Mast cell exosomes promote lung adenocarcinoma cell proliferation – role of KIT-stem cell factor signaling

Hui Xiao1,2, Cecilia Lässer2, Ganesh Vilas Shelke2, Juan Wang1, Madeleine Rådinger2, Taral Rameshchand Lunavat2, Carina Malmhäll2, Li Hui Lin1, Jia Li1, Li Li1* and Jan Lötvall2*†

Abstract

Background: Human cells release nano-sized vesicles called exosomes, containing mRNA, miRNA and specific proteins. Exosomes from one cell can be taken up by another cell, which is a recently discovered cell-to-cell communication mechanism. Also, exosomes can be taken up by different types of cancer cells, but the potential functional effects of mast cell exosomes on tumor cells remain unknown.

Methods and results: Exosomes were isolated from the human mast cell line, HMC-1, and uptake of PKH67-labelled exosomes by the lung epithelial cell line, A549, was examined using flow cytometry and fluorescence microscopy. The RNA cargo of the exosomes was analyzed with a Bioanalyzer and absence or presence of the c-KIT mRNA was determined by RT-PCR. The cell proliferation was determined in a BrdU incorporation assay, and proteins in the KIT-SCF signaling pathway were detected by Western blot. Our result demonstrates that exosomes from mast cells can be taken up by lung cancer cells. Furthermore, HMC-1 exosomes contain and transfer KIT protein, but not the c-KIT mRNA to A549 cells and subsequently activate KIT-SCF signal transduction, which increase cyclin D1 expression and accelerate the proliferation in the human lung adenocarcinoma cells.

Conclusions: Our results indicate that exosomes can transfer KIT as a protein to tumor cells, which can affect recipient cell signaling events through receptor-ligand interactions.

Keywords: Exosomes, Extracellular vesicles, KIT, Lung cancer, Mast cell, Transfer

Introduction

Exosomes are nano-sized membrane vesicles (30–100 nm) constitutively released by various cells, such as B lymphocytes [1], dendritic cells [2,3], mast cells [4], natural killer (NK) cells [5], intestinal epithelial cells [6], tumor cells [7,8], and neuronal cells [9]. These extracellular vesicles have a lipid bilayer structure, and carry multiple proteins and RNA molecules [10]. Exosomes are known to shuttle their cargo, including proteins and lipids between cells [11], and in addition, we showed in 2007, that exosomes also shuttle functional RNA from one cell to another [12]. Importantly, the transfer of these molecules can change a recipient cell phenotype in many ways, and the exosome-mediated signal from the original cell may be different under different circumstances [13]. Exosomes are present in all human body fluids so far investigated, including saliva [14], blood plasma [15], breast milk [16], cerebrospinal fluid [17] and urine [18], and are abundant in the tumor microenvironment [19,20], indicating their importance for tumor biology.

The human c-KIT oncogene codes for the protein mast/stem cell growth factor receptor Kit (KIT), a member of the tyrosine kinase family of growth receptors [21]. KIT is expressed on a variety of hematopoietic cells, such as mast cells and bone marrow progenitor cells. Stem cell factor (SCF) dependent activation of KIT is critical to maintain homeostasis and function of mast cells [22]. In clinical lung cancer research, it has been
shown that non-small cell lung cancer more rapidly leads to death if the tumor is KIT positive [23]. For example, if tumors are positive for KIT at the time of surgery, the disease is associated with short term survival, compared to those that are KIT negative [24]. In addition, co-expression of KIT and other tumor-promoting molecules such as EGFR tend to increase mortality further [25]. During some circumstances it is less clear how tumor cells become KIT positive, but one possibility is that non-tumor cells in the tumor microenvironment could shuttle such molecules between cells [26].

Tumors also harbor many other cells beside tumor cells, including inflammatory cells such as dendritic cells and mast cells [27,28], as well as fibroblasts and endothelium [29,30]. Furthermore, co-cultures of mast cells and non-small cell lung cancer leads to increased proliferation of the cancer cells both in vitro and in vivo [31]. In this study we therefore hypothesized that KIT could possibly be transferred to tumor cells via exosomes from one or several of the surrounding cells. To test this, we used a mast cell line (HMC-1) constitutively expressing the active form of the KIT receptor, and a non-small cell cancer lung epithelial tumor cell line (A549), to determine whether KIT can be transferred from mast cells to the epithelial cancer cell via exosomes, and whether those exosomes can influence the function of the recipient cell.

Materials and methods

Cell cultures

The lung adenocarcinoma cell line, A549, was obtained from the ATCC and the human mast cell line, HMC-1 (Dr Joseph Butterfield, Mayo Clinic, Rochester, MN, USA) was a kind gift from professor Gunnar Nilsson at the Karolinska Institute, Stockholm, Sweden. Control exosomes were derived either from the mouse embryonic fibroblast cell line, NIH 3T3 (Cell lines service, Eppelheim, Germany), or the human embryonic kidney 293 cell line, HEK 293 (from ATCC and a kind gift from Jonas Nilsson at the Sahlgrenska University Hospital, Gothenburg, Sweden). HMC-1 cells were maintained in Iscove's (from ATCC and the human mast cell line, HMC-1, as well as the control cells HEK 293 and NIH 3T3, were labelled with the green fluorescent dye PKH67 (Sigma-Aldrich) according to the adjusted protocol outlined in Lässer et al. [14]. Briefly, 20 μg of the PKH67-stained exosomes were washed five times using 300 kDa Vivaspin filters (Sartorius AG, Göttingen, Germany) to remove excess dye and then added to 2 × 10^5 A549 cells in culture. Cells were harvested at different time points (1, 2, 4, 8, 12, 24 and 48 hours), washed three times and analyzed by flow cytometry using a BD FACSAria (BD Biosciences). Additionally, cells were visualized at 8 or 12 hours with a fluorescence microscopy (Zeiss Axiosplan 2 microscope, Carl Zeiss) or at 4 hours with confocal laser scanning microscope (LSM 700, Carl Zeiss). As control for non-specific labelling of the

Exosomes

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Exosomes were isolated from the supernatant of HMC-1, HEK 293 and NIH 3T3 cells by differential centrifugation and a filtration step as described by Lässer et al. [32]. In brief, cell supernatant were harvested, centrifuged at 300 × g for 10 minutes to eliminate cells and at 16,500 × g for 20 minutes, followed by filtration through 0.2 μm filter (Sarstedt, Numbrecht, Germany) to eliminate cellular debris and larger vesicles. Exosomes were pelleted by ultracentrifugation at 120,000 × g for 70 minutes. Exosomes were measured for their protein content using the BCA protein assay kit (Thermo Scientific Pierce, Rockford, IL, USA).

Electron microscopy

Isolated exosomes from HMC-1 cells were resuspended in PBS, and loaded onto UV-light pre-treated formvar/carbon-coated nickel grids (Ted Pella Inc., Redding, CA, USA). The exosomes were pre-fixed in 2% paraformaldehyde, before immuno-staining with anti-human CD63 antibody (BD Biosciences, San José, CA, USA). The exosomes were then immuno-stained with a 10 nm gold-labelled secondary antibody (Sigma-Aldrich) prior to being post- fixed in 2.5% glutaraldehyde and contrasted in 2% uranyl acetate. Preparations were examined in a LEO 912AB Omega electron microscope (Carl Zeiss Jena GmbH, Eching, Germany).

Uptake of HMC-1 exosomes by A549 cells

To monitor uptake kinetics, exosomes derived from HMC-1, as well as the control cells HEK 293 and NIH 3T3 cells, were labelled with the green fluorescent dye PKH67 (Sigma-Aldrich) according to the adjusted protocol outlined in Lässer et al. [14]. Briefly, 20 μg of the PKH67-stained exosomes were washed five times using 300 kDa Vivaspin filters (Sartorius AG, Göttingen, Germany) to remove excess dye and then added to 2 × 10^5 A549 cells in culture. Cells were harvested at different time points (1, 2, 4, 8, 12, 24 and 48 hours), washed three times and analyzed by flow cytometry using a BD FACSAria (BD Biosciences). Additionally, cells were visualized at 8 or 12 hours with a fluorescence microscopy (Zeiss Axiosplan 2 microscope, Carl Zeiss) or at 4 hours with confocal laser scanning microscope (LSM 700, Carl Zeiss). As control for non-specific labelling of the
cells, PBS was PKH67 stained, washed and added to the cells in a parallel experiment. For analysis with flow cytometry, the A549 cells were washed twice with PBS, treated with 0.25% trypsin-EDTA solution (Sigma-Aldrich) to detach the cells and washed twice with 1% FBS in PBS before acquired in a BD FACSARia flow cytometry running BD FACS Diva version 6.0 Software (BD Biosciences) and analyzed with the FlowJo Software (Tree Star Inc., Ashland, OR, USA). For microscopy, the cells were washed twice with PBS, fixed with 4% formaldehyde solution for 15 minutes and washed again twice with PBS. For the fluorescence microscopy the cells were mounted with Vectashield (Vector Laboratories Inc., Burlingame, CA, USA) supplemented with 3% 7-Aminoactinomycin (7-ADD; BD Biosciences) to label cell nuclei and for confocal microscopy cells were mounted with ProLong® Gold Antifade Mountant containing 4’,6-diamidino-2-phenylindole (DAPI; Life Technology).

**Transfer of KIT protein and c-KIT mRNA to A549 cells**

To determine if the KIT protein could be transferred via exosomes, HMC-1 exosomes (80 μg) were added to A549 cells (4 × 10^5) and the cells were incubated for 24 hours. The A549 cells were harvested, washed, and the total proteins were isolated using RIPA buffer (Cell Signaling Technology, Danvers, Massachusetts, USA). As a control, cells were treated with only media. The presence of KIT was determined using Western blot.

To determine if the c-KIT mRNA could be transferred via exosomes, HMC-1 exosomes (80 μg) were added to A549 cells (4 × 10^5). The cells were harvested at different time points (30 minutes, 2, 4, 24 and 48 hours), washed and isolation of total RNA was performed. Presence of c-KIT mRNA was determined using reverse transcription polymerase reaction (RT-PCR).

**RT-PCR**

Total RNA was isolated using miRCURY™ RNA Isolation Kit (Exiqon, Vedbaek, Denmark) according to the manufacturer’s protocol and as previously described [33]. Detection and quantity of RNA was determined using a Bioanalyzer and RNA 6000 Nano chips according to the manufacturer’s protocol (Agilent Technologies, Santa Clara, CA, USA).

For examination of the c-KIT gene expression, 500 ng of total RNA was converted into cDNA using RT® first Strand Kit (Qiagen, Hilden, Germany) according to the manufacturer’s recommendations. For PCR amplification 1 μl of diluted cDNA (<500 ng) was used as a template to make 50 μl reactions containing the reagents from HotStar™Taq Master Mix (Qiagen). KiQStart™ primer pairs (Sigma-Aldrich) used were as follows: GAPDH (forward) 5’-CTTTTGCCTGCGCAG-3’, (reverse) 5’-TTGATGGCAACAATTCCAC-3'; c-KIT (forward) 5’-ACAAAAACCA GAAATCCTGAC-3’, (reverse) 5’-CAGTTCCTGGACAA AAATACC-3’. The length of the expected amplicon for c-KIT and GAPDH were 109 and 139 base pair, respectively. PCR amplification involved 30 cycles at 94°C for 30 seconds, 55°C for 45 seconds, and 72°C for 30 seconds. PCR products were resolved on a 1.5% agarose gel (by loading 5 μl of the PCR product) and detected utilizing Gel Star® Nucleic Acid Gel Stain (Lonza, Rockland, ME, USA) and a VersaDoc 4000 MP (Bio-Rad Laboratories, Hercules, CA, USA).

**Reverse migration assay**

To evaluate the functional effect on lung epithelial cells (A549) induced by HMC-1 derived exosomes, reverse migration assay was performed using a 46 well Boyden chamber (Neuroprobe Inc.). Briefly, A549 cells (32,500 cells/well) were added in the lower chamber and allowed to adhere onto the gelatin (0.1%) coated polycarbonate membrane by inverting the Boyden chamber upside down (Neuroprobe Inc.). After 3 hours of incubation the assembly was placed in correct orientation and exposed to various HMC-1 exosomes dosage on the upper side of the membrane for 12 hours. Migrating cells towards the upper side of membrane were fixed in ethanol, and stained with Giemsa (Histolab, Gothenburg, Sweden) and images were acquired using a light microscope (Zeiss Axioplan, Germany). Adhered cells, present on lower side of membrane (non-migrated side), were wiped out carefully before imaging.

**Detection of cell proliferation**

Bromodeoxyuridine (BrdU) is incorporated into the newly synthesized DNA strands of actively proliferating cells. We therefore determined cell proliferation using a BrdU labeling ELISA kit (Calbiochem, San Diego, CA, USA) according to the manufacturer’s protocol. Shortly A549 cells were seeded at the density of 1 × 10^5 in a 96-well plate and incubated overnight to allow cells to attach to the plate. Cells received BrdU (20 μl) and 20 μg of either the HMC-1 exosomes or the control exosomes (isolated from NIH 3T3 cells) at the same time. After 4 hours of incubation the absorbance was obtained at dual wavelength (450 nm and 595 nm) with a spectrophotometer (Spectra Max; Molecular Devices, Sunnyvale, CA, USA). The results were normalized as percent of control.

**Western blot analysis**

The cellular and exosomal proteins were separated by SDS-PAGE, transferred to nitrocellulose membrane (Bio-Rad Laboratories), blocked (5% BSA-TBST; bovine serum albumin in TBS tween 20) for 2 hours. The membrane were then incubated with the following primary
rabbit anti-human antibodies: anti-CD81 (Santa Cruz Biotechnology, Dallas, TX, USA), anti-calnexin (Santa Cruz Biotechnology), anti-KIT (Abcam, Cambridge, UK), anti-SCF (Santa Cruz Biotechnology), anti-PI3K and anti-p-PI3K P85(T458)/P55(T199), anti-AKT and anti-p-AKT(T308), anti-GSK3β and anti-p-GSK3β(S9), and anti-cyclin D1 and anti-p-cyclin D1(T286) and primary mouse anti-human antibody; TSG101 (Abcam) diluted in 5% BSA -TBST at 4°C overnight. All antibodies were purchased from Cell Signaling Technology Inc., (Danvers, MA, USA) unless stated otherwise. The membrane was washed 3 × 5 minutes before incubation with the secondary antibody for 2 hours. The secondary antibodies used were goat F(ab)2 anti-rabbit IgG or donkey anti-mouse IgG (HRP conjugated, Harlan Sera-Lab, Loughborough, UK) diluted in 1% BSA-TBST. The membrane was washed 3 × 5 minutes before being analyzed with the Amersham™ECL Plus™ Western Blotting Detection System (GE Healthcare, Buckinghamshire, UK) and a VersaDoc 4000 MP (Bio-Rad Laboratories). To normalize protein loading, identical loaded samples were probed for GAPDH. Relative intensity was calculated as follows for p-PI3K, p-AKT and p-GSK3β; (phosphorylated protein/GAPDH)/(total protein/GAPDH), and for cyclin D1; cyclin D1/GAPDH.

Statistical methods
The statistical analyses were performed using SPSS 16.0 statistical software (SPSS, Chicago, IL, USA). Student’s t-test was used to analyze the difference between two groups, and one-way ANOVA followed by Dunnett’s test or Kruskal-Wallis test followed by Dunn’s multiple comparisons test was employed for the comparisons among three or more groups. To estimate correlation, a Spearman ranked correlation test was performed. Data are presented as the mean ± SEM for all statistical tests. Results were considered to be statistically significant when p ≤0.05.

Results
Identification of mast cell exosomes and their effect on lung adenocarcinoma cells
After isolation of exosomes from the supernatant of cultured HMC-1 cells, using ultracentrifugation, CD63 positive vesicular round structures could be visualized by electron microscopy (Figure 1A). The protein fraction isolated from the pellet after ultracentrifugation was positive for the traditional exosome markers CD81 and TSG101, while negative for a endoplasmic reticulum protein, calnexin (Figure 1B), confirming our previous data showing that HMC-1 cells can release exosomes [12,34,35]. Furthermore, exosomes isolated from HEK 293 and NIH 3T3 cells were also positive for the exosomal markers and negative for the endoplasmic reticulum protein calnexin (Figure 1B). These exosomes were therefore considered to be appropriate to be used as control exosomes in this study.

To examine whether exosomes from human mast cells can be taken up by lung cancer cells, the HMC-1 exosomes were labeled with PKH67 dye and added to cultures of A549 cells. Flow cytometry analysis (Figure 2A) showed an increased fluorescence intensity of the A549 cells after the addition of mast cell-derived exosomes, indicating cellular uptake. This uptake was reduced at 4°C, arguing that the uptake was an active process (Figure 2B). The fluorescence of A549 cells was obvious already two hours after addition of the stained exosomes, and increased over time (Figure 2C and D), indicating initiation of uptake of the exosomes by A549 cells. The uptake of the fluorescent exosomes by the A549 cells was also visualized using fluorescence microscopy (Figure 2E) and confocal microscopy (Figure 2F). Additionally, fluorescence microscopy demonstrated that control exosomes isolated from HEK 293 and NIH 3T3 cells were taken up by A549 cells in a similar manner as HMC-1 exosomes (Figure 2G). This verifies their validity to be used as control exosomes in the later functional experiments.

To determine whether mast cell exosomes can influence lung adenocarcinoma, both proliferation and migration was assessed in the A549 cells in the presence of HMC-1 exosomes. Firstly, HMC-1 exosomes were added to A549 cells in culture for 4 hours in the presence of BrdU to evaluate their capacity to influence proliferation. The incorporation of BrdU was significantly increased by 142% in the presence of HMC-1 exosomes compared to the medium control and by 38% compared to the control exosomes, as quantified by an ELISA kit (Figure 3A). Secondly, the A549 cells were seeded on the membrane of the lower chamber with different doses of HMC-1 exosomes present in the upper chamber to evaluate the exosomal capacity to induce migration. Significantly more cells migrated into the upper chamber in a dose response dependent manner in the presence of HMC-1 exosomes, with the highest doses being statistically significant compared to the control and with a correlation coefficient of 0.91 (p <0.0001) (Figure 3B).

The presence of KIT protein in mast cell exosomes and their shuttling to lung adenocarcinoma cells
The presence of the KIT protein in HMC-1 exosomes is illustrated in Figure 4A. By contrast, the A549 cells had no detectable KIT, as determined by Western blot. However, when HMC-1 exosomes were incubated with the A549 cells for 24 hours, KIT could be detected in the A549 cells by Western blot (Figure 4A). Stem cell factor (SCF), the ligand for KIT, was however present in the A549 cells, but absent in HMC-1 exosomes. By contrast,
we were unable to detect any c-KIT mRNA in the exosomes, but only in the HMC-1 cells (Figure 3B). Furthermore, the c-KIT mRNA was not detected in the A549 cells at any of the time points analyzed. GAPDH were used as positive controls, and GAPDH mRNA was present in all samples, except in the HMC-1 exosomes (Figure 3B). Our identification of KIT protein, but not c-KIT mRNA, in the HMC-1 exosomes, and the transfer of the protein to lung adenocarcinoma cells, suggests that the presence of KIT in the A549 cells after exosomes depend on transfer of KIT protein, rather than transfer of its mRNA (Figure 4A and B).

**Mast cell exosomes activate the KIT-SCF signaling pathway in recipient lung adenocarcinoma cells**

Next, we sought to determine whether transfer of KIT by HMC-1 exosomes could influence A549 intracellular KIT-associated pathways, proposing a molecular explanation for the observed enhanced proliferation seen in Figure 3A. As shown in Figure 5A-E, we indeed found that PI3K and its downstream targets, AKT, and GSK3β showed increased phosphorylation in A549 cells when HMC-1 exosomes were added, compared to media alone or control exosomes. However, the total amounts of these three proteins were unchanged according to the Western blot analysis. When PI3K is phosphorylated and thus activated it phosphorylates AKT, which in turn inactivates GSK3β by phosphorylating the S9 residue on GSK3β. Cyclin D1 is a cell-cycle regulator downstream of GSK3β and is inhibited by GSK3β. Therefore an increase in deactivation of GSK3β by phosphorylation will lead to an increase in cyclin D1, which will result in cells moving from the G1 phase into the S phase of the cell cycle. Importantly, cyclin D1, was indeed increased in A549 cells after the addition of HMC-1 exosomes, which then can be associated to increased proliferation of the lung adenocarcinoma cells.

When cyclin D1 is phosphorylated it is transported out of the nucleus and degraded, and as shown in Figure 5A, this inactivated stage is attenuated by HMC-1 exosomes, which thus further can enhance the activation of cyclin D1.

These experiments together indicate that KIT positive exosomes from HMC-1 cells have the capability to induce PI3K/AKT signaling and influence cell proliferation in A549 cells.

**Discussion**

Tumor tissues include many different cells, and little is known how they interact in a clinical situation to influence cancer development. It has been suggested that exosomes and other extracellular vesicles can shuttle many molecules between cells, which is a process likely to be very active in cell-to-cell signaling in tumors. Tumor tissue also contains many inflammatory cells, and it is known that mast cells can be part of the tumor microenvironment in lung tumors such as non-small cell lung cancer. However, little is known how mast cells might act to specifically promote cancer proliferation. Here, we found that exosomes derived from the human mast cell line, HMC-1, contain the receptor KIT, which can be transferred to lung adenocarcinoma cells by exosomes. In particular, we show that mast cells release exosomes with typical exosome markers such as TSG101 and CD81. The mast cell exosomes are rapidly taken up by lung adenocarcinoma cells (A549 cells), a process that peaks after approximately 12 hours. Importantly, the exosomes released by human mast cells contain the natural growth factor receptor KIT, but not c-KIT mRNA. Addition of mast cell exosomes containing KIT enhance proliferation and migration of the lung adenocarcinoma cells, and enhance the KIT-SCF signaling pathway activity. Overall, these data suggest that exosomes from mast cell
can enhance proliferation of lung adenocarcinoma cells, putatively by enhancing KIT-SCF signaling in tumor cells.

In this study, we confirm the ability of the mast cell line HMC-1 to release exosomes into their microenvironment [34], and extend these findings by demonstrating their ability to be taken up by human lung adenocarcinoma cells (A549 cells). The observed uptake is rapid, as more than 70% of cells have taken up some exosomes.
Figure 3 Mast cell-derived exosomes induce proliferation and migration in A549 cells. (A) BrdU cell proliferation assays were used to detect proliferating A549 cells after co-culturing for 4 hours with HMC-1 exosomes or control exosomes (derived from NIH 3T3 cells). *P <0.05. (B) A549 cells were added to the lower chamber of a Boyden chamber (32 500 cells/well). To the upper chamber 30 μl of the different doses of HMC-1 exosomes were added. Media was used as a control. After 12 hours the number of cells migrated to the lower chamber of the 8 μm pore-sized membrane were analyzed by taking photos and counting the number of cells per visual field. Kruskal-Wallis test followed by Dunn’s multiple comparisons test were used to determine significant differences where all concentrations were only compared to the control. Spearman’s rank correlation coefficient was 0.91 (p <0.0001). p-values; * <0.05, *** <0.001.

Figure 4 HMC-1 exosomes transfer KIT protein to A549 cells. Eighty microgram of HMC-1 exosomes were added per 4 ×10^5 A549 cells and incubated at 37°C for 24 hours for the Western blot experiments (A) and for 30 minutes - 48 hours for the RT-PCR experiments (B). A) Western blot confirmed the presence of the KIT protein in HMC-1 exosomes, but not in the A549 cells. SCF expression was detected in A549 cell but not in the HMC-1 exosomes. SCF and KIT were both detected in A549 cells 24 hours after the addition of HMC-1 exosomes. B) RT-PCR showed that c-KIT mRNA was present in HMC-1 cells, whereas it was absent in both HMC-1 exosomes and A549 cells. GAPDH mRNAs were detected as positive control and were detected in all samples except in HMC-1 exosomes.
already after one hour, and after four hours almost all cells are positive for exosomes. However, the uptake continues over time, measured as relative fluorescent intensity, and peaks at 12–24 hours after addition of exosomes. This time course of uptake is slightly faster than what we have previously reported [34].

Less is known about the functionality of mast cell exosomes. In the current experiments, we have described the presence of the KIT tyrosine kinase receptor in mast cell exosomes, a molecule that sometimes, but not always, is expressed in lung tumor tissues [36]. Importantly, we were unable to detect the c-KIT mRNA in the exosomes, suggesting that any functional shuttling of this molecule between cells is at the protein level. KIT was present in the lung adenocarcinoma cell line after addition of mast cell exosomes, documented using Western blot analysis. KIT has been considered to be a marker of progenitor cells [37] and can be more expressed in preneoplastic tissue and tumors, such as gastrointestinal stromal tumor (GIST) [38], small cell lung cancer (SCLC) [39], colorectal cancer [40,41] and pancreatic neoplasms [42]. KIT has also been implied in non-small cell lung cancer mortality, indicating that it may influence tumor growth and metastasis [23].

In the present study we confirm that addition of KIT-containing mast cell exosomes can enhance the proliferation of lung adenocarcinoma cells, shown as uptake of BrdU. A similar finding was recently published in which patient tumor exosomes containing KIT protein could transfer that protein to gastrointestinal stromal tumor cells and smooth muscle cells in vitro, which enhanced signs of invasiveness [26]. In our study, we have also included an appropriate exosome control in all

![Figure 5 Mast cell-derived exosomes can activate the KIT-SCF signaling pathway in A549 cells. A549 cells treated with exosomes from control cells (HEK 293) or HMC-1 cells were analyzed using Western blot. Phosphorylated and total PI3K, AKT, GSK3β and cyclinD1 were measured by Western blot and to normalize protein loading, samples were also probed for GAPDH. (A). Relative intensity was calculated as follows for p-PI3K (B), p-AKT (C) and p-GSK3β (D): (phosphorylated protein/GAPDH)/(total protein/GAPDH), and for cyclin D1 (E), cyclin D1/GAPDH. All the above data are representative of three independent experiments (n = 3). *P < 0.05 and **P < 0.01.](http://www.biosignaling.com/content/12/1/64)
experiments, and provided evidence that the KIT containing mast cell exosomes can enhance the KIT-SCF signaling pathways intracellularly.

We could here detect enhanced KIT-SCF signaling pathways, by up-regulation of phosphorylated PI3K as well as AKT and GSK3β. These data suggest, collectively, that the KIT containing mast cell exosomes indeed can activate cascades downstream from the tyrosine kinase receptor KIT. We therefore propose that the tumor cell-produced SCF can help tumor growth via a PI3K dependent pathway, if mast cell KIT-containing exosomes are delivered to the cells. Mast cell exosomes carrying KIT may bind SCF molecules produced by the tumor cells, and activate the cell in an autocrine fashion via PI3K and AKT. It is known that AKT can enhance tumor growth, by phosphorylating and inactivating GSK3β. This can in turn lead to a decrease in the nuclear exclusion of cyclin D1, which thereby allow the molecule to functioning as a cell cycle promoting by driving the cells from G1 phase to S phase [43,44]. Overall, our data imply that mast cell KIT-containing exosomes can influence the PI3K signaling pathway in recipient tumor cells.

There are of course shortcomings with in vitro studies such as our, as results may or may not be applicable for clinical disease. However, in a previous clinical study of non-small cell lung tumors, we found that KIT positivity was associated with higher mortality [23].

Our current series of experiments have demonstrated the release of KIT-containing exosomes from a human mast cell line, and shows that these exosomes can be taken up by lung adenocarcinoma cells. This leads to enhanced proliferation in recipient tumor cells, by the activation of the PI3K signaling pathway. Future work can highlight the molecular mechanisms leading to the release of exosomes from mast cells, and their uptake into tumor cells. Further, it is essential to understand the diverse possibilities of exosome-based intercellular communication among many different types of cells in the tumor microenvironment.

Acknowledgement
This work is supported by the National Natural Science Foundation of China (NO. 81072448 and NO. 81273276), the Shanghai Jiao Tong University training fund for major projects and the Shanghai Municipal Science and Technology Commission Foundation (NO.11JC1410300). This work was also funded by the IVB Group Herman Keffring Foundation for Asthma and Allergy Research, the Swedish Cancer Foundation (120772/CAN 2012/690) and the Swedish Research Council (k2011-56X-20676-04-6).

Received: 4 April 2014 Accepted: 30 September 2014

Published online: 14 October 2014

References
1. Raposo G, Nijman HI, Stoovogel W, Liejedekker R, Harding CJ, Melief CJ, Gruze HJ. B lymphocytes secrete antigen-presenting vesicles. J Exp Med 1996, 183:1161–1172.
2. Nolte’t Hoen EN, Buschow SI, Anderton SM, Stoorvogel W, Wauben MH. Activated T cells recruit exosomes secreted by dendritic cells via LFA-1. Blood 2009, 113:1977–1981.
3. Zitvogel L, Regnault A, Lasser A, Wolbers J, Flamant C, Tenza D, Ricciardi-Castagnoli P, Raposo G, Amigorena S. Eradication of established murine tumors using a novel cell-free vaccine: dendritic cell-derived exosomes. Nat Med 1998, 4:594–600.
4. Raposo G, Tenza D, Mecheri S, Peronet R, Bonnerot C, Desaymard C. Accumulation of major histocompatibility complex class II molecules in mast cell secretory granules and their release upon degranulation. Mol Biol Cell 1997, 8:2631–2645.
5. Fais S. NK cell-released exosomes: Natural nanobullets against tumors. Oncoimmunology 2013, 2:e22337.
6. Buning J, von Smolinski D, Tafazzoli K, Zimmer KP, Strobel S, Apostolaki M, Kollis G, Heath JK, Ludwig D, Gebert A. Multivesicular bodies in intestinal epithelial cells: responsible for MHC class II-restricted antigen processing and origin of exosomes. Immunology 2008, 125:510–521.
7. Hood JL, San RS, Wickline SA. Exosomes released by melanoma cells prepare sentinel lymph nodes for tumor metastasis. Cancer Res 2011, 71:3792–3801.
8. Friel AM, Corcoran C, Crown J, O’Driscoll L. Relevance of circulating tumor cells, extracellular nucleic acids, and exosomes in breast cancer. Breast Cancer Res Treat 2010, 123:613–625.
9. Bellingham SA, Coleman BM, Hill AF. Small RNA deep sequencing reveals a distinct miRNA signature released in exosomes from fractionated neuronal cells. Nucleic Acids Res 2012, 40:10937–10949.
10. Chaput N, Thery C. Exosomes: Immune properties and potential clinical implementations. Semin Immunopathol 2011, 33:419–440.
11. Luga V, Zhang L, Wiloria-Pettit AM, Ogunjimi AA, Ivanhui NJ, Chiu E, Buchanan M, Hosein AN, Basik M, Warna JL. Exosomes mediate stromal mobilization of autocrine Wnt-PCP signaling in breast cancer cell migration. Cell 2012, 151:1542–1556.
12. Valadi H, Ekstrom K, Bossios A, Jöst Strand M, Lee JJ, Lotvall JO. Exosome-mediated transfer of miRNAs and microRNAs is a novel mechanism of genetic exchange between cells. Nat Cell Biol 2007, 9:654–669.
13. Aliotta M, Pereira M, Li M, Amaral A, Sorokina A, Dooner MS, Sears EH, Brilliant K, Ramratnam B, Hixson DC, Quesenberry PJ. Stable cell fate changes in marrow cells induced by lung-derived microvesicles. J Extracell Vesicles 2012, 1.
14. Lasser C, Allikman VS, Ekstrom K, Edikh M, Paredes PT, Bossios A, Jöst Strand M, Gabrielson S, Lotvall J, Valadi H. Human saliva, plasma and breast milk exosomes contain RNA: uptake by macrophages. J Transl Med 2011, 9:5.
15. Caby MP, Lankar D, Vincendeau-Scherrer C, Raposo G, Bonnerot C. Exosomal-like vesicles are present in human blood plasma. Int Immunol 2005, 17:879–887.
16. Admrye C, Johansson SM, Qazi R, Field J, Lahesmaa R, Norman M, Neve EP, Scheynis A, Gabrielson S. Exosomes with immune modulatory features are present in human breast milk. J Immunol 2007, 179:1969–1978.
17. Street JM, Barran PE, Mackay CL, Wiedt S, Balimuth C, Walsh TS, Chalmers RT, Webb DJ, Dear JW: Identification and proteomic profiling of exosomes in human cerebrospinal fluid. J Transl Med 2012, 10:55.

18. Pietkun T, Shen RF, Knepper MA: Identification and proteomic profiling of exosomes in human urine. Proc Natl Acad Sci USA 2004, 101:13568–13573.

19. Kalhuri C, Skulli B: Exosomes in tumor microenvironment influence cancer progression and metastasis. J Mol Med (Berl) 2013, 91:431–457.

20. Park JE, Tan HS, Datta A, Lai RC, Zhang H, Meng W, Lim SK, Sae HK. Hypoxic tumor microenvironment modulates its microenvironment to enhance angiogenic and metastatic potential by secretion of proteins and exosomes. Mol Cell Proteomics 2010, 9:1085–1099.

21. Jensen BM, Akin C, Gilfillan AM: Pharmacological targeting of the KIT growth factor receptor: a therapeutic consideration for mast cell disorders. Br J Pharmacol 2013, 154:572–1582.

22. Rådinger M, Kuehn HS, Kim MS, Metcalfe DD, Gilfillan AM: Glycogen synthase kinase 3β activation is a prerequisite signal for cytokine production and chemotaxis in human mast cells. J Immunol 2010, 184:564–572.

23. Xiao H, Wang J, Liu Y, Li L: Relative influence of c-Kit expression and epidermal growth factor receptor gene amplification on survival in patients with non-small cell lung cancer. Oncology Letter 2014, 8:582–588.

24. Regan JL, Kendrick H, Magnay FA, Vaafaezadeh V, Groner B, Smalley MJ: c-Kit is required for growth and survival of the cells of origin of Brca1-mutation-associated breast cancer. Oncogene 2012, 31:8869–8883.

25. Macher-Greppinger S, Perzel R, Roth W, Dienermann H, Thomas M, Schnabel PA, Schirmacher P, Blaker H: Expression and mutation analysis of EGFR, c-Kit, and beta-catenin in pulmonary blastoma. J Clin Pathol 2011, 64:349–353.

26. Atay S, Banksota S, Crow J, Sethi G, Rink L, Godwin AK: Oncogenic KIT-containing exosomes increase gastrointestinal stromal tumor cell invasion. Proc Natl Acad Sci USA 2014, 111:711–716.

27. Jacobs B, Ullrich E: The interaction of NK cells and dendritic cells in the tumor environment: how to enforce NK cell & DC action under immunosuppressive conditions? Curr Med Chem 2012, 19:1771–1779.

28. Johansson A, Rudolfsson S, Hammarsten P, Halin S, Pietras K, Jones J, Stattin P, Egevad L, Granfors T, Wikstrom P, Bergh A: CD133, BCRP1/ABCG2 and CD117/c-KIT are not associated with prognosis in resected early-stage non-small cell lung cancer. Int J Cancer 2004, 111:259–263.

29. Ham M, Moon A: Inflammatory and microenvironmental factors involved in breast cancer progression. Arch Pharm Res 2013, 36:1419–1431.

30. Evans SJ, Prabhu RG, Gnananuban V, Findl EA, Ramasubramanian AK: Monocytes mediate metastatic breast tumor cell adhesion to endothelium under flow. FASEB J 2013, 27:3017–3029.

31. Stoyanov E, Uddin M, Manikuta D, Dubinitz SM, Levi-Schaffer F: Mast cells and histamine enhance the proliferation of non-small cell lung cancer cells. Lung Cancer 2012, 75:38–44.

32. Lässer C, Eldh M, Lötavall J: Isolation and characterization of RNA-containing exosomes. J Vis Exp 2012, 9:59.

33. Lässer C, D’Onè SE, Ekerljung L, Ekström K, Sjöstrand M, Lötavall J: RNA-containing exosomes in human nasal secretions. Am J Rhinol Allergy 2011, 25:89–93.

34. Ekström K, Valadi H, Sjöstrand M, Malmhall C, Bossios A, Eldh M, Lötavall J: Characterization of mRNA and microRNA in human mast cell-derived exosomes and their transfer to other mast cells and blood CD4+ progenitor cells. J Extracellular Vesicles 2012, 1.

35. Crescielli R, Lasser C, Szabo TG, Kittel A, Eldh M, Danzani I, Buas E, Lötavall J: Distinct RNA profiles in subpopulations of extracellular vesicles: apoptotic bodies, micovesicles and exosomes. J Extracellular Vesicles 2013, 2.

36. Herpel E, Jensen K, Muley T, Warth A, Schnabel PA, Meister M, Henth FJ, Dienermann H, Thomas M, Gottschling S: The cancer stem cell antigens CD133, BCRP1/ABCG2 and CD117/c-Kit are not associated with prognosis in resected early-stage non-small cell lung cancer. Anticancer Res 2011, 31:4491–4500.

37. Ikin T, Ludin A, Gradus B, Gur-Cohen S, Kalinkovich A, Schajnovitz A, Ovadya Y, Kollet O, Canani J, Shezen E, Coffin DJ, Enkolopoulos GN, Berg T, Picciello W, Hornstein E, Lisacek T, FGF-2 expands hematopoietic stem and progenitor cells via proliferation of stromal cells, c-Kit activation, and CXCL12 down-regulation. Blood 2012, 120:1843–1855.

38. Heinrich MC, Cofess CL, Dernethi GD, Blanke CD, von Mehren M, Joensuu H, McGreevey LS, Chen CJ, Van den Abbeele AD, Drucker BJ, Kiese B, Eisenberg B, Roberts PJ, Singer S, Fletcher CD, Silberman S, Dimitrijevic S, Fletcher JA; Kinase mutations and imatinib response in patients with metastatic gastrointestinal stromal tumor. J Clin Oncol 2003, 21:4342–4349.

39. Rohr UP, Reifeld N, Pfugfelder L, Geddert H, Müller W, Steidl U, Fenk R, Graf T, Schott M, Thiele RP, Gabbett HE, Germing U, Kronenwett R, Haas R: Expression of the tyrosine kinase c-Kit is an independent prognostic factor in patients with small cell lung cancer. Int J Cancer 2013, 91:431–457.

40. Attoub S, Rivat C, Rodrigues S, Van Boclaer S, Bedin M, Bruyneel E, Louvet C, Kompriot M, Andre T, Mareel M, Mester J, Gespach C: The c-Kit tyrosine kinase inhibitor ST1571 for colorectal cancer therapy. Cancer Res 2002, 62:4879–4883.

41. El-Serag HM, Bahnassy AA, Ali NM, Eid SM, Kamel NM, Abdel-Hamid NA, Zeiki AR: The prognostic value of c-Kit, K-ras codon 12, and p53 codon 72 mutations in Egyptian patients with stage II colorectal cancer. Cancer 2010, 116:4954–4964.

42. Yauuda A, Sawai H, Takahashi H, Ochi N, Matsu Y, Funahashi H, Sato M, Okada Y, Takeyama H, Manabe T: The stem cell factor/c-Kit receptor pathway enhances proliferation and invasion of pancreatic cancer cells. Mol Cancer 2006, 5:46.

43. Engelman JA: Targeting PI3K signalling in cancer: opportunities, challenges and limitations. Nat Rev Cancer 2009, 9:550–562.

44. Koyasu S: The role of PI3K in immune responses. Nat Immunol 2003, 4:313–319.