Synergistic cooperation involving TGFβ1-mediated crosstalk enhances the invasiveness of genetically distant cancer clones

Caroline S. Carneiro (0000-0002-9984-2106), Jorian D. Hapeman (0000-0002-6223-5126), Aurora M. Nedelcu* (0000-0002-7517-2419)

University of New Brunswick, Department of Biology, Fredericton, NB, Canada, E3B 5A3

*Corresponding author; anedelcu@unb.ca

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ABSTRACT

Intratumour heterogeneity is often associated with poor response to treatment and bad prognosis. In addition to constitutive genetic/epigenetic sources, phenotypic and functional heterogeneity can reflect cell plasticity due to changes in gene expression patterns induced by signals from the tumour microenvironment or other cells. Positive interactions between cancer clones can increase their fitness and contribute to tumour growth, resistance to drugs and metastasis. Consequently, understanding the pathways involved in such behaviours is of great significance for cancer treatment. To explore if and how genetically distant clones can synergistically enhance each other's metastatic potential, this study used three (two breast and one lung) cancer cell lines with different aggressiveness levels. We found that (i) the conditioned media from the breast and lung aggressive lines induce mesenchymal features and increase the migration and invasion potential of the poorly metastatic breast line, and (ii) in both cases, this interclonal communication is based on the same soluble factor – namely, the tumour growth factor TGF-β1. Furthermore, when the two breast lines are mixed and co-cultured, the invasive potential of both lines is enhanced, and this outcome is dependent on the recruitment of the less aggressive clone into expressing a malignant phenotype. Based on our findings, we propose a two-tier model whereby highly metastatic clones can recruit weakly metastatic clones into acquiring an invasive phenotype, which in turn augments the invasion ability of the former (i.e., a “help me help you” strategy) through shared proteases and/or ECM remodelling. We suggest that such synergistic cooperation can easily emerge via cross-talk involving metastatic clones able to constitutively secrete signalling molecules that induce and maintain their own malignant state (i.e., autocrine/cell-autonomous signalling) and clones that have the ability to respond to those signal (i.e., paracrine/non-cell-autonomous signalling) and express a metastatic phenotype. Taking into account the lack of therapies to directly affect the metastatic process, interfering with such cooperative behaviours that tumour cells engage in during the early steps in the metastatic cascade could provide an additional strategy to increase patient survival.

Keywords: cooperation, conditioned media, TGF-β1, intratumour heterogeneity, metastasis, EMT, invasion

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INTRODUCTION

Despite intensive research, cancer remains the second cause of death worldwide [1]. The difficulties in curing cancer or preventing tumour progression reflect the complex nature of this disease, including high levels of heterogeneity between and within tumours. Increased heterogeneity is linked to poor prognosis, as it poses a direct problem to targeted therapies (i.e., not all clones will be sensitive) and can facilitate the evolution of drug resistance [2,3]. High levels of intratumour heterogeneity are the result of both genetic mutations or epigenetic alterations as well as phenotypic plasticity [3].

Transitory changes in cancer cell state or phenotype can be triggered by either physicochemical modifications in the tumour microenvironment (fluctuations in pH, oxygen, and glucose levels) or signalling molecules released from other cancer or non-cancer cells [4]. Malignant cells are known to secrete molecules that can regulate their own genetic/cellular machinery (i.e., autocrine signalling) but can also affect surrounding cells (i.e., paracrine signalling) [5,6]. Most common paracrine signalling factors include metabolites, miRNAs [7], cytokines (e.g., IL-6, Tumour necrosis factor-α), chemokines (e.g., CXCL1, CCL2) [8–10], and growth factors – such as the epidermal growth factor (EGF) and the transforming growth factor-β (TGF-β) family [11,12].

According to the clonal evolution model of cancer, intratumour heterogeneity should create the conditions for interclonal competition and selection, which would result in selective sweeps and a reduction in levels of heterogeneity [13]. However, in addition to competing, cancer clones could also cooperate with each other or with non-cancer cells [46]. Such cooperative behaviours can contribute to maintaining the heterogeneity of tumours [14] by increasing the fitness of the interacting clones and ultimately result in drug resistance [15,16] and tumour growth [17,18].

During tumour progression, cancer cells can acquire several mutations that activate the epithelial-mesenchymal transition (EMT) pathway and enable them to metastasize to new tissues and locations within the body [19]. The classical view of metastasis envisions single cells migrating and invading neighbouring tissues, dispersing via the vascular system, and disseminating to distant locations. However, the more recent findings that some of these steps (e.g., migration, invasion, dispersal) might, in fact, involve groups of cells (that could include distinct clones) rather than single cells allows for the possibility that cooperative interactions can also take place during the early stages of this complex process (i.e., local invasion and intravasation) [20].

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Cooperative interactions during metastasis can be strictly based on adhesion (such as in the formation of circulating tumour cell clusters) [21]. However, they can also involve sharing of signals (i.e., public goods) or changes in phenotype/state (phenotypic/cellular plasticity) through paracrine signalling. For instance, the secretion of paracrine signals by aggressive cancer cells was suggested to induce EMT and enhance the metastatic potential of non-metastatic clones in lung [22,23] and breast cancers [24,25]. Moreover, cell cooperation can also involve remodelling of the tumour microenvironment (TME) as cells that have undergone EMT can degrade the surrounding matrix and lead the way to invasion and intravasation, which then allows non-EMT cells to enter the bloodstream and establish colonies in secondary sites [26].

Although several examples of possible inter-clonal cooperative interactions have been reported, the type of cooperation (in terms of the outcome for the interacting clones) and the mechanisms through which cancer clones cooperate, especially during the initial steps of the metastatic process, are not fully understood. For instance, subclones derived from the highly aggressive breast line MDA-MB-231 were shown to interact and enhance each other's growth and migration/invasion. Still, the mechanisms and factors involved in this interclonal communication were not identified [27]. Information about the molecules and pathways involved in initiating and maintaining such interactions could provide new therapeutic targets to inhibit the progression of metastasis.

In this study, we (i) addressed whether aggressive clones can recruit less aggressive (and genetically distinct) clones into expressing a more malignant phenotype that would enhance the metastatic potential of both clones (i.e., synergistic cooperation involving a "help me help you" strategy) and (ii) investigated the signalling molecules involved in such interclonal cooperation. To do so, we used two molecularly and cytologically different breast cancer cell lines that reflect the most common breast cancer cell types – basal (MDA-MB-231; aggressive) and luminal (MCF7; less aggressive) as well as a highly metastatic experimentally evolved non-small lung cell line (derived from NCI-H2122). In a heterogeneous tumour, different cell types can be in direct cell-cell contact or affect each other through diffusible factors (paracrine crosstalk). To simulate such interactions, we either subjected each line to the conditioned medium of the other line or grew the two cell lines in co-culture systems (as separate groups sharing the medium or as mixed populations/direct contact). To assess changes in metastatic behaviour, we assessed morphological changes associated with EMT and investigated the capacity of cells to migrate and invade.

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MATERIALS AND METHODS

Cell lines and culturing conditions

The two human breast cancer cell lines used in this study, MCF7 and MDA-MB-231, were obtained from ATCC. An adherent non-small cell lung cancer cell line (H2122 AS) was derived from a line that grows as a mixture of adherent and suspension cells (NCI-H2122; ATCC) through selective passages of the adherent population [28]. All cell lines were grown in low glucose (5.5 mM) Dulbecco's Modified Eagle Medium (Gibco) supplemented with 10 % fetal bovine serum (FBS; ATCC) and 1 % Penicillin/Streptomycin (Gibco) at 37 °C with 5 % CO2. Cells were passaged by enzymatic dissociation with 0.05% trypsin-EDTA (Gibco) when 80% confluence was reached.

Conditioned media

To collect the conditioned media (CM), cells were grown in 5.5 mM glucose DMEM with 10% FBS until the monolayer reached around 90% confluence. Cells were then washed with PBS and maintained in DMEM with low FBS (1%) for 24 hours (low FBS reduces the level of proteins and growth factors present in the conditioned medium). The collected conditioned medium was centrifuged at 1000xg for 10 minutes, and the supernatant was passed through a 0.2 µm syringe filter and stored at -20 °C. To test the effect of CM, cells were incubated for up to 72 hours with 25% CM (diluted in regular media); the CM was refreshed daily.

The conditioned media was also filtered using Amicon® Ultra Centrifugal Filter Units (*Millipore*) with different cut-offs (100 kDa, 30 kDa, and 10 kDa) according to the manufacturer's instructions. The fractions were tested for their ability to induce migration.

Reagents

Human Recombinant TGF-β1 (R&D systems; at 10 ng/ml final concentration) was used as a positive control. A TGF-β receptor I inhibitor (SB431542; EMD Millipore; 10 µM final concentration) and antibodies against TGF-β1, 2, 3 (1D11; R&D systems; at 2 µg/ml final concentration) were used to block the response to TGF-β1 or the TGF-β1 itself.

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Fluorescence staining and microscopy

For co-culture experiments, cells were stained with either DiO or DiD (Invitrogen™ Vybrant™) following the manufacturer's instructions. The stained cells were co-cultured for 72 hours and fixed with 2% paraformaldehyde. Slides were photographed at 20X magnification using a LEICA DM R (Epi-Fluorescence Microscope) with FITC and TRICT filters.

Wound healing assays

To assess migration, we performed wound healing assays. Gaps within cell monolayers were achieved using cross-shaped inserts (*Ibidi*). Cells were seeded at 10^5 cells/cm^2 in DMEM with 1% FBS (low levels of FBS inhibit cells' proliferative abilities during migration assays) and were allowed to adhere overnight. After the inserts were lifted, wells were washed with PBS, and fresh medium was added. The cells were allowed to migrate and fill in the gap while the medium was refreshed daily. For co-culture assays, each cell line was seeded in a separate insert in the same well. Once cells were attached, the inserts were removed, and DMEM with 1% FBS was added. The plates were placed on a shaker (45 rpm) to ensure the diffusion and mixing of the medium. Radom fields of the gap were imaged every 24 hours. At the end of the assay, when cells occupied around 80% of the gap (24h for MDA, 48h for H2122 AS, and 48 to 72h for MCF7), the cell layers were fixed with 70% ethanol and stained with 0.2% crystal violet. Gap width was measured using the Wound Healing Tool implemented in Fuji Image J. The % of the area that was occupied (Af) relative to the area at the start of the assay (Ai) was calculated ($\%\, occupied\ area = (Ai - Af)/(Ai * 100)$).

Transwell assays

Migration and invasion were also assayed using transwell inserts (Corning). 10^5 cells were seeded in the inserts and placed in contact with media containing a chemoattractant (i.e., DMEM with 10% FBS). Cells were allowed to migrate through the 8 μm porous membrane on the underside of the insert towards the chemoattractant. After 48 hours, cells that migrated on the underside of the insert were fixed with 70% ethanol and stained with 0.2% crystal violet. Five random fields of view from the stained insert were photographed using brightfield microscopy at 10x magnification. Cells attached to the underside of the insert were counted for each field of view and averaged. The same protocol was followed to assess invasive abilities. Cells were placed onto inserts containing

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a layer of 1mg/ml Matrigel (Corning), and the number of cells that invaded was assessed after 72 hours. In the co-culture experiments, one cell line (MCF7) was pre-labelled with DiO, and the other (MDA) was unstained. At the end of the assay, the cells were fixed with 2% paraformaldehyde. As above, the number of invaded fluorescent cells was assessed, and all invaded cells were stained with crystal violet. The number of MDA cells that invaded was calculated by subtracting the number of invaded MCF7 cells (i.e., fluorescent) from the total number of invaded cells (i.e., stained with crystal violet).

Statistical Analyses

The experiments were repeated several times independently, with three biological replicates each. Data were expressed as mean ± SEM. Unpaired, two-tailed Student's t-tests or one-way ANOVA with Sidak's test for multiple comparisons were performed to assess the statistical significance of the relevant comparisons. Data analysis was performed using Prism GraphPad 8; $p \leq 0.05$ was considered statistically significant.

RESULTS

1. MDA-MB-231 induces an EMT-like change in MCF7

To address whether the highly metastatic clone, MDA-MB-231 (abbreviated as MDA from herein on), can induce an EMT-like change in the less metastatic clone, MCF7, we either subjected MCF7 to the conditioned medium (CM) collected from MDA or co-cultured the two cell lines (as a mixture, in a 1:1 ratio). We found that in the presence of MDA or its conditioned medium, MCF7 cells acquired a mesenchymal-like morphology (Fig. 1a and 1b). Specifically, cells extensions associated with an EMT were observed in MCF7 cultures after 72h of treatment with MDA conditioned media (MDA_CM) or exogenous TGF-β1 (as a positive control) – but not when exposed to their own CM (MCF7_CM) (Fig. 1a). Similar extensions were also developed by MCF7 cells when co-culture with MDA cells for 72h; on the other hand, the morphology of MDA did not change when in the presence of MCF7 (Fig. 1b).

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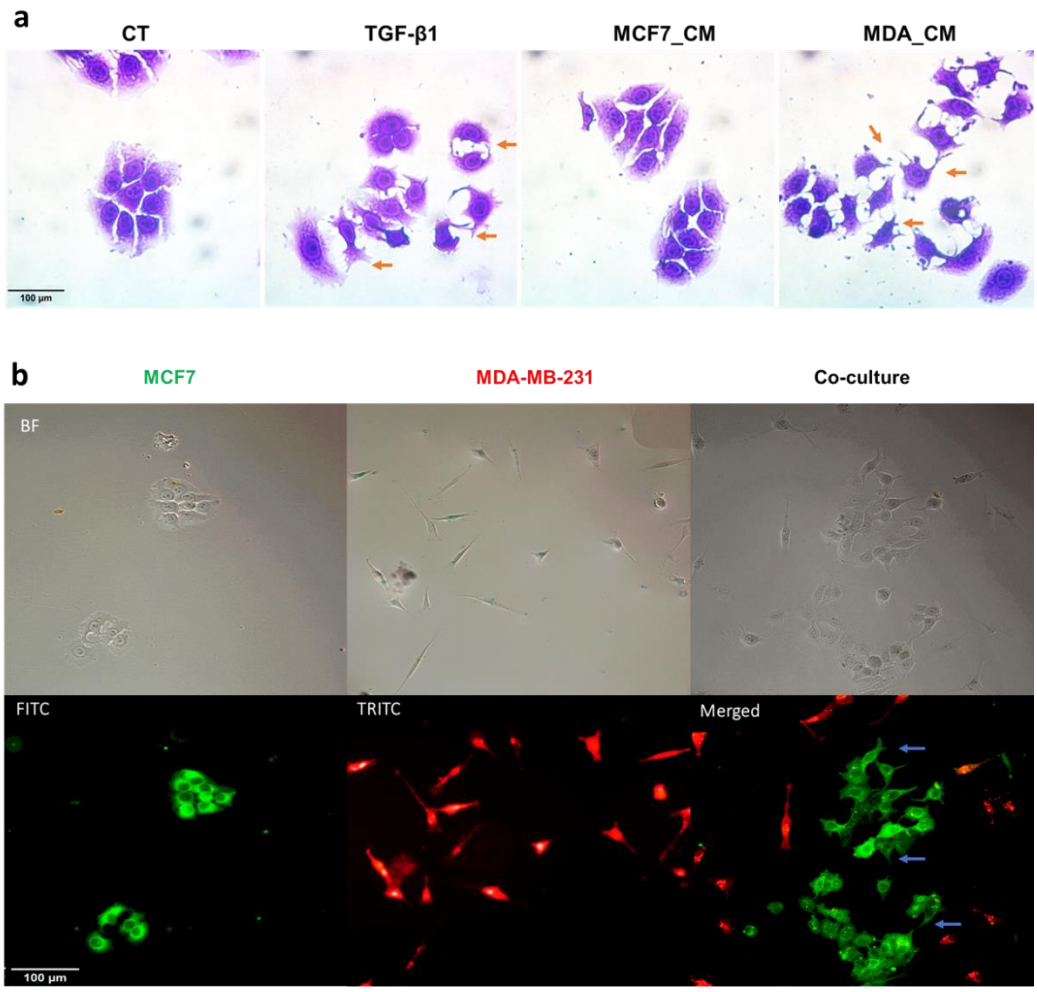

Fig. 1. MDA induced an EMT-like change in MCF7. (a) MCF7 cells were treated with MCF7_CM or MDA_CM and TGF-β1 (positive control) for 72 hours. Cells were fixed with ethanol and stained with 0.2% crystal violet; (b) MCF7 cells were cultured in direct contact with MDA cells for 72 hours. Cells were stained with DiO and DiD, respectively, and fixed with paraformaldehyde 2% and then examined in bright field (BF; top row) and with fluorescence microscopy using FITC or TRITC filters. Arrows indicate cell extensions; 20X original magnification.

2. MDA-MB-231 increases the migration potential of MCF7

To address whether the observed EMT-like changes induced by MDA are associated with an enhanced ability to migrate, we assessed the migration potential of MCF7 in the presence of the

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conditioned medium from MDA (using both wound-healing and transwell assays) as well as in co-culture with MDA (wound-healing assay). Overall, the migration of MCF7 increased in all contexts. Specifically, the conditioned medium from MDA significantly increased the migration of MCF7 (compared to the effect induced by its own CM) to a level similar to that promoted by TGF-β1 (Fig. 2a and 2b). The migration of MCF7 was also significantly increased when MCF7 and MDA cells were co-cultured (in separate patches in the same well) (Fig. 2c). On the other hand, the migration of MDA was not affected in the presence of MCF7_CM or when in co-culture with MCF7 (Fig.2d and 2e).

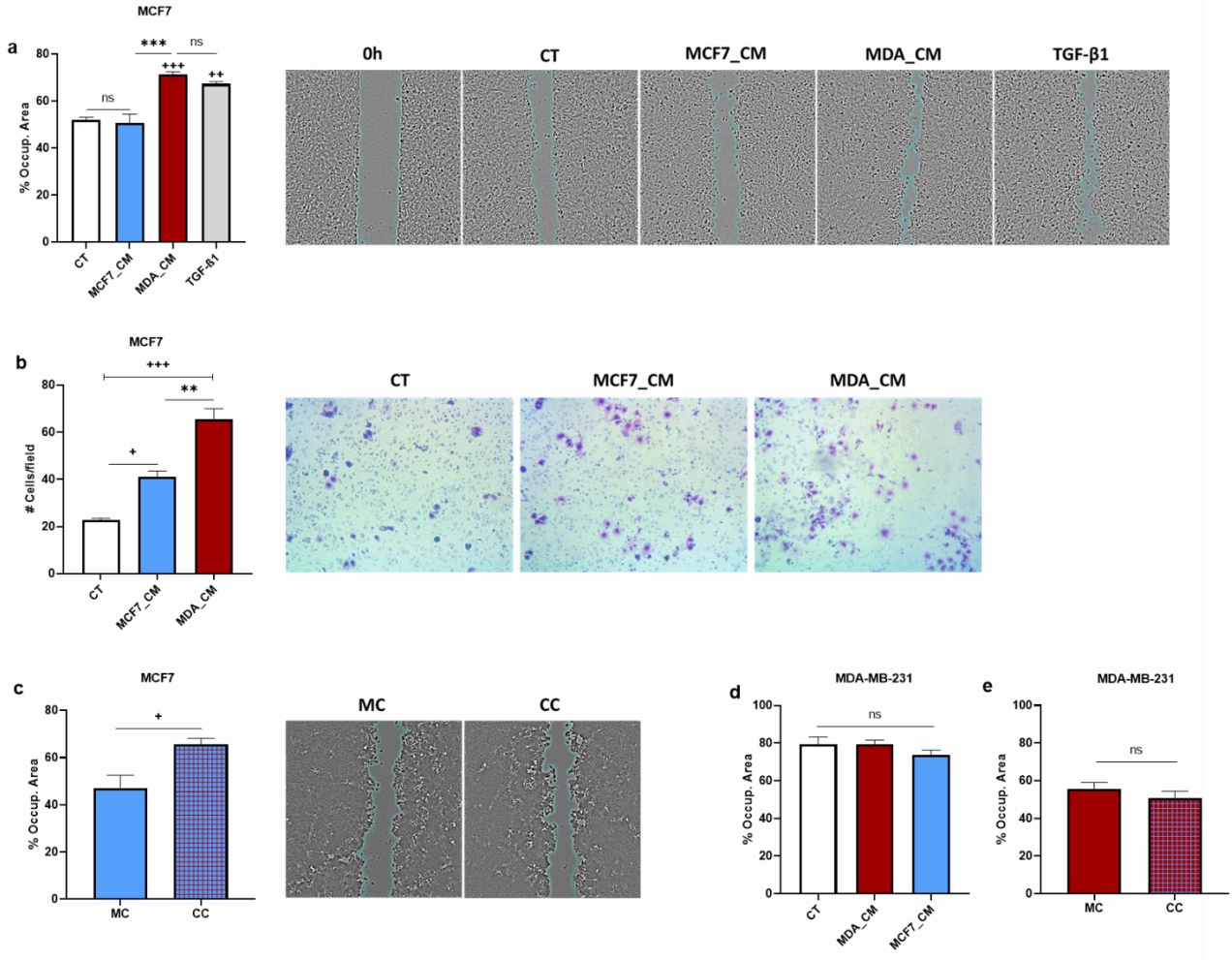

Fig.2. MDA enhanced the migration of MCF7. The migration of MCF7 was assessed in the presence of the CM collected from MCF7 (MCF7_CM) or MDA (MDA_CM) – using wound healing for 48h **(a)** or transwell 72h **(b)** assays, as well as in monoculture (MC) and co-culture (CC) with MDA for 72h – using wound-healing assays **(c)**. MDA migration (using transwell

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assays) was also assessed after 24 hours of contact with MCF7 CM **(d)** or in co-culture with MCF7 **(e)**. Y-axis indicates the percentage of the occupied area (a, c, d and e) or the number of cells per field (b). Representative images of the gap at the end of the wound healing assays and the migrated cells through the transwell inserts are also shown. TGF-β1 (10 ng/ml) was used as a positive control. Error bars indicate SEM (n = 3). + signs indicate differences relative to control (CT), and * signs refer to differences between treatments. ns means no significant; + or * $p<0.05$; ++ or **$p<0.01$; +++ or *** $p<0.001$.

3. TGF-β1 is involved in the MDA-induced migration of MCF7

As MDA is known to secrete TGF-β1 (one of the most important inducers of EMT), we tested whether this growth factor is responsible for the increased migration of MCF7 when in the presence of MDA. To do so, we used either a TGF-β receptor I inhibitor or antibodies against TGF-β. We found that inhibiting either the TGF-β receptor or the TGF-β1 itself significantly decreased the effect of MDA_CM on the migration of MCF7, with the TGF-β receptor I inhibitor completely blocking its effect (Fig. 3a and 3b). Consistent with MDA secreting and responding to its own TGF-β1 (i.e., autocrine signalling), the TGF-β receptor I inhibitor also decreased the migration of MDA (Fig. 3c). On the other hand, the TGF-β receptor I inhibitor did not affect the un-induced migration of MCF7 (Fig. 3a), suggesting that the constitutive MCF7 migration does not rely on TGF-β1.

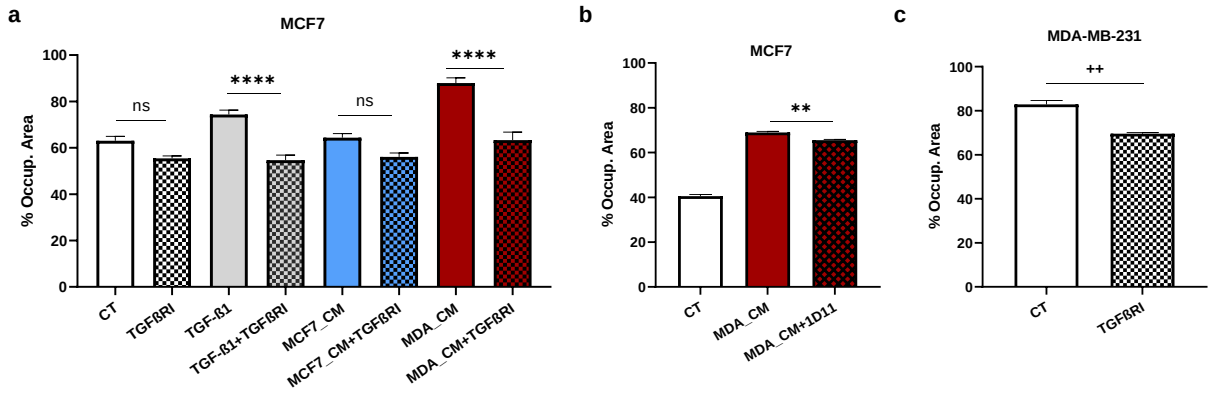

Fig. 3. The effect of TGF-β pathway inhibition on the migration of MDA and MCF7. (a) The effect of a TGF-β receptor I inhibitor (TGFβRI; 10 µM) on the migration of MCF7 control (CT) cultures as well as of cultures exposed to either TGF-β1 (as a positive control), MCF7_CM or

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MDA_CM. **(b)** The effect of MDA_CM neutralized with antibodies against TGF-β 1, 2, and 3 (CM+1D11; 2 µg/ml) on the migration of MCF7 (wound-healing assay; 72 hours). **(c)** The effect of a TGF-β receptor 1 inhibitor on the migration of MDA (wound-healing assay; 24 hours). Error bars indicate SEM (n = 3). + refers to control (CT), and * refers to treatments. ns means no significant; ++ or **p<0.01; or **** p<0.0001.

To further confirm that TGF-β1 is involved in the effect of MDA_CM on MCF7, we fractionated the CM based on the molecular weight and exposed MCF7 to the various fractions. We found that the most effective fractions were those containing molecules between 30 and 100kDa, and above 100kDa (Fig. 4a), which is consistent with the size of the various TGF-β1 forms – including an active form around 28 kDa and a latent form of 220 kDa. Furthermore, the activity of these fractions was decreased by the addition of the TGF-β receptor I inhibitor (Fig. 4b). Also, as acidification can activate latent forms of TGF-β1, we lowered the pH of the MDA_CM and observed a slight increase in its migratory effect on MCF7 (Fig. 4c).

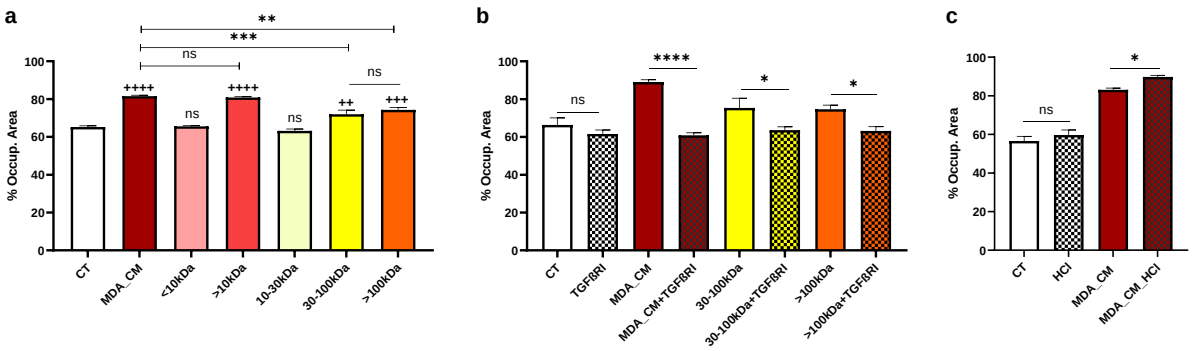

Fig. 4. MDA_CM contains both active and inactive TGF-β1 forms. (a) Wound-healing assay was performed with MCF7 incubated with full MDA_CM and various MDA_CM fractions. **(b)** The effect of the TGF-β receptor I inhibitor on the ability of the full MDA_CM and its 30-100 kDa and >100 kDa fractions to induce migration in MCF7. **(c)** The effect of activated MDA_CM on the migration of MCF7 in the absence (control – CT) and presence of MDA_CM; to activate TGF-β latent forms, pH was lowered to 4.5 with HCl and then neutralized with NaOH (wound-healing assay; 72 hours). Y axis: Percentage of the occupied area. Error bars indicate SEM (n =

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3). + refers to control (CT), and * refers to treatments. ns means no significant; * p<0.05; ++ or **p<0.01; +++ or *** p<0.001; ++++ or **** p<0.0001.

4. TGFβ1-mediated inter-clonal cooperation is not specific to breast cancer

To explore the possibility that the interaction we observed was not specific to these lines, we used a lung cancer cell line – H2122 AS, in conjunction with either MCF7 or MDA. First, we tested the effect of the conditioned medium from H2122 AS on the migration abilities of MCF7. We found that the inducing effect of H2122AS_CM on MCF7 migration was even higher than that of the MDA_CM (Fig. 5a). Consistent with this effect involving TGF-β1, the migration of MCF7 decreased (but was not entirely blocked) in the presence of the TGF-β receptor I inhibitor (Fig. 5b). Second, we tested whether the MDA_CM was also able to affect the H2122 AS, and found a significant increase in its migration potential (Fig. 5c). Again, consistent with this effect involving TGF-β1, the receptor inhibitor fully blocked the effect of the MDA_CM on H2122 AS migration (Fig. 5d). However, in contrast to MDA, the TGF-β receptor I inhibitor (at the same concentration) did not affect the constitutive migration of H2122 AS (Fig. 5d and 3c).

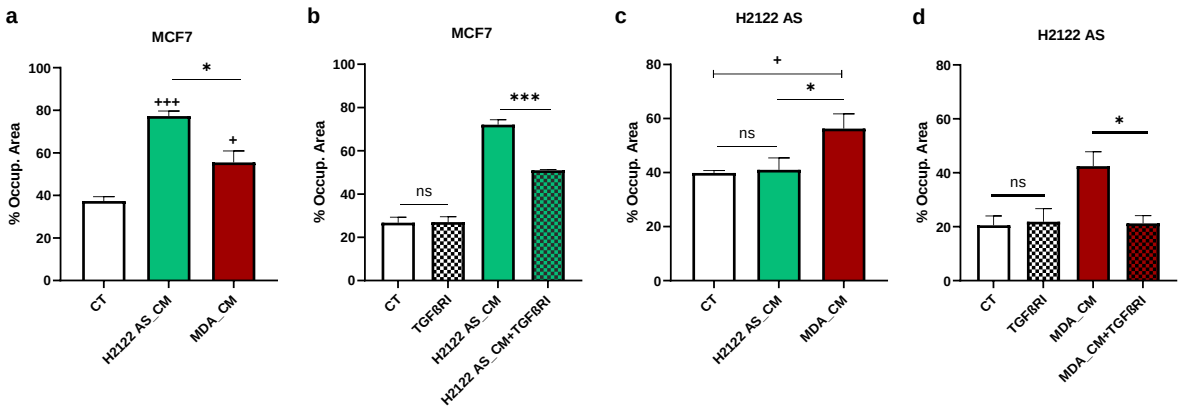

Fig. 5. Cooperation between breast and lung cancer cell lines. (a) The effect of CM collected from MDA or H2122 AS on the migration of MCF7. **(b)** The effect of the TGF-β receptor I inhibitor (TGFβRI) on the MCF7 migration induced by H2122AS_CM (wound-healing assay; 48 hours). **(c)** The effect of MDA_CM on the migration of H2122 AS; **(d)** The effect of the TGF-β receptor I inhibitor on the H2122 AS migration induced by MDA_CM (wound-healing assay; 48

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hours). Y axis: Percentage of the occupied area. CT – Control/Constitutive migration. Error bars indicate SEM (n = 3). + refers to control (CT), and * refers to treatments. ns means no significant; + or * $p<0.05$; +++ or *** $p<0.001$.

5. The invasion of both MDA and MCF7 increases when in co-culture

To address whether the presence of MDA can also enhance the invasive potential of MCF7, we first assessed the number of invaded MCF7 cells in the presence of MDA_CM. We found that the MCF7 invaded significantly more when exposed to MDA_CM (relative to both control cells and cells exposed to their own CM; Fig. 6a). In contrast, the MCF7 conditioned media did not affect the invasion of MDA (Fig. 6b). To further evaluate the interaction between the two cell types, we mixed fluorescently labelled MCF7 and unlabelled MDA cells in a 1:1 ratio and allowed them to invade in a co-culture transwell assay. As controls, we used monocultures of each line containing the same number of cells as the total number in the co-culture (i.e., both mono and co-cultures were initiated with the same number of cells). We found that the overall number of invaded cells in the co-culture was higher than in either of the two monocultures, indicating that this heterogeneous population had an overall higher invasion potential relative to either of the two corresponding homogenous populations of the same size (Fig. 6c). Then, to assess the direct effects of the interaction on each of the two lines, we compared the numbers of invaded cells in monocultures and co-culture, for each line (Fig. 6d). We found that the number of invaded MCF7 cells more than doubled when MCF7 and MDA were co-cultured. Nevertheless, surprisingly, we observed that the number of invaded MDA cells also increased in the presence of MCF7 cells (to almost double, relative to when in monoculture) (Fig. 6d), suggesting that MDA also benefited from this interaction. To test whether the increased invasive ability of MDA was dependent on the acquired invasive phenotype of MCF7, we pre-incubated (for 1 hour) MCF7 with the TGFβRI inhibitor prior to co-culturing the two lines. We found that when MCF7 was pretreated with the inhibitor, the invasive abilities of both lines were lower relative to control co-cultures (Fig. 6e), indicating that the invasive benefit of MDA is dependent on the MCF7 being able to respond to the TGF-β1 released by MDA.

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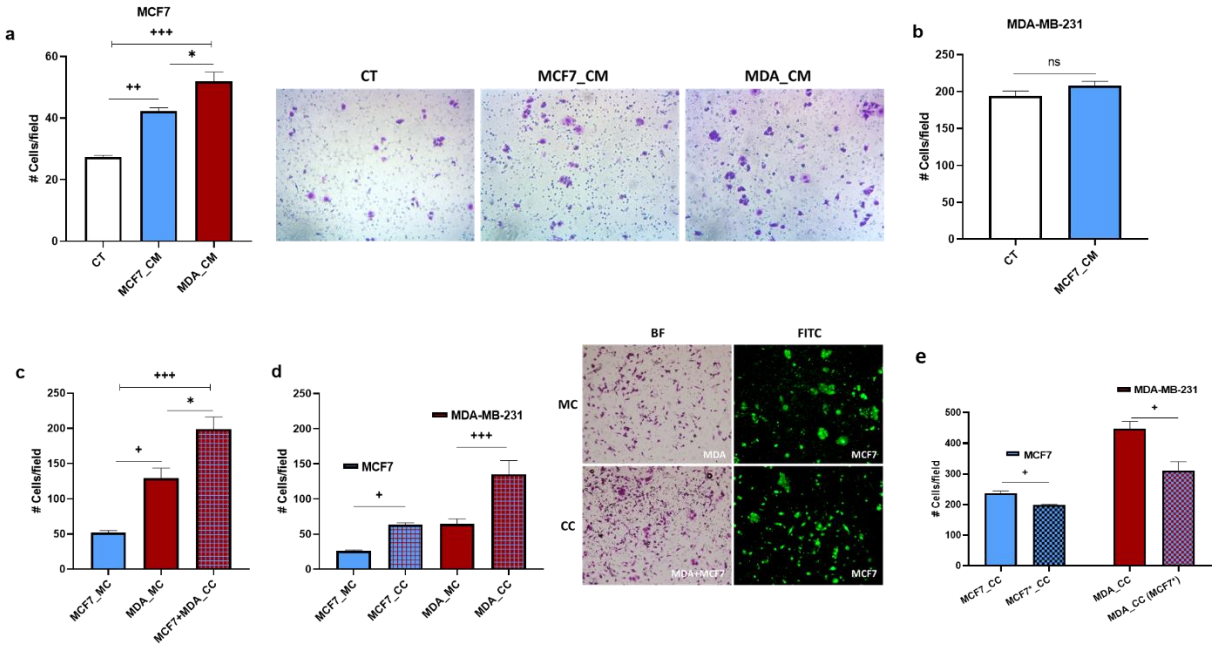

Fig. 6. The invasive capabilities of MCF7 and MDA in the presence of CM and in co-culture. **(a)** MCF7 was treated with CM from either MCF7 or MDA, and **(b)** MDA was treated with CM from MCF7, and the number of invaded cells was assessed after 72 hours using a transwell assay (CT – untreated control cells; constitutive invasion); representative images are also shown. **(c)** The same total number of MCF7 or MDA cells in monoculture (MC) and co-culture (CC; 1:1 ratio) were allowed to invade for 72 hours (transwell assay), and the overall number of invaded cells was assessed. **(d)** Comparison between the numbers of invaded cells when in monoculture (MC) and co-culture (CC) for both MCF7 and MDA; MCF7_MC and MDA_CM represent half of the invaded cells in monoculture, to allow direct comparisons with the number of invaded cells of each line when in co-culture (MCF7_CC and MDA_CC), as the co-culture contained only 50% of the cells in each monoculture. **(e)** Comparison between the numbers of invaded cells for both MCF7 and MDA when in co-culture (CC) with naïve or pretreated (with the TGFβRI inhibitor; MCF7*) MCF7. To assess the total number of invaded cells, membranes were stained with crystal violet. In co-cultures, MCF7 was stained with DiO, and the number of MDA cells was calculated by subtracting the fluorescent MCF7 cells from the total number of cells. Y axis: Number of cells per field at x10 original magnification; Error bars indicate SEM (n = 3). + refers to control (CT) or monocultures, and * refers to comparisons between treatments. ns means no significant; + or * $p<0.05$; ++ $op<0.01$; +++ $p<0.001$.

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DISCUSSION

Intratumour heterogeneity is often associated with poor response to treatment and unfavourable prognosis. In addition to genetic and epigenetic sources, phenotypic heterogeneity can also reflect plastic responses to physical factors in the TME or signals from other cells – of the same or different genotype. The latter can be mediated by various cell-cell interactions, from antagonistic (i.e., competition) to commensalistic or cooperative (synergistic, mutually beneficial or altruistic). Positive exchanges can increase the fitness of clones and contribute to tumour growth, resistance to drugs and metastasis. Consequently, understanding the pathways involved in these interactions is of great significance for cancer treatment. This is particularly relevant to metastasis – for which treatment options are limited, despite the fact that it is the most lethal phase during cancer progression [29].

Several cooperative interactions that can affect the metastatic potential of tumours have been reported, and they differ greatly in their nature and the signalling pathways and mechanisms involved. For instance, such interactions can be based on the secretion of exosomes (e.g., exosomes from a highly metastatic melanoma cell line significantly increased the metastatic potential of a poorly metastatic tumour cell line injected into mice [30]) and/or miRNAs (e.g., hsa-miR21 secreted by one lung cancer cell line stimulated EMT in another clone [22]). Additionally, mesenchymal cells can secrete growth factors (e.g., fibroblast growth factor 2 [23]) or other proteins that increase the invasive abilities of other clones. For instance, in prostate cancer, mesenchymal clones secreted a matricellular protein that increased the invasive capacity of epithelial clones after inducing EMT [31]. In breast cancer, several subclones derived from the highly metastatic cell line MDA-MB-231 enhanced each other's migration and invasion through unknown secreted factors [27].

In this study, we set up to test whether highly aggressive clones can recruit less aggressive and genetically distant clones into expressing a metastatic behaviour and whether and how such interaction can synergistically benefit both clones. To do so, we chose two breast cancer cell lines with different aggressiveness levels and very different secretome profiles. MDA-MB-231 (a basal and more aggressive subtype) overexpresses more than 25 specific proteins, while MCF7 (a luminal and less aggressive subtype) secretes fifteen unique proteins [32]. Moreover, MDA releases factors that can maintain its metastatic potential (i.e., autocrine signalling) [33]. In addition, to test

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if such interactions can be generalized to other cancer types and involve genetically distant clones, we also used a lung metastatic cell line.

The TGF-β1 pathway mediates the recruitment of a less aggressive clone into expressing a metastatic behaviour

Our data show that MDA was able to recruit MCF7 into expressing a metastatic behaviour. These findings are consistent with other studies reporting that more aggressive breast lines can enhance the metastatic potential of less metastatic lines. For instance, several aggressive breast cell lines were shown to induce EMT in non-metastatic cells and increase their metastatic abilities through the secretion of miR-200 packaged in extracellular vesicles [24] or through paracrine GLI activation involving Hedgehog ligands (without inducing EMT) [25]. However, in our system, we found that MDA induced EMT and increased the migration of MCF7 through secreted TGF-β1. The conditioned medium from MDA contained both latent and active forms of TGF-β1, and both the neutralization of TGF-β1 with antibodies and the inhibition of the TGF-β receptor I interfered with the ability of MDA to recruit MCF7. MDA is known to secrete large amounts of TGF-β1 to maintain its own metastatic potential [34], and consistent with such studies, the inhibition of MDA's TGF-β receptor I suppressed its migration abilities. Interestingly, the non-small cell lung cancer we used is also able to secrete TGF-β1, although its constitutive migration does not seem to be affected by the TGF-β receptor I inhibitor (at least not at the concentration that is effective for MDA). Notably, TGF-β1 in conjunction with miRNAs was previously reported to be involved in interclonal paracrine signalling between two non-small lung cancer cell lines, resulting in EMT induction [22].

Overall, our findings suggest that both aggressive clones can release increased levels of TGF-β1, and this secreted TGF-β1 can contribute to the recruitment of the less metastatic clone through paracrine signalling. TGF-β1 is broadly present in breast TME and is associated with maintaining the metastatic tumour potential in an autocrine manner [35–37]. Also, the serum levels of TGF-β1 are known to increase following tumour progression in patients with colorectal carcinoma [37], prostatic cancer [38], and breast cancer [39]; such increase is frequently associated with poor prognosis [40]. Thus, TGF-β1 might be implicated in similar interactions in other systems.

However, a response to TGF-β1 implies that the recruited clone expresses TGF-β1 receptors and can activate migration pathways through a TGF-β1 signalling pathway. Indeed, we found that

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MCF7 was able to respond to exogenous TGF-β1, and the inhibition of the TGF-β receptor I suppressed the effect of both TGF-β1 and the conditioned medium from MDA. Interestingly, a previous study exploring the transmission of aggressive traits between breast cancer lines found that the expression of metastatic features in MCF7 in response to conditioned medium from MDA did not involve TGF-β1 paracrine signalling [41]. Instead, the effect was attributed to the secretion of cytokines, G-CSF, GM-CSF, MCP-1, and IL-8. In addition, although the conditioned media from MDA increased MCF7 invasion, it only induced a partial EMT in MCF7; and MCF7 was found to not respond to exogenous TGF-β1 [41]. However, consistent with our findings, many studies have reported that TGF-β1 does induce EMT (and the expression of mesenchymal markers) and migration in MCF7 [42–44]. These conflicting results might be due to the observation that MCF7 differentially expresses TGF-β receptor II depending on the cell passage number and the downregulation of Sp1 [43,44]. In our MCF7 cell line, both TGF-β receptors I and II are constitutively expressed (unpublished data).

Our finding that the conditioned medium from a lung cancer cell line can enhance the metastatic potential of a breast cancer line (and *vice versa*) suggests that this type of recruitment is more general and can involve distantly related clones. We suggest that such synergistic cooperative interactions can easily emerge between any clones that express the TGF-β1 receptors, regardless of the degree of overall genetic relatedness. This scenario is consistent with the so-called "green beard effect" that has been proposed to facilitate the evolution of cooperation between genealogically unrelated individuals that express the same phenotypic marker (i.e., a "green beard") linked to a cooperative behaviour [45].

Interclonal synergistic cooperative interactions can increase tumour invasion potential

Cancer cell-cell cooperative interactions can be defined as commensalism (one partner passively benefits from the activity of the other) or mutualistic (both partners benefit) [46]. In this study, we have investigated the effect of interclonal interactions in the context of both migration and invasion. Interestingly, although MCF7 benefited from the presence of MDA in terms of increasing its own migration potential, the migration of MDA was not affected by MCF7. In other words, in terms of individual migration, this interaction did not appear mutually beneficial. Rather, MCF7 increased its metastatic abilities by taking advantage of TGF-β1 released by MDA in a

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commensalistic-like manner. Nevertheless, in the context of a tumour, such an interaction resulting in an increased metastatic potential of the recipient can still provide indirect benefits to the producer. Indeed, we found that the invasive potentials of both lines were enhanced when the two lines were co-cultured in direct contact, suggesting that *in vivo*, such interclonal interactions can be mutually beneficial and synergistic. Notably, basal and luminal cell clones are known to co-exist in mammary tumours (see [18] and references therein).

Several types of collective behaviours have been previously reported during the invasion phase in various models and contexts. In most studies, leader cells can enhance the invasion of follower cells through ECM [47,48]. In most cases, the cells with low invasiveness passively benefit from the microenvironmental remodelling ability of the highly invasive cells. In one case, however, poorly invasive melanoma cells were found to take advantage of the more invasive cells by secreting an unidentified factor that induces a switch in the mode of invasion of the invasive cells, from proteolytic-independent to MT1-MMP-dependent [49]. A comparable synergistic interaction was also observed in a rat mammary carcinoma cell line with two stable subtypes, where a soluble factor released by one subtype induced collagenase secretion by the other clone, such that collagenase could only be sufficiently secreted when both cellular types were present [50]. Similarly, several MDA-MB_231 subclones released soluble factors that increased each other's invasiveness *in vitro* and *in vivo* [27].

However, in our system, we did not detect such an interaction, as the conditioned media from non-induced MCF7 did not affect the migratory or invasive ability of MDA. Also, MCF7 which was previously treated with an inhibitor of the TGF-β receptor I did not facilitate the migration of MDA when in co-culture. These findings suggest that MCF7 can benefit MDA only if exposed to the TGF-β1 secreted by MDA. Following this activation step, the two clones act synergistically to enhance their own invasion potentials either through the shared secretion of soluble factors (e.g., proteases) or taking advantage of each other's overall ECM remodelling, or both. Notably, an increase in MMP-9 secretion was found when MCF7 was previously stimulated with TGF-β1 for 24 h and co-cultured (though not in direct contact) with MDA-MB-231 [42]. In the melanoma system mentioned above [49], both protease and ECM remodelling have been found to facilitate collective invasion, though an increase in the invasion of the highly metastatic line was not reported (i.e., the interaction was not synergistic).

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Based on our findings, we propose a model whereby highly metastatic clones can recruit non-metastatic clones into expressing metastatic features (including the ability to invade), which in turn will indirectly enhance the invasion ability of the former and result in an increase in the overall invasion potential of the tumour (i.e., a "help me help you" strategy) (Fig. 7). Overall, we suggest that such cooperative interactions can easily emerge via cross-talk involving metastatic clones that are able to constitutively secrete signalling molecules that induce and maintain their own malignant state (i.e., autocrine/cell autonomous signalling) and clones that have the ability to respond (express the corresponding receptors) to those signal (i.e., paracrine/non-autonomous signalling) and switch to a malignant phenotype.

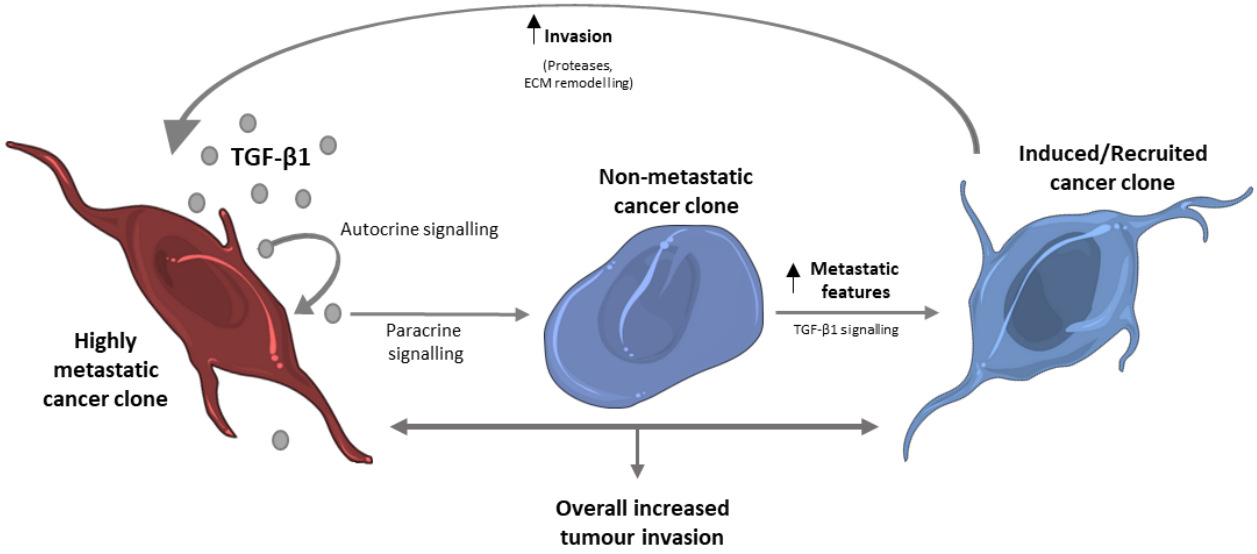

Fig. 7. Proposed model for the TGFβ1-mediated synergistic cooperative interaction between two clones with different aggressivity levels. Diagram created with free online Servier Medical Art at www.servier.com.

CONCLUSION

This study underscores the importance of deciphering the nature and mechanistic basis of interclonal cooperative interactions that could take place during the early stages of metastasis (i.e., local invasion and intravasation). Identifying the main players and signalling pathways involved in interclonal crosstalk can help develop new targets to slow down the metastatic process. Notably, Metformin – a drug used to treat type 2 diabetes, has been reported to interfere with the TGF-β1

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signalling pathway by inhibiting the response to TGF-β1 or inactivating TGF-β1 itself [51]. These mechanisms might also provide a means to affect the TGFβ1-mediated crosstalk that can underlie cooperative interactions within tumours. Taking into account the negative impact that metastasis has on cancer prognosis and the lack of therapies to directly affect this process, interfering with the specific cooperative behaviours that tumour cells engage in should provide an additional strategy to increase patient survival.

Author contributions:

Caroline S. Carneiro: designed and performed experiments, analyzed data, wrote the manuscript.

Jorian D. Hapeman: contributed to experiments, data analysis and manuscript preparation.

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

Statements and Declarations

All authors certify that they have no affiliations with or involvement in any organization or entity with any financial interest or non-financial/competing interest in the subject matter or materials discussed in this manuscript.

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