

PML–RARA modulates the vascular signature of extracellular vesicles released by acute promyelocytic leukemia cells

Yi Fang^{1,2} · Delphine Garnier¹ · Tae Hoon Lee¹ · Esterina D'Asti¹ · Laura Montermini¹ · Brian Meehan¹ · Janusz Rak¹

Received: 27 May 2015 / Accepted: 10 September 2015 / Published online: 15 September 2015
© Springer Science+Business Media Dordrecht 2015

Abstract Oncogenic transformation is believed to impact the vascular phenotype and microenvironment in cancer, at least in part, through mechanisms involving extracellular vesicles (EVs). We explored these questions in the context of acute promyelocytic leukemia cells (NB4) expressing oncogenic fusion protein, PML–RARA and exquisitely sensitive to its clinically used antagonist, the all-*trans* retinoic acid (ATRA). We report that NB4 cells produce considerable numbers of EVs, which are readily taken up by cultured endothelial cells triggering their increased survival. NB4 EVs contain PML–RARA transcript, but no detectable protein, which is also absent in endothelial cells upon the vesicle uptake, thereby precluding an active intercellular trafficking of this oncogene in this setting. ATRA treatment changes the emission profile of NB4-related EVs resulting in preponderance of smaller vesicles, an effect that occurs in parallel with the onset of cellular differentiation. ATRA also increases IL-8 mRNA and protein content in NB4 cells and their EVs, while decreasing the levels of VEGF and tissue factor (TF). Endothelial cell uptake of NB4-derived EVs renders these cells more TF-positive and procoagulant, and this effect is diminished by pre-treatment of EV donor cells

with ATRA. Profiling angiogenesis-related transcripts in intact and ATRA-treated APL cells and their EVs reveals multiple differences attributable to cellular responses and EV molecular packaging. These observations point to the potential significance of changes in the angiogenic signature and activity associated with EVs released from tumor cells subjected to targeted therapy.

Keywords Acute promyelocytic leukemia · PML–RARA · Oncogene · Extracellular vesicles · Exosomes · Angiogenesis · Tissue factor · Endothelial cells · ATRA · Targeted agents · Angiogenic signature · Extracellular RNA · Cancer biomarker

Abbreviations

APL	Acute promyelocytic leukemia
ATO	Arsenic trioxide
ATRA	All- <i>trans</i> retinoic acid
EVs	Extracellular vesicles
IL-8	Interleukin 8
NTA	Nanoparticle tracking analysis
PML–RARA	Promyelocytic leukemia–retinoic acid receptor alpha
TF	Tissue factor
TF-PCA	TF-dependent procoagulant activity
VEGF	Vascular endothelial growth factor

Electronic supplementary material The online version of this article (doi:10.1007/s10456-015-9486-1) contains supplementary material, which is available to authorized users.

✉ Janusz Rak
janusz.rak@mcgill.ca

¹ The Research Institute of the McGill University Health Centre, Montreal Children's Hospital, McGill University, 1001 Decarie Blvd, Montreal, QC H4A 3J1, Canada

² Department of Hematology, Renji Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai 200127, China

Introduction

Acute promyelocytic leukemia (APL), a subtype of acute myelogenous leukemia (AML), is characterized by a balanced reciprocal translocation between chromosomes 17 and 15 [*t*(15;17)(q22;q21)]. This genetic event results in

oncogenic fusion between promyelocytic leukemia (PML) and retinoic acid receptor alpha (RAR α) genes [1], thereby altering their physiological roles. RAR α acts as the nuclear receptor for the retinoic acid (RA), a function that triggers transcriptional activation of genes involved in granulocytic differentiation. While PML–RAR α and other less frequent RAR α fusion products obliterate myeloid differentiation, the exposure of leukemic cells to all-*trans* retinoic acid (ATRA) can restore RAR α -dependent transcription, also leading to degradation of the PML–RAR α oncoprotein by proteasome and autophagy pathways [2]. In combination with arsenic trioxide (ATO), which targets PML–RAR α in leukemic stem cells, and chemotherapy, ATRA imparts complete and lasting remission in the majority of APL cases [3].

APL can be viewed as a paradigm of not only “oncogene addiction” and the efficacy of oncogene-directed (differentiation) therapy with ATRA, but also a multifaceted nature of vascular involvement in the leukemic process. Thus, coagulopathy is a common and early event in APL patients, as manifested by severe bleeding tendency with a considerable risk of mortality [4, 5]. This diathesis results from disseminated intravascular coagulation (DIC) largely attributed to procoagulant characteristics of leukemic cells themselves, including their Annexin 2 (A2) and tissue factor (TF) expression, but may also involve emission of TF exposing microparticles/extracellular vesicles (EV) into the circulation [6–8]. Moreover, bone marrow of patients with APL exhibits increased features of angiogenic activation, such as elevated microvascular density, while leukemic cells are known to produce proangiogenic and proinflammatory mediators [9]. This phenotype includes PML–RAR α -dependent up-regulation of vascular endothelial growth factor (VEGF) [10], and the concomitant activation of hypoxia response pathways [11]. These characteristics are consistent with the proposed role of the angiogenic circuitry in the progression of hematopoietic malignancies [12]. The related mechanisms include bone marrow perfusion, trophic and endothelial niche effects, which support the expansion of leukemic stem cells [11, 13, 14]. These effects may also entail autocrine stimulation of malignant cells by angiogenic growth factors [15], immunomodulation [16] and possibly other processes [17].

ATRA profoundly alters the cellular phenotype, gene expression [18–20] and interactions between APL cells and the vascular system. While the nature of these effects remains controversial [21], it is well documented that the exposure to ATRA triggers a reversal of TF-related procoagulant activity of APL cells and suppresses coagulopathy in vivo [22, 23]. These responses also include down-regulation of VEGF and of other APL-associated angiogenic activities [10]. However, ATRA also possesses potentially proangiogenic activities, such as direct stimulation of

endothelial cell responses [21], and production of proangiogenic mediators by APL cells. The latter includes ATRA-dependent deregulation of cytokines and chemokines, such as interleukin 8 (IL-8/CXCL8), IL-6 and IL-1 β , some of which may directly interact with endothelial cells (IL-8), while others promote inflammatory responses, potentially with vascular consequences. These paradoxical effects are exemplified by the drug-related differentiation syndrome (DS) associated with respiratory distress, pulmonary infiltration and other perturbations affecting lung microcirculation in ATRA-treated patients [20, 24].

It is now recognized that in addition to cytokines and soluble mediators the secretome of cancer and leukemic cells also contains other more complex bioactive constituents, including heterogeneous membrane structures known as extracellular vesicles (EVs) [25]. EVs are generated through diverse biogenetic mechanisms [25], some of which involve membrane blebbing leading to abscission and shedding of larger vesicles (100–1000 nm) known as ectosomes, microvesicles or microparticles (MPs). In contrast, smaller vesicles (30–150 nm in diameter) may emerge within endosomal multivesicular bodies (MVB) and undergo a regulated release into the extracellular space as exosomes, rich in tetraspanins, integrins, membrane receptors and nucleic acids. Several other vesiculation pathways may also exist and result in formation of multiple distinct EV subsets [25–27].

EVs are believed to mediate molecular transfer and intercellular communication between hematopoietic, stromal, vascular and leukemic cells. This includes the maintenance of cellular equilibrium within the stem cells hierarchy [28], reprogramming cellular differentiation states [29], coagulation system activation, communication with stromal and endothelial cells, and transfer of signaling receptors, nucleic acids and other cargo in various settings of chronic and acute leukemia [29–33]. While such EV-mediated processes have been described in the case of APL, their nature remains relatively unexplored [7].

Of particular relevance in this context is the link between EVs and oncogenic pathways [34, 35]. Thus, cellular vesiculation is often increased or otherwise deregulated in cells expressing mutant oncogenes, while the respective oncogenic proteins, transcripts, micro-RNA and DNA are found in the cargo of EVs produced by cancer cells [34, 36–38]. Moreover, the emission of such EVs containing cancer-specific cargo may serve as a mechanism of intercellular horizontal transfer of biological activity leading to responses that may resemble cellular transformation, including increase in survival and angiogenic potential in the recipient cells [34, 36, 38–41]. For example, EV-mediated transfer of oncogenic epidermal growth factor receptor (EGFR) to normal endothelial cells may result in the onset of VEGF production and autocrine

reprogramming of these cells [42]. While some of these effects have also been investigated in models of leukemia [32, 43], the links between PML–RAR α -mediated transformation, ATRA therapy and vesiculation pathways in APL cells remain relatively unstudied.

Here we report that PML–RAR α and ATRA treatment alter the emission profile and cargo of EVs produced by APL cells. APL-derived NB4 cells produce EVs with endothelial stimulating activity. However, unlike in other tumor contexts, we observe no incorporation of the bioactive PML–RAR α oncoprotein itself into the EV cargo, or its direct transfer to target endothelial cells. Instead, EVs released by APL (NB4) cells contain several PML–RAR α (ATRA)-regulated vascular effector proteins and transcripts (TF, VEGF, IL-8). Thus, we conclude that oncogenic effects of PML–RAR α modulate EV productions and angiogenic cargo, but do not lead to production of PML–RAR α -containing EVs.

Materials and methods

Cells and ATRA treatment

Human APL cell line NB4 was generously provided by Dr. Minden of the University of Toronto and cultured in RPMI-1640 medium supplemented with 10 % fetal bovine serum (FBS) (Multicell Wisent, Inc., St-Bruno, Quebec, Canada). Human umbilical vein endothelial cells (HUVEC) were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and cultured in complete endothelial cell growth medium-2 (Lonza, Allendale, NJ). Unless otherwise indicated, NB4 cells were treated with ATRA (Sigma, Oakville, ON, Canada) at the final concentration of 1 μ mol/L which was established as non-toxic and capable of inducing myeloid differentiation measured by positivity for the CD11b antigen (data not shown).

Preparation of extracellular vesicles

EVs were isolated as described elsewhere [34]. Briefly, the assay medium was prepared using EV-depleted FBS obtained by ultracentrifugation at 150,000 $\times g$ for 5 h and passaged through 0.2- μ m-pore-size filters. NB4 cells were cultured in the assay medium for the lengths of time indicated (usually 2–3 days), and such conditioned medium was collected through centrifugation at 400 $\times g$ for 10 min. The supernatant was passed through 0.45- or 0.2- μ m filters (Thermo Scientific; Mississauga, ON), followed by centrifugation at 100,000 $\times g$ for 1 h to pellet EVs. The EV pellet was washed extensively in phosphate-buffered saline (PBS) and then centrifuged at 100,000 $\times g$ for 1 h and analyzed immediately.

Transmission electronic microscope (TEM)

Cells and EVs were fixed 2.5 % glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4). Samples were processed according to standard protocols and imaged on the Tecnai 12 BioTwin 120 kV TEM.

Nanoparticle tracking analysis (NTA)

EV profiling entailed the measurements of size and number of particles shed from cells cultured in EV-depleted medium using the NS500 nanoparticle tracking analysis system (#NS500, Nanosight, Amesbury, UK).

Co-culture assays

NB4 cells and HUVECs were surface labeled with PKH26 (red fluorescent dye) and PKH67 (green fluorescent dye; both from Sigma-Aldrich, Oakville, ON), respectively, as described earlier [44] and according to manufacturer's instructions. The labeled cells were co-cultured in 4-well chamber slides for 24 h. Suspended NB4 cells were settled on the surface of the slide by gentle cytopsin (Thermo Scientific) centrifugation. Upon incubation, the cells were fixed for 10 min in 4 % paraformaldehyde (PF) and imaged using Zeiss LSM 780 laser scanning confocal microscope.

EV transfer assays

NB4 cells were surface labeled with PKH26 (red fluorescent dye; Sigma-Aldrich) [44] and allowed to release fluorescent EVs into the culture media. Labeled EVs were purified as above and incubated for 24 h with HUVECs following the measurement of the fluorescence uptake using FACSCalibur flow cytometer (BD Biosciences, Mississauga, ON). For EV-mediated RNA and protein transfer, recipient cells were lysed and tested for the respective molecular targets. Biological responses to the EV transfer were recorded using MTS assay (Promega, San Luis Obispo, CA) as described earlier [38]. Briefly HUVEC cells were seeded in gelatin pre-coated 96-well plates and then incubated with different concentration of NB4 EVs at 0, 5, 25, 50, 100 and 200 μ g/ml, followed by addition of MTS reagents and reading of the metabolic activity at 405 nm. The changes in viability and cell numbers were inferred from the change in MTS signal.

RNA analysis

EVs were harvested from conditioned medium of NB4 cells at 72 h and surface pre-treated with 100 μ g/ml RNase (Sigma) for 15 min at 37 °C to remove non-luminal RNA contaminants. EV-associated RNA was extracted using

RNeasy[®] mini kit (Qiagen) according to manufacturer's instructions, following conversion into cDNA using QuantiTect[®] Reverse Transcription kit (Qiagen, Mississauga, ON). The following primers spanning the fusion site were obtained from Integrated DNA Technologies (Coralville, IA): PML–RARa (forward 5'-TCTTCCTGCC CAACAGCAA-3' and reverse 5'-GCTTGTAGATGCGG GGTAGAG-3'); GAPDH (forward 5'-GAGTCAACGGA TTTGGTTCGT-3' and reverse 5'-TTGATTTTGGAGGG ATCTCG-3'). For PCR array analysis, reverse transcription was conducted using the RT² First Strand kit (Qiagen). PCR array (SABiosciences/Qiagen, Mississauga, ON) analyses were carried out according to manufacturer's instructions, the result of which was illustrated in MultiExperiment Viewer (MEV) and further analyzed by Vennplex [45]. Fold change of ≥ 2.0 or ≤ 2.0 was chosen as cutoff values to define significantly increased and decreased gene expression, respectively.

Western blotting

Protein lysates were obtained in RIPA buffer containing protease inhibitors (Roche, Mississauga, ON), or nuclear protein extraction buffer for EVs and cells. Protein concentrations were qualified by BCA Protein Assay (Thermo Scientific) resolved by SDS-PAGE and transferred to PVDF membranes. The membranes were incubated with antibodies against either rabbit anti-human RARa (Sigma), mouse anti-human RARa (R&D Systems/Cedarlane, Burlington, ON), mouse anti-human b-actin (Sigma), mouse anti-human flotillin-1, mouse anti-human TSG101 (both from BD Biosciences, Mississauga, ON), mouse anti-human Rab27a (Abnova, Corp. Walnut CA), rabbit anti-human CREB (Santa Cruz Biotech, Santa Cruz, CA) followed by corresponding horseradish peroxidase-coupled secondary antibodies (Cell Signaling Technology Inc., Danvers, MA and Bio-Rad, Mississauga, ON). The signal was visualized using enhanced chemiluminescence (GE Healthcare, Toronto, ON), imaged and quantified with ChemiDoc software (Bio-Rad).

Elisa

Vascular regulators were quantitatively detected using ELISA assays as described earlier [34]. IMUBIND TF ELISA kit (Sekisui Diagnostics, Stamford, CT), human VEGF ELISA kit (R&D Systems, Minneapolis, MN) and human IL-8 ELISA kit (R&D Systems, Minneapolis, MN) were used to quantify the levels of TF, VEGF and IL-8, respectively. TF procoagulant activity was measured as described earlier [46] by Actichrome[®] TF kit (Sekisui) according to manufacturer's instructions.

Statistical analysis

All experiments were reproduced at least twice with similar results and presented as mean value of replicates \pm SD. Statistical significance was evaluated using SPSS software, and differences were considered significant for $p < 0.05$.

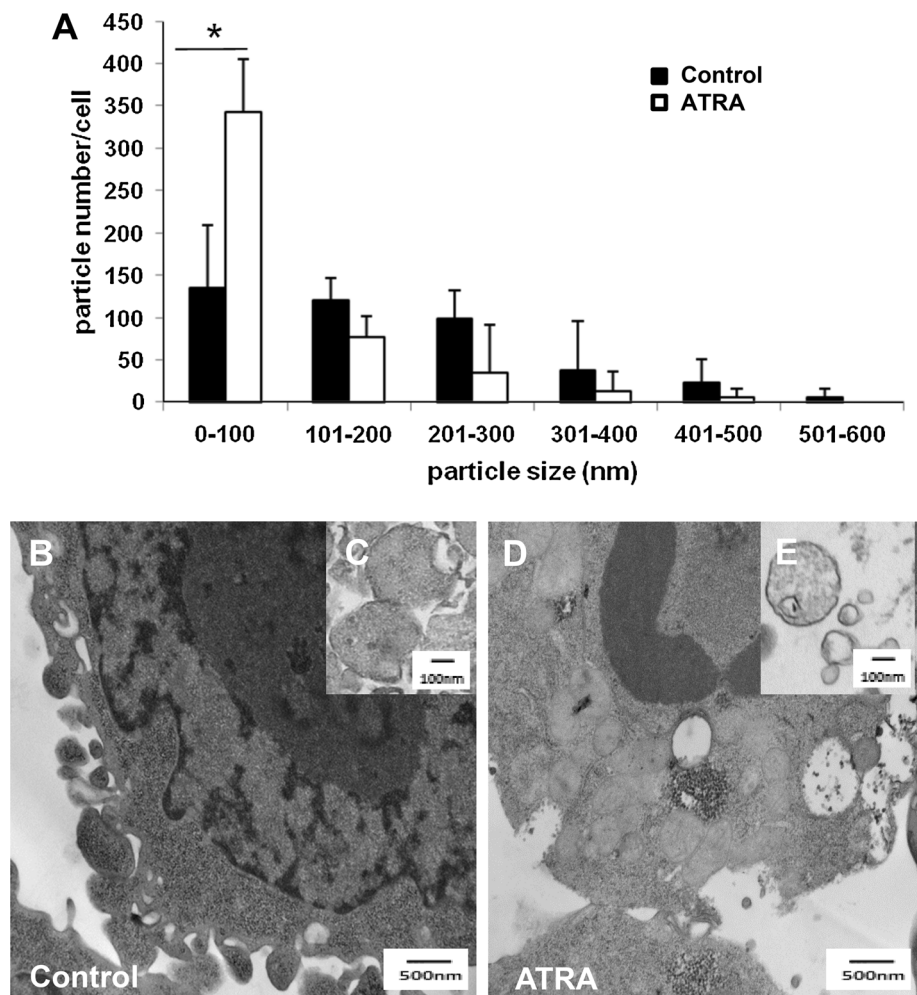
Results

Impact of ATRA on vesiculation of cultured APL cells

While APL cells are known to release bioactive EVs [7], the link between this process and malignant transformation remains presently unknown. Given the impact of oncogenic pathways on vesiculation in other cancer contexts [27], we surmised that PML–RARa driver mutation may influence EV emission by APL cells, both quantitatively and qualitatively. Therefore, we chose NB4 cells, a well-characterized model of human APL, to examine the profile of EV emission in the presence or absence of ATRA-mediated inhibition of the PML–RARa transforming activity. We first performed cell viability (MTS) and myeloid differentiation assays (FACS), using different dosing and timing protocols. As expected, following 72 h of exposure to 1 μ mol/L of the drug, ATRA induced a measurable reversal of PML–RARa-dependent transformation of NB4 cells, as indicated by the increase in CD11b positivity and in the absence of any apparent cytotoxic effects (Fig. S1).

Interestingly, in the presence of ATRA the number and size of EVs produced by NB4 cells also underwent a remarkable change. Thus, as measured by NTA (Nanosight) assays and revealed by electron microscopy (TEM), untreated NB4 cells constitutively release readily detectable EVs, heterogeneous in size and shape but largely below 300 nm in diameter (Fig. 1a). While there is a preponderance of larger vesicles (100–300 nm) in this material, smaller, exosome-sized EVs can also be visualized, either in conditioned media (inset) or in association with cellular membranes (Fig. 1b). This profile underwent a profound change following the exposure to ATRA, which resulted in a sharp and selective increase in emission of small exosomal-sized EVs (below 100 nm in diameter; $p = 0.02$, Fig. 1a). The shape of NB4-derived EVs has also changed as a result of this treatment. In this regard, TEM images reveal a greater number of smaller and more vacuolar EVs in the conditioned medium of treated cells (Fig. 1b–e), along with larger EVs containing intraluminal vesicular structures. The surface of ATRA-treated NB4 cells contained fewer protrusions and blebs relative to control cells, and a greater number of pits reminiscent of exocytosis. More apparent and less electron dense vesicular

Fig. 1 Impact of ATRA on vesiculation of APL cells in culture. **a** Nanoparticle tracking (NTA/Nanosight) profiling of EV emission by NB4 cells cultured in the presence or absence of ATRA (1 μ M) for 72 h. ATRA triggers a change in numbers and size distribution of leukemic EVs; mean particle counts \pm SD ($n = 3$ independent runs); * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **b–e** Transmission electron microscopy of vesiculating NB4 cells and their EVs (*insets*) in the absence (**b, c**) and presence (**d, e**) of ATRA. Features of more differentiated nuclear morphology, vacuolar cytoplasm, changes in cell surface and shape of EV are apparent in ATRA-treated cells (*representative images*)



structures were also present within the cytoplasm of these cells along with features of cellular differentiation. Notably nuclei of ATRA-treated cells assumed a more granulocytic polylobular appearance with reduced nuclear-to-cytoplasm ratio (Fig. 1c). These experiments suggest that PML-RAR α affects the pattern of cellular vesiculation in APL cells in a manner that relates to their differentiation status and can be reversed in the presence of ATRA.

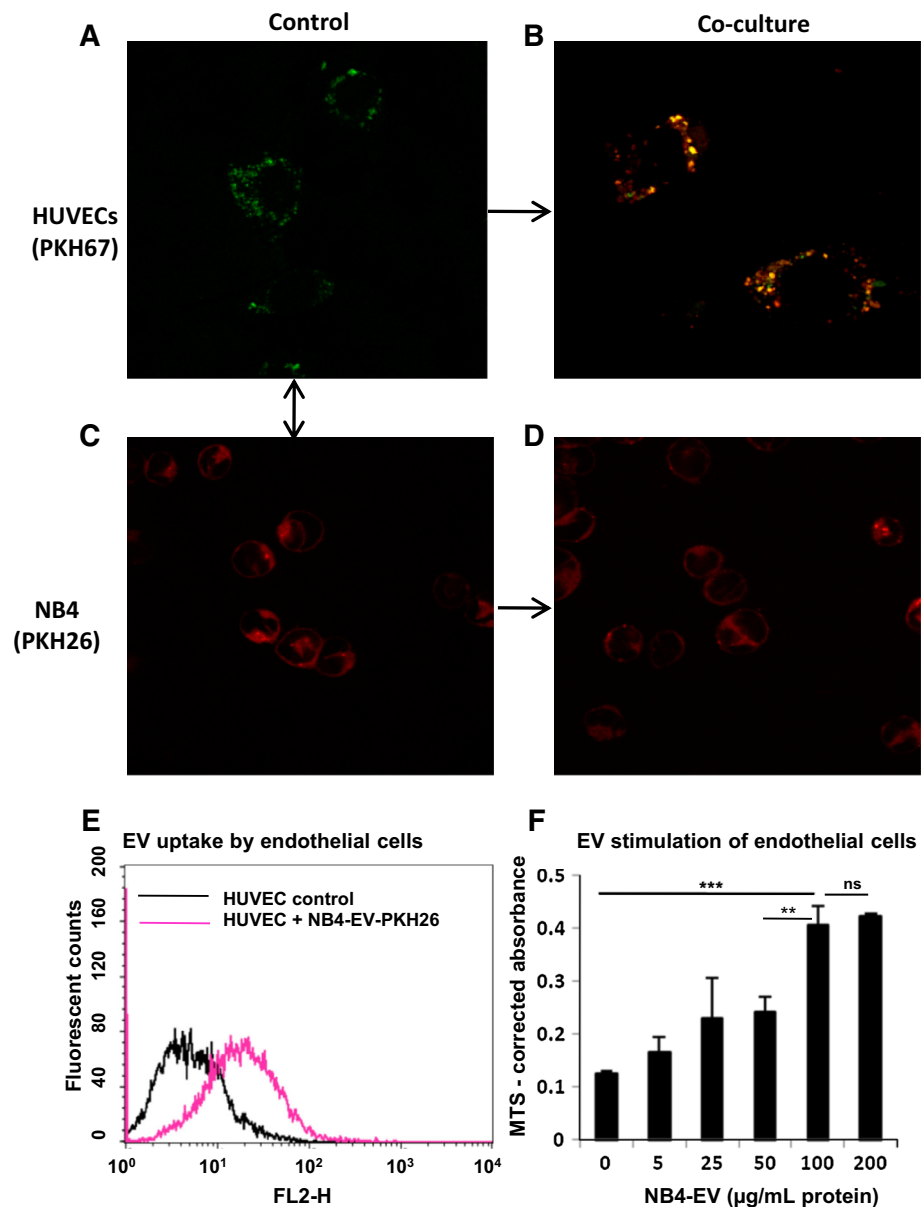
Responses of endothelial cells to the uptake of NB4-derived EVs

Oncogenes are known as drivers of the angiogenic phenotype in cancer [47], in part though their impact on cellular vesiculation [34]. We asked whether this could be applicable to APL cells. To explore this question, we first examined whether co-culture of NB4 cells with normal endothelial cells (HUVEC) leads to a detectable transfer of membrane material between these two cell populations. Membranes of NB4 cells were labeled with the PKH26, red

fluorescent dye, and these cells were seeded in dishes containing HUVEC monolayers pre-labeled with PKH67 (green dye). Following co-culture for 24 h, the cells were visualized using fluorescent confocal microscopy (Fig. 2a–d). Indeed, both cell types could be readily located using the corresponding fluorescent signals (Fig. 2a, c). Interestingly, in the course of co-culture, some HUVECs exhibited a mixture of green (original) and red (NB4-derived) fluorescence on their surfaces (Fig. 2b). In contrast, NB4 cells remained only labeled with the red fluorescence throughout the experiment (Fig. 2d) and clusters of NB4 and HUVEC cells were not readily observed.

We reasoned that this membrane exchange could occur through the uptake of shed EVs and in the absence of direct cell–cell contact. To explore this possibility more directly, fluorescent EVs were purified from cultures of NB4 cells that were pre-labeled with PKH26. HUVEC cells incubated with these EV preparations revealed the acquisition of robust red fluorescence, as documented by FACS analysis indicating the ability of endothelial cells to avidly take up

Fig. 2 Uptake and responses of endothelial cells to leukemic EVs. **a–d** Uptake of fluorescently labeled cellular membranes from NB4 cells to HUVECs. NB4 cells were surface labeled with PKH26 (red), while adherent HUVECs were labeled with PKH67 (green) and the cells were imaged using confocal microscopy individually (control; **a, c**) or upon co-culture for 24 h (co-culture; **b, d**). HUVECs acquired red fluorescence in this co-culture system (yellow; **b**), suggesting the uptake of EVs. **e** Treatment of HUVEC cells with fluorescent (PKH26) NB4-derived EVs results in their uptake and cell fluorescence (FACS). **f** Stimulation of survival (MTS) of starved HUVEC cells by treatment with NB4-derived EVs; mean absorbance \pm SD ($n = 3$ independent runs); * $p < 0.05$; ** $p < 0.01$, *** $p < 0.001$



leukemic EVs (Fig. 2e). Since such EVs were previously postulated to possess proangiogenic properties, we used MTS assay to test responses of growth-factor-starved HUVEC to the addition of increasing concentrations of EVs derived from NB4 cell cultures. This treatment increased the viability signal in HUVEC cultures, in a significant and dose-dependent manner with the maximal effect achieved at the EV protein concentration of 100 $\mu\text{g}/\text{ml}$ (Fig. 2f). Since these responses were achieved in the absence of exogenous endothelial survival factors, MTS readings likely reflect cell survival in this case. This observation suggests that leukemic cells can interact with endothelium via the exchange of EVs.

EV-mediated emission and intercellular transfer of PML-RAR α transcript

It has previously been suggested that horizontal stimulation and transformation may occur by EV-mediated intercellular trafficking of oncogenic mRNA and protein from cancer cells to their untransformed neighbors [36]. A similar process may also be involved in tumor-vascular interactions and angiogenesis [42]. To explore whether such oncogene transfer may occur in the context of APL cells and to explain their aforementioned ability to stimulate endothelial cell survival (Fig. 2f), we first assessed the expression and EV-mediated release of the oncogenic

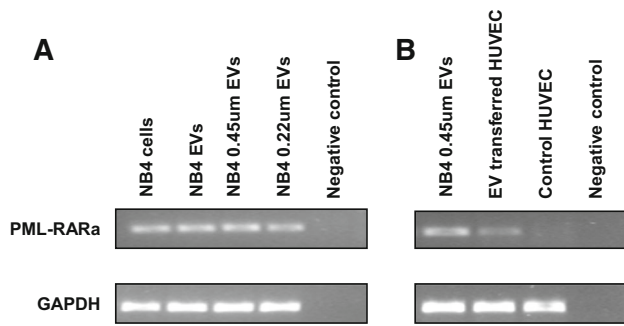


Fig. 3 EV-mediated emission and intercellular transfer of PML–RARA transcript. **a** Detection of PML–RARA mRNA in lysates of NB4 cells and their EVs (RT-PCR). EVs were surface pre-treated with RNase to remove external contamination and used to extract RNA, either directly or upon passage through either 0.45- or 0.22-µm-pore-size filters, to remove debris and cells, and to restrict the sizes of EVs to near exosomal range. In all cases PML–RARA transcript was readily detected. **b** HUVEC cells were used as recipients of NB4-derived EVs, resulting in the transfer of PML–RARA mRNA signal (RT-PCR; as in “Methods” section)

PML–RARA transcript by NB4 leukemic cells (Fig. 3). NB4-derived EVs were externally pre-treated with RNase to remove surface contamination with soluble RNA [48], and the lysates of this material were used to test for the expression of PML–RARA transcript by RT-PCR. Indeed, this analysis revealed the presence of PML–RARA fusion gene product in both NB4 cells and their derived EVs, the latter either tested collectively, or size fractionated by passage through either 0.45-, or 0.22-µm-pore-size filters. This filtration step was intended to enrich the EV preparation for exosome-like small vesicles and also to preclude the contamination with live cancer cells and cellular debris. Furthermore, we found that leukemic EVs are capable of delivering oncogenic mRNA into normal endothelial cells, since HUVECs incubated with NB4-derived EV preparations contained the PML–RARA transcript, which was undetectable in EV-untreated control cultures (Fig. 3b). These experiments document that APL cells release EVs containing oncogenic mRNA, which can be taken up by non-transformed primary endothelial cells.

The absence of functional PML–RARA protein in leukemic EVs or endothelial cells following vesicle transfer

Stimulation of endothelial cells with leukemic EVs (Fig. 1), and their content of PML–RARA transcripts are reminiscent of earlier findings suggesting the horizontal transfer and expression of active oncogenes [42]. For PML–RARA oncogene to exert such an effect would necessitate the expression of the bioactive oncoprotein in recipient endothelial cells. This could occur either through translation of the EV-transferred mRNA in recipient cells,

or through the intercellular transfer of preformed oncoprotein (regardless of mRNA), as demonstrated for oncogenic membrane receptors [34, 39]. In either case, the recipient cells would be expected to become responsive to PML–RARA inhibition by ATRA. To test these possibilities, we first determined that PML–RARA protein is readily detected in NB4 cells and its expression could be inhibited somewhat by the exposure to ATRA for extended periods of time (72 h; Fig. 4a), while the cells underwent myeloid differentiation (data not shown).

We next examined the NB4 EVs in a similar manner. However, no PML–RARA protein could be detected by Western blotting in this material with or without pre-treatment with ATRA (Fig. 4b). This suggests that this particular oncoprotein is not released from APL cells as cargo of EVs. Of note is the fact that our EV preparations contained abundant protein cargo and were positive for several markers associated with exosomes (TSG-101, RAB27a) and other vesicles (Flotillin-1), suggesting that PML–RARA protein is selectively excluded from the vesiculation process (Fig. 4c). To explore the possibility that PML–RARA transcript may, nonetheless, trigger the expression of the bioactive oncoprotein in EV recipient endothelial cells, we cultured HUVECs in the presence or absence of NB4-derived EVs for 24, 48 or 72 h (Fig. 4d). Once again, no PML–RARA protein was found in HUVEC lysates treated in this manner. Collectively, these results suggest that, unlike in the case of other oncogenes [42], PML–RARA is not incorporated into the EV cargo [34], or directly transferred to recipient endothelial cells.

Indirect effects of PML–RARA on vascular mediators present in the leukemic secretome

Since stimulation of HUVECs with leukemic vesicles occurs without a direct intercellular transfer of the bioactive PML–RARA, we reasoned that this oncogene may impact the biological activity of EVs more indirectly, namely through reprogramming the repertoire of vascular mediators incorporated into these vesicles prior to their release. To this end, we explored the known PML–RARA targets with vascular activity, namely VEGF, IL-8 and TF. Their levels in the cargo of NB4 EVs and cellular supernatants were analyzed using ELISA and activity assays.

Thus, as expected NB4 cells produced appreciable amounts of VEGF protein, which was mostly associated with the soluble fraction of the conditioned medium and only in minor amounts retained in cell lysates or EVs (Fig. 5a). Treatment of NB4 cells with ATRA led to a significant reduction in the residual EV content of this angiogenic growth factor (Fig. 5b). Similarly, NB4 cells

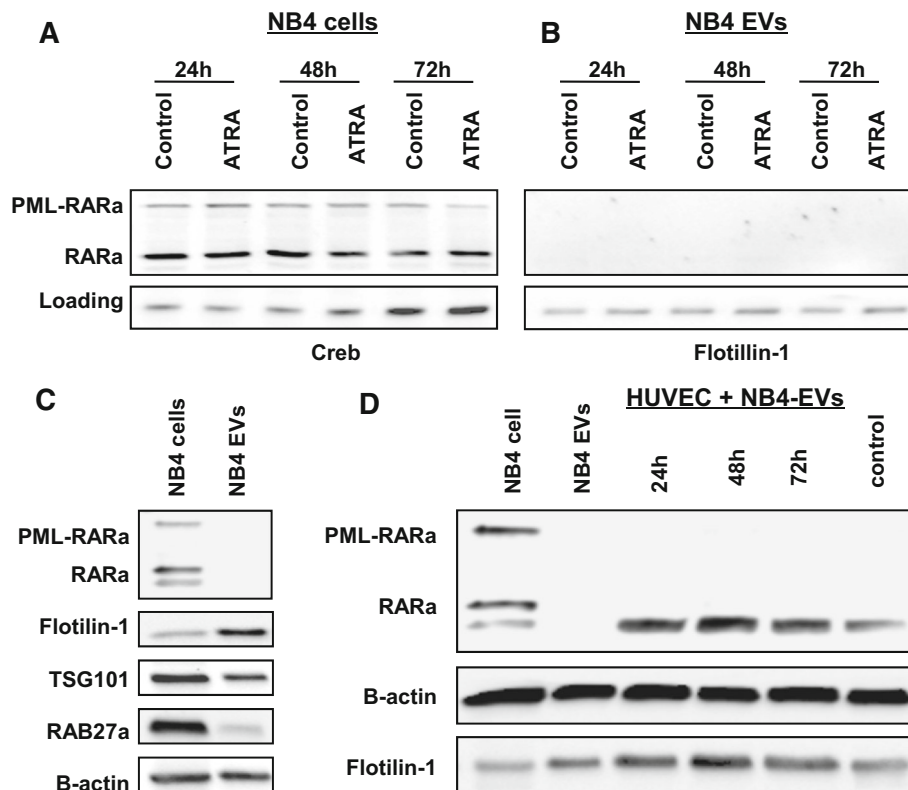


Fig. 4 Absence of PML–RARa protein expression in leukemic EVs and in endothelial cells following the vesicle transfer. **a** PML–RARa (*top band*) and RARa proteins (*bottom band*) are readily detected in nuclear lysates of NB4 cells (Western blotting). Addition of ATRA for different lengths of time results in a slight diminution of the PML–RARa signal after 72 h of treatment. **b** The absence of PML–RARa and RARa proteins in lysates of NB4-derived EVs. The respective protein loading is documented by signal for Creb (cells) and Flotillin-

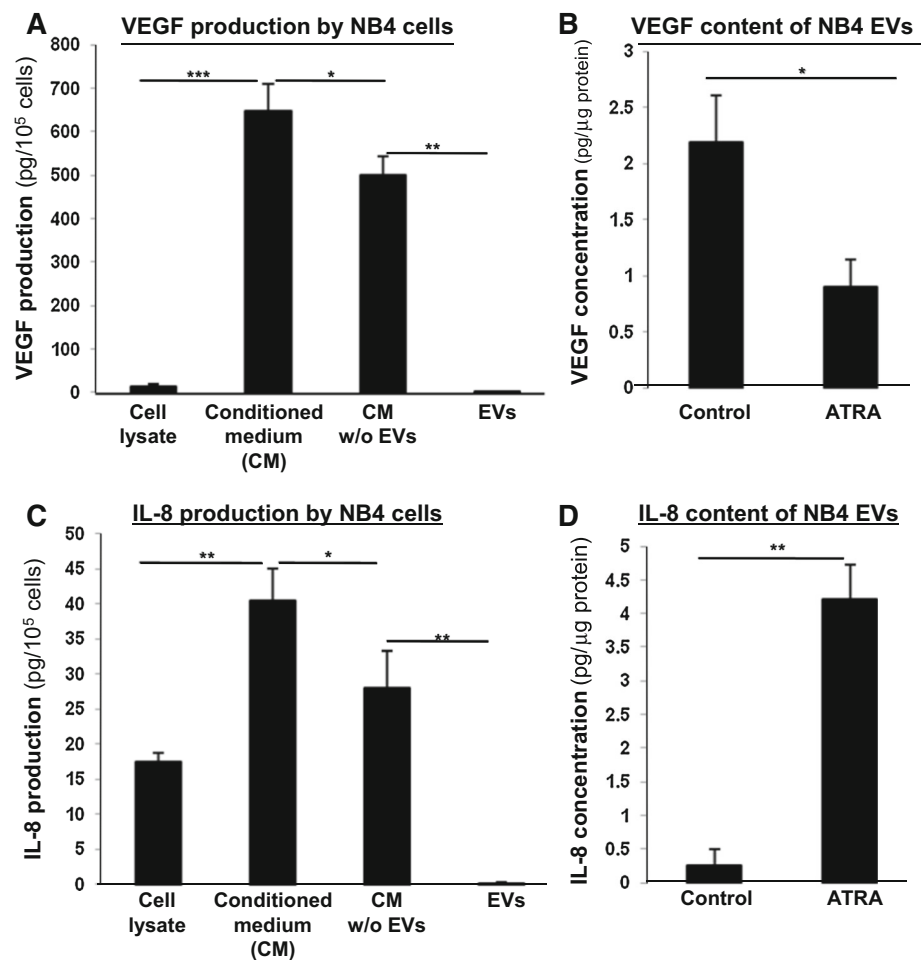
1 (EVs). **c** NB4 cells and their derived EVs express vesicular, raft and exosomal proteins (Flotillin-1, TSG101, RAB27a), but EVs are negative for PML–RARa; beta (b)-actin was used as loading control. **d** Transfer of NB4-derived EVs to HUVECs does not result in expression of PML–RARa by recipient endothelial cells, suggesting that neither functional protein, mRNA nor DNA is expressed in the latter cellular background (Western blotting; as in “Methods” section)

also produced considerable amounts of cell-associated and soluble IL-8. While some of the secreted IL-8 immunoreactivity could be removed from the conditioned medium by ultracentrifugation, suggesting the association with the fraction of EVs, the recovery of IL-8 from purified EVs was minimal (as in the case of VEGF), possibly through losses during filtration and preparation steps or by association with cell debris or larger vesicles. Nonetheless, unlike in the case of VEGF, treatment of donor NB4 cells with ATRA resulted in a robust, near ten-fold up-regulation of the IL-8 content within leukemic EVs (Fig. 5d). Thus, angiogenic factors produced by NB4 cells are mostly contained in the soluble fraction and the immediate biological effects related to their content in EVs are likely negligible, especially in the case of VEGF. However, the EV content of angiogenic factors reflects ATRA-induced reprogramming of the vascular phenotype of leukemic cells, from proangiogenic (VEGF) to angiogenic/inflammatory (IL-8) state, a notion with conceivable implications for the use of EV as biomarkers of the vascular phenotype.

Impact of ATRA on procoagulant properties of leukemic EVs

Unlike in the case of angiogenic growth factors, TF, the potent procoagulant and signaling transmembrane receptor with multiple vascular activities [49] is mostly cell-associated in the case of NB4 leukemic cells (Fig. 6a). However, these cells also release measurable amounts of TF into the culture media, of which the vast majority can be recovered with the EV fraction. We observed no obvious release of soluble or alternatively spliced TF (asTF) in this setting [46, 50]. Treatment with ATRA markedly reduced the EV-associated TF pool, in keeping with earlier reports linking the expression of this receptor with the oncogenic activity of PML–RARa [22] (Fig. 6b). While the presence of TF in the cargo of tumor-related EVs is often thought to contribute to cancer coagulopathy, this area is mired in controversy over the extent of the actual procoagulant activity of these circulating particles in various disease settings [51]. One way to reconcile these observations is to

Fig. 5 Secretion of angiogenic growth factors and EVs from cultured leukemic cells. **a** VEGF release by cultured NB4 leukemic cells (ELISA). Leukemic cells produce appreciable amounts of mostly soluble VEGF, of which only a small proportion was depleted by ultracentrifugation of conditioned media (w/o EVs). **b** The residual expression of EV-associated VEGF is reduced by NB4 cell pre-treatment with ATRA (1 μ mol/L). **c** IL-8 release by cultured NB4 cells (ELISA). While some IL-8 appears to be cell-associated, a large proportion is secreted into the culture media. A minor fraction of IL-8 can be depleted from conditioned medium by ultracentrifugation (w/o EVs) and is poorly recovered from purified EVs, possibly due to association with cell debris and larger vesicles. **d** ATRA induces a dramatic increase in the IL-8 content of NB4-derived EVs; mean absorbance \pm SD ($n = 3$ independent runs); * $p < 0.05$; ** $p < 0.01$, *** $p < 0.001$



consider the role of TF taken up (as EV cargo) by normally non-coagulant endothelial cells [52]. Indeed, we observed that cultured HUVECs readily incorporate NB4 EVs (Fig. 2) leading to the increased exposure of the TF antigen on their surfaces and the elevation of the related procoagulant activity (TF-PCA). Notably, this uptake was markedly diminished by pre-treatment of NB4 cells with ATRA, suggesting that this procoagulant conversion of the recipient endothelial cells is ultimately influenced by the oncogenic PML–RARA activity within tumor cells (NB4) acting as EV and TF donors (Fig. 6).

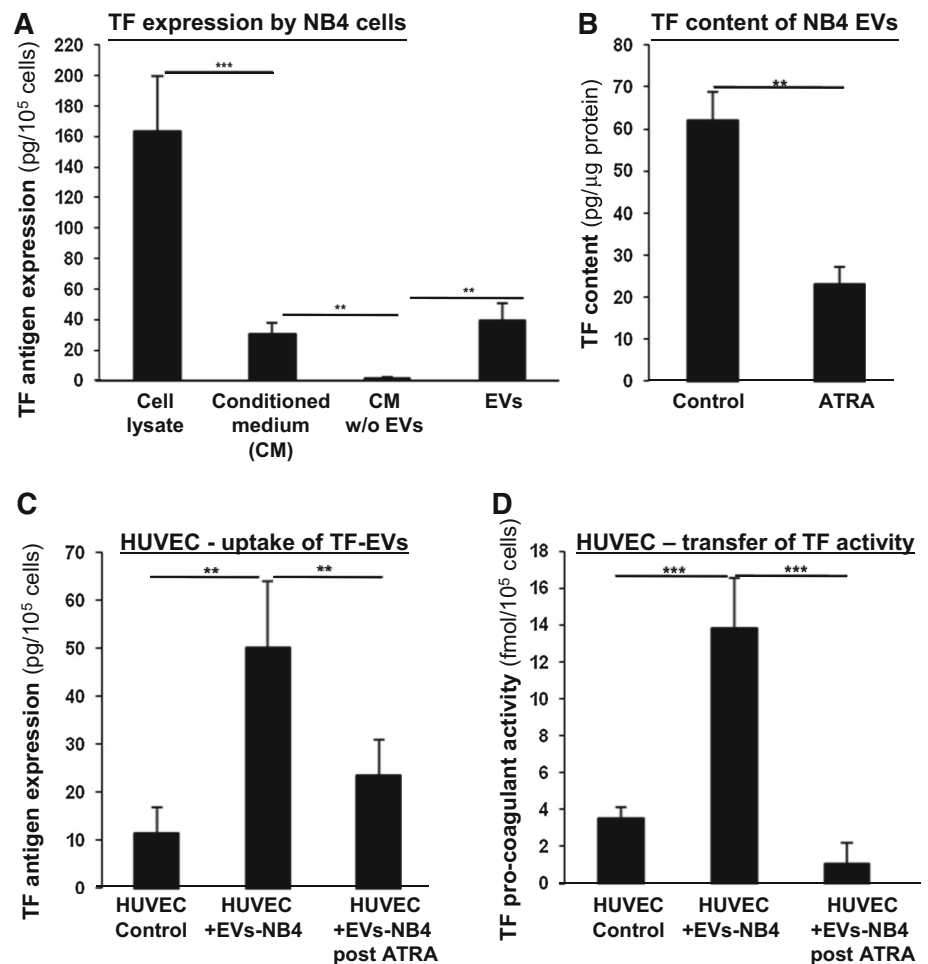
Impact of PML–RARA on angiogenesis-related gene expression profile in NB4 cells and EVs

To obtain more comprehensive insights as to vascular mediators affected by the PML–RARA-induced transformation of APL cells, we generated profiles of angiogenesis-related transcripts expressed in NB4 cells, or in their EVs, and in the presence or absence of ATRA. Using targeted RT-PCR array platform, we evaluated the levels of 84 key genes implicated in angiogenesis and vascular

regulation including growth factors and their receptors, adhesion molecules, cytokines, chemokines, proteases, enzyme inhibitors and other regulatory molecules (Fig. 7). Several notable differences in distribution of these transcripts were observed between NB4 cells and their EVs, and between the cells and EVs following the treatment with ATRA. Thus, in comparison with their parental NB4 cells, 17 angiogenic transcripts were significantly up-regulated and 13 down-regulated in NB4 EVs. After ATRA treatment, 42 angiogenic transcripts were up-regulated while 18 transcripts were down-regulated in NB4 EVs compared to the corresponding cells (Fig. S2–S4).

To assess the influence of ATRA on the expression of angiogenic transcripts in NB4 EVs specifically, we compared the profiles of EVs collected from untreated or ATRA-treated NB4 cells. In this case, 37 genes were significantly up-regulated and 18 genes down-regulated in EVs as a result of ATRA treatment. In this regard, the transcript for chemokine (C–C motif) ligand 2 (CCL2) was found to be the most remarkably up-regulated, and EPH receptor B4 (EPHB4) was the most strongly ATRA down-regulated gene under these experimental conditions

Fig. 6 ATRA-modulated expression of tissue factor in NB4 cells and their EVs. **a** Tissue factor (TF) is released into NB4 cell-conditioned medium in relatively small quantities, mostly within the EV fraction (ELISA). **b** The EV-associated TF content is markedly reduced (threefold) by NB4 cell pre-treatment with ATRA (1 $\mu\text{mol/L}$). **c** HUVECs treated with NB4-derived EVs acquire the expression of TF antigen (ELISA), which is reduced if donor cells are pre-treated with ATRA. **d** HUVECs become highly procoagulant upon the uptake of NB4-derived EVs, and ATRA pre-treatment of donor cells dramatically suppresses this conversion; mean absorbance \pm SD ($n = 3$ independent runs); * $p < 0.05$; ** $p < 0.01$, *** $p < 0.001$



(Fig. S2-4). We also observed that IL-8 mRNA was up-regulated in ATRA-treated EVs with the fold change of 72.15, ranking as fourth among the most up-regulated genes. VEGF and TF were found to be down-regulated by ATRA at the mRNA level (in EVs), with the fold change of 1.83 and 4.32, respectively. These results are consistent with our analysis at the protein level and, in a wider sense, demonstrate that EVs are a rich source of information as to the reprogramming of the angiogenic phenotype of APL cells following the inhibition of the PML–RARA oncogene by ATRA.

Discussion

Our present study brings forward several novel observations. First, unlike in the case of oncogenic receptor tyrosine kinases (EGFR, HER2, MET) or GTP-ases (RAS) [27, 34, 38], the transforming PML–RARA oncoprotein is not included in the vesicular cargo of APL cells, neither intact nor upon treatment with ATRA. Moreover, PML–RARA transcript present in the cargo of leukemic EVs

does not induce any detectable expression of the PML–RARA oncoprotein upon the EV uptake by recipient endothelial cells. Thus, from the functional standpoint, NB4 cells do not produce oncogenic EVs (oncosomes) capable of horizontal transfer of the APL driver gene, at least to endothelial cell recipients. Second, PML–RARA transformation does influence cellular vesiculation profile, by inducing a wider size range and lower emission rates of leukemic EVs. This can be inferred from the opposite trend in the NTA profile in the presence of ATRA. Whether this is due to the effect of the PML–RARA inhibition on effectors of EV biogenesis in NB4 cells, or represents a secondary reflection of their myeloid differentiation remains presently unclear. It is also plausible that the change in EV profile results from some off-target effects of ATRA. This is unlikely given well-documented specificity of this agent, but all possibilities remain to be carefully investigated. Third, ATRA (PML–RARA) profoundly changes the molecular cargo of leukemic EVs, especially as it relates to multiple vascular effectors. These influences could reveal novel PML–RARA-dependent interactions between APL cells and the vascular

Angiogenesis related mRNA in NB4 cells and EVs

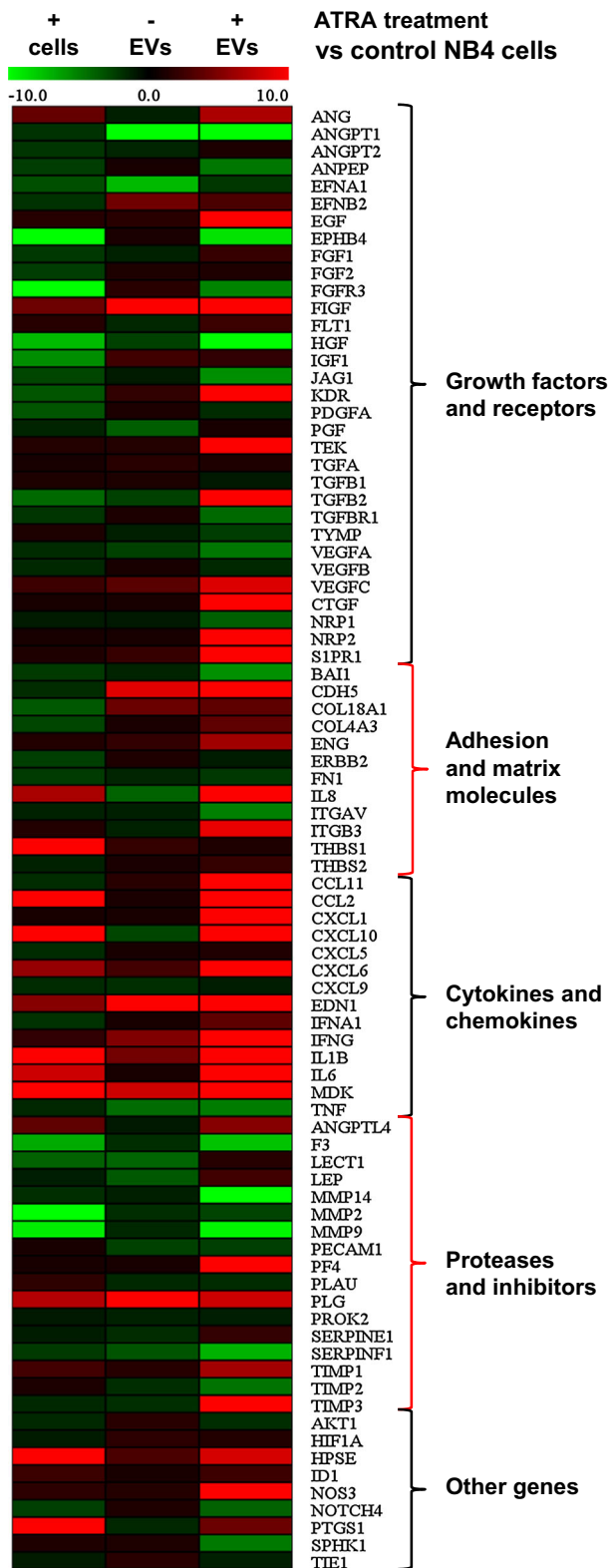


Fig. 7 Impact of ATRA on the expression of angiogenesis-related transcripts in NB4 cells and their derived EVs. Angiogenesis-targeted PCR-based gene expression array (SAB) analysis was conducted on lysates of intact or ATRA-treated NB4 cells and their corresponding EVs, and the fold change was computed using untreated NB4 cells as baseline. Fold change of ≥ 2.0 or ≤ 2.0 was chosen as the basis for cutoff values ($n = 2$ independent runs, see text for details)

system and the related consequences of the ATRA therapy [3, 10, 11, 17, 53].

These results may be informative as one element in considering the merits of EV-based biomarker development to monitor vascular effects in leukemia and other malignancies [27]. For example, even though VEGF is released from NB4 cells predominantly as a soluble protein, its content in defined EV subsets may reflect the change in production of this factor by a specific cell type (APL cells in this case) and thereby could help distinguish this from other sources of the angiogenic activity. Similar inferences can be made in the case of TF or the corresponding (vascular) EV-associated mRNA, as indicated by our results involving ATRA-induced shift in the profile of transcripts contained in NB4-derived EVs.

The oncogene and therapy-related changes in the cargo of leukemic EVs are also of interest in view of their possible biological activity. In this regard, interactions of APL cells with the vascular system are complex and unlikely reducible to angiogenesis stimulation or inhibition, or solely reflected by endothelial growth responses in vitro. For example, while we observed stimulation of HUVEC survival by NB4-derived EVs, this activity was not significantly affected by ATRA (Fig S5). This is not surprising, as decline in VEGF content following ATRA exposure was paralleled by the up-regulation of IL-8, another endothelial and inflammatory agonist, along with multiple other changes. While the exceedingly low content of VEGF and IL-8 associated with EVs may argue against the involvement of these factors in biological effects of EVs, they illustrate the underlying trends. We posit that at least some of these effects could be relevant to the ATRA-induced inflammatory side effects, such as differentiation syndrome (DS), some of which may be linked to the biological activity of APL-related EVs. It also cannot be presently excluded that EVs may possess a unique ability to transport certain cytokines, such as IL-8 and others (even in low amounts) to remote organs and cellular populations. In this manner EVs could, perhaps, still contribute to the recruitment of inflammatory cells or exert other effects qualitatively different from those attributable to extracellular diffusion gradients of the respective soluble mediators.

It is also presently unclear whether angiogenic transcripts found in APL-derived EVs merely reflect the state of parental cells and responses to ATRA or are capable of generating functional proteins in recipient cells. In some cases, these changes, indeed, parallel the levels of the corresponding bioactive mediators. One of the most striking functional effects of this kind was the ability of ATRA to diminish the ability of leukemic EVs to trigger procoagulant conversion of endothelial cells through the transfer of TF.

Notably, a somewhat different pattern of vesiculation and intercellular TF trafficking was observed in the case of the ATO treatment. In this case, greater cytotoxicity of this compound at higher doses resulted in undiminished coagulant phenotype of NB4 cells and of endothelial recipients receiving NB4-derived EVs (Fig. S6). This observation exemplifies the diversity and drug specificity of EV responses to PML–RARA inhibitors, a hitherto unknown detail which could be diagnostically and biologically informative. It is possible that the uptake and not the mere presence of TF-containing EVs in the circulation is among mechanisms driving coagulopathy in APL and other malignancies, as we suggested earlier [40, 44, 52]. Some of these effects may also depend on EV-unrelated mechanisms of membrane exchange between cells (e.g., trogocytosis) [54], processes that still remain to be investigated in the context of vascular regulation.

We reasoned that the absence of PML–RARA oncoprotein in EVs either may represent a common feature associated with transformation mediated by fusion gene products with nuclear location, or be unique to this particular gene and/or to APL cells. Indeed, recent studies revealed the presence of DNA, RNA and protein corresponding to other fusion oncogenes in the cargo of EVs. For example, the breakpoint cluster region–Abelson leukemia gene human homolog 1 (BCR–ABL1) was found in EVs isolated from K562 cells derived from blast crisis of human chronic myelogenous leukemia CML [32, 55]. BCR–ABL1-positive EVs were implicated in the induction of genetic instability and leukemia-like malignant growth in mice exposed to this material [55]. This finding is reminiscent of the phenomenon known as donor cell leukemia, a rare hematopoietic malignancy occurring in recipients of therapeutic bone marrow transplants [56]. These observations may suggest that, unlike in our study, the BCR–ABL1 oncogene may possess a direct horizontal transforming ability due to its EV-mediated intercellular transfer.

While ATRA has revolutionized the treatment of APL, the effects of monotherapy are not curative. Interestingly, the increased efficacies of agents capable of inducing long-term APL remission, such as ATO, can be recapitulated in the NB4 model in which ATO (but not ATRA) markedly decreases cell survival. This effect is paralleled by a

distinctive pattern of cellular vesiculation, which is dominated by the upsurge in release of larger particles (Fig. S6). It is of interest to determine the biological basis of this differential EV release profile and whether measurements of the EV output and cargo could be informative as to responses or resistance of APL cells and their stem cell subsets to different targeted therapeutics [3].

We suggest that EV-mediated cell–cell communication may be a part of a larger network. EVs are critical in the intercellular transfer of MET receptor to myeloid cells during dissemination of melanoma [39]. AXL receptor is known to traffic as cargo of EVs in chronic B cell leukemia (CLL) and influence bone marrow stroma [30]. Exosomal trafficking of WNT regulates stem cell equilibrium in the context of diffuse large B-cell lymphoma (DLBCL) [28]. In a similar manner, perturbations in the EV content in the context of APL could impact various tissue responses and side effects of APL-directed agents, such as ATRA, ATO or chemotherapy. Whether such effects contribute to angiogenesis, inflammation or DS syndrome associated with ATRA therapy is presently unclear. DS mediators were postulated to be involved in chemotactic transmigration of ATRA-treated APL cells toward alveolar epithelial cells [57], and it is possible that the EV uptake by epithelial, inflammatory or stromal cells may play an important role in these events.

In conclusion, our study documents a novel link between oncogenic PML–RARA and vesiculation of APL cells. These observations are not without limitations and await extension through the use of multiple APL models and patient material. Future studies will explore whether EVs contribute to the intercellular regulatory network that likely exists between PML–RARA-transformed leukemic cells and the various facets of the vascular system, including endothelial and inflammatory cells, stem cell niches, as well as components of the coagulation system. The effects of ATRA, ATO and other therapeutic agents on these interactions require further study, while EVs may present themselves as a conceivable source of predictive or pharmacodynamic biomarkers in this context [31].

Acknowledgments We thank our McGill colleagues for their assistance: J. Mui (Electron Microscopy) and M. Fu (Confocal Microscopy). The project was supported by operating grants to J. R. from the Canadian Institutes of Health Research (CIHR Foundation Grant; MOP 111119, 133424). The infrastructure support was provided by Fonds de Recherche en Santé du Québec (FRSQ). J. R. is Jack Cole Chair in Pediatric Hematology/Oncology. Y. F. was supported by Postdoctoral Fellowship from the Cole Foundation, National Natural Science Foundation of China for Young Scientists (30801062), China Scholarship Council (2009831055) and Scholarship of Renji Hospital Sponsored Overseas Project; DG was supported by Michael Whitehead Fellowship Endowment to Montreal Children's Hospital Foundation, and EDA was a recipient of the FRSQ Doctoral Studentship and Piccoli Research Fund.

Contributions YF—designed, conducted and interpreted the experiments, and wrote the manuscript. This included culturing and analysis of NB4 and HUVEC cells, Western blotting ELISA, NTA and other assays. DG, THL, EDA, LM and BM—established the assays, conducted experiments provided intellectual input and revised the manuscript. This included calibration of NTA assays, RNA and Western protocols, EV transfer experiments, FACS and other experiments; JR—conceived and designed the study, interpreted the data, administered and supervised the project.

Compliance with ethical standards

Conflict of interests J.R is the inventor on a pending patent application describing identification of oncogenes in the cargo of extracellular vesicles. Otherwise authors declare no competing interests.

References

- Vardiman JW, Thiele J, Arber DA, Brunning RD, Borowitz MJ, Porwit A, Harris NL, Le Beau MM, Hellstrom-Lindberg E, Tefferi A, Bloomfield CD (2009) The 2008 revision of the World Health Organization (WHO) classification of myeloid neoplasms and acute leukemia: rationale and important changes. *Blood* 114:937–951
- Isakson P, Bjoras M, Boe SO, Simonsen A (2010) Autophagy contributes to therapy-induced degradation of the PML/RARA oncoprotein. *Blood* 116:2324–2331
- Dos Santos GA, Kats L, Pandolfi PP (2013) Synergy against PML–RARa: targeting transcription, proteolysis, differentiation, and self-renewal in acute promyelocytic leukemia. *J Exp Med* 210:2793–2802
- Choudhry A, DeLoughery TG (2012) Bleeding and thrombosis in acute promyelocytic leukemia. *Am J Hematol* 87:596–603
- Chang H, Kuo MC, Shih LY, Dunn P, Wang PN, Wu JH, Lin TL, Hung YS, Tang TC (2012) Clinical bleeding events and laboratory coagulation profiles in acute promyelocytic leukemia. *Eur J Haematol* 88:321–328
- Arbuthnot C, Wilde JT (2006) Haemostatic problems in acute promyelocytic leukaemia. *Blood Rev* 20:289–297
- Ma G, Liu F, Lv L, Gao Y, Su Y (2013) Increased promyelocytic-derived microparticles: a novel potential factor for coagulopathy in acute promyelocytic leukemia. *Ann Hematol* 92:645–652
- Gheldof D, Mullier F, Bailly N, Devalet B, Dogne JM, Chatelain B, Chatelain C (2014) Microparticle bearing tissue factor: a link between promyelocytic cells and hypercoagulable state. *Thromb Res* 133:433–439
- Hatfield KJ, Evensen L, Reikvam H, Lorens JB, Bruserud O (2012) Soluble mediators released by acute myeloid leukemia cells increase capillary-like networks. *Eur J Haematol* 89:478–490
- Kini AR, Peterson LC, Tallman MS, Lingen MW (2001) Angiogenesis in acute promyelocytic leukemia: induction by vascular endothelial growth factor and inhibition by all-*trans* retinoic acid. *Blood* 97:3919–3924
- Coltella N, Percio S, Valsecchi R, Cuttano R, Guarmerio J, Ponzoni M, Pandolfi PP, Melillo G, Pattini L, Bernardi R (2014) HIF factors cooperate with PML–RARalpha to promote acute promyelocytic leukemia progression and relapse. *EMBO Mol Med* 6:640–650
- Perez-Atayde AR, Sallan SE, Tedrow U, Connors S, Allred E, Folkman J (1997) Spectrum of tumor angiogenesis in the bone marrow of children with acute lymphoblastic leukemia. *Am J Pathol* 150:815–820
- Jothilingam P, Basu D, Dutta TK (2014) Angiogenesis and proliferation index in patients with acute leukemia: a prospective study. *Bone Marrow Res*. doi:10.1155/2014/634874
- Rak JW, Hegmann EJ, Lu C, Kerbel RS (1994) Progressive loss of sensitivity to endothelium-derived growth inhibitors expressed by human melanoma cells during disease progression. *J Cell Physiol* 159:245–255
- Dias S, Hattori K, Heissig B, Zhu Z, Wu Y, Witte L, Hicklin DJ, Tateno M, Bohlen P, Moore MA, Rafii S (2001) Inhibition of both paracrine and autocrine VEGF/VEGFR-2 signaling pathways is essential to induce long-term remission of xenotransplanted human leukemias. *Proc Natl Acad Sci USA* 98:10857–10862
- Heine A, Held SA, Bringmann A, Holderried TA, Brossart P (2011) Immunomodulatory effects of anti-angiogenic drugs. *Leukemia* 25:899–905
- Trujillo A, McGee C, Cogle CR (2012) Angiogenesis in acute myeloid leukemia and opportunities for novel therapies. *J Oncol*. doi:10.1155/2012/128608
- Zheng PZ, Wang KK, Zhang QY, Huang QH, Du YZ, Zhang QH, Xiao DK, Shen SH, Imbeaud S, Eveno E, Zhao CJ, Chen YL, Fan HY, Waxman S, Auffray C, Jin G, Chen SJ, Chen Z, Zhang J (2005) Systems analysis of transcriptome and proteome in retinoic acid/arsenic trioxide-induced cell differentiation/apoptosis of promyelocytic leukemia. *Proc Natl Acad Sci USA* 102:7653–7658
- Wang D, Jensen R, Gendeh G, Williams K, Pallavicini MG (2004) Proteome and transcriptome analysis of retinoic acid-induced differentiation of human acute promyelocytic leukemia cells, NB4. *J Proteome Res* 3:627–635
- Shibakura M, Niiya K, Niiya M, Asami N, Yoshida C, Nakata Y, Tanimoto M (2005) Induction of CXCL1 and CXCL2 chemokines by all-*trans* retinoic acid in acute promyelocytic leukemia cells. *Leuk Res* 29:755–759
- Saito A, Sugawara A, Uruno A, Kudo M, Kagechika H, Sato Y, Owada Y, Kondo H, Sato M, Kurabayashi M, Imaizumi M, Tsuchiya S, Ito S (2007) All-*trans* retinoic acid induces in vitro angiogenesis via retinoic acid receptor: possible involvement of paracrine effects of endogenous vascular endothelial growth factor signaling. *Endocrinology* 148:1412–1423
- Tallman MS, Lefebvre P, Baine RM, Shoji M, Cohen I, Green D, Kwaan HC, Paietta E, Rickles FR (2004) Effects of all-*trans* retinoic acid or chemotherapy on the molecular regulation of systemic blood coagulation and fibrinolysis in patients with acute promyelocytic leukemia. *J Thromb Haemost* 2:1341–1350
- Zhu J, Guo WM, Yao YY, Zhao WL, Pan L, Cai X, Ju B, Sun GL, Wang HL, Chen SJ, Chen GQ, Caen J, Chen Z, Wang ZY (1999) Tissue factors on acute promyelocytic leukemia and endothelial cells are differently regulated by retinoic acid, arsenic trioxide and chemotherapeutic agents. *Leukemia* 13:1062–1070
- Shibakura M, Niiya K, Kiguchi T, Shinagawa K, Ishimaru F, Ikeda K, Namba M, Nakata Y, Harada M, Tanimoto M (2002) Simultaneous induction of matrix metalloproteinase-9 and interleukin 8 by all-*trans* retinoic acid in human PL-21 and NB4 myeloid leukaemia cells. *Br J Haematol* 118:419–425
- Bobrie A, Thery C (2013) Exosomes and communication between tumours and the immune system: are all exosomes equal? *Biochem Soc Trans* 41:263–267
- Vizio D, Kim J, Hager MH, Morello M, Yang W, Lafargue CJ, True LD, Rubin MA, Adam RM, Beroukhim R, Demichelis F, Freeman MR (2009) Oncosome formation in prostate cancer: association with a region of frequent chromosomal deletion in metastatic disease. *Cancer Res* 69:5601–5609
- Rak J (2013) Extracellular vesicles—biomarkers and effectors of the cellular interactome in cancer. *Front Pharmacol* 4:21. doi:10.3389/fphar.2013.00021

28. Koch R, Demant M, Aung T, Diering N, Cicholas A, Chapuy B, Wenzel D, Lahmann M, Guntzsch A, Kiecke C, Becker S, Hupfeld T, Venkataramani V, Ziepert M, Opitz L, Klapper W, Trumper L, Wulf GG (2014) Populational equilibrium through exosome-mediated Wnt signaling in tumor progression of diffuse large B-cell lymphoma. *Blood* 123:2189–2198
29. Ratajczak J, Miekus K, Kucia M, Zhang J, Reca R, Dvorak P, Ratajczak MZ (2006) Embryonic stem cell-derived microvesicles reprogram hematopoietic progenitors: evidence for horizontal transfer of mRNA and protein delivery. *Leukemia* 20:847–856
30. Ghosh AK, Secreto CR, Knox TR, Ding W, Mukhopadhyay D, Kay NE (2010) Circulating microvesicles in B-cell chronic lymphocytic leukemia can stimulate marrow stromal cells: implications for disease progression. *Blood* 115:1755–1764
31. Huan J, Hornick NI, Shurtleff MJ, Skinner AM, Goloviznina NA, Roberts CT Jr, Kurre P (2013) RNA trafficking by acute myelogenous leukemia exosomes. *Cancer Res* 73:918–929
32. Cai J, Wu G, Tan X, Han Y, Chen C, Li C, Wang N, Zou X, Chen X, Zhou F, He D, Zhou L, Jose PA, Zeng C (2014) Transferred BCR/ABL DNA from K562 extracellular vesicles causes chronic myeloid leukemia in immunodeficient mice. *PLoS One* 9:e105200
33. Corrado C, Raimondo S, Saieva L, Flugy AM, De LG, Alessandro R (2014) Exosome-mediated crosstalk between chronic myelogenous leukemia cells and human bone marrow stromal cells triggers an interleukin 8-dependent survival of leukemia cells. *Cancer Lett* 348:71–76
34. Al-Nedawi K, Meehan B, Micallef J, Lhotak V, May L, Guha A, Rak J (2008) Intercellular transfer of the oncogenic receptor EGFRvIII by microvesicles derived from tumour cells. *Nat Cell Biol* 10:619–624
35. Yu X, Harris SL, Levine AJ (2006) The regulation of exosome secretion: a novel function of the p53 protein. *Cancer Res* 66:4795–4801
36. Skog J, Wurdinger T, van RS, Meijer DH, Gainche L, Curry WT Jr, Carter BS, Krichevsky AM, Breakefield XO (2008) Glioblastoma microvesicles transport RNA and proteins that promote tumour growth and provide diagnostic biomarkers. *Nat Cell Biol* 10:1470–1476
37. Chen C, Skog J, Hsu CH, Lessard RT, Balaj L, Wurdinger T, Carter BS, Breakefield XO, Toner M, Irimia D (2010) Microfluidic isolation and transcriptome analysis of serum microvesicles. *Lab Chip* 10:505–511
38. Lee TH, Chennakrishnaiah S, Audemard E, Montermini L, Meehan B, Rak J (2014) Oncogenic ras-driven cancer cell vesiculation leads to emission of double-stranded DNA capable of interacting with target cells. *Biochem Biophys Res Commun* 451:295–301
39. Peinado H, Aleckovic M, Lavotshkin S, Matei I, Costa-Silva B, Moreno-Bueno G, Hergueta-Redondo M, Williams C, Garcia-Santos G, Ghajar CM, Nitoro-Hoshino A, Hoffman C, Badal K, Garcia BA, Callahan MK, Yuan J, Martins VR, Skog J, Kaplan RN, Brady MS, Wolchok JD, Chapman PB, Kang Y, Bromberg J, Lyden D (2012) Melanoma exosomes educate bone marrow progenitor cells toward a pro-metastatic phenotype through MET. *Nat Med* 18:833–891
40. Garnier D, Magnus N, Lee TH, Bentley V, Meehan B, Milsom C, Montermini L, Kislinger T, Rak J (2012) Cancer cells induced to express mesenchymal phenotype release exosome-like extracellular vesicles carrying tissue factor. *J Biol Chem* 287:43565–43572
41. Luga V, Zhang L, Vitoria-Petit AM, Ogunjimi AA, Inanlou MR, Chiu E, Buchanan M, Hosein AN, Basik M, Wrana JL (2012) Exosomes mediate stromal mobilization of autocrine Wnt-PCP signaling in breast cancer cell migration. *Cell* 151:1542–1556
42. Al-Nedawi K, Meehan B, Kerbel RS, Allison AC, Rak J (2009) Endothelial expression of autocrine VEGF upon the uptake of tumor-derived microvesicles containing oncogenic EGFR. *Proc Natl Acad Sci USA* 106:3794–3799
43. Jaworski E, Narayanan A, Van DR, Shabbeer-Meyering S, Iordanskiy S, Saifuddin M, Das R, Afonso PV, Sampey GC, Chung M, Popratiloff A, Shrestha B, Sehgal M, Jain P, Vertes A, Mahieux R, Kashanchi F (2014) Human T-lymphotropic virus type 1-infected cells secrete exosomes that contain Tax protein. *J Biol Chem* 289:22284–22305
44. Yu J, May L, Milsom C, Anderson GM, Weitz JI, Luyendyk JP, Broze G, Mackman N, Rak J (2008) Contribution of host-derived tissue factor to tumor neovascularization. *Arterioscler Thromb Vasc Biol* 28:1975–1981
45. Cai H, Chen H, Yi T, Daimon CM, Boyle JP, Peers C, Maudsley S, Martin B (2013) VennPlex—a novel Venn diagram program for comparing and visualizing datasets with differentially regulated datapoints. *PLoS One* 8:e53388
46. 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* 2:2065–2067
47. Rak J, Mitsushashi Y, Bayko L, Filmus J, Sasazuki T, Kerbel RS (1995) Mutant ras oncogenes upregulate VEGF/VPF expression: implications for induction and inhibition of tumor angiogenesis. *Cancer Res* 55:4575–4580
48. Valadi H, Ekstrom K, Bossios A, Sjostrand M, Lee JJ, Lotvall JO (2007) Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. *Nat Cell Biol* 9:654–659
49. Mackman N (2008) Triggers, targets and treatments for thrombosis. *Nature* 451:914–918
50. Davila M, Robles-Carrillo L, Unruh D, Huo Q, Gardiner C, Sargent IL, Adam M, Woodhams BJ, Francis JL, Bogdanov VY, Amirkhosravi A (2014) Microparticle association and heterogeneity of tumor-derived tissue factor in plasma: is it important for coagulation activation? *J Thromb Haemost* 12:186–196
51. Geddings JE, Mackman N (2013) Tumor-derived tissue factor-positive microparticles and venous thrombosis in cancer patients. *Blood* 122:1873–1880
52. D’Asti E, Magnus N, Meehan B, Garnier D, Rak J (2014) Genetic basis of thrombosis in cancer. *Semin Thromb Hemost* 40:284–295
53. Taverna S, Amodeo V, Saieva L, Russo A, Giallombardo M, De LG, Alessandro R (2014) Exosomal shuttling of miR-126 in endothelial cells modulates adhesive and migratory abilities of chronic myelogenous leukemia cells. *Mol Cancer* 13:169. doi:10.1186/1476-4598-13-169
54. Poupot M, Fournie JJ (2003) Spontaneous membrane transfer through homotypic synapses between lymphoma cells. *J Immunol* 171:2517–2523
55. Zhu X, You Y, Li Q, Zeng C, Fu F, Guo A, Zhang H, Zou P, Zhong Z, Wang H, Wu Y, Li Q, Kong F, Chen Z (2014) BCR-ABL1-positive microvesicles transform normal hematopoietic transplants through genomic instability: implications for donor cell leukemia. *Leukemia* 28:1666–1675
56. Wiseman DH (2011) Donor cell leukemia: a review. *Biol Blood Marrow Transplant* 17:771–789
57. Tsai WH, Hsu HC, Lin CC, Ho CK, Kou YR (2007) Role of interleukin-8 and growth-regulated oncogene-alpha in the chemotactic migration of all-trans retinoic acid-treated promyelocytic leukemic cells toward alveolar epithelial cells. *Crit Care Med* 35:879–885