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

G Xie¹,², A Ji³, Q Yuan⁴, Z Jin⁵, Y Yuan⁶, C Ren¹, Z Guo⁵, Q Yao¹, K Yang⁶, X Lin¹ and L Chen¹

¹Department of Radiation Oncology, Nanfang Hospital, Southern Medical University, Guangzhou, Guangdong 510515, PR China; ²Department of Pharmaceutical Science, Zhujiang Hospital, Southern Medical University, Guangzhou, Guangdong 510282, PR China; ³Jules Stein Eye Institute, David Geffen School of Medicine, University of California, Los Angeles, CA 90095, USA; ⁴Department of Pharmacy, Jining First People’s Hospital, Jining, Shandong 272111, PR China; ⁵Breast Center, Nanfang Hospital, Southern Medical University, Guangzhou, Guangdong 510515, PR China and ⁶Department of Neurosurgery, Institute of Neurosurgery, Nanfang Hospital, Southern Medical University, Guangzhou, Guangdong 510515, PR China

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⁻/CD105⁻ 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 program 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 overlap with TICs. Several studies have shown that normal and transformed breast epithelial cell lines that had undergone EMT lose numerous epithelial characteristics accompanied by acquisition of therapeutic resistance (Mani et al., 2009; 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 U ml\(^{-1}\) penicillin, 100 \(\mu\)g ml\(^{-1}\) streptomycin, and 2 \(\mu\)M \(\alpha\)-glutamine. MCF7 cells were maintained in DMEM (Gibco) supplemented with 10% FBS, 100 U ml\(^{-1}\) penicillin, 100 \(\mu\)g ml\(^{-1}\) streptomycin, and 2 \(\mu\)M \(\alpha\)-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-\(\beta\) (TGF-\(\beta\); Peprotech) at 37°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 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 PCR assays 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 shown below:

- For E-cadherin: forward, 5'-CCACCAACGCTACAAGGGTGC-3'; reverse, 5'-CTGGGATATTGGGGGCATC-3'; vimentin: forward, 5'-CGCCAGATGGCTGAAATGG-3', reverse, 5'-ACCAAGAGGA GTAATTCCACA-3'; N-cadherin: forward, 5'-TTAAACGGCCT GACAATGAC-3', reverse, 5'-CCCCCAGCCATGTCAGGATAT-3'; fibronectin: forward, 5'-CAGTGGGAGACCTGGAGAAG-3', reverse, 5'-TCCTCTGGAACATCAGAAA-3'; twist: forward, 5'-TCGCCAGACCTTACGAGGAG-3', reverse, 5'-GGGGAGGAG TCTTACCTGCT-3'; CDA4: forward, 5'-CAGCAACACCTA CTGATGAC-3', reverse, 5'-GCCTAGAGGGATCTGCAA ATGA-3'; CD24: forward, 5'-TTTACAACCTGCCTGACACA-3', reverse, 5'-CGATCTGTGGTGGCAGTTTCCATGT-3'; 18S: forward, 5'-CCTGGATACCCGACCTAGGA-3', reverse, 5'-GGGCGGCT AAATA GATGCCAC-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°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'-CACACAGAGGGTCGAGGATG TCCCCAGGACCTC-3' and a reverse primer: 5'-ATTCTGTACAGG ATGTCACAGCTCTGACTCAG-3'. The PCR products of 332 bp were reclained 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 directlyonto the culture surface, spreading it evenly with a pipette tip and incubating for 30 min at 37°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°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-\(\beta\). 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.
Epithelial–mesenchymal transition induces the CD44<sup>+</sup> or CD44<sup>-/CD24<sup>-/low</sup></sup> 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, BT-474, 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<sup>+/CD24<sup>-</sup></sup> 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<sup>+</sup> / CD24<sup>-/low</sup> cells and thus better assess the role of EMT in tumour-initiating capacity, we isolated CD44<sup>+</sup>/CD24<sup>-</sup> cells in T47D, MCF7, ZR-75-1, and BT474 lines or CD44<sup>+</sup>/CD24<sup>-/low</sup> 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<sup>+</sup> cells or CD44<sup>-/CD24<sup>-/low</sup></sup> cells in the cultured cells after 10 days of exposure (Figure 1B). To determine whether these resulting CD44<sup>+</sup> cells or CD44<sup>-/CD24<sup>-/low</sup></sup> 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 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 upregulation of CD44 expression and repression of CD24 expression. Immunofluorescence analyses showed that the induced MCF-10A 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. EMT-related markers between the CD44<sup>-/CD24<sup>-/low</sup></sup> cells and parental untreated cells from MCF7 cells or CD44<sup>+</sup>/CD24<sup>-/low</sup> 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<sup>-/CD24<sup>-/low</sup></sup> 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<sup>-/CD24<sup>-/low</sup></sup> 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<sup>-/CD24<sup>-/low</sup></sup> 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<sup>-/CD24<sup>-/low</sup></sup> 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<sup>-1</sup> bovine insulin, 2% B27 supplement, 20 ng ml<sup>-1</sup> bFGF, and 10 ng ml<sup>-1</sup> EGF to determine whether the resulting...
Tumour-initiating capacity and EMT status

A. Control, IL-6, EGF+bFGF, TGF-β

B. Control, IL-6, EGF+bFGF, TGF-β

C. Graph showing relative mRNA levels for E-cadherin, Vimentin, N-cadherin, Fibronectin, Twist, CD44, CD24, and GAPDH.

D. Western blots for E-cadherin, Vimentin, CD44, CD24, and GAPDH.

E. Immunofluorescence for E-cadherin and Vimentin.

www.bjcancer.com | DOI:10.1038/bjc.2014.153 | 2517
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
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 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 present study 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 (Celis-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

---

Table 1. Tumour-forming rates of the CD44+/CD24−/low cells induced by cytokines

| Cells per injection | 80 Days* | 120 Days* |
|---------------------|----------|----------|
|                     |          |          |
| **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      | 4/4      | 2/4      |
| TGF-β               | 2/3      | 1/4      | 0/4      | 2/3      | 2/4      | 0/4      |

**MCF-10A**

| Cells per injection | 80 Days* | 120 Days* |
|---------------------|----------|----------|
|                     |          |          |
| 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.

**The values denote number of tumours/number of injections.**
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 higher 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

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.
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.

ACKNOWLEDGEMENTS

This work was supported by the Natural Science Foundation of China grants (81272508 and 81302326), Natural Science Foundation of Guangdong grant (S2012040008087), Key Applied and Basic Projects of Guangzhou Science and Technology Program (11C22120714), National Specialized Research Fund for the Doctoral Program of Higher Education (20114433110015), and Integration Project of Production, Teaching and Research of Ministry of Education of Guangdong Province (2011B090400019).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES

Ackland ML, Newgreen DF, Fridman M, Waltham MC, Arvanitis A, Minichiello J, Price JT, Thompson EW (2003) Epidermal growth factor-induced epithelio-mesenchymal transition in human breast carcinoma cells. Lab Invest 83: 435–448.

Acloque H, Adams MS, Fishwick K, Bronner-Fraser M, Nieto MA (2009) Epithelial-mesenchymal transitions: the importance of changing cell state in development and disease. J Clin Invest 119: 1438–1449.

Ailles LE, Weissman IL (2007) Cancer stem cells in solid tumors. Curr Opin Biotechnol 18: 460–466.

Al-Hajj M, Becker MW, Wicha M, Weissman I, Clarke MF (2004) Therapeutic implications of cancer stem cells. Curr Opin Genet Dev 14: 43–47.

Al-Hajj M, Wicha MS, Benito-Hernandez A, Morrison SJ, Clarke MF (2003) Prospective identification of tumorigenic breast cancer cells. Proc Natl Acad Sci USA 100: 3983–3988.

Albino D, Longoni N, Curti L, Mello-Grand M, Pinton S, Civenni G, Thalmann G, D’Ambrosio G, Sarti M, Sessa F, Chiorino G, Catapano CV, Carbone GM (2012) ESE3/EHF controls epithelial cell differentiation and its loss leads to prostate tumors with mesenchymal and stem-like features. Cancer Res 72: 2889–2900.

Balanis N, Wendt MK, Schiemann BJ, Wang Z, Schiemann WP, Carlin CR (2013) Epithelial to mesenchymal transition promotes breast cancer progression via a fibronectin-dependent STAT3 signaling pathway. J Biol Chem 288: 17954–17967.

Baum B, Settleman J, Quinlan MP (2008) Transitions between epithelial and mesenchymal states in development and disease. Semin Cell Dev Biol 19: 294–308.

Blick T, Hugo H, Widodo E, Waltham M, Pinto C, Mani SA, Weinberg RA, Neve RM, Lenburg ME, Thompson EW (2010) Epithelial mesenchymal transition traits in human breast cancer cell lines parallel the CD44(hi)/CD24(lo) stem cell phenotype in human breast cancer. J Mammary Gland Biol Neoplasia 15: 235–252.

Celia-Terrassa T, Meca-Cortes O, Mateo F, de Paz AM, Rubio N, Arnal-Estape A, El BJ, Bermudo R, Diaz A, Guerra-Rebollo M, Lozano JJ, Estaras C, Ulloa C, Alvarez-Simon D, Mila J, Villiclara F, Paciucci R, Martinez-Balbas M, de Herreros AG, Gomis RR, Karg Y, Blanco J, Fernandez PL, Thomson KM (2012) Epithelial-mesenchymal transition can suppress major attributes of human epithelial tumor-initiating cells. J Clin Invest 122: 1849–1868.

Chang CC, Hsu WH, Wang CC, Chou CH, Kuo MY, Lin BR, Chen ST, Tai SK, Kuo ML, Yang MH (2013) Connective tissue growth factor activates pluripotency genes and mesenchymal-epithelial transition in head and neck cancer cells. Cancer Res 73: 4147–4157.
Chang CJ, Chao CH, Xia W, Yang JY, Xiong Y, Li CW, Yu WH, Rehman SK, Hou JL, Lee HH, Liu M, Chen CT, Yu D, Hung MC (2015) p53 regulates epithelial-mesenchymal transition and stem cell properties through modulating miRNAs. Nat Cell Biol 13: 317–323.

Chao YL, Shepard CR, Wells A (2010) Breast carcinoma cells re-express E-cadherin during mesenchymal to epithelial reversion transition. Mol Cancer 9: 179.

Chen SC, Kung ML, Hu TH, Chen HY, Wu JC, Kuo HM, Tsai HE, Lin YW, Wen ZH, Liu JK, Yeh MH, Tai MH (2012) Hepatoma-derived growth factor regulates breast cancer cell invasion by modulating epithelial-mesenchymal transition. J Pathol 228: 158–169.

Chiou SH, Wang ML, Chou YT, Chang CJ, Chen YS, Lin TW, Hsu HS, Wu CW (2010) Coexpression of Oct4 and Nanog enhances malignancy in lung adenocarcinoma by inducing cancer stem cell-like properties and epithelial-mesenchymal transdifferentiation. Cancer Res 70: 10433–10444.

Creighton CJ, Li X, Landis M, Dixon JM, Neumeister VM, Dixon JM, Landis M, Neumeister VM, Sjolund A, Creighton CJ, Li X, Landis M, Neumeister VM, Sjolund A, (2010) Breast carcinoma cells re-express E-cadherin during mesenchymal to epithelial reversion transition. Mol Cancer 9: 179.

Dasgupta P, Rizwani W, Pillai S, Kinkade R, Kovacs M, Rastogi S, Banerjee S, Devarajan E, Song YH, Krishnappa S, Alt E (2012) Epithelial-mesenchymal transition pathway in metastatic breast cancer cells. Int J Oncol 40: 1858–1864.

Li X, Lewis MT, Huang J, Gutierrez C, Osborne CK, Wu MF, Hilsenbeck SG, Pavlik A, Zhang X, Channess GC, Wong H, Rosen J, Chang JC (2008) Intrinsic resistance of tumorigenic breast cancer cells to chemotherapy. J Natl Cancer Inst 100: 672–679.

Liu M, Casimiro MC, Wang C, Shirley LA, Jiao X, Katiyar S, Ju X, Li Z, Yu Z, Zhou J, Johnson M, Fortina P, Hyslop T, Windle JJ, Festel RG (2009) p21CIP1 attenuates Ras- and c-Myc-dependent breast tumor epithelial mesenchymal transition and cancer stem cell-like gene expression in vivo. Proc Natl Acad Sci USA 106: 19035–19039.

Liu Y, El-Naggar S, Darling DS, Higashi Y, Dean DC (2008) Zeb1 links epithelial-mesenchymal transition and cellular senescence. Development 135: 579–588.

Mani SA, Guo W, Liao MJ, Eaton EN, Ayyanan A, Zhou AY, Brooks M, Reinhard F, Zhang CC, Shipitsin M, Campbell LL, Polyak K, Brisken C, Yang J, Weinberg RA (2008) The epithelial-mesenchymal transition generates cells with properties of stem cells. Cell 133: 704–715.

Meng X, Wang X, Wang Y (2009) More than 45% of AS45 and H446 cells are cancer initiating cells: evidence from cloning and tumorigenic analyses. Oncol Rep 21: 995–1000.

Miao Z, Luker KE, Summers BC, Berarovich R, Bhojani MS, Rehemtulla A, Kleer CG, Essner J, Nasevicus A, Luker GD, Howard MC, Schall TJ (2007) CXXCR7 (RDC1) promotes breast and lung tumor growth in vivo and is expressed on tumor-associated vasculature. Proc Natl Acad Sci USA 104: 15735–15740.

Minn AJ, Gupta GP, Siegel PM, Bos PD, Shu W, Giri DD, Viale A, Olshen AB, Gerald WL, Massague J (2005) Genes that mediate breast cancer metastasis to lung. Nature 436: 518–524.

Mored AP, Lievre M, Thomas C, Hinkel G, Ansieau S, Puisieux A (2008) Generation of breast cancer stem cells through epithelial-mesenchymal transition. PLoS One 3: e2888.

Nakaya Y, Kuroda S, Katagiri YT, Kaibuchi K, Takahashi Y (2004) Mesenchymal-epithelial transition during somitic segmentation is regulated by differential roles of Cdc42 and Rac1. Dev Cell 7: 425–438.

Nguyen PT, Tsumetatsu M, Tanagisawa S, Kudo Y, Miyauchi M, Kamata N, Takata T (2013) The FGFR1 inhibitor PD173074 induces mesenchymal-epithelial transition through the transcription factor AP-1. Br J Cancer 109: 2248–2258.

Oliveras-Ferraros C, Corominas-Faja B, Cufi S, Vaquez-Martian A, Martin-Castillo B, Iglesias JM, Lopez-Bonet E, Martin AG, Menendez JA (2012) Epithelial-to-mesenchymal transition (EMT) confers primary resistance to trastuzumab (Herceptin). Cell Cycle 11: 4020–4032.

Park SM, Gaur AB, Lengyl E, Peter ME (2008) The miR-200 family determines the epithelial phenotype of cancer cells by targeting the E-cadherin repressors ZEB1 and ZEB2. Genes Dev 22: 894–907.

Phillips TM, McBride WH, Pajonk F (2006) The response of CD24(−) and CD24(+) breast cancer cells to paclitaxel is modulated by ERalpha downregulation. J Clin Invest 119: 1420–1428.

Kim J, Jeong H, Lee Y, Kim C, Kim H, Kim A (2013) HRG-beta1-driven ErbB3 signaling induces epithelial-mesenchymal transition in breast cancer cells. BMC Cancer 13: 383.

Kim SJ, Yang KM, Jung Y, Lee JM, Kim W, Cho JK, Jeong J (2013) Loss of TRB1 induces epithelial-mesenchymal transition in the breast cancer cells by ESRalpha downregulation. Cancer Res 73(22): 6679–6689.

Kong D, Banerjee S, Ahmad M, Li Y, Wang Z, Sethi S, Sarkar FH (2010) Epithelial to mesenchymal transition is mechanistically linked with stem cell signatures in prostate cancer cells. PLoS One 5: e12445.

Kutner RH, Zang XZ, Reiner J (2009) Production, concentration and titration of pseudotyped HIV-1-based lentiviral vectors. Nat Protoc 4: 495–505.

Leonovich AA, Zhang S, Quatraro C, Iankov I, Veroux PF, Gambino MW, Degnim A, McCubrey J, Ingle J, Galanis E, D’Assoro AB (2012) Raf-1 oncogenic signaling is linked to activation of mesenchymal to epithelial transition pathway in metastatic breast cancer cells. Int J Oncol 40: 1858–1864.

Price JE, Polyzos A, Zhang RD, Daniels LM (1990) Tumorigenicity and metastasis of human breast carcinoma cell lines in nude mice. Cancer Res 50: 717–721.

Rosen JM, Jordan CT (2009) The increasing complexity of the cancer stem cell paradigm. Science 324: 1670–1673.

St. Croix B, Kerbel RS (1997) Cell adhesion and drug resistance in cancer. Curr Opin Oncol 9: 549–556.

Tumour-initiating capacity and EMT status
Supplementary Information accompanies this paper on British Journal of Cancer website (http://www.nature.com/bjc)