475.
- [7] E.B. Pasquale, Eph receptors and ephrins in cancer: bidirectional signalling and beyond, *Nat. Rev. Cancer* 10 (2010) 165–180.
- [8] J.G. Albeck, J.S. Brugge, Uncovering a tumor suppressor for triple-negative breast cancers, *Cell* 144 (2011) 638–640.
- [9] T. Sun, N. Aceto, K.L. Meerbrey, J.D. Kessler, C. Zhou, I. Migliaccio, D.X. Nguyen, N.N. Pavlova, M. Botero, J. Huang, R.J. Bernardi, E. Schmitt, G. Hu, M.Z. Li, N. Dephore, S.P. Gygi, M. Rao, C.J. Creighton, S.G. Hilsenbeck, C.A. Shaw, D. Muzny, R.A. Gibbs, D.A. Wheeler, C.K. Osborne, R. Schiff, M. Bentires-Alj, S.J. Eledge, T.F. Westbrook, Activation of multiple proto-oncogenic tyrosine kinases in breast cancer via loss of the PTPN12 phosphatase, *Cell* 144 (2011) 703–718.
- [10] D.M. Brantley-Sieders, G. Zhuang, D. Hicks, W.B. Fang, Y. Hwang, J.M. Cates, K. Coffman, D. Jackson, E. Bruckheimer, R.S. Muraoka-Cook, J. Chen, The receptor tyrosine kinase EphA2 promotes mammary adenocarcinoma tumorigenesis and metastatic progression in mice by amplifying ErbB2 signaling, *J. Clin. Invest.* 118 (2008) 64–78.
- [11] N. Munarini, R. Jager, S. Abderhalden, G. Zuercher, V. Rohrbach, S. Loercher, B. Pfanner-Meyer, A.C. Andres, A. Ziemiecki, Altered mammary epithelial development, pattern formation and involution in transgenic mice expressing the EphB4 receptor tyrosine kinase, *J. Cell Sci.* 115 (2002) 25–37.
- [12] D.P. Zelinski, N.D. Zantek, J.C. Stewart, A.R. Irizarry, M.S. Kinch, EphA2 overexpression causes tumorigenesis of mammary epithelial cells, *Cancer Res.* 61 (2001) 2301–2306.
- [13] S.R. Kumar, J. Singh, G. Xia, V. Krasnopetrov, L. Hassanieh, E.J. Ley, J. Scheinet, N.G. Kumar, D. Hawes, M.F. Press, F.A. Weaver, P.S. Gill, Receptor tyrosine kinase EphB4 is a survival factor in breast cancer, *Am. J. Pathol.* 169 (2006) 279–293.
- [14] K.A. Mohammed, X. Wang, E.P. Goldberg, V.B. Antony, N. Nasreen, Silencing receptor EphA2 induces apoptosis and attenuates tumor growth in malignant mesothelioma, *Am. J. Cancer Res.* 1 (2011) 419–431.
- [15] N.K. Noren, M. Lu, A.L. Freeman, M. Koolpe, E.B. Pasquale, Interplay between EphB4 on tumor cells and vascular ephrin-B2 regulates tumor growth, *Proc. Natl. Acad. Sci. U. S. A.* 101 (2004) 5583–5588.
- [16] S. Sawamiphak, S. Seidel, C.L. Essmann, G.A. Wilkinson, M.E. Pitulescu, T. Acker, A. Acker-Palmer, Ephrin-B2 regulates VEGFR2 function in developmental and tumour angiogenesis, *Nature* 465 (2010) 487–491.
- [17] Y. Wang, M. Nakayama, M.E. Pitulescu, T.S. Schmidt, M.L. Bochenek, A. Sakakibara, S. Adams, A. Davy, U. Deutsch, U. Luthi, A. Barberis, L.E. Benjamin, T. Makinen, C.D. Nobes, R.H. Adams, Ephrin-B2 controls VEGF-induced angiogenesis and lymphangiogenesis, *Nature* 465 (2010) 483–486.
- [18] D.M. Brantley-Sieders, W.B. Fang, D.J. Hicks, G. Zhuang, Y. Shyr, J. Chen, Impaired tumor microenvironment in EphA2-deficient mice inhibits tumor angiogenesis and metastatic progression, *FASEB J.* 19 (2005) 1884–1886.
- [19] M.S. Duxbury, H. Ito, M.J. Zinner, S.W. Ashley, E.E. Whang, EphA2: a determinant of malignant cellular behavior and a potential therapeutic target in pancreatic adenocarcinoma, *Oncogene* 23 (2004) 1448–1456.
- [20] X.D. Ji, G. Li, Y.X. Feng, J.S. Zhao, J.J. Li, Z.J. Sun, S. Shi, Y.Z. Deng, J.F. Xu, Y.Q. Zhu, H.P. Koefler, X.J. Tong, D. Xie, EphB3 is overexpressed in non-small-cell lung cancer and promotes tumor metastasis by enhancing cell survival and migration, *Cancer Res.* 71 (2011) 1156–1166.
- [21] D. Jackson, J. Gooya, S. Mao, K. Kinneer, L. Xu, M. Camara, C. Fazanbaker, R. Fleming, S. Swamynathan, D. Meyer, P.D. Senter, C. Gao, H. Wu, M. Kinch, S. Coats, P.A. Kiener, D.A. Tice, A human antibody-drug conjugate targeting EphA2 inhibits tumor growth *in vivo*, *Cancer Res.* 68 (2008) 9367–9374.
- [22] J.W. Lee, H.D. Han, M.M. Shahzad, S.W. Kim, L.S. Mangala, A.M. Nick, C. Lu, R.R. Langley, R. Schmandt, H.S. Kim, S. Mao, J. Gooya, C. Fazanbaker, D. Jackson, D.A. Tice, C.N. Landen, R.L. Coleman, A.K. Sood, EphA2 immunoconjugate as molecularly targeted chemotherapy for ovarian carcinoma, *J. Natl. Cancer Inst.* 101 (2009) 1193–1205.
- [23] J.W. Lee, R.L. Stone, S.J. Lee, E.J. Nam, J.W. Roh, A.M. Nick, H.D. Han, M.M. Shahzad, H. S. Kim, L.S. Mangala, N.B. Jennings, S. Mao, J. Gooya, D. Jackson, R.L. Coleman, A.K. Sood, EphA2 targeted chemotherapy using an antibody drug conjugate in endometrial carcinoma, *Clin. Cancer Res.* 16 (2010) 2562–2570.
- [24] C.M. Annunziata, E.C. Kohn, P. LoRusso, N.D. Houston, R.L. Coleman, M. Buzoiaru, G. Robbie, R. Lechleider, Phase 1, open-label study of MEDI-547 in patients with relapsed or refractory solid tumors, *Invest. New Drugs* 31 (2013) 77–84.
- [25] S. Imai, K. Nagano, Y. Yoshida, T. Okamura, T. Yamashita, Y. Abe, T. Yoshikawa, Y. Yoshioka, H. Kamada, Y. Mukai, S. Nakagawa, Y. Tsutsumi, S. Tsunoda, Development of an antibody proteomics system using a phage antibody library for efficient screening of biomarker proteins, *Biomaterials* 32 (2011) 162–169.
- [26] H.C. Aasheim, S. Patzke, H.S. Hjorthaug, E.F. Finne, Characterization of a novel Eph receptor tyrosine kinase, EphA10, expressed in testis, *Biochim. Biophys. Acta* 1723 (2005) 1–7.
- [27] K.E. Huber, L.A. Carey, D.E. Wazer, Breast cancer molecular subtypes in patients with locally advanced disease: impact on prognosis, patterns of recurrence, and response to therapy, *Semin. Radiat. Oncol.* 19 (2009) 204–210.
- [28] P.J. Roberts, C.J. Der, Targeting the Raf-MEK-ERK mitogen-activated protein kinase cascade for the treatment of cancer, *Oncogene* 26 (2007) 3291–3310.
- [29] I. Zachary, VEGF signalling: integration and multi-tasking in endothelial cell biology, *Biochem. Soc. Trans.* 31 (2003) 1171–1177.
- [30] H. Zhong, J.P. Bowen, Molecular design and clinical development of VEGFR kinase inhibitors, *Curr. Top. Med. Chem.* 7 (2007) 1379–1393.
- [31] B. Boilly, A.S. Vercouter-Edouart, H. Hondermarck, V. Nurcombe, X. Le Bourhis, FGF signals for cell proliferation and migration through different pathways, *Cytokine Growth Factor Rev.* 11 (2000) 295–302.
- [32] M.B. Buck, C. Knabbe, TGF-beta signaling in breast cancer, *Ann. N. Y. Acad. Sci.* 1089 (2006) 119–126.
- [33] H.J. Johansson, B.C. Sanchez, F. Mundt, J. Forshed, A. Kovacs, E. Panizza, L. Hultin-Rosenberg, B. Lundgren, U. Martens, G. Mathe, Z. Yakhini, K. Helou, K. Krawiec, L. Kanter, A. Hjerpe, O. Stal, B.K. Linderholm, J. Lehtio, Retinoic acid receptor alpha is associated with tamoxifen resistance in breast cancer, *Nat. Commun.* 4 (2013) 2175.
- [34] M. Scaltriti, P.J. Eichhorn, J. Cortes, L. Prudkin, C. Aura, J. Jimenez, S. Chandralapaty, V. Serra, A. Prat, Y.H. Ibrahim, M. Guzman, M. Gili, O. Rodriguez, S. Rodriguez, J. Perez, S. R. Green, S. Mai, N. Rosen, C. Hudis, J. Baselga, Cyclin E amplification/overexpression is a mechanism of trastuzumab resistance in HER2+ breast cancer patients, *Proc. Natl. Acad. Sci. U. S. A.* 108 (2011) 3761–3766.
- [35] S.L. Tilghman, I. Townley, Q. Zhong, P.P. Carriere, J. Zou, S.D. Llopis, L.C. Preyan, C.C. Williams, E. Skripnikova, M.R. Bratton, Q. Zhang, G. Wang, Proteomic signatures of acquired letrozole resistance in breast cancer: suppressed estrogen signaling and increased cell motility and invasiveness, *Mol. Cell. Proteomics* 12 (2013) 2440–2455.