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Ephrin type-A receptor 2 on tumor-derived exosomes enhances angiogenesis through the activation of MAPK signaling

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Exosomes are potent players in the development of metastases and they play an important role in cancer angiogenesis and exacerbation. However, it is unclear how proteins on exosomes affect development of blood vessel networks. In this study, we focused on relationships between membrane proteins on exosomes and angiogenesis using human umbilical vein endothelial cells (HUVEC). Lung tumor cell-derived exosomes induced tube formation and growth of endothelial cells *in vitro* in a dose-dependent manner involving MAPK activation, but this was not seen in normal lung epithelial cells. Ephrin type-A receptor 2 (EphA2) was identified by proteomic analysis and an inhibition assays showed it is a major MAPK activator on exosomes. Thus EphA2 on exosomes participates in angiogenesis as a ligand of the ephrin signaling pathway. These results support the development of novel therapeutic strategies such as blockade of remote cancer communications through exosomes.

1. Introduction

Exosomes, which are endogenous nano-vesicles (40–100 nm in diameter), are secreted from various cells and they contain mRNAs, miRNAs, as well as soluble and transmembrane proteins derived from their host cells (Shtam et al. 2013; Deng et al. 2012; Kosaka et al. 2012). These internal and membrane contents of exosomes transfer to their target cells where they can regulate cellular functions (McDonald et al. 2014; Zhang et al. 2014; Mathivanan et al. 2010). Therefore, exosomes are regarded as functional mediators of cell-cell communication without requiring direct interactions, e.g. effecting communication through endogenous liposomes in a cancer microenvironment. However, it is still not clear whether vesicular stimulation is cell-type specific, and if it occurs by vesicular uptake into cells or by direct interactions between target cells and the exosomal membrane. Thus, elucidation of the mechanisms involved in exosome stimulation remains an important challenge.

Recently, it has been proposed that cancer-derived exosomes are an important element for the progression of cancer characteristics (neoplastic growth, invasion, metastases and epithelial-to-mesenchymal transitions). A clinical study has shown that the amount of exosomes in plasma increases in parallel with the cancer stage (Taylor and Gercel-Taylor 2008). On the other hand, a basic science research study by Takahashi et al. (2013) reported that B16BL6-derived exosomes transfer signals first to the liver and then to the lungs. Moreover, Hood et al. (2009) have reported that tumor exosomes can promote endothelial angiogenic responses, which could in turn contribute to tumor metastatic potential. To summarize, various relationships between exosomes and cancer metastasis have been noted by many research groups, suggesting that exosomes released from cancer cells may play an important role in cancer metastasis, contributing to the propagation of transformed endothelial cells (ECs) or other types of cells.

Recent studies have pointed to a previously unknown role of exosomes as important signaling vesicles facilitating cross-talk between cancer cells and ECs (Ekstrom et al. 2014; Corrado et al. 2012). In principle, the cancer-derived fluid from exosomes could contribute to the tumor microenvironment and to cell-cell communication between malignant and non-malignant cells of the host (Liu et al. 2012; Utoguchi et al. 1995). Tumor angiogenesis is initiated through activation of endothelial cells by angiogenic factors such as vascular endothelial cell growth factor (VEGF) and fibroblast growth factors (FGF) (Strimpakos et al. 2013). However, little information on the angiogenic properties of exosomes is available.

In this study, we investigated the biological effects of exosomes derived from cancer cells on human umbilical vein endothelial cells (HUVEC), to test the hypothesis that cancer exosomes promote angiogenesis. Furthermore, we carried out a proteomic analysis in an attempt to identify key proteins that stimulate HUVEC. This analysis identified ephrin family proteins as MAPK activators and indicated that the ephrin type-A receptor 2 (EphA2) is a component of exosomes and that it is a major MAPK activator for HUVEC.

2. Investigations and results

2.1. Effect of exosomes derived from HARA-B or HPAEpiC on tube formation

Tube formation assays were performed using the lung tumor (HARA-B)-derived or normal lung epithelial cell (HPAEpiC)-derived exosomes to evaluate the effects of exosomes on the formation of a vascular network. Figure 1a shows a picture of tube formation in HUVEC treated with VEGF (positive control), HARA-B-derived exosomes and HPAEpiC-derived exosomes. These results showed that HARA-B-derived exosomes enhanced tube formation

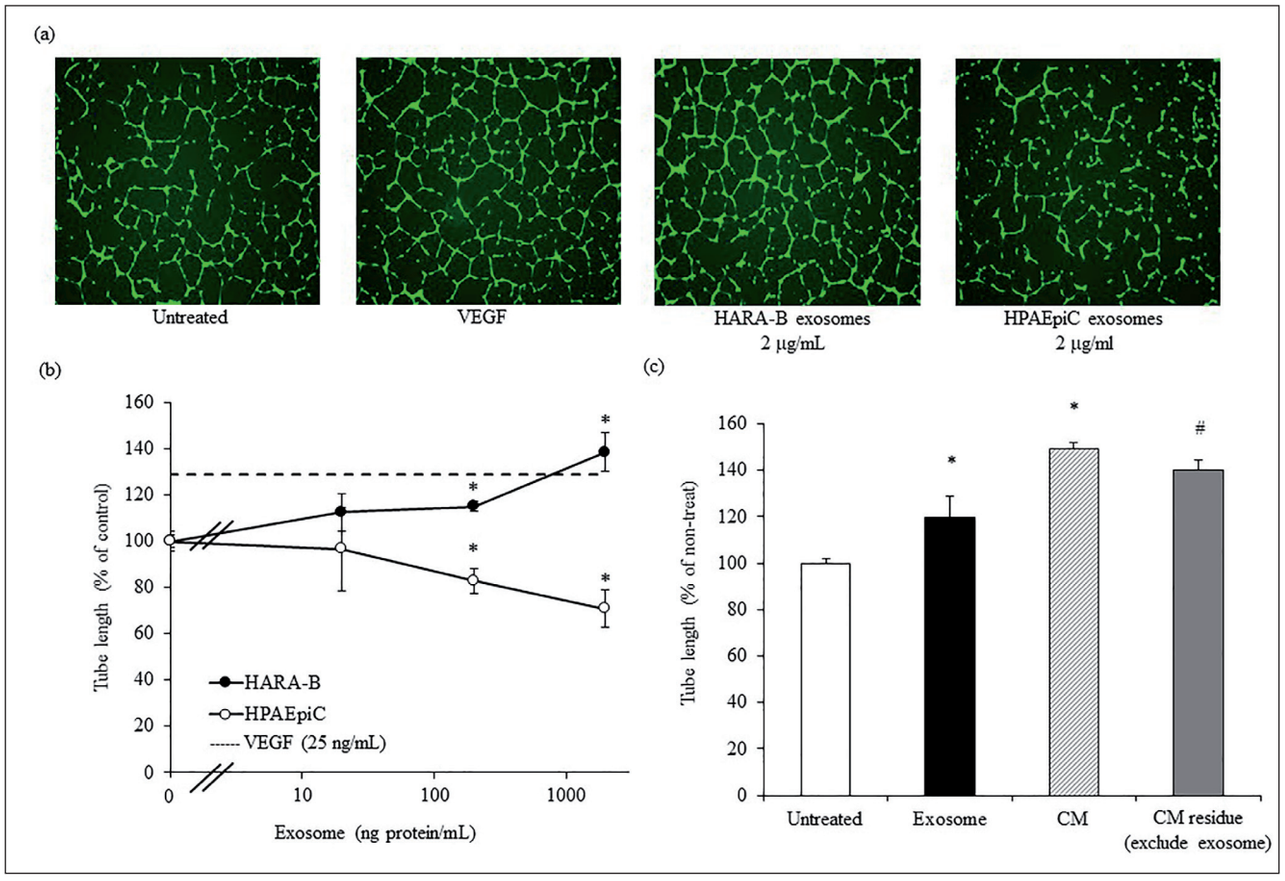

Fig. 1: Enhancement of tube formation by treatment with exosomes and CM derived from HARA-B or HPAEpiC on HUVEC. Effect on tube formation of treatment with exosomes and CM derived from HARA-B or HPAEpiC was assessed by tube formation assay. Different concentration of exosomes (0.02-2 mg protein/mL) were added to each well. HUVEC stimulated with VEGF (25 ng/mL) was used as positive control. CM residue were prepared from CM of HARA-B by centrifugation. Tube formation was quantified from eight randomly selected fields per experiment by tube length per set using an image analyzer. Data are shown as means and standard deviations (n=3). *P<0.01 (vs. non-treat group). #P<0.05 (vs. CM treated group).

dose-dependently (Fig. 1b). The enhanced effect was similar for the 200 to 2000 ng/ml HARA-B-derived exosome-treated groups and the 25 ng/ml VEGF-treated groups. Surprisingly, the HPAEpiC–derived exosome-treated group showed suppression of tube formation (Fig. 1b). Moreover, to evaluate the effects of adding different quantities of exosomes on tube formation in conditioned medium (CM), the tube formation assay was carried out using purified exosomes, CM and exosome-free CM, which was the remainder of the exosome preparation. The amount of each sample was derived from 250 ml of CM. The purified exosomes induced 119 % of the tube extension measured for the untreated HUVEC compare to the untreated cells (Fig. 1c). These results was positively correlated with the cell proliferation of HUVEC in a dose-dependent manner (data not shown). Furthermore, CM strongly induced tube extension (150 % of untreated HUVEC). On the other hand, tube formation was decreased 10 % by removal of exosomes from the CM (Fig. 1c).

2.2. Comparison of p-ERK1/2, p-p38 and p-VEGF expression in HUVEC stimulated with exosomes derived from HARA-B

To determine how these exosomes can transduce signals into cells, the signaling pathways were analyzed by evaluating the phosphorylation of signaling molecules. Phosphorylation of the MAPK family (ERK1/2, p38) and VEGFR2 were measured by western blotting after treating HUVEC with HARA-B exosomes. The phosphorylation of ERK1/2 and p38 were remarkably increased after a 30 min treatment (Fig. 2a). However, no phosphorylation of VEGFR2 was observed following exosome treatment. Furthermore, the phosphorylation and proliferation effects were blocked by a MEK1/2 inhibitor U0126 (Fig. 2b, c). These data suggested that VEGF is not involved in the proliferation signal from HARA-B exosomes. To examine the effects of exosome-derived membrane proteins, the exosomes were digested with trypsin. ERK1/2 phosphorylation was analyzed by western blot analysis following HUVEC treatment with trypsin-digested exosomes. The structure of the exosomes was not destroyed by trypsin digestion (data not shown). Phosphorylation of ERK1/2 was weakly inhibited by this treatment compared to the control group (Fig. 2d). This result indicated that membrane proteins on exosomes include activators of MAPK.

2.3. Identification of membrane proteins activating MAPK on exosomes derived from HARA-B

To identify the MAPK activators on the exosomes, shotgun membrane proteome analysis comparing cells treated with exosomes derived from HPAEpiC and HARA-B was performed by LC-MS/MS. Eph A2, B2, B3, B4 and ephrin B1 were identified as MAPK activators following HARA-B exosome treatment but not following HPAEpiC exosome treatment by proteomic analysis (data not shown). We focused on EphA2 because it has been reported that EphA2 plays an important role in cancer angiogenesis (Zhou et al. 2011; Merritt et al. 2010). Therefore, we confirmed the expression levels of EphA2 on cells and on exosomes derived from HARA-B and HPAEpiC cells. EphA2 was highly expressed on HARA-B cells compared to HPAEpiC and the other human lung cancer cell lines (Fig. 3a). The expression level of EphA2 was significantly higher HARA-B exosomes compared to HPAEpiC

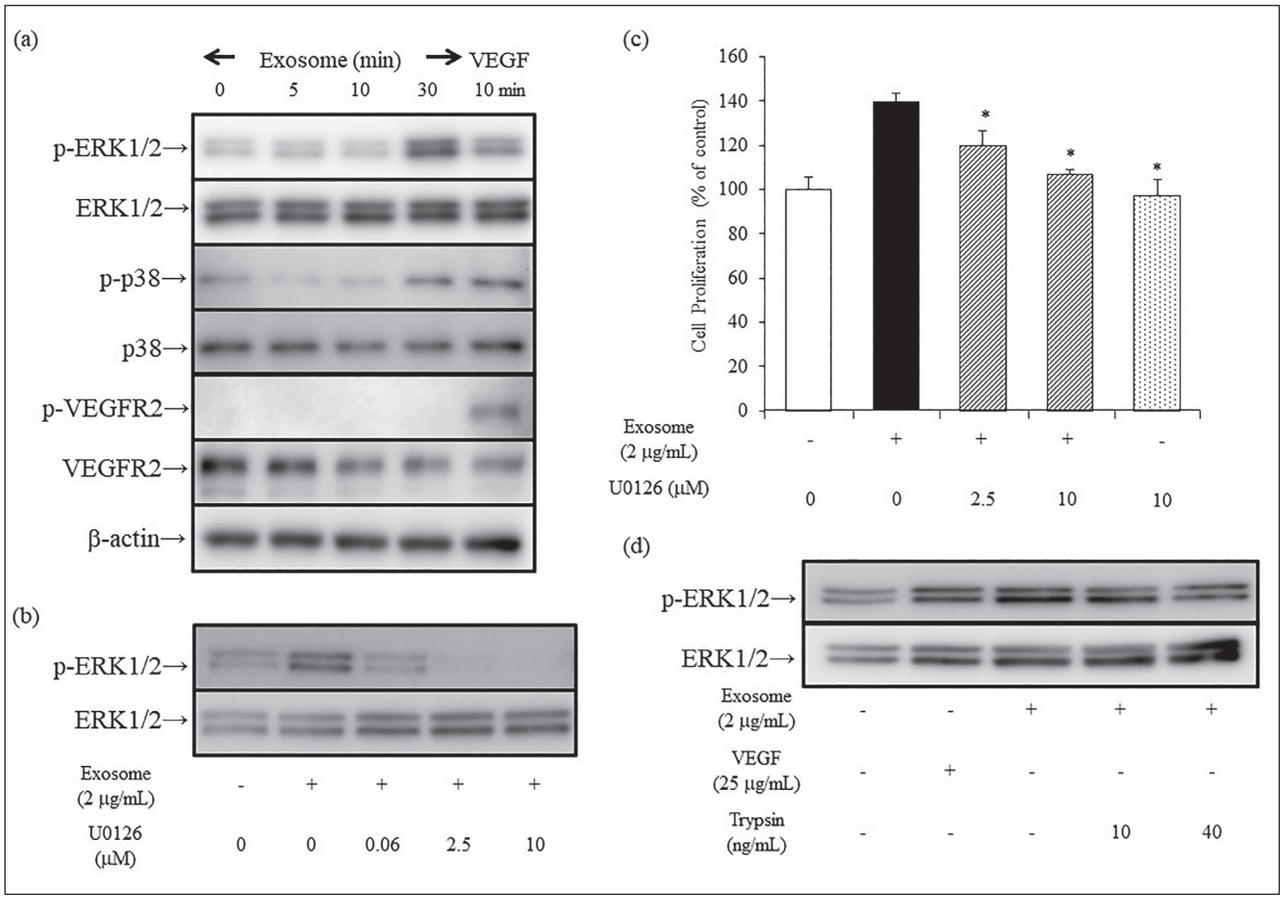

Fig. 2: Relationship between MAPK/VEGFR phosphorylation and treatment of exosomes derived from HARA-B on HUVEC. ERK, p-ERK, p38, p-p38, VEGFR2 and p-VEGFR2 were detected by western blot in HUVEC. Exosomes (2 mg/mL), VEGF (25 ng/mL), U0126 (0.06-10 mM) or trypsinized exosomes (2 mg/mL) were added to each well, the plates were incubated for 0-30 min. HUVEC treated various conditions (a:exosomes/VEGF, b:exosomes/U0126, d:exosomes/VEGF/trypsin) were resolved by SDS buffer and performed western blot. Equal amounts of protein loading were confirmed by parallel b-actin immunoblotting. Effect on proliferation of treatment with exosomes and U0126 was assessed by WST-8 assay (c). Data are shown as means and standard deviations (n=3). *P<0.01 (vs. Exosome + U0126 – group).

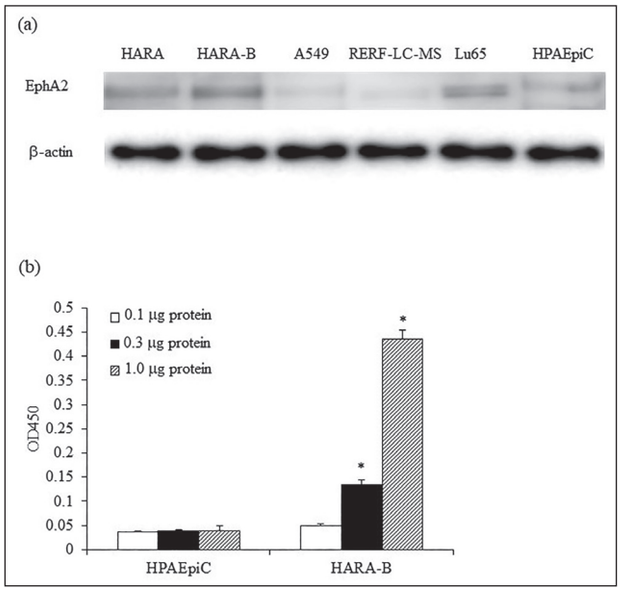

Fig. 3: Expression of EphA2 on exosomes derived from HARA-B. Expression analysis of EphA2 on lung cancer cells or HPAEpiC was detected by western blot (a). Equal amounts of protein loading were confirmed by parallel b-actin immunoblotting. Expression analysis of EphA2 was detected by ELISA (b). ELISA plate was coated with exosomes and then detected using an anti-EphA2 antibody. Data are shown as means and standard deviations (n=3). *P<0.01 (vs. HPAEpiC group).

exosomes in a concentration dependent manner (Fig. 3b). Accordingly, the ligands of EphA2 (ephrin A1, A4, A5) were detected in HUVEC using RT-PCR (data not shown).

2.4. Evaluation of HUVEC proliferation in response to EphA2 on exosomes

Many ephrin receptor family proteins can bind to ephrin family molecules. The proliferation effect of EphA2 on exosomes was examined by blocking it with an anti-EphA2 antibody. Treatment with EphA2 Fc chimera and exosomes induced proliferation of HUVECs dose-dependently (Fig. 4a). Furthermore, anti-EphA2 antibody blocked the proliferation induced by the EphA2 Fc chimera (Fig. 4b). Finally, the results of proliferation assays indicated that the proliferation effect of EphA2 on exosomes was blocked by an anti-EphA2 (Fig. 4c). Taken together, these results demonstrated that EphA2 on exosomes directly induced proliferation of HUVEC.

3. Discussion

Recently, we reported the possible utility of determining epidermal growth factor receptor (EGFR) expression on exosomal membranes for lung cancer diagnosis (Yamashita et al. 2014). Furthermore, other groups reported that the transfer of exosomal genetic material and signaling proteins increases angiogenesis (Zhang et al. 2013; van Balkom et al. 2013). Angiogenesis is a fundamental process that allows cancer-derived exosomes from the tissue niche to enter the general circulation. However, the angiogenic process cannot be recapitulated using only soluble angiogenic factors such as VEGF and FGF. These

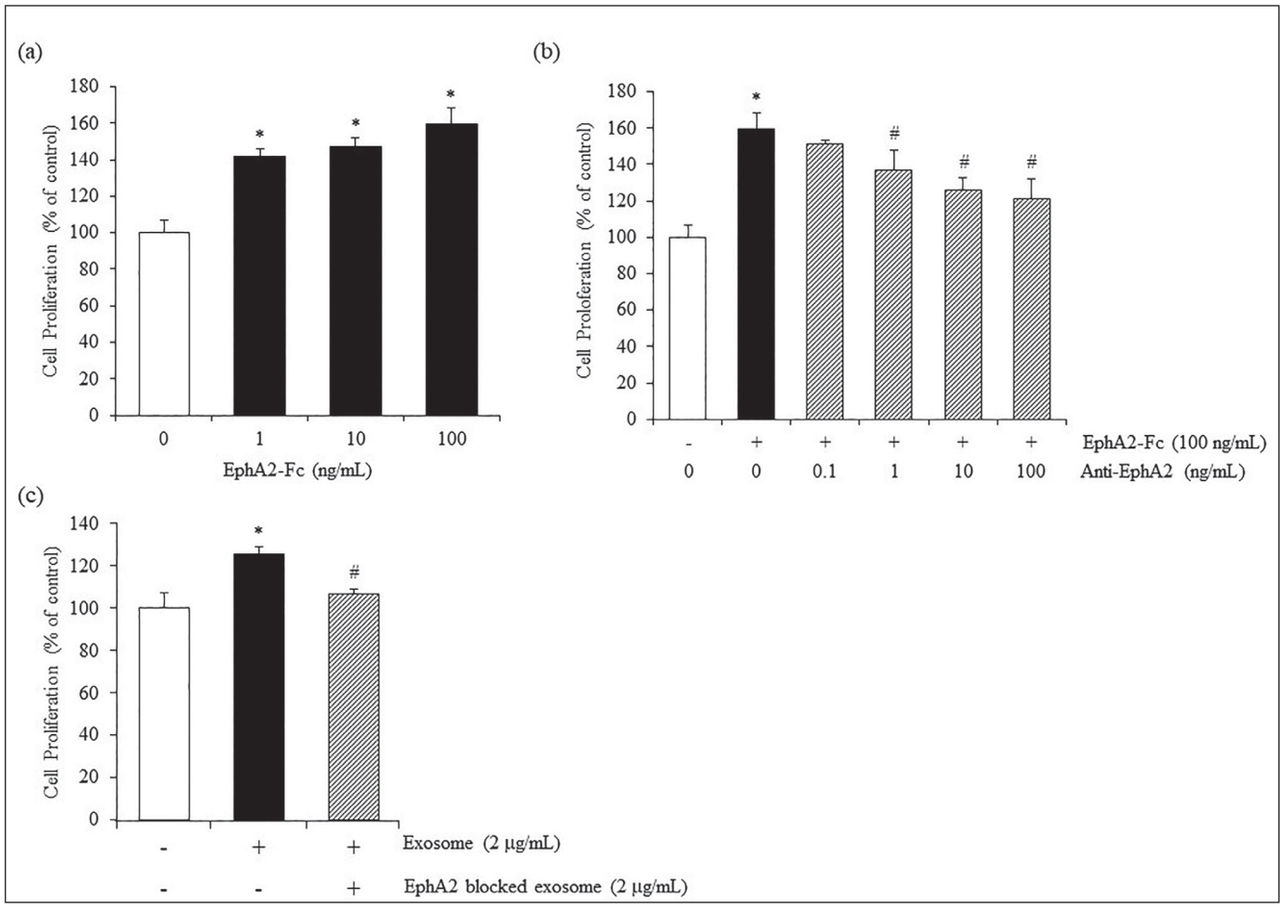

Fig. 4: Neutralization of EphA2-mediated cell growth on exosome by anti-EphA2 antibody. Cell proliferation was observed by WST-8 assay. HUVEC treated with EphA2-Fc (a), EphA2-Fc/anti-EphA2 antibody (b) and exosomes/anti-EphA2 antibody(c) for 12 h. Data are shown as means and standard deviations (n=3). *P<0.01 (vs. non-treat group). #P<0.01 (vs. EphA2-Fc or Exosome treated group).

observations indicated that released exosomes may promote cancer angiogenesis through functional proteins on their surfaces. However, there is little evidence demonstrating the relevance of trans membrane proteins on exosomes derived from cancer cells to angiogenesis.

We found that EphA2 on exosomes derived from lung cancer cells acts as a major activator of MAPK in HUVEC. Ephrin receptors are a family of tyrosine kinase receptors. In general, A-class ephrins (ephrin A1-A6) bind to the A-class Eph receptors (EphA1-A10) and B-class ephrins (ephrin B1-B3) bind to the B-class receptors (EphB1-B6), although cross-reactions have also been described. Interactions between ephrin receptors and their ligands (ephrins) occur at sites of cell-to-cell contact (Holland et al. 1996). Furthermore, their soluble forms show some functionality as carriers for signal propagation (He et al. 2005; Brantley et al. 2002). The cellular response to Eph stimulation by ligands includes a wide range of biological effects exerted through several bi-directional signaling pathways (Zimmer et al. 2003). This is considered an unique property of the Eph-ephrin interaction as it consists of both forward and reverse signaling components. Eph-ephrin signaling has been implicated primarily in angiogenesis, cell shape formation, adhesion and motility in normal developmental processes (Miao et al. 2000; McBride et al. 1998). EphA2 is overexpressed in many human cancers, and it is often associated with poor prognostic features (Strimpakos et al. 2013; Miyazaki et al. 2013). It is involved in many processes fundamental to malignant progression, such as migration, invasion, metastasis, proliferation, survival and angiogenesis. EphA2 inhibition by an anti-EphA2 antibody has been shown to decrease tumor growth, prolong survival and inhibit angiogenesis in multiple preclinical cancer models Merritt et al. 2010; Ansuini et al. 2009). Considering previous reports and our findings, the therapeutic effect of blocking EphA2 might include not only direct inhibition of cancer cell growth but also blockade of cancer communication through EphA2 on exosomes.

This study demonstrated that EphA2 worked as a major MAPK activator in angiogenesis. It has been reported that ephrin A1 and its primary receptor, EphA2, play a vital role in normal angiogenesis and cancer angiogenesis through MAPK activation (Miao et al. 2001; Cheng and Chen 2001). Moreover, ephrin A1 was strongly expressed on HUVEC compare to other ephrins in our experiments (unpublished data). Thus, we hypothesized that EphA2 on exosomes directly stimulates ephrin A1 on HUVEC. A previous study reported EphA2 expression on exosomes derived from colon cancer using a proteomic approach (Tauro et al. 2012). However, there is little evidence regarding the biological function of EphA2 on exosomes. This study demonstrates for the first time that EphA2 on exosomes directly regulates development of the cancer blood vessel network *in vitro,* without utilizing the VEGF pathway.

Figures 1 and 4 show that exosomes contribute to the tube formation effect of CM on HUVEC. It is likely that EphA2 on exosomes correlates with the development of the cancer blood vessel networks, as it is a direct ligand of the ephrin signaling pathway. Our results have demonstrated that direct communication between membrane proteins on exosomes and recipient cells leads to stimulation of tumor ECs. Regarding remote signal communication, it has been thought that tumor angiogenesis is initiated through activation of EC by soluble factors such as VEGF and FGF in the cancer fluid. However, this study demonstrates that exosomes may be important participants in remote signal communication resulting in cancer angiogenesis. Further comprehensive studies of the biological significance of exosomes should offer new therapeutic strategies, such as methods to block remote communication in cancer and various other diseases.

4. Experimental

4.1. Cell culture

Human umbilical vain endothelial cells (HUVEC, Takara) were maintained at 37 °C in a humidified atmosphere of 5 % CO_2 in EGM-2 BulletKit (Takara). Primary human pulmonary alveolar epithelial cells (HPAEpiC, Science Cell Research Laboratory) were maintained in Alveolar Epithelial Cell Medium (Science Cell Research Laboratory) under a 5 % CO_2 atmosphere at 37 °C. The human lung cancer cell lines, HARA, HARA-B, A549, RERF-LC-MS, Lu65 were purchased from the Japanese Collection of Research Bioresources Cell Bank. The human lung cancer cell lines were cultured at 37 °C in a humidified atmosphere of 5 % CO_2 by provider's protocol and medium condition.

4.2. Preparation of conditioned media and exosomes

Exosomes were prepared from the conditioned media (CM) of HARA-B and HPAEpiC by centrifugation. HARA-B and HPAEpiC were grown to 80-90 % confluence, washed two times with 10 mL of PBS, and incubated for 72 h in serum-free medium. Six hundred ml volumes of supernatants containing 6 x 10^8 lung cancer cells or 1 x 10^8 HPAEpiC were centrifuged at 300 x g for 5 min to pellet the cells and at 16,000 x g for 20 min, followed by filtration through a 0.22 µm filter to clear the supernatant of cell debris. Exosomes were precipitated by ultracentrifugation at 140,000 x g for 70 min. The exosome pellets were washed once in PBS and their protein contents were measured using a Micro BCA protein assay kit (Thermo Fisher Scientific). Particle sizes were measured by dynamic light scattering using a Zetasizer nano (Malvern). The shapes of the exosomes were then observed using an electron microscope. Exosome images were obtained by floating a carbon-coated 400-mesh Formvar EM grid on top of 5 ml of freshly prepared exosomes in deionized water for 20 min. The grid was then briefly washed with deionized water and floated on a drop of 2 % uranyl acetate. Samples were examined using an H-7650 Transmission Electron Microscope (Hitachi High-Technologies). These characteristic data reported in our previous study (Yamashita et al. 2014).

4.3. In vitro angiogenesis assay

For analysis of invasion, 24-well culture plates were coated with 100 µl of Matrigel (BD Bioscience) per well and then allowed to polymerize for 30 min at 37 °C. HUVEC suspensions of 250 µl of medium containing 10 % FCS and 250 µl of CM (3 x 10^4 cells/well) were seeded on polymerized Matrigel. After incubation at 37 °C for 6 h, each culture was fluorescent stained by Calcein-AM (DOJINDO) and was photographed at x 100 magnification using a fluorescence microscope (Power IX81, OLYMPUS). Tube formation was quantified from eight randomly selected fields per experiment by analyzing tube length per set using an image analyzer (MetaXpress, Molecular Devices, Inc.).

4.4. Proteomic analysis

Exosomes (containing ~1 mg protein) were incubated with 5 mM Tris [2-carboxymethyl] phosphine for 15 min at room temperature and at 37 °C, respectively. The exosomes were then treated with iodoacetamide for 15 min at room temperature. Trypsin (Promega, 20 ng) was added to each exosome solution and these solutions were incubated for 16 h at 37 °C. Extracted peptides were analyzed by liquid chromatography mass spectrometry (LTQ Orbitrap XL: Thermo Scientific). The Mascot search engine (http://www.matrixscience.com) was initially used to query the entire theoretical tryptic peptide database as well as SwissProt (http://www.expasy.org/, a public domain database provided by the Swiss Institute of Bioinformatics). The search query assumed the following: (i) the peptides were mono-, di- or tri-isotopic (ii) methionine residues may be oxidized (iii) all cysteines were modified with carbamidomethyl.

4.5. Cell proliferation assay

HUVEC were seeded into 96-well microplates (5 x 10^4 cells/well) cultured at 37 °C for 12 h. Culture medium was changed to fresh medium (without FCS and growth factors). After incubation at 37 °C for 12 h, various concentrations of exosomes, human EphA2 Fc chimera (R&D systems), anti-human EphA2 antibody (R&D systems) or U0126 were added to each well, the plates were incubated for 12 h, and cell viability was measured using Cell count reagent SF (Nacalai tesque) containing WST-8. EphA2 on exomes was blocked by anti-human EphA2 antibody at 37 °C for 1 h. Free anti-human EphA2 antibody and trypsin were removed by centrifugation. Absorbance was measured using a microplate reader (Bio-Rad) at test and reference wavelengths of 450 and 650 nm, respectively.

4.6. Western blot analysis

HUVEC were seeded into 12-well microplates (1 x 10^5 cells/well) and cultured at 37 °C for 12 h. Each medium was changed to fresh medium (without FCS and growth factors). After incubation at 37 °C for 12 h, exosomes (2 mg/mL), VEGF (25 ng/mL), U0126 (0.06-10 mM) or trypsinized exosomes (2 mg/mL) were added to each well, the plates were incubated for 0-30 min. The HUVEC cell lysates in RIPA buffer (Wako) were separated in 10 % SDS-polyacrylamide gels and transferred to Immobilon membranes (Millipore). After blocking with 4 % block ace (DS Pharma Biomedical) or 5 % BSA for 1 h at room temperature, the blots were reacted with primary antibodies in a buffer containing 0.4 % block ace or 5 % BSA, and then with the appropriate peroxidase-conjugated secondary antibodies in the same buffer. Expression of EphA2, ERK, p-ERK, p38, p-p38, VEGFR2 and p-VEGFR2 was detected by goat anti-human EphA2 (AF3035: R&D systems) and rabbit anti-human ERK, p-ERK, p38, p-p38, VEGFR2, p-VEGFR2 polyclonal antibody (Cell Signalling Technology) followed by an HRP-conjugated polyclonal anti-goat (Jackson Immuno Research) or anti-rabbit (Cell Signalling Technology) IgG antibody using the ECL-plus system (GE Healthcare Biosciences). Equal amounts of protein loading were confirmed by parallel b-actin immunoblotting.

4.7. Detection of EphA2 on the human lung cancer cell lines using western blot analysis

The human lung cancer cell lines were solubilized in RIPA buffer. Cell lysates were separated in 10 % SDS-polyacrylamide gels and transferred to Immobilon membranes. After blocking with 4 % block ace for 1 h at room temperature, the blots were reacted with goat anti-human EphA2 (1 mg/mL) in a buffer containing 0.4 % block ace and then with the appropriate peroxidase-conjugated secondary antibodies in the same buffer. Equal amounts of protein loading were confirmed by parallel b-actin immunoblotting.

4.8. Detection of EphA2 on exosomes derived from conditioned media using ELISA

Ninety-six well Maxisorp plates (Nunc) were coated with several concentrations of purified exosomes in volumes of 100 ml/well of carbonate buffer (pH 9.6) and incubated overnight at 4 °C. After incubation, 100 ml/well of 4 % block ace were added and the plates were incubated overnight at 4 °C. After 2 washes with PBS, anti-human EphA2 antibody was added and incubated for 60 min at 37 °C. After 3 washes with PBS, the plates were incubated with 100 ml of HRP-conjugated anti-goat IgG antibody per well diluted 1: 5,000 in 0.4 % block ace for 60 min at room temperature. After the final 3 washes with PBS, the reaction was developed with tetramethyl benzidine reagents, blocked with H_2SO_4 and optical densities were recorded at 450 nm.

4.9. Statistical analysis

Differences in tumor volumes between the control and target groups were compared using the one-way ANOVA followed by the Tukey post-hoc test.

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Author contributions: All the authors planned experiments and analyzed data; T. Y and S, K performed experiments; T. Y, H. K and S. T wrote the paper.

Conflicts of interest: None declared.

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