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Cancer Cells Induced to Express Mesenchymal Phenotype Release Exosome-like Extracellular Vesicles Carrying Tissue Factor*

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Background: Cross-talk of oncogenic and differentiation pathways in cancer coagulopathy is poorly understood.
Results: EGFR activation and blockade of E-cadherin in cancer cell lines induce mesenchymal phenotype and tissue factor (TF) shedding, as exosomes, capable of transferring procoagulant activity to endothelium.
Conclusion: Mesenchymal and procoagulant phenotypes are linked in cancer.
Significance: Epithelial-to-mesenchymal transition (EMT) may influence tumor-vascular interactions via TF-containing exosomes.

Aggressive epithelial cancer cells frequently adopt mesenchymal characteristics and exhibit aberrant interactions with their surroundings, including the vasculature. Whether the release/uptake of extracellular vesicles (EVs) plays a role during these processes has not been studied. EVs are heterogeneous membrane structures that originate either at the surface (microparticles), or within (exosomes) activated or transformed cells, and are involved in intercellular trafficking of bioactive molecules. Here, we show that epithelial cancer cells (A431, DLD-1) adopt mesenchymal features (epithelial-to-mesenchymal transition-like state) upon activation of epidermal growth factor receptor (EGFR) coupled with blockade of E-cadherin. This treatment leads to a coordinated loss of EGFR and tissue factor (TF) from the plasma membrane and coincides with a surge in emission of small, exosome-like EVs containing both receptors. TF (but not EGFR) is selectively up-regulated in EVs produced by mesenchymal-like cancer cells and can be transferred to cultured endothelial cells rendering them highly procoagulant. We postulate that epithelial-to-mesenchymal transition-like changes may alter cancer cell interactions with the vascular systems through altered vesiculation and TF shedding.

Oncogenic pathways not only overwhelm the intracellular signaling circuitry but also profoundly alter patterns of communication between cells (1). Notable manifestations of the latter property can be found at the tumor vascular interface where transformed cells orchestrate the recruitment of endothelial, perivascular, and inflammatory cells, as well as platelets and clotting factors, thereby altering several aspects of vascular homeostasis (2). One process that contributes to these perturbations is the frequent deregulation of tissue factor (TF),[4] the key transmembrane receptor for the coagulation factor VII/VIIa, and the essential trigger of both the coagulation cascade and intracellular signaling (3). In cancer, TF is often up-regulated by external stimuli and oncogenic mutations, including those affecting epidermal growth factor receptor (EGFR) (4–6). The resulting responses have been implicated in cancer-related thrombosis, angiogenesis, metastasis, tumor initiation, and other crucial events (7, 8). TF is also shed from cancer cells to the circulation, as procoagulant microparticles (MPs), more broadly referred to as extracellular vesicles (EVs). This property may contribute to systemic coagulopathy often associated with cancer (9).

EVs represent a unique mechanism of cell-to-cell communication. Indeed, these organelle-like structures mediate intercellular trafficking of molecules traditionally regarded as insoluble or "cell-associated," including membrane receptors, cytoplasmic and nuclear proteins, or nucleic acids (10). EVs are released from cells either constitutively or under the influence of exogenous stimuli, stress, or malignant transformation (11). At least two different subcellular regions are viewed as major sources of EV biogenesis (vesiculation), namely the cellular plasma membrane blebs, including regions known as lipid rafts (12) and the network of intracellular vesicles comprising the endosomal system (13).

It is believed that formation of plasma membrane blebs on the cell surface may lead to their scission and detachment as

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[4] The abbreviations used are: TF, tissue factor; EGFR, epidermal growth factor receptor; EMT, epithelial-to-mesenchymal transition; EV, extracellular vesicle; MV, microvesicle; MP, microparticles; HUVEC, human umbilical vein endothelial cell(s); TAT, thrombin-antithrombin complex.

large EVs (100–1000 nm in diameter), often referred to as microparticles (MPs) or ectosomes, and known to expose phosphatidylserine, integrins, lineage antigens, and certain functional receptors, including TF. MPs containing tissue factor (TF-MPs) are thought to mediate transfer of this procoagulant activity between nucleated cells and platelets (11) and are investigated as prospective inducers and biomarkers of thrombosis in cancer (14, 15).

In contrast to MPs, endosomal EVs (exosomes) are relatively small (30–100 nm) and emerge as secondary, intraluminal vesicles within intracellular multivesicular bodies. As a part of the endosomal system, these structures control internalization, recycling, and signaling activity of several surface proteins, as exemplified by EGFR and other receptor tyrosine kinases (16). Multivesicular bodies may be either directed toward lysosomal degradation or reach plasma membrane, leading to the release of their intraluminal vesicles, as exosomes, into the extracellular space. Exosomes are enriched in certain molecular cargo such as tetraspanins, heat shock proteins, and nucleic acids (mRNA, microRNA) (13).

Aggressive cancer cells tend to emit elevated quantities of EVs (both MPs and exosomes), as compared with their non-transformed or indolent counterparts. This is, at least in part, attributed to the activation of oncogenic and growth factor pathways, including EGFR, which appears to modulate mechanisms of EV biogenesis (17–19). Tumor cell vesiculation has been implicated in thrombosis, angiogenesis, immunomodulation metastasis (20–23), and in the intercellular transfer of oncogenic EGFR (17).

Cancer cells harboring constitutive oncogenic alterations are known to alternate between epithelial and mesenchymal phenotypes. This process is often described as epithelial-to-mesenchymal transition (EMT) and thought to transiently render epithelial cancer cells more migratory, metastatic and capable of tumor initiation (24, 25). EMT-like states are induced by diverse signaling pathways involving cytokines (TGFβ), oncoproteins (Ras) and transcription factors (Twist, Snail), or loss of E-cadherin adhesion. The related hallmarks include disruption of cell-cell adhesion and the onset of mesenchymal features, including vimentin expression and elongated morphology (24). Interestingly, these changes modulate EGFR-dependent TF up-regulation in cancer cells (4), but their role in TF shedding, cellular vesiculation, and intercellular exchange of bioactive molecules remains unknown.

Here, we show that induction of the mesenchymal-like state in epithelial cancer cells (A431 and DLD-1) leads to changes in their vesiculation profiles. Thus, stimulation of EGFR coupled with blockade of E-cadherin results in the adoption of mesenchymal morphology associated with emission of exosome-size EVs with elevated TF content. Such TF-rich EVs interact with endothelial cells causing their exaggerated procoagulant conversion. Moreover, tumor-derived circulating TF and elevated markers of coagulation (thrombin-antithrombin complexes (TATs)) are detectable in blood of tumor bearing mice. Our observations suggest that mesenchymal conversion may modulate the way cancer cells vesiculate and interact with the vascular system.

EXPERIMENTAL PROCEDURES

Cell Culture and Treatments—The human squamous cell carcinoma A431 and colorectal adenocarcinoma DLD-1 cell lines were obtained from American Type Culture Collection (ATCC, Manassas, VA) and cultured in DMEM or RPMI 1640 medium (Invitrogen), respectively, supplemented with 10% fetal bovine serum (FBS) (Multicell Wisent, Inc., St-Bruno, Quebec, Canada) depleted of EVs. Cells were serum-starved overnight prior to treatments with 50 ng/ml TGFα (Invitrogen), and 2 μg/ml SHE78-7 anti-E-cadherin neutralizing antibody (Invitrogen) for 48 h. Human umbilical vein endothelial cells (HUVEC; ATCC) were cultured in complete endothelial cell growth medium-2 (Lonza, Allendale, NJ) and serum-starved prior to transfer experiments.

Preparation of Extracellular Vesicles—EVs were obtained by ultracentrifugation as described previously (12, 26–31). Briefly, culture supernatants were centrifuged for 10 min at 400 $\times$ *g*, followed by 2 h at 100,000 $\times$ *g* to pellet EVs, which were washed extensively in phosphate-buffered saline (PBS). Differential centrifugation was performed when indicated through the following steps: 15 min at 1,000 $\times$ *g*, 20 min at 2,500 $\times$ *g* (MV/MP fraction-P2), and 2 h at 100,000 $\times$ *g* (exosome fraction-P4).

Western Blotting—As described previously (6), membranes were prepared and probed with the following primary antibodies: mouse anti-human TF (Sekisui, Stamford, CT), rabbit anti-EGFR (Cell Signaling, Danvers, MA), mouse anti-vimentin (Abcam, Cambridge, MA); and mouse anti-β-actin (Sigma) or mouse anti-flotillin-1 (BD Biosciences). Horseradish peroxidise (HRP)-conjugated secondary antibodies (Dako, Mississauga, ON, Canada) and chemiluminescence (GE Healthcare) were used for detection.

TF Procoagulant Activity Assay—Factor Xa generation assay was performed as described previously (6). Briefly, cells were exposed to 5 nM recombinant FVIIa, 150 nm Factor X (Enzyme Research Laboratories, South Bend, IN), 5 mN $CaCl_2$, and 2 mM Factor Xa chromogenic substrate (Chromogenix, Bedford, MA). After a 30-min incubation, the reaction was stopped with 50% acetic acid and read under 405 nm using an Epoch microplate spectrophotometer (Biotek, Winooski, VT).

ELISA—IMUBIND TF ELISA kit (Sekisui) and human EGFR ELISA kit (R&D Systems, Minneapolis, MN) were used to quantify the levels of secreted TF and EGFR, respectively.

Immunohistochemistry—A vulvar and a head/neck cancer tissue microarrays (US Biomax, Inc., Rockville, MD) or A431 xenograft sections were stained, as indicated. A431 tumors were removed 3 weeks after subcutaneous injection of 2×10^6 cells per immunodeficient (SCID) mouse. Tissues were embedded in paraffin and sectioned (5 μm) (28). Slides were stained with rabbit or mouse anti-vimentin antibody (Abcam) or sheep anti-TF as described previously (6).

Immunofluorescence—Cultured cells were fixed for 10 min in 4% paraformaldehyde, permeabilized with 0.2% Triton X-100, and stained overnight at 4 °C with sheep anti-human TF antibody (Affinity Biologicals, Ancaster, ON, Canada), rabbit anti-human EGFR (Cell Signaling), mouse anti-human β-catenin (Thermo Scientific, Rockford, IL), rabbit anti-human γ-catenin (Abcam) or FITC-phalloidin (Sigma) followed by their respec-

tive secondary Alexa Fluor antibodies (Invitrogen). Imaris software (Bitplane) was used for confocal image analysis (colocalization).

Nanoparticle Tracking Analysis—EV profiling entailed measurements of size and numbers of particles shed from cells cultured in exosome depleted medium using the NS500 nanoparticle tracking analysis system (Nanosight, Amesbury, UK) (29).

Membrane Labeling with PKH26 and Flow Cytometry—A431 cells were surface labeled with PKH26 (red fluorescent dye; Sigma) as per manufacturer's instructions (26). EVs from labeled A431 cells were incubated for 24 h with HUVEC, and the fluorescence uptake was detected using FACSCalibur flow cytometer (BD Biosciences).

Electron Microscopy—For scanning electron microscopy (SEM), cells were fixed with 2.5% glutaraldehyde for 24 h, mounted on SEM stubs, sputtered with gold/palladium, and imaged using Hitachi FEG SEM model S-4700. For transmission electron microscopy, EVs were fixed for at least 1 h at room temperature in 1% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, post-fixed in 1% osmium tetroxide, and embedded in Epon 812. Cells were also stained with anti-TF and immunogold-labeled secondary antibodies. Ultrathin sections were cut and imaged using the on Philips CM100 instrument (28).

Data Analysis—All experiments were reproduced at least twice with similar results and presented as number of replicates (n) and mean value of replicates ± S.D. Statistical significance was evaluated using SPSS software, differences were considered significant for $p < 0.05$.

RESULTS

Mesenchymal Phenotype within Epithelial Tumors—Epidermoid cancers of vulvar, or head-and-neck origin (Fig. 1, *A* and *B*) exemplify the interplay between constitutive genetic transformation and phenotypic plasticity, as epithelial morphology often coexists with various degrees of positivity for the mesenchymal marker, vimentin (vim+). Similarly, injection of epithelial (vim−) squamous cell carcinoma cells (A431) into immunodeficient (SCID) mice leads to formation of heterogeneous tumors containing both epithelial (vim−) and mesenchymal (vim+) cellular subsets (Fig. 1*C*), even though these cells harbor a constitutive amplification, overexpression, and activation of the oncogenic EGFR.

Induction of Mesenchymal Phenotype in Cancer Cells in Vitro—Interestingly, a transient mesenchymal (EMT-like) state can be induced in cultured A431 cells in the presence of the E-cadherin blocking antibody (SHE78-7), and this is further exacerbated upon stimulation of EGFR, in the presence of the transforming growth factor α (TGFα) (Fig. 2*A*). This (SHE/TGFα) treatment leads to the dissolution of cell-cell contacts, loss of the "cobblestone" morphology, cellular elongation, reorganization of the actin cytoskeleton, and expression of vimentin (Fig. 2, *A* and *B*), with no significant change in cell proliferation (Fig. 2*C*).

Subcellular Localization of EGFR and TF During Mesenchymal Transition—On the surface of the intact (epithelial) A431 cells, EGFR is co-expressed with TF, its regulatory target, which mediates coagulation and signaling effects of EGFR-transformed cells. Stimulation with the SHE/TGFα mixture or TGFα alone triggers EGFR down-regulation (Fig. 3*A*), resulting in largely intracellular EGFR immunostaining, due to ligand-dependent internalization (Fig. 3*B*) (16). These effects were also observed in the presence of SHE78-7 antibody alone suggesting a link between EGFR and E-cadherin signaling in these cells. Predictably, TF levels increased upon stimulation of A431 cells with SHE/TGFα and TGFα, but paradoxically, this led to a diminution of the surface staining for TF, which has also occurred in the presence of the SHE78-7 antibody (Fig. 3*B*). Curiously, although in the mesenchymal state (SHE/TGFα) both EGFR and TF are mainly localized in the cytoplasm, they are poorly co-localized (Fig. 3*C*), suggesting their retention in different subcellular compartments. It is also of note that in intact A431

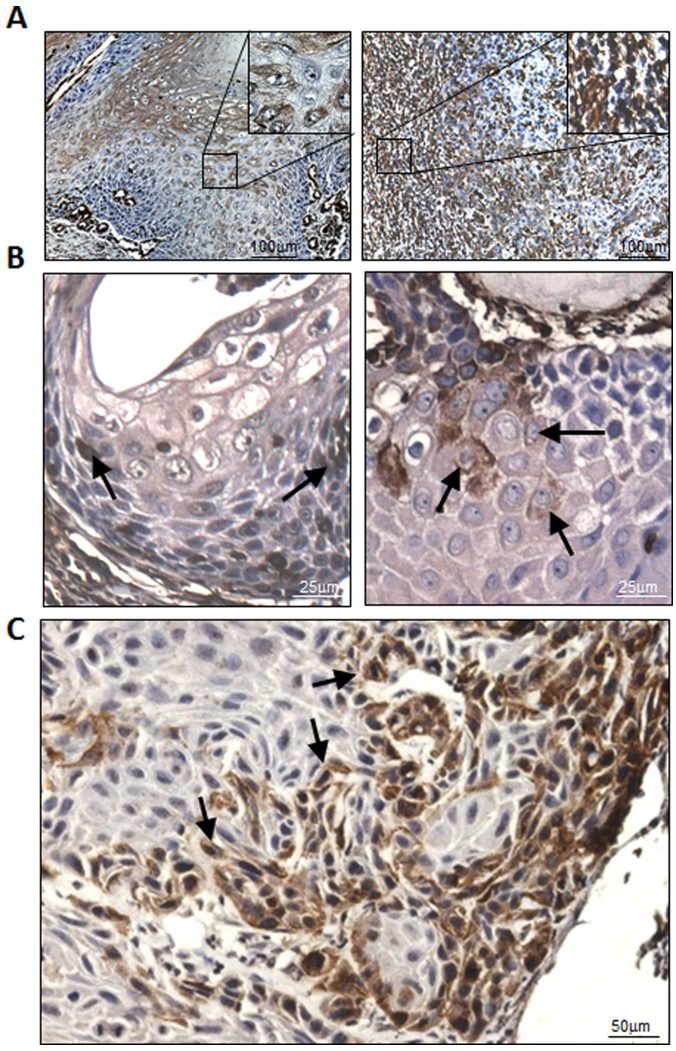

FIGURE 1. **Mesenchymal cell subpopulations within epithelial cancers.** Staining for vimentin expression in sections of human head and neck and vulvar cancer (*A* and *B*), as well as A431 xenografts in immunodeficient (SCID) mice (*C*). *A*, examples of grade I (*left*) or grade II (*right*) head and neck tumors: notable presence of vimentin-positive cells in both stromal and tumor cell compartments. *B*, transitory phenotypes in epidermoid vulvar carcinoma with different degrees of vimentin-positivity among cells with epithelial morphology (*arrows*). *C*, cellular heterogeneity of subcutaneous A431 tumor xenografts in immunodeficient mice. Tumors, which originate from morphologically uniform cancer cells driven by amplification of EGFR, contain both epidermoid vimentin-negative and mesenchymal vimentin-positive (*arrows*) cellular subpopulations, the latter indicative of an ongoing EMT.

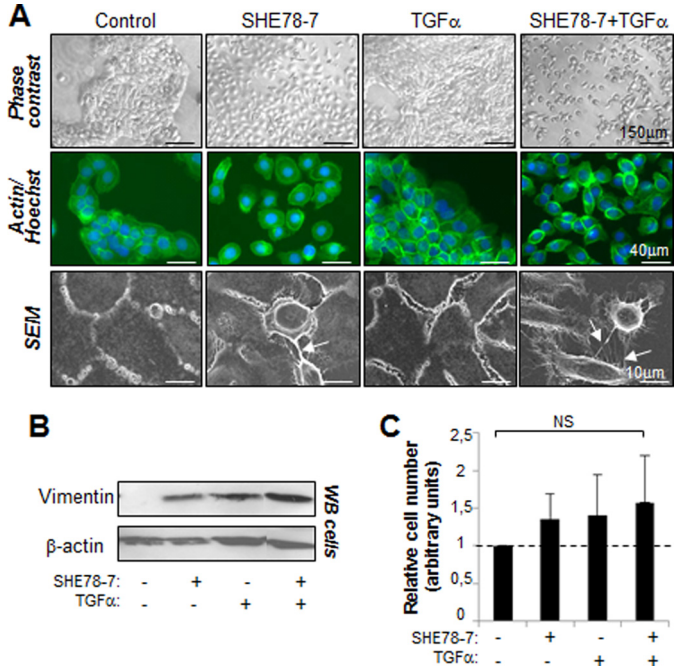

FIGURE 2. **Induction of the mesenchymal phenotype in A431 cancer cells.** *A*, induction of the EMT-like phenotype *in vitro* by a combined blockade of E-cadherin (SHE78-7 antibody) and stimulation of EGFR (TGFα, 50 ng/ml). The consequences of either individual or combined treatments on the cobblestone morphology of A431 colonies (*upper row*; phase contrast microscopy), architecture of the actin cytoskeleton (*middle panel*, phalloidin and DAPI fluorescent staining), and on the cellular morphology (*bottom panel*, *SEM*) are shown as indicated. Of note is the dissociation of cell-cell contacts, residual cytoneme-like protrusions (*white arrows*), and elongated morphology in cells treated with both SHE78-7 and TGFα. *Scale bars* are given in the respective panels. *B*, induction of vimentin expression in A431 cells treated as indicated. SHE78-7 and TGFα induce vimentin expression individually, but their effects are most pronounced under co-treatment conditions inducing EMT-like morphology. *C*, EMT-like changes are not associated with major changes in cell number (cell count). *NS*, non-significant statistically ($p > 0.05$; $n = 3$). *WB*, Western blot.

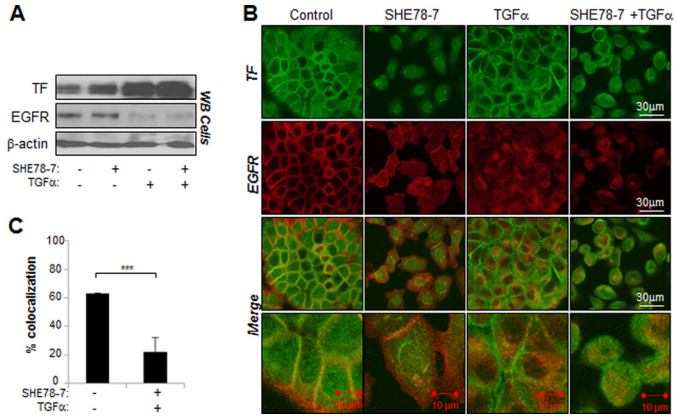

FIGURE 3. **The impact of mesenchymal phenotype induction on the expression patterns of EGFR and TF in cancer cells.** *A*, differential impact of EMT inducing treatments on the levels of EGFR and TF in A431 cells (Western blotting; *WB*). There is a notable and expected decrease in levels of EGFR upon treatment with TGFα alone or in combination with SHE78-7, whereas these treatments up-regulate TF expression. *B*, changes in subcellular localization of EGFR and TF upon induction of EMT-like state with both receptors depleted from the plasma membrane. The cells were treated as indicated and subjected to immunofluorescent staining for EGFR and TF. *C*, computation of TF and EGFR co-localization in confocal images of A431 cells under epithelial and mesenchymal growth conditions. There is a reduction in the overlap between the respective signals in SHE78-7/TGFα-treated cells (***, $p < 0.0005$) ($n = 3$).

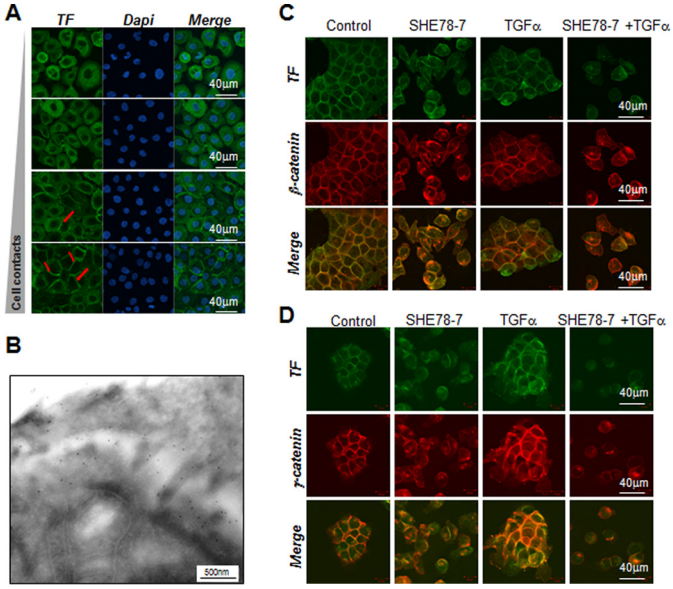

FIGURE 4. **Accumulation of TF at cell junctions in A431 epithelial cells.** *A*, staining for TF in A431 cells. At low density, even unstimulated A431 cells exhibit weaker presence of TF at the plasma membrane and this changes at the cell-cell interface once the cells make contact. *B*, transmission electron microscopy image following immunogold staining for TF shows a more pronounced accumulation of TF signal (*fine black dots*) around cell-to-cell junction. *C* and *D*, TF localization at the cell-cell junctions co-localizes with junction proteins such as β-catenin (*C*) and especially γ-catenin (*D*).

cells, TF is highly expressed at intercellular junctions (Fig. 4). Indeed, we observed that when the cells come into contact with each other in sparse cultures, their interfaces are especially positive for TF (Fig. 4, *A* and *B*), as is the interior, but not the edges, of multicellular colonies. Junctional localization is reinforced by TF colocalization with β- and γ-catenins (Fig. 4, *C* and *D*), which is abrogated upon blockade of E-cadherin (SHE78-7, SHE/TGFα). These observations suggest that the dynamics of intercellular contacts may play a role in TF retention at the plasma membrane. Because SHE/TGFα-induced mesenchymal phenotype leads to reduced cell-cell contacts and diminished TF association with the plasma membrane, but also to higher overall TF levels, it is possible that TF is perpetually shed from the cell surface as cargo of membrane EVs.

Mesenchymal Phenotype Impacts Vesiculation of Cancer Cells—To assess the state of cellular shedding under epithelial and mesenchymal conditions, we performed transmission electron microscopy analysis, which revealed that intact A431 cells constitutively produce ample EVs ranging between 100–300 nm in size, with few exosome-like particles (Fig. 5*A*). SEM imaging revealed that the mesenchymal phenotype profoundly alters cell surfaces, disrupts intercellular contacts, and leads to formation of filopodia-like processes (Fig. 5*B*). We also measured the number and sizes of EVs emitted under these conditions using nanoparticle tracking analysis, which documented heterogeneity of EV sizes. In intact cells, EVs varied between 20 and 1000 nm and peaked at 150–200 nm, whereas mesenchymal-like cells exhibited a marked shift in their EV profile, with a prominent peak in the exosomal size range of 20–50 nm (Fig. 5*C*).

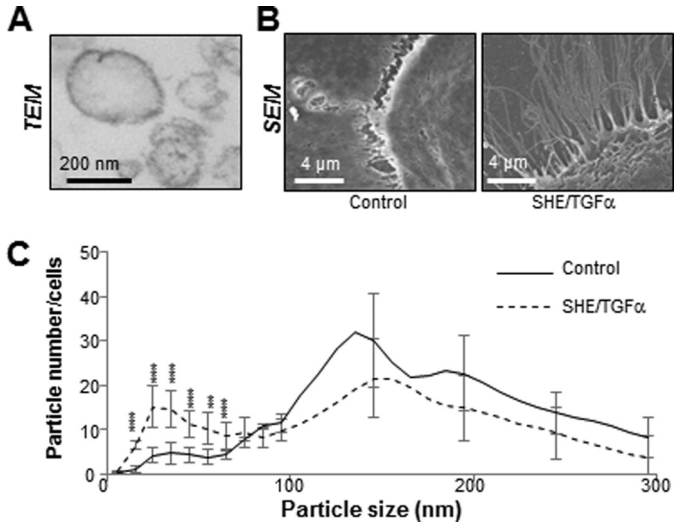

FIGURE 5. **Quantitative and qualitative changes in cancer cell vesiculation profile as a function of mesenchymal transition.** *A*, heterogeneity of EVs produced by A431 cells in culture (transmission electron microscopy; *TEM*). Of note is the presence of EVs ranging in size between 50 and 200 nm. *B*, Surface characteristics of A431 cells in their epithelial (*left*) and mesenchymal state (*right*). Untreated A431 cells (*left panel*) exhibit relatively smooth apical surfaces, form tight intercellular contacts, and deploy multiple intercellular membrane bridges. In contrast, many of the A431 cells in SHE78-7/TGFα treated cultures (*right panel*) are isolated from one another, assume elongated morphology, and exhibit a very complex and rough surface architecture with deployment of numerous filopodia-like processes. *C*, nanoparticle tracking analysis (*NTA*) of control and SHE78-7/TGFα-treated A431 cells. Untreated cells emit a wide spectrum of EVs of different sizes (as in *A*), whereas cells treated with SHE/TGFα, a mesenchymal phenotype inducing mixture, produce a distinct peak within the size range of exosomes (20–50 nm). Only particles between 0 to 300 nm are shown to maintain the resolution. (**, $p < 0.005$; ***, $p < 0.0005$) ($n = 3$).

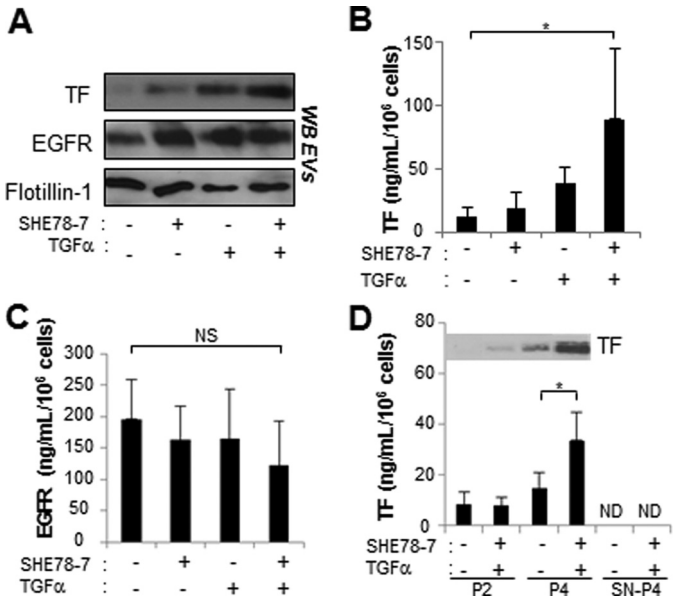

FIGURE 6. **The impact of epithelial and mesenchymal culture conditions on the EV-mediated emission of EGFR and TF by A431 cancer cells.** *A*, differential content of EGFR and TF in EVs isolated from supernatants of control A431 cells and upon indicated treatments (Western blotting (*WB*); flotillin-1 present in EVs is used as a loading control, also compare Fig. 5*A*). *B*, quantification of TF in the conditioned medium of A431 cells induced to undergo mesenchymal transition (TF ELISA). The effects of individual treatments are markedly less pronounced than the combined exposure to SHE78-7/TGFα ($n = 3$). *C*, sustained emission of EGFR as cargo of EVs into the A431 conditioned media ($n = 3$). *D*, concentration of the TF signal as a function of the SHE78-7/TGFα treatment in the P4 fraction of the conditioned media corresponding to small (exosome-like) EVs, relative to larger EVs (P2 fraction; TF ELISA). The removal of small EVs (P4) by ultracentrifugation resulted in depletion of the TF signal from the cell culture supernatants, suggesting the absence of soluble (EV-unrelated) TF in this material. P4 and P2 fractions were defined as in the text by differential centrifugation protocols ($n = 2$) (*, $p <$ 0,05). *NS*, not significant; *ND*, not detectable.

Mesenchymal Phenotype Promotes Emission of Small Vesicles Containing TF—A431 cells are known to emit both EGFR and TF via the vesiculation pathway (17, 26). In this regard, the EV content of EGFR did not change significantly as a function of various treatments (Fig. 6, *A* and *C*), but the overall levels of the cell-associated EGFR declined under these conditions (Fig. 3*A*). In contrast, Western blot of purified EV lysates (Fig. 6*A*) and ELISA analysis of the corresponding cell culture supernatants (Fig. 6*B*) both indicated a massive increase in EV-associated (and cellular) TF in the presence of the SHE/TGFα mixture, which induces mesenchymal features in these cells.

To determine the contribution of exosomal EVs to this TF shedding, conditioned medium of A431 cells was subjected to differential centrifugation intended to separate larger EVs (P2 fraction) from exosomal EVs (P4 fraction) (31). Interestingly, SHE/TGFα treatment selectively stimulated TF release with the exosomal fraction of A431 supernatants (Fig. 6*D*). Moreover, depletion of exosome-like EVs from this material using ultracentrifugation (100,000 × *g*) resulted in a complete removal of the TF immunoreactivity.

We have also tested these events in an independent cancer cell line, DLD-1, known to harbor K-ras mutation, but responsive to EGFR stimulation and blockade of E-cadherin (34). Indeed, these cells also scattered and assumed mesenchymal morphology in the presence of the SHE/TGFα mixture (Fig. 7*A*). Moreover, this change was coupled with the emission of exosome-sized particles containing high levels of TF (Fig. 7, *B*

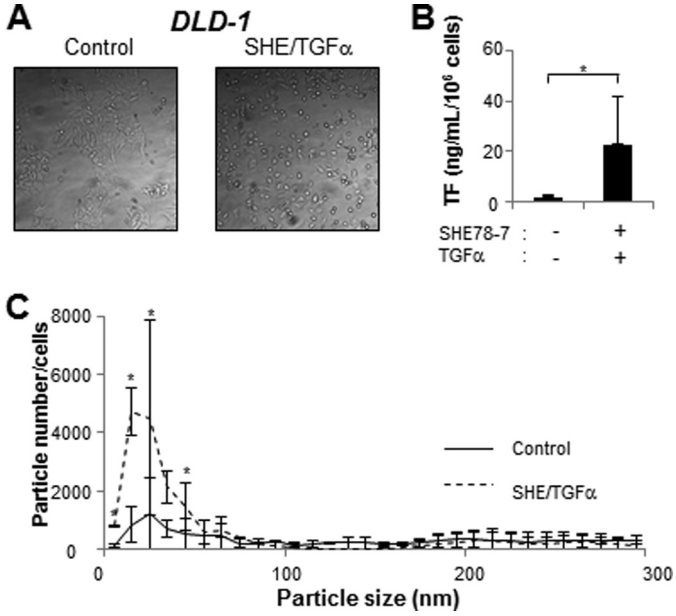

FIGURE 7. **Stimulation of EGFR and blockade of E-cadherin induce mesenchymal phenotype and exosomal TF emission in DLD-1 cells.** *A*, stimulation of DLD-1 colorectal adenocarcinoma cells with SHE78-7 antibody and TGFα provokes cell scattering, elongation, and mesenchymal appearance. *B*, marked release of TF into conditioned medium of DLD-1 cells stimulated with the SHE/TGFα mixture (TF ELISA) ($n = 3$) (*, $p <$ 0,05). *C*, increase in production of exosomal-like EVs by DLD-1 cells stimulated with SHE78-7 and TGFα ($n = 2$) (*, $p < 0.05$).

and *C*). These observations suggest in cancer cells the release of TF occurs primarily in association with EVs and not as a soluble ectodomain, as described in the literature (32). The EGFR/E-cadherin pathway regulates mesenchymal phenotype in some of these cells and may shift TF release toward small exosome-like particles.

Procoagulant Conversion of Endothelial Cells Exposed to TF-containing Extracellular Vesicles—Biological activity of EVs can be measured by their ability to transfer their molecular cargo between cells (26). To assess whether TF-containing EVs generated by A431 cells under epithelial or mesenchymal culture conditions differ in this regard, we incubated HUVEC with these EVs and tested for their overall EV uptake, as well as transfer of the TF antigen and procoagulant activity (Fig. 8). HUVEC are normally TF-negative, but they readily incorporated PKH26-labeled EVs produced by A431 cells, regardless of their epithelial or mesenchymal phenotype (Fig. 8*A*). However, the EV-mediated transfer of TF immunoreactivity and procoagulant activity to HUVEC were both dramatically elevated (relative to control) in the case of EVs isolated from A431 cancer cells induced to undergo mesenchymal transition by treatment with the SHE/TGFα mixture (Fig. 8, *B* and *C*). These results suggest that the mesenchymal phenotype may modulate ways in which EV-associated TF can traffic between cancer cells and endothelium. Notably, upon such transfer, TF becomes exposed on the cell surface in an active coagulation-compatible (decrypted) configuration.

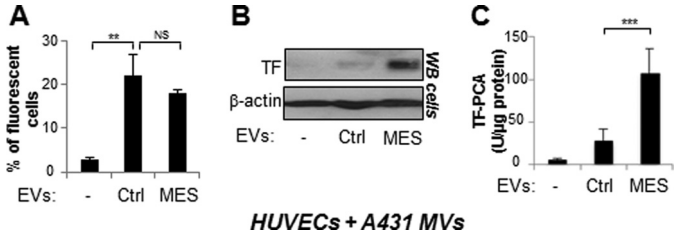

FIGURE 8. **Extracellular vesicle-mediated transfer of tumor-derived TF to endothelial cells.** *A*, EV-mediated transfer of membrane fluorescence from PKH26-labeled A431 cells to cultured endothelium (HUVEC). Following a 24-h incubation with EVs generated by control (*EVs Ctrl*) or SHE78-7/TGFα-stimulated/mesenchymal tumor cells (*EVs MES*), HUVEC cells avidly incorporated EV-associated fluorescence (~20% cells were gated as positive), regardless of treatment. *B*, differential acquisition of TF positivity by HUVEC cells exposed to EVs from control and SHE78-7/TGFα-treated A431 cells (Western blotting; *WB*). The incorporated TF signal is severalfold stronger in the case of HUVEC incubated with EVs released by stimulated A431 cells relative to control. *C*, TF-containing EVs isolated from A431 cells cultured under mesenchymal conditions transfer their procoagulant phenotype to HUVEC more robustly than their control counterparts (EVs from untreated A431 cells). TF procoagulant activity assay that measures activation of the coagulation factor X to Xa was conducted on HUVEC cells treated as in *B* ($n = 2$) (**, $p < 0.005$; ***, $p < 0.0005$). *NS*, not significant statistically.

Detection of Circulating TF and Procoagulant Activity in Vivo—In A431 xenografts, the expression of both vimentin (Fig. 1*C*) and TF (Fig. 9*A*) is heterogeneous (4), with islands of high positivity interspersed with negative regions. We observed that human TF circulates in plasma of mice bearing such tumors (Fig. 9*B*), and this is coupled with detectable levels of TATs (Fig. 9*C*), indicative of the systemic coagulation system activation (and absent in tumor-free animals). This may suggest that the release of TF containing EVs from TF-expressing cancer cells has a potential to trigger procoagulant effects *in vivo*.

DISCUSSION

This work brings forward several novel aspects of tumor cell vesiculation. First, we observed a previously unrecognized link between the induction of mesenchymal features in cancer cells and TF emission as pro-coagulant exosome-like EVs. The retention of TF on the plasma membrane has thus far been studied in the context of TF internalization, as a result of stimulation with its natural ligand, factor VIIa (33). Our study implicates another mechanism involving TF extracellular shedding regulated by EGFR and E-cadherin and coupled with the mesenchymal phenotype. E-cadherin is involved in several biological processes, including cell-cell adhesion, formation of intercellular junctions, organization of the cytoskeleton, modulation of signals emanating from receptor tyrosine kinases (*e.g.* EGFR) (34), regulation of the canonical Wnt pathway activity, and many others (35). A constitutive blockade of E-cadherin in epithelial cancer cells induces EMT and stem cell-like phenotype (25). Our observations highlight the possible link between these events and pathways of cellular vesiculation, TF shedding, and coagulation. It is of note that the pioneering studies of Rao and co-workers (36, 37) have already revealed the existence of several subcellular pools of TF, some of which were associated with the plasma membrane, lipid rafts and Golgi. We describe

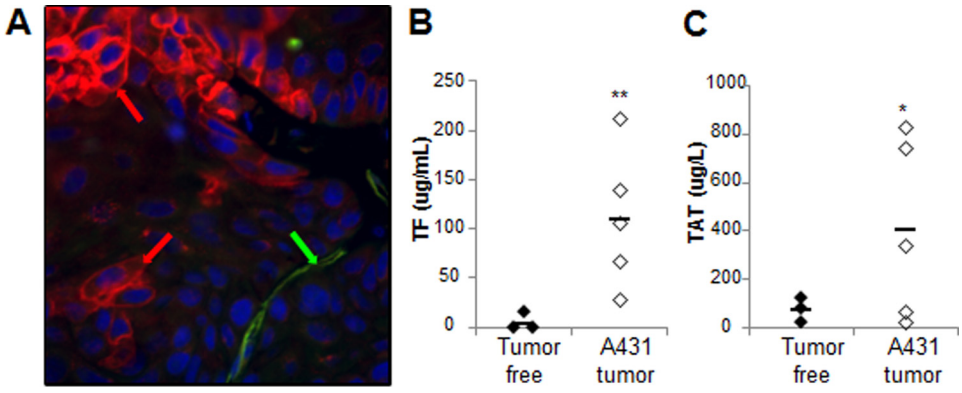

FIGURE 9. **Circulating TF and TAT complexes in tumor bearing mice.** *A*, heterogeneous pattern of TF staining (*red*) in A431 tumor xenografts resembles the heterogeneity of vimentin expression (compare Fig. 1*C* and (4)). TF-positive cells are found in the proximity of blood vessels (*green*, CD105 staining; *blue*, DAPI (nuclei)). *B*, circulating human (tumor-derived) TF in plasma of mice harboring A431 tumors measured by ELISA. *C*, TAT complexes indicative of the activated coagulation system parallel the presence of TF in plasma of tumor bearing mice and are absent in tumor-free mice (TAT ELISA). *, $p < 0.05$; **, $p < 0.005$.

another aspect of this regulation with TF accumulating at the cell-cell junctions alongside with β- and γ-catenins or within the EV compartment. In two different cell lines the mesenchymal transition led to disruption of cell-cell contacts and TF exit from cells via exosome-like vesicles. Notably, this form of EV-mediated receptor exit is also observed in the case of EGFR, which may suggest that EV production may regulate the strength of the EGFR signaling in mesenchymal cancer cells, in addition to classical processes of intracellular trafficking (16).

Second, our study speaks to the heterogeneity of extracellular particles containing tumor-derived TF. This "shedding" process has traditionally been linked with larger, membrane-derived EVs (MPs) (12), and only few recent reports implicated exosomes as possible extracellular carriers of coagulation-competent TF (38). This is despite a growing interest in detection of TF-exposing MPs in plasma of cancer patients, with hopes to develop predictive biomarkers for venous thromboembolism and predictors of disease outcomes (14). Indeed, circulating TF-MPs have been recorded in several cancer settings, including through sophisticated flow cytometry approaches, but the results of these studies have been variable (15). If exosomes constitute a significant proportion of the TF pool present in plasma, a direct detection of this material by flow cytometry could be very challenging due to small sizes of these particles, their uptake by host cells and other factors (39). Our results suggest that this could be relevant in the context of tumors with a significant component of EMT, *e.g.* in certain subtypes of epithelial or glial malignancies (40) or under hypoxia (24). However, these effects are unlikely uniform between cells driven by different oncogenic pathways and under variable EMT-inducing stimuli. Still, it is thought-provoking that unstimulated DLD-1 cells harboring mutant K-ras emit mainly exosomal-size particles, whereas EGFR-driven A431 cells produce mainly ectosomes, and in both cases, the SHE/TGFα mixture selectively enhances production of exosomes containing TF.

Third, although endothelial cells normally exhibit anti-coagulant functions (41) and do not express TF *in vivo*, these properties may be compromised in the context of malignancy. This is illustrated by studies demonstrating endothelial cell expression of TF in invasive breast cancer (42) or the impact of their genetic status on their anticoagulant potential (43). It remains to be studied whether TF trafficking from mesenchymal cancer cells to endothelium could produce similar effects and contribute to thrombosis or metastasis.

To the best of our knowledge, this is the first comprehensive analysis of the link between vesiculation and the onset of the EMT-like phenotype. EMT is increasingly recognized as a process involved in cancer cell mobility, metastasis, and stemness (36). Whether emission of EVs containing TF, EGFR, or other cargo contributes to these respective biological processes is a fascinating but presently unanswered question. At the same time, the link between EGFR and EMT induction has already been explored. For example, in another squamous cell carcinoma cell line, SSC10A, EGFR stimulation induces EMT through the up-regulation of matrix metalloproteinase 9 and the related cleavage of E-cadherin (44). In some of these instances, E-cadherin becomes internalized (45). Other oncogenes (Ras), cytokines (TGFβ), transcription factors (Twist), signaling receptors (Met), and mechanisms obliterating E-cadherin signaling have also been explored (24) as inducers of EMT. These pathways may also affect cellular vesiculation in ways that are worthy of further exploration.

Overall, this study documents the hitherto unsuspected changes in EV emission profiles as a function of mesenchymal and epithelial transdifferentiation states. Consequently, the messages encapsulated in tumor cell-derived EVs, including their content of TF, are clearly not constitutive but rather depend on the heterogeneity and plasticity of parental cancer cell populations. This should be considered when EVs are studied as biological effectors and biomarkers in cancer.

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REFERENCES

1. Rak, J. (2009) Ras oncogenes and tumour vascular interface in *Cancer Genome and Tumor Microenvironment* (Thomas-Tikhonenko, A., ed) pp. 150–170, Springer, New York
2. Carmeliet, P., and Jain, R. K. (2011) Molecular mechanisms and clinical applications of angiogenesis. *Nature* **473,** 298–307
3. Ruf, W., Disse, J., Carneiro-Lobo, T. C., Yokota, N., and Schaffner, F. (2011) Tissue factor and cell signalling in cancer progression and thrombosis. *J. Thromb. Haemost.* **9,** 306–315
4. Milsom, C. C., Yu, J. L., Mackman, N., Micallef, J., Anderson, G. M., Guha, A., and Rak, J. W. (2008) Tissue factor regulation by epidermal growth factor receptor and epithelial-to-mesenchymal transitions: effect on tumor initiation and angiogenesis. *Cancer Res.* **68,** 10068–10076
5. Rong, Y., Belozerov, V. E., Tucker-Burden, C., Chen, G., Durden, D. L., Olson, J. J., Van Meir, E. G., Mackman, N., and Brat, D. J. (2009) Epidermal growth factor receptor and PTEN modulate tissue factor expression in glioblastoma through JunD/activator protein-1 transcriptional activity. *Cancer Res.* **69,** 2540–2549
6. Magnus, N., Garnier, D., and Rak, J. (2010) Oncogenic epidermal growth factor receptor up-regulates multiple elements of the tissue factor signaling pathway in human glioma cells. *Blood* **116,** 815–818
7. Rickles, F. R. (2006) Mechanisms of cancer-induced thrombosis in cancer. *Pathophysiol. Haemost. Thromb.* **35,** 103–110
8. Versteeg, H. H., Schaffner, F., Kerver, M., Petersen, H. H., Ahamed, J., Felding-Habermann, B., Takada, Y., Mueller, B. M., and Ruf, W. (2008) Inhibition of tissue factor signaling suppresses tumor growth. *Blood* **111,** 190–199
9. Falanga, A. (2005) Thrombophilia in cancer. *Semin. Thromb. Hemost.* **31,** 104–110
10. Rak, J., and Guha, A. (2012) Extracellular vesicles–vehicles that spread cancer genes. *Bioessays* **34,** 489–497
11. Al-Nedawi, K., Meehan, B., and Rak, J. (2009) Microvesicles: messengers and mediators of tumor progression. *Cell Cycle* **8,** 2014–2018
12. Del Conde, I., Shrimpton, C. N., Thiagarajan, P., and López, J. A. (2005) Tissue-factor-bearing microvesicles arise from lipid rafts and fuse with activated platelets to initiate coagulation. *Blood* **106,** 1604–1611
13. Théry, C., Ostrowski, M., and Segura, E. (2009) Membrane vesicles as conveyors of immune responses. *Nat. Rev. Immunol.* **9,** 581–593
14. Zwicker, J. I. (2010) Predictive value of tissue factor bearing microparticles in cancer associated thrombosis. *Thromb. Res.* **125,** S89–S91
15. Lacroix, R., Judicone, C., Poncelet, P., Robert, S., Arnaud, L., Sampol, J., and Dignat-George, F. (2012) Impact of pre-analytical parameters on the measurement of circulating microparticles: towards standardization of protocol. *J. Thromb. Haemost.* **10,** 437–446
16. Citri, A., and Yarden, Y. (2006) EGF-ERBB signalling: towards the systems

level. *Nat. Rev. Mol. Cell Biol.* **7,** 505–516
17. Al-Nedawi, K., Meehan, B., Micallef, J., Lhotak, V., May, L., Guha, A., and Rak, J. (2008) Intercellular transfer of the oncogenic receptor EGFRvIII by microvesicles derived from tumour cells. *Nat. Cell Biol.* **10,** 619–624
18. Di Vizio, D., Kim, J., Hager, M. H., Morello, M., Yang, W., Lafargue, C. J., True, L. D., Rubin, M. A., Adam, R. M., Beroukhim, R., Demichelis, F., and Freeman, M. R. (2009) Oncosome formation in prostate cancer: association with a region of frequent chromosomal deletion in metastatic disease. *Cancer Res.* **69,** 5601–5609
19. Muralidharan-Chari, V., Clancy, J. W., Sedgwick, A., and D'Souza-Schorey, C. (2010) Microvesicles: mediators of extracellular communication during cancer progression. *J. Cell Sci.* **123,** 1603–1611
20. Zwicker, J. I., Trenor, C. C., 3rd, Furie, B. C., and Furie, B. (2011) Tissue factor-bearing microparticles and thrombus formation. *Arterioscler. Thromb. Vasc. Biol.* **31,** 728–733
21. Peinado, H., Alečković, M., Lavotshkin, S., Matei, I., Costa-Silva, B., Moreno-Bueno, G., Hergueta-Redondo, M., Williams, C., García-Santos, G., Ghajar, C., Nitadori-Hoshino, A., Hoffman, C., Badal, K., Garcia, B. A., Callahan, M. K., Yuan, J., Martins, V. R., Skog, J., Kaplan, R. N., Brady, M. S., Wolchok, J. D., Chapman, P. B., Kang, Y., Bromberg, J., and Lyden, D. (2012) Melanoma exosomes educate bone marrow progenitor cells toward a prometastatic phenotype through MET. *Nat. Med.* **18,** 883–891
22. Skog, J., Würdinger, T., van Rijn, S., Meijer, D. H., Gainche, L., Sena-Esteves, M., Curry, W. T., Jr., Carter, B. S., Krichevsky, A. M., and Breakefield, X. O. (2008) Glioblastoma microvesicles transport RNA and proteins that promote tumour growth and provide diagnostic biomarkers. *Nat. Cell Biol.* **10,** 1470–1476
23. Wolfers, J., Lozier, A., Raposo, G., Regnault, A., Théry, C., Masurier, C., Flament, C., Pouzieux, S., Faure, F., Tursz, T., Angevin, E., Amigorena, S., and Zitvogel, L. (2001) Tumor-derived exosomes are a source of shared tumor rejection antigens for CTL cross-priming. *Nat. Med.* **7,** 297–303
24. Thiery, J. P., Acloque, H., Huang, R. Y., and Nieto, M. A. (2009) Epithelial-mesenchymal transitions in development and disease. *Cell.* **139,** 871–890
25. Scheel, C., Eaton, E. N., Li, S. H., Chaffer, C. L., Reinhardt, F., Kah, K. J., Bell, G., Guo, W., Rubin, J., Richardson, A. L., and Weinberg, R. A. (2011) Paracrine and autocrine signals induce and maintain mesenchymal and stem cell states in the breast. *Cell.* **145,** 926–940
26. Yu, J., May, L., Milsom, C., Anderson, G. M., Weitz, J. I., Luyendyk, J. P., Broze, G., Mackman, N., and Rak, J. (2008) Contribution of host-derived tissue factor to tumor neovascularization. *Arterioscler. Thromb. Vasc. Biol.* **28,** 1975–1981
27. Yu, J. L., May, L., Lhotak, V., Shahrzad, S., Shirasawa, S., Weitz, J. I., Coomber, B. L., Mackman, N., and Rak, J. W. (2005) Oncogenic events regulate tissue factor expression in colorectal cancer cells: implications for tumor progression and angiogenesis. *Blood* **105,** 1734–1741
28. Al-Nedawi, K., Meehan, B., Kerbel, R. S., Allison, A. C., and Rak, J. (2009) Endothelial expression of autocrine VEGF upon the uptake of tumor-derived microvesicles containing oncogenic EGFR. *Proc. Natl. Acad. Sci. U.S.A.* **106,** 3794–3799
29. Dragovic, R. A., Gardiner, C., Brooks, A. S., Tannetta, D. S., Ferguson, D. J., Hole, P., Carr, B., Redman, C. W., Harris, A. L., Dobson, P. J., Harrison, P., and Sargent, I. L. (2011) Sizing and phenotyping of cellular vesicles using Nanoparticle Tracking Analysis. *Nanomedicine* **7,** 780–788
30. Taylor, P., Nielsen, P. A., Trelle, M. B., Hørning, O. B., Andersen, M. B., Vorm, O., Moran, M. F., and Kislinger, T. (2009) Automated 2D peptide separation on a 1D nano-LC-MS system. *J. Proteome Res.* **8,** 1610–1616
31. Bianco, F., Perrotta, C., Novellino, L., Francolini, M., Riganti, L., Menna, E., Saglietti, L., Schuchman, E. H., Furlan, R., Clementi, E., Matteoli, M., and Verderio, C. (2009) Acid sphingomyelinase activity triggers microparticle release from glial cells. *EMBO J.* **28,** 1043–1054
32. Bogdanov, V. Y., Balasubramanian, V., Hathcock, J., Vele, O., Lieb, M., and Nemerson, Y. (2003) Alternatively spliced human tissue factor: a circulating, soluble, thrombogenic protein. *Nat. Med.* **9,** 458–462
33. Hansen, C. B., Pyke, C., Petersen, L. C., and Rao, L. V. (2001) Tissue factor-mediated endocytosis, recycling, and degradation of factor VIIa by a clathrin-independent mechanism not requiring the cytoplasmic domain of tissue factor. *Blood* **97,** 1712–1720
34. St Croix, B., Sheehan, C., Rak, J. W., Flørenes, V. A., Slingerland, J. M., and Kerbel, R. S. (1998) E-Cadherin-dependent growth suppression is mediated by the cyclin-dependent kinase inhibitor p27(KIP1). *J. Cell Biol.* **142,** 557–571
35. Heuberger, J., and Birchmeier, W. (2010) Interplay of cadherin-mediated cell adhesion and canonical Wnt signaling. *Cold Spring Harb. Perspect. Biol.* **2,** a002915
36. Mandal, S. K., Pendurthi, U. R., and Rao, L. V. (2007) Tissue factor trafficking in fibroblasts: involvement of protease-activated receptor-mediated cell signaling. *Blood* **110,** 161–170
37. Mandal, S. K., Pendurthi, U. R., and Rao, L. V. (2006) Cellular localization and trafficking of tissue factor. *Blood* **107,** 4746–4753
38. Davila, M., Amirkhosravi, A., Coll, E., Desai, H., Robles, L., Colon, J., Baker, C. H., and Francis, J. L. (2008) Tissue factor-bearing microparticles derived from tumor cells: impact on coagulation activation. *J. Thromb. Haemost.* **6,** 1517–1524
39. Wang, J. G., Geddings, J. E., Aleman, M. M., Cardenas, J. C., Chantrathammachart, P., Williams, J. C., Kirchhofer, D., Bogdanov, V. Y., Bach, R. R., Rak, J., Church, F. C., Wolberg, A. S., Pawlinski, R., Key, N. S., Yeh, J. J., and Mackman, N. (2012) Tumor-derived tissue factor activates coagulation and enhances thrombosis in a mouse xenograft model of human pancreatic cancer. *Blood* **119,** 5543–5552
40. Verhaak, R. G., Hoadley, K. A., Purdom, E., Wang, V., Qi, Y., Wilkerson, M. D., Miller, C. R., Ding, L., Golub, T., Mesirov, J. P., Alexe, G., Lawrence, M., O'Kelly, M., Tamayo, P., Weir, B. A., Gabriel, S., Winckler, W., Gupta, S., Jakkula, L., Feiler, H. S., Hodgson, J. G., James, C. D., Sarkaria, J. N., Brennan, C., Kahn, A., Spellman, P. T., Wilson, R. K., Speed, T. P., Gray, J. W., Meyerson, M., Getz, G., Perou, C. M., and Hayes, D. N. (2010) Integrated genomic analysis identifies clinically relevant subtypes of glioblastoma characterized by abnormalities in PDGFRA, IDH1, EGFR, and NF1. *Cancer Cell* **17,** 98–110
41. van Hinsbergh, V. (2012) Endothelium–role in regulation of coagulation and inflammation. *Semin. Immunopathol.* **34,** 93–106
42. Contrino, J., Hair, G., Kreutzer, D. L., and Rickles, F. R. (1996) *In situ* detection of tissue factor in vascular endothelial cells: correlation with the malignant phenotype of human breast disease. *Nat. Med.* **2,** 209–215
43. Horowitz, N. A., Blevins, E. A., Miller, W. M., Perry, A. R., Talmage, K. E., Mullins, E. S., Flick, M. J., Queiroz, K. C., Shi, K., Spek, C. A., Conway, E. M., Monia, B. P., Weiler, H., Degen, J. L., and Palumbo, J. S. (2011) Thrombomodulin is a determinant of metastasis through a mechanism linked to the thrombin binding domain but not the lectin-like domain. *Blood.* **118,** 2889–2895
44. Zuo, J. H., Zhu, W., Li, M. Y., Li, X. H., Yi, H., Zeng, G. Q., Wan, X. X., He, Q. Y., Li, J. H., Qu, J. Q., Chen, Y., and Xiao, Z. Q. (2011) Activation of EGFR promotes squamous carcinoma SCC10A cell migration and invasion via inducing EMT-like phenotype change and matrix metalloproteinase 9-mediated degradation of E-cadherin. *J. Cell. Biochem.* **112,** 2508–2517
45. Cowden Dahl, K. D., Symowicz, J., Ning, Y., Gutierrez, E., Fishman, D. A., Adley, B. P., Stack, M. S., and Hudson, L. G. (2008) Matrix metalloproteinase 9 is a mediator of epidermal growth factor-dependent e-cadherin loss in ovarian carcinoma cells. *Cancer Res.* **68,** 4606–4613