Cancer cells induced to express mesenchymal phenotype release exosome-like extracellular vesicles carrying tissue factor

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**Keywords:** Mesenchymal state; Exosomes; Tissue Factor; Coagulation

**Background:** Cross-talk of oncogenic and differentiation pathways in cancer coagulopathy is poorly understood.

**Results:** EGFR activation and blockade of E-cadherin induce mesenchymal phenotype and exosomal shedding of tissue factor (TF) from cancer cells, coupled with transfer of 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.

**SUMMARY**

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 (EMT-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 upregulated in EVs produced by mesenchymal-like cancer cells, and can be transferred to cultured endothelial cells rendering them highly procoagulant. We postulate that EMT-like changes may alter cancer cell interactions with the vascular systems through altered vesiculation and TF shedding.

Oncogenic pathways not only overwhelm the intracellular signalling circuitry, but also profoundly alter patterns of communication between cells (1). Notable manifestations of the latter 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 upregulation of tissue factor (TF) the key transmembrane receptor for the coagulation factor VII/VIIa and the essential trigger of both the coagulation cascade and intracellular signalling (3). In cancer, TF is often upregulated by external stimuli and
E-cadherin regulates tissue factor release in cancer

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), otherwise known as extracellular vesicles (EVs). This property may contribute to systemic coagulopathy 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 large EVs (100 – 1000 nm in diameter), often referred to as microparticles (MPs) or ectosomes, and known to expose phosphatidylserine (PS), 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 (ILVs) within intracellular multivesicular bodies (MVBs). Being a part of the endosomal system these structures control internalization, recycling and signalling activity of several surface proteins, as exemplified by EGFR and other receptor tyrosine kinases (RTKs)(16). MVBs may be either directed toward lysosomal degradation, or reach plasma membrane leading to the release of ILVs, 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 to 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 or metastasis (20-23), as well as 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 signalling 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 upregulation 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 mesenchymal-like state in epithelial cancer cells (A431 and DLD-1) leads to changes in the cellular vesiculation profile. Thus, stimulation of EGFR coupled with blockade of E-cadherin results in 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 (TAT) 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 (SCC) A431 and colorectal adenocarcinoma DLD-1 cell lines were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in DMEM or RPMI-1640 media (Life Technologies, Grand Island, NY, USA) respectively, supplemented with 10% fetal bovine serum (FBS)(Multicell Wisent Inc., St-Bruno, QC, Canada) depleted of EVs. Cells were serum-starved overnight, prior to treatments with 50 ng/mL of TGF\(\alpha\) (Life Technologies), 2 \(\mu\)g/mL SHE78-7 anti-E-cadherin neutralizing antibody (Life Technologies) for 48h. Human umbilical vein endothelial cells (HUVEC; ATCC) were cultured in complete endothelial cell growth medium-2 (EGM-2)(Lonza, Allendale, NJ, USA), and serum-starved prior to transfer experiments.

Preparation of extracellular vesicles - EVs were obtained by ultracentrifugation as described earlier (12;26;27). Briefly, culture supernatants were centrifuged for 10min at 400g, followed by 2h at 100,000g to pellet EVs, which were washed extensively in phosphate buffered saline (PBS). Differential centrifugation was performed, when indicated, through the following steps: 15min at 1,000g, 20min at 2,500g (MV/MP fraction-P2), 2h at 100,000g (exosome fraction-P4).

Western blotting – As described earlier (6), membranes were prepared and probed with the following primary antibodies: mouse anti-human TF (Sekisui, Stamford, CT, USA), rabbit anti-EGFR (Cell Signaling, Danvers, MA, USA), mouse anti-vimentin (Abcam, Cambridge, MA, USA); and mouse anti-\(\beta\)-actin (Sigma Chemical Co., St Louis, MO, USA) or mouse anti-Flotillin-1 (BD Biosciences, Mountain View, CA, USA). Horseradish peroxidase (HRP)–conjugated secondary antibodies (Dako, Mississauga, ON, Canada) and chemiluminescence (GE Healthcare, Piscataway, NJ, USA) were used for detection.

TF procoagulant activity assay (TF-PCA) - Factor Xa generation assay was performed as previously described (6). Briefly, cells were exposed to 5nM recombinant FVIIa, 150nm FX (Enzyme Research Laboratories, South Bend, Indiana,) 5mM CaCl\(_2\), and 2mM FXa chromogenic substrate (Chromogenix, Bedford, Massachusetts, USA). After 30min incubation, the reaction was stopped with 50% acetic acid and read under 405 nm using an Epoch microplate spectrophotometer (Biotek, Winooski, VT, USA).

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

Immunohistochemistry – A vulvar and a head/neck cancer tissue microarrays (TMAs; US Biomax, inc, Rockville, MD, USA) or A431 xenograft sections were stained, as indicated. A431 tumors were removed 3 weeks after s.c. injection of 2x10\(^6\) cells per immunodeficient (SCID) mouse. Tissues were embedded in paraffin, and sectioned (5\(\mu\)m) (28). Slides were stained with rabbit or mouse anti-vimentin antibody (Abcam), or sheep anti-TF as previously described (6).

Immunofluorescence - Cultured cells were fixed for 10min in 4% paraformaldehyde, permeabilized with 0.2% Triton X and stained overnight at 4\(^{\circ}\)C with sheep anti-human TF antibody (Affinity Biologicals, Ancaster, ON, Canada), rabbit anti-human EGFR (Cell Signaling), mouse anti-human \(\beta\)-catenin (Thermo Scientific, Rockford, IL, USA), rabbit anti-human \(\gamma\)-catenin (Abcam) or FITC-Phalloidin (Sigma) followed by their respective secondary Alexa Fluor antibodies (Life Technologies). Imaris software (Bitplane) was used for confocal image analysis (colocalization).

Nanoparticle tracking analysis (NTA) - EV profiling entailed measurements of size and numbers of particles shed from cell cultured in exosome depleted medium, using the NS500 NTA system (Nanosight, Amesbury, UK) (29).

Membrane labelling with PKH26 and flow cytometry - A431 cells were surface labelled with PKH26 (red fluorescent dye; Sigma) as per manufacturer’s protocol (26). EVs from labelled A431 cells were incubated for 24 h with HUVECs 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 24h, mounted on SEM stubs, sputtered with gold/palladium and imaged using Hitachi FEG SEM model S-4700. For transmission electron microscopy (TEM), EVs were fixed for at least 1h at room temperature in 1% glutaraldehyde in 0.1M pH7.4 cacodylate buffer, post-fixed in 1% osmium tetroxide and embedded in Epon 812. Cells were also stained with anti-TF and
immunogold labelled 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 ± SD. 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 (H&N) origin (Fig. 1AB) 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. 1C), even though these cells harbour 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 alpha (TGFα)(Fig. 2A). 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. 2A and B), with no significant change in cell proliferation (Fig. 2C).

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 signalling effects of EGFR-transformed cells. Stimulation with the SHE/TGFα cocktail, or TGFα alone, triggers EGFR downregulation (Fig. 3A), resulting in largely intracellular EGFR immunostaining, due to ligand-dependent internalization (16)(Fig. 3B). These effects were also observed in the presence of SHE78-7 antibody alone suggesting a link between EGFR and E-cadherin 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. 3B). Curiously, while in the mesenchymal state (SHE/TGFα) both EGFR and TF are mainly localized in the cytoplasm, they are poorly co-localized (Fig. 3C), suggesting their retention in different subcellular compartments. It is also of note that in intact A431 cells TF is highly expressed at intercellular junctions (Fig. 4). Indeed, we observed that when the cells come into contact in sparse cultures their interfaces are especially positive for TF (Fig. 4AB), as is the interior, but not the edges, of multicellular colonies. Junctional localization is reinforced by TF colocalization with β- and γ-catenins (Fig.4CD), 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. Since SHE/TGFα-induced mesenchymal phenotype leads to reduced cell-cell contacts and TF association with the plasma membrane, but 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 TEM analysis, which revealed that intact A431 cells constitutively produce ample EVs ranging between 100 – 200 nm in size, with few exosome-like particles (Fig. 5A). SEM imaging revealed that the mesenchymal phenotype profoundly alters cell surfaces, disrupts intercellular contacts and leads to formation of filopodia-like processes (Fig. 5B). We also measured the number and sizes of EVs emitted under these conditions using nanoparticle tracking analysis (NTA), which documented heterogeneity of EV sizes. In intact cells EVs varied between 20 and 1000 nm and peaked at 150-200 nm, while 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. 5C).
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. 6A and C), but the overall levels of the cell-associated EGFR declined under these conditions (Fig. 3A). In contrast, western blot of purified EV lysates (Fig. 6A) and ELISA analysis of the corresponding cell culture supernatants (Fig. 6B) both indicated a massive increase in EV-associated (and cellular) TF in the presence of the SHE/TGFα cocktail, 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, the SHE/TGFα treatment selectively stimulated TF release with the exosomal fraction of the A431 supernatants (Fig. 6D). Moreover, depletion of exosome-like EVs from this material using ultracentrifugation (100,000g) 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 harbour 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 cocktail (Fig. 7A). Moreover, this change was coupled with the emission of exosome-sized particles containing high levels of TF (Fig. 7BC). 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 human endothelial cells (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 labelled EVs produced by A431 cells, regardless of their epithelial or mesenchymal phenotype (Fig. 8A). 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 cocktail (Fig. 8BC). 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.

DISCUSSION

Our study 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, 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.
E-cadherin regulates tissue factor release in cancer

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 et al have already revealed the existence of several subcellular pools of TF, some of which were associated with the plasma membrane, lipid rafts and Golgi (36; 37). We describe 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 EVs may regulate the strength of the EGFR signalling in mesenchymal cancer cells, in addition to classical processes of intercellular 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 in spite of a growing interest in detection of TF-exposing MPs in plasma of cancer patients, with hopes to develop predictive biomarkers for venous thromboembolism (VTE) 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 harbouring mutant K-ras emit mainly exosomal-size particles, while EGFR-driven A431 cells produce mainly ectosomes while in both cases the SHE/TGFα cocktail selectively enhances production of exosomes containing TF.

Third, while 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 upregulation of matrix metalloproteinase 9 (MMP-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), signalling receptors (Met) and mechanisms obliterating E-cadherin signalling 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, our 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 ought to be considered when EVs are studied as biological effectors and biomarkers in cancer.
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FOOTNOTES

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4The abbreviations used are: EGFR, Epidermal Growth Factor Receptor; EMT, Epithelial-to-Mesenchymal Transition; EVs, Extracellular Vesicles; MVs, Microvesicles; MPs, Microparticles; TF, Tissue Factor; TGFα, Transforming Growth Factor alpha.

FIGURE LEGENDS

FIGURE 1. Mesenchymal cell subpopulations within epithelial cancers. Staining for vimentin expression in sections of human head and neck and vulvar cancer (A, 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 protrusion (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).
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). There is a notable, and expected, decrease in levels of EGFR upon treatment with TGFα alone, or in combination with SHE78-7, while these treatments upregulate 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. TEM image following immunogold staining for TF shows a more pronounced accumulation of TF signal around cell-to-cell junction. C-D. TF localization at the cell-cell junctions co-localizes with junction proteins such as β-catenin (C) and γ-catenin (D).

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 (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 panel A) while cells treated with SHE/TGFα mesenchymal phenotype inducing cocktail 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; flotilin-1 present in EVs is used as a loading control, also compare Fig. 5A). 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).

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 cocktail (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).
FIGURE 8. Extracellular vesicle – mediated transfer of tumor-derived TF to endothelial cells. A. EV-mediated transfer of membrane fluorescence from PKH26-labelled A431 cells to cultured endothelium (HUVEC). Following 24h 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 (approximately 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). Incorporated TF signal is several fold 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 (TF-PCA) that measures activation of the coagulation factor X to Xa was conducted on HUVEC cells treated as in panel B (n=2)(**p<0.005; ***p<0.0005, NS: not significant statistically).

FIGURE 9. Circulating TF and thrombin-antithrombin (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, 1C and (4)]. TF positive cells are found, in the proximity to 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. Thrombin-antithrombin (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).
Figure 2
Figure 3
Figure 4
Figure 5
Figure 6
Figure 7

A

DLD-1

Control

SHE/TGFα

B

TF (ng/mL/10^6 cells)

0 20 40 60

Control

SHE/TGFα

Particles number/cells

C

Particles number/cells

Particles size (nm)

Control

SHE/TGFα

SHE78-7 : - +

TGFα : - +

Figure 7
Figure 8

A

% of fluorescent cells

EVs: - Ctrl MES

** NS

B

HUVECs + A431 EVs

TF

β-actin

WB cells

C

TF-PCA (U/µg protein)

EVs: - Ctrl MES

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