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Interclonal mutually beneficial cooperation mediated by TGF-β1 enhances invasion of breast cancer cells

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ABSTRACT

Intratumour heterogeneity is often associated with poor response to treatment and bad prognosis. In addition to genetic and epigenetic sources, phenotypic heterogeneity can also reflect plastic responses to signals from other cells. The latter can be mediated by various cell-cell interactions, from antagonistic (i.e., competition) to commensalistic or cooperative (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 such interactions is of great significance for cancer treatment. This study used two breast cancer cell lines with different aggressiveness levels and very different secretome profiles (i.e., MDA-MB-231 and MCF7) to address the nature and mechanistic basis of interclonal crosstalk through paracrine signalling involving soluble factors during the early stages of metastasis. Our data show that MDA-MB-231 is able to recruit MCF7, through TGFβ1-mediated paracrine signalling, into expressing mesenchymal features and increased migration. On the other hand, MCF7 has no effect on the migration of MDA, suggesting a passive/commensalistic interaction. However, we found that the invasive potentials of both lines are enhanced when they are co-cultured, indicating that the two lines act synergistically and that such interclonal interactions can be mutually beneficial in vivo. 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 during tumour progression should 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 are related to 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 resistance to chemotherapies [2, 3]. High levels of intratumour heterogeneity are the result of both genetic mutations or epigenetic alterations and phenotypic plasticity [3].

Transitory changes in cancer cell state or phenotype can be triggered by either physicochemical modifications in the tumour microenvironment (e.g., 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 hat 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 inter-clonal selection that would result in selective sweeps and a reduction in levels of heterogeneity [13]. However, in addition to competing, cancer clones are also known to cooperate with each other or with other non-cancer cells in several ways. 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, more recently, the finding 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 also take place during this complex process [20].

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 paracrine signalling (and phenotypic plasticity) or microenvironmental remodelling. 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 cancer [24, 25]. Moreover, cell cooperation can involve remodelling the tumour microenvironment (TME). For

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example, 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 many examples of possible inter-clonal cooperative interactions have been reported, the extent and the means through which different cancer clones can cooperate, especially during the initial steps of the metastatic process, are not fully understood. Identifying and understanding 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) explored the type of interactions (e.g., competitive, mutually beneficial, altruistic or commensalistic) that can take place between clones with different metastatic potential and (ii) investigated the underlying signalling pathways. 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) and luminal (MCF7). 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 and investigate the signalling molecules and pathways involved, we either subjected each line to the conditioned medium of the other line or grew the two cell lines in a coculture system. To assess changes in metastatic behaviour, we assessed morphological changes associated with EMT and investigated the capacity of cells to migrate and invade.

MATERIALS AND METHODS

Cell lines and culturing conditions

Two human breast cancer cell lines, MCF7 and MDA-MB-231, were obtained from ATCC. An adherent lung cancer cell line (H2122 AS) was obtained 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 % 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.

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Reagents

Human Recombinant TGF-β1 (R&D systems; at 10 ng/ml final concentration) was used as a positive control. A TGF-β1 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 TGF-β1 activity.

Fluorescence staining and microscopy

Cells were stained with either DiO or DiD (Invitrogen™ Vybrant™) for coculture experiments following the manufacturer's instructions. The stained cells were cocultured 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 coculture 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 released factors. Radom fields (using a 4x objective) 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, from 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 a layer of 1mg/ml Matrigel (Corning), and the number of cells that invaded was assessed after 72 hours. In the coculture 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

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number of invaded MCF7 cells (i.e., fluorescent) from the total number of invaded cells (i.e., stained with crystal violet).

Ultrafiltration

Conditioned media was 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.

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 a highly metastatic clone (such as MDA-MB-231; abbreviated as MDA from herein on) can induce an EMT-like change in a less metastatic clone (such as MCF7), we either subjected MCF7 to the conditioned medium collected from MDA or cocultured the two cell lines (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 transition 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 coculture 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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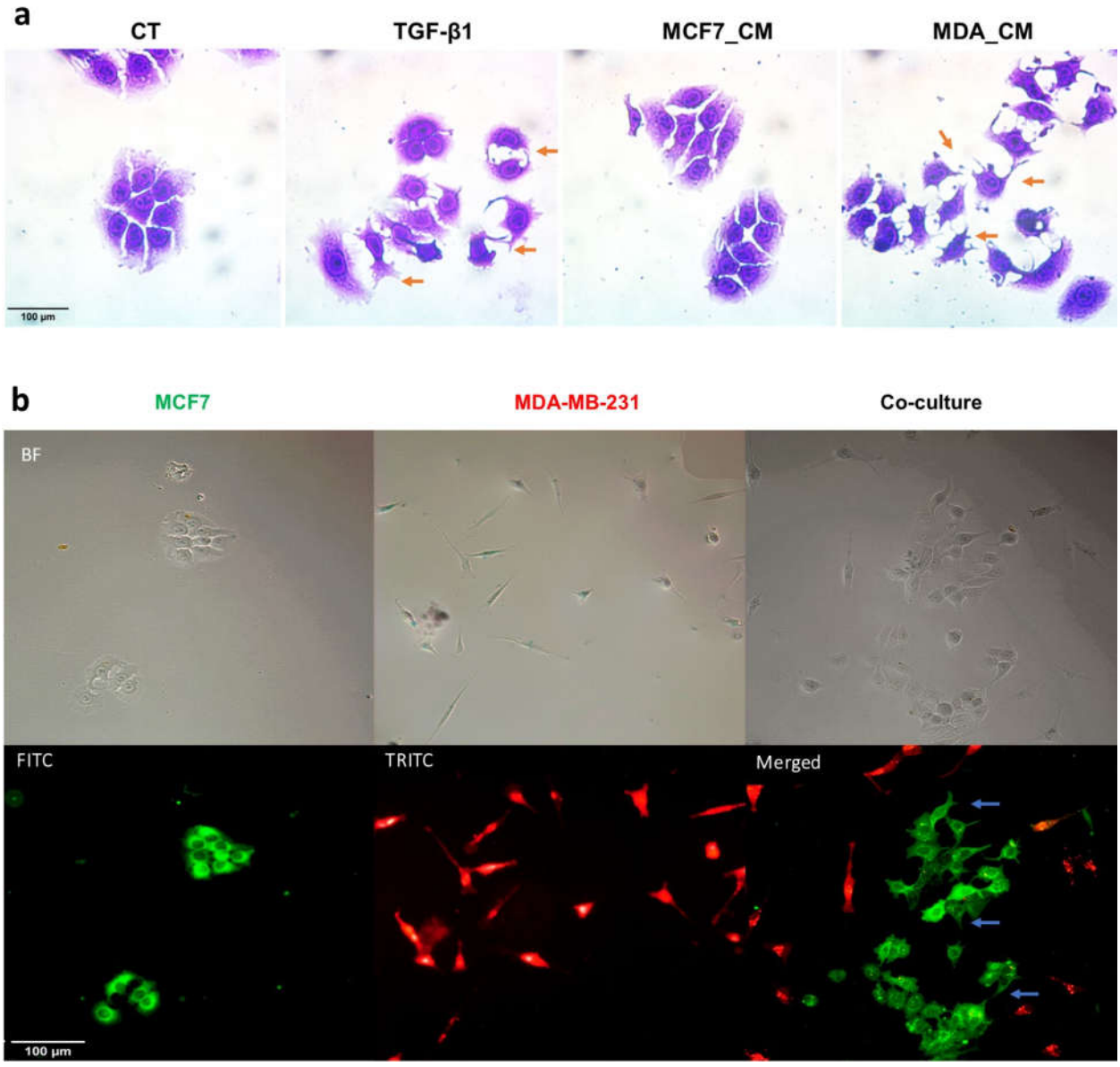

Fig. 1. MDA induced an EMT-like change in MCF7. (a) MCF7 cells were treated with MCF7 CM or MDA CM and TGF-β1 (positive control) for 72 hours. Cells were fixed with ethanol and stained with 0.2% crystal violet; (b) MCF7 cells were cultured in direct contact with MDA cells for 72 hours. Cells were stained 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 conditioned medium from MDA (using both wound-healing and transwell assays) as well as in

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coculture with MDA (wound-healing assay). Overall, the migration of MCF7 was increased in all contexts. Specifically, the MDA-conditioned media significantly increased the migration of MCF7 (compared to the effect induced by its own CM) to a level similar to that induced by TGF-β1 (Fig. 2a and 2b). The migration of MCF7 was also significantly increased when MCF7 and MDA cells were cocultured (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 coculture with MCF7 (Fig.2d and 2e).

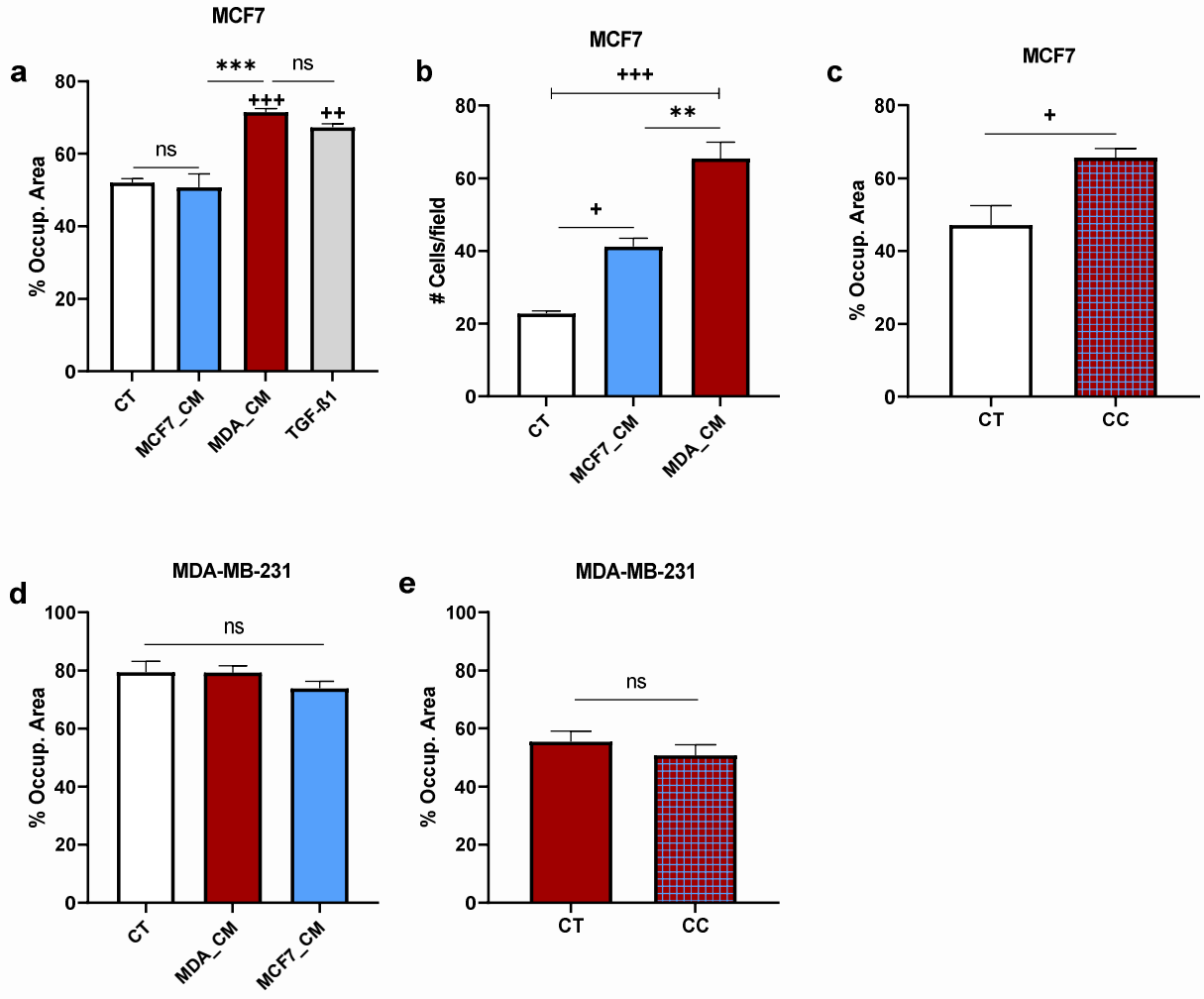

Fig.2. MDA enhanced the migration of MCF7. The migration of MCF7 was assessed in the presence of the CM collected from MCF7 (MCF7_CM) or MDA (MDA_CM) – using wound healing for 48h **(a)** or transwell 72h **(b)** assays, as well as in coculture with MDA for 72h – using wound-healing assays **(c)**. Y-axis indicates the percentage of the occupied area (a, c, d and e) or the number of cells per field (at x10 original magnification) (b). TGF-β1 (10 ng/ml) was used as a positive control. MDA migration was also tested after 24 hours of contact with MCF7 CM **(d)** or in coculture with MCF7 **(e)**. 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 *** $p<0.001$ ++++ or **** $p<0.0001$.

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3. The invasion of both MDA and MCF7 increases when in coculture

First, to address whether the presence of MDA can also enhance the invasive potential of MCF7, we 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. 3a). In contrast, the MCF7 conditioned media did not affect the invasion of MDA (Fig. 3b). To further evaluate the interaction between the two cell types, we mixed labelled MCF7 and MDA cells in a 1:1 ratio and allowed them to invade in a coculture transwell assay. As controls, we used monocultures of each line containing the same number of cells as the total number in the coculture (i.e., both mono and cocultures were initiated with the same number of cells). We found that the overall number of invaded cells in the coculture 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. 3c). 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 coculture, for each line (Fig. 3d). We found that the number of invaded MCF7 cells more than doubled when MCF7 and MDA were cocultured. Nevertheless, surprisingly, we observed that the number of invaded MDA cells also increased (to almost double, relative to when in monoculture) in the presence of MCF7 cells (Fig. 3d), suggesting that MDA also benefited from this interaction.

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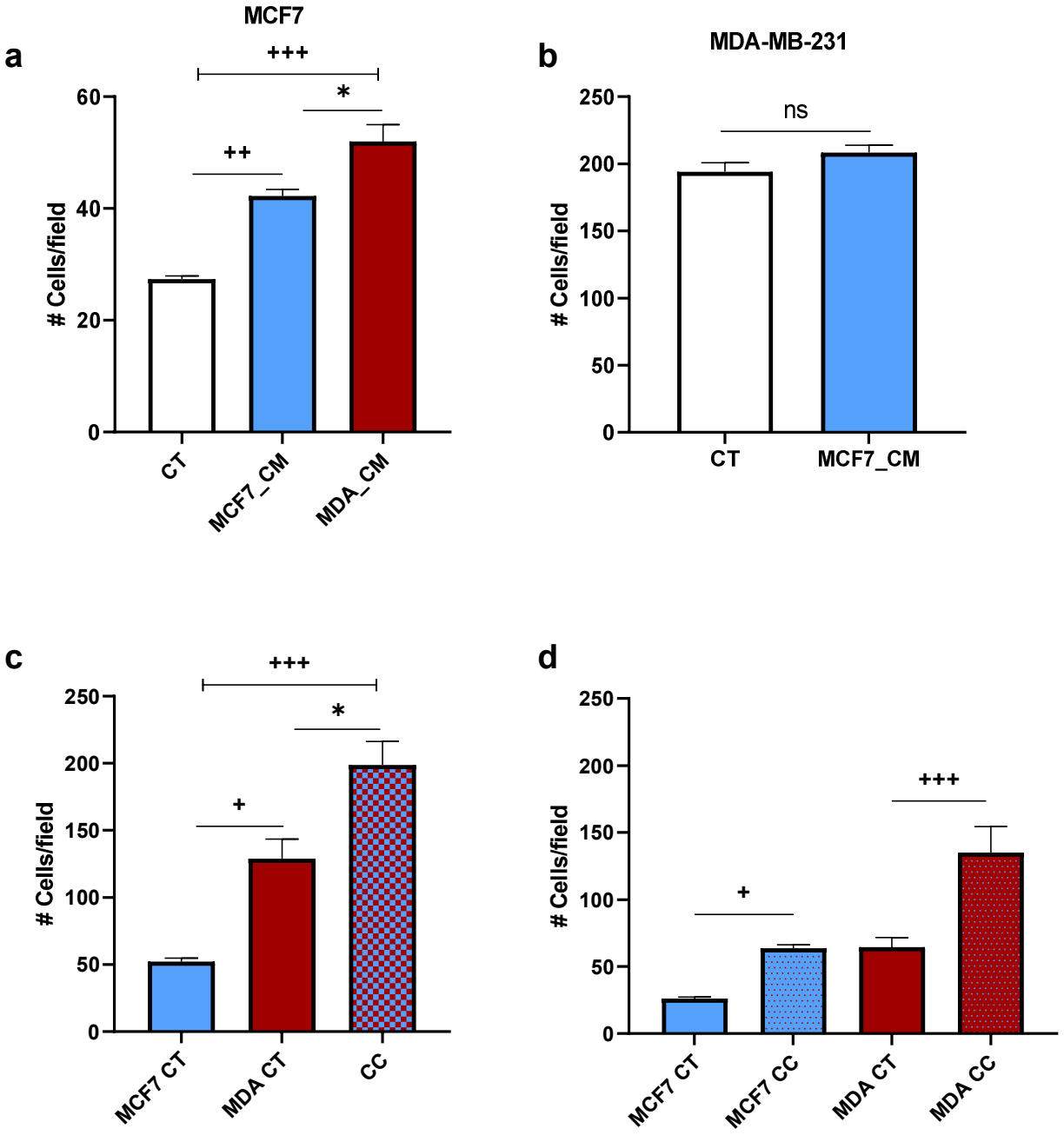

Fig. 3. The invasive capabilities of MCF7 and MDA in the presence of CM and in coculture. (a) MCF7 was treated with CM from either MCF7 or MDA, and **(b)** MDA was treated with CM from MCF7, and the number of invaded cells was assessed after 72 hours using a transwell assay (CT – untreated control cells; constitutive invasion). **(c)** The same total number of cells in monoculture (MCF7 or MDA) and coculture (MCF7+MDA; 1:1) 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 (control – CT) and coculture (CC) for both MCF7 and MDA; MCF7 CT and MDA CT represent half of the invaded cells in monoculture, to allow direct comparisons with the number of invaded cells of each line when in coculture (MCF7 CC and MDA CC), as the coculture contained only 50% of the cells in each

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monoculture. To assess the total number of invaded cells, membranes were stained with crystal violet. In cocultures, 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), and * refers to treatments. ns means no significant; + or * $p<0.05$; ++ or **$p<0.01$; +++ or *** $p<0.001$ ++++ or **** $p<0.0001$.

4. 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-β1 receptor inhibitor or antibodies against TGF-β. We found that inhibiting either the TGF-β1 receptor or the TGF-β itself significantly decreased the effect of MDA_CM on the migration of MCF7, with the inhibitor completely blocking its effect (Fig. 4a and 4b). Consistent with MDA secreting and responding to its own TGF-β1 (i.e., autocrine signalling), the TGF-β1 receptor inhibitor decreased the migration of MDA (Fig. 4c). On the other hand, the TGF-β1 receptor inhibitor did not affect the un-induced migration of MCF7 (Fig. 4a), suggesting that the constitutive MCF7 migration does not rely on TGF-β1.

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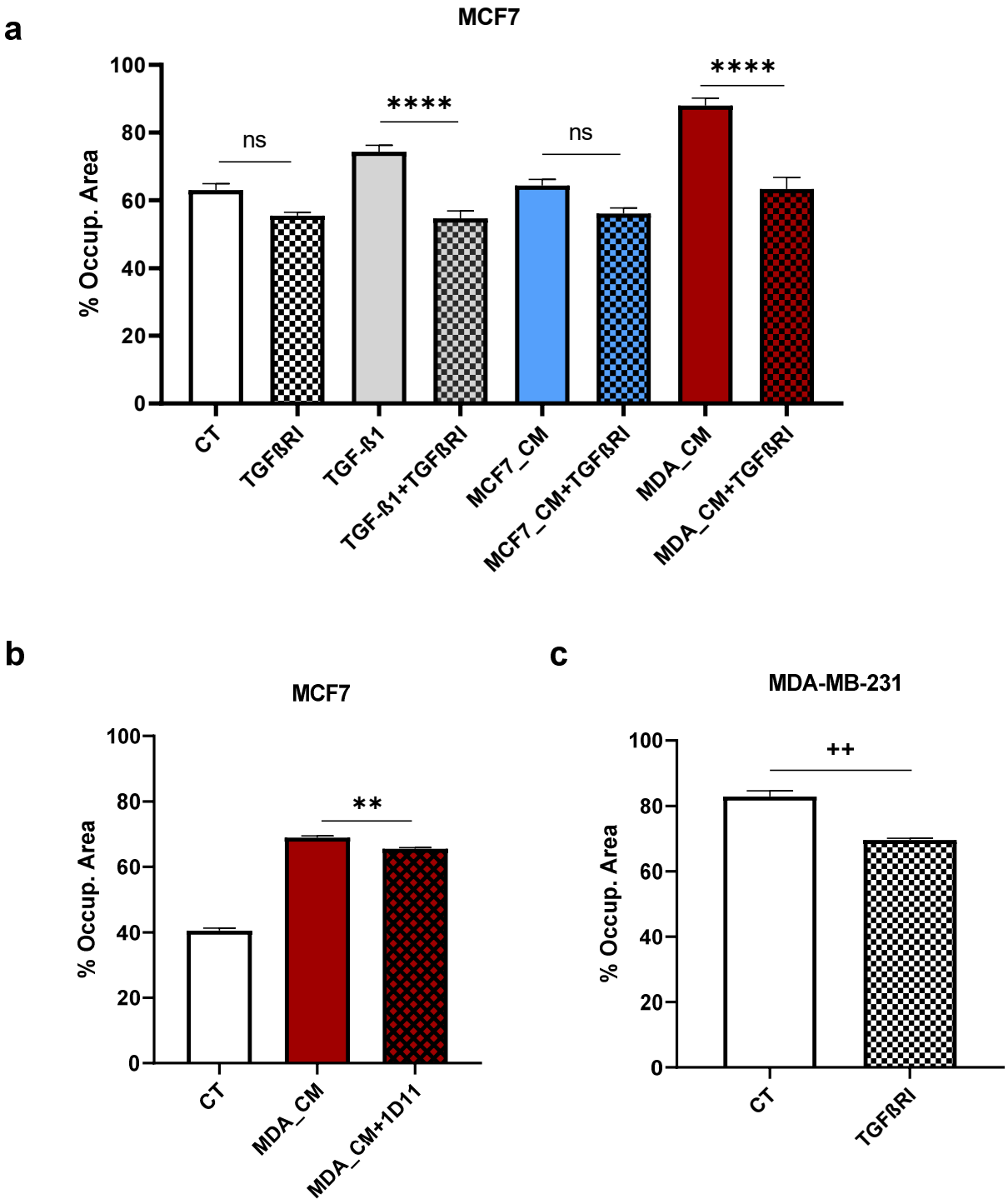

Fig. 4. The effect of TGF-β pathway inhibition on the migration of MDA and MCF7. (a) The effect of a TGF-β receptor I inhibitor (10 µM; TGFβRI) on the migration of MCF7 control (CT) cultures as well as cultures exposed to either TGF-β1 (as a positive control), MCF7_CM or MDA_CM. **(b)** The effect of MDA_CM neutralized with antibodies against TGF-β 1, 2, and 3 (2 µg/ml; CM+1D11) on the migration of MCF7 (wound-healing assay; 72 hours). **(c)** The effect of a TGF-β receptor 1 inhibitor on the migration of

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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 * $p<0.05$; ++ or **$p<0.01$; +++ or *** $p<0.001$ ++++ 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. Consistent with the size of the various TGF-β1 forms (including an active form around 28 kDa and a latent form of 220 kDa), we found that the most effective fractions were those containing molecules between 30 and 100kDa, and above 100kDa (Fig. 5a). Furthermore, their activity was decreased by the addition of the TGF-β1 receptor inhibitor (Fig. 5b). 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. 5c).

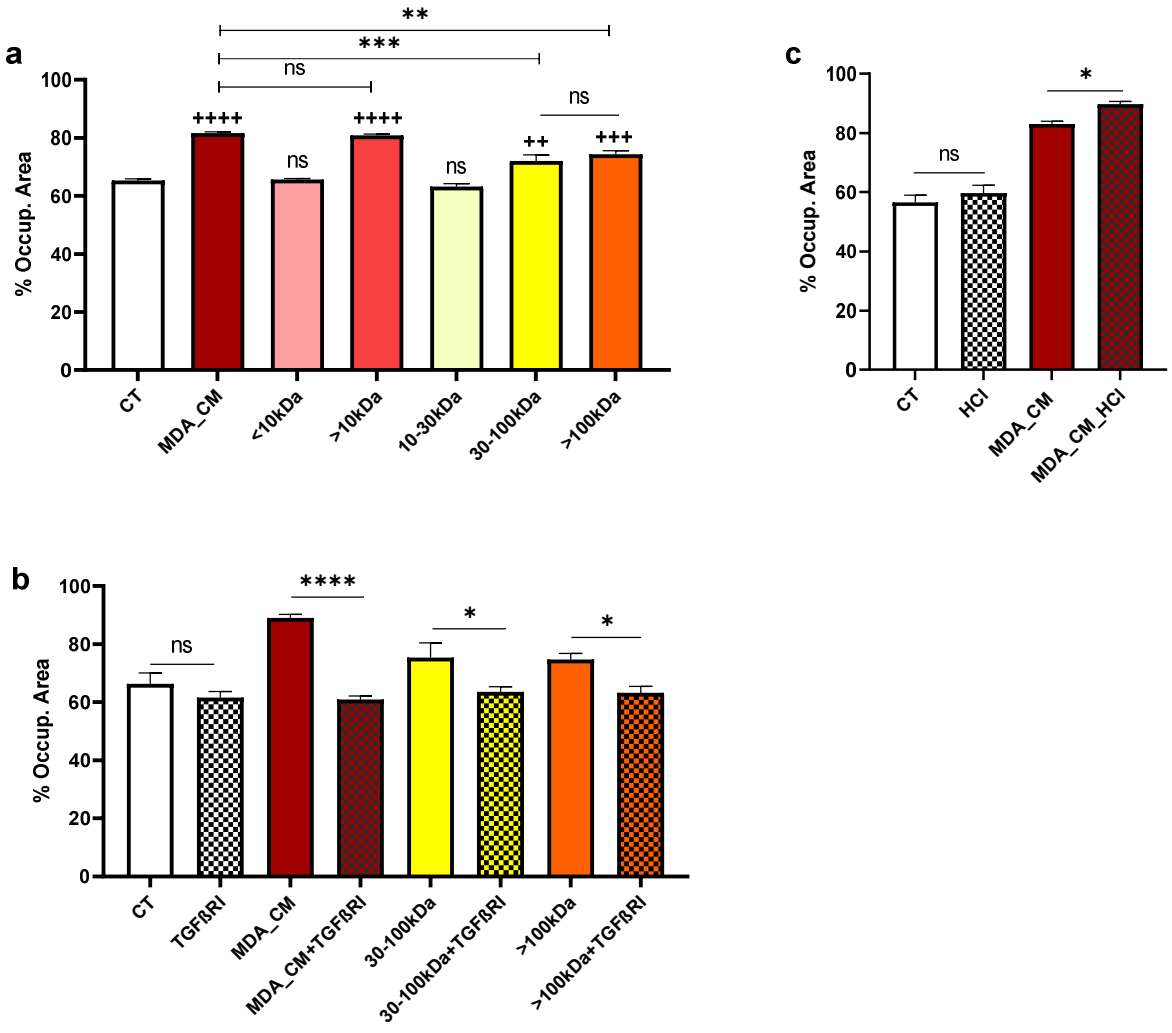

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Fig. 5. MDA_CM contains both active and inactive TGF-β1 forms. (a) Wound-healing assay performed with MCF7 incubated with full MDA_CM and various MDA_CM fractions. **(b)** The effect of the TGF-β1 receptor inhibitor on the ability of the full MDA_CM 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 = 3). + refers to control (CT), and * refers to treatments. ns means no significant; + or * $p<0.05$; ++ or ** $p<0.01$; +++ or *** $p<0.001$ ++++ or **** $p<0.0001$.

5. TGF-β-mediated inter-clonal cooperation is not specific to breast cancer

To explore the possibility that other cancer cell types might affect each other's metastatic capabilities using TGF-β signalling, 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. 6a). 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 inhibitor (Fig. 6b). 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. 6c). Again, consistent with this effect involving TGF-β1, the receptor inhibitor fully blocked the effect of the MDA_CM on H2122 AS migration (Fig. 6d). However, in contrast to MDA, the TGF-β receptor inhibitor did not affect the constitutive migration of H2122 AS (Fig. 6d and 4c).

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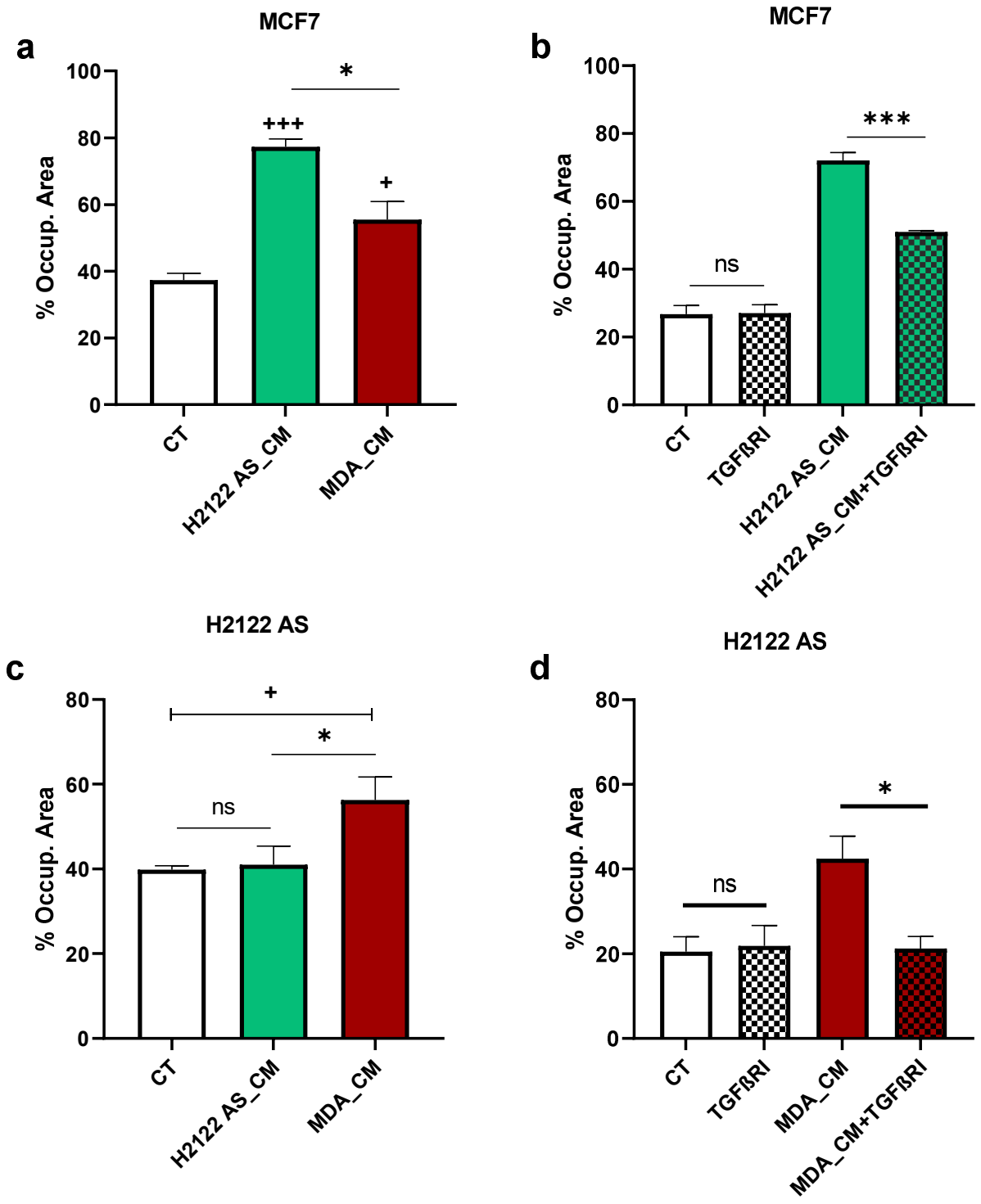

Fig. 6. Inter-clonal cooperation between breast and lung cancer cell lines. (a) The effect of CM collected from MDA or H2122 AS on the migration of MCF7. **(b)** The effect of the TGF-β1 receptor inhibitor (TGFβRI) on the MCF7 migration induced by H2122AS_CM (wound-healing assay; 48 hours). **(c)** The effect of MDA_CM on the migration of H2122 AS; **(d)** The effect of the TGF-β1 receptor inhibitor on the H2122 AS migration induced by MDA_CM (wound-healing assay; 48 hours). Y axis: Percentage of the occupied area. CT – Control/Constitutive migration. Error bars indicate SEM (n = 3). + refers to control

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DISCUSSION

Intratumour heterogeneity is often associated with poor response to treatment and bad 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 (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]) or miRNAs (e.g., hsa-miR21 secreted by one lung cancer cell line stimulated EMT in another clone [22]). Additionally, mesenchymal cells can secrete 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 clones derived from the highly metastatic cell line MDA-MB-231 enhanced each other's migration and invasion through unknown secreted factors [32].

In this study, we set up to address both the nature and mechanistic basis of interclonal crosstalk through paracrine signalling involving soluble factors during the early stages of metastasis. 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 [33]. Moreover, MDA releases factors that can maintain its metastatic potential (i.e., autocrine signalling) [34].

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

Our data show that MDA is able to recruit MCF7 (through the secretion of soluble factors) into expressing a metastatic behaviour by inducing EMT, which consequently increased its migration. These findings are consistent with other reports from the literature on breast cancer in the sense that more aggressive breast lines can enhance the metastatic potential of less metastatic lines. For instance, aggressive breast cell lines can 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 Hh ligands [25].

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However, we found that in our system, MDA induced EMT and migration in MCF7 through the release of 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-β1 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 [35], and consistent with such studies, the inhibition of TGF-β1 receptor I on MDA suppressed its migration abilities. TGF-β is broadly present in breast TME and is associated with maintaining the metastatic tumour potential in an autocrine manner [36–38]. The serum levels of TGF-β1 increase following tumour progression in patients with colorectal carcinoma [39], prostatic cancer [40], and breast cancer [41]; such increase is frequently associated with poor prognosis [42]. Our findings suggest that increased levels of TGF-β1 released by the more aggressive clones can contribute to the recruitment of less metastatic clones through paracrine signalling.

A response to TGF-β1 implies that the recruited clone expresses TGF-β1 receptors and can activate EMT through a TGF-β1 signalling pathway. Indeed, we found that MCF7 responded to exogenous TGF-β1, and the inhibition of the TGF-β1 receptor I suppressed the effect of both TGF-β1 and the conditioned medium from MDA. Furthermore, consistent with the activation of the EMT pathway, we found a downregulation of the gene encoding the epithelial marker cadherin 23 and an upregulation of the mesenchymal marker vimentin gene in MCF7 cells exposed to the MDA_CM for 24 hours (our unpublished data).

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 [43]. Instead, the effect was attributed to the secretion of cytokines, G-CSF, GM-CSF, MCP-1, and IL-8. In addition, although MDA_CM increased MCF7 invasion, it only induced a partial EMT in MCF7; and MCF7 was found to not respond to exogenous TGF-β1 [43]. 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 [44, 45]. Notably, consistent with our findings, many studies have reported that TGF-β1 does induce EMT and migration in MCF7 [46–48].

In addition to our study, TGF-β1 was previously reported to also be involved in interclonal paracrine signalling between two lung cancer cell lines, resulting in EMT induction [22]. Furthermore, 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.

The TGFβ1-mediated interclonal interaction is mutually beneficial during invasion

Cancer cell-cell interactions are generally classified based on their effects on the fitness of interacting cells, often in the framework of ecological interactions between different species (e.g., competition, mutualism, symbiosis, commensalism) [49]. Interactions within species are generally discussed in the framework of cooperation (mutually beneficial/public goods or altruistic), selfishness or spite. 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

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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 commensalistic-like manner. Nevertheless, in the context of a tumour, such an interaction resulting in increased migratory abilities of the recipient can provide indirect benefits to the producer. Indeed, we found that the invasive potentials of both lines are enhanced when the two lines are cocultured, suggesting that in vivo, such interclonal interactions can be mutually beneficial.

Several types of collective behaviours have been reported during the invasion phase in various models and contexts. In most reported cases, leader cells can enhance the invasion of follower cells through ECM [50, 51]. In these cases, the cells with low invasiveness passively benefit from the microenvironmental remodeling ability of the highly invasive cells. In one case, the poorly invasive cells were found to take advantage of the more invasive cells by secreting a factor that induces a switch in the mode of invasion of the invasive cells [52]. Similarly, several MDA-MB_231 subclones released soluble factors that increased each other's invasiveness *in vitro* and *in vivo* [32]. 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. These findings suggest that MCF7 can benefit MDA only after it responds to the MDA secreted factors (including TGF-β1). Following this activation step, the two clones act synergistically to increase their invasion potential either through the secretion of soluble factors (e.g., proteases) or the overall ECM remodelling. A similar scenario was reported in a rat mammary carcinoma cell line with two stable subtypes, where a soluble factor released by one subtype induces collagenase secretion by the other clone, such that collagenase could only be sufficiently secreted when both cellular types were present [27].

CONCLUSION

This study underscores the importance of understanding the nature and mechanistic basis of interclonal cooperative interactions that could take place during the early stages of metastasis. Identifying the main players and signalling pathways involved in interclonal crosstalk can help develop new targets to slowdown the metastatic process. 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.

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