Phenotypic plasticity during the dissemination of circulating tumour cell clusters: A model involving TGFβ1-mediated cluster dissociation, adherence and single-cell extravasation

Jorian D. Hapeman, Caroline S. Carneiro, Aurora M. Nedelcu*

University of New Brunswick, Department of Biology, Fredericton, NB, Canada, E3B 6J4

*Corresponding author; anedelcu@unb.ca

Jorian D. Hapeman 0000-0002-6223-5126

Caroline S. Carneiro 0000-0002-9984-2106

Aurora M. Nedelcu 0000-0002-7517-2419

Author contributions:

Jorian D. Hapeman: designed and performed experiments, analyzed data, wrote the manuscript.

Caroline S. Carneiro: contributed to experiments, data analysis and manuscript preparation.

Aurora M. Nedelcu: contributed to project and experimental design and manuscript preparation.

Acknowledgements: JDH was supported by funds from the New Brunswick Innovation Foundation; support from the University of New Brunswick Research Fund is also acknowledged.

Key words: cancer, circulating tumour cell clusters, metastasis, TGF-β1, extravasation, phenotypic plasticity

 doi:10.20944/preprints202210.0046.v1

ABSTRACT

Metastasis – the ability of cancer cells to disperse and colonize distant locations in the body, is responsible for the majority of cancer-related deaths. While in the vasculature, tumour cells are referred to as circulating tumour cells (CTCs) and can manifest either as single cells or clusters of cells, with the latter being the most aggressive. Despite their significant role in the metastatic process, the mechanisms through which CTC clusters extravasate and disseminate remain largely unknown. Notably, CTC clusters have been found to contain platelets, which are known to secrete many factors, including Transforming Growth Factor Beta 1 (TGF-β1) – a signaling molecule that has been widely implicated in many aspects of cancer, including the extravasation of single CTCs. To address whether the interaction between platelets and CTC clusters might also facilitate the extravasation of CTC clusters, we evaluated the effect of exogenous TGF-β1 on an experimentally evolved lung cancer cell line that grows as cell clusters that we previously developed and used to investigate the biology of CTC clusters. We found that exogenous TGF-β1 induces the dissociation of clusters and cell adherence. Furthermore, once adhered, cells release their own TGF-β1 and are able to migrate and invade in the absence of exogenous TGF-β1. Based on these findings we propose a model that involves both paracrine and autocrine TGFβ1-mediated phenotypic plasticity resulting in the acquisition of traits that enable the extravasation of CTC clusters as single cells.

 doi:10.20944/preprints202210.0046.v1

INTRODUCTION

Metastasis involves a complex series of events through which cancer cells break off the primary tumour, travel through the circulatory system, and ultimately disseminate and colonize new tissues [1]. While in the vasculature, tumour cells are referred to as circulating tumour cells (CTCs) and can manifest either as single cells or as groups of cells known as CTC clusters [2]. Although CTC clusters only make up 2-5% of a typical CTC population [3], several studies suggest that they have a much higher metastatic potential compared to single CTCs [3–6], and thus have important clinical significance.

CTC clusters typically consist of 2-100 cells organized in grape-like morphologies [7]. They often display significant phenotypic heterogeneity, including cells expressing various levels of epithelial and mesenchymal markers [8]. Clusters of tumour cells have been isolated from the blood of patients with a wide range of malignancies such as cancer of the brain [9], pancreas [10], breast [11], colon [12], kidney [13], liver [14], lung [5] and skin [15]. Their presence is generally associated with bad prognosis [4] and several factors are thought to contribute to the increased metastatic potential of CTC clusters compared to single CTCs. For instance, while in circulation, CTC clusters might have increased resistance to shear stress [16], programmed cell death, anoikis [17], immune attacks [18] and drug therapies [19]. Furthermore, it has been shown that CTC clusters feature DNA methylation patterns that promote the expression of stem-cell related genes [20], and that the formation of clusters promotes a metabolic switch that increases the efficiency of metastasis [21].

Despite their significant role in the metastatic process, the mechanisms through which CTC clusters disperse remain largely unknown. The processes that are used to describe the dispersal of single CTCs often lack explanatory power when applied to cell clusters. For example, the initial dissociation of single cells from the primary tumour is often attributed to the conversion of epithelial cells into migratory mesenchymal-like cells during the epithelial-mesenchymal transition (EMT) [2]. Due to the hybrid phenotype exhibited by CTC clusters (i.e., a mixture of cells with epithelial and mesenchymal traits), the role that EMT plays in their initial dissociation if at all, is not clear. Divergent lines of evidence argue that CTC clusters arise either via the aggregation of single cells in the vasculature or just outside of blood vessels [22] or by

 doi:10.20944/preprints202210.0046.v1

the cohesive shedding of clusters from the tumour followed by collective movement into the bloodstream [3, 6], which can be facilitated by hypoxic tumour microenvironments [23].

Even less is known about the mechanisms through which CTC clusters exit the vasculature and disseminate to colonize new tissues. When CTCs enter and exit the bloodstream, they must traverse the endothelial wall in what is known as transendothelial migration [24]. Due to local angiogenesis, the blood vessels that populate a tumour generally have weak cell-cell junctions through which cancer cells, possibly including CTC clusters, can enter the vasculature [24]. This principle cannot necessarily be applied when cells exit the vasculature to colonize new tissues as the blood vessels in these tissues are more secure.

Once in capillaries, single CTCs form stable attachments with the endothelial wall [25]. Some studies suggested that single tumour cells will then cross the endothelial barrier in a similar manner to leukocytes, by inserting between endothelial cells and inducing a temporary weakening of the cell-cell junctions (i.e., diapedesis) [26]. Contrarily, other reports have shown single CTCs to induce either necroptosis in surrounding endothelial cells [27] or vascular remodelling through a process known as angiopellosis [28], both of which create openings in the vessel wall allowing cells to leave the vasculature. It is not clear if/how similar processes can explain the extravasation of CTC clusters. It has been suggested that due to their size, CTC clusters are arrested in small capillaries and give rise to metastases through proliferation at their site of arrest [29]. Alternatively, multicellular aggregates caught in capillaries have been shown to induce angiopellosis allowing collective extravasation and movement into new tissues [30]. Other studies point to the possibility that CTC clusters rearrange into a single-cell file and can move through thin capillaries [31]. However, none of these possibilities have been extensively investigated, and if/how CTC clusters can exit the vasculature and develop into distant metastases, is still not well understood.

CTC clusters in the bloodstream may contain other cell types such as platelets, endothelial cells, fibroblasts, leukocytes and pericytes [32]. The association of CTC clusters with such cells provides ample opportunity for crosstalk. Of interest is the close association between platelets and CTC clusters because of the platelets' ability to release TGF-β1 (Transforming Growth Factor Beta 1) – a signalling molecule that, in addition to its complex roles in normal activities, also plays a major role in many aspects of human cancers [33]. Platelet-derived TGF-

 doi:10.20944/preprints202210.0046.v1

β1 has a regular function in wound healing [34]. However, it has been shown that single CTCs can activate platelets in mouse models, causing release of TGF-β1, which in turn enhances the metastatic tumour seeding abilities of single CTCs by promoting migratory and invasive properties [35].

Notably, a recent study found that 80% of the CTC clusters isolated from patients with pancreatic cancer (especially in those with rapid progression in metastasis) were cloaked with platelets [10]. Based on the observed *in vivo* association between CTC clusters and platelets, we hypothesized that platelet-derived TGF-β1 might also have an impact on the extravasation and dissemination of CTC clusters into new tissues. To address this possibility, we used an *in vitro* model-system that we developed and previously used to investigate the biology of CTC clusters [36, 37]. Specifically, we used an experimentally evolved non-small cell lung cancer line that grows as clusters in suspension and evaluated the effect of exogenous TGF-β1 (at a physiologically relevant concentration) on the adherence, migration, and invasion of cell clusters. Based on our data we propose a model that involves phenotypic plasticity through both paracrine and autocrine TGF-β1 signaling resulting in the acquisition of traits required for the extravasation and dissemination of CTC clusters.

MATERIALS AND METHODS

Cell line and culture conditions

We used an experimentally evolved cancer cell line derived from a non-small cell lung cancer cell line obtained from the American Type Culture Collection – ATCC (Manassas, VA, USA). This original cell line (NCI-H2122) was established from the pleural effusion of a 46-year-old female with stage-4 adenocarcinoma and grows as a mixture of two distinct phenotypes: clusters of cells growing in suspension and adherent cells that grow as a monolayer. By selectively passaging only the adherent or suspension cell populations, two cell lines were experimentally evolved: one that grows as cell clusters in suspension (referred to as the H2122 Suspension-Selected or SS line) and one that grows as adherent cells (referred to as the H2122 Adherent-Selected or AS line) (Jong et al., 2019). The SS line has been previously shown to be a viable model-system for CTC clusters [36]. The SS cells were grown at 37°C and 5% CO_2 in RPMI-

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 5 October 2022 doi:10.20944/preprints202210.0046.v1

1640 media (MP Biomedicals) supplemented with 10% FBS and 1% Penicillin/Streptomycin mix. The concentrations of glucose and glutamine in the media were adjusted to 5 mM and 0.5 mM respectively. Cells were passaged every 2-3 days at a 1:3, 1:4 or 1:5 ratio depending on the desired cell density.

General Experimental Set-up

Cultures were seeded (in triplicates) in tissue-treated 12-well plates (Sarstedt) at a density of $1x10^5$ cells/ml for all experiments (except for Transwell assays – see below, and the conditioned media experiments in which cells were seeded at $5x10^4$ cells/ml – see results). Several independent trials were conducted for each experiment.

Reagents

Human recombinant TGF-β1 (R&D Systems - 7754-BH-005) was reconstituted in 4 mM HCl at 10 mg/ml and was added directly to the culture medium at 10 ng/ml. A TGF-β receptor 1 (TGFβRI) inhibitor (EMD Millipore - 616464) was used at a concentration of 10 μM.

Cell Counting and Viability Assessment

Cells were stained with Syto-9 (3.34 mM in DMSO, Invitrogen) and Propidium Iodide (20 mM in DMSO, Invitrogen) at a final concentration of 10 μM and 60 μM, respectively. Numbers of live and dead cells were assessed (4 technical replicates for each biological replicate) using the Countess™ II FL Automated Cell Counter (Invitrogen). The suspension and adherent cell populations for each biological replicate were counted separately.

Conditioned Media Collection

To collect conditioned media from TGF-β1-treated cells, naïve SS cells were placed in a 12-well plate at $1x10^5$ cells/ml and treated with TGF-β1 for 48 hours. The media was removed, the adherent cells were washed with PBS, and fresh media containing 1% FBS was added to the cells. After 24 hours, this conditioned medium was collected, centrifuged at 1000xg for 10 min, and the supernatant was filter sterilized before being used in experiments at 1:1 ratio with fresh medium.

 doi:10.20944/preprints202210.0046.v1

Transwell Assays

Cell migration and invasion were assessed using the Transwell assay [38, 39]. Cells were suspended in FBS-deficient RPMI media at $1x10^6$ cells/ml, and aliquots of 100 µl of this suspension were added to 6.5 mm Transwell inserts (Corning). For invasion, 100 µl of Matrigel (Corning - 354277) (1.5 mg/ml) was first added to the inserts and allowed to solidify for 8 hours.

The inserts were each placed into individual wells of 24-well plates containing RPMI-1640 media with FBS, which was used as chemoattractant. Cells were then allowed to migrate/invade through the membrane towards the chemoattractant media for 48 or 72 hours and the cells trapped on the membrane were fixed and stained (see below). Stained membranes were then visualized at 100x magnification, and 10 random fields of view were photographed. The number of cells for each photo was assessed using the ImageJ cell counter tool [40] and the average number of cells migrated was calculated.

Staining Adherent Cells and Transwell Inserts

TGF-β1-induced adherent cells and cells attached to Transwell inserts were initially fixed to their substrate by coating them with 70% ethanol for 5 minutes after which the ethanol was removed, and the cells were dried for 15 minutes. They were then stained with a 0.2% crystal violet (in 20% ethanol) solution for 10 minutes, washed with PBS five times and imaged.

Microscopy and Image Processing

Photos were taken with an OMAX USB digital microscope camera. Background noise was removed using the Calculator plus tool in FIJI [40] the smear tool available in the GIMP 2.10 software.

Statistical Analyses

The mean of each group was calculated from the three replicates and expressed as mean ± SE. Welch's two sample t-test was used to compare the mean of two groups and assess statistical significance, while ANOVA was used for 3 or more groups. The difference between groups was considered statistically significant at $p < 0.05$.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 5 October 2022 doi:10.20944/preprints202210.0046.v1

RESULTS

TGF-β1 induces the dissociation and transition of H2122 SS clusters to an adherent mesenchymal-like phenotype

To address whether TGF-β1 can induce the adherence and migration of the H2122 SS cell line, cells were exposed to TGF-β1 (10 ng/ml) for 48 hours, and changes in their phenotypic state and morphology were recorded. In its native state, the H2122 SS cell line grows as clusters of cells in suspension (Fig. 1a-c). However, following exposure to TGF-β1, most clusters dissociated into single cells that transitioned to an adherent phenotype which often featured cytoplasmic extensions characteristic of mesenchymal cells (Fig. 1d-f).

To quantify this change in phenotypic state, the adherent and suspension cells were counted separately and the proportion of the two cell populations was determined. We found that treatment with TGF-β1 resulted in a significant increase in the proportion of adherent cells (Fig. 1g). Specifically, compared to control cultures, TGF-β1-treated cultures displayed a significantly higher proportion of adherent cells (70.60% vs. 0.81%; $p < 0.05$), indicating that most cells transitioned to an adherent state in the presence of TGF-β1. Interestingly, the treated cultures also showed an overall lower number of live cells (with no change in the number of dead cells – Fig. 1h), indicating that TGF-β1 has an inhibitory effect on cell proliferation.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 5 October 2022 doi:10.20944/preprints202210.0046.v1

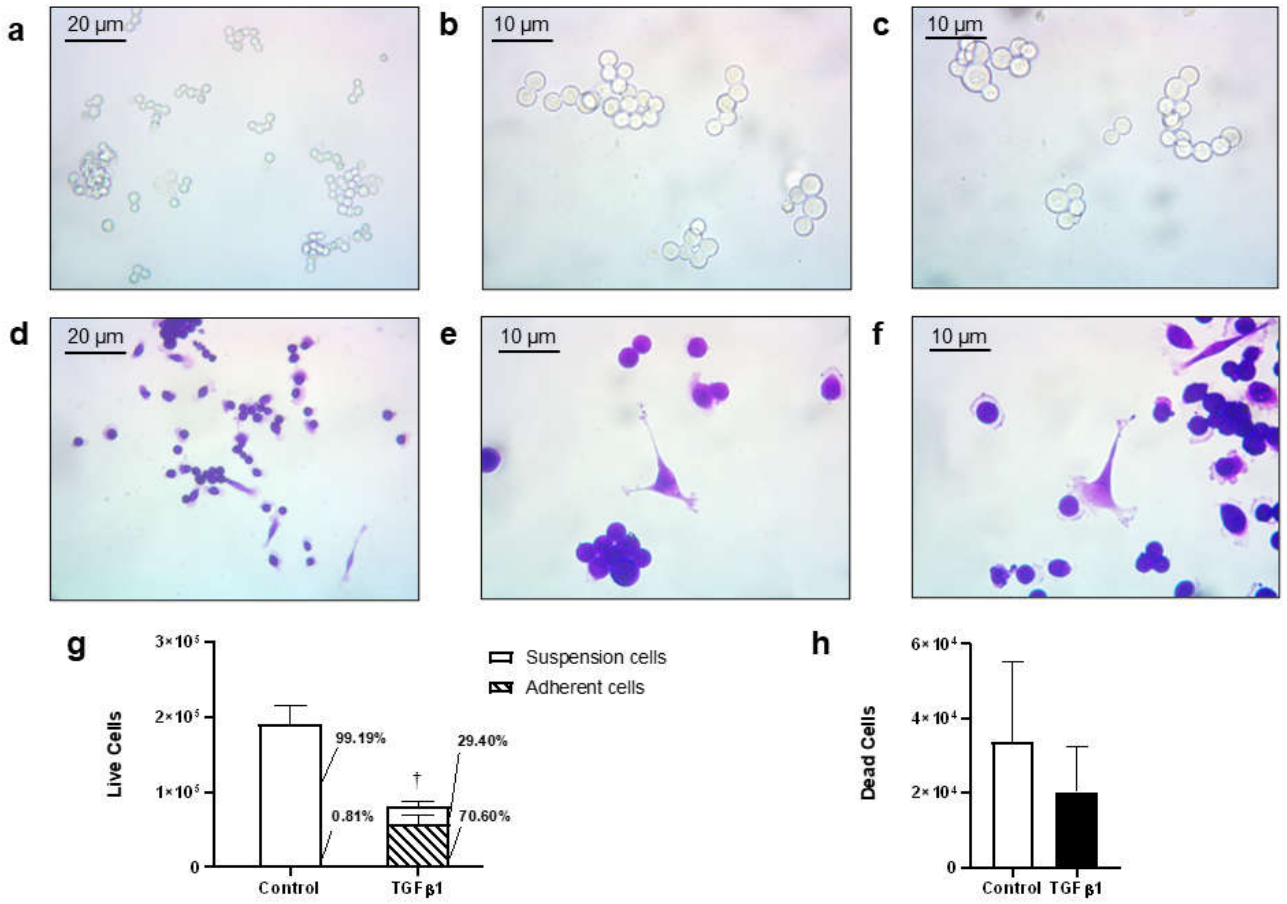

Fig. 1. The effect of TGF-β1 on the phenotype of H2122 SS cells. Micrographs comparing the morphology of H2122 SS cells before **(a-c)** and after **(d-f)** treatment (cells were stained with 0.2% crystal violet); and the number and proportion of adherent and suspension live cells **(g)** and the number of dead cells **(h)** in the H2122 SS cell line treated with 10 ng/ml TGF-β1 and untreated (Control) for 48 hours. Error bars represent standard error, n = 3. Cross (†) indicates significant difference in the proportion of adherent cells between the treatment and the control groups ($p < 0.001$; Welch's two sample t-test).

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 5 October 2022 doi:10.20944/preprints202210.0046.v1

TGF-β1 induces the migration of H2122 SS cells

To determine whether the morphology of TGF-β1-treated cells reflects an ability to migrate, we used the Transwell assay and assessed the migration of H2122 SS in the presence and absence of TGF-β1, at 24 and 48 hours (Fig. 2). Compared to control cultures, significantly more cells migrated in the cultures treated with TGF-β1 (60 cells vs 0.2 cells per field of view at 24 hours, $p < 0.05$; and 174 cells vs 0.4 cells per field of view at 48 hours, $p<0.05$). Overall, these data indicate that TGF-β1 can also induce the migration of the H2122 SS cells that adhered.

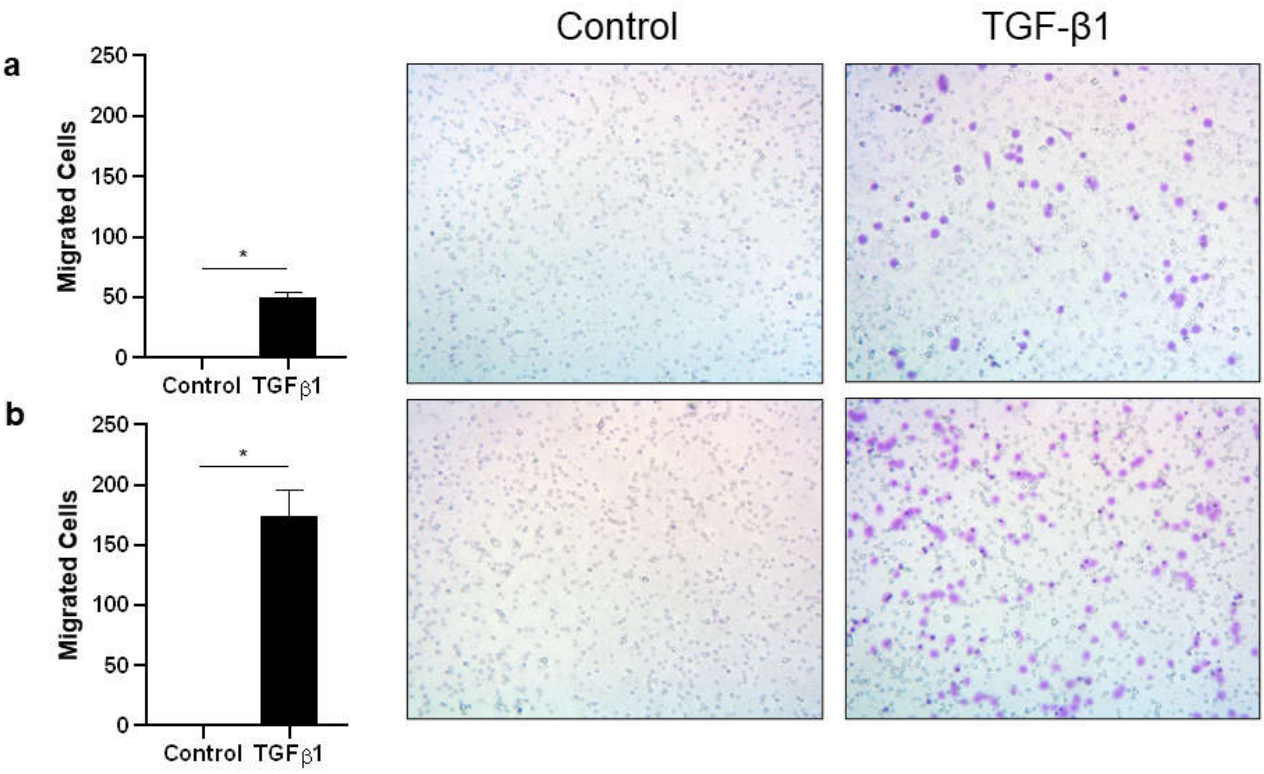

Fig. 2. The effect of TGF-β1 on the migration of H2122 SS cells. The number of H2122 SS cells that migrated through a Transwell insert after 24 hours **(a)** and 48 hours **(b)** post-seeding. Pictures are sample fields of view showing cells that have migrated and adhered to the underside of the insert (cells were stained with 0.2% crystal violet). Error bars represent standard error, n = 3; asterisks (*) indicate $p < 0.05$ (Welch's two sample t-test).

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 5 October 2022 doi:10.20944/preprints202210.0046.v1

TGF-β1-treated H2122 SS cells have increased invasive abilities

We also used the Transwell assay to evaluate the invasive abilities of H2122 SS cells treated with TGF-β1, after 48 and 72 hours (Fig. 3). Compared to the control cultures, significantly more cells were able to break down the Matrigel matrix and migrate in the presence of TGF-β1 (176 cells vs 0.5 cells per field of view at 48 hours, $p < 0.05$; 359 cells vs 1 cell per field of view at 72 hours, $p<0.05$) indicating that TGF-β1 increases invasive abilities in H2122 SS cells.

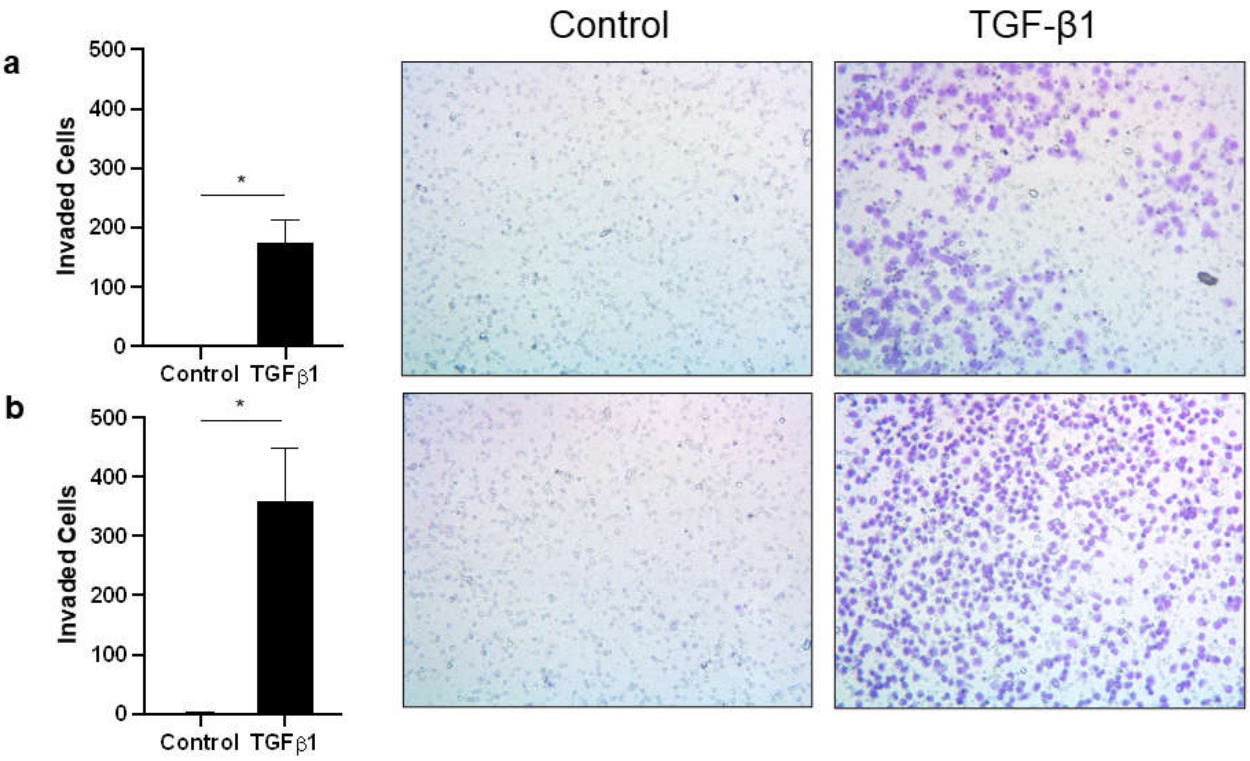

Fig. 3. The effect of TGF-β1 on the invasiveness of H2122 SS cells. The number of invaded H2122 SS cells after 48 hours **(a)** and 72 hours **(b)**. Pictures are sample fields of view showing cells that have migrated through a layer of Matrigel and adhered to the underside of the Transwell insert (cells were stained with 0.2% crystal violet). Error bars represent standard error, n = 3. Asterisks (*) indicate $p < 0.05$ (Welch's two sample t-test).

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 5 October 2022 doi:10.20944/preprints202210.0046.v1

The TGF-β1-induced adherent phenotype is stable in the absence of exogenous TGF-β1

To evaluate whether the TGF-β1-induced adherent phenotype is stable in the absence of exogenous TGF-β1, we first treated H2122 SS cells with 10 ng/ml TGF-β1 for 48 hours to induce the adherent phenotype. We then removed the cells that were still in suspension and assessed the number and proportion of adherent and suspension cells. Next, we washed the adherent cells with PBS and maintained them for another 7 days in RPMI media without TGF-β1 (with media being refreshed every 2 days). After 7 days in the absence of TGF-β1, we recorded the numbers and proportions of adherent and suspended cells and compared them with those in the population initially treated with TGF-β1 for 48-hours (Fig. 4). Interestingly, compared to the initial cultures treated with TGF-β, the cultures maintained in media without exogenous TGF-β1 for 7 days did not show a statistically significant difference in the proportion of adherent cells (88.73% vs 92.59% adherent cells), suggesting that most of the TGF-β1-induced adherent cells do not lose their adherent phenotype in the absence of exogenous TGF-β1. Furthermore, there was no additional cell proliferation after the removal of the TGF-β1.

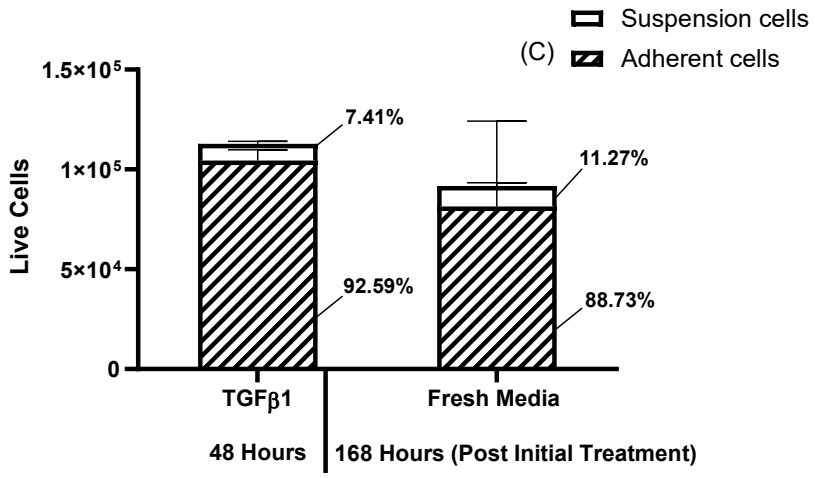

Fig. 4. The stability of the TGF-β1-induced adherent phenotype in the absence of exogenous TGF-β1. The number and proportion of live adherent and suspension H2122 SS cells in cultures treated with TGF-β1 for 48 hours and cultures grown in the absence of TGF-β1 for an additional 7 days (168 hours) after their initial treatment. Error bars represent standard error, n = 3. No significant difference in the proportion of adherent cells in either population (Welch's two sample t-test).

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 5 October 2022 doi:10.20944/preprints202210.0046.v1

TGF-β1-induced adherent cells release TGF-β1

To test if the stability of the TGF-β1-induced phenotype might be due to the ability of TGF-β1-induced adherent cells to release their own TGF-β1, we collected conditioned media from TGF-β1-induced adherent cells and added it to naïve H2122 SS cells in the presence or absence of a TGFβ1 receptor 1 (TGFβRI) inhibitor. After 48 hours, the suspension and adherent cell populations were counted. The addition of conditioned medium (1:1 ratio with fresh medium) resulted in a significant increase in the proportion of adherent cells compared to the control (4.64% in control vs 71.30% in treatment, $p < 0.05$) (Fig. 5). Furthermore, the addition of the TGFβRI inhibitor (10 μM) significantly decreased the proportion of adherent cells (10.64% in the presence of inhibitor vs 71.30% without the inhibitor, $p < 0.05$) and restored cell proliferation. Collectively, the data suggest that the ability of the conditioned medium to induce the transition of the naïve SS cells to an adherent phenotype is due to the secretion of TGF-β1 by the adherent cells.

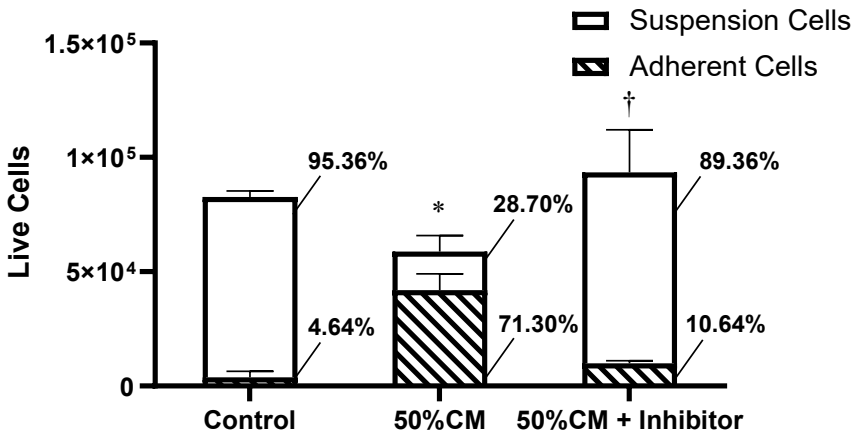

Fig. 5. The effect of the conditioned medium from TGF-β1-induced adherent cells on the phenotype of naïve H2122 SS cells. Number and proportion of live adherent and suspension H2122 SS cells in cultures treated with 50% conditioned media (CM) from TGF-β1-induced adherent H2122 SS cells in the absence and presence of 10 μM TGFβRI inhibitor, relative to control. Error bars represent standard error, n = 3. Asterisk (*) denotes a significant difference in the proportion of adherent cells in the total population of the control compared to the 50% conditioned media group and cross (†) indicates significant differences between the 50% CM + inhibitor group compared to the 50% CM group ($p < 0.001$).

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 5 October 2022 doi:10.20944/preprints202210.0046.v1

Autocrine TGF-β1 signaling is not required for the stability of the adherent phenotype

To address whether the TGF-β1 released by the adherent cells is required to maintain their adherent phenotype, naïve H2122 SS cells were initially treated with 10 ng/ml TGF-β1 for 48 hours to induce the adherence. Then, after the adherent cells were washed with PBS, RPMI media alone or containing either 10 ng/ml TGF-β1 or a TGFβRI inhibitor was added to the adherent cells. After 48 hours, the numbers of adherent and suspension cells were assessed and compared. The proportion of adherent cells did not change significantly between the three cultures (Fig. 6). Specifically, in fresh media alone, 85.32% of the cells were adherent; however, the cultures grown in medium with exogenous TGF-β1 or the TGF-β1 inhibitor still showed a high proportion of adherent cells (84.96% and 83.89%, respectively) (Fig. 6). Overall, these data suggest that although H2122 SS cells treated with TGF-β1 release their own TGF-β1, autocrine TGF-β1 signaling is not required to maintain the adherent phenotype.

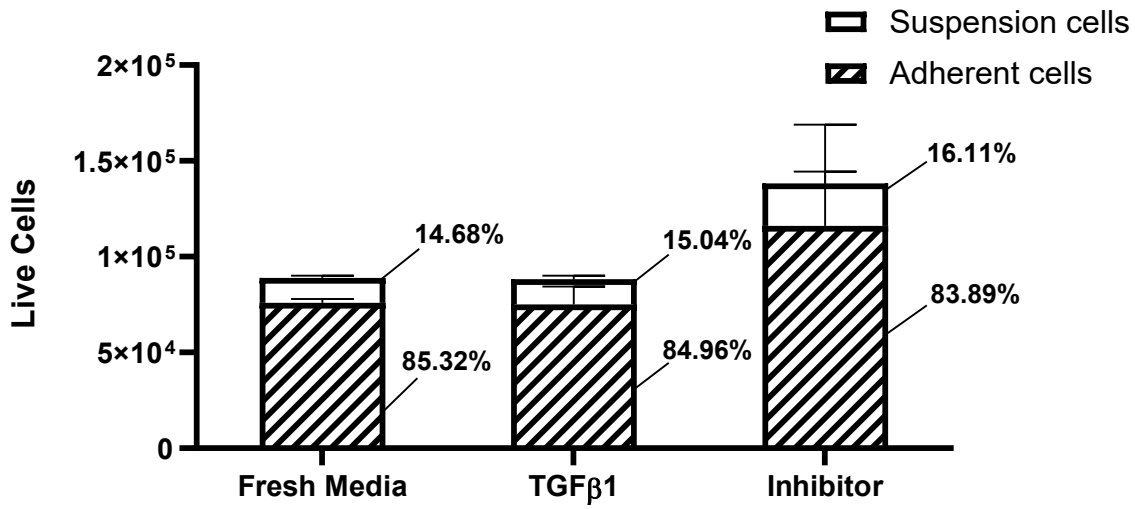

Fig. 6. The stability of the TGF-β1-induced adherent phenotype in the absence of autocrine TGF-β1 signaling. Number and proportion of live adherent and suspension H2122 SS cells in TGF-β1-induced adherent cultures following 48 hours in fresh media alone or fresh media with either TGF-β1 or a TGFβRI inhibitor. Error bars represent standard error, n = 3. No significant difference in the proportion of adherent cells in either population (One way ANOVA followed by post-hoc Tukey test).

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 5 October 2022 doi:10.20944/preprints202210.0046.v1

Autocrine TGF-β1 signaling allows cells to migrate in the absence of exogenous TGF-β1

To further explore the role of the TGF-β1 secreted by the TGF-β1-induced adherent cells, we addressed whether the released TGF-β1 is required for the migration of the adherent cells in the absence of exogenous TGF-β1. H2122 SS cells were seeded in Transwell inserts in the presence of TGF-β1 to induce the adherent phenotype within the insert, and cell migration was evaluated after 24 hours. Then, after the adherent cells on the top side of the inserts were washed with PBS, either RPMI media, RPMI media with TGF-β1, or RPMI media with a TGFβRI inhibitor was added to the inserts. The inserts were subsequently placed in a new well with RPMI media containing FBS as a chemoattractant, and the adherent cells were allowed to migrate for another 24 hours, before the migration abilities of each group were assessed (Fig. 7). Cells in RPMI media without exogenous TGF-β1 migrated as much as cells in the presence of TGF-β1, presumably due to the secretion of TGF-β1 (autocrine signaling). Furthermore, the addition of a TGFβRI inhibitor reduced the number of cells that migrated, confirming that autocrine TGF-β1 signaling plays a role in maintaining a migratory state in the absence of exogenous TGF-β1.

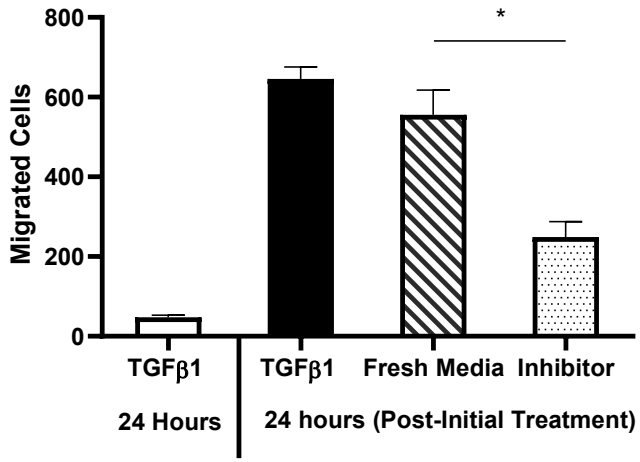

Fig. 7. The role of autocrine TGF-β1 signaling in the migration of TGF-β1-induced adherent H2122 cells in the absence of exogenous TGF-β1. Number of TGF-β1-induced adherent H2122 SS cells that migrated to the underside of the insert (per field of view), after the initial treatment with TGF-β1 for 24 hours, as well as after an additional 24 hours in either the presence of 10 ng/ml TGF-β1, the absence of exogenous TGF-β1, or in the presence of 10 μM TGFβRI inhibitor. Error bars represent standard error, n = 3. Asterisk (*) indicates a significant difference between migrated cells in the absence of exogenous TGF-β1 and the TGF-β1 inhibitor ($p < 0.05$).

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 5 October 2022 doi:10.20944/preprints202210.0046.v1

DISCUSSION

The goal of this study was to explore the possibility that platelet-derived TGF-β1 plays a role in the extravasation and dissemination of CTC clusters. This scenario is consistent with the fact that RNA sequencing revealed strong levels of TGF-β signatures in human breast CTC clusters coated with platelets [41]. To mimic CTC clusters, we used an experimentally evolved lung cancer cell line that grows as clusters in suspension and exhibits similarities with real CTC clusters in terms of size, cell-cell connections, and the expression of mesenchymal and epithelial markers [36]. To simulate the presence of platelets, we used exogenous TGF-β1 at a concentration of 10 ng/ml. Reported TGF-β1 levels in human plasma vary greatly (from 0.1 ng/ml to more than 25 ng/ml), with ca. 4 ± 2 ng/ml being the most common [42, 43]. However, the presence of platelets can greatly increase these levels as platelets contain between 2,500-4,000 TGF-β1 molecules/cell and contribute up to 45 ng TGF-β1 per 1 ml of blood [42, 44]. Furthermore, TGF-β1 levels might increase during cancer progression, as elevated TGF-β1 levels have been reported in patients with advanced breast cancer [45].

Using our *in vitro* model-system, we found that TGF-β1 can induce a switch from a suspension cell-cluster phenotype to an adherent single-cell state. Importantly, once adhered, these cells do not require the presence of TGF-β1 to maintain their adherent state. In addition, while the cell clusters have almost no migration and invasion potential, the TGF-β1-induced adherent cells have greatly enhanced abilities to migrate and invade relative to the untreated cell clusters. Furthermore, once adherent, the cells produce and release their own TGF-β1 that can induce a migratory behaviour in the absence of exogenous TGF-β1. Based on these findings, below we propose a step-by-step model for the extravasation and dissemination of CTC clusters in which platelet-derived TGF-β1 causes the dissociation of clusters into individual adherent cells that can migrate and invade even in the absence of paracrine TGF-β1 signaling, and can ultimately act as the seeds for new tumours (Figure 8).

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 5 October 2022 doi:10.20944/preprints202210.0046.v1

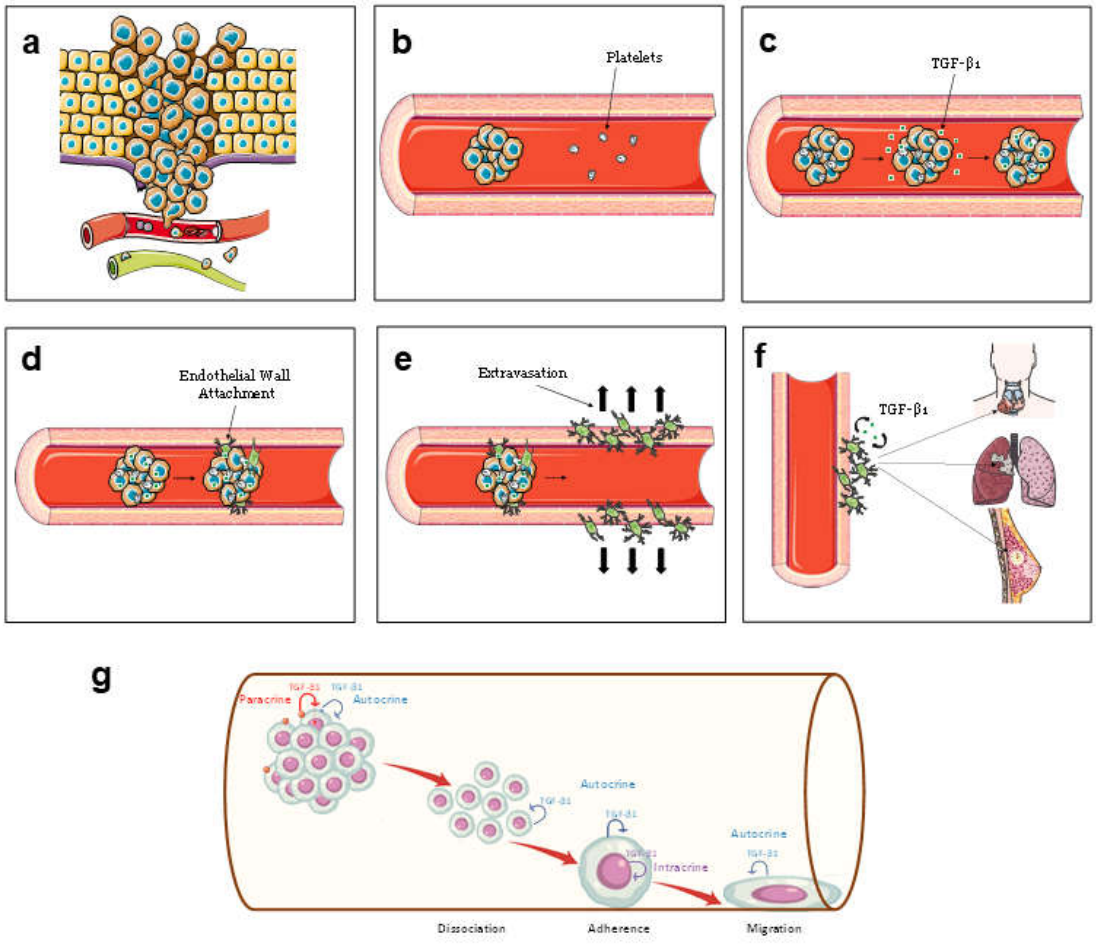

Fig. 8. Proposed mechanism for the extravasation and dissemination of CTC clusters involving phenotypic plasticity mediated by para- and autocrine TGF-β1 signaling. Envisioned steps during the dispersal of cancer cells from a primary tumour to new locations in the body. **(a)** A cluster of cells initially breaks off the primary tumour and enters the vasculature as a CTC cluster. **(b)** In the bloodstream, CTC clusters encounter platelets that become embedded into the cluster. **(c)** Platelets release TGF-β1 that interacts with cancer cells (paracrine signaling) in the CTC cluster. **(d)** In response to the TGF-β1released from platelets, individual CTCs dissociate from the cluster and adhere to the endothelial wall. **(e)** These individual adherent cells migrate and pass through the endothelial wall and start invading the adjacent tissues. **(f)** Once in the tissue, cell secrete their own TGF-β1 (autocrine signalling), which allows them to migrate to locations where they can resume proliferation and form secondary tumours. **(g)** Paracrine and autocrine (and possibly, intracrine) TGF-β1 signaling during the extravasation

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 5 October 2022 doi:10.20944/preprints202210.0046.v1

of CTC clusters. Diagram created with free online Servier Medical Art at www.servier.com and Vecteezy.com.

(i) Platelet TGF-β1-induced CTC cluster dissociation and adherence

Based on our finding that upon treatment with TGF-β1, the H2122 SS cell clusters dissociated and developed an adherent phenotype, we suggest that in the bloodstream, crosstalk between platelets and CTC clusters may cause a TGF-β1-mediated transition from a clustered phenotype to individual adherent cells (Fig. 8a, d-e). Since TGF-β1 is known to be involved in regulating EMT by downregulating the expression of cell-cell adhesion proteins such as E-cadherin [46], it is possible that the downregulation of such proteins may also be responsible for the dissociation of H2122 SS cell clusters into single cells after treatment with TGF-β1.

Adhesion to the endothelial wall is an important step in CTCs dissemination; in fact, it was suggested that such cell-cell interactions, and not mechanical entrapment, are responsible for the arrest of CTCs in microvessels [47]. Nevertheless, a role for TGF-β1 in inducing adherence to a substrate is relatively less known. However, in certain cell lines, TGF-β1 has been described as being involved in the expression of proteins that promote adhesion to the extracellular matrix during migration [48, 49]. Furthermore, a similar response to what we observed has been reported in a gastric cancer cell line that grows predominantly as clusters in suspension; in this case, the TGF-β1-induced adherence (at as low as 1 ng/ml) was found to involve the expression of integrin alpha-3 subunit [50]. Given that H2122 SS is derived from a cell line that grows as a mixture of adherent and suspension cells, it is possible that this evolved line still maintains the ability to express an adherent phenotype. Overall, this predisposition towards an adherent phenotype is not unlike CTC clusters *in vivo* as CTC clusters isolated from the bloodstream and cultured in treated tissue flasks often transition to an adherent phenotype [51].

Although the attached cells might remain in contact with platelets, we found that the adherent phenotype can be stable in the absence of any source (exogenous or endogenous) of TGF-β1. One possibility is that the stability of the phenotype is the result of trans-generational epigenetic changes. In fact, it has been suggested that TGF-β1-induced EMT can involve epigenetic changes. For example, TGF-β1 treatment of certain lung cancer cell lines results in EMT being induced in part by demethylation of *Slug*, a gene coding for a prominent EMT-inducing transcription factor [46]. Alternatively, there is evidence that cell-substratum adhesion in some

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 5 October 2022 doi:10.20944/preprints202210.0046.v1

cell lines is mediated by intracellular feedback regulation stimulated by integrin-substrate interaction [52]. Therefore, the adherent phenotype's stability even in the absence of TGF-β1 may be the result of positive feedback loops responding to the initial attachment to the substrate. Another possibility involves intracrine TGF-β1 signaling, whereby latent TGF-β1 is activated intracellularly [53] and through a "private" loop [54] active TGF-β1 maintain its own expression and the expression of the pathways it regulates.

(ii) TGF-β1-induced extravasation

Based on the TGF-β1-induced migratory and invasive abilities seen *in vitro, w*e suggest that, in response to TGF-β1 (involving either para-or autocrine signaling; discussed below), the cells that adhered to the endothelial wall can migrate/invade as single cells through the endothelium into new tissues (Fig. 8e). TGF-β1 is known to induce EMT and has been shown to increase migration and invasion in several adherent cancer cell lines such as breast [55], lung [46] and colon [56], among others. Interestingly, a link between TGF-β1 and the switch from cohesive to single cell motility has already been reported. Specifically, using a breast cancer cell line that grows as distinct adherent colonies when seeded at low density, Giampieri et al. showed that treatment with TGF-β1 caused the cells to dissociate and migrate as single cells both *in vitro* and *in vivo* [57].

The dissociation of clusters into single cells would allow for easier passage through the endothelial wall and prevent the need for very large openings in the blood vessel. In the single cell state, cells could extravasate using any of the known mechanisms proposed for single CTCs, such as through diapedesis [26] by inducing necroptosis [27], or by causing vascular remodelling through angiopellosis [28]. Overall, significant remodelling of the vasculature would not be necessary because there is no need for the entire cluster to move through the opening at once. If CTC clusters dissociate at the site of extravasation, tumour cells could still benefit from travelling through the vasculature in a clustered state, but also exploit the benefits of extravasating as single cells through the endothelial wall. However, our model does not exclude the possibility that once adhered to the endothelial wall, cells could aggregate through homotypic interactions [47] and extravasate as multicellular clusters, through angiopellosis [30]. Alternatively, cells can aggregate following extravasation, in the perivascular space [22].

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 5 October 2022 doi:10.20944/preprints202210.0046.v1

(iii) Autocrine TGF-β1 signalling-mediated dissemination

The initial steps in the migration and invasion of single cells during the extravasation step might be mediated by platelet-derived TGF-β1 if the association with platelets is maintained after adherence. However, once cells leave the bloodstream, the limited availability of TGF-β1 in tissues (e.g., [58]) can restrict their migration/invasion capabilities (although some tissues – especially bones, can contain high levels of TGF-β1; [59]. Based on the fact that TGF-β1-induced adherent cells secrete their own TGF-β1 (which allows them to migrate in response to a chemoattractant in the absence of exogenous TGF-β1), we suggest that a similar TGF-β1 autocrine signalling ensures that once CTCs leave the bloodstream (and the exogenous sources of TGF-β1) they can maintain migratory/invasive capabilities until reaching appropriate locations for proliferation (Figure 8f).

This autocrine loop can be initiated by the platelet-derived TGF-β1 (Figure 8g), as TGF-β1 is known to be able to induce its own expression [60, 61] and TGF-β signaling can be regulated by various feedback loops that provide versatile and context-dependent functions [62]. For instance, in certain types of breast cancer, invasiveness and tumour progression are promoted by autocrine TGF-β signalling [63, 64]. Similarly, the mesenchymal state of MCF10A (a non-tumorigenic human epithelial cell line) is maintained through an autocrine mechanism involving TGF-β, Snail1 and miR-200 [65].

Interestingly, we also found that adherent cells in the presence of either exogenous or endogenous TGF-β1 have a low proliferation potential. This is consistent with known anti-proliferative effects of TGF-β1 [66] and the proliferation-migration trade-off thought to characterize cancer cells [67]. Obviously, to develop into new tumours, cells need to proliferate, which will likely require to discontinue the release of, and/or response to, TGF-β1. This could be achieved in response to tissue microenvironmental conditions and represents a testament to the "seed and soil" hypothesis first proposed by Paget in 1889 [68].

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 5 October 2022 doi:10.20944/preprints202210.0046.v1

CONCLUSION

Despite increasing research efforts, the five-year survival rate for most patients with metastatic cancer remains distressingly low [69]. Our inability to improve survival rates underscores the need for a deeper understanding of the metastatic process as well as for developing strategies to suppress it. The significance of CTC clusters in metastatic disease is becoming increasingly recognized and elucidating the mechanisms involved in their extravasation and dissemination has the potential to yield new therapeutic targets.

Our study provides *in vitro* evidence for a new model for the extravasation and dissemination of CTC clusters involving phenotypic plasticity mediated by crosstalk between platelets and cancer cells and a combination of paracrine and autocrine (and possibly intracrine) TGF-β1 signaling (Figure 8g). Current models propose that CTC clusters are arrested in small capillaries and either proliferate at their site of arrest [29], remodel the surrounding vasculature and exit as multicellular clusters [30], or re-organize themselves in a single cell file that can leave the capillaries [31]. These are all viable possibilities, and we suggest that given the complexity and diversity of cancer, multiple mechanisms might be at play. That is, CTC clusters may make use of any one of these extravasation and dissemination mechanisms depending on cancer type, the composition of CTC clusters in terms of cancer cell phenotypes (i.e., epithelial, mesenchymal, mixed) and non-cancer cells types (e.g., platelets), the expression of certain receptors (e.g., TGF-β1 receptors), their location (blood vs lymphatic vessels) etc. Our proposed model applies to CTC clusters that travel through bloodstream and interact with platelets, and can respond to TGF-β1 (i.e., express TGF-β1 receptors). This model opens the possibility that TGF-β1 signalling components can be developed as targets to inhibit the development of metastatic tumours by specifically inhibiting the extravasation and dissemination of CTC clusters. Additionally, if the expression of TGF-β1 receptors correlates with the metastatic potential of CTC clusters, specific markers with prognostic and therapeutic value could be developed. Our results call for studies in other systems as well as *in vivo* to confirm this model and evaluate whether TGF-β signalling components can be used as potential therapeutic targets to suppress the metastatic abilities of certain types of CTC clusters.

REFERENCES

1. Massagué J, Obenauf AC (2016) Metastatic colonization by circulating tumour cells. Nature 529:298–306. https://doi.org/10.1038/nature17038

2. Lambert AW, Pattabiraman DR, Weinberg RA (2017) Emerging Biological Principles of Metastasis. Cell 168:670–691. https://doi.org/10.1016/j.cell.2016.11.037

3. Aceto N, Bardia A, Miyamoto DT, Donaldson MC, Wittner BS, Spencer JA, Yu M, Pely A, Engstrom A, Zhu H, Brannigan BW, Kapur R, Stott SL, Shioda T, Ramaswamy S, Ting DT, Lin CP, Toner M, Haber DA, Maheswaran S (2014) Circulating tumor cell clusters are oligoclonal precursors of breast cancer metastasis. Cell 158:1110–1122. https://doi.org/10.1016/j.cell.2014.07.013

4. Jansson S, Bendahl PO, Larsson AM, Aaltonen KE, Rydén L (2016) Prognostic impact of circulating tumor cell apoptosis and clusters in serial blood samples from patients with metastatic breast cancer in a prospective observational cohort. BMC Cancer 16:433. https://doi.org/10.1186/s12885-016-2406-y

5. Hou JM, Krebs MG, Lancashire L, Sloane R, Backen A, Swain RK, Priest LJC, Greystoke A, Zhou C, Morris K, Ward T, Blackhall FH, Dive C (2012) Clinical significance and molecular characteristics of circulating tumor cells and circulating tumor microemboli in patients with small-cell lung cancer. Journal of Clinical Oncology 30:525–532. https://doi.org/10.1200/JCO.2010.33.3716

6. Cheung KJ, Padmanaban V, Silvestri V, Schipper K, Cohen JD, Fairchild AN, Gorin MA, Verdone JE, Pienta KJ, Bader JS, Ewald AJ (2016) Polyclonal breast cancer metastases arise from collective dissemination of keratin 14-expressing tumor cell clusters. Proc Natl Acad Sci U S A 113:E854–E863. https://doi.org/10.1073/pnas.1508541113

7. Au SH, Edd J, Stoddard AE, Wong KHK, Fachin F, Maheswaran S, Haber DA, Stott SL, Kapur R, Toner M (2017) Microfluidic isolation of circulating tumor cell clusters by size and asymmetry. Sci Rep 7:2433. https://doi.org/10.1038/s41598-017-01150-3

8. Hou JM, Krebs M, Ward T, Sloane R, Priest L, Hughes A, Clack G, Ranson M, Blackhall F, Dive C (2011) Circulating tumor cells as a window on metastasis biology in lung cancer. American Journal of Pathology 178:989–996. https://doi.org/10.1016/j.ajpath.2010.12.003

9. Krol I, Castro-Giner F, Maurer M, Gkountela S, Szczerba BM, Scherrer R, Coleman N, Carreira S, Bachmann F, Anderson S, Engelhardt M, Lane H, Jeffry Evans TR, Plummer R, Kristeleit R, Lopez J, Aceto N (2018) Detection of circulating tumour cell clusters in human glioblastoma. Br J Cancer 119:487–491. https://doi.org/10.1038/s41416-018-0186-7

10. Lim M, Park S, Jeong HO, Park SH, Kumar S, Jang A, Lee S, Kim DU, Cho YK (2021) Circulating tumor cell clusters are cloaked with platelets and correlate with poor prognosis

in unresectable pancreatic cancer. Cancers (Basel) 13:5272. https://doi.org/10.3390/cancers13215272

11. Krol I, Schwab FD, Carbone R, Ritter M, Picocci S, de Marni ML, Stepien G, Franchi GM, Zanardi A, Rissoglio MD, Covelli A, Guidi G, Scarinci D, Castro-Giner F, Mazzarella L, Doglioni C, Borghi F, Milani P, Kurzeder C, Weber WP, Aceto N (2021) Detection of clustered circulating tumour cells in early breast cancer. Br J Cancer 125:23–27. https://doi.org/10.1038/s41416-021-01327-8

12. Zhang D, Zhao L, Zhou P, Ma H, Huang F, Jin M, Dai X, Zheng X, Huang S, Zhang T (2017) Circulating tumor microemboli (CTM) and vimentin+ circulating tumor cells (CTCs) detected by a size-based platform predict worse prognosis in advanced colorectal cancer patients during chemotherapy. Cancer Cell Int 17:6. https://doi.org/10.1186/s12935-016-0373-7

13. Klezl P, Pospisilova E, Kolostova K, Sonsky J, Maly O, Grill R, Pawlak I, Bobek V (2020) Detection of circulating tumor cells in renal cell carcinoma: Disease stage correlation and molecular characterization. J Clin Med 9:1372. https://doi.org/10.3390/jcm9051372

14. Yu JJ, Shu C, Yang HY, Huang Z, Li YN, Tao R, Chen YY, Chen Q, Chen XP, Xiao W (2021) The Presence of Circulating Tumor Cell Cluster Characterizes an Aggressive Hepatocellular Carcinoma Subtype. Front Oncol 11:734564. https://doi.org/10.3389/fonc.2021.734564

15. Luo X, Mitra D, Sullivan RJ, Wittner BS, Kimura AM, Pan S, Hoang MP, Brannigan BW, Lawrence DP, Flaherty KT, Sequist L v, Mcmahon M, Bosenberg MW, Stott SL, Ting DT, Ramaswamy S, Toner M, Fisher DE, Maheswaran S, Haber DA (2014) Isolation and Molecular Characterization of Circulating Melanoma Cells. Cell Rep 7:645–653

16. Maeshiro M, Shinriki S, Liu R, Nakachi Y, Komohara Y, Fujiwara Y, Ohtsubo K, Yoshida R, Iwamoto K, Nakayama H, Matsui H (2021) Colonization of distant organs by tumor cells generating circulating homotypic clusters adaptive to fluid shear stress. Sci Rep 11:6150. https://doi.org/10.1038/s41598-021-85743-z

17. Zhao Q, Barclay M, Hilkens J, Guo X, Barrow H, Rhodes JM, Yu LG (2010) Interaction between circulating galectin-3 and cancer-associated MUC1 enhances tumour cell homotypic aggregation and prevents anoikis. Mol Cancer 9:1937–1947. https://doi.org/10.1186/1476-4598-9-154

18. Lo HC, Xu Z, Kim IS, Muscarella A, Liu J, Hein S, Wang H, Krupnick A, Neilson J, Paust S, Rosen J, Zhang X (2020) Circulating tumor cell clusters exhibit enhanced immune evasion from natural killer cells. The Journal of Immunology 204:88.18

19. Bithi SS, Vanapalli SA (2017) Microfluidic cell isolation technology for drug testing of single tumor cells and their clusters. Sci Rep 7:41707. https://doi.org/10.1038/srep41707

20. Gkountela S, Castro-Giner F, Szczerba BM, Vetter M, Landin J, Scherrer R, Krol I, Scheidmann MC, Beisel C, Stirnimann CU, Kurzeder C, Heinzelmann-Schwarz V, Rochlitz C, Weber WP, Aceto N (2019) Circulating Tumor Cell Clustering Shapes DNA Methylation to Enable Metastasis Seeding. Cell 176:98-112.e14. https://doi.org/10.1016/j.cell.2018.11.046

21. Labuschagne CF, Cheung EC, Blagih J, Domart MC, Vousden KH (2019) Cell Clustering Promotes a Metabolic Switch that Supports Metastatic Colonization. Cell Metab 30:720-734.e5. https://doi.org/10.1016/j.cmet.2019.07.014

22. Liu X, Taftaf R, Kawaguchi M, Chang YF, Chen W, Entenberg D, Zhang Y, Gerratana L, Huang S, Patel DB, Tsui E, Adorno-Cruz V, Chirieleison SM, Cao Y, Harney AS, Patel S, Patsialou A, Shen Y, Avril S, Gilmore HL, Lathia JD, Abbott DW, Cristofanilli M, Condeelis JS, Liu H (2019) Homophilic CD44 interactions mediate tumor cell aggregation and polyclonal metastasis in patient-derived breast cancer models. Cancer Discov 9:96–113. https://doi.org/10.1158/2159-8290.CD-18-0065

23. Donato C, Kunz L, Castro-Giner F, Paasinen-Sohns A, Strittmatter K, Szczerba BM, Scherrer R, di Maggio N, Heusermann W, Biehlmaier O, Beisel C, Vetter M, Rochlitz C, Weber WP, Banfi A, Schroeder T, Aceto N (2020) Hypoxia Triggers the Intravasation of Clustered Circulating Tumor Cells. Cell Rep 32:108105. https://doi.org/10.1016/j.celrep.2020.108105

24. Reymond N, d'Água BB, Ridley AJ (2013) Crossing the endothelial barrier during metastasis. Nat Rev Cancer 13:858–870. https://doi.org/10.1038/nrc3628

25. Strilic B, Offermanns S (2017) Intravascular Survival and Extravasation of Tumor Cells. Cancer Cell 32:282–293. https://doi.org/10.1016/j.ccell.2017.07.001

26. Reymond N, Im JH, Garg R, Vega FM, D'agua BB, Riou P, Cox S, Valderrama F, Muschel RJ, Ridley AJ (2012) Cdc42 promotes transendothelial migration of cancer cells through β1 integrin. Journal of Cell Biology 199:653–668. https://doi.org/10.1083/jcb.201205169

27. Strilic B, Yang L, Albarrán-Juárez J, Wachsmuth L, Han K, Müller UC, Pasparakis M, Offermanns S (2016) Tumour-cell-induced endothelial cell necroptosis via death receptor 6 promotes metastasis. Nature 536:215–218. https://doi.org/10.1038/nature19076

28. Allen TA, Gracieux D, Talib M, Tokarz DA, Hensley MT, Cores J, Vandergriff A, Tang J, de Andrade JBM, Dinh PU, Yoder JA, Cheng K (2017) Angiopellosis as an Alternative Mechanism of Cell Extravasation. Stem Cells 35:170–180. https://doi.org/10.1002/stem.2451

29. Giuliano M, Shaikh A, Lo HC, Arpino G, de Placido S, Zhang XH, Cristofanilli M, Schiff R, Trivedi M v. (2018) Perspective on circulating tumor cell clusters: Why it takes a village to metastasize. Cancer Res 78:845–852. https://doi.org/10.1158/0008-5472.CAN-17-2748

30. Allen TA, Cullen MM, Hawkey N, Mochizuki H, Nguyen L, Schechter E, Borst L, Yoder JA, Freedman JA, Patierno SR, Cheng K, Eward WC, Somarelli JA (2021) A Zebrafish Model of Metastatic Colonization Pinpoints Cellular Mechanisms of Circulating Tumor Cell Extravasation. Front Oncol 11:641187. https://doi.org/10.3389/fonc.2021.641187

31. Au SH, Storey BD, Moore JC, Tang Q, Chen YL, Javaid S, Sarioglu AF, Sullivan R, Madden MW, O'Keefe R, Haber DA, Maheswaran S, Langenau DM, Stott SL, Toner M (2016) Clusters of circulating tumor cells traverse capillary-sized vessels. Proc Natl Acad Sci U S A 113:4947–4952. https://doi.org/10.1073/pnas.1524448113

32. Umer M, Vaidyanathan R, Nguyen NT, Shiddiky MJA (2018) Circulating tumor microemboli: Progress in molecular understanding and enrichment technologies. Biotechnol Adv 36:1367–1389. https://doi.org/10.1016/j.biotechadv.2018.05.002

33. Syed V (2016) TGF-β Signaling in Cancer. J Cell Biochem 117:1279–1287. https://doi.org/10.1002/jcb.25496

34. Locatelli L, Colciago A, Castiglioni S, Maier JA (2021) Platelets in Wound Healing: What Happens in Space? Front Bioeng Biotechnol 9:716184. https://doi.org/10.3389/fbioe.2021.716184

35. Labelle M, Begum S, Hynes RO (2011) Direct Signaling Between Platelets and Cancer Cells Induces an Epithelial-Mesenchymal-Like Transition and Promotes Metastasis. Cancer Cell 20:576–590. https://doi.org/10.1016/j.ccr.2011.09.009

36. May AN, Crawford BD, Nedelcu AM (2018) In vitro model-systems to understand the biology and clinical significance of circulating tumor cell clusters. Front Oncol 8:63. https://doi.org/10.3389/fonc.2018.00063

37. Jong ED, Chan ICW, Nedelcu AM (2019) A model-system to address the impact of phenotypic heterogeneity and plasticity on the development of cancer therapies. Front Oncol 9:683. https://doi.org/10.3389/fonc.2019.00842

38. Justus CR, Leffler N, Ruiz-Echevarria M, Yang L v. (2014) In vitro cell migration and invasion assays. Journal of Visualized Experiments 88:51046. https://doi.org/10.3791/51046

39. Boyden S (1962) The chemotactic effect of mixtures of antibody and antigen on polymorphonuclear leucocytes. J Exp Med 115:453–466. https://doi.org/10.1084/jem.115.3.453

40. Schneider CA, Rasband WS, Eliceiri KW (2012) NIH Image to ImageJ: 25 years of Image Analysis HHS Public Access. Nat Methods 9:671–675. https://doi.org/10.1038/nmeth.2089

41. Yu M, Bardia A, Wittner BS, Stott SL, Smas ME, Ting DT, Isakoff SJ, Ciciliano JC, Wells MN, Shah AM, Concannon KF, Donaldson MC, Sequist L v., Brachtel E, Sgroi D, Baselga J, Ramaswamy S, Toner M, Haber DA, Maheswaran S (2013) Circulating breast

tumor cells exhibit dynamic changes in epithelial and mesenchymal composition. Science (1979) 339:580–584. https://doi.org/10.1126/science.1228522

42. Grainger DJ, Mosedale DE, Metcalfe JC (2000) TGF-b in blood: a complex problem. Cytokine Growth Factor Rev 11:133–145. https://doi.org/10.1016/s1359-6101(99)00037-4

43. Wakefield LM, Letterlo JJ, Chen T, Danielpour D, Allison RSH, Pai LH, Denicoff AM, Noone MH, Cowan KH, O'shaughnessy JA, Sporn MB, Branch M (1995) Transforming Growth Factor-B1 Circulates in Normal Human Plasma and Is Unchanged in Advanced Metastatic Breast Cancer. Clin Cancer Res 1:129–136

44. Assoian RK, Sporn MB (1986) Type B Transforming Growth Factor in Human Platelets: Release during Platelet Degranulation and Action on Vascular Smooth Muscle Cells. J Cell Biol 102:1217–1223. https://doi.org/10.1083/jcb.102.4.1217

45. Ivanovicá V, Todorovic N, Demajo M, Nesˇkovic Z, Subota V, Ivanisěvic O, Nikolic D (2003) Elevated plasma levels of transforming growth factor-B1 (TGF-B1) in patients with advanced breast cancer: association with disease progression. European journal of cancer 39:454–461. https://doi.org/10.1016/s0959-8049(02)00502-6

46. Kim BN, Ahn DH, Kang N, Yeo CD, Kim YK, Lee KY, Kim TJ, Lee SH, Park MS, Yim HW, Park JY, Park CK, Kim SJ (2020) TGF-β induced EMT and stemness characteristics are associated with epigenetic regulation in lung cancer. Sci Rep 10:10597. https://doi.org/10.1038/s41598-020-67325-7

47. Glinskii O v., Huxley VH, Glinsky G v., Pienta KJ, Raz A, Glinsky V v. (2005) Mechanical entrapment is insufficient and intercellular adhesion is essential for metastatic cell arrest in distant organs. Neoplasia 7:522–527. https://doi.org/10.1593/neo.04646

48. Wang H, Radjendirane V, Wary KK, Chakrabarty S (2004) Transforming growth factor β regulates cell-cell adhesion through extracellular matrix remodeling and activation of focal adhesion kinase in human colon carcinoma Moser cells. Oncogene 23:5558–5561. https://doi.org/10.1038/sj.onc.1207701

49. Xiong Shen M, Zhu DM, Ying Wang L, Liang Zha X (2003) TGF-β1-promoted epithelial-to-mesenchymal transformation and cell ad-hesion contribute to TGF-β1-enhanced cell migration in SMMC-7721 cells. Cell Res 13:343–350. https://doi.org/10.1038/sj.cr.7290179

50. Kim H-P, Lee M-S, Park J-A, Jong H-S, Kim T-Y, Weon LEE J, Bang Y-J, Korea S (2004) TGF-β1 (transforming growth factor-β1)-mediated adhesion of gastric carcinoma cells involves a decrease in Ras/ERKs (extracellular-signal-regulated kinases) cascade activity dependent on c-Src activity. Biochem J 379:141–150. https://doi.org/10.1042/bj20031408

51. Yu M, Bardia A, Aceto N, Bersani F, Madden MW, Donaldson MC, Desai R, Zhu H, Comaills V, Zheng Z, Wittner BS, Stojanov P, Brachtel E, Sgroi D, Kapur R, Shioda T, Ting DT, Ramaswamy S, Getz G, Iafrate AJ, Benes C, Toner M, Maheswaran S, Haber

DA (2014) Ex vivo culture of circulating breast tumor cells for individualized testing of drug susceptibility. Science (1979) 345:216–220. https://doi.org/10.1126/science.1253533

52. Sjaastad MD, Angres B, Lewis RS, Nelson WJ (1994) Feedback regulation of cell-substratum adhesion by integrin-mediated intracellular Ca2+ signaling (epithelial cells/signal transduction/substrate adhesion). Proc Natl Acad Sci U S A 91:8214–8218. https://doi.org/10.1073/pnas.91.17.8214

53. Gressner OA, Lahme B, Siluschek M, Rehbein K, Herrmann J, Weiskirchen R, Gressner AM (2008) Activation of TGF-β within cultured hepatocytes and in liver injury leads to intracrine signaling with expression of connective tissue growth factor. J Cell Mol Med 12:2717–2730. https://doi.org/10.1111/j.1582-4934.2008.00260.x

54. Ungefroren H (2021) Autocrine TGF-β in cancer: Review of the literature and caveats in experimental analysis. Int J Mol Sci 22:1–22. https://doi.org/10.3390/ijms22020977

55. Melzer C, von der Ohe J, Hass R, Ungefroren H (2017) TGF-β-dependent growth arrest and cell migration in benign and malignant breast epithelial cells are antagonistically controlled by Rac1 and Rac1b. Int J Mol Sci 18:1574. https://doi.org/10.3390/ijms18071574

56. Lu C, Yang Z, Yu D, Lin J, Cai W (2020) RUNX1 regulates TGF-β induced migration and EMT in colorectal cancer. Pathol Res Pract 216:153142. https://doi.org/10.1016/j.prp.2020.153142

57. Giampieri S, Manning C, Hooper S, Jones L, Hill CS, Sahai E (2009) Localized and reversible TGFβ signalling switches breast cancer cells from cohesive to single cell motility. Nat Cell Biol 11:1287–1296. https://doi.org/10.1038/ncb1973

58. Khan SA, Joyce J, Tsuda T (2012) Quantification of active and total transforming growth factor-β levels in serum and solid organ tissues by bioassay. BMC Res Notes 5:636. https://doi.org/10.1186/1756-0500-5-636

59. Trivedi T, Pagnotti GM, Guise TA, Mohammad KS (2021) The role of tgf-β in bone metastases. Biomolecules 11:1643. https://doi.org/10.3390/biom11111643

60. van Obberghen-Schilling E, Roche NS, Flanders KC, Sporn MB, Roberts AB (1988) Transforming Growth Factor 81 Positively Regulates Its Own Expression in Normal and Transformed Cells*. J Biol Chem 263:7741–7746

61. Zhang M, Fraser D, Phillips A (2006) ERK, p38, and Smad signaling pathways differentially regulate transforming growth factor-β1 autoinduction in proximal tubular epithelial cells. American Journal of Pathology 169:1282–1293. https://doi.org/10.2353/ajpath.2006.050921

62. Yan X, Xiong X, Chen YG (2018) Feedback regulation of TGF-β signaling. Acta Biochim Biophys Sin (Shanghai) 50:37–50. https://doi.org/10.1093/abbs/gmx129

63. Daroqui MC, Vazquez P, Bal De Kier Joffé E, Bakin A v., Puricelli LI (2012) TGF-β autocrine pathway and MAPK signaling promote cell invasiveness and in vivo mammary adenocarcinoma tumor progression. Oncol Rep 28:567–575. https://doi.org/10.3892/or.2012.1813

64. Liu Z, Bandyopadhyay A, W. Nichols R (2012) Blockade of Autocrine TGF-β Signaling Inhibits Stem Cell Phenotype, Survival, and Metastasis of Murine Breast Cancer Cells. J Stem Cell Res Ther 02:1–8. https://doi.org/10.4172/2157-7633.1000116

65. Silveira DA, Gupta S, Mombach JCM (2020) Systems biology approach suggests new miRNAs as phenotypic stability factors in the epithelial-mesenchymal transition: Systems biology approach suggest new miRNAs as phenotypic stability factors in the epithelial-mesenchymal transition. J R Soc Interface 17:20200693. https://doi.org/10.1098/rsif.2020.0693

66. Li J, Ballim D, Rodriguez M, Cui R, Goding CR, Teng H, Prince S (2014) The anti-proliferative function of the tgf-β1 signaling pathway involves the repression of the oncogenic tbx2 by its homologue tbx3. Journal of Biological Chemistry 289:35633–35643. https://doi.org/10.1074/jbc.M114.596411

67. Gallaher JA, Brown JS, Anderson ARA (2019) The impact of proliferation-migration tradeoffs on phenotypic evolution in cancer. Sci Rep 9:2425. https://doi.org/10.1038/s41598-019-39636-x

68. Paget S (1889) Distribution of secondary growths in cancer of the breast. Cancer Metastasis Rev 8:98–101

69. Siegel RL, Miller KD, Fuchs HE, Jemal A (2022) Cancer statistics, 2022. CA Cancer J Clin 72:7–33. https://doi.org/10.3322/caac.21708