Heterogeneity of microvesicles from cancer cell lines under inflammatory stimulation with TNF-α

Frank Gieseler 1*, Corinna Plattfaut1, Tabea Quecke1, Annika Freund1, Hendrik Ungefroren1,2 and Fanny Ender1

1 Section Experimental Oncology, University Hospital and Medical School (UKSH), University of Luebeck, Luebeck 23538, Germany
2 Department of General and Thoracic Surgery, University Hospital Schleswig-Holstein, Kiel 24105, Germany

Abstract

Microvesicles (MVs) represent a subgroup of extracellular vesicles (EVs) emerging from various cells by blebbing of their outer membrane. Therefore, they share features such as membrane composition and antigenicity with their parental cells. Released by many immune and tumor cells, MVs act as intercellular messengers, account for horizontal gene transfer and can activate the coagulation system. With the aim to investigate their relevance for tumor cell biology, we characterized MVs released by human tumor cell lines of various origins in the absence or presence of TNF-α. After stimulation, we used the combination of low and high-speed centrifugation to enrich MVs from cell culture supernatants. We analyzed the presentation of phosphatidylserine (PS) and tissue factor (TF) activity on the cell surface and investigated their potency to induce tumor cell migration. In all tumor cell lines, TNF-α stimulation enhanced the release of MVs. While the expression of PS was universally increased, an elevated activity of procoagulant TF could be detected on MVs from lung, pancreatic, and colon carcinoma, but not from breast and ovarian cancer cell lines. Functionally, TNF-α stimulation significantly increased the potency of MVs to induce tumor cell migration. In conclusion, inflammatory conditions promote the release of MVs with increased procoagulant activity from tumor cell lines in vitro. PS-containing and TF-expressing MVs may account for systemic activation of the coagulation system as seen in cancer patients and, since they induce tumor cell migration, they may serve as biomarkers for tumor progression.

Keywords: cancer; coagulation; extracellular vesicles; inflammation; microvesicles; thrombosis

Introduction

Since Rudolf Virchow reported the formation of neoplastic tissue on sites of previous inflammation in 1863 (Virchow, 1862), the association between inflammation and cancer has been of interest to scientists and clinicians alike. The interaction of tumor cells with their microenvironment is essential for tumor growth and expansion, as well as their escape from the immune system. Tumor progression and the development of metastatic disease can be better understood in the context of the tumor microenvironment including stromal cells, different effectors of the immune system, blood platelets, growth factors, cytokines, hormones, and other humoral factors (Upadhyay et al., 2018; Valilou et al., 2018). Inflammation mediators such as interleukin 6 (IL-6) or tumor necrosis factor-α (TNF-α) may enhance cancer progression and trigger pro-thrombotic changes in the circulation and vasculature (Gonda et al., 2009). Hemostatic factors such as TF complexes can both drive inflammation and promote cancer progression and dissemination (Gonda et al., 2009). It has also been reported that altered expression of certain oncogenes or tumor suppressor genes, as modulated by inflammation or hypoxia, may promote the procoagulant properties of cancer cells (Prandoni et al., 2005).

In this context, information and signal transmission between tumor cells and non-tumor cells within the...
microenvironment by EVs is a currently studied system (Ciardiello et al., 2016; Clancy et al., 2015b). In the extracellular space, besides apoptotic bodies, several different classes of EVs including exosomes, microvesicles, and oncosomes with different sizes are present and their membrane structures, content and ways of generation have been described (van der Pol et al., 2016). The vesicles can transfer genetic information (DNA, RNA) as well as non-coding RNA and proteins by interacting with cell membrane structures and antigens (Fatima and Nawaz, 2017a). Several cells including thrombocytes, immune cells, and tumor cells release EVs, especially under inflammatory conditions that characterize the tumor microenvironment (Thery et al., 2009). Plasmatic EVs are considered to play a decisive role in the pathogenesis of several inflammatory conditions, such as infections and autoimmune diseases (Buzas et al., 2014); they have also been correlated with high venous thromboembolism (VTE) rates in cancer patients (Manly et al., 2010) as well as tumor progression (Muralidharan-Chari et al., 2010). The contribution of inflammatory mechanisms to VTE and cancer mortality differs across cancer types and stages (Key et al., 2016).

The composition and antigenicity of the membrane of EVs is of crucial importance. For instance, EVs have the potential to activate the coagulation system by specific surface structures, such as coagulation factors (e.g., TF) or phospholipids (Baj-Krzyworzeka et al., 2006; Penfornis et al., 2016). In particular, microvesicles (MVs), a subgroup of EVs formed by the bud-like projection and constriction of the outer cell membrane (0.2–1 μm) (Nawaz et al., 2014), have a greater ability to accomplish this in comparison to other EVs, such as oncosomes and exosome-like vesicles, because of the exposure of PS as well as the presentation of TF on their surface (Zwicker, 2008; Zwicker et al., 2011; Geddings and Mackman, 2013). Cancer-associated thrombosis, including pulmonary embolism (PE) and VTE, is a major challenge for cancer patients, with severe impact on prognosis and life quality (Fuentes et al., 2016). The risk of VTE is increased in cancer patients treated with chemotherapy, and it predicts poorer survival (Key et al., 2016). In fact, thromboembolism has been described as a leading cause of death in cancer patients receiving outpatient chemotherapy (Khorana et al., 2007). TF-positive MVs as well as increased levels of leukocytes and platelets are suspected to be factors that alone or in combination increase cancer-associated thrombosis (Bharthuar et al., 2013; Hisada and Mackman, 2017).

In this study, we examined MVs formed and released by tumor cell lines of various origins. We compared MVs released under normal cell culture conditions with those released in the presence of TNF-α, which mimics the inflammatory in vivo situation. TNF-α has been described as “a master switch for inflammation to cancer” (Sethi et al., 2008). The examined cancer cell lines were of various origins, including those highly associated with VTEs such as ovarian, pancreatic, and lung cancer. MVs were enriched by sequential centrifugation steps including high-speed centrifugation at 10,000g and characterized by high-resolution flow cytometry and scanning electron microscopy. Subsequently, we looked for their PS presentation and their potency to form procoagulant complexes (extrinsic tenase), as well as their ability to promote migration of the pancreatic tumor cell line Colo357.

Material and methods

Cell culture

All cell lines were cultured under serum-free conditions with 10% panexin NTA as serum supplement (see Table S1 for product descriptions). Since fetal calf serum contains large amounts of MVs that can contaminate the experimental preparations, it was not added to the medium (Thery et al., 2006). Instead, the concentration of panexin NTA was increased stepwise over several weeks. Cells were split using biotase depending on their growth rate when a confluence of about 80% was reached. All cell lines were free of contamination and regular tests for detection of mycoplasma infections were performed.

Inflammatory stimulation

For the simulation of an inflammatory environment, TNF-α (100 ng/mL) was added to the cell culture medium. In previous experiments, we compared this method of MV stimulation with other stimulating agents, such as transforming growth factor-β (TGF-β) and the induction of hypoxia by detaNO; however, TNF-α was the most potent inducer of MVs (data not shown). Due to its important role in cancer-induced inflammation, all further experiments were performed with TNF-α. For stimulation experiments, 9 × 10⁵ cells were seeded into a T75 flask and incubated for 3 days with serum-free, panexin NTA-supplemented cell culture medium (RPMI-1640) +/− 100 ng/mL TNF-α at 37°C, 5% CO₂. After 96 h, the supernatant was collected, spun at 1300 g to remove cellular debris and samples were stored at −20°C. Prior to further experiments, cells were detached from the cell culture flask by biotase and stained with trypan blue to check for viability.

Proliferation

To determine the proliferation rate, 3 × 10⁵ cells were seeded into T25 flasks and incubated for 96 h with serum-free, panexin NTA-supplemented cell culture medium (RPMI-1640) +/− 100 ng/mL TNF-α at 37°C, 5% CO₂. After 24, 48,
72, and 96 h, the supernatant of three flasks was collected and cells were removed by biotase. Cells were counted using the NovoCyte Flow Cytometer.

Apoptosis
To check for apoptosis, 9 \times 10^5 cells were seeded into T75 flasks and incubated for 96 h under the same conditions as mentioned above. After 96 h, human caspase-3 assay was performed according to the manufacturer’s instructions. In short, cells were scraped from the culture flask, washed twice, pelleted, and lysed with cell extraction buffer. Cell lysates were spun at 13,000g for 10 min at 4°C and the supernatants were transferred to a clean tube. Assay’s standards and controls or samples were introduced into the wells of antibody-coated plates and incubated for 2 h at room temperature. After washing, the caspase-3 detection antibody was introduced and incubated for 1 h at room temperature. After another washing step, HRP-coupled anti-IgG antibody was introduced and incubated for 30 min at room temperature. After washing, the stabilized chromogen substrate was introduced and incubated for 30 min at room temperature in the dark. After 30 min, the stop solution was introduced and absorbance was measured at 450 nm. Changes in absorbance were directly proportional to the amount of active caspase-3 present in the sample/control/standard. A standard curve was used to determine the caspase-3 concentration of the sample.

Enrichment of MVs
Several methods of EV separation have been described (Nawaz et al., 2014). In our study, MVs were enriched using the protocols previously published by Lacroix et al. (Lacroix et al., 2013) and Muralidharan-Chari et al. (Clancy et al., 2015a). In short, samples were thawed at room temperature. For depletion of cells and large cell debris including most apoptotic bodies and large oncosomes, samples were spun twice at a low-speed centrifugation of 2,500 g for 20 min and the supernatant was collected. Subsequently, the supernatant was spun at 10,000g for 90 min. The pellet was then collected and re-suspended in filtered PBS (0.22 μm filters). Since MVs, especially activated MVs, tend to stick together and are hard to homogenize, purified MVs, at concentrations depending on the cell line of origin, were directly administered to the following assays without intermittent storing.

Scanning electron microscopy of MVs
Isolated MVs intended for scanning electron microscopy (SEM) were fixed with a glutaraldehyde at a final concentration of 2.5% in filtered PBS and stored at 4°C until further preparation. After homogenization, 10–20 μL of each sample were placed onto a Thermostat coverslip and allowed to settle for 90–120 min in a humid chamber to prevent drying. For dehydration, the samples were then placed into solutions with increasing acetone concentration (70–100%) and subsequently fully dried via critical point drying using CO2 to avoid shrinkage effects and loss of structure from air drying. The dehydrated samples were sputtered with gold and analyzed with a Zeiss EVO LS15 scanning electron microscope.

Flow cytometric analysis of MVs
MV membranes were stained with carboxyfluorescein succinimidyl ester (CFSE) to separate vesicle signals from debris. For CFSE staining, samples were incubated with 40 μM CFSE for 1 h prior to high-speed centrifugation. This concentration and incubation time were titrated in preceding experiments (not shown). The pellet was then suspended in filtered PBS. The amount of MV was determined using a NovoCyte Flow Cytometer in a fluorescein isothiocyanate (FITC)-triggered measurement mode (Arraud et al., 2016). This allows discarding all recorded events below the fluorescence detection limit, such as background noise and unstained buffer signals. We used CFSE-stained PBS as negative control and subtracted that signal from the samples. For size gate calibration, we used the Megamix, the Megamix Plus FSC, and Megamix Plus SSC beads, which contain six fluorescent bead populations of defined sizes (0.16 μm; 0.20 μm; 0.24 μm; 0.30 μm; 0.50 μm; 0.90 μm; 3.00 μm). The Megamix Plus gate was set according to the manufacturer’s instructions including the 0.16 μm and 0.90 μm bead populations (Poncelet et al., 2016). Analysis was performed in triplicates.

PS content of MVs
The content of PS in MVs was measured using the Zymuphen MP-Activity assay according to manufacturer’s instructions; samples were diluted 1:20 prior to use. In short, standards, controls (both provided in the kit) or samples were diluted with sample diluent, which contains FXa and thrombin inhibitors. Diluted samples were then introduced to the streptavidin and biotinylated annexin V-coated microtiter plate and incubated for 1 h at 37°C. After washing, FXa, FVa, calcium, and prothrombin were introduced and incubated for 10 min at 37°C. Thrombin-specific chromogenic substrate was introduced and after 3 min, substrate turnover stopped by the addition of 2% citric acid. After 10 min stabilization time, the absorbance was measured at 405 nm. Since PS is a cofactor for the FXa-FVa complex, catalyzing the activation of prothrombin to thrombin, the thrombin substrate turnover is proportional.
to the amount of PS bound to the microtiter plate. Assays were performed in triplicates. ELISA results were calibrated to the exact MV numbers and expressed in relation to 10^6 MVs.

**TF-expression of MVs**

MV-associated TF activity was measured using the Zymu-phen MP-TF assay according to manufacturer’s instructions; samples were diluted 1:10 prior to use. In brief, standards, controls (both provided in the kit) and samples were diluted with provided sample diluent, introduced with assay enhancer into TF antibody-coated microtiter plates and incubated overnight at room temperature. After washing, FVIIa and FX were introduced and incubated for additional 2 h at 37°C. Without further washing, FXa chromogenic substrate was introduced and after 2 h incubation at 37°C, substrate turnover was stopped with citric acid (2%). Absorbance was measured at 405 nm. FXa substrate turnover was directly proportional to the amount of active TF present in the sample. Assays were performed in quadruplicates. ELISA results were calibrated to the exact MV numbers and expressed in relation to 10^6 MVs.

**Migration-induction potency of MVs and inhibition experiments**

Cell-based Ori8™ migration assay was performed to examine the effect of MVs on tumor cells. Assays were performed according to the manufacturer’s instructions with the human pancreatic carcinoma Colo357 cell line. In short, 3 x 10^4 cells/well were seeded using cell culture medium supplemented with 10% panexin NTA. After overnight incubation, cells formed an adherent layer. Twenty-four hours after seeding, stoppers were pulled, cell culture medium was removed and 100 μL cell culture medium (20% panexin NTA) + 100 μL sample/control were introduced. The number of MVs within these 100 μL samples can be estimated by the data in Figure 1. Protease-activated receptor 2 (PAR2) agonistic peptide (100 μM) and transforming growth factor-β1 (TGF-β1) 10 ng/mL served as positive control, and PBS as negative control. For inhibitory experiments, cells were co-incubated with MVs of the cell lines +/+ GB88 (10 μM). GB88 is a specific PAR2 inhibitor, which competitively inhibits the activation of PAR2 by trypsin and other PAR2 agonists (Suen et al., 2012). After 24 h at 37°C, 5% CO2, cells received additional 100 μL cell culture medium (supplemented with 10% panexin NTA). Cells were left for 48 h to migrate into the free space in the middle of the well, then fixed and stained with DiffQuick Cell Staining kit. After staining, Ori8™ detection mask was clipped to the bottom of the plate and images were taken with a blackfly camera on an Axioskop HBO 50 microscope. Images were analyzed with Fiji module of ImageJ software. Assays were performed in quadruplicates. Results were then calibrated to the exact MV numbers and expressed in relation to 10^6 MVs.

**Statistics**

Statistical analysis was performed using MS Excel (Microsoft Excel for Mac 2011, version 14.6.3). All data were analyzed using two-tailed student’s t-test. Values of P < 0.05 were considered statistically significant. All data are described as mean ± SD.

**Results**

**Response of cancer cell lines to inflammatory stimulation**

To determine if an inflammatory environment alters the biological properties of cancer cells, we determined the proliferation rate in lung (A549), pancreatic (Colo357), colon (CaCo2), breast (MDA-MB231), and ovarian (OV-CAR3) carcinoma cell lines cultures using TNF-α to generate an inflammatory environment. Since TNF-α is a known inhibitor of epithelial cell growth (Fräther-Schröder et al., 1987), it reduced the proliferation rate of all the tested cell lines (Table 2 and Table S2). As the reduced proliferation was most pronounced in the pancreatic cancer cell line Colo357, we exemplarily tested this cell line for signs of apoptosis. After 96 h of incubation, there was no significant difference in caspase-3 activity between the TNF-α-stimulated and the non-stimulated cells (Table 2).
Different types of immune cells have been shown to release MVs into the extracellular space under steady-state conditions. As shown in Figure 1, cancer cells of various origins have comparable capabilities to release MVs in a non-inflammatory environment. Specifically, under steady-state cell culture conditions, ovary, colon, and breast carcinoma cells released lower amounts of MVs than pancreas and lung carcinoma cells. Inflammatory stimulation of cancer cell lines with TNF-α resulted in a significantly increased MV release in most of the cancer cell lines tested (Figure 1). The effect of TNF-α on breast carcinoma cells was highly potent, inducing the cells to release amounts of MVs at the upper detection level. In pancreas and ovary carcinoma cells, the inflammatory micro-milieu also led to a strong but intermediate MV release. Lung carcinoma cells, with the highest release rate of MVs under steady-state conditions, could not be stimulated further with TNF-α under the given conditions.

These data suggest that inflammatory stimulation of cancer cell lines results in a change in the cells’ metabolism since proliferation was reduced in favor of an increased MV production and altered MV properties as described in following sections.

**Structural characterization of isolated MVs**

Tumor cell-derived MVs were purified by a sequential centrifugation process as described in the materials and methods section, and further characterized by high-resolution flow cytometry. Besides the quantification of released MVs (Figure 1), we also wished to determine their size, as well as structural parameters. Therefore, we used fluorescent “Megamix beads” that represented six distinct size populations in the range from 0.16 to 3.0 μm. The upper panel of Figure 2 shows that more than 96% of purified MVs were found within the Megamix Plus gate representing MVs in the size range of 0.16–0.9 μm under both steady-state and inflammatory cell culture conditions. In addition to high-resolution flow cytometry, we performed SEM of purified MVs to get a structural impression of their nature. Representative SEM figures show the higher number of MVs being released from cancer cells upon TNF-α.

![Figure 2](image-url)
stimulation. Furthermore, activated MVs from stimulated cancer cell line cultures seemed to have a greater potential to adhere to each other (Figure 2, lower panel).

In summary, the combination of sequential centrifugation and high-resolution flow cytometry is suitable for detecting MVs with high sensitivity. Microscopically, the isolated MVs were within the suggested size range and their phenotypes seem to be dependent on their micro-milieu.

**Functional characterization of isolated MVs**

As we got the impression of MVs switching to a sticky phenotype within an inflammatory microenvironment (Figure 2, lower panels), we tested the MVs for their PS content. We found a certain amount of PS already under steady-state culture conditions on MVs from all cell lines tested. While MVs from lung carcinoma cells presented with the lowest, MVs from pancreas carcinoma cells presented with the highest amount of PS on their surface. MVs from all cell lines showed a significantly higher amount of PS upon stimulation with TNF-α (Figure 3). The increase in PS content under inflammatory conditions was most pronounced in lung carcinoma cells.

Due to their expression of surface structures such as PS and tissue factor (TF), MVs are capable of activating the coagulation system. Under steady-state cell culture conditions, TF activity could be detected on MVs from all cancer cell lines tested (Figure 4). Among those, lung carcinoma cells produced MVs with the lowest level of active TF, while breast carcinoma cells shed MVs with the highest TF activity. Surprisingly, stimulation of the cell cultures with TNF-α had rather diverse effects on the regulation of TF activity on MVs. MVs originating from pancreas, colon, or lung carcinoma cells showed an increase in active TF. In contrast, a decreased TF activity was detected on MVs from ovary and breast carcinoma cells under inflammatory conditions.

Importantly, TF is not only involved in the activation of the extrinsic coagulation cascade but can also activate cellular receptors such as PAR2 (Gieseler et al., 2013) and thus induce pro-migratory signaling. To test the effect of tumor cell line-derived MVs on tumor cell migration, we used the Oris™ Cell Migration assay. Representative images of MVs co-incubated with pancreas carcinoma cells for 48 h are shown in Figure 5. It can be clearly seen that MVs derived from TNF-α-stimulated cells (Figure 5B) induced significantly stronger tumor cell migration as compared to those from unstimulated cells (Figure 5A). Controls are shown in Figure S1.

Overall, there was a differential effect of MVs isolated from non-stimulated and stimulated cancer cell lines on tumor cell migration (Figure 6). In comparison to the basic migratory activity of pancreas carcinoma cells used as test cell line in this study (Figure S2A), MVs from all cell lines induced migration (Figure S2B). MVs derived from TNF-α-stimulated colon (CaCo2) and lung carcinoma cells (A549) showed an increased potency to induce tumor cell migration compared to the MVs derived from the non-stimulated cell culture. Once again, MVs derived from the gynecological cell lines MDA-MB-231 (breast cancer) and OVCAR3 (ovary carcinoma) exerted a reduced pro-migratory effect when cultures were stimulated with TNF-α compared to those derived from unstimulated cells. This seems to be consistent with their reduced expression of active TF as shown in Figure 4 and points to the importance of TF activity for the migration-inducing effect (via PAR2 stimulation).
It has been discussed before that the PAR2 signaling pathway via TF-complex activation through MVs is a possible signaling pathway for the induction of tumor cell migration (Schaffner and Ruf, 2009; Gieseler et al., 2013; Witte et al., 2016). In Figure 7, it is shown that GB88, a specific inhibitor of PAR2 signaling (Suen et al., 2012), inhibits MV-induced tumor cell migration. In line with the assumption that the formation of the TF complex on the surface of MVs is crucial for the induction of migration through the activation of PAR2, the inhibitory effect was not significant for the MVs isolated from the gynecological tumor cell lines, in contrast to MVs from the other cell lines.

In summary, PS content is upregulated on MVs of tumor cell lines in an inflammatory environment. However, an additional increase in TF activity seems to be essential for the induction of MV-mediated tumor cell migration via PAR2 as inhibition of this pathway (with GB88) has an inhibitory effect upon MV-induced migration.

Discussion

In this study, we characterized MVs, the sub-group of EVs formed by the plasma membrane (ectosomes), that present

![Figure 5 Oris migration assay using tumor cell-derived MVs.](image)

The pancreas carcinoma cell line Colo357 was used as migrating cells and seeded at a density of $3 \times 10^5$ cells per well in the presence of a stopper covering the migration area. After overnight incubation, stoppers were pulled, and cells were co-incubated with the purified MVs for 48h. After fixation and DiffQuick staining, the migration area was analyzed under the microscope using the Oris™ detection mask. Shown is an exemplary picture of Colo357 cells co-incubated with MVs derived from (A) unstimulated or (B) TNF-$\alpha$-stimulated CaCo2 cells. Control experiments with PBS, PAR2 agonist, TGF-$\beta$, and GB88 are shown in Figures S1 and S2 in the supplemental data files.

![Figure 6 Impact of tumor cell-derived MVs on tumor cell migration.](image)

Cells from the pancreas carcinoma cell line Colo357 were used as migrating cells. After co-incubation with the tumor cell line-derived MVs for 48h, migration was determined and is expressed in $\text{pixel}^2$ per $10^6$ MVs (pixel$^2 = 1.69 \text{ mm}^2$). Displayed is the migration after co-incubation with MVs derived from unstimulated (light gray columns) or TNF-$\alpha$-stimulated (dark gray columns) tumor cell lines. Values shown are the mean ± SD; $n = 4$ per group; * indicates significant differences between unstimulated and stimulated treatment groups, $^*P < 0.01$, $^{**}P < 0.001$.

![Figure 7 Inhibition of MV-induced tumor cell migration by a specific inhibitor of PAR2 signaling (GB88).](image)

Colo357 were used as migrating cells. The migration upon co-incubation with purified MVs derived from TNF-$\alpha$-stimulated tumor cell lines was determined in the absence (dark gray columns) or presence (diagonally striped columns) of GB88. Control experiments (pooled data) with MVs from unstimulated cells w/wo GB88 are shown in the left group of columns. Migration is expressed in % of the negative control. Values shown are the mean ± SD; $n = 4$ per group; * indicates significant differences between unstimulated and stimulated treatment groups, $^*P < 0.01$, $^{**}P < 0.001$. Further control experiments for GB88 are shown in the suppl. Figures S1 and S2.
antigens and membrane structures on the outer leaflet of the cell membrane. We separated MVs from other EVs, such as the smaller exosomes, by serial centrifugation steps. The predominance of MVs after the separation protocol is shown by validating the expected size range as well by high-resolution flow cytometry as by SEM (Figure 2). Of note, regarding the high specificity of MV purification that we obtained, this method is more suitable as compared to the heterogeneous EV population after centrifugation at 100,000 g (Buzas et al., 2014). It has been shown that MVs can not only promote leukocyte aggregation, but also induce macrophage apoptosis, thus severely hampering the immune system (Ardoin et al., 2007). Stressed or injured cells release MVs showing damage-associated molecular patterns, which induce and promote further inflammation (Hezel et al., 2017). MVs can also present auto-antigens in auto-inflammatory diseases (Buzas et al., 2014). Furthermore, it has been reported that tumor MVs induce the formation of pro-metastatic niches in remote organs (Hoshino et al., 2015). MVs themselves can carry and secrete membrane-bound TNF-α and can induce other cells to release inflammatory cytokines (Obregon et al., 2009; Yanez-Mo et al., 2015). Thus, induction of MVs might be a clinically relevant link between inflammation, cancer-associated thrombosis and, finally, cancer progression and metastasis.

We investigated different cancer cell lines, described in Table 1, under steady-state as well as under inflammatory cell culture conditions and compared properties of the induced MVs regarding tumor progression and involvement in cancer-associated development of thrombosis or VTE. We used TNF-α to mimic the inflammatory in vivo conditions. TNF-α has been described as the "a master switch for inflammation to cancer" (Sethi et al., 2008). In addition, it has been described to induce strong inflammatory reactions as well as EV release in various cell lines including COLO357 cells (Raitano et al., 1990; Yamamoto et al., 2015). Stimulation with TNF-α resulted in a significantly increased release of MVs in almost all tumor cell lines tested (Figure 1) without inducing apoptosis under the used concentration (Table 2). Interestingly, the amount of MVs released by lung cancer cells was very high as compared to the other tested cell lines already under steady-state cell culture conditions and was not further increased by TNF-α treatment (Figure 1). The high amounts of MVs in lung carcinoma cells correlate with the high thrombosis rate of lung carcinoma patients. Elevated platelet-derived MV, endothelial cell-derived MV, and monocyte-derived MV concentrations are documented in almost all thrombotic diseases; however, the significance of MVs derived from tumor cells remains controversial (Nomura and Shimizu, 2015). Moreover, the increase of MVs after TNF-α stimulation might also indicate an escape mechanism from immune surveillance by tumor cells in vivo, as several cells, including tumor cells, release

### Table 1 Overview of cell lines used

| Name    | Origin         | Organ          | Characteristics                                      | Doubling time |
|---------|----------------|----------------|------------------------------------------------------|---------------|
| OVCAR3  | ATCC: HTB-161  | Ovary          | Adenocarcinoma                                       | 69 h          |
| Colo357 | ECACC: 94072245| Pancreas       | Adenocarcinoma, lymph node, metastasis,              | 21 h          |
| AS49    | ATCC: CRM-CCL-185| Lung           | Epithelial carcinoma (KRAS CRM)                     | 22 h          |
| CaCo2   | ATCC: HTB-37   | Colon          | Adenocarcinoma                                       | 62 h          |
| MDA-MB-231 | ATCC: HTB-26 | Breast         | Epithelial carcinoma pleural effusion               | 38 h          |

All cell lines were free of contamination and regular tests for detection of mycoplasma infections were performed. More information about the used ATCC cell lines including genomic profile, etc. can be found here: https://www.lgcstandards-atcc.org; and for the ECACC cell line Colo357: https://web.expasy.org/cellosaurus/CVCL_0221

### Table 2 Cell count and caspase 3 levels of unstimulated and stimulated Colo357 cells

|                      | Unstimulated | Stimulated (TNF-alpha) | P-value |
|----------------------|--------------|------------------------|---------|
| Cell count (x10⁵ cells) |              |                        |         |
| 48 h                 | 4.20 ± 0.01  | 4.95 ± 0.54            | n.s.    |
| 72 h                 | 16.59 ± 0.59 | 11.69 ± 0.29           | <0.001  |
| 96 h                 | 24.34 ± 1.28 | 18.12 ± 0.41           | <0.05   |
| Caspase 3 (pg/10⁵ cells) |              |                        |         |
| 96 h                 | 25.90 ± 2.27 | 27.43 ± 1.14           | n.s.    |

Mean and standard deviation, n = 4; Student’s t-test two-tailed P-value. GB88 did not reduce proliferation or viability (trypan blue exclusion); therefore, a caspase 3 was not performed with GB88.
MV to constrain and evade the immune system, for example, via FAS-ligand-exposing vesicles that induce T-cell apoptosis (Abrahams et al., 2003; Fatima and Nawaz, 2017b); this mechanism has been described, for instance, in ovarian cancer (Nawaz et al., 2016).

Overall, the varying numbers of MVs induced by different cancer cell lines with and without inflammatory stimulus reflects differences in the underlying tumor biology. Indeed, a correlation of MV counts with tumor stage has been found in patients with breast cancer, where late-stage ascites contained substantially more vesicles than those in early-stage disease (Graves et al., 2004). Similarly, in breast cancer cell lines, the number of shed MVs correlated with their invasive capacity (Ginestra et al., 1998).

Remarkably, TNF-α not only induced MV release but there was significantly higher PS presentation on MVs from all tumor cell lines (Figure 3) and higher TF expression in most tumor cell lines (Figure 4). In general, PS presentation on membranes is a known adhesion factor, which is shown, for instance, for erythrocytes in sickle cell anemia (Setty et al., 2002). Furthermore, PS is a potent cofactor of the coagulation factors Xa (FXa) and Va (FVa); it accelerates the rate of thrombin generation and consequently clot formation (Lentz, 2003; Leventis and Grinstein, 2010). With regard to MVs, it is known that they express functional cytoadhesins, bioactive phospholipids, cytoplasmic components, and various antigens that are characteristic for the cell of their origin, and for the type of stimulus (Freyssinet, 2003; Hussein et al., 2003). The amount of PS on the surface of MVs has been associated with their way of generation by budding of the outer leaflet of the cell membrane. Therefore, the membrane composition of MVs reflects the membranous elements of the cell of origin, with phospholipids being particularly important because they are involved in their bioactive function (Leventis and Grinstein, 2010). Notably, the increased PS content on MVs from TNF-α-stimulated cancer cell line cultures might be the explanation for the sticky phenotype of MVs after cell stimulation as shown in the laser scanning microscopy (Figure 2).

Besides PS presentation, MVs have the potency to activate the coagulation system, which is considerably increased by the membrane components, namely P-selectin and TF. Several studies have described the frequent expression of TF on the surface of MVs (Yu and Rak, 2004; Hron et al., 2007; Rauch and Antoniak, 2007; Langer et al., 2008; Nieuwland, 2008; Zwicker, 2008). However, in contrast to the universal increase in PS on MVs from tumor cell lines upon TNF-α stimulation, the activity of TF was elevated especially on MVs from pancreas, colon and lung carcinoma cells but not on the two gynecological cell lines Ovcar3 and MDA-MB-231 (Figure 4). Co-expression of PS and TF is a strong signal for the activation of the TF-pathway of the coagulation system (Muralidharan-Chari et al., 2010) as well as for the activation of the PAR2 signaling pathway (Gieseler et al., 2013; Langer and Ruf, 2014). Initiation of the TF-pathway through the release of tumor cell-derived MVs into the blood stream is suggested to be a pathophysiological explanation for the systemic activation of the coagulation system in cancer patients (Manly et al., 2010; Thaler et al., 2012b) with deleterious consequences, such as thrombosis and VTE (Khorana et al., 2007). Nevertheless, one must keep in mind that strong inhibitors of the TF/FVIIa complex such as tissue factor pathway inhibitor (TFPI) play an important role in the in vivo situation and allow possibilities for therapeutic interventions, for instance, by TFPI-releasing low molecular weight heparins (LMWHs) such as tinzaparin (Depasse et al., 2003; Mousa and Mohamed, 2004; Gamperl et al., 2016). Besides the TF-pathway, TF/FVIIa complexes, especially in conjunction with PS in the membranes, are potent inducers of tumor cell migration through their ability to activate PAR2 (Schaffner and Ruf, 2009; Gieseler et al., 2013; Witte et al., 2016).

PAR2 and other PARs belong to the family of G protein-coupled receptors and are abundantly expressed on tumor cells (Ikeda et al., 2003; Elste and Petersen, 2010; Gieseler et al., 2013). In our study, we determined the impact of MVs derived from TNF-α-stimulated cancer cell lines on the migration of Colo357 cells, as they persistently express PAR2 (Witte et al., 2016). In line with the diverse expression of active TF, MVs from TNF-α-stimulated carcinoma cells differentially induced tumor cell migration in vitro (Figure 6). As the two gynecologic tumor cell lines released MVs expressing lower levels of active TF with a lower potency to induce migration of Colo357 cells, we suggest that the expression of active TF is essential for the induction of tumor cell migration. Accordingly, we could show that the migration of Colo357 cells is regulated via PAR2 signaling as the administration of a specific inhibitor of PAR2 (GB88) significantly reduced the migration-inducing effect of tumor cell-derived MVs (Figure 7). MV-mediated induction of tumor cell migration is of potential clinical relevance, since it can be seen as a prerequisite for metastatic spread (Ikeda et al., 2003; Geho et al., 2005). In addition, it has been shown that PAR2 activation induces the release of even more TF-bearing MVs, which might further increase the effect in vivo (Ettelaie et al., 2016).

A direct correlation between the pure amount of MVs in the plasma and VTEs could not be established in all cancers (Thaler et al., 2012a), which might be due to the complex set of factors that define the clinical situation as well as the diversity of MVs from different tumor cells. This should be taken into account in the interpretation of our in vitro data.
Conclusions

The strength of this project lies in the structural and functional characterization of MVs from various cell lines with and without inflammatory stimulation. The quantification has been done by using a high-resolution flow cytometer and all functional results have been expressed in relation to the exact number of MVs. Although the results of in vitro experiments using cell lines must be interpreted with caution, our observations are potentially of clinical relevance, as patients with progressive tumors have high VTE rates with deleterious effects and, cancer patients with an activated coagulation system have a worse prognosis. Our results also show that MVs released by tumor cells of various entities are highly diverse, underlining the need for further research with ex vivo material to find clinically relevant surrogate parameters for cancer-associated VTEs and cancer progression.

Acknowledgments and funding

We thank Monica Vollmert, Sylvia Grammerstorff and Detlev Schult-Badusche for their technical assistance. We thank Matthias Klinger and Hristo Örun for performing the scanning electron microscopy. This work was in part funded by Annelise-Asmussen Stiftung and LEO-Pharma, Germany.

Author contributions

FG and CP planned the project, conceived and designed experiments, analyzed data, FG, CP, and FE wrote the manuscript. CP, AF, FE, HU, and TQ conducted the experiments. All authors controlled and corrected the manuscript.

Conflicts of interest

The authors declare no conflict of interest.

References

Abrahams VM, Straszewski SL, Kamsteeg M, Hanczarak B, Schwartz PE, Rutherford TJ, Mor G (2003) Epithelial ovarian cancer cells secrete functional Fas ligand. Cancer Res 63: 5573–81.

Ardoin SP, Shanahan JC, Pisetsky DS (2007) The role of microparticles in inflammation and thrombosis. Scand J Immunol 66: 159–65.

Arraud N, Gounou C, Turpin D, Brisson AR (2016) Fluorescence triggering: a general strategy for enumerating and phenotyping extracellular vesicles by flow cytometry. Cytometry A 89: 184–95.

Baj-Krzyworzeka M, Szatanek R, Weglarczyk K, Baran J, Urbanowicz B, Branski P, Ratajczak MZ, Zembala M (2006) Tumour-derived microvesicles carry several surface determinants and mRNA of tumour cells and transfer some of these determinants to monocytes. Cancer Immunol Immunother 55: 808–18.

Bharthuar A, Khorana AA, Hutson A, Wang J-G, Key NS, Mackman N, Iyer RV (2013) Circulating microparticle tissue factor, thromboembolism and survival in pancreaticobiliary cancers. Thrombosis Res 132: 180–4.

Buza E, György B, Nagy G, Falus A, Gay S (2014) Emerging role of extracellular vesicles in inflammatory diseases. Nat Rev Rheumatol 10: 356–64.

Ciardiello C, Cavallini L, Spinelli C, Yang J, Reis-Sobreiro M, De Candia P, Minciaccchi VR, Di Vizio D (2016) Focus on extracellular vesicles: new frontiers of cell-to-cell communication in cancer. Int J Mol Sci 17: 175.

Clancy JW, Sedgwick A, Rosse C, Muralidharan-Chari V, Raposo G, Method M, Chavrier P, D’Souza-Schorey C (2015a) Regulated delivery of molecular cargo to invasive tumour-derived microvesicles. Nat Commun 6: 6919.

Clancy JW, Tricarico CJ, D’Souza-Schorey C (2015b) Tumor-derived microvesicles in the tumor microenvironment: how vesicle heterogeneity can shape the future of a rapidly expanding field. Bioessays 37: 1309–16.

Depasse F, de Suso MG, Lagouette I, Fontcuberta J, Borrell M, Samama M (2003) Comparative study of the pharmacokinetic profiles of two LMWHS—bemiparin (3500 IU, anti-Xa) and tinzaparin (4500 IU, anti-Xa)—administered subcutaneously to healthy male volunteers. Thrombosis Res 109: 109–17.

Elste AP, Petersen I (2010) Expression of proteinase-activated receptor 1–4 (PAR 1–4) in human cancer. J Mol Histol 41: 89–99.

Etteiaie C, Collier ME, Featherby S, Benelhaj NE, Greenman J, Maraveyas A (2016) Analysis of the potential of cancer cell lines to release tissue factor-containing microvesicles: correlation with tissue factor and PAR2 expression. Thrombosis J 14: 2.

Fatima F, Nawaz M (2017a) Long distance metabolic regulation through adipose-Derived circulating exosomal miRNAs: a trail for RNA-Based therapies? Front Physiol 8: 545.

Fatima F, Nawaz M (2017b) Nexus between extracellular vesicles, immunomodulation and tissue remodeling: for good or for bad? Ann Transl Med 5.

Fräter-Schröder M, Risau W, Hallmann R, Gautschi P, Bühlen P (1987) Tumor necrosis factor type alpha, a potent inhibitor of endothelial cell growth in vitro, is angiogenic in vivo. Proc Natl Acad Sci 84: 5277–81.

Freyssinet JM (2003) Cellular microparticles: what are they bad or good for? J Thromb Haemost 1: 1655–62.

Fuentes HE, Tafur AJ, Caprini JA (2016) Cancer-associated thrombosis. Disease-a-Month 62: 121–58.

Gampfer H, Plattfaut C, Freund A, Quecke T, Theophil F, Gieseler F (2016) Extracellular vesicles from malignant effusions induce tumor cell migration: inhibitory effect of LMWH tinzaparin. Cell Biol Int 40: 1050–61.

Geddings JE, Mackman N (2013) Tumor-derived tissue factor-positive microparticles and venous thrombosis in cancer patients. Blood 122: 1873–80.
Microvesicles from cancer cell lines

Geho DH, Bandle RW, Clair T, Liotta LA (2005) Physiological mechanisms of tumor-cell invasion and migration. Physiology 20: 194–200.

Gieseler F, Ungefroren H, Settmacher U, Hollenberg MD, Kaufmann R (2013) Proteinase-activated receptors (PARs)-focus on receptor-receptor-interactions and their physiological and pathophysiological impact. Cell Commun Signal 11: 86.

Ginestra A, La Placa M, Saladinio F, Cassara D, Nagase H, Vittorelli M (1998) The amount and proteolytic content of vesicles shed by human cancer cells correlate with their in vitro invasiveness. Anticancer Res 18: 3433–7.

Gonda TA, Tu S, Wang TC (2009) Chronic inflammation, the tumor microenvironment and carcinogenesis. Cell Cycle 8: 2005–13.

Graves LE, Ariztia EV, Navari JR, Matzel HJ, Stack MS, Fishman DA (2004) Proinvasive properties of ovarian cancer ascites-derived membrane vesicles. Cancer Res 64: 7045–9.

MEv Hezel, Nieuwland R, Bruggen Rv, Juffermans NP (2017) The ability of extracellular vesicles to induce a pro-inflammatory host response. International Journal of Molecular Sciences 18: 1285.

Hisada Y, Mackman N (2017) Cancer-associated pathways and biomarkers of venous thrombosis. Blood. 130: 1499–506.

Hoshino A, Costa-Silva B, Shen TL, Rodrigues G, Hashimoto A, Tesic Mark M, Molina H, Kohsaka S, Di Giannatale A, Ceder S, Singh S, Williams C, Soplop N, Uryu K, Pharrer L, King T, Bojmar L, Davies AE, Ararso Y, Zhang T, Zhang H, Hernandez J, Weiss JM, Dumont–Cole KD, Kramer K, Wexler LH, Narendran A, Schwartz GK, Healey JH, Sandstrom P, Labori KJ, Kure EH, Grandgenett PM, Hollwadge MA, de Sousa M, Kaur S, Jain M, Mallya K, Batra SK, Jarnagin WR, Brady MS, Fodstad O, Muller V, Pantel K, Minn AJ, Bissell MJ, Garcia BA, Kang Y, Rajasekhar VK, Ghajar CM, Mati E, Peinado H, Bromberg J, Lyden D (2015) Tumour exosome integrins determine organotropic metastasis. Nature 527: 329–35.

Hron G, Kollars M, Weber H, Sagaster V, Quehenberger P, Eichinger S, Kyrlie PA, Weltermann A (2007) Tissue factor-positive microparticles: Cellular origin and association with coagulation activation in patients with colorectal cancer. Thromb Haemost 97: 119–23.

Hussein A, Meesters EW, Osmanovic N, Romijn F, Nieuwland R, Sturk A (2003) Antigenic characterization of endothelial cell-derived microparticles and their detection ex vivo. J Thromb Haemost 1: 2434–43.

Ikeda O, Egami H, Ishiko T, Ishikawa S, Kamohara H, Hidaka H, Mita S, Ogawa M (2003) Expression of proteinase-activated receptor-2 in human pancreatic cancer: a possible relation to cancer invasion and induction of fibrosis. Int J Oncol 22: 295–300.

Key NS, Khorana AA, Mackman N, McCarty OJ, White GC, Francis CW, McCrae KR, Palumbo JS, Raskob GE, Chan AT (2016) Thrombosis in cancer: research priorities identified by a national cancer institute/national heart, lung, and blood institute strategic working group. AACR.

Khorana A, Francis C, Culakova E, Kuderer N, Lyman G (2007) Thromboembolism is a leading cause of death in cancer patients receiving outpatient chemotherapy. J Thromb Haemost 5: 632–4.

Lacroix R, Judicone C, Mooberry M, Bouckeine M, Key NS, Dignat-George F (2013) Standardization of pre-analytical variables in plasma microparticle determination: results of the International Society on Thrombosis and Haemostasis SSC Collaborative workshop. J Thromb Haemost.

Langer F, Ruf W (2014) Synergies of phosphatidylserine and protein disulfide isomerase in tissue factor activation. Thromb Haemost 111: 590–7.

Langer F, Spath B, Haubold K, Holstein K, Marx G, Wierecky J, Brummendorf TH, Dierlamm J, Bokemeyer C, Eifrig B (2008) Tissue factor procoagulant activity of plasma microparticles in patients with cancer-associated disseminated intravascular coagulation. Ann Hematol 87: 451–7.

Lentz BR (2003) Exposure of platelet membrane phosphatidylserine regulates blood coagulation. Prog Lipid Res 42: 423–38.

Leventis PA, Grinstein S (2010) The distribution and function of phosphatidylserine in cellular membranes. Annu Rev Biophys 39: 407–27.

Manly DA, Wang J, Glover SL, Kashturi R, Liebnam HA, Key NS, Mackman N (2010) Increased microparticle tissue factor activity in cancer patients with venous thromboembolism. Thromb Res 125: 511–2.

Mossa SA, Mohamed S (2004) Inhibition of endothelial cell tube formation by the low molecular weight heparin, tinzaparin, is mediated by tissue factor pathway inhibitor. J Thromb Haemost 92: 627–33.

Muralidharan-Chari V, Clancy JW, Sedgwick A, D’Souza-Schorey C (2010) Microvesicles: mediators of extracellular communication during cancer progression. J Cell Sci 123: 1603–11.

Nawaz M, Camussi G, Valadi H, Nazarenko I, Ekstrom K, Wang X, Principe S, Shah N, Ashraf NM, Fatima F (2014) The emerging role of extracellular vesicles as biomarkers for urogenital cancers. Nat Rev Urol 11: 688–701.

Nawaz M, Fatima F, Nazarenko I, Ekstrom K, Murtaza I, Anees M, Sultan A, Neder L, Camussi G, Valadi H (2016) Extracellular vesicles in ovarian cancer: applications to tumor biology, immunotherapy and biomarker discovery. Expert Rev Proteomics 13: 395–409.

Nieuwland R (2008) Cellular origin of microparticles exposing tissue factor in cancer: a mixed double? J Thromb Haemost 6: 1514–6.

Nomura S, Shimizu M (2015) Clinical significance of procoagulant microparticles. J Intensive Care 3: 2.

Obregon C, Rothen-Rutishauser B, Gerber P, Gehr P, Nicod LP (2009) Active uptake of dendritic cell-derived exovesicles by epithelial cells induces the release of inflammatory mediators through a TNF-alpha-mediated pathway. Am J Pathol 175: 696–705.

Penfornis P, Vallabhaneni KC, Whitt J, Pochampally R (2016) Extracellular vesicles as carriers of microRNA, proteins and lipids in tumor microenvironment. Int J Cancer 138: 14–21.
Poncelet P, Robert S, Bouriche T, Bez J, Lacroix R, Dignat-George F (2016) Standardized counting of circulating platelet micro-particles using currently available flow cytometers and scatter-based triggering: forward or side scatter? Cytometry A 89: 148–58.

Prandoni P, Falanga A, Piccioli A (2005) Cancer and venous thromboembolism. Lancet Oncol. 6: 401–10.

Raitano AB, Scuderi P, Korc M (1990) Binding and biological effects of tumor necrosis factor and gamma interferon in human pancreatic carcinoma cells. Pancreas 5: 267–77.

Rauch U, Antoniak S (2007) Tissue factor-positive microparticles in blood associated with coagulopathy in cancer. Thromb Haemost 97: 9–10.

Schaffner F, Ruf W (2009) Tissue factor and PAR2 signaling in the tumor microenvironment. Arterioscler Thromb Vasc Biol 29: 1999–2004.

Sethi G, Sung B, Aggarwal BB (2008) TNF: a master switch for inflammation. Front Biosci 13: 5094-107.

Setty BY, Kulkarni S, Stuart MJ (2002) Role of erythrocyte phosphatidylserine in sickle red cell-endothelial adhesion. Blood 99: 1564–71.

Suen JY, Barry GD, Lohman RJ, Halili MA, Cotterell AJ, Le GT, Fairlie DP (2012) Modulating human proteinase activated receptor 2 with a novel antagonist (GB88) and agonist (GB110). Br J Pharmacol 165: 1413–23.

Thaler J, Ay C, Pabinger I (2012a) Microparticle-associated tissue factor activity, venous thromboembolism and mortality in pancreatic, gastric, colorectal and brain cancer patients. J Thromb Haemost 10: 1363–70.

Thaler J, Ay C, Pabinger I (2012b) Clinical significance of circulating microparticles for venous thromboembolism in cancer patients. Hamostaseologie 32: 127–31.

Thery C, Amigorena S, Raposo G, Clayton A (2006) Isolation and characterization of exosomes from cell culture supernatants and biological fluids. Curr Protoc Cell Biol. Chapter 3:Unit 3.22

Thery C, Ostrowski M, Segura E (2009) Membrane vesicles as conveyors of immune responses. Nat Rev Immunol 9: 581–93.

Upadhyay S, Sharma N, Gupta KB, Dhiman M (2018) Role of immune system in tumor progression and carcinogenesis. J Cell Biochem. 119(7): 5028–42.

Valioli SF, Keshavarz-Fathi M, Silvestris N, Argentiero A, Rezaei N (2018) The role of inflammatory cytokines and tumor associated macrophages (TAMs) in microenvironment of pancreatic cancer. Cytokine Growth Factor Rev 39: 46–61.

van der Pol E, Boing AN, Gool EL, Nieuwland R (2016) Recent developments in the nomenclature, presence, isolation, detection and clinical impact of extracellular vesicles. J Thromb Haemost 14: 48–56.

Virchow R (1862) Die krankhaften Geschwüste: 30 Vorlesungen, gehalten während des Wintersemesters 1862-1863 an der Universität zu Berlin. Berlin: Hirschwald.

Witte D, Zeeh F, Gadeken T, Gieseler F, Rauch BH, Settmacher U, Kaufmann R, Lehnhert H, Ungefroren H (2016) Proteinase-activated receptor 2 is a novel regulator of TGF-β signaling in pancreatic cancer. J Clin Med 5: 111.

Yamamoto S, Niida S, Azuma Y, Yanagibashi T, Muramatsu M, Huang TT, Sagara H, Higaki S, Ikutani M, Nagai Y (2015) Inflammation-induced endothelial cell-derived extracellular vesicles modulate the cellular status of pericytes. Sci Rep 5: 8505.

Yanez-Mo M, Siljander PR, Andreu Z, Zavec AB, Borras FE, Buzas EI, Buzas K, Casal E, Cappello F, Carvalho J, Colas E, Cordeiro-da Silva A, Fais S, Falcon-Perez JM, Ghoibrial IM, Giebel B, Gimona M, Graner M, Gursel I, Gursel M, Heegaard NH, Hendrix A, Kierulf P, Kokubun K, Kosanovic M, Kralj-Iglic V, Kramer-Albers EM, Laitinen S, Lasser C, Lener T, Ligeti E, Line A, Lippis G, Llorente A, Lotvall J, Mancek-Keber M, Marcilla A, Mittelbrunn M, Nazarenko I, Nolte-'t Hoen EN, Nyman TA, O'Driscoll L, Olivan M, Oliveira C, Pallinger E, Del Portillo HA, Reventos J, Rigau M, Rohde E, Sammar M, Sanchez-Madrid F, Santarem N, Schallmoser K, Ostenfeld MS, Stoovogel W, Stukelj R, Van der Grein SG, Vasconcelos MH, Wauben MH, De Wever O (2015) Biological properties of extracellular vesicles and their physiological functions. J Extracell Vesicles 4: 27066.

Yu JL, Rak JW (2004) Shedding of tissue factor (TF)-containing microparticles rather than alternatively spliced TF is the main source of TF activity released from human cancer cells. J Thromb Haemost 2: 2065–7.

Zwicker JI (2008) Tissue factor-bearing microparticles and cancer. Semin Thromb Hemost 34: 195–8.

Zwicker JI, Trenor CC, 3rd, Furie BC, Furie B (2011) Tissue factor-bearing microparticles and thrombus formation. Arterioscler Thromb Vasc Biol 31: 728–33.

Received 9 March 2018; accepted 29 July 2018. Final version published online 23 August 2018.

Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1. Exemplary migration assay using controls.

Figure S2. Controls for Oris cell migration.

Table S1. List of equipment and reagents.