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Tumour-initiating capacity is independent of epithelial–mesenchymal transition status in breast cancer cell lines

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Background: Epithelial–mesenchymal transition (EMT) and cancer stem cells (CSCs) are considered to be crucial for cancer biology. The purpose of this study was to determine whether EMT directly led to the acquisition of tumour-initiating capacity in breast cancer cell lines.

Methods: Epithelial–mesenchymal transition was induced in five breast cancer cell lines and one normal breast cell line by EMT-related cytokine stimulation. Mesenchymal–epithelial transition (MET) was induced by stably overexpressing miR-200c in three mesenchymal-like breast cancer cell lines. Molecular expression and cell function analysis were performed to evaluate the effect of EMT or MET on tumour-initiating capacity and other biological characteristics.

Results: The induction of EMT did not enhance tumour-initiating capacity but, instead, conferred a $CD44^+/CD24^{-/low}$ phenotype as well as cell proliferation, migration, and resistance to doxorubicin and radiation on breast cancer cell lines. Furthermore, MET did not lead to inhibition or loss of the tumour-initiating capacity in mesenchymal-like breast cancer cell lines, but it markedly attenuated other malignant properties, including proliferation, invasion, and resistance to therapy.

Conclusions: Epithelial–mesenchymal transition does not alter tumour-initiating capacity of breast cancer cells but some other biological characteristics. Therefore, EMT and tumour-initiating capacity may not be directly linked in breast cancer cell lines.

Recently, studies of neoplastic tissues have provided evidence indicating that cancer originates from a small fraction of cancer cells capable of initiating and developing tumours—usually termed tumour-initiating cells (TICs), tumorigenic cells, or cancer stem cells (CSCs) (Al-Hajj et al, 2003a, 2004). It has been suggested that TICs are the source of tumour formation and are also responsible for tumour progression, metastasis, resistance to therapy, and subsequent tumour recurrence (Ailles and Weissman, 2007; Rosen and Jordan, 2009). Breast cancer is the first human tumour from which TICs were identified and isolated as a subpopulation of breast cancer cells with markers of $CD44^+/CD24^{-/low}$ (Al-Hajj et al, 2003a) or high intracellular aldehyde dehydrogenase activity (ALDH+) (Ginestier et al, 2007). Currently, the existence of TICs is based on the important evidence that these cells have tumour-initiating capacity when transplanted into highly immunocompromised mice, whereas non-TICs inefficiently produce tumours.

Epithelial–mesenchymal transition (EMT) is a highly coordinated process and a multi-step event during which epithelial cells

lose numerous epithelial characteristics accompanied by acquisition of mesenchymal characteristics (Thiery et al, 2009). Accumulating evidence suggests that aberrant activation of the EMT developmental programme contributes to tumour initiation, invasion, metastatic dissemination, and acquisition of therapeutic resistance (Yang and Weinberg, 2008; Acloque et al, 2009; Kalluri and Weinberg, 2009; Thiery et al, 2009). Moreover, this process can be reversed through mesenchymal–epithelial transition (MET), where a migratory cell becomes anchored at distant sites, loses its migratory potential, and colonises to macrometastases (Takebe et al, 2011). These lines of evidence indicate that EMT appears to functionally overlap with TICs. Several studies have shown that normal and transformed breast epithelial cell lines that had undergone EMT formed tumours more efficiently in vivo than control cells (Mani et al, 2008; Morel et al, 2008). Hence, it will be very meaningful to explore whether EMT directly leads to the acquisition of tumour-initiating capacity of cells in breast cancer cell lines. In this study, we attempted to investigate the effect of different EMT status on tumour-initiating capacity, proliferation, invasion, and resistance to therapy in several breast cancer cell lines.

MATERIALS AND METHODS

Cell culture. T47D, MCF7, ZR-75-1, BT474, MDA-MB-231, MDA-MB-435S, and BT549 cells were obtained from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). MCF-10A cells were obtained from American Tissue Culture Collection (ATCC, Manassas, VA, USA). T47D, ZR-75-1, BT474, MDA-MB-231, and BT549 cells were maintained in RPMI 1640 (Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco), $100 \, IU \, ml^{-1}$ penicillin, $100 \, \mu g \, ml^{-1}$ streptomycin, and 2 mM L-glutamine. MCF7 cells were maintained in DMEM (Gibco) supplemented with 10% FBS, $100 \, IU \, ml^{-1}$ penicillin, $100 \, \mu g \, ml^{-1}$ streptomycin, and 2 mM L-glutamine; MDA-MB-435S cells were maintained in Leibovitz's L-15 (Gibco) supplemented with 10% FBS; MCF-10A cells were maintained in DMEM/F12 (1:1) (HyClone, Logan, UT, USA) supplemented with 5% horse serum (HyClone), $20 \, ng \, ml^{-1}$ epidermal growth factor (EGF; Peprotech, Rocky Hill, NJ, USA), $100 \, ng \, ml^{-1}$ cholera toxin (Sigma, St Louis, MO, USA), $10 \, \mu g \, ml^{-1}$ insulin (Sigma), and $0.5 \, \mu g \, ml^{-1}$ hydrocortisone (Sigma).

For EMT induction, cells were cultured in media with $50 \, ng \, ml^{-1}$ IL-6 (Peprotech), $20 \, ng \, ml^{-1}$ EGF (Peprotech) and $20 \, ng \, ml^{-1}$ bFGF (Peprotech), or $10 \, ng \, ml^{-1}$ transforming growth factor-β1 (TGF-β1; Peprotech) at $37 \, ^{\circ}C$ in 5% CO_2.

Real-time RT-PCR analysis. Cells were harvested, and RNA was extracted using Trizol (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol. One microgram of total RNA was reverse transcribed into cDNA and real-time PCRs using the SYBR Green PCR Master Mix (Takara, Dalian, China) were performed using an ABI PRISM 7500 Sequence Detection System (Perkin-Elmer/Applied Biosystems, Rotkreuz, Switzerland). Data were analysed and normalised to 18S expression. Primer sequences are as follows:

E-cadherin: forward, 5′-CCCACCACGTACAAGGGTC-3′, reverse, 5′-CTGGGGTATTGGGGGCATC-3′; vimentin: forward, 5′-CGCCAGATGCGTGAAATGG-3′, reverse, 5′-ACCAGAGGGAGTGAATCCAGA-3′; N-cadherin: forward, 5′-TTAAAGCGGCTGACAATGAC-3′, reverse, 5′-CCCCCAGTCGTTCAGGTAAT-3′; fibronectin: forward, 5′-CAGTGGGGAGACCTCGAGAAG-3′, reverse, 5′-TCCCTCGGAACATCAGAAAC-3′; twist: forward, 5′-GTCCGCAGTCTTACGAGGAG-3′, reverse, 5′-GCTTGAGGGTCTGAATCTTGCT-3′; CD44: forward, 5′-CAGCAACCCTACTGATGATGACG-3′, reverse, 5′-GCCAAGAGGGATGCCAAGATGA-3′; CD24: forward, 5′-TTTACAACTGCCTCGACACA-3′,

reverse, 5′-CGATCTGTTTGTTCCCATGT-3′; 18S: forward, 5′-CCTGGATACCGCAGCTAGGA-3′, reverse, 5′-GCGGCGCAATA CGAATGCCCC-3′.

Western blotting analysis. Primary antibodies included mouse anti-E-cadherin (1:5000; BD Biosciences, San Jose, CA, USA), mouse anti-vimentin (1:100; Clone V9, Dako, Glostrup, Denmark), rabbit anti-CD44 (1:100; GeneTex Inc., Irvine, CA, USA), and mouse anti-CD24 (1:100; Lifespan Biosciences, Seattle, WA, USA). Secondary antibodies included rabbit anti-mouse IgG-HRP (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and goat anti-rabbit IgG-HRP (1:1000; GE Healthcare, Chalfont St Giles, UK). Horseradish peroxidase-conjugated monoclonal mouse anti-GAPDH (Kangchen, Shanghai, China) was used as an internal parameter. All antibodies were diluted with 5% milk in phosphate-buffered saline (PBS) containing 0.1% Tween-20 (PBS-T) and incubated for either 1 h at room temperature or overnight at $4 \, ^{\circ}C$. All western blots were visualised with ECL Western blotting substrate (Pierce, Rockford, IL, USA).

Construction of the pGIPZ-hsa-mir-200c plasmid. The hsa-mir-200c sequence was amplified from the genomic DNA of humans by a forward primer: '5′-CAACAGAAGGCTCGAGGAAGTGT CCCCAGGGACTC-3′' and a reverse primer: '5′-ATTCTGATCA GGATCCAACGCTCTCAGCTCAAGACG-3′'. The PCR products of 332 bp were reclaimed from agarose gel electrophoresis and cloned into lentivirus shuttle plasmid pGIPZ (Open Biosystems, Huntsville, AL, USA) between the enzyme sites XhoI and BamHI.

All constructs were verified by sequencing. Lentivirus packaging followed the standard instruction (Kutner et al, 2009). Titre of lentivirus was measured by qPCR experiment in 293T cells (Kutner et al, 2009).

Flow cytometry. Cells were trypsinised, suspended into single-cell mixtures and washed with PBS. Next, cells were incubated on ice for 30 min with monoclonal antibodies specific for human cell surface markers including CD44-FITC/CD24-PE or CD44-PE/CD24-APC (eBioscience, San Diego, CA, USA). In negative control experiments, cells were incubated with fluorescence-labelled isotype-matched pre-immune IgG instead. The cells were washed and analysed using a flow cytometer (BD FACSAria, San Jose, CA, USA).

Three-dimensional invasion assays. The pre-chilled culture surface was coated with a thin layer of matrigel (BD Biosciences) by slowly pipetting $60–100 \, \mu l$ of matrigel directly onto the culture surface, spreading it evenly with a pipette tip and incubating for 30 min at $37 \, ^{\circ}C$ to allow the matrigel to gel. Cells were mixed with complete culture medium of 5% matrigel and were then seeded into pre-coated plates. The cells were cultured at $37 \, ^{\circ}C$ in 5% CO_2.

In vivo **tumorigenicity assays.** Cells were resuspended in a 1:1 (v/v) mixture of culture media and matrigel (BD Biosciences), and cells were injected into the breast of 4-week-old female NOD/SCID mice based on limiting dilution assays. To continue to acquire the stimulation of cytokines for some time in vivo, we suspended the cytokine-induced cells in the matrigel containing IL-6, EGF/bFGF, or TGF-β1. Tumour growth was monitored every 5 days with calipers at the site of injection. The tumour volume was calculated as follows: tumour volume $(mm^3) = length \times (width)^2 \times 0.5$. Animal maintenance and experiments were performed in accordance with the animal care guidelines of the Southern Medical University, Guangzhou, China. All animal experiments were approved by the Animal Care and Use Committee of the Southern Medical University, Guangzhou, China.

Statistical analyses. Differences among groups were analysed using a chi-squared or variance (ANOVA) method using SPSS (13.0) software (SPSS, Chicago, IL, USA). A probability level of 0.05 was chosen for statistical significance.
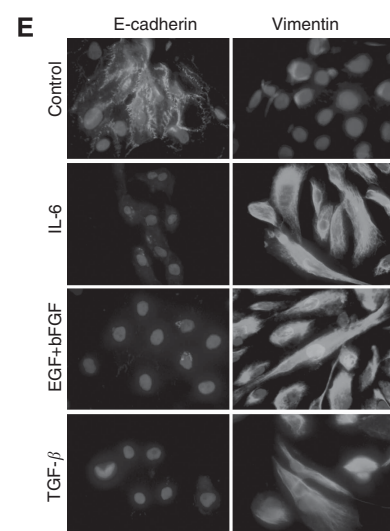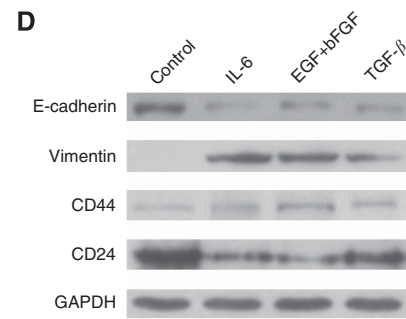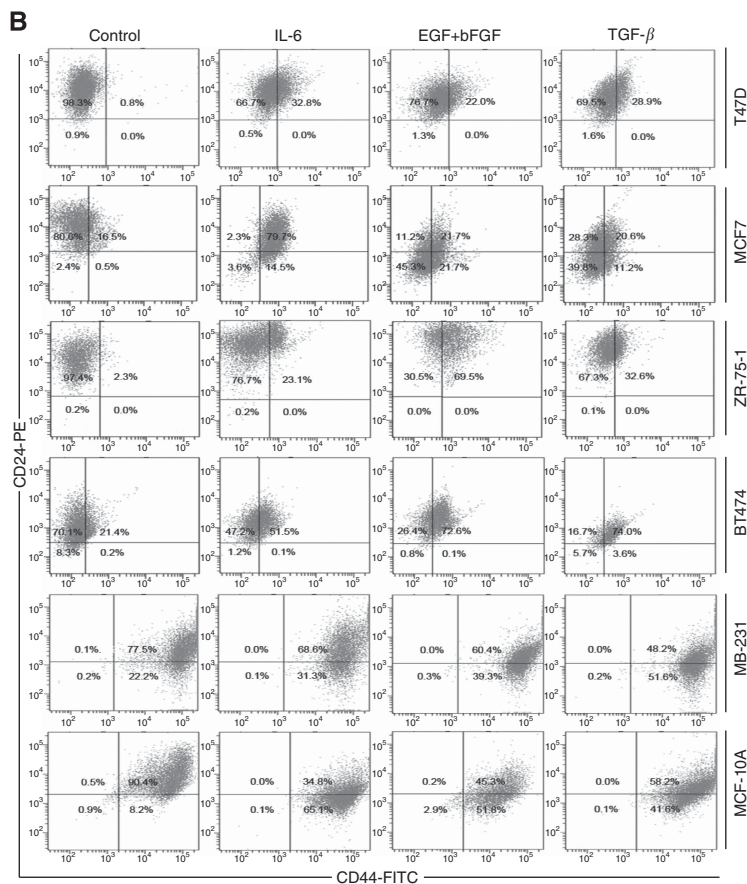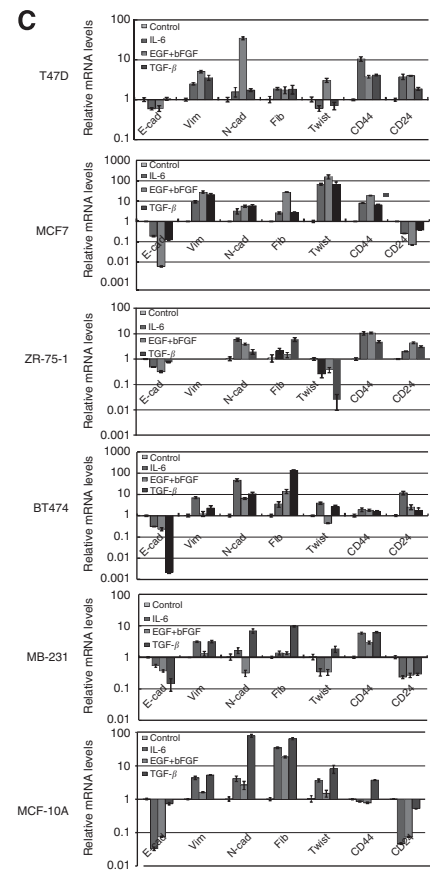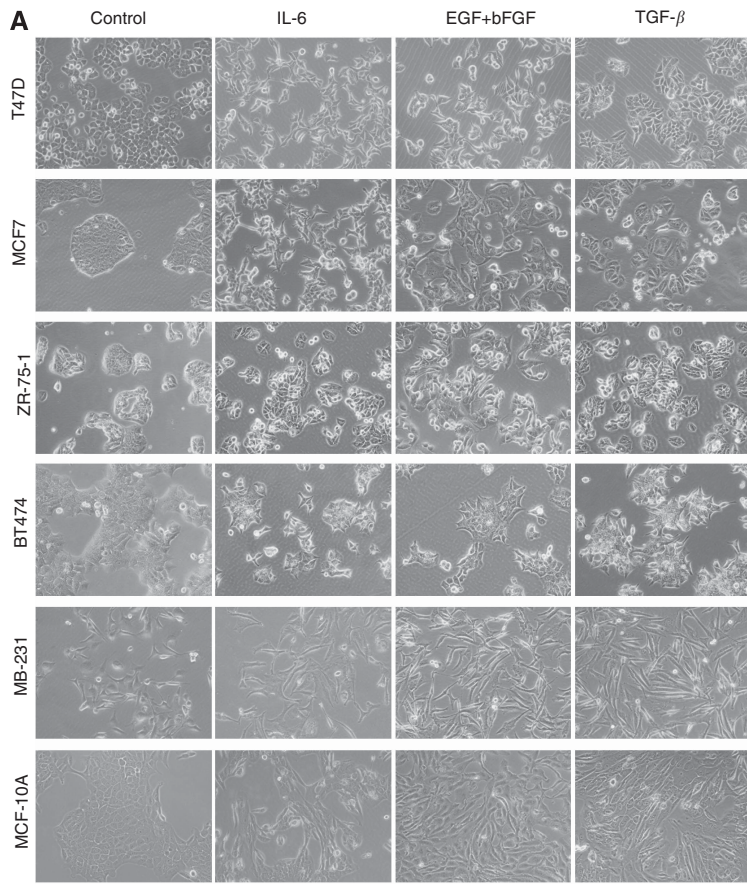
RESULTS

Epithelial–mesenchymal transition induces the CD44$^+$ or CD44$^+$/CD24$^{-/low}$ phenotype in breast epithelial cells. We first induced an EMT process by exposing cells to EMT-inducing cytokines, including IL-6 (Sullivan *et al*, 2009), EGF/bFGF (Ackland *et al*, 2003), and TGF-β1 (Xu *et al*, 2009), in five breast cancer cell lines (T47D, MCF7, ZR-75-1, BT474, and MDA-MB-231) and one normal breast cell line (MCF-10A). In previous studies (Al-Hajj *et al*, 2003; Ponti *et al*, 2005; Phillips *et al*, 2006; Li *et al*, 2008), the CD44$^+$/CD24$^-$ cells existing in breast cancer cell lines have been considered to be a subpopulation defined as TICs or CSCs that have the enhanced tumour-initiating capacity. Herein, to exclude the possible interference effect of CD44$^+$/CD24$^{-/low}$ cells and thus better assess the role of EMT in tumour-initiating capacity, we isolated CD44$^-$/CD24$^+$ cells in T47D, MCF7, ZR-75-1, and BT474 lines or CD44$^+$/CD24$^+$ cells in MDA-MB-231 and MCF-10A lines by flow cytometry to assess the role of EMT in tumour-initiating capacity. After that, these isolated cells (parental cells) were exposed to complete media containing IL-6, EGF/bFGF, or TGF-β1. As shown in Figure 1A, a morphological change in the cultured cells, from a cobblestone-like to a spindle-like morphology, was clearly observed even at 48 h after exposure to these cytokines. Interestingly, all these cytokines induced the generation of CD44$^+$ cells or CD44$^+$/CD24$^{-/low}$ cells in the cultured cells after 10 days of exposure (Figure 1B). To determine whether these resulting CD44$^+$ cells or CD44$^+$/CD24$^{-/low}$ cells represented a population of cells that underwent the EMT process in response to exposure of these EMT-inducing cytokines, the gene expression patterns associated with EMT were analysed in these cells compared with parental cells without cytokine exposure. As expected, these cells exhibited a gene expression pattern associated with EMT compared with parental untreated cells (Figure 1C and Supplementary Figure S1), including E-cadherin repression and concomitant activated expression of major mesenchymal markers (vimentin, N-cadherin, fibronectin, and twist) accompanied by induction of CD44 (in T47D, MCF7, ZR-75-1, and BT474 cells) or repression of CD24 (in MCF7, MDA-MB-231, and MCF-10A cells). The results were further verified by western blotting in the induced MCF7 cells (Figure 1D) and immunofluorescence analyses in the induced MCF-10A cells by 10-day exposure to cytokines (Figure 1E). Furthermore, we analysed the gene expression of the CD44$^-$/CD24$^+$ cells isolated from the MCF7 cells treated with cytokines for 10 days compared with parental untreated cells. As shown in Supplementary Figure S2, there was no significant difference in the expression levels of EMT-related markers between the CD44$^-$/CD24$^+$ cells isolated from cytokine-treated cells and parental untreated cells. This result suggests that the cytokines have no definitive effect on gene expression in the cultured cells; instead, EMT induced by cytokines *per se* has a crucial role in the gene expression of the resulting CD44$^+$/CD24$^-$ cell population. Therefore, in line with previous reports (Mani *et al*, 2008; Morel *et al*, 2008), EMT indeed induced the CD44$^+$ or CD44$^+$/CD24$^{-/low}$ phenotype *in vitro* in breast cancer cells and untransformed breast epithelial cells.

Epithelial–mesenchymal transition does not enhance tumour-initiating capacity but rather imparts other malignant characteristics on cancer cells. To determine whether EMT, *per se*, enhances tumour-initiating capacity on the resulting CD44$^+$/CD24$^{-/low}$ cells, we isolated the CD44$^+$/CD24$^{-/low}$ cells from MCF7 or MCF-10A lines at 10 days after exposure to cytokines. To continue to acquire the *in vivo* stimulation of cytokines for some time, the isolated cells were first resuspended in the matrigel containing IL-6, EGF/bFGF, or TGF-β1, and then injected into the mammary fat pad of NOD/SCID mice using limiting dilution assays. As shown in Figure 2A and B, the resulting CD44$^+$/CD24$^{-/low}$ cells induced by IL-6 or EGF/bFGF exposure spent less time in forming palpable tumours and grew faster in NOD/SCID mice than their parental cells, and a little higher tumour-forming rates were observed in the resulting cells than in the parental cells on 80 days only at the injection of 1×10^4 cells (Table 1). However, on 120 days after injections, we did not find significantly different tumour-forming rates between the resulting CD44$^+$/CD24$^{-/low}$ cells and parental cells without cytokine exposure (Table 1). Importantly, the resulting CD44$^+$/CD24$^{-/low}$ cells from untransformed MCF-10A cells did not form any palpable tumours even at 120 days after injections. These results indicate that the CD44$^+$/CD24$^{-/low}$ cells generated by EMT did not acquire enhanced tumour-initiating capacity compared with parental untreated cells.

To validate these results above, we further used a cloning and tumorigenic analysis (CTA) to determine the frequencies of clonogenic cells and TICs in the resulting CD44$^+$/CD24$^{-/low}$ cells and parental cells (Supplementary Figure S3A and Supplementary Materials and Methods). Cloning and tumorigenic analysis assesses tumour-initiating capacity on the basis of one-single cell and has been used to determine the fractions of TICs in previous reports (Zheng *et al*, 2007; Meng *et al*, 2009). This method allows one-single cell to expand extensively *in vitro* and then make these expanded cells injected into immunocompromised mice to develop a solid tumour. A cell that had the potential to expand unlimitedly *in vitro* was defined as clonogenic cell. To keep continuously stimulating EMT, the resulting CD44$^+$/CD24$^{-/low}$ cells were kept cultured in media containing cytokines during *in vitro* expansion, whereas parental cells were cultured in common medium. Consistent with the results obtained by limiting dilution tumour formation assays, no significantly different frequencies of clonogenic cells and TICs were observed between the resulting CD44$^+$/CD24$^{-/low}$ cells and parental untreated cells from MCF7 cells (Supplementary Figure S3B and Supplementary Table S1). Moreover, for untransformed MCF-10A cells, clonogenic cells and TICs were detected neither in parental untreated cells nor in the resulting CD44$^+$/CD24$^{-/low}$ cells. Therefore, EMT does not lead to enhancement or acquisition of tumour-initiating capacity.

However, these resulting cells, except for the TGF-β1 group, acquired enhanced proliferation potential (Figure 2C), reduced cell death after doxorubicin treatment (Figure 2D), increased resistance to radiation (Figure 2E), and increased migration (Figure 2F) compared with control parental cells. In addition, we performed spheroid formation assay in serum-free medium containing 5 μg ml^{-1} bovine insulin, 2% B27 supplement, 20 ng ml^{-1} bFGF, and 10 ng ml^{-1} EGF to determine whether the resulting

Figure 1. Epithelial–mesenchymal transition-inducing cytokines induce the generation of CD44$^+$ or CD44$^+$/CD24$^{-/low}$ cells. (A) Morphological changes from a cobblestone-like to a spindle-like morphology were observed at 48 h after exposure to cytokines. **(B)** The 10-day exposure to cytokines induced CD44$^+$ cells or CD44$^+$/CD24$^{-/low}$ cells. **(C)** The CD44$^+$ cells or CD44$^+$/CD24$^{-/low}$ cells induced by cytokines exhibited a gene expression pattern consistent with EMT, including E-cadherin repression and concomitant activated expression of major mesenchymal markers (vimentin, N-cadherin, fibronectin, and twist), accompanied by induction of CD44 (in T47D, MCF7, ZR-75-1, and BT474 cells) or repression of CD24 (in MCF7, MDA-MB-231, and MCF-10A cells). **(D)** Western blot analyses verified that the induced MCF7 cells by 10-day exposure to cytokines repressed E-cadherin expression and activated expression of vimentin, accompanied by upregulation of CD44 expression and repression of CD24 expression. **(E)** Immunofluorescence analyses showed that the induced MCF-10A cells by 10-day exposure to cytokines repressed E-cadherin expression and activated expression of vimentin.

CD44$^+$/CD24$^{-/low}$ cells acquired enhanced spheroid-forming capability. As shown in Supplementary Figure S4, the resulting cells from both MCF7 cancer cells and MCF-10A untransformed cells indeed significantly increased the frequencies of the formed mammospheres, suggesting that EMT promotes spheroid-forming capability not only for breast cancer cells but also for untransformed breast epithelial cells.

Mesenchymal–epithelial transition was induced by stable ectopic expression of miR-200c in mesenchymal-like breast cancer cells. To further determine whether the reverse process of EMT, MET, can lead to loss of tumour-initiating capacity in mesenchymal-like breast cancer cells, we also induced the MET process by stable ectopic expression of miR-200c, which is a small non-coding regulatory RNA that has been well established to be a potent MET inducer through direct targeting the E-cadherin repressors ZEB1 (Park *et al*, 2008), in three mesenchymal-like breast cancer cell lines (MDA-MB-231, MDA-MB-435S, and BT549) (Supplementary Figure S5A and B). A distinct morphological change from a spindle-like to a cobblestone-like appearance was observed in miR-200c-overexpressed cells (Figure 3A). As shown in Figure 3B, the miR-200c-overexpressed MDA-MB-231 cells exhibited gene expression consistent with MET, including

induction of E-cadherin and repression of most mesenchymal markers. More importantly, the MET induction led to a significantly decreased proportion of CD44$^+$/CD24$^{-/low}$ cells that was measured in MDA-MB-231 by flow cytometry (Figure 3C). Western blotting further confirmed that MET induction in MDA-MB-231 activated expression of E-cadherin and repressed the expression of vimentin, coinciding with the repression of CD44 expression (Figure 3D). Functionally, the miR-200-overexpressed MDA-MB-231 cells markedly slowed down growth (Figure 3Ea), increased susceptibility to doxorubicin (Figure 3Eb) and radiation treatment (Figure 3Ec), and repressed migration and invasion activities (Figure 3F and Supplementary Figure S6).

Mesenchymal–epithelial transition does not result in inhibition or loss of tumour-initiating capacity in mesenchymal breast cancer cells. To further evaluate the influence of MET on tumour-initiating capacity of cancer cells, the tumour-initiating ability of cells that had undergone an MET-induced transition was assessed by limiting dilution assays. Our results showed that the miR-200c-overexpressed cells efficiently initiated tumour in NOD/SCID mice on 80 days, almost the same as miR-NC-overexpressed cells (Figure 4A). However, it is noteworthy that these cells that had undergone an MET delayed time to form palpable tumours and
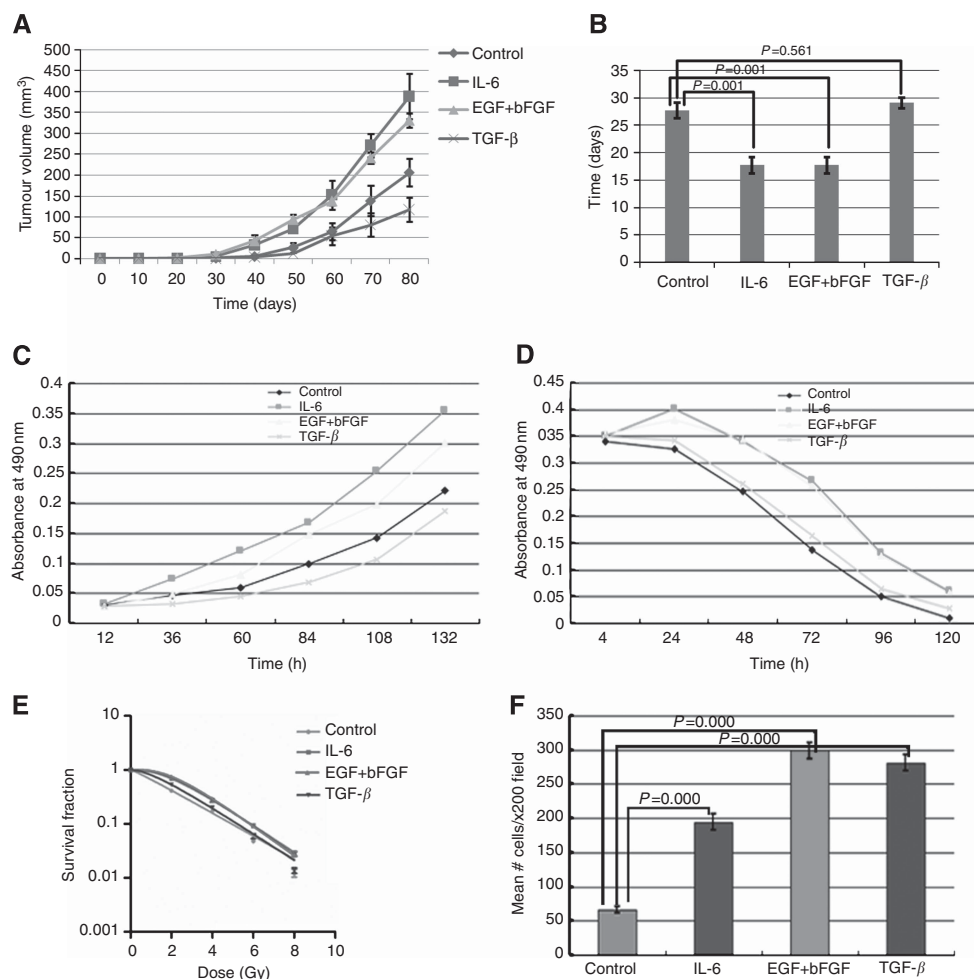

Figure 2. The CD44$^+$/CD24$^{-/low}$ cells induced (from MCF7 cells) by cytokines, except for TGF-β1, acquired some more malignant properties but not tumour-initiating capacity. (A) The tumour volumes were measured after 1×10^6 induced cells were injected into NOD/SCID mice. **(B)** The induced cells spent less time to form palpable tumours than control parental cells. **(C)** The induced cells displayed enhanced proliferation potential compared with control parental cells. **(D)** The induced cells had reduced doxorubicin-induced death compared with control parental cells. **(E)** The induced cells had increased resistance to radiation compared with control parental cells. **(F)** The induced cells had increased migration compared with control parental cells.

Table 1. Tumour-forming rates of the CD44$^+$/CD24$^{-/low}$ cells induced by cytokines

Cells	80 Days[a]			120 Days[a]		
	Cells per injection			Cells per injection		
	1×10^6	1×10^4	1×10^3	1×10^6	1×10^4	1×10^3
MCF7						
Parental cells	3/3	1/4	0/4	3/3	4/4	2/4
IL-6	3/3	2/4	0/4	3/3	4/4	1/4
EGF + bFGF	3/3	2/4	0/4	3/3	3/4	2/4
TGF-β	2/3	1/4	0/4	2/3	2/4	0/4
MCF-10A						
Parental cells	0/3	0/4	—	0/3	0/4	—
IL-6	0/3	0/4	—	0/3	0/4	—
EGF + bFGF	0/3	0/4	—	0/3	0/4	—
TGF-β	0/3	0/4	—	0/3	0/4	—

Abbreviations: bFGF = basic fibroblast growth factor; EGF = epidermal growth factor; IL-6 = interleukin-6; TGF-β = transforming growth factor-β.
[a]The values denote number of tumours/number of injections.

exhibited slower growth *in vivo* than did the control cells (Figure 4B). These results suggest that the transition from the mesenchymal phenotype to the epithelial phenotype does not lead to inhibition or loss of tumour-initiating capacity but markedly attenuates other malignant properties, including proliferation, invasion, and resistance to therapy, at least in our transition induced by miR-200c. Therefore, tumour-initiating capacity of breast cancer cells may be independent of their mesenchymal properties.

DISCUSSION

Epithelial–mesenchymal transition is a critical developmental process that has recently come to the forefront of cancer biology (Polyak and Weinberg, 2009). In breast cancers, the acquisition of a mesenchymal-like phenotype is associated with enhanced migration, invasiveness, elevated resistance to apoptosis, and cancer recurrence (Creighton *et al*, 2009; Thiery *et al*, 2009). In parallel, breast CSCs are increasingly thought to have a major role in breast cancer growth, the formation of metastases, resistance to therapy, and subsequent tumour recurrence (Ailles and Weissman, 2007; Rosen and Jordan, 2009; Takebe *et al*, 2011). Since Weinberg and colleagues (Mani *et al*, 2008) reported empirical evidence connecting EMT to generation of CSCs, increasing studies have firmly established the phenotypic association between EMT and CSCs (Morel *et al*, 2008; Liu *et al*, 2009; Fang *et al*, 2011; Chen *et al*, 2012; Devarajan *et al*, 2012; Oliveras-Ferraros *et al*, 2012; Balanis *et al*, 2013; Kim *et al*, 2013; Nguyen *et al*, 2013). These studies suggest that EMT promotes cellular proliferation and invasion *in vitro*, and tumour growth *in vivo*, while the reversion of EMT inhibits cellular proliferation and invasion *in vitro*, and tumour growth *in vivo*. Conversely, recent studies by Tsai (Tsai *et al*, 2012) and Chang (Chang *et al*, 2013) reported that MET promoted colonisation, cellular proliferation, and tumour growth. In any case, these studies still support that EMT or MET promotes tumour generation. However, it is still unknown whether EMT or MET directly leads to acquisition or loss of tumour-initiating capacity. In the present study, we performed EMT induction by cytokines and found that EMT generated cells with the

CD44$^+$/CD24$^{-/low}$ phenotype, which displayed a repressed expression state of E-cadherin and an increased expression state of mesenchymal markers. Interestingly, these EMT-generated CD44$^+$/CD24$^{-/low}$ cancer cells exhibited just a little higher tumour-forming rates compared with control cells in only a relatively short observation period. When the observation period was extended, there was little difference in tumour-forming rates between these EMT-generated cells and untreated cells. More importantly, the EMT-generated cells from the normal immortalised MCF-10A cells could not form any palpable tumour in immune-deficient mice. Therefore, our results indicate that EMT does not lead to the enhancement of tumour-initiating capacity in cancer cells or the acquisition of tumour-initiating capacity in normal epithelial cells, which was further vindicated by the results from CTAs. Consistent with previous studies (Dasgupta *et al*, 2009; Thiery *et al*, 2009), EMT indeed promoted some abilities including cell proliferation, migration, and resistance to apoptosis induction. Epithelial–mesenchymal transition also promoted spheroid formation not only for breast cancer cells but also for untransformed breast epithelial cells. However, the enhanced spheroid-forming ability by EMT does not necessarily represent the enhanced tumour-initiating capacity of cells because the resulting CD44$^+$/CD24$^{-/low}$ cells from untransformed MCF-10A cells that cannot initiate any tumour *in vivo* also acquired the enhanced spheroid-forming ability after being treated with cytokines. It is noteworthy that the enhanced proliferation of tumour cells by EMT might decrease the latency of tumour formation so that the breast tumour cells that had undergone EMT spent less time forming tumours in immunocompromised mice. In addition, TGF-β1-induced cells in our results presented the repressed proliferative activities and were more sensitive to treatment compared with control cells although the CD44$^+$/CD24$^{-/low}$ phenotype was generated. However, we still could not exclude the effects of TGF-β1-induced EMT on pro-proliferation and pro-resistance because EMT-mediated pro-proliferation and pro-resistance may be counteracted by TGF-β1 direct anti-proliferative and pro-apoptotic effects that may be mediated through another signalling pathway (Docherty *et al*, 2006).

Mesenchymal–epithelial transition is also a critical process for the formation of the body plan and in the differentiation of multiple tissues and organs during embryonic development (Davies, 1996; Nakaya *et al*, 2004). Does MET lead to inhibition or loss of tumour-initiating capacity of cancer cells? Our results showed that breast cancer cells that had undergone MET process could form tumours in immunocompromised mice identically as their counterparts, although they delayed time to form palpable tumours and displayed slower tumour growth *in vivo*, suggesting that the MET process does not lead to inhibition or loss of tumour-initiating capacity of the breast cancer cells. Recent studies have demonstrated that MET was crucial for circulating tumour cells in the blood to seed and establish early metastatic lesions at distant sites (Baum *et al*, 2008; Leontovich *et al*, 2012). Therefore, MET may be essential for disseminated tumour cells from primary tumours to colonise at distant sites rather than for depriving them of tumour-initiating capacity.

Notably, several recent reports showing that a more epithelial phenotype or MET provides properties of TICs, as demonstrated in cancer cells such as some prostate and bladder cancer cell lines (Celia-Terrassa *et al*, 2012) and head and neck cancer cell lines (Chang *et al*, 2013). The results in these studies showed that a more epithelial subpopulation had a much higher potential for *in vitro* cell proliferation, *in vivo* local tumour growth, and even displayed a very active self-renewal/pluripotency gene programme compared with a mesenchymal subpopulation. However, these results contrast to the results observed by other studies showing that a more epithelial phenotype or MET repressed cell proliferation *in vitro* and decreased potential of tumour formation in primary
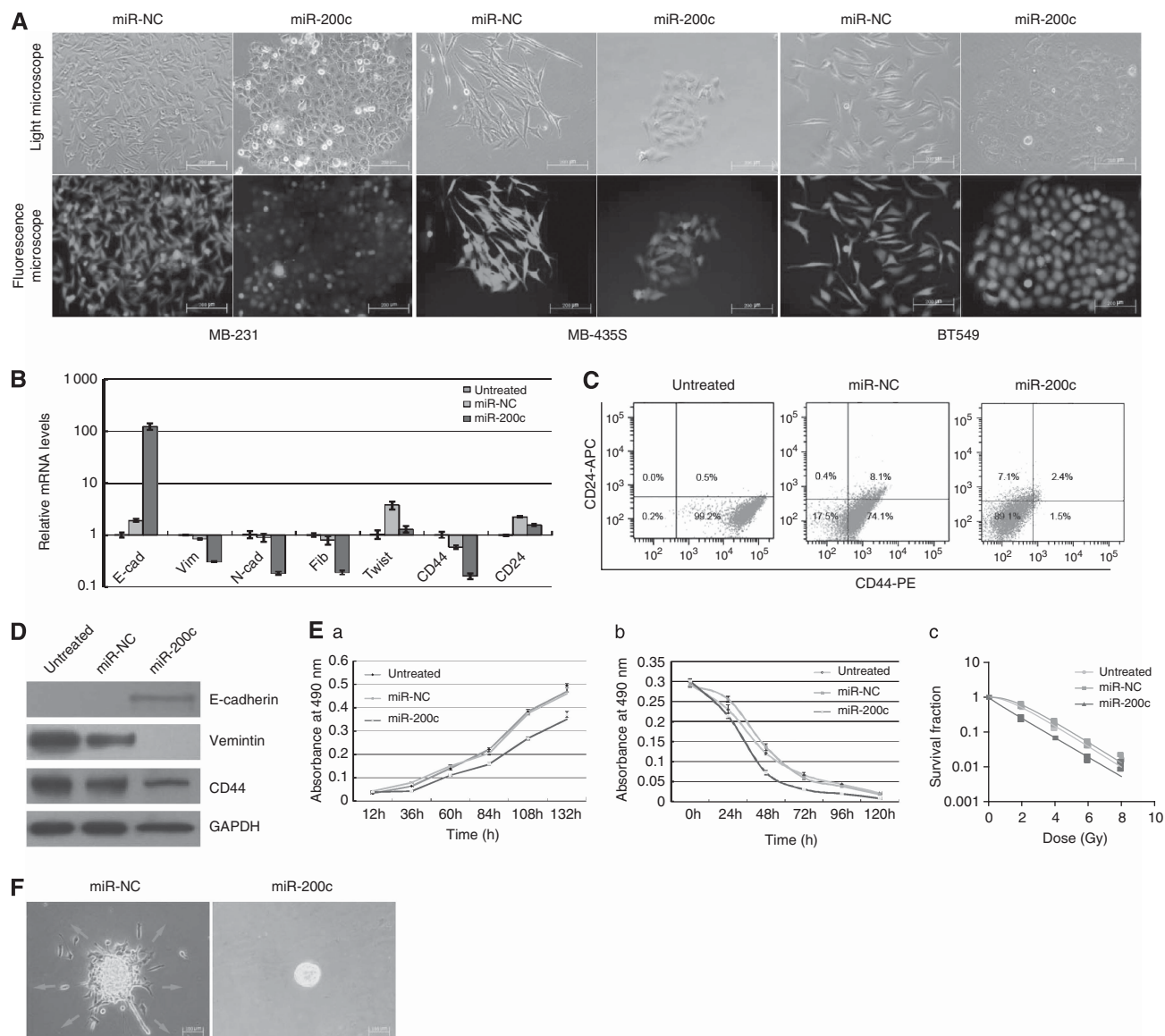
Figure 3. Mesenchymal–epithelial transition was induced by stable ectopic expression of miR-200c in mesenchymal-like breast cancer cell lines. (A) Morphological changes from a spindle-like to a cobblestone-like morphology are shown in cells that stably overexpressed MET-inducing miR-200c. (B) Real-time RT-PCR analysis of miR-200-overexpressed MDA-MB-231 (MB-231) cells showed a gene expression pattern consistent with MET, accompanied by repression of CD44 expression. (C) The MET induction of MDA-MB-231 cells led to a significantly decreased proportion of CD44$^+$/CD24$^{-/low}$ cells that was measured by flow cytometry. (D) Western blotting further confirmed that MET induction of MDA-MB-231 cells activated expression of E-cadherin and repressed expression of vimentin, coinciding with repression of CD44 expression. (E) Mesenchymal–epithelial transition significantly slowed down growth (a, F = 82.757, P = 0.000) and increased susceptibility to doxorubicin (b, F = 28.129, P = 0.000)) or radiation (c, F = 19.726, P = 0.002) in MDA-MB-231 cells. (F) Mesenchymal–epithelial transition markedly repressed migration and invasion in MDA-MB-231 cells.

sites (St Croix and Kerbel, 1997; Liu et al, 2008; Albino et al, 2012; Fendrich et al, 2012) and that EMT promotes cell proliferation, tumour formation, and even the establishment of metastases in distant organs (Chang et al, 2011; Fendrich et al, 2012; Zhang et al, 2012; Kim et al, 2013). Meanwhile, a number of studies have confirmed that some cancer cell lines with a stable mesenchymal phenotype, such as MDA-MB-231 and MDA-MB-435S, had a much high potential to proliferate in vitro, form tumours in primary sites, and even establish metastases in distant organs (Price et al, 1990; Minn et al, 2005; Miao et al, 2007; Iorns et al, 2012). Furthermore, Chao et al (2010) have validated that the primary tumours formed by mesenchymal-like MDA-MB-231 breast cancer cells in immunocompromised mice still display a mesenchymal phenotype but not a more epithelial phenotype in

both the central and peripheral areas of the tumours, which was consistent with expression pattern in vitro of MDA-MB-231 cells. These results suggest that the epithelial phenotype may not be necessary for these mesenchymal-like breast cancer cells to initiate primary tumours in vivo. Moreover, several previous studies have shown that the cancer cells with mesenchymal phenotype were also characterised by increased expression of multiple pluripotent genes such as of Sox2, Nanog, Oct4, Lin28B, and Notch1 (Blick et al, 2010; Chiou et al, 2010; Kong et al, 2010; Prat et al, 2010; Han et al, 2012). Interestingly, our results herein suggest that in some breast cancer cell lines, neither EMT nor MET status is responsible for tumour-initiating capacity that is more likely to be an inherent ability of cancerous cells. Therefore, it is a great controversy as to whether EMT or MET endows cancer cells with the increased
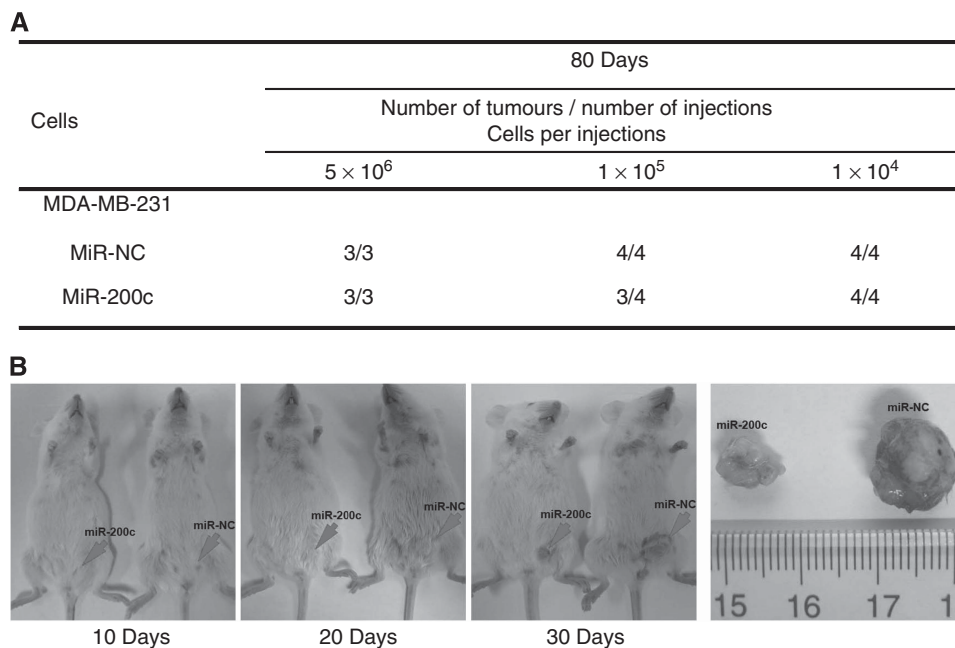
A

Cells	80 Days		
	Number of tumours / number of injections Cells per injections		
	5×10^6	1×10^5	1×10^4
MDA-MB-231			
MiR-NC	3/3	4/4	4/4
MiR-200c	3/3	3/4	4/4

B

Figure 4. Mesenchymal–epithelial transition does not result in loss of tumour-initiating capacity in mesenchymal-like breast cancer cell lines. (**A**) The miR-200c-overexpressed cells had almost the same frequencies of tumour formation in NOD/SCID mice on 80 days as compared with miR-NC-overexpressed cells. (**B**) The miR-200c-overexpressed cells exhibited slower tumour growth *in vivo* than did the control cells when 5×10^6 cells were injected into the breast of 4-week-old female NOD/SCID mice.

tumour-initiating capacities. These inconsistent conclusions may indeed, at least partially, represent complex biological functions of cancer cells that are usually influenced by multiple factors, such as the cellular and microenvironmental context, animal models, existence of intermediate phenotype of EMT/MET, distinct critical molecular events activated in EMT or MET, multiple functions of EMT/MET mediators, and even interaction of biological signalling pathways. Although our findings extend understanding of the relationship between EMT/MET status and tumour-initiating capacity, further researches are still needed to demonstrate cellular, molecular, and microenvironmental mechanism mediating more intricate relationships between EMT/MET status and malignant biological behaviours of cancer cells.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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