An EMT spectrum defines an anoikis-resistant and spheroidogenic intermediate mesenchymal state that is sensitive to e-cadherin restoration by a src-kinase inhibitor, saracatinib (AZD0530)

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The phenotypic transformation of well-differentiated epithelial carcinoma into a mesenchymal-like state provides cancer cells with the ability to disseminate locally and to metastasise. Different degrees of epithelial–mesenchymal transition (EMT) have been found to occur in carcinomas from breast, colon and ovarian carcinoma (OC), among others. Numerous studies have focused on bona fide epithelial and mesenchymal states but rarely on intermediate states. In this study, we describe a model system for appraising the spectrum of EMT using 43 well-characterised OC cell lines. Phenotypic EMT characterisation reveals four subgroups: Epithelial, Intermediate E, Intermediate M and Mesenchymal, which represent different epithelial–mesenchymal compositions along the EMT spectrum. In cell-based EMT-related functional studies, OC cells harbouring an Intermediate M phenotype are characterised by higher N-cadherin and ZEB1 expression and lower E-cadherin and ERBB3/HER3 expression and are more anoikis-resistant and spheroidogenic. A specific Src-kinase inhibitor, Saracatinib (AZD0530), restores E-cadherin expression in Intermediate M cells in in vitro and in vivo models and abrogates spheroidogenesis. We show how a 33-gene EMT Signature can sub-classify an OC cohort into four EMT States correlating with progression-free survival (PFS). We conclude that the characterisation of intermediate EMT states provides a new approach to better define EMT. The concept of the EMT Spectrum allows the utilisation of EMT genes as predictive markers and the design and application of therapeutic targets for reversing EMT in a selective subgroup of patients.

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Epithelial–mesenchymal transition (EMT), a fundamental mechanism in embryonic development, is crucial in carcinoma progression.1 The EMT programme contributes to the dissemination of carcinoma cells from solid tumours and the formation of micro-metastatic foci that subsequently develop into clinically detectable metastases.2 EMT describes a process that controls the progressive loss of epithelial characteristics and the acquisition of mesenchymal features,3 as well as the acquisition of chemo-resistance,4 immune escape5 and the maintenance of cancer stemness.6,7 Fluctuations in several molecular markers are commonly used to assess the EMT status, such as an increase in the mesenchymal marker, vimentin and/or a decrease in the epithelial markers, E-cadherin and cytokeratin.1,3 However, studies usually overlook the progressive changes during intermediate cellular states.8,9 An intermediate state implies a spectrum of heterogeneity for EMT (rather than two extreme ends of the process), with potentially metastable, plastic intermediate cells. Indeed, cells within intermediate states express traits of a mixed lineage, with double positivity of cytokeratins and vimentins.8 However, few studies have addressed the functionality of these transitory states, an oversight partly caused by the lack of a detailed understanding of EMT and the current dearth of reliable readouts for its progression. Thus, the existence of intermediate states, which may also occur during carcinoma progression,10 has not been well appreciated.

Cancer cell line collections, such as the US National Cancer Institute (NCI) 60 human tumour cell line (NCI60), serve as models for drug discovery,11 and as robust tools for modelling...
in vivo tumours. Indeed, breast and ovarian cancer cell line collections, for example, have retained molecular characteristics corresponding to those of their in vivo counterparts, thus providing powerful options for modelling cancer heterogeneity in vitro\textsuperscript{12,13} and for studying the several hallmarks of cancer\.\textsuperscript{14}

However, EMT phenotyping in these in vitro models has not been systematically explored. One protocol proposes the use of morphological and molecular features to indicate EMT status, including the loss of cell–cell contact, elongation of cell shape, increased scattering migration/invasion and resistance to anoikis.\textsuperscript{15} Other in vitro studies have also demonstrated the importance of characterising EMT phenotypes in cancer cell lines\textsuperscript{16–18} to provide insight into the biological relevance of the EMT status.

Anoikis describes apoptotic cell death induced by anchorage-free/cell-matrix-disrupted conditions.\textsuperscript{19,20} To achieve distant dissemination, cancer cells must overcome anoikis thought to be achieved by an increase in the expression of integrins compatible with the surrounding extracellular matrix (ECM), overexpression of pro-survival receptor tyrosine kinases that can compensate for missing integrins, cytoskeletal rearrangement for mechano-sensing or sustainability of an EMT phenotype.\textsuperscript{21} Indeed, EMT induction via silencing E-cadherin\textsuperscript{22} or sFRP1\textsuperscript{23} can protect mammary epithelial cells against anoikis. These results indicate that the gain of a mesenchymal phenotype confers anoikis resistance, with possibly common regulators between these two systems.\textsuperscript{21,24}

Ovarian carcinoma (OC) is a unique entity among cancers with EMT involvement.\textsuperscript{25–27} Metastasis in OC is established by the EMT-driven delamination of OC cells from the primary tumour in situ and their penetration into the surrounding peritoneal cavity. EMT and its reversed process, mesenchymal–epithelial transition (MET), are frequently and actively involved in different phases of OC progression.\textsuperscript{26}

Although several EMT markers are correlated with clinical significance in OC,\textsuperscript{27} a global clinical view of EMT and its potential intermediate state(s) has not been elucidated. In this study, we describe a model system for appraising the heterogeneous spectrum of EMT using a panel of well-characterised OC cell lines.\textsuperscript{13,28} Our detailed phenotypic characterisation of their epithelial–mesenchymal compositions

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**Figure 1** Identification of epithelial–mesenchymal phenotypes and EMT Spectrum in SGOCL(43). (a) The EMT phenotypic characterisation was achieved using IF staining of E-cadherin (E-cad), pan-cytokeratin (PCK) and Vimentin (Vim). Four phenotypes were identified: Epithelial (E-cad-positive, PCK-positive, Vim-negative), Intermediate E (E-cad-positive, PCK-positive, Vim-positive), Intermediate M (E-cad-negative, PCK-positive, Vim-positive) and Mesenchymal (E-cad-negative, PCK-negative, Vim-positive). (b) Phase contrast images (Phase) and IF staining of E-cadherin (E-cad), Pan-cytokeratin (PCK) and Vimentin (Vim) in Caov3, OVCA432, DOV13 and OVCAR10, representing Epithelial, Intermediate E, Intermediate M and Mesenchymal phenotypes, respectively. Scale bar = 200 \(\mu\)m. (c) Pie chart of the number and percentage distribution of four phenotypes in SGOCL(43). (d) Plot of QPCR expressions (2^{-\Delta\Delta C_{T}}) of the key EMT genes E-cadherin (CDH1), cytokeratin 19 (KRT19) and vimentin (VIM), showed a gradient along Epithelial (e), Intermediate E (Int E), Intermediate M (Int M) and Mesenchymal (M) phenotypes. Statistical significance at **\(P<0.05\) in both ANOVA and Kruskal–Wallis test.
describes an intermediate phenotype with both epithelial and mesenchymal characteristics that confers a more aggressive phenotype.

**Results**

**Four phenotypic subgroups identified by epithelial–mesenchymal status.** An OC library comprising 43 cell lines (SGOCL(43); Supplementary Table 1), was utilised to explore EMT heterogeneity. The epithelial–mesenchymal phenotype for each cell line was characterised by morphological examination and immunofluorescence (IF) staining for prototypic EMT markers. A decision rule was established to determine the phenotype of each line based on the IF pattern of E-cadherin, pan-cytokeratin and vimentin (Figure 1a; Materials and Methods). SGOCL(43) was characterised into four epithelial–mesenchymal phenotypes: Epithelial, Intermediate Epithelial (Intermediate E), Intermediate Mesenchymal (Intermediate M) and Mesenchymal (Figures 1a and b; Supplementary Table 2), with 9 (20.9%) Epithelial, 18 (41.9%) Intermediate E, 8 (18.6%) Intermediate M and 7 (18.6%) Mesenchymal phenotypes.

**Establishing an EMT spectrum.** To validate EMT gene expression among the four phenotypes, we utilised a commercially available quantitative PCR (QPCR) amplification array of 84 EMT-related genes. Of these, 34 genes showed significant differences in expression among the four EMT phenotypes (ANOVA or Kruskal–Wallis test; Table 1 and Supplementary Table 3), with significant differences observed for CDH1, KRT19 and VIM expression. A gradient pattern was observed among the four phenotypes (Figure 1d) – for instance, CDH1 and VIM displayed descending and ascending trends, respectively (Figure 1d and Table 1) – with a significant negative correlation (Spearman correlation coefficient: $-0.66$; $P$-value: 2.65411e-006). Thus, the phenotypic categorisation of SGOCL(43) created an EMT Spectrum within which EMT markers adhere to an EMT gradient. Several other EMT-related genes also demonstrated a gradient of expression, including $CDH2$, $CDH1$, and $VIM$, with $CDH2$ showing significant differences in expression among the four phenotypes, we utilised a

| Gene symbol | $P$-value (KW) | Expression pattern |
|-------------|---------------|--------------------|
| CDH1        | 8.79E-06**    | Descending         |
| DSP         | 0.0004348**   | Descending         |
| F11R        | 0.0153598**   | Descending         |
| FGFBP1      | 0.0008591**   | Descending         |
| IL1RN       | 0.0007688**   | Descending         |
| JAG1        | 0.0133748**   | Descending         |
| KRT19       | 4.85E-05**    | Descending         |
| MST1R       | 0.0013871**   | Descending         |
| WNT11       | 0.0495988**   | Descending         |
| AKT1        | 0.0328503**   | Nadir at Int E    |
| WNT5A       | 0.0473454*    | Nadir at Int E    |
| ERBB3       | 0.000465**    | Nadir at Int M    |
| TWIST1      | 0.0019854**   | Ascending         |
| VIM         | 2.68E-05**    | Ascending         |
| WNT5B       | 0.0329648*    | Ascending         |
| ZEB2        | 0.0001512**   | Ascending         |
| GNG11       | 0.003524**    | Peak at Int E     |
| SNA1        | 0.032298**    | Peak at Int E     |
| BMP7        | 0.0493499**   | Peak at Int M     |
| CDH2        | 0.0138235**   | Peak at Int M     |
| FN1         | 0.0193777**   | Peak at Int M     |
| ILK         | 0.0069188**   | Peak at Int M     |
| ITGA5       | 0.0019977**   | Peak at Int M     |
| MAP1B       | 0.0037885**   | Peak at Int M     |
| MMP2        | 0.0209024*    | Peak at Int M     |
| SERPIN1     | 0.0161845*    | Peak at Int M     |
| SOX10       | 0.0336869*    | Peak at Int M     |
| VCAN        | 0.0496592*    | Peak at Int M     |
| ZEB1        | 2.20E-05**    | Peak at Int M     |
| ESR1        | 0.0186407*    | No pattern        |
| MTF         | 0.0087447**   | No Pattern        |
| TGFBI       | 0.0382433*    | No Pattern        |

FOXC2, GSC and SNAI2 showed no significant difference in expression (Supplementary Table 3), whereas SNAI1, TWIST1, ZEB1 and ZEB2 showed sequential expression along the EMT Spectrum, with variable peaks in expression of SNAI1 and ZEB1 (Intermediate E) and TWIST1 and ZEB2 (Mesenchymal) (Figure 2f). Furthermore, CDH2 and ERBB3 at Intermediate M were apparently correlated with ZEB1 expression (Figures 2a and d). These results suggest the existence of different waves of expression of EMT drivers along the EMT Spectrum.

**Intermediate M phenotype has higher anoikis resistance and spheroid-forming ability in vitro.** To explore the biological functions of these different phenotypes along the EMT Spectrum, several EMT-related functional analyses were performed on selected lines. OC aggressiveness is often marked by the presence of malignant ascites, where OC cells are shed and survive in suspension as single cells or spheroids once overcoming anoikis. Thus, we compared the viability index (VI) of cells grown under conditions of ultra-low attachment suspension (ULAS) to those grown on normal tissue culture plastic (TCP). Generally, all four phenotypes survived well on TCP, (VI $>1.0$; Supplementary Figure 2), whereas in ULAS, the VI dropped significantly in most cell lines, indicating that the suspension was unfavourable for growth. However, for all Mesenchymal (100%) and the majority of Intermediate M (75%) lines, the VI was above the majority of Intermediate M (75%) lines, the VI was above the majority of Intermediate M (75%) lines, the VI was above
Very few Epithelial (33.3%) or Intermediate E (25%) lines had an VI above 1.0 (Figures 3a and b). These data indicate that Mesenchymal and Intermediate M phenotypes might be more resistant to anoikis. We also observed morphological differences after 96 h in ULAS cultures. The majority of the tested lines formed ‘grape-like’, irregularly shaped aggregates (marked as A; Figures 3c and d) in ULAS, whereas almost all Intermediate M lines formed smooth contoured spheroids (S). Only one Epithelial line, OV90, showed spheroid formation but with significantly lower efficiency than that in Intermediate M SKOV3 line, which showed high spheroidogenic efficiency (SKOV3 expanded from 10 spheroids to 50 spheroids/1000 cells following sequential passages in ULAS; Figure 3e). Cell migration and invasion assays in vitro showed that Intermediate M cells had a tendency toward higher migratory and invasive potential than other cell lines (Supplementary Figures 3A–D), and using a subcutaneous xenograft model in nude mice, we showed that the SKOV3 line caused tumour formation after 5 weeks in the absence of Matrigel (Figure 3f and Supplementary Figure 3F).

Collectively, we conclude that Intermediate M cells are more anoikis-resistant, display increased spheroidogenic potential and have a tendency toward increased migration and invasion. Thus, the Intermediate M phenotype might represent an aggressive category in vitro.

Relevance of EMT Spectrum in other cancers. To demonstrate the relevance of the EMT Spectrum in other cancers, we tested the lung adenocarcinoma cell line, A549, which exhibits an Intermediate E phenotype, as determined by its co-expression of E-cadherin, N-cadherin, cytokeratins and vimentin. Performing single clone selection, we isolated two clones exhibiting epithelial (A549E-A6) or mesenchymal (A549M-L3) morphology from the parental A549 (A549P) cells. A549E-A6 exhibited a rounded shape in sparse cultures and a cobblestone-like monolayer in confluent cultures, whereas A549M-L3 exhibited an elongated cell shape in sparse and confluent cultures (Figure 4a). From QPCR, we found that A549P expressed CDH1 and CDH2 at comparable levels, whereas A549E-A6 and A549M-L3 preferentially
expressed CDH1 and CDH2, respectively (Figure 4b and Supplementary Table 4). In addition, A549M-L3 showed a higher expression of ZEB1 and lower ERBB3 as compared with A549E-A6 and A549P (Figure 4b and Supplementary Table 4). Thus, the A549M-L3 clone may resemble an Intermediate M phenotype. In spheroid-forming assays, A549M-L3 displayed high spheroid-forming efficiency, whereas A549E-A6 only formed huge cell clumps in ULAS (Figures 4c and d). These data suggest that an Intermediate M-like sub-clone of A549 was more aggressive in vitro and that the phenomenon is not exclusive to OC.

Saracatinib (AZD0530) restores E-cadherin expression in intermediate M phenotype. Inhibitors targeting ALK5, MEK and Src kinases can induce EMT reversal. We utilised these inhibitors to test whether we could alter the four EMT phenotypes. As compared with OVCA3 (Epithelial), OVCA433 (Intermediate E) and OVCA110 (Mesenchymal) cells, only SKOV3 (Intermediate M) cells underwent reversal after treatment with the Src-kinase inhibitor Saracatinib (AZD0530) (Figure 5a and Supplementary Figure 4).

Furthermore, in AZD0530-treated SKOV3 cells, QPCR analysis showed a significant, dose-dependent upregulation in CDH1 transcripts (Figure 5b, Supplementary Figure 5A), decreases in SNAI1 and SNAI2 (Supplementary Figure 4B) but no change in ZEB1, ZEB2 or TWIST1 (Supplementary Figure 4B). We also measured a fivefold increase in E-cadherin promoter activity in AZD0530-treated SKOV3 cells, with a concomitant increase in E-cadherin protein levels (Figures 5c and d). The AZD0530-treated SKOV3 tumour xenograft in mice also showed a dramatic increase in E-cadherin immunoreactivity (Figure 5e). These data demonstrate that the Intermediate M phenotype of SKOV3 cells could be reversed by inhibiting Src kinase.

Saracatinib (AZD0530) affects spheroid formation in intermediate M SKOV3 cells. We next tested whether AZD0530 would render the Intermediate M cells anoikis-sensitive and tested its effects on spheroid formation. As demonstrated above, VI in ULAS was significantly lower than cells on TCP with or without AZD0530. In addition, VI was significantly reduced with AZD0530 treatment in both
conditions; however, the difference in VI was larger for cells on TCP (Figure 5f). This suggests that Src kinase may not have a major role in anoikis resistance. However, AZD0530 significantly decreased spheroid formation efficiency and average spheroid size in ULAS cultures (Figures 5g–i), although administering AZD0530 did not alter the size or weight of mice following SKOV3 xenograft (data not shown).

EMT signature identifies an Intermediate subgroup of OC patients with worse progression-free survival (PFS). To verify the clinical relevance of the intermediate EMT states identified in vitro, we utilised an EMT Signature to stratify OC patients based on different EMT states. Three genes, CDH1, ZEB1, and ERBB3, were used to generate gene expression signatures: CDH1 represents the descending EMT gradient; ZEB1, the peak at Intermediate M; and ERBB3, the nadir at Intermediate M. A Venn diagram was then used to compare commonly encountered gene IDs that we believed represented the key components in EMT (Figure 6a). Table 2 shows the complete list of 36 probe-set IDs representing 33 identified genes.

Three genes overlapped with the genes that showed peak expression at Intermediate M in the EMT array: ITGA5, VIM and ZEB1 (Figure 2a and Table 1). To further test how well their expression patterns fit with our IF-based classification scheme, we validated the selected genes from the EMT Signature that have been reported to be involved in EMT and not included in the initial EMT array (Table 2) in SGOCL(43). PRSS8 and RAB25 showed a gradient pattern similar to the EMT Spectrum (Supplementary Figure 7A), whereas EPCAM, ESRP1 (Supplementary Figure 7B), DDR1 and GRHL2 (data not shown) expression levels were lowest at Intermediate M. This further confirmed the robustness of our IF-based EMT Spectrum and the validity of utilising the EMT Signature to stratify OC patients.

Hierarchical clustering of the expression profiles of an OC data set GSE9891 using the 33-gene EMT Signature identified four clusters: Epithelial (E), Intermediate E (IE), Intermediate M (IM) and Mesenchymal (M) (Figure 6b). From the PFS analysis, these four clusters showed interesting trends in PFS (Figure 6c). The 5-year PFS difference was significant in E versus M and IE versus M clusters (log-rank test; Figures 6c and d), with no other differences observed. We also found that the restricted mean survival time (RMST) difference at 60 months indicated a survival advantage of IE4E4IM4M (Figure 6e), with only a slightly better PFS in the M cluster than in the IM (IM versus M: 2.185 at 60 months, Figure 6e; Supplementary Figure 7). Collectively, we conclude that the EMT Signature could be applied to identify a subcategory of OC tumours with an intermediate EMT status correlated with worse clinical outcome.

Discussion

The SGOCL(43) in vitro OC cell line model system was used to identify an EMT Spectrum. Only 20.9% of SGOCL(43) cell lines maintained the full epithelial phenotype, and 18.6% a full mesenchymal phenotype, with a complete loss of epithelial features. As such, most of the SGOCL(43) lines were in intermediate EMT states (60.5%), suggesting that most carcinoma cells gain mesenchymal features without completely losing their epithelial characteristics during EMT in vitro.
For example, A2780 cells stain negatively for E-cadherin but positively for N-cadherin at the junctions (Supplementary Figure 8), with weak cytoplasmic E-cadherin staining observed. As such, A2780 cells were categorised into the Mesenchymal subgroup, even though they display an 'epithelial'-like morphology. During the establishment or propagation of cell lines in vitro, clones that have undergone partial EMT within the heterogeneous population might already have more advantages during clonal selection as compared with cells that have not. As approximately two-thirds of the cell lines screened showed an intermediate phenotype, this suggests that a fully executed EMT might not be necessary for gaining growth advantages during clonal expansion. Indeed, others have shown that promotion of cell survival could be achieved via partial EMT. Thus, the difference in the growth advantage for transitioned cells could explain the relatively limited cell lines with a solely Epithelial phenotype. However, the difference between Intermediate E and Intermediate M in resisting anoikis and forming spheroids suggests that a more complete EMT execution endows cells with increased aggressiveness.

During EMT, cells undergo a series of sequential events to disintegrate cell–cell contacts. With the established EMT Spectrum, we were able to detect changes in gene expression patterns, with the sequential activation of major EMT drivers: for instance, SNAI1 might be required early to downregulate CDH1 followed by SNAI2, ZEB1/2 and TWIST to further accomplish the cadherin switch. These sequential changes support previous studies, where SNAI1 is expressed at the onset of EMT and SNAI2, ZEB1/2 and TWIST are induced later. This EMT Spectrum will serve as a very informative tool, given that most of the current knowledge about EMT derives from binary separation between the epithelial and mesenchymal phenotypes.
Interestingly, despite the comparable anoikis resistance between Mesenchymal and Intermediate M phenotypes, the Mesenchymal cells lacked spheroid-forming ability in suspension cultures. This might be because of their decreased expression of genes important for cell–microenvironment interactions, such as ITGAV and MMP2. Interactions between α5β1-integrin and fibronectin are reported to mediate OC spheroidogenesis and adhesion to ECM proteins at sites of secondary metastasis. In the OC clinical data set, however, the PFS was similar between the Mesenchymal and Intermediate M groups. One plausible explanation is that clinical tumours designated as Mesenchymal are unlikely to be completely devoid of epithelial characteristics. Therefore, these ‘clinically’ Mesenchymal tumours would resemble Intermediate M lines in vitro.

From our data, we also noticed that two Epithelial lines OVCAR8 and OV90 and one Intermediate E line IGROV1 demonstrated high VI in suspension cultures (Figure 3a). We previously showed that the IGROV1 cell line was molecularly characterised as ‘Stem-A’ as it demonstrated anchorage-independent growth in soft agar and others have shown that OVCAR8 has spheroid-forming property in a hanging droplet method. In the current study, OV90 also displayed spheroidogenic properties in ULAS cultures, albeit with limited efficiency (Figure 3e). These studies suggest that both the molecular properties and the EMT phenotype may contribute to anoikis resistance in OC cells and could explain why the three ‘epithelial’-like cell lines showed high anoikis resistance in our study. Interestingly, IGROV1 and OVCAR8 did not form spheroids in ULAS as compared with the reported soft agar and hanging droplet systems, perhaps suggesting that the ULAS culture condition is less favourable for these ‘epithelial’-like cell lines to form spheroids despite their ability to overcome anoikis.

Two of three genes used to create the EMT Signature, CDH1 and ZEB1, are well-characterised EMT factors, on the other hand, has been less associated with EMT. ERBB3, which codes for the HER3 receptor, has a neuregulin-binding domain but not an active kinase domain. ERBB3 is significantly increased in non-small cell lung cancer cell lines with an epithelial phenotype and our data provide further support for the preferential expression of ERBB3 in OC Epithelial cells. This is of special interest because the neuregulin/ERBB3 axis was recently shown to be a promising therapeutic target in OC. Therefore, our findings illustrate that targeting ERBB3/HER3 might only be effective in tumours that have not undergone EMT. Among the 33 genes in the EMT Signature, we noticed a higher frequency of epithelial over mesenchymal genes. This suggests that the threshold for losing epithelial characteristics during EMT might be lower than that for acquiring mesenchymal characteristics. This probably explains why the control of EMT is mainly via multiple negative regulatory loops, such that lifting the suppression can allow the efficient execution of EMT. However, it is still intriguing whether these epithelial and mesenchymal genes are regulated by common or separate mechanisms. Among the 33 genes, 16 genes have been shown to be involved in EMT (Table 2), whereas 17
Genes have not. Gaining a further understanding of how these 17 EMT Signature genes are regulated and their functional role in EMT will be crucial in the quest for EMT-reversing agents. One possibility is that these EMT Signature genes can act as reporters for screening of EMT-reversing compounds. As AZD0530 had a differential effect on EMT Signature genes, with maximal effect on CDH1, it would be beneficial to find compounds that could also restore the expression of other epithelial genes for EMT reversal.

Finally, our subclassification of OC demonstrated that there might be intrinsic heterogeneity contributing to EMT in tumours. Our previous work, showing that the enrichment of EMT-related genes can define the Mesenchymal subtype, is very robust and reproducible. Furthermore, the enrichment of EMT-related genes has been demonstrated in large OC data sets such as the Australian and TCGA OC cohorts. Indeed, the Mesenchymal molecular subtype in the TCGA data set significantly correlated with this 33-gene EMT Signature. Using gene set enrichment analysis (GSEA), the Mesenchymal molecular subtype of the TCGA cohort was enriched in the Mesenchymal gene set (P = 0.046) obtained from this EMT Signature (data not shown).

Phenotypic heterogeneity has been suggested in OC, as evidenced by subpopulations of transitory cells. We propose that tumour heterogeneity can be summarised and represented as an EMT Status describing cells that undergo full, partial, or no EMT. The EMT Status is a quantitative measure of the extent of EMT in tumours, ranging from 0 (no EMT) to 1 (full EMT).

Table 2: Summary of 33 key EMT component genes

| Affy transcript ID | Symbol | Entrez gene name | Phenotype | Ref |
|--------------------|--------|-----------------|-----------|-----|
| 8030484            | AP1M2  | Adaptor-related protein complex 1, mu 2 subunit | E         |     |
| 8136897            | ARHGEF5| Rho guanine nucleotide exchange factor (GEF) 5 | E         | 40  |
| 8143610            | ARHGEF5| Rho guanine nucleotide exchange factor (GEF) 5 | E         | 40  |
| 8024013            | C19orf21| Chromosome 19 open reading frame 21 | E         |     |
| 7996837            | CDH1   | Cadherin 1, type 1, E-cadherin (epithelial) | E         | 7   |
| 7996819            | CDH3   | Cadherin 3, type 1, P-cadherin (placental) | E         |     |
| 8129560            | CTAGE6P| CTAGE family, member 6, pseudogene | E         |     |
| 8117900            | DDR1   | Discoidin domain receptor tyrosine kinase 1 | E         | 49  |
| 8117786            | DDR1   | Discoidin domain receptor tyrosine kinase 1 | E         | 49  |
| 8081853            | EPCAM  | Epithelial cell adhesion molecule | E         | 7,48.56 |
| 8098439            | EPCAM  | Epithelial cell adhesion molecule | E         | 7,48.56 |
| 8147351            | ESRP1  | Epithelial splicing regulatory protein 1 | E         | 42.57,58 |
| 8040190            | GRHL1  | Grainyhead-like 1 (drosophila) | E         | 43  |
| 8147967            | GRHL2  | Grainyhead-like 2 (drosophila) | E         |     |
| 7901765            | HOOK1  | Hook homologue 1 (drosophila) | E         |     |
| 8035412            | JUP    | Junction plakoglobin | E         | 53  |
| 8009844            | LLGL2  | Lethal giant larvae homologue 2 (drosophila) | E         |     |
| 8027793            | LSR    | Lipolysis stimulated lipoprotein receptor | E         |     |
| 8148040            | MAL2   | mal, I-cell differentiation protein 2 | E         | 44  |
| 8129783            | MAP7   | Microtubule-associated protein 7 | E         |     |
| 8022367            | MYO5B  | Myosin VB | E         |     |
| 7962212            | PKP2   | Plakophilin 2 | E         |     |
| 8001007            | PRSS8  | protease, serine, 8 | E         | 45  |
| 7906079            | RAB25  | RAB25, member RAS oncogene family | E         | 41  |
| 7920297            | ST100A14| S100 calcium binding protein A14 | E         |     |
| 8005672            | SLCA4A2| Solute carrier family 44, member 2 | E         |     |
| 7982829            | SPINT1 | Serine peptidase inhibitor, Kunitz type 1 | E         | 46.47 |
| 79452604           | ST14   | Suppression of tumorigenicity 14 (colon carcinoma) | E         | 48.48 |
| 7980891            | TC2N   | Tandem C2 domains, nuclear | E         |     |
| 8117630            | ZNF165 | Zinc-finger protein 165 | E         |     |
| 8175647            | CD99L2  | CD99 molecule-like 2 | M         |     |
| 8030007            | EMP3   | Epithelial membrane protein 3 | M         |     |
| 7963786            | ITGA5  | Integrin, alpha 5 | M         | 50–52 |
| 8026407            | SYDE1  | Synapse defective 1, Rho GTPase, homologue 1 (C. elegans) | M         |     |
| 7926916            | VIM    | Vimentin | M         | 7.55 |
| 7926916            | ZEB1   | Zinc-finger E-box binding homeobox 1 | M         | 37.54 |

E, epithelial; M, mesenchymal

Figure 7: A proposed simplified scheme of EMT heterogeneity in carcinoma representing tumours harbouring a different EMT status as a function of undergoing various degrees of EMT process (No, Partial and Full EMT) under the cue of EMT gradient to acquire different phenotypes (Epithelial, Intermediate, and Mesenchymal)
partial or no EMT and leading to tumours with Epithelial, Intermediate or Mesenchymal phenotypes, respectively (Figure 7). EMT heterogeneity can be modelled by cell line characterisation to predict the in vivo tumour phenotype and outcome. Indeed, the reversal potential of the Intermediate M phenotype by ABD0530 may shed light on the potential clinical use of these compounds in EMT reversal; for instance, Intermediate M patients could be considered for trials of EMT-reversing agents postoperatively to better control PFS.

In conclusion, we identified an intermediate EMT phenotype with enhanced aggressiveness in vitro, characterised by high N-cadherin and ZEB1 expression and low E-cadherin and ERBB3/HER3 expression. An EMT Signature, derived from the molecular characterisation of this intermediate phenotype, can permit the subclassification of OC patients into four EMT States with differing clinical outcomes.

Materials and Methods

OC cell line library. An OC cell line library, termed SGOCCL(43), consisting of 43 different OC cell lines of serous, endometroid and undifferentiated histology, was acquired via various sources and maintained in house. Detailed cell line names and growth conditions can be found in Supplementary Table 1. Supplementary Figure 1 shows the schematic presentation of the experimental design in establishing and characterising the ovarian cancer cell line library described in this study.

Immunofluorescence staining of EMT markers. Cells were grown on 15-mm glass coverslips (Pialt Marienfeld GmbH & Co.KG, Germany) until 70–80% confluent. Cells were then fixed in cold acetone at −20 °C for 10 min, rehydrated with 1 x PBS (thrice, 5 min each) and blocked with 3% (w/v) BSA (Fraction V, Sigma-Aldrich, St. Louis, MO, USA) in 1 x PBS for 1 h at room temperature. After washing with PBS, incubations with primary antibodies against E-cadherin (no. 610182, BD Biosciences, San Jose, CA, USA, 1:200), pan-cytokeratin (no. M3515, AE1/AE3, Takara Bio Inc, Shiga, Japan, 1:200), N-cadherin (no. M142, Takara Bio Inc, Shiga, Japan; 1:200), pan-cytokeratin (no. M3515, AE1/AE3, Dako, Denmark; 1:100) and vimentin (no. M7020, Dako; 1:100) were performed at 37 °C for 1 h. After washing with PBS, incubation with secondary antibodies conjugated with Alexa-488 (no. A11029, no. A11034, Invitrogen, Eugene, OR, USA) was performed at room temperature for 1 h in the dark. Slides were washed again with PBS, and coverslips were subsequently mounted onto the glass slides with anti-fading mounting media (Vector Laboratories Inc., Burlingame, CA, USA). All images were viewed under an Olympus IX71 fluorescent microscope and images were taken under ×10 magnification using the Olympus DP71 camera (Olympus Optical Co. Ltd, Tokyo, Japan). The staining results were read by three independent researchers (JPT, SWJ and LYF). For E-cadherin and N-cadherin, only membranous junctional immunolabelling was regarded as positive (coded as 1 in Supplementary Table 2).

EMT phenotypic characterisation of SGOCCL(43). E-cadherin immunoreactivity was utilised to determine the general epithelial (E-Cad-positive) or mesenchymal (E-Cad-negative) category. Subsequently, pan-cytokeratin and vimentin immunoreactivities were used to determine the differentiation subcategory. Cells assigned into the epithelial category that were also for vimentin immunoreactivity were designated as having a true epithelial (E) phenotype. Cells assigned into the mesenchymal category that were also negative for pan-cytokeratin immunoreactivity were designated as a true mesenchymal (M) phenotype. Cells that co-expressed pan-cytokeratin and vimentin were designated as Intermediates.

Nucleic acid isolation. RNA was isolated based on the protocols described in the miRNeasy Kit (no. 217004, Qiagen, Gmbhl, Germany). Briefly, cells were lysed in QiaZol reagent (800 µl/10 cm² culture surface area), and the lysate vigorously mixed with 160 µl of chloroform before separating the aqueous phase via centrifugation (12,000 × g, 15 min, 4 °C). RNA was precipitated with 1.5 x volume of absolute ethanol (of aqueous phase) and purified using the spin columns provided in the kit. Isolated RNA was subsequently resuspended in nuclease-free water provided in the kit. RNA concentration was determined using the NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, San Jose, CA, USA) and RNA integrity was assessed using the Agilent Bioanalyzer 2100 (Agilent, Santa Clara, CA, USA).

ELISA assay for HER3. Total protein lysates of SGOCCL(43) (25 µg) were subjected to enzyme-linked immunosorbent assay (ELISA) tests using PathScan Total HER3/ErbB3 Sandwich ELISA Kit (no. 7888) and PathScan Phospho HER3/ErbB3 (panTyr) Sandwich ELISA Kit (no. 7890) (Cell Signalling Technology, Beverly, MA, USA). Phospho-HER3/ErbB3 (Tyr1289) (21D3) rabbit monoclonal antibody (no. 4791) (Cell Signalling Technology) was used as the detection antibody replacing the panTyr antibody provided in the Phospho Kit. The absorbance at 450 nm wavelength was measured using TECAN Infinite 200 PRO microplate reader (Tecan, Crailsheim, Germany).

Anokias assays and VI. Selected cell lines (CAOV3, OV90, OV2008, OVCAR3, OVCAR8, PEO1, IGROV1, OVCA429, OVCA433, PEO4, CH1, DOV13, Hey, HeyC2, SKOV3, A2780, A1847, HeyA88 and TYKNU) were used for the anokias assay. Anokias resistance assays were performed by seeding 5 x 10⁵ cells into the wells of six-well plates of either normal tissue culture grade (TCP; no. 140675, Nunc, Denmark) or ultra-low attachment grade (UL; no. 3471, Corning). Cells were incubated for 48 and 96 h prior to the MTT assay (no. G4100, Promega, Madison, WI, USA). Absorbance was read using a microplate reader (Tecan, Manfred, Switzerland). The degree of anokias resistance, termed VI,
was measured by the ratio of the absorbance readouts of MTT between 96 and 48 h. Anoikis resistance was determined if the VI measured above 1.0.

Spheroid assays and morphology examination. Cells of interest were trypsin-dissociated and strained through a 40-μm pore-strainer (no. 352340, BD Falcon, Franklin Lakes, NJ, USA). During quantification, the cell suspension was visually inspected for the presence of clumps using microscopy. Clumps were removed by additional rounds of straining until a single cell suspension was obtained. Single cells were seeded at a concentration of ~18 cells/mm² (~10⁵ cells in a 100-mm dish, 55 cm² surface area) in ULA dishes (no. 3262, Corning Inc.). Cell suspension was incubated overnight with full media under standard conditions (37°C, 5% CO₂) and strained as above after overnight to remove the presence of clumps. This was to prevent false-positive aggregates that may be mistaken for spheroids over prolonged culture. The cell suspension was incubated for a further period of 1–2 weeks for spheroid formation. Spheroids were determined by exhibiting a smooth surface of the compact multicellular masses by examining under a light microscope with proper phase contrast ring fitting (Nikon, Yokohama, Japan). Multicellular masses displaying uneven surface were determined as aggregates.

In vivo subcutaneous xenograft model. The human OC cell lines, SKOV3, OVCAR3, OVCAR10 and OVCAR433, were maintained in complete high-glucose Dulbecco’s modified Eagle’s medium (DMEM), supplemented with 10% fetal bovine serum (FBS) and 1% penicillin and streptomycin; the cells were cultured at 37°C in a humidified atmosphere containing 5% CO₂ and 95% air. The cells were harvested utilising 0.25% trypsin. Trypsin was inhibited using the same volume of complete medium. Cells were pelleted and resuspended in PBS in preparation for inoculation. All animal work adhered to the Agency of Science Technology and Research (A*STAR), Institutional Animal Care and use Committee (IACUC), guidelines on animal use and handling. Xenografts were generated by injecting 0.1 ml of 5 × 10⁶ cells/mouse into the dorsal flanks of female BALB/c nude mice (6–8 weeks old). Animal body weight and physical signs were monitored during the experiments. Tumour size was measured with vernier caliper every week. The tumour volume was calculated, with the formula: (length × width²)/2.

In vitro EMT reversal assays. Cells of interest were grown in complete media in either six-well plates (no. 140675, Nunc) or 100-mm dishes (no. 150350, Nunc) to allow growth until 60% confluence prior to drug treatment. DMSO control (no. D8418, Sigma-Aldrich; 0.05%) or AZD0530 at various concentrations were added for 24 h prior to downstream assays. Cells were examined under a light microscope with phase contrast rings (Olympus) to document morphological changes. RNA and protein were harvested and subjected to QPCR analysis and western blotting, respectively. For E-cadherin promoter assays, cells were seeded into 96-well plates (no. 3904, Corning Inc.) at a density of 0.5–1.0 × 10⁴ cells per well. After 24 h, the cells were transfected with E-cadherin promoter (a kind gift from Dr. Alice Wong, Hong Kong University) or vector control plasmid using XtremeGENE HP (no. 6366236001, Roche, Mannheim, Germany) with 2:1 HP:DNA ratio. The cells were treated with each drug on day 3 at a final concentration of 5 μM per well. The dual luciferase assay (no. E1960, Promega) was conducted on day 4 according to the manufacturer's protocol.

In vitro functional assays of AZD0530 treatment in SKOV3 cells. SKOV3 cells (ATCC, Manassas, VA, USA) were cultured in high-glucose DMEM (Nacalai Tesque) supplemented with 10% (v/v) FBS (Biowest SAS, Nuaille, France). Cells were pretreated with either AZD0530 at 5 μM (final concentration) or a similar volume of DMSO (for control conditions, final concentration: 0.05% v/v) for 3 days prior to the respective assays. After the respective treatments, SKOV3 cells were trypsin-dissociated, sorted using a 50-μm-mesh strainer and seeded at varying concentrations for the respective assays. During these assays, cells were treated as described before. For spheroid formation assays, pretreated SKOV3 cells were seeded at a concentration of 200 cells/well onto a neutrally charged, ultra-low attachment surface (ULA) 96-well plate (Corning Inc.) and treated as described above. Cells were incubated (2 weeks at 37°C, 5% CO₂) and the spheroids that formed were visually counted using an inverted microscope (Olympus). For anoikis assays, pretreated SKOV3 cells were seeded at a concentration of 1 × 10⁴ cells/well in six-well plates and treated as per the conditions mentioned above. Each treatment was carried out on both Nunclon-A surface (Nunc) for adherent cultures or on neutral-charged ULA for suspension culture. Cells were incubated for 48 and 96 h and an MTT assay (Promega) was carried out as per the instructions on the product user guide.

In vivo assays of AZD0530 treatment in SKOV3-luc-D3 cells. All animal work adhered to the Agency of Science Technology and Research (A*STAR), IACUC guidelines on the use and handling of animals. SKOV3-Luc-D3 cells (Xenogen Co., Alameda, CA, USA) at a density of 3.5 × 10⁶ in 100 μl of PBS were injected into the intraperitoneal cavity of 4-week-old female BALB/c nude mice. At 6 weeks post implantation, the mice were randomly divided into control and treatment groups (n = 5 animals per group). For the treatment group, mice were administered orally via gavage with 50 mg/kg AZD0530 (Selleck Chemicals, Houston, TX, USA) for 5 days a week for 2 weeks. The drug was re-suspended in 0.5% hydroxypropyl methylcellulose (Sigma-Aldrich) and 0.1% polysorbate buffer (Sigma-Aldrich). The control group received the vehicle buffer alone. The growth of tumour xenografts was monitored by bioluminescence using the IVIS system 2000 series (Xenogen Co.). The xenografts were harvested at 8 weeks post implantation for paraffin embedding followed by immunohistochemical staining for E-cadherin (no. 51955; Cell Signalling Technology, Beverly, MA, USA).

Gene expression microarrays and generation of epithelial signatures. Affymetrix GeneChip Human Gene 1.0ST Array was used for gene expression analysis according to the protocols from the manufacturer. Data were pre-processed and RMA-normalised using Affymetrix Gene Expression Console. Expressions for genes were mean-aggregated for each gene based on Affymetrix probes annotation. Six cell lines with the highest expressions and six with the lowest expressions of CDH1, ESRB3 and ZEB1, respectively, were selected to perform expression microarrays. The cell lines used in each signature are summarised below as are the expression cut-off levels: CHD1: High: Caov3, OVCAR42, OVCAR43, OAW42, OVCAR433, C13; Low: Hey, TykNu, OVCAR10, A2780, BG1, HeyA8, ESRB3: High: A1847, JHOS4, OVCAR420, PE01, OVCAR42, OVC3; Low: TykNu, HeyC2, OV7, OV56, HeyA8, SKOV3 and ZEB1: High: OV7, TykNu, SKOV3, Hey, COLO720E, BG1; Low: OVCAR3, OV2008, A2008, C13, OVAR8, JHOS4). CDH1, ESRB3 and ZEB1 signatures were generated by Partek Genomic Suites version 6.6 by using Student's t-test with FDR of 0.05.

EMT status identification in GSE8981 and Kaplan–Meier survival analysis. To identify the EMT subtype in the GSE8981 cohort, unsupervised hierarchical clustering (correlation similarity metric; average-linkage) was selected to perform expression microarrays. The cell lines used in each signature are summarised below as are the expression cut-off levels: CHD1 (High: Caov3, OVCAR432, OVCAR420, OAW42, OVCAR433, C13; Low: Hey, TykNu, OVCAR10, A2780, BG1, HeyA8), ESRB3 (High: A1847, JHOS4, OVCAR420, PE01, OVCAR42, OVC3; Low: TykNu, HeyC2, OV7, OV56, HeyA8, SKOV3) and ZEB1 (High: OV7, TykNu, SKOV3, Hey, COLO720E, BG1; Low: OVCAR3, OV2008, A2008, C13, OVAR8, JHOS4). CDH1, ESRB3 and ZEB1 signatures were generated by Partek Genomic Suites version 6.6 by using Student's t-test with FDR of 0.05.

Conflict of Interest

The authors declare no conflict of interest.

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Author Contributions

RY-JH and JPT designed and conceived the study. RY-JH wrote the manuscript. MKW performed the cell line expansion experiments and, together with YFL and VYC, performed QPCR experiments. TZZ and SM performed the microarray
analysis. HCL, JLL, MC provided clinical interpretation. NM provided some of the functional experiments. YFL, together with WJS, performed the immunofluorescence experiments and analysed the data. KTK, AHN and CT performed the ABD 0530 reversal experiments. YSC isolated the A549 clones.

1. Thiey JP. Epithelial-mesenchymal transitions in tumour progression. Nat Rev Cancer 2002; 2: 442–454.
2. Thiey JP. Metastasis: alone or together? Curr Biol 2009; 19: R1211–R1213.
3. Thiey JP, Siaman JP. Complex networks orchestrate epithelial-mesenchymal transitions. Nat Rev Mol Cell Biol 2008; 9: 131–142.
4. Kurrey NK, Malacinski PJ, Joglekar AV, Ghanate AD, Chaskar PD, Doiphode RY et al. Snail and slug mediate radioresistance and chemoresistance by antagonizing p53-mediated apoptosis and inducing a stem-like phenotype in ovarian cancer cells. Stem Cells 2009; 27: 2059–2068.
5. Kudo-Saito C, Shirako H, Takeuchi T, Kawakami Y. Cancer metastasis is accelerated through immunosuppression during Snail-induced EMT of cancer cells. Cancer Cell 2009; 15: 195–206.
6. Guo W, Liang MJ, Shute EN, Aysaan A, Zhou AY et al. The epithelial-mesenchymal transition generates cells with properties of stem cells. Cell 2008; 133: 704–715.
7. Guo W, Keckesova Z, Donaher JL, Shube T, Tischier V, Reinhardt F et al. Slug and Sox2 cooperatively determine the mammary stem cell state. Cell 2012; 148: 1015–1028.
8. Lee JM, Dedhar S, Kaluri R, Thompson EW. The epithelial-mesenchymal transition: new insights in signaling, development, and disease. J Cell Biol 2006; 172: 973–981.
9. Huang RY, Guilford P, Thiey JP. Early events in cell adhesion and polarity during epithelial-mesenchymal transition. J Cell Sci 2012; 125: 4417–4422.
10. Thiey JP, Acloque H, Huang RY, Nieto MA. Epithelial-mesenchymal transitions in development and disease. Cell 2009; 139: 671–890.
11. Shoemaker RH. The NCICD human tumour cell line anticancer drug screen. Nat Rev Cancer 2008; 8: 813–823.
12. Neve RM, Chin K, Fridyand J, Yeh J, Baehner FL, Fevr T et al. The miR-200 family and miR-205 regulate epithelial to mesenchymal transition by targeting ZEB1 and ZEB2. Nat Rev Cancer 2008; 8: 563–571.
13. Thiey JP, Siaman JP, Slamovits CH, Cao T, Sato TK, Nabet B, Hogenesch JB, Carstens RP. ESRP1 and ESRP2 are epithelial cell-type-specific regulators of FGFR2 splicing. Mol Cell 2009; 33: 591–601.
14. Casey RC, Burleson KM, Skubitz KM, Pambuccian SE, Oegema TR Jr., Ruff LE et al. Beta 1-integrins regulate the formation and adhesion of ovarian carcinoma multicellular spheroids. Am J Pathol 2001; 159: 2071–2080.
15. Sheng G, Liu X, Fleming E, Yuan K, Piao H, Chen J et al. An activated ErbB3/Erk1/2 autocrine loop supports in vivo proliferation of ovarian cancer cells. Cancer Cell 2010; 17: 298–310.
16. Davidson B, Trope CG, Reich R. Epithelial-mesenchymal transition in ovarian carcinoma. J Cell Physiol 2006; 206: 619–626.
17. Royston P, Parmar MK. The use of restricted mean survival time to estimate the treatment effect in randomized clinical trials when the proportional hazards assumption is in doubt. Statist Bull 2011; 80: 2409–2417.
18. Leroy P, Mostov KE. Slug is required for cell survival during partial epithelial-mesenchymal transition of HGF-induced tubulogenesis. Mol Biol Cell 2007; 18: 1943–1952.
19. Penaudo H, Olmeda D, Cano A. Snail and βH-Leg factors in tumour progression: an alliance against the epithelial phenotype? Nat Rev Cancer 2007; 7: 415–428.
20. Tothill RW, Tinker AV, George J, Brown R, Fox SB, Lade S et al. Novel molecular subtypes of senescent and endometrioid ovarian cancer linked to clinical outcome. Clin Cancer Res 2008; 14: 5198–5206.
21. Kalluri R. The role of the extracellular matrix in cancer progression. Annu Rev Physiol 2007; 69: 415–428.
22. Tothill RW, Tinker AV, George J, Brown R, Fox SB, Lade S et al. Novel molecular subtypes of senescent and endometrioid ovarian cancer linked to clinical outcome. Clin Cancer Res 2008; 14: 5198–5206.
23. Lee Y, Park S, Lee JW, Kim S. ZEB2 upregulates integrin alpha5 expression in recurrent ovarian cancer. Int J Cancer Suppl 2009; 11: 911–916.
24. Matsumura N, Huang Z, Mori S, Baba T, Fuji S, Konishi I et al. Epigenetic suppression of the TGF-beta pathway revealed by transcriptome profiling in ovarian cancer. Genome Res 2011; 21: 74–82.
25. Ahmed N, Thompson EW, Quinn MA. Epithelial-mesenchymal interconversions in normal ovarian surface epithelium and ovarian carcinomas: an exception to the norm. J Cell Physiol 2007; 213: 581–588.
26. Chun KA, Sim JW, Racine V, Lee SY, Goh BC, Thiery JP. A cell-based small molecule screening method for identifying inhibitors of epithelial-mesenchymal transition in carcinoma. PLoS One 2012; 7: e33183.
27. Gerson AG, Trinke AV, George J, Brown R, Fox SB, Lade S et al. Novel molecular subtypes of senescent and endometrioid ovarian cancer linked to clinical outcome. Clin Cancer Res 2008; 14: 5198–5206.
28. Prapiot JC, Krzymowski K, Mulligan BM, Pambuccian SE, Gaffney TR, Thiery JP. Reversible transition towards a fibroblastic phenotype in a rat carcinoma cell line. Int J Cancer Suppl 1989; 5: 69–75.
29. Bratbliz S, Bratbliz T. The ZEB/miR-200 feedback loop—a motor of cellular plasticity in development and cancer? EMBO Rep 2010; 11: 670–677.

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55. Kokkinos MI, Wafai R, Wong MK, Newgreen DF, Thompson EW, Waltham M. Vimentin and epithelial-mesenchymal transition in human breast cancer—observations in vitro and in vivo. Cells Tissues Organs 2007; 185: 191–203.
56. Evseenko D, Zhu Y, Schenke-Layland K, Kuo J, Latour B, Ge S et al. Mapping the first stages of mesoderm commitment during differentiation of human embryonic stem cells. Proc Natl Acad Sci USA 2010; 107: 13742–13747.
57. Warzecha CC, Jiang P, Amirikian K, Dittmar KA, Lu H, Shen S et al. An ESRP-regulated splicing programme is abrogated during the epithelial-mesenchymal transition. Embo J 2010; 29: 3286–3300.
58. Warzecha CC, Carstens RP. Complex changes in alternative pre-mRNA splicing play a central role in the epithelial-to-mesenchymal transition (EMT). Semin Cancer Biol 2012; 22: 417–427.
59. Tothill RW, Tinker AV, George J, Brown R, Fox SB, Lade S et al. Novel molecular subtypes of serous and endometrioid ovarian cancer linked to clinical outcome 2008; 14: 5198–5208.
60. Network TCGAR. Integrated genomic analyses of ovarian carcinoma. Nature 2011; 474: 609–615.
61. Strauss R, Li ZY, Liu Y, Beyer I, Persson J, Sova P et al. Analysis of epithelial and mesenchymal markers in ovarian cancer reveals phenotypic heterogeneity and plasticity. PLoS One 2011; 6: e16186.

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