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The EphA3 Receptor Is Expressed in a Subset of Rhabdomyosarcoma Cell Lines and Suppresses Cell Adhesion and Migration

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ABSTRACT

Elevated expression of the Eph receptor tyrosine kinase EphA3 is associated with lymphocytic leukaemia, but little is known about its expression or function in solid tumours. Out of a panel of cancer cell lines, we found that EphA3 was expressed only on two rhabdomyosarcoma (RMS) cell lines of the embryonal histological subtype and on one of the alveolar RMS subtype, whereas it was not detected on two other cell lines of the alveolar subtype. Other EphA receptors (1–7) were, either not expressed in any, or expressed in all five RMS cell lines. Stimulation of EphA3-expressing TE671 and RD RMS cells with ephrinA5 resulted in loss of adhesion to fibronectin, decreased migration towards the stromal cell-derived growth factor-I (SDF-I), increased EphA3 phosphorylation, and increased Rho GTPase activity. In contrast, ectopic expression of EphA3 in the EphA3 negative CRL2061 cell line resulted in decreased cell adhesion. Finally, suppression of EphA3 expression by siRNA in RD cells results in increased SDF-I-mediated motility. These data indicate that EphA3 expression may define subsets of RMS tumours, and that EphA3 suppresses motility through regulation of Rho GTPases in RMS cells. J. Cell. Biochem. 105: 1250–1259, 2008.

KEY WORDS: EphA3; RHABDOMYOSARCOMA; MIGRATION

With 16 members, the Eph receptor family is the largest subgroup of the receptor tyrosine kinase (RTK) family. It is divided into two subclasses, A and B, based on distinct structural properties of their ligands, the ephrins [Committee, 1997]. The Eph receptors and ephrins are membrane-bound proteins whose interactions initiate unique bidirectional signalling events in both the receptor- and the ligand-expressing cells [Dodelet and Pasquale, 2000]. Ephs and ephrins are frequently expressed on adjacent or even the same cell populations and exhibit a characteristic promiscuity, with the possibility for ephrin ligands to ligate and activate more than one member of the Eph receptor family [Gale et al., 1996; Smith et al., 2004a]. Crosstalk between Ephs and other receptors has also been reported as well as the formation of stable receptor complexes between different members of the Eph family, all of which adds to the complexity of Eph/ephrin bidirectional signalling [Murai and Pasquale, 2003].

Altered expression of Ephs and ephrins is associated with angiogenesis and tumour vasculature in many types of human cancers, including breast, lung, prostate cancers, melanoma, and leukaemia [Dodelet and Pasquale, 2000; Clevers and Batlle, 2006], reviewed in Surawska et al. [2004]. EphA2 expression is associated with aggressive cancer progression and metastasis [Easty et al., 1995; Zelinski et al., 2001]. The EphA3 receptor (also called HEK) is increased without apparent amplification or rearrangement in human lymphoid tumour cell lines, which raises the possibility that overexpression of EphA3 is a contributing factor in lymphoid malignancy [Wicks et al., 1992]. EphB4 was found to be highly expressed in the outer layer of the tumour cell mass in grade III

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breast carcinomas, which is the most likely part of the tumour mass to metastasise, [Berclaz et al., 1996]. Both EphB4 and EphA2 were found to be expressed in invasive mammary tumours but not in non-metastatic mammary tumours [Andres et al., 1994].

Although many reports document increased expression of Ephs in tumours, suppressed expression of the Eph receptor/ligand system is also associated with cancer. EphB4 is considered to be a tumour suppressor because its expression is suppressed in colorectal carcinoma [Batlle et al., 2005; Davalos et al., 2006]. Loss of expression of EphB6, which lacks kinase activity [Shimoyama et al., 2000], correlates with metastatic melanoma and poor prognosis in human neuroblastoma [Hafner et al., 2003, 2006]. Eph receptor signalling outputs include the generation of adhesive or repulsive signals associated with cell migration [Wimmer-Kleikamp and Lackmann, 2005; Parri et al., 2007], axon guidance [Egea and Klein, 2007], tissue patterning [Poliakov et al., 2004] and angiogenesis [Kuijper et al., 2007]. These effects are mediated largely through recruitment of signalling molecules associated with cytoskeletal organization and integrin signalling [Miao et al., 2000], Rho GTPase [Parri et al., 2007] or Ras-Map signalling [Miao et al., 2001]. The mechanisms underlying the repulsive responses are complex and are not fully characterised. However, they involve loss of Eph-ephrin interactions, either by cleavage [Hattori et al., 2000] or internalisation of receptors [Marston et al., 2003; Zimmer et al., 2003; Cowan et al., 2005]. They also involve increased contractility of Eph-expressing cells resulting in the withdrawal of cellular extensions [Wahl et al., 2000; Parri et al., 2007].

We previously reported that the expression of EphA3 was induced by IGF-I in neoplastic but not in normal T cells [Smith et al., 2004b]. EphA3 expression is known to be associated with B and T cell malignancies [Wicks et al., 1992; Dottori et al., 1999; Fox et al., 2006], but the expression or function of EphA3 in solid tumours has not been widely studied. Since IGF-I signalling is associated with many different tumours [Smith et al., 2004b], we were interested to investigate EphA3 expression and function in non-lymphocytic tumour cells. We found that out of a series of cancer cell lines, EphA3 was detected only in a subset of rhabdomyosarcoma (RMS) cell lines.

RMS is a fast-growing, highly malignant tumour, which accounts for over half of the soft tissue sarcomas in children. RMS tumours are classified according to their histology, with two major histological subtypes: the more prevalent embryonal RMS (ERMS) subtype and the more aggressive alveolar RMS (ARMS) subtype. At the molecular level, ERMS is frequently associated with loss of heterozygosity of 11p15.5 [Gil-Benso et al., 2003], a region thought to encode a tumour suppressor gene [Xia et al., 2002]. In contrast, ARMS is normally characterized by a specific chromosomal translocation which gives rise to a novel chimeric fusion protein between the 5′ DNA binding domain of PAX3 [t(2;13)] or PAX7 [t(1;13)] and the 3′ transactivation domain of FKHR [Davis and Barr, 1997; Kelly et al., 1998; Bennicelli et al., 1999]. Members of the PAX (paired box) gene family PAX3 and PAX7 have critical roles as master regulators of organogenesis [Dahl et al., 1997; Mansouri et al., 1999], and mutations of the *pax* genes can cause profound developmental defects [Chi and Epstein, 2002]. The PAX-FKHR fusion protein is a much more potent transcriptional activator than wild-type PAX and this deregulation has been correlated with increased metastasis [Kouraklis et al., 1999].

Here we investigated the function of EphA3 and the effects of modulating its expression in RMS cell lines. Our data indicate that EphA3 expression in a subset of RMS cells is associated with decreased directional migration and increased Rho GTPase signalling.

MATERIALS AND METHODS

REAGENTS

Monoclonal anti-phosphotyrosine antibody (PY20) was obtained from BD Transduction Laboratories (Cowley, Oxford, UK) and the anti-phospho-p42/44 MAP kinase and p42/44 MAP kinase monoclonal antibodies were from Cell Signalling Technologies (Danvers, MA). The anti-EphA3 polyclonal antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-β-actin and tubulin antibodies were purchased from Sigma (Dublin, Ireland). EphrinA5/Fc human Fc chimeric fusion protein, control human proteins, and SDF-1β were from R&D Systems Europe Ltd (Abingdon, UK). Goat anti-human Fc antibody was from Jackson Laboratories (Soham, Cambridgeshire, UK). The Rho activation kit was purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Protein G sepharose was purchased from GE Healthcare (Giles, Bucks, UK).

CELL CULTURE AND TRANSFECTION

Two haematopoietic cell lines HL-60 and Jurkat, the breast cancer cell line MCF-7, the prostate cancer cell line DU-145, the neuroblastoma cell line SHSY5Y, and the colon cancer cell lines SW620 and SW480 were used in this study, together with five human RMS cell lines comprising three ARMS lines (KM77, CRL2061 and FLOH-1) established in University of Würzburg and two ERMS lines (RD and TE671). These cell lines and the 293T human embryonic kidney cell line were maintained in RPMI 1640 or DMEM supplemented with 10% heat inactivated FCS, 2 mM each of penicillin, streptomycin and L-glutamine (all from Biowhittaker). The cells were cultured in a humidified atmosphere of 5% CO_2, and 37°C.

CRL2061 cells (10 cm plate; 70% confluent) were transiently transfected with pEFBOS-EphA3 wild-type, pEFBOS-EphA3 3YF (inactive EphA3, having the three major critical tyrosines mutated to phenylalanine) and pEFBOS-Vector (a kind gift from Dr. A. Lackmann) using 8 μg of DNA and LipofectAMINE Plus (Invitrogen, Paisley, UK).

Hek293T cells in 10 cm tissue culture plates were transfected with 6 μg total plasmid DNA using the calcium phosphate transfection method. Briefly, plasmid DNA was added to 61 μl $CaCl_2$ and 438 μl H_2O, and this was then added drop-wise to 500 μl Hank's Buffered Salt Solution (HBSS). A final volume of 1 ml of DNA solution was added drop-wise to a 10 cm tissue culture plate containing 10 ml of culture media.

IMMUNOFLUORESCENCE

Cell surface expression of EphA3 on various tumour cell lines was evaluated by fluorescence-activated cell sorting (FACS) analysis as previously described [Smith et al., 2004b]. Briefly cells (5×10^5 per

sample) were suspended in 100 μl RPMI containing 25 mM HEPES, 10% horse serum and 0.01% azide (FACS buffer) with the indicated antibodies (2 μg/ml). Cells were incubated for 1 h, washed and resuspended in FACS buffer containing FITC-labelled secondary antibody specific for mouse IgG. Following 30 min incubation at 4°C, cells were again washed and the cell-associated fluorescence was quantified with a FACScan flow cytometer and Cell quest software (Becton Dickinson).

RT-PCR

First strand cDNA synthesis from total RNA, was carried out by reverse-transcription of equal amounts (2 μg) of DNA-free RNA using a cDNA synthesis kit from Roche (Boehringer-Mannheim, East Sussex, UK). Unless otherwise indicated Polymerase Chain Reaction (PCR) using HotStarTaq (Qiagen, Crawley, UK) was then carried out using the primers listed in Table I, with the following conditions: incubation at 95°C for 15 min to activate the polymerase followed by cycling at 94°C for 30 s, 55°C for 30 s, and 72°C for 60 s. The primers were designed to ensure amplification of specific Eph family members using amplifying regions that spanned between 150 and 250 bp (base pairs).

CELL ADHESION ASSAYS

Fibronectin (0.5 and 1.0 μg/well) in phosphate-buffered saline (PBS) was coated onto 96-well microtitre plates overnight at 4°C. Non-specific binding sites were blocked with 1% bovine serum albumin (BSA) in PBS for 2 h at 37°C. Where appropriate cells were stimulated with ephrinA5/Fc as described previously [Smith et al., 2004b] and reseeded onto fibronectin-coated plates at a density of 2×10^4 cells/well. Following incubation at 37°C for 30 min non-adherent cells were washed away with PBS. Cells were fixed with 100% methanol for 5 min. Crystal violet (0.1%) was then added for 15 min and the cells were destained by extensive washing in tap water and allowed to air dry. The dye was solublised by addition of using 0.5% Triton X-100 in PBS, and the absorbance was measured at 590 nm in a microtitre plate reader. Triplicate samples were examined for each condition and each experiment was repeated at least three times with similar results.

CELL MOTILITY ASSAYS

Directional movement of cells toward stromal-cell derived growth factor-1 (SDF-I) across a 5 μM pore polycarbon membranes of Boyden chambers/transwells (Costar, Cambridge, MA) was determined. RMS cells were serum starved for 4 h by incubating with 0.5% BSA in serum-free DMEM. Top and bottom of membranes were coated by incubating in fibronectin and ephrinA5/Fc for 2 h at 37°C and discarding the solution prior to use. Cells were detached from plates by incubating with 0.5 mM EDTA/PBS, washed with DMEM medium and resuspended in DMEM containing 0.5% BSA. Cells (1×10^5) were seeded into the upper chamber of a transwell insert at a density of SDF-I, at a concentration of 300 ng/ml in serum-free medium, was added to the lower chamber. After 24–48 h the insert was removed from the transwell. Cells remaining in the upper chamber were removed by scraping, and the number of cells that had transmigrated were fixed with methanol, stained with 0.5% crystal violet, and air-dried for counting under microscope. For Hek293T transfected cells, the same procedure were applied except directional movement toward 10% foetal bovine serum (FBS) after 16 h was examined. The data are presented as the averages of counts from five fields of triplicate wells for each test condition and the experiments were repeated at least three times.

WESTERN BLOTTING

Whole cell lysates were prepared using lysis buffer (150 mM NaCl, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0, 1 mM PMSF, 1 μM pepstatin, 2 mg/ml aprotinin, 1 μM $NaVO_4$) and were separated by SDS/PAGE, transferred to nitrocellulose membranes (Schleicher and Schuell, GmbH, Dassel, Germany) and were probed with the appropriate antibodies according to the manufacturer's instructions. Polyclonal anti-IGF-IR (insulin-like growth factor-1) and anti-EphA3 antibodies were obtained from Santa Cruz Biotechnology. Membranes were either incubated with horseradish peroxidase (HRP)-conjugated secondary antibody at room temperature for 1 h, and antibody reactive bands were detected using enhanced chemiluminescence solution (Amersham Pharmacia Biotech, Buckinghamshire, UK) or using a LI-COR Odyssey infrared imaging system (LI-COR Biosciences UK Ltd, Cambridge, UK). For quantification purposes, Western blot analysis was performed and fluorescent dye-conjugated (IR Dye™ 800-labeled or IR Dye™ 680-labeled, goat anti-rabbit or anti-mouse IgG) secondary antibodies were employed and detected with the LI-COR Odyssey Infrared Imaging System according to the manufacturer's instruction (LI-COR Biosciences). Densitometry was directly performed on the blot using the LI-COR Odyssey Analysis program.

SUPPRESSION OF EphA3 USING siRNA

The siRNA sequence targeting human EphA3 (from mRNA sequence Gen-Bank accession number NM_005233) and mouse EphA3 (from mRNA sequence Gen-Bank accession number XM_148529)

TABLE I. Primer Sequences and Expected size of Amplified Products

Transcript	Forward primer	Reverse primer	Size of product
EphA1	TgCAAggTgTCTgACTTTgg	TCATCTCCCCATAAggCTTg	205
EphA2	gAgggCgTCATCTCCAAATA	TCAgACACCTTgCAgACCAg	236
EphA4	gCTTCACCCAAgTggACATT	gACgTATCAgCCCCTgTgAT	213
EphA5	TTgCATTgCTCTggTTTCTg	CATCggTCACAgAATggTTg	151
EphA6	AAgCCATCgCCTACAgAAAA	TggAgCATCAgCTggTgTAg	195
EphA7	AAgCAggCTACCAgCAAAAA	ggTCAgATggAgCCCTgTAA	171

DNA oligonucleotides used as forward and reverse primers were designed to specifically amplify each of the indicated EphA receptors in RT-PCR reactions. The sequence of each oligonucleotide is indicated and the predicted size of the transcript amplification product (base pairs) for each set of primers is indicated in right column.

corresponds to the coding region relative to the first nucleotide of the start codon. As recommended by Dharmacon, EphA3-targeted regions were selected beginning 50–100 nucleotides downstream from the start codon. Sequences close to 50% G/C content were chosen. Specifically, human EphA3; 5′-aagagaucaguggugguggaug-3′; mouse EphA3; 5′-aagagaucaguggugu**u**gaug-3′ (corresponding to nucleotides 155–175 after the start codon for both human and mouse EphA3). Nucleotide typed in bold indicate where the mouse siRNA differs from the human. The mouse siRNA was used as a negative control.

For transfection, cells (30–50% confluent) were incubated with 200 pmol oligonucleotide using the OligofectAMINE transfection reagent according to the protocol provided by Invitrogen. Cells were incubated for at least 48 h before biochemical experiments and/or functional assays were conducted as described.

Rho/Rac1 ACTIVATION ASSAY AND IMMUNOPRECIPITATION

Rho and Rac1 pulldown assays were performed with GST-Rhotekin or GST-PAK (Upstate Biotechnology, Inc.) according to the manufacturer's instructions. TE671 cells were serum starved for 4 h and stimulated with pre-clustered ephrinA5/Fc as described previously [Smith et al., 2004b]. SDS–PAGE resolved samples, transferred to nitrocellulose membranes (Schleicher and Schuell, GmbH) were probed with anti-RhoA/B/C or anti-Rac1. A blot of the cell lysate was also probed with anti-RhoA/B/C or anti-Rac1 as loading control.

For immunoprecipitation experiments, TE671 cells were serum starved for 4 h and stimulated with pre-clustered ephrinA5/Fc as described previously [Smith et al., 2004b]. Extracts from stimulated or unstimulated cells were initially pre-cleared using protein G sepharose beads (15 μl beads per 400 μg of total protein in 700 ml lysis buffer) by incubation at 4°C for 1 h with gentle rocking. The lysates were recovered from the beads by centrifugation at 3,000 rpm for 3 min and transferred to fresh tubes for incubation with primary antibody (5 μg of each antibody) for 16 h at 4°C with gentle rocking. Immune complexes were obtained by adding 30 μl of protein G sepharose beads for 1 h at 4°C. The beads were washed (three times) with ice-cold lysis buffer, and the immune complexes were then removed from the beads by boiling for 5 min in 20 μl of 2× SDS–PAGE sample buffer for electrophoresis and Western blot analysis.

RESULTS

LIGATION OF EphA3 ECTOPICALLY EXPRESSED IN Hek293T RESULTS IN DECREASED ADHESION AND MIGRATION

We have previously reported that ligation of EphA3 results in decreased attachment of Jurkat leukaemic T cell lines to fibronectin [Smith et al., 2004b]. To further investigate if EphA3 can generally regulate cell adhesion and indeed cell migration in adherent cells, and to eliminate the possibility of contributions from other Ephs, the EphA3 receptor was transiently expressed in Hek293T cells. Hek293T cells lack endogenous type A Eph receptors and ephrins [Nakamoto and Bergemann, 2002], and have previously been used to study Eph function [Holmberg et al., 2000]. Ephrin-A5/Fc stimulation of 293T cells overexpressing the EphA3 receptor resulted in decreased attachment to fibronectin compared to Vector-transfected cells (Fig. 1A). Directional migration towards FBS was also decreased in EphA3-expressing cells, whereas no significant change in migration was detected when Vector cells were exposed to ephrinA5/Fc (Fig. 1B). The expression levels of EphA3 in cells tested in Figure 1A,B is shown (Fig. 1C). These data indicate that ephrinA5 stimulation decreases adhesion and migration of Hek293T cells overexpressing EphA3.

EphA3 IS EXPRESSED IN A SUBSET OF RHABDOMYOSARCOMA CELL LINES

We next sought to examine whether EphA3 is widely expressed and active in different cancer cell lines. To do this EphA3 expression was analysed by flow cytometry in a panel of cell lines derived from different tumours. The results demonstrated that EphA3 was not detectable on the myeloid leukaemia cell line HL-60, on the breast cancer cell line MCF-7, the prostate cancer cell line DU-145, the neuroblastoma cell line SHSY5Y, or the colon cancer cell lines SW620 and SW480 (Fig. 2A). EphA3 expression was however detected on the RMS cell line, TE671 (Fig. 2B).

To determine whether EphA3 expression is a general feature of RMS, another ERMS cell line, RD and three primary RMS cell lines, FLOH-1, CRL2061 and KM77 were examined. EphA3 was not expressed on the t(2;13) translocation positive ARMS cell lines KM77 and CRL2061, but was abundantly expressed on a translocation negative ARMS cell line, FLOH-1, and also on the ERMS cell line, RD (Fig. 2B).

As EphA3 can be induced by insulin-like growth factor-1 (IGF-I) in Jurkat cells [Smith et al., 2004b], and since IGF-I signalling is associated with many different tumours [Smith et al., 2004b], we were interested to investigate whether EphA3 expression was also induced by IGF-I in the solid tumour cell lines TE671 and RD. We found that EphA3 expression is upregulated in these cell lines after 2 h IGF-I stimulation (Fig. 2C).

Overall these data indicate that EphA3 expression is not widely expressed in different cancer cell lines but that it is associated with a subset of RMS cells where it can be induced by IGF-I.

RT-PCR CONFIRMS RESTRICTED EphA3 EXPRESSION AND WIDESPREAD EXPRESSION OF OTHER EphA RECEPTORS IN RMS CELL LINES

Due to the potential for promiscuous signalling interactions between Eph family members it was next necessary to determine which other EphA receptors were expressed in the RMS cell lines. RT-PCR was carried out using gene specific primers (Table I) for each of the Eph receptors A1–A7 and the relative abundance of EphA transcripts was quantified by densitometry. The results shown in Table II are in agreement with results in the cell surface expression of EphA3 observed by flow cytometry analysis (Fig. 2B). EphA3 receptor mRNA was expressed in TE671 and RD cells but not in KM77 or CRL2061 cells. EphA1, EphA2 and EphA4 receptors were detected in all cell lines tested albeit at a low level, whereas EphA5, EphA6 and EphA7 receptors were all present at low levels in CRL2061 cells, and not generally in the other cell lines. These data indicate that out of all the Eph family members tested, EphA3 alone exhibits a pattern of restricted expression in RMS cells.

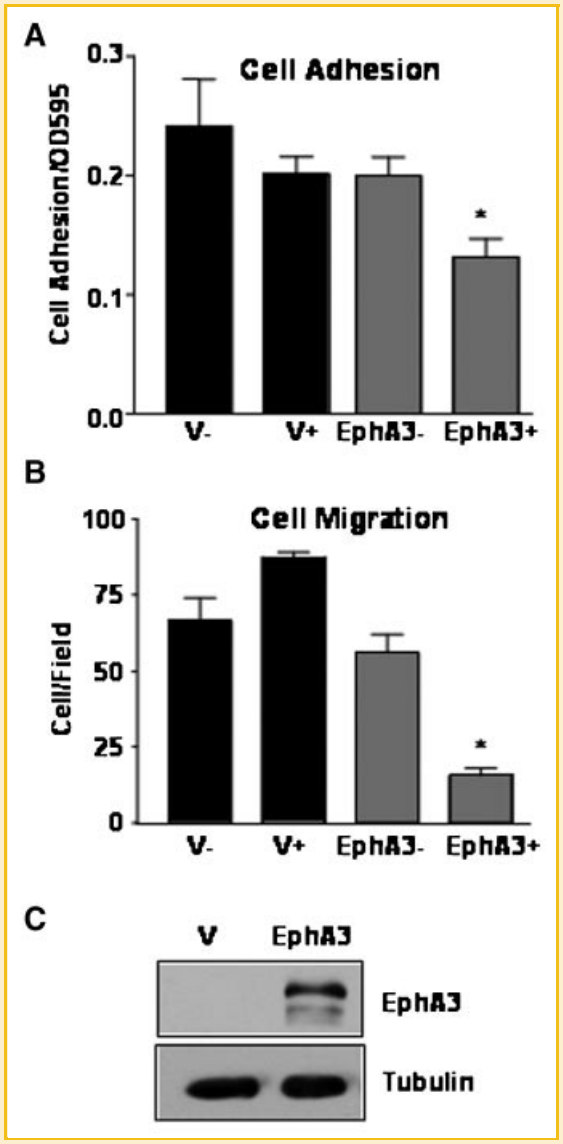

Fig. 1. Ligation of EphA3 ephrinA5/Fc inhibits cell migration and cell adhesion in Hek293T cells overexpressing the EphA3 receptor. Hek293T cells were transiently transfected with Vector control (V) or EphA3. A: Hek293T-Vector (V) and Hek293T-EphA3 cells were detached with PBS/EDTA and reseeded onto fibronectin-coated 96-well plates with (+) or without (−) pre-clustered ephrin-A5/Fc (1.5 μg/ml). Cells were allowed to attach for 10 min and were then fixed with methanol and stained with crystal violet. The absorbance for each sample was read at 595 nm. These data are represented as the means ± SD of absorbance from triplicate samples ($^{*}P < 0.05$ Student's *t*-test). B: The migratory potentials of Hek293T-Vector and Hek293T-EphA3 were assessed in modified Boyden chambers. The transwell membrane was coated with 10 μg/ml fibronectin (Fn). Cells were serum starved and plated at 1×10^5 cells/chamber with (+) or without (−) pre-clustered ephrin-A5/Fc (1.5 μg/ml). Cells were allowed to migrate towards 10% FBS overnight. Cells on the upper surface of the membrane were removed by scraping, and the attached cells were fixed with methanol and stained with crystal violet. The absorbance for each sample was read at 595 nm ($^{*}P < 0.05$ Student's *t*-test). C: Western blots analysis was performed using anti-EphA3 antibody to determine levels of EphA3 expression. Equal loading was assessed using an anti-tubulin antibody.

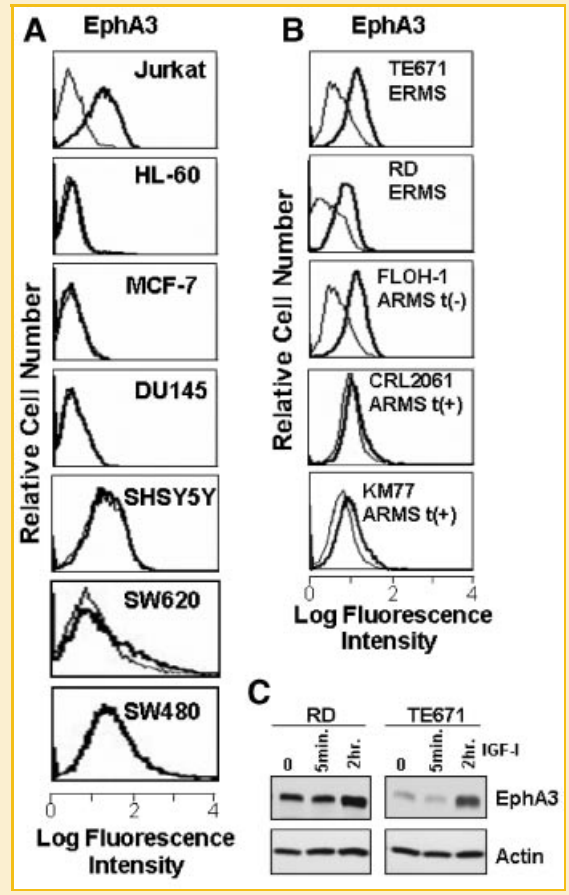

Fig. 2. Expression of EphA3 is restricted to a subset of RMS cell lines. Cell surface expression of EphA3 was determined by flow cytometry analysis in (A) the indicated tumour cell lines and (B) a panel of rhabdomyosarcoma cell lines as described in Materials and Methods Section. The thin line represents staining with the secondary antibody alone and the bold line represents staining with the anti-EphA3 antibody. C: TE671 and RD cell lines were serum starved for 4 h and stimulated with IGF-1 (100 ng/ml) for the indicated times and lysed. Expression of EphA3 was analysed by Western blot. Equal loading was measured using anti-actin antibody on the same blot.

EphrinA5/Fc STIMULATION OF EphA3 DECREASES ADHESION AND MIGRATION OF RMS CELLS

We next sought to investigate the function of EphA3 in RMS cells. We previously found that activation of EphA3 promotes detachment of T cells and Hek293T-EphA3 cells from fibronectin cells [Smith et al., 2004b]. We therefore investigated whether stimulation of RMS cells with the preferred ligand for EphA3 (soluble pre-clustered ephrinA5/Fc), would have similar affects in RMS. Stimulation of the TE671 and RD (ERMS cell lines), both of which express EphA3, with ephrinA5/Fc resulted in decreased adhesion to fibronectin compared with unstimulated cells (Fig. 3A). In contrast, stimulation of CRL2061 cells (which do not express EphA3) with ephrin-A5/Fc resulted in a slight increase in cell adhesion (Fig. 3A).

To further test whether EphA3 is the critical EphA receptor in promoting de-adhesion in RMS cells we introduced wild-type EphA3 (WT), and 3YF mutant EphA3 (EphA3 Y596, Y602 and Y779)

TABLE II. Expression of EphA Receptors in RMS Cells

Receptor	TE671	KM77	RD	CRL2061
EphA1	+/−	+	+/−	++
EphA2	+	+	+	+
EphA3	++	−	++	−
EphA4	++	++	+/−	+
EphA5	−	−	−	+/−
EphA6	−	+	−	+
EphA7	+	−	−	+

RT-PCR analysis was used to determine expression pattern of Eph receptors in RMS cell lines, TE671, KM77, RD and CRL2061. Relative expression levels: −, not expressed; +/− poorly expressed; +, expressed; ++, highly expressed. The data represent one of at least two independent experiments that gave similar results.

[Lawrenson et al., 2002] into CRL2061 cells. As reported by Lawrenson et al. [2002] the 3YF mutant has two conserved tyrosines in the juxtamembrane region of Eph receptors, which function as SH2-domain docking sites, mutated to phenylalanine. In addition, the third tyrosine in the activation-loop tyrosine is mutated to phenylalanine. Phosphorylation of these three tyrosines is required for full enzymatic activity [Binns et al., 2000; Zisch et al., 2000]. Cells transfected with wild-type EphA3 showed decreased adhesion to fibronectin while cells expressing 3YF mutant EphA3 adhered at similar levels to Vector-transfected cells (Fig. 3B). These data indicate that ephrinA5/Fc-mediated ligation of a fully active EphA3, but not an impaired EphA3, causes decreased adhesion of the cells. This rules out the possibility of promiscuous effects of the ligand on adhesion.

To determine if the decreased adhesion was associated with altered cell migration in TE671 and RD cells, we measured the ability of cells to migrate towards the chemokine stromal-derived growth factor-I (SDF-I), which was previously shown to promote robust migration of RMS cells through fibronectin covered membranes [Libura et al., 2002]. As shown in Figure 4, stimulation of TE671 and RD cells with ephrinA5/Fc resulted in decreased directional migration towards SDF-I, compared with control Fc-stimulated cells. Overall these data indicate that activation of EphA3 in RMS cells leads to decreased adhesion and decreased directional migration.

SUPPRESSION OF EphA3 INCREASES MIGRATION OF RMS CELLS

We next tested the effects of suppression of EphA3 on SDF-I-mediated cell migration. RD RMS cells were transfected with siRNA

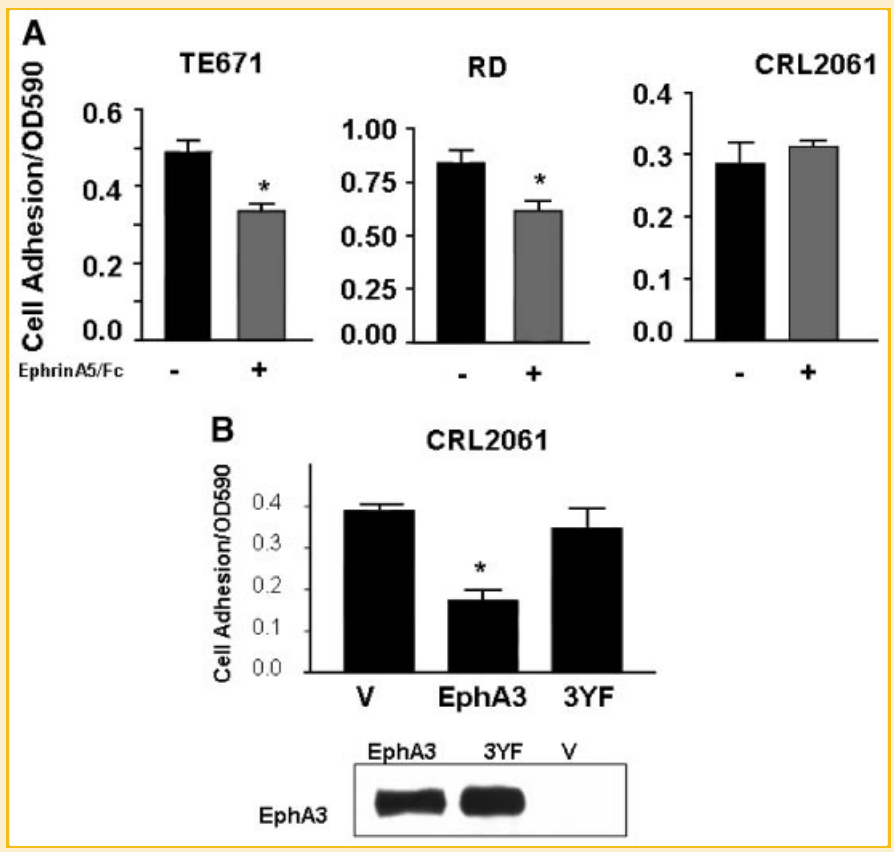

Fig. 3. EphA3 expression regulates cell adhesion. A: TE671, RD and CRL2061 cells were detached with PBS/EDTA, reseeded onto fibronectin-coated 96-well plates with or without ephrin-A5/Fc, and allowed to attach for the indicated times. Cells were then fixed with methanol and stained with crystal violet. The absorbance for each sample at 590 nm was obtained and the data are represented as the mean ± SD of absorbance from triplicate samples ($^*P < 0.05$ Student's *t*-test). B: CRL2061 cells were transiently transfected with control empty Vector (V), or Vectors encoding wild-type EphA3 or mutant EphA3 (3YF). To measure adhesion, cells were allowed to attach for 30 min and were then fixed with methanol and stained with crystal violet. The absorbance for each sample was read at 590 nm. These data are represented as the means ± SD of absorbance from triplicate samples ($^*P < 0.05$ Student's *t*-test). The inset Western blot shows the expression levels of EphA3 in transfected cells.

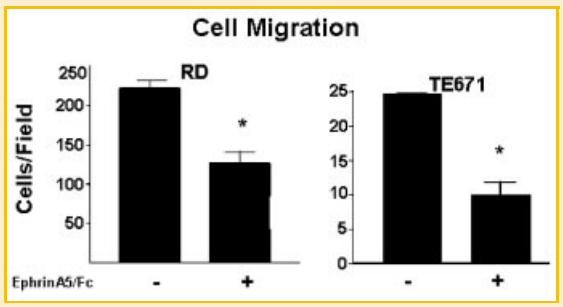

Fig. 4. Ligation of EphA3 with ephrinA5/Fc suppresses cell migration in RMS cells. The migratory potential of TE671 cells was assessed using modified Boyden chambers. The transwell membrane was coated with 10 μg/ml fibronectin (Fn) and with ephrin-A5/Fc (1.5 μg/ml) or control Fc proteins. TE671 cells were serum starved, plated at 1×10^5 cells/chamber and allowed to migrate toward towards SDF-I for 24 (TE671) or 48 (RD) h. Cells on the upper surface of the membrane were removed by scraping, and remaining cells were fixed with methanol, stained with crystal violet, and microscopically examined under a magnification of 100× (TE671) or 10× (RD). The data are represented as the averages of total cell counts from five fields of triplicate samples for each test condition ($^*P < 0.05$ Student's *t*-test).

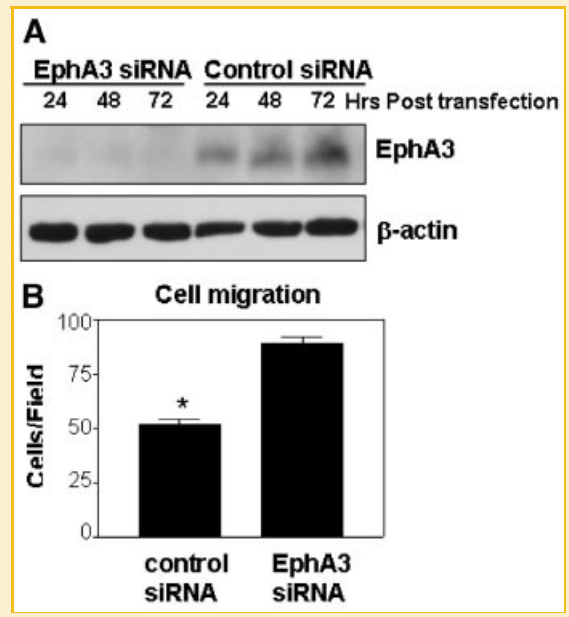

Fig. 5. Suppression of EphA3 using siRNA results in increased migration. RD cells were transfected with control siRNA or siRNA directed to EphA3. A: Expression of EphA3 was analysed 24–72 h after transfection by Western blot analysis using an anti-EphA3 antibody. Protein loading was measured using an anti-actin antibody. B: Cells were plated at 1×10^5 cells/chamber and migration towards SDF-I across transwell membranes coated with fibronectin was measured after 48 h. Cells on the upper surface of the membrane were removed by scraping and remaining cells were fixed with methanol, stained with crystal violet, and examined using a microscope under a magnification of 10×. The data are represented as the averages of total cell counts from five fields of triplicate samples for each test condition.

directed against either human EphA3 or mouse EphA3 (as a control). Effective suppression of EphA3 occurred 24–72 h following siRNA transfection (Fig. 5A). Migration of cells towards SDF-I was assessed in transwell assays. RD cells with reduced expression of EphA3 showed significantly increased chemotactic activity towards SDF-I compared with control-transfected cells (Fig. 5B).

Taken together the data indicate that EphA3 expression levels may regulate the migratory potential of RMS cells. When EphA3 is active cell motility is decreased, and when EphA3 is suppressed cell motility is increased.

LIGATION OF EphA3 BY EphrinA5/Fc ACTIVATES Rho

Next we investigated signalling pathways underlying the effects of EphA3 on cell adhesion and migration. Phosphorylation of EphA3 was observed after 5 min and up to 1 h in TE671 cells stimulated with soluble pre-clustered ephrinA5/Fc (Fig. 6A). We investigated the activation of the Rho GTPase proteins as candidate suppressors of cell adhesion and migration [Sharfe et al., 2002; Murai and Pasquale, 2005]. As shown in Figure 6B, ephrinA5/Fc induced a rapid increase in Rho activity by 2 min and this peaked at 10 min. In similar assays activation of the small G protein Rac1 was slightly decreased and there was a slight increase in Akt and ERK phosphorylation after 20 min stimulation (Supplementary Fig. 2). Altogether, these results indicate that ephrinA5/Fc-mediated ligation of EphA3 may regulate RMS cell adhesion and migration through modulation of Rho GTPases.

DISCUSSION

The Eph family of receptor tyrosine kinases and their cell-presented ligands, the ephrins, are frequently overexpressed in a wide variety of cancers, including breast, small-cell lung and gastrointestinal cancers, melanomas, and neuroblastomas. Previous studies have shown that EphA3 is expressed in B and T cell malignancies [Wicks et al., 1992; Dottori et al., 1999; Smith et al., 2004b; Fox et al., 2006], however the expression and function of EphA3 in solid tumours has not been widely studied. Our findings in this manuscript demonstrate that EphA3 is not widely expressed in cell lines derived from tumours of different origin, but it is highly expressed in a subset of RMS cell lines where it can be induced by IGF-I.

Previously, EphA3 was found to be upregulated by IGF-I in Jurkat cell in our laboratory. The fact that EphA3 is expressed on a T lymphocytic tumour cell line and not on primary T cells suggests that EphA3 may be associated with transformation of T cells to a malignant phenotype [Smith et al., 2004b]. However, there was no correlation between IGF-IR expression and EphA3 expression, as many of the cell lines (such as MCF-7) that did not express detectable EphA3 are highly responsive to IGF-I.

While both ERMS and ARMS overexpress the transcription factor PAX3, some cases of ARMS have a characteristic translocation that fuses the PAX3 gene with the FKHR gene both of which encode transcription factors in normal tissues. It is thought that the resulting fusion transcription factor inappropriately activates transcription of genes that contribute to transformation [Dagher and Helman, 1999]. Interestingly, both subtypes also overexpress

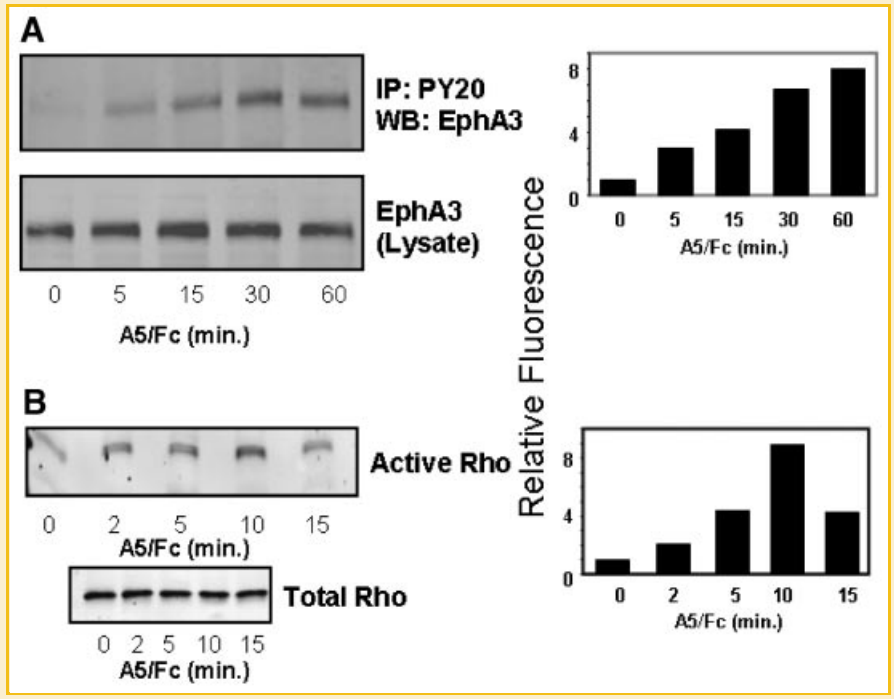

Fig. 6. EphrinA5/Fc stimulates phosphorylation of EphA3 and activates the small G protein Rho. TE671 cells were starved from serum for 4 h and incubated with 1.5 μg/ml pre-clustered ephrin-A5/Fc (A5/Fc) for the indicated times. A: Tyrosine phosphorylated proteins were immunoprecipitated (IP) with the anti-PY20 antibody and the presence of phosphorylated EphA3 in the immunoprecipitates was measured by Western blotting with the anti-EphA3 antibody. Levels of EphA3 protein in total cell lysates are also shown. Panels on the right are a numerical representation of the amount of protein in each lane calculated from densitometric measurements (using the LI-COR Odyssey Analysis program) normalized to EphA3 in lysates. B: Activated Rho was pulled down with GST-rhotekin and detected by immunoblotting with anti-Rho. Fold induction was quantified by densitometry normalized to total Rho levels in lysates. The data represent one of two (A) or three (B) independent experiments that gave similar results.

IGF-II, which is a ligand for the IGF-IR and can stimulate RMS tumour cell growth [Kalebic et al., 1994]. IGF-I induces EphA3 expression in RMS tumours as it does in Jurkat cells. Therefore it is possible that EphA3 expression also plays a role in RMS tumour development. A report by Wang et al. [1998] indicates tumour cells derived from IGF-II-transfected cells shows no local invasion whereas tumour cells derived from IGF-II and PAX-FKHR co-tranfected cell were invasive. Our observations that EphA3 can decrease the adhesive and migratory capacity of RMS cells suggest that its expression in the 'translocation negative' RMS may have a role in inhibiting the development of the migratory or metastatic phenotype. The expression of PAX3-FKHR oncogene together with IGF-II may override the effects of IGF-I-induced EphA3 expression and facilitate tumour progression. From our results we propose that EphA3 expression and activity restricts migration, and thus perhaps invasiveness of these cells.

Interestingly, out of all seven EphA receptors examined, EphA3 was the only receptor that demonstrated a restricted expression pattern in RMS cell lines. EphA3 was expressed in two cell lines that do not harbour chromosomal translocations (TE671 and RD) and in one translocation-negative ARMS cell line (FLOH-1), whereas it was not expressed in two ARMS cell lines (CRL2061 and KM77), both of which harbour chromosomal translocations and are predicted to be more aggressive with respect to metastasis. This raises the possibility that EphA3 expression may be suppressed in chromosomal translocation-positive or metastatic RMS cells. However, this idea needs to be tested in a larger panel of tumours, especially in ARMS cell lines that are reported to have a more invasive phenotype and that contain the PAX-FKHR oncogene.

Our finding that EphA3 expression may be correlated with non-metastatic RMS cells was initially unexpected, but is not unprecedented. High expression levels of EphA receptors have usually correlated with more malignant and metastatic tumours [Easty et al., 1995; Zelinski et al., 2001], and we previously observed EphA3 in T-cell lymphomas but not in normal peripheral T cells or in any subset of thymus-derived developing T cells [Smith et al., 2004b]. However, loss of expression of the EphB6 has previously been correlated with a poor prognosis in human neuroblastoma and in progression to metastatic disease in melanoma [Hafner et al., 2003]. More recently, loss of expression of EphB has also been identified as a critical step in colorectal cancer progression in the transition from adenoma to carcinoma with reduced expression accelerating tumourigenesis in the colon and rectum of $Apc^{Min/+}$ mice [Batlle et al., 2005].

Ligation of EphA3 by ephrinA5/Fc stimulation caused decreased cell adhesion to fibronectin and decreased migration towards SDF-I. In addition, suppression of EphA3 in RMS cells resulted increased cell migration towards SDF-I. The effects on adhesion and chemotaxis were similar to those observed in T cells, so our subsequent studies in RMS cells were aimed at understanding the role of EphA3 signalling in suppressing cell migration. Our results indicate that ligation of the EphA3 receptor in RMS cells leads to

modulation of Rho GTPases. Previous studies have shown that stimulation of Eph receptors can either activate or repress Rho GTPases depending on cell type and physiological context [Noren et al., 2006]. In 293T and melanoma cells ephrin-A-induced Rho activation is thought to mediate retraction of cell processes, cell rounding/detachment, and membrane blebbing [Lawrenson et al., 2002]. Rho activity may mediate migratory events by stabilizing actin filaments and promoting actomyosin contractility [Luo, 2000; Dickson, 2001]. Groeger and Nobes [2007] recently reported that cooperative Cdc42 and Rho signalling mediates ephrin-B triggered endothelial cell retraction. Similarly, ligation of EphA receptors with ephrin-A1 in T cells activates RhoA, while concomitantly blocking chemokine induced activation of Cdc42, resulting in the inhibition of chemotaxis [Sharfe et al., 2002]. Although SDF-I has been reported to activate Rho in some cells [Vicente-Manzanares et al., 2002; Tan et al., 2006], SDF-I did not enhance Rho activity in TE671 RMS (data not shown). Therefore, our data suggest that ephrinA5/Fc enhances Rho activity through activation of the EphA3 receptor.

In summary, the data presented demonstrate that EphA3 expression is associated with a subset of RMS cells, where is suppresses cell adhesion and cell migration through activation of Rho. Thus EphA3 may act as a suppressor of the motile or metastatic phenotype.

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