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ORIGINAL ARTICLE

Transcriptome profiling of a TGF-β-induced epithelial-to-mesenchymal transition reveals extracellular clusterin as a target for therapeutic antibodies

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Transforming growth factor (TGF)-β plays a dual role in tumorigenesis, switching from acting as a growth inhibitory tumor suppressor early in the process, to a tumor promoter in late-stage disease. Since TGF-β's prometastatic role may be linked to its ability to induce tumor cell epithelial-to-mesenchymal transition (EMT), we explored TGF-β's EMT-promoting pathways by analysing the transcriptome changes occurring in BRI-JM01 mammary tumor epithelial cells undergoing a TGF-β-induced EMT. We found the clusterin gene to be the most highly upregulated throughout most of the TGF-β time course, and showed that this results in an increase of the secreted form of clusterin. By monitoring several hallmark features of EMT, we demonstrated that antibodies targeting secreted clusterin inhibit the TGF-β-induced EMT of BRI-JM01 cells, as well as the invasive phenotype of several other breast and prostate tumor cell lines (4T1, NMuMG, MDA-MB231LM2 and PC3), without affecting the proliferation of these cells. These results indicate that secreted clusterin is a functionally important EMT mediator that lies downstream within TGF-β's EMT-promoting transcriptional cascade, but not within its growth-inhibitory pathways. To further investigate the role played by secreted clusterin in tumor metastasis, we assessed the effect of several anti-clusterin monoclonal antibodies *in vivo* using a 4T1 syngeneic mouse breast cancer model and found that these antibodies significantly reduce lung metastasis. Taken together, our results reveal a role for secreted clusterin as an important extracellular promoter of EMT, and suggest that antibodies targeting clusterin may inhibit tumor metastasis without reducing the beneficial growth inhibitory effects of TGF-β.
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Introduction

Transforming growth factor (TGF)-β is a multifunctional cytokine that controls a plethora of cellular processes during embryonic development and adult tissue homeostasis (Siegel and Massague, 2003). TGF-β acts by binding to the TGF-β type-II/TGF-β type-I (TβRII/TβRI) serine/threonine kinase receptor complex, which results in the recruitment of Smad-2 and 3. Once phosphorylated these Smads interact with Smad-4 and this complex then translocates into the nucleus, where, in conjunction with other nuclear cofactors, target genes are activated or repressed (Feng and Derynck, 2005).

In normal epithelial cells, and during early stages of tumor development, TGF-β functions as a growth inhibitor, thereby acting as a tumor suppressor. Lack of responsiveness to TGF-β's antiproliferative signals is therefore considered a key step in tumor development (Wakefield and Roberts, 2002). Conversely, TGF-β's role as a tumor promoter is supported by many *in vitro* and *in vivo* studies (Pardali and Moustakas, 2007). TGF-β's ability to enhance tumor cell invasiveness and migration (Dalal *et al*., 1993), and to modulate the vascular and immune cell compartment of the tumor stroma (Nam *et al*., 2008), are mechanisms proposed to underlie this tumor-promoting activity.

Epithelial-to-mesenchymal transition (EMT) is a process during which epithelial cells acquire the attributes of mesenchymal cells, including increased motility and invasiveness (Shook and Keller, 2003; Thiery, 2003; Hay, 2005; Peinado *et al*., 2007; Gregory *et al*., 2008). Although EMT is clearly critical for the vertebrate embryonal gastrulation process (Hay, 2005), its role in tumor progression has been debated (Tarin *et al*., 2005). Nonetheless, considerable evidence has

accumulated over the years supporting a role for EMT in tumor invasion and dissemination (Brabletz *et al*., 2001; Hugo *et al*., 2007; Scheel *et al*., 2007; Yang *et al*., 2008). Depending on the context, EMT can be induced by TGF-β alone (Moustakas and Heldin, 2007) or in concert with other signaling pathways (Oft *et al*., 1996; Bates and Mercurio, 2003). Smads, as well as other effectors, have been implicated in the TGF-β-induced EMT process (Yu *et al*., 2002; Moustakas and Heldin, 2007) and are thus common to both the tumor-inhibitory and tumor-promoting aspects of TGF-β. Inhibitors targeting these components may therefore disrupt not only late-stage tumor progression and metastasis, but may also inadvertently accelerate the growth of preneoplastic lesions. For these reasons it is important to identify downstream targets that lie specifically in TGF-β's EMT-promoting cascades.

Here we present a transcriptional screen that identifies secreted clusterin (sCLU) as a candidate EMT-pathway-specific mediator. We confirm that sCLU is a downstream target specific to TGF-β's EMT-promoting transcriptional cascade, by demonstrating that anti-clusterin antibodies inhibit the TGF-β-induced EMT of BRI-JM01 cells, as well as the invasive phenotype of several other cell lines, without affecting TGF-β's growth-inhibitory activity. Additionally, using a 4T1 syngeneic mouse breast cancer model, which is considered to be one of the best models of postoperative stage-IV breast cancer (Aslakson and Miller, 1992; Lelekakis *et al*., 1999), we found that clusterin monoclonal antibodies significantly suppress lung metastasis. These results strongly suggest that sCLU promotes EMT of breast tumor cells, thereby enhancing their ability to form pulmonary metastases.

Results

Transcriptome analysis of BRI-JM01 cells undergoing TGF-β-induced EMT

The BRI-JM01 cell line, which has an intact canonical Smad signaling cascade (Supplementary Figure 1), provides an attractive model for the study of TGF-β's EMT and growth-inhibitory pathways (Lenferink *et al*., 2004). Although this cell line was isolated from a mammary tumor from a bigenic mouse (activated Neu + TGF-β type-II receptor antisense RNA), these cells do not express either transgene (Lenferink *et al*., 2004). The EMT that occurs in the BRI-JM01 cell line is characterized by acquisition of a spindle-shaped morphology, rearrangement of F-actin fibers, dissolution of adherens and tight junctions and increase in cell motility (Figure 1; see also references Lenferink *et al*. (2004)). To identify EMT mediators downstream of TGF-β, we generated a DNA microarray data set from BRI-JM01 cells exposed for 0.5–24 h to TGF-β1. Transcriptional changes were evaluated and 328 significantly modulated genes were identified (Supplementary Table 1). Two-dimensional hierarchical clustering (Eisen *et al*., 1998) grouped these modulated genes into clusters corresponding to 'early' (0.5–1 h), 'middle' (2–6 h) and 'late' (12–24 h) phases of EMT (Figure 1b). Several known TGF-β1-responsive genes (for example, *idb2*, *idb3*, *myc*) were identified. Gene ontology term classification indicated that the majority of the genes encode for proteins with structural and extracellular matrix (for example, *fn1*,

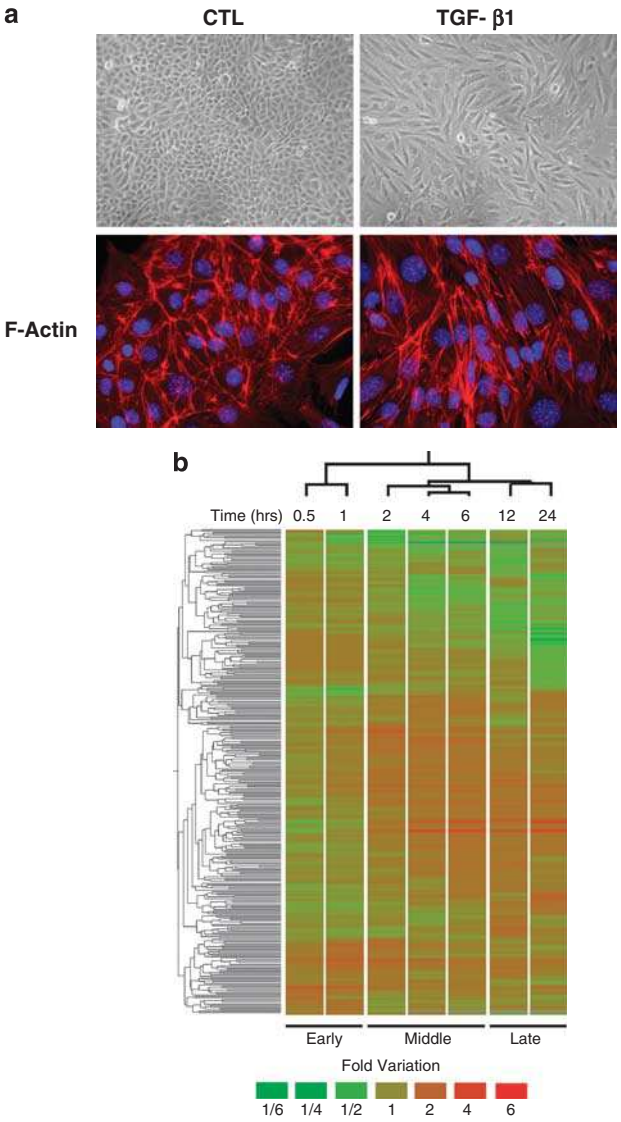

Figure 1 cDNA microarray analysis of the TGF-β1 induced EMT in BRI-JM01 cells. (**a**) BRI-JM01 cells switch from an epithelial (CTL) to a mesenchymal morphology (magnification ×40) when treated with 100 pM TGF-β1 (24 h). This morphology change is accompanied by a rearrangement of the F-actin filaments (magnification ×400). (**b**) Two-dimensional hierarchical clustering of the 328 statistically significant TGF-β1 modulated genes in the BRI-JM01 cell line (upregulated, red; downregulated, green) that were identified using the SAM algorithm with a 1.3-fold cut-off and a false discovery rate <10%. Vertical dendrograms illustrate similarities between gene expression profiles; horizontal dendrograms show similarities between various time points. A complete list of all modulated genes can be found in Supplementary Table 1. EMT, epithelial-to-mesenchymal transition; TGF-β, transforming growth factor-β.

itgb1, *sdc1-3*) or signal transduction (for example, *abl1*, *rgs2*, *sgk*, *ghr*)-associated functions.

The genes we identified overlap with the publicly available EMT data sets from TGF-β1-treated NMuMG mouse mammary epithelial cells (Xie *et al*., 2003; Valcourt *et al*., 2005), whereas 16 of our upregulated transcripts can also be found in a subset of human NCI-60 cancer cell lines displaying a mesenchymal phenotype (Ross *et al*., 2000) (Supplementary Table 2).

Validation of selected cDNA microarray data at the RNA and protein level

Selected transcripts were validated using semi-quantitative reverse transcription–PCR (Figure 2a and Supplementary Figure 2). Clusterin was confirmed to be significantly upregulated during the middle and late EMT phase. Western blots of BRI-JM01 cell extracts demonstrated that TGF-β1 treatment upregulated the levels of clusterin protein (Figure 2b). Two bands

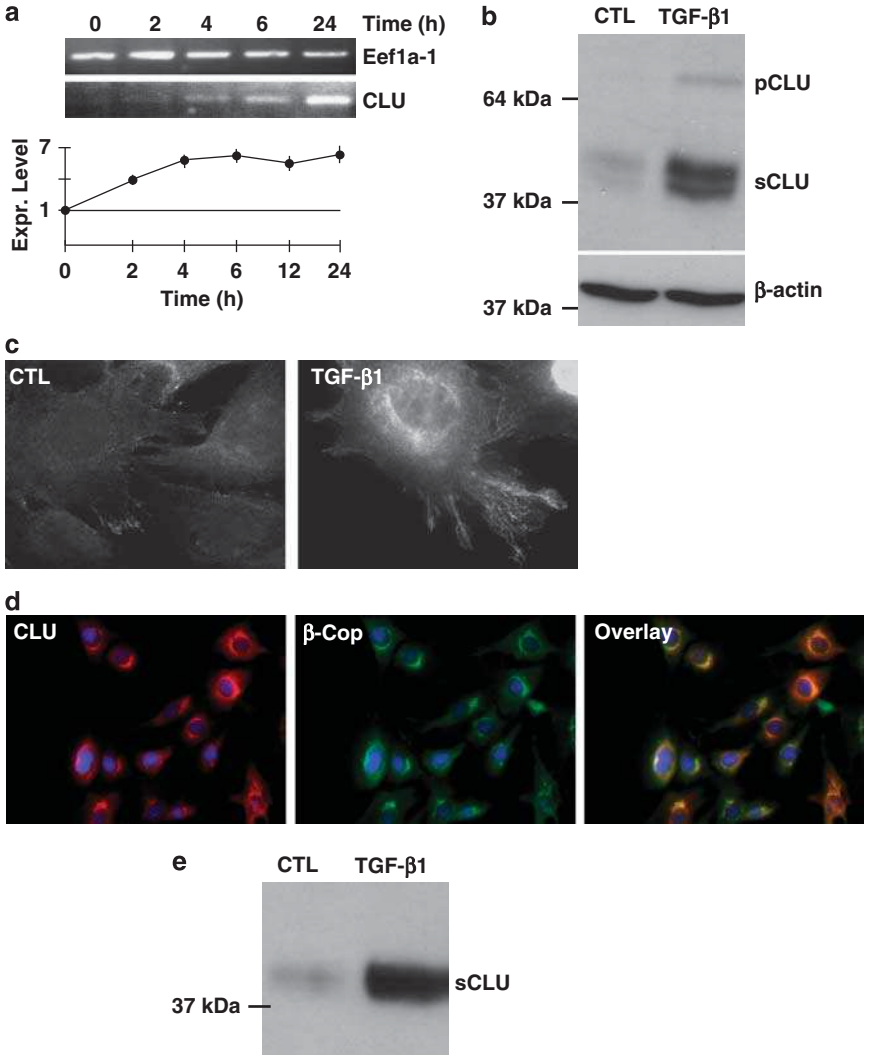

Figure 2 Validation of clusterin upregulation by semi-quantitative reverse transcription–PCR, western blot analysis and immunofluorescence microscopy. (**a**) Semi-quantitative reverse transcription–PCR analysis (bottom panels) of the clusterin transcript (CLU) in BRI-JM01 cells induced after 2, 4, 6 and 24 h of TGF-β1 (100 pM) exposure confirming the clusterin transcript modulation observed in the microarray experiments (The graph underneath represents normalized data obtained from the microarray experiments. Bars represent the t-test *P*-value of 4–6 experiments performed for each time point.) The Eef1a-1 transcript (top panel), which does not change upon TGF-β1 treatment, was used as loading control. (**b**) Western blot analysis of whole-cell lysates (WCL) from BRI-JM01 cells grown in the absence or presence of 100 pM TGF-β1 (24 h) confirming the upregulation of clusterin and showing the uncleaved precursor form (pCLU) of clusterin in the WCL, and the the mature processed form (sCLU) in both the WCL and the CM. Membranes containing the WCL samples were reprobed for β-actin to confirm equal loading. (**c**) Immunofluorescence microscopy (magnification × 1000) of BRI-JM01 cells treated with 100 pM TGF-β1 (24 h) show clusterin to be close to the cell's outer membrane (top panels) and (**d**) colocalized with the Golgi marker β-COP (bottom) (red, clusterin; green, β-COP; blue, diamidino phenylindole (DAPI)-stained nuclei; magnification × 400). (**e**) Western blot of the CM confirming the increased levels of secreted clusterin (sCLU) in the medium of BRI-JM01 cells treated with 100 pM TGF-β1 (24 h). CM, conditioned medium; sCLU, secreted clusterin; TGF-β, transforming growth factor-β.

were detected; a ~60-kDa band corresponding to the uncleaved clusterin precursor (termed pCLU) and a 40-kDa band representing mature clusterin (composed of α and β-chains, each with a molecular weight of ~40 kDa; Figure 2b). We refer to the 40-kDa band as sCLU, a term that was recommended by Trougakos *et al*., 2009, since this form of clusterin is often secreted. Immunofluorescence microscopy demonstrated that clusterin was present in the perinuclear area of the cell and in vesicles at the cell boundary (Figure 2c). The perinucler colocalization with the Golgi marker β-COP (Figure 2d), indicates that clusterin is likely secreted by these cells. Indeed, a dose-dependent increase in the amount of sCLU in the conditioned media (CM) from BRI-JM01 cells treated with 6.25–100 pM TGF-β1 was observed (Figure 2e and Supplementary Figure 3).

sCLU acts as an extracellular mediator of TGF-β1-induced EMT in BRI-JM01 cells

We chose to adopt an antibody-based neutralization approach to assess whether sCLU acts as an extracellular mediator promoting EMT in BRI-JM01 cells. Polyclonal IgG (raised against a peptide sequence in the C-terminus of clusterin) significantly reduced several hallmark features of the TGF-β1-induced EMT, including acquisition of a spindle-shape morphology (data not shown), loss of junctional zona occludens-1 (ZO-1) (monitored by immunofluorescence microscopy; Figure 3a) and loss of junctional E-cadherin (monitored by flow cytometry and immunofluorescence microscopy; Figure 3c and Supplementary Figure 4). Antibody specificity was demonstrated by blocking polyclonal IgG with its antigen peptide (Supplementary Figure 4c). These results indicate that sCLU acts as a critical extracellular EMT-promoting mediator downstream of TGF-β1 in BRI-JM01 cells, and that the C-terminal region of clusterin is involved in this function.

To evaluate whether BRI-JM01 cells maintain a requirement for either TGF-β1 or clusterin at later stages of the EMT process, we tested whether CM obtained from cells cultured for 24 h in the absence (CM-CTL) or presence of TGF-β1 (CM-TGF-β1) induced EMT, and whether this induction could be blocked by TGF-β or clusterin antibodies. As expected, the CM-CTL did not induce EMT, whereas the CM-TGF-β1 promoted the loss of junctional ZO-1 (Figure 3b) and E-cadherin (Supplementary Figure 4b). Interestingly, a TGF-β-neutralizing antibody did not prevent CM-TGF-β1-induced EMT, even though the concentration of the TGF-β antibody was sufficient to block the EMT induced directly by TGF-β1 (Figure 3a). The clusterin polyclonal IgG on the other hand blocked the CM-TGF-β1-induced loss of junctional ZO-1 (Figure 3b) and E-cadherin (Supplementary Figure 4b). These results demonstrate that (1) after 24 h of TGF-β1 treatment, BRI-JM01 cells have reached a point in their transition at which secreted factors are able to promote the EMT independent of extracellular TGF-β1; (2) sCLU is one of these critical EMT-promoting factors and (3) sCLU acts through a mechanism that does not involve extracellular TGF-β.

Purified human clusterin induces EMT in BRI-JM01 cells

The next question we addressed was whether clusterin alone could promote EMT in the BRI-JM01 cells, or whether other TGF-β1-induced factors are also required. BRI-JM01 cells exposed to purified human clusterin (either expressed in human embryonic kidney (HEK)-293 cells (Figure 4a) or isolated from human serum (data not shown)) acquired a spindle-shaped morphology (Figure 4b, top panels) and exhibited loss of junctional ZO-1 (Figure 4b, bottom panels) and E-cadherin (Supplementary Figure 5), confirming that clusterin alone can promote EMT in these cells. One possible mechanism through which clusterin may stimulate EMT is by transactivation of the TβRI/TβRII receptor complex in a TGF-β-independent manner. SB431542, a specific TβRI-kinase inhibitor, did however not prevent the clusterin-induced change in cell morphology (as expected, it did when the change was TGF-β1-induced) (Supplementary Figure 6), indicating that clusterin is not acting through TβRI.

sCLU promotes the motility and invasion of BRI-JM01 cells, but does not cause growth inhibition

The ability of clusterin to promote two additional EMT features, cell motility and invasion, was evaluated using a wound-closure motility assay (Dumont *et al*., 2003), a black cellular spreading and motility (BCSM) assay (al Moustafa *et al*., 1999) and a Transwell invasion assay. In the wound-closure motility assay, BRI-JM01 cells treated with TGF-β1 or purified clusterin migrated into the wounded area more rapidly than untreated cells. Clusterin polyclonal IgG largely prevented TGF-β1-induced cell migration (Figure 5a). Similar results were obtained in the BCSM assay; exposure to both TGF-β1 and clusterin doubled the average area cleared by motile cells, whereas clusterin IgG reduced motility to that of control cells (Figure 5b). Finally, the Transwell invasion assay (Figure 5c) demonstrated that TGF-β1 and purified clusterin induced a twofold increase in the invasion of BRI-JM01 cells, whereas clusterin polyclonal IgG blocked the effect of TGF-β1.

To assess whether matrix metalloproteinases (MMPs) play a role in the increase in invasion induced by TGF-β1 and clusterin, we analysed CMs from TGF-β1- or clusterin-treated BRI-JM01 cells using a zymogram. MMP9 activity was upregulated under both the conditions, albeit to a somewhat lesser extent by clusterin. As expected, clusterin polyclonal IgG completely blocked clusterin-induced MMP9 upregulation; however, it only partly blocked the upregulation induced by TGF-β1. These results indicate that MMP9 is induced by TGF-β1 through clusterin-dependent and independent routes, and that regulation of MMP9 secretion may account at least in part for the effects of TGF-β1 and clusterin on the invasion of BRI-JM01 cells.

Previously we have shown that BRI-JM01 cells are growth inhibited by TGF-β1 (Lenferink *et al*., 2004). Using a thymidine incorporation assay we observed that

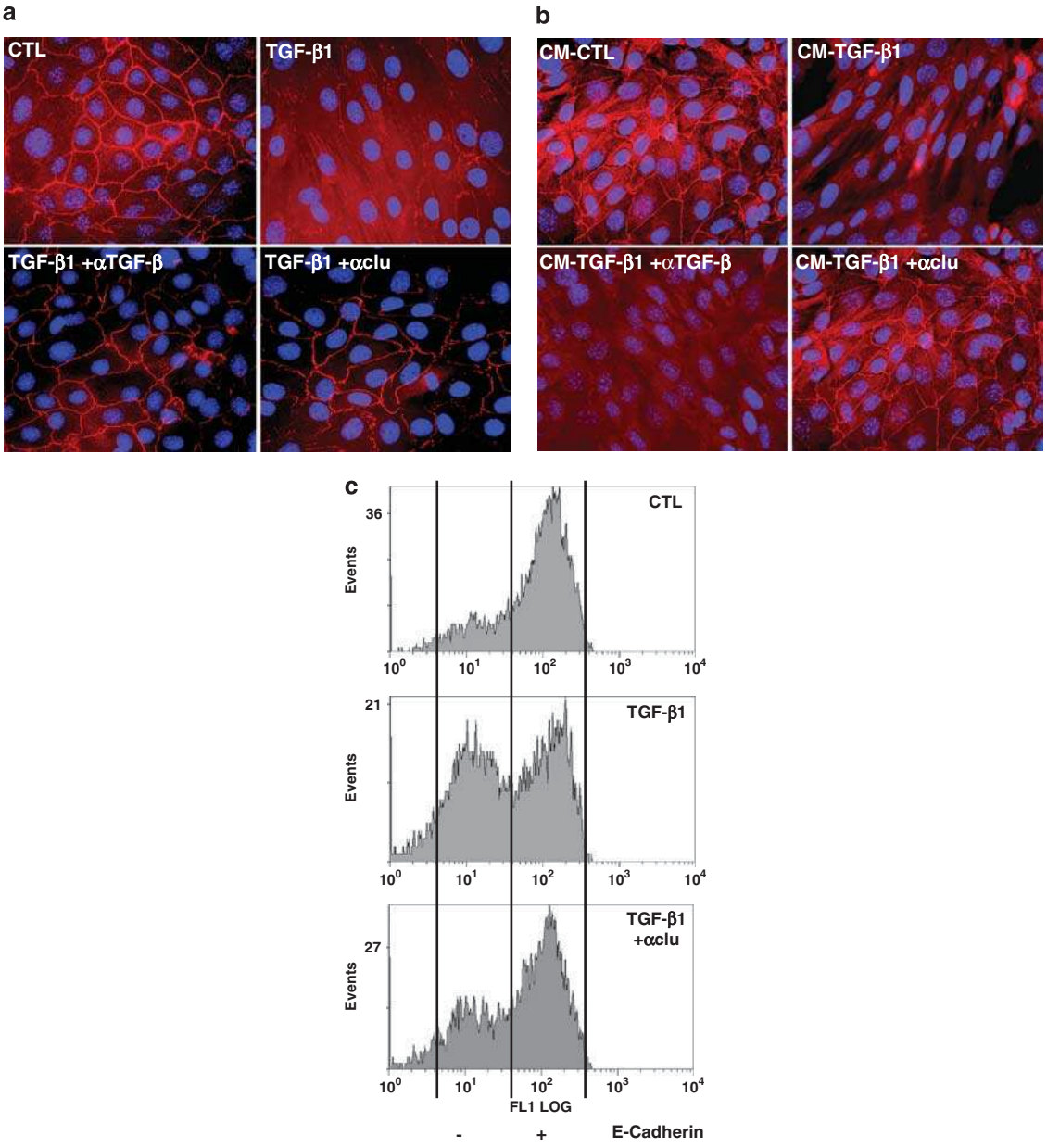

Figure 3 Antibodies against sCLU block the TGF-β1-induced loss of junctional ZO-1 and E-cadherin in BRI-JM01 cells. Immunofluorescence microscopy of ZO-1 in BRI-JM01 cells grown (**a**) in the absence or presence of 100 pM TGF-β1 with or without antibodies against TGF-β (anti-TGF-β, 10 nM) or clusterin (anti-clu, 8 μg/ml), or (**b**) in CM from untreated BRI-JM01 cells (CM-CTL) or from cells treated with 100 pM TGF-β1 for 24 h (CM-TGF-β1) both in the absence or presence of antibodies against (anti-TGF-β, 10 nM) or clusterin (anti-clu, 8 μg/ml). For all the panels: red, ZO-1; blue, diamidino phenylindole (DAPI)-stained nuclei (magnification: ×400). (**c**) Flow cytometric evaluation of the cell-surface levels of E-cadherin expressed by BRI-JM01 cells exposed to TGF-β1 (100 pM) in the absence or presence of clusterin polyclonal IgG (anti-clu, 8 μg/ml). Cell populations expressing high (+) and low (−) levels of cell-surface E-cadherin are indicated. CM, conditioned medium; sCLU, secreted clusterin; TGF-β, transforming growth factor-β; ZO, zona occludens.

clusterin polyclonal IgG did not block the growth inhibition induced by TGF-β1, and clusterin did not inhibit the growth of BRI-JM01 cells (Figure 5e). These results demonstrate that sCLU is not involved in TGF-β1's growth-inhibitory pathways in these cells, and suggest that clusterin is an effector that is relatively EMT-pathway-specific.

Clusterin polyclonal IgG inhibits the motility and invasiveness of several epithelial cell lines without affecting proliferation

We next determined whether clusterin plays a role in the invasive behavior and growth of two additional mouse mammary epithelial cell lines, the tumor 4T1 (Lou *et al*., 2008) and immortalized NMuMG cell lines, which have

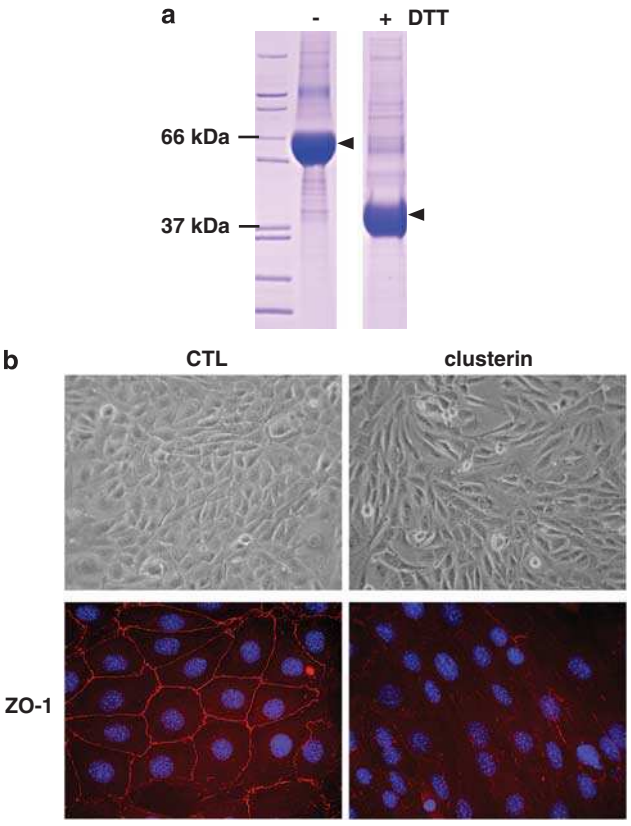

Figure 4 Purified clusterin promotes a spindle-shaped morphology and loss of junctional ZO-1 in BRI-JM01 cells. (**a**) Coomassie blue-stained gel under non-reducing (−DTT (dithiothreitol)) and reducing (+DTT) conditions of purified recombinant human clusterin (◀) expressed and secreted by HEK-293-E6 cells. (**b**) BRI-JM01 cells treated with 200 nM purified clusterin (24 h) show a spindle-shaped morphology (top; magnification ×40) and loss of tight-junctional ZO-1 (red, bottom; magnification ×400). HEK, human embryonic kidney; ZO, zona occludens.

been used previously as EMT models (Miettinen *et al*., 1994; Xie *et al*., 2003; Valcourt *et al*., 2005; Nam *et al*., 2006), and which both secrete detectable levels of clusterin (Figure 6a). We also assessed the human prostate tumor PC3 cell line, which secretes clusterin (Figure 6a) and, which has been used in many studies investigating the role of clusterin in prostate cancer (Sintich *et al*., 1999; Moretti *et al*., 2007).

When seeded in Matrigel, the 4T1 and PC3 tumor cell lines displayed a stellate morphology with protrusions sprouting into the Matrigel (Figure 6b), a feature correlated with increased invasive potential (Thompson *et al*., 1992). Clusterin polyclonal IgG significantly reduced this stellate morphology (Figure 6b), implying that the secretion of clusterin by these cells contributes to their invasive phenotype. This was confirmed using a Transwell invasion assay (Figure 6c), which showed that clusterin polyclonal IgG reduced the invasiveness of the 4T1 and PC3 cells by ∼60 and 25%, respectively. In addition, we also used the MDA-MB231LM2 cell line (Minn *et al*., 2005), which secretes clusterin (Figure 6e) and has been used in EMT studies (Buijs *et al*., 2007a, 2007b; Hunakova *et al*., 2009; Jo *et al*., 2009). We found that clusterin polyclonal IgG also reduced the invasiveness of these cells by ∼30%.

In contrast to the tumor cell lines, NMuMG cells did not display a stellate morphology in Matrigel (Figure 6a). However, there moderate potential to invade, as shown in the Transwell invasion assay was reduced by ∼25% in the presence of clusterin polyclonal IgG (Figure 6c), which indicates that sCLU promotes invasion of these cells. With regard to proliferation (Figure 6d), TGF-β1 inhibited the growth of 4T1 cells only slightly (∼20%), whereas proliferation of both NMuMG and PC3 cells was significantly inhibited (∼70 and ∼55%, respectively). The TGF-β1-induced growth inhibition of these cell lines was not affected by clusterin polyclonal IgG, further supporting the idea that sCLU functions as an EMT-pathway-specific effector.

Clusterin monoclonal antibodies reduce 4T1 cell metastasis to lung after orthotopic implantation

To this point we made use of polyclonal IgG (raised against a clusterin peptide antigen) to investigate the EMT-promoting effects of sCLU in tumor cell lines. To further investigate the role of sCLU in promoting tumor metastasis *in vivo*, we generated 12 clusterin monoclonal antibodies (CLU mAbs) using full-length clusterin as antigen. These CLU mAbs were tested for their clusterin-neutralizing ability using 4T1 cells in the BCSM motility assay (Figure 7a). We found that several of the 12 mAbs (including 11E2, 16B5 and 16C11) inhibited cell motility to the same extent or more than the clusterin anti-peptide polyclonal IgG (C18) or its monoclonal counterpart (B5), whereas others, such as 20G3, did not (Figure 7a). Interestingly, the majority of the EMT-blocking mAbs have epitopes that overlap with that of the anti-peptide polyclonal IgG (paper in preparation), supporting the premise that an epitope at the C-terminus of clusterin is important for clusterin's EMT-promoting activity.

We evaluated the effect of the 16B5, 16C11, 11E2 and 20G3 CLU mAbs on lung metastasis in BALB/c mice with orthotopically implanted 4T1 cells (experimental design; see Figure 7b). We found that the EMT-neutralizing CLU mAbs 16B5, 16C11 and 11E2 significantly reduced lung metastasis, whereas the effect of 20G3 was not statistically different from the saline-injected control animals (Figure 7c). These results suggest that sCLU can serve as a target for antimetastatic mAbs, and that the antimetastatic activity of these mAbs is due, at least in part, to their ability to block the EMT-promoting function of sCLU.

Discussion

To investigate the dual tumor-suppressing and promoting roles played by TGF-β, we used transcriptional profiling to identify targets that lie in its EMT-promoting pathway. The transcriptome analysis of the TGF-β1-induced EMT in the BRI-JM01 cell line contributes to existing EMT-related transcriptional data

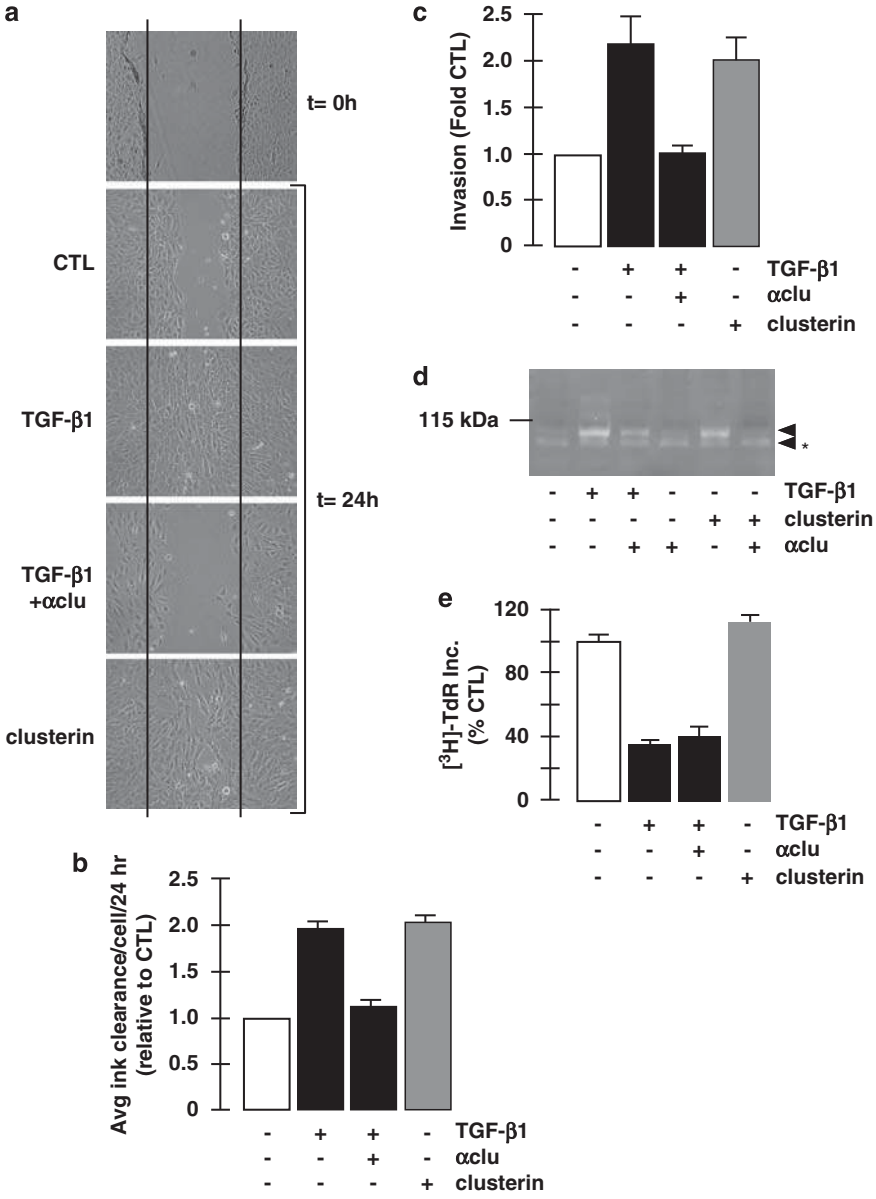

Figure 5 sCLU promotes cell motility and invasion, but not growth inhibition of BRI-JM01 cells. (**a**) A wound-healing assay depicting BRI-JM01 cells exposed to 100 pM TGF-β1 (24 h) in the presence or absence of clusterin polyclonal IgG (anti-clu, 8 μg/ml) or purified clusterin (200 nM) alone. Non-treated cells (CTL) were used as control for endogenous motility. The initial wound edge is shown as a black line (0 h). (**b**) Using the BCSM) assay, the motility of BRI-JM01 cells treated for 24 h with 100 pM TGF-β1 (black bars) ± clusterin polyclonal IgG (anti-clu), or with purified clusterin (gray bar) was determined by evaluating the ink clearance in 10 independent microscopic fields per treatment using the ImageJ Freeware (http://rsb.info.nih.gov/ij/). The average motility is expressed as average ink clearance/cell/24 h and is relative to non-treated control cells (± s.d.). (**c**) The ability of BRI-JM01 cells to invade Matrigel while exposed to TGF-β1 ± clusterin polyclonal IgG (anti-clu), or purified clusterin was evaluated using a Transwell invasion assay. Results (expressed relative to non-treated cells) are shown as the average (± s.d.) of two independent experiments. (**d**) Zymogram showing MMP9 gelatinase activity (◀) secreted in the CM by BRI-JM01 cells treated with 100 pM TGF-β1 or with 200 nM purified clusterin, either alone or in the presence of clusterin polyclonal IgG (anti-clu, 8 μg/ml). The bottom band (◀*) represents bovine MMP9 present in the cell culture medium containing 5% FBS. (**e**) Growth inhibition of BRI-JM01 treated with 100 pM TGF-β1 (black bars) ± clusterin polyclonal IgG (anti-clu, 8 μg/ml), or with 200 nM purified clusterin (gray bar) was monitored by measuring the tritiated thymidine ([3H]-TdR) incorporation. Results are expressed as a percentage of non-treated cells and shown as the average (± s.d.) of two independent experiments carried out in triplicate. BCSM, black cellular spreading and motility; FBS, fetal bovine serum; MMP, matrix metalloproteinase; sCLU, secreted clusterin; TGF-β, transforming growth factor-β.

sets and which can be used to identify molecular EMT signatures, and to search for correlations with the metastatic potential of human tumor cell lines/tumors (Chin *et al*., 2006; Neve *et al*., 2006; Nguyen and Massague, 2007). We further evaluated clusterin as a candidate EMT mediator since it was (1) the most significantly upregulated gene in our data set; (2) selectively expressed in a subset of human NCI-60

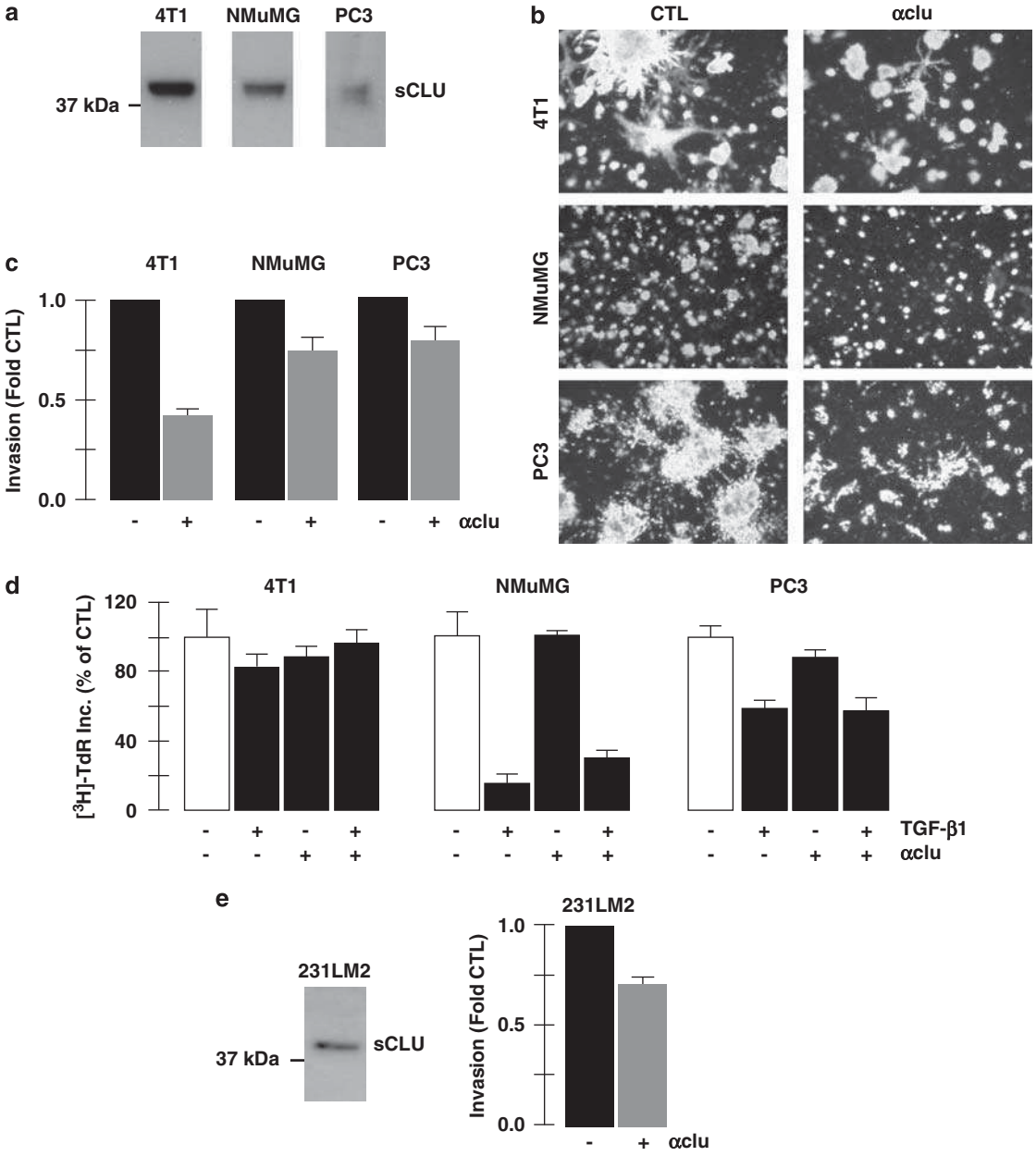

Figure 6 Clusterin polyclonal IgG inhibits the invasive behavior of cell lines other than the BRI-JM01 cell line. Clusterin polyclonal IgG (anti-clu, 8 μg/ml) inhibits (**a**) the stellate morphology of 4T1 and PC3 tumor cells when cultured in Matrigel for 3 weeks (magnification ×40). (**b**) Western blot analysis confirming the presence of sCLU in the CM of 4T1, NMuMG and PC3 cells. (**c**) Clusterin polyclonal IgG (anti-clu, 8 μg/ml) inhibits the Matrigel invasion of 4T1, NMuMG and PC3 cell lines ing a Transwell invasion assay. Results (expressed relative to non-treated cells) are shown as the average (±s.d.) of two independent experiments. (**d**) Clusterin polyclonal IgG (anti-clu, 8 μg/ml) does not significantly affect the growth-inhibitory response induced by TGF-β1 in 4T1, NMuMG and PC3 cells. Results are expressed relative to non-treated cells and are shown as the average (±s.d.) of two independent experiments performed in triplicate. (**e**) Western blot analysis showing the presence of sCLU in the CM of MDA-MB231LM2 cells (left panel). Clusterin polyclonal IgG (anti-clu, 8 μg/ml) inhibits the Matrigel invasion of these cells in a Transwell assay (right panel). Results (expressed relative to non-treated cells) are shown as the average (±s.d.) of two independent experiments. CM, conditioned medium; sCLU, secreted clusterin; TGF-β, transforming growth factor-β.

cancer cell lines displaying a mesenchymal phenotype and (3) secreted by the TGF-β1-treated BRI-JM01 cells, rendering it amenable for antibody neutralization.

Clusterin was identified as a protein promoting cell aggregation (Blaschuk *et al*., 1983; Fritz *et al*., 1983), but lately has been implicated in diverse cellular processes (Trougakos and Gonos, 2002; Trougakos *et al*., 2005; Shannan *et al*., 2006). These diverse functions can be attributed to the existence of two alternatively spliced forms, encoding secreted and nuclear clusterin (Shannan *et al*., 2006; Andersen *et al*., 2007; Cochrane *et al*., 2007). sCLU is a 70- to 80-kDa heterodimeric glycoprotein, which confers chemoresistance to cancer cells (Miyake *et al*., 2000;

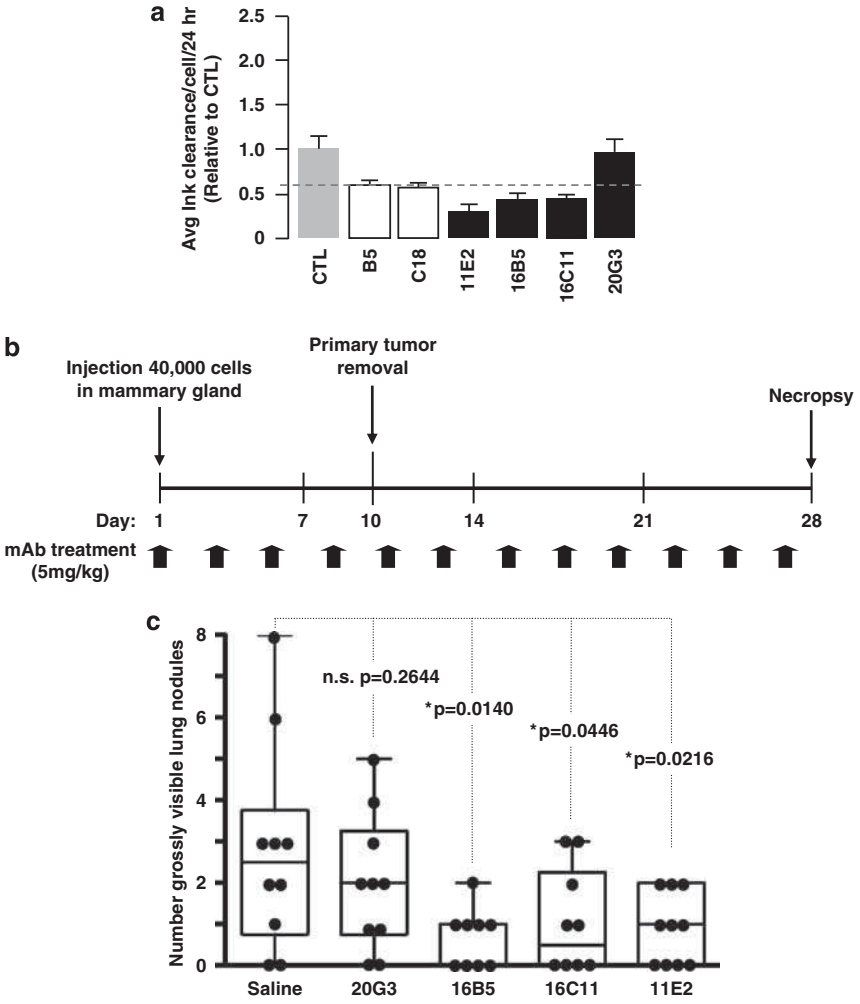

Figure 7 Clusterin monoclonal antibodies inhibit the motility of 4T1 cells and significantly reduce 4T1 cell metastasis to lungs after orthotopic implantation. (**a**) Evaluation of the ability of in-house-generated clusterin monoclonal antibodies 11E2, 16B5, 16C11 and 20G3 at a concentration of 8 μg/ml (black bars), and a commercially available clusterin monoclonal antibody B5 and anti-peptide polyclonal IgG, C18 (white bars), to block 4T1 cell motility in a BCSM assay. The average motility (± s.d.) was determined by measuring ink clearance in 10 independent microscopic fields (per treatment) and is expressed as average ink clearance/cell/24 h relative to non-treated control cells (gray bar). The hatched line depicts the cut-off that we used to define an antibody as having neutralizing activity, which was based on the degree of inhibition caused by B5 antibody. (**b**) Experimental design. 4T1 cells (4×10^4) were injected in the left # 4 inguinal mammary gland. Animals were treated (5 mg/kg) with neutralizing (16B5, 16C11 and 11E2) clusterin antibody, non-neutralizing (20G3) clusterin antibody or saline (control) thrice a week (intraperitoneally), starting the day of cell implantation. Mice were killed on day 28. (**c**) The number of macroscopically grossly visible lung metastasis in the mice were quantified 28 days post tumor cell implantation and statistically analysed using the non-parametric Mann–Whitney *U*-test ($n = 10$ for the saline, 20G3 and 11E2 groups; $n = 9$ for the 16B5 and 16C11 groups). BCSM, black cellular spreading and motility.

Gleave *et al*., 2001; Chi *et al*., 2005). Nuclear clusterin, which is translated from an alternative AUG codon (Leskov *et al*., 2003), exhibits tumor-suppressing activities (Miyake *et al*., 2000; Leskov *et al*., 2003; Scaltriti *et al*., 2004; Chi *et al*., 2005). The fact that clusterin has been reported to be overexpressed in various human tumors and that Custirsen (OGX-011), a clusterin-expression-inhibiting antisense oligonucleotide (Zellweger *et al*., 2001; So *et al*., 2005a, b, c), is currently in cancer clinical trials (Chi *et al*., 2005), support the notion that clusterin has a tumor-promoting role (Steinberg *et al*., 1997; Redondo *et al*., 2000; Miyake *et al*., 2002b; Park *et al*., 2008).

We demonstrated that BRI-JM01 cells secrete large amounts of clusterin upon TGF-β1 treatment, whereas basal clusterin secretion in NMuMG, 4T1, PC3 and MDA-MB231LM2 cells can be upregulated by TGF-β1. Antibodies targeting the C-terminal region of clusterin inhibited several aspects of the EMT process in BRI-JM01 cells and the invasive potential of these other cell lines, indicating that sCLU promotes a mesenchymal phenotype. In addition, purified clusterin induced EMT in BRI-JM01 cells without affecting cell proliferation, while *in vivo* studies using a 4T1 cell orthotopic tumor mouse model indicated that EMT-neutralizing CLU mAbs reduced pulmonary metastases. To our knowledge, this is the first report identifying sCLU as a downstream mediator in TGF-β's EMT-promoting pathway, even though clusterin has been linked previously to TGF-β as a transcriptional target (Jin and

Howe, 1997, 1999) and as a marker for TGF-β1-mediated thyrocyte dedifferentiation (Wegrowski *et al*., 1999). In addition, intracellular clusterin has also been shown to interact with the C-terminus of the TGF-β receptors (Reddy *et al*., 1996) and to regulate Smad2/3 stability (Lee *et al*., 2008). Since our neutralizing antibodies can only target sCLU, it is unlikely that these other interactions are responsible for clusterin's EMT-promoting effects.

sCLU has been shown to interact extracellularly with the LRP-2/megalin receptors (Lakins *et al*., 2002). However, we did not detect LRP-2 receptor transcripts in the BRI-JM01 cell line (data not shown). Also, mAb G7, which inhibits binding of clusterin to LRP-2 (Lakins *et al*., 2002), did not prevent the clusterin-induced EMT. We were not able to detect cell-surface-specific binding using iodinated clusterin (data not shown) suggesting that sCLU may promote EMT by interacting with a soluble ligand. Clusterin has been shown to bind to a wide range of soluble ligands (Wilson and Easterbrook-Smith, 2000; Ammar and Closset, 2008; Jo *et al*., 2008), which likely results from clusterin's ability to chaperone stressed proteins (Humphreys *et al*., 1999; Poon *et al*., 2002). Although we have not yet been able to identify the extracellular interaction underliying sCLU's EMT-promoting activity, we have established that residues 421–443 of the clusterin β-subunit are important for this activity. This sequence, which defines the epitope of our EMT-blocking antibodies, lies within a region that is predicted to be intrinsically disordered (Dunker *et al*., 2001), suggesting that these antibodies block the interaction of a ligand by constraining the conformation of this disordered region.

MMP9 is a known mediator acting downstream of TGF-β1. We demonstrated that purified clusterin increased MMP9 activity in BRI-JM01 cells, whereas EMT-blocking clusterin antibodies at least partially reduced this increase, implying that MMP9 activity is important for EMT induction by both clusterin and TGF-β.

Clusterin's effects on cell motility have been reported previously to be either inhibitory (Santilli *et al*., 2003), stimulatory (Miyake *et al*., 2002a; Lau *et al*., 2006) or neutral (Moretti *et al*., 2007). It is unclear whether variations in the expression of nuclear versus cytosolic/sCLU underlie these differences. Our results, showing that clusterin antibodies inhibit the motility/invasion of BRI-JM01, NMuMG, 4T1, PC3 and MDA-MB231LM2 cell lines, provide evidence that sCLU stimulates cell motility and invasion. In accordance with our study, Chou *et al*. (2009) recently reported that clusterin promotes EMT in lung A549 cells. It is, however, not clear whether nuclear clusterin or sCLU is involved in promoting EMT in these cells.

In summary, we identified sCLU as an extracellular mediator, which promotes multiple hallmark features of EMT in several cell lines, but which does not participate in the growth-inhibitory pathways of TGF-β. This, together with our *in vivo* data demonstrating an antimetastatic effect of clusterin antibodies, suggest that sCLU could act as an accessible therapeutic target whose neutralization will inhibit metastasis while sparing TGF-β's beneficial tumor-suppressing, growth-inhibitory effects.

Materials and methods

Cell culture, antibodies and reagents

BRI-JM01 cells were isolated, characterized and cultured as described by Lenferink *et al*. (2004). Mouse mammary 4T1and NMuMG (ATCC), human prostate PC3 (NCI) and human MDA-MB231LM2 (provided by Dr P Siegel) cells were cultured accordingly. Human recombinant TGF-β1- and pan-TGF-β-neutralizing antibody 1D11 (R&D Systems, Minneapolis, MN, USA), and TGF-β type-I inhibitor SB431542 (Sigma, Oakville, Canada), were reconstituted according to the manufacturer's instructions. Human serum clusterin was kindly provided by Professor MR Wilson (Wilson and Easterbrook-Smith, 1992). Antibodies against the following proteins were purchased: clusterin (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), E-cadherin (Sigma, Minneapolis, MN, USA; R&D Systems, Oakville, Canada), ZO-1 (anti-ZO-1; Chemicon, Billerica, MA, USA; Zymed, San Francisco, CA, USA), β-COP (anti-β-COP; Cedarlane, Burlington, Canada) smad2 and phospho-smad2 (both Cell Signaling Technology, Danvers, MA, USA) and β-actin (Sigma). Horseradish peroxidase-conjugated antibodies were obtained from Jackson ImmunoResearch Laboratories Inc. (West Grove, PA, USA) and Alexa-488-labeled antibodies and Texas-red-labeled phalloidin were from Molecular Probes (Eugene, OR, USA).

RNA isolation and labeling

Monolayers of BRI-JM01 cells were grown in full medium in the absence or presence of 100 pM TGF-β1 for 30 min, 1, 2, 4, 6, 12 or 24 h. PolyA + mRNA was extracted (4×150 mm dishes) using the FastTrack 2.0 kit (Invitrogen, Burlington, Canada) as described by the manufacturer. Labeled cDNA was purified using the QIAquick PCR purification kit (Qiagen, Mississauga, Canada) according to the manufacturer's guidelines, with modifications (see Supplementary information).

Hybridization and data analysis

cDNA microarrays (15 264 sequence-verified mouse ESTs) were obtained from the University Health Network Microarray Centre (http://www.microarrays.ca/). Slides were hybridized with Cy3- or Cy5-labeled cDNA as described by Enjalbert *et al*. (2003), scanned at a 10-micron resolution (ScanArray 5000, version 2.11; Perkin Elmer, Waltham, MA, USA) and quantified (16-bit TIFF files, QuantArray, verson 3.0; Perkin Elmer). Normalization (Lowess algorithm) and hierarchical clustering were performed as described by Enjalbert *et al*. (2003) using GeneSpring (Agilent Technologies, Santa Clara, CA, USA). The SAM one-class algorithm (Tusher *et al*., 2001) was used to identify significantly modulated genes (false discovery rate $< 10\%$; at least a 1.3-fold variation at one or more time points).

Semi-quantitative reverse transcription–PCR

Cells were grown in 100-mm dishes and treated for 24 h with TGF-β1 (100 pM). Total RNA was isolated and 3–5 μg was amplified (20 μl first-strand reverse transcription–PCR reaction) using 50 U of SuperScript II (Invitrogen), according to the manufacturer's guidelines, with modifications (see Supplementary information).

Expression and purification of recombinant human clusterin
HEK-293-6E cells were grown and transiently transfected with the human clusterin gene containing the pTT vector (Durocher *et al*., 2002), using PEI as a transfection reagent (Tom *et al*., 2007). For further details see the Supplementary information.

Western blot
Cells grown in 35-mm dishes were treated for 24 h with TGF-β1 (100 pM) and CM was collected. Monolayers were rinsed (phosphate-buffered saline (PBS)), lysed and protein concentrations were determined using a BCA protein assay. Total cell lysates (50 μg) or CM normalized for total protein in the corresponding lysate was resolved by sodium dodecyl sulfate–polyacryladmide gel electrophoresis (10%) under reducing conditions. Nitrocellulose membranes were probed for total Smad2/3 (1/500) or phospho-Smad2 (1/500) antibodies, or anti-clusterin (1/500) antibodies (CM). Immunoreactive bands were visualized by chemiluminescence (Perkin-Elmer).

Immunofluorescence microscopy
Cells were seeded in glass chamber slides (Lab-Tek) and treated with purified clusterin (200 nM) or TGF-β1 (100 pM) with or without anti-clusterin (8 μg/ml) or anti-TGF-β (10 nM). CM obtained from cells grown for 24 h with or without TGF-β1 (100 pM) was preincubated (30 min) with anti-clusterin and/or anti-TGF-β prior to addition to non-treated BRI-JM01 cells. After 24 h, cells were fixed, permeabilized, blocked, incubated with primary antibody (anti-ZO-1 (1/100), anti-clusterin (1/50), anti-E-cadherin (1/200) and anti-β-COP (1/300)) and further processed as described by Lenferink *et al*. (2004).

Thymidine incorporation
Cells (2.5×10^4 cells/well) were seeded in 24-well plates in 5% fetal bovine serum (FBS). The medium was replenished the next day with medium containing 1% FBS after which purified clusterin (200 nM), TGF-β1 (100 pM), TGF-β1 + anti-TGF-β (100 nM) or TGF-β1 + anti-clusterin (8 μg/ml) was added. The next day cells were processed as described by Lenferink *et al.* (2004).

Cell motility
Wound-healing assays were performed as described by Dumont *et al*. (2003), whereas several modifications (see Supplementary information) were made to the BCSM assay (al Moustafa *et al*., 1999). Cells (2×10^4 cells/well) were immediately seeded in 500 μl of medium ± TGF-β1 (100 pM), TGF-β1 + anti-clusterin (8 μg/ml), anti-clusterin alone (8 μg/ml) or purified clusterin (200 nM) and images were captured 24 h later.

Flow cytometry
Cells were grown (48 h) in 100-mm dishes with or without TGF-β1 (100 pM) ± anti-clusterin (8 μg/ml), harvested (non-enzymatic cell dissociation buffer; Sigma), washed twice (ice-cold PBS + 10% FBS (PBS/10%)) and incubated (20 min, 4 °C), pelleted, resuspended in PBS/10% and divided in three equal portions and incubated with E-cadherin antibody (1:1600), an isotype-equivalent control antibody (anti-hemagglutinin, 1:1600) or secondary Alexa-488 antibody (1:400) in PBS/10% (1 h, 4 °C). Cells were rinsed twice (ice-cold PBS/10%), incubated with Alexa-488 secondary antibody in the dark (1 h, 4 °C), washed twice (ice-cold PBS/10%) and resuspended (1 ml PBS). Viable cell populations were selected and cell-surface E-cadherin expression was detected using a Coulter EPICSTM XL-MCL flow cytometer (Beckman-Coulter, Mississauga, Canada).

Matrigel outgrow
Twelve-well plates were coated with growth factor-reduced Matrigel (Becton Dickinson, Franklin Lakes, NJ, USA). Cells (2.5×10^4) were resuspended in culture medium/Matrigel (1:3), layered on top of the Matrigel-covered bottom and covered with cell-specific growth medium. Where indicated, anti-clusterin (8 μg/ml) was added to each layer. Plates were incubated (37 °C) for up to 3 weeks during which growth medium (± anti-clusterin) was replenished weekly.

Transwell invasion
Twenty-four-well Biocoat Matrigel invasion chambers (8 μm; BD Biosciences, Franklin Lakes, NJ, USA) were used according to the manufacturer's protocol. Top chambers were seeded with 5×10^4 viable BRI-JM01, NMuMG, PC3 and 4T1 cells in culture medium containing 0.2% FBS, and bottom chambers were filled with culture medium containing 10% FBS. TGF-β1 (100 pM) ± anti-clusterin (8 μg/ml) was added to both the compartments. After 24 (4T1) or 48 h (JM01, NMuMG, PC3), non-invasive cells were removed with a cotton swab. Cells that migrated through the membrane were fixed and stained with 0.2% crystal violet (w/v) in anhydrous ethanol.

Gelatin zymography
Cells grown in 12-well dishes were treated for 24 h with TGF-β1 (100 pM) after which the CM was collected. Monolayers were rinsed (PBS), lysed and protein concentrations were determined using a BCA protein assay (Pierce, Rockford, IL, USA). Volumes of CM, normalized for total cell protein, were loaded and gels were processed as described by Matsubara *et al*. (1991a,b).

In vivo *metastasis study*
All animals were maintained under approved animal protocols according to the Biotechnology Research Institute Animal Care Committee guidelines. Female BALB/c animals (6–8 weeks old; Charles River, Wilmington, MA, USA) were anesthetized and 20 μl of Matrigel/sterile saline (1:1, v/v) containing 4×10^4 4T1 cells were injected into the left # 4 inguinal mammary gland. Mice were randomized into five treatment groups (10 animals) and clusterin monoclonal antibodies 16B5, 16C11, 11E2 and 20G3 (5 mg/kg bodyweight in 100 μl sterile saline), or sterile saline (100 μl), were injected (intraperitoneally) thrice a week starting the day of tumor cell implantation. Primary tumors were surgically removed 10 days post tumor cell injection. Mice were killed on day 28 and the lungs evaluated. Statistical analysis was performed using the non-parametric Mann–Whitney *U*-test (Two-tailed, confidence interval of 99%).

Conflict of interest

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc)