Abstract. Background: We have previously shown that the selection of cancer cell lines with chemotherapeutic agents can alter the invasive potential of the cells, resulting in a superinvasive phenotype, where cells not only invade through matrigel and migrate through membrane pores, but also subsequently detach from the underside of the invasion chamber, survive in suspension and ultimately attach to and grow on the bottom of the well beneath the insert. In order to determine the significance of this in vivo, the following experiments were performed. Materials and Methods: 4T1-GFP mouse mammary adenocarcinoma cells were pulse-selected with doxorubicin or paclitaxel. Three variants with differing invasiveness were isolated and their metastatic potential in vivo was compared to the parental cell line, through injection into the mammary fat pad of BALB/c mice and subsequent primary tumour growth and metastasis to the lungs measurement. Results: Increasing superinvasiveness was inversely linked to tumour diameter (p<0.005). The superinvasive status predicted the incidence of lung nodules in 2/3 variant groups, with significant differences in the number of nodules (p<0.001). Conclusion: The in vitro superinvasive phenotype coupled with decreased adhesion may predict for metastatic potential in vivo.

Patients who have received chemotherapy and shown a complete or partial response often remain disease-free for a period, only for the cancer to reappear at a later stage. The tumour may recur at the same site or appear at other sites, possibly arising from micrometastases that had been undetectable and/or dormant at the time of treatment.

A tumour is a heterogeneous collection of cells. After chemotherapeutic reduction of the tumour mass, many of the cells remaining will have either acquired resistance to the treatment or are intrinsically resistant to toxic insult. It is also possible that the therapy may "select" for a subset of cells which are invasive or may actually induce an invasive phenotype (1-4). In this paper, cell populations with differing invasive potential were selected by pulse selection of the cells, mimicking clinical therapy.

There are various reports suggesting a link between multiple drug resistance and invasive potential in carcinoma cells (2, 3). The majority of studies focused on the direct effect of drug exposure on invasive potential (invasion in the presence of the drug), as opposed to the long-term effects of drug treatment on the invasive phenotype of the cell, coupled with emerging drug resistance. Paclitaxel was shown to have an inhibitory effect on the invasiveness of the melanoma cell lines B16F10 and B16BL6 (5), and was also shown to inhibit the migration of irradiated glioblastoma cells and to induce the migration of non-irradiated glioblastoma cells. This suggests that, depending on the treatment history of the cells, paclitaxel can either induce or suppress motility (6). We have previously shown that selection of the human breast carcinoma cell line MDA-MB-435S-F with paclitaxel and doxorubicin induced a more aggressive “superinvasive” phenotype (4). This phenotype is defined as the ability of the cells to invade through the matrigel to the underside of the invasion chamber and detach, followed by relocation to the lower surface of the plate. Although this phenotype was accompanied by decreased adhesion, the expression of the...
matrix metalloproteases (MMP), MMP-2 and MMP-9 remained unaffected (4). Similar results have been previously described. Exposure of SW620 colon cancer-derived metastatic cells to paclitaxel resulted in huge aneuploid, polynuclear cells, with increased invasive capacity (7). This increase did not coincide with the induction or release of proteolytic enzymes. A neuroblastoma cell line resistant to doxorubicin exhibited more than a 2-fold increase in clonal growth in vitro, accelerated adhesion and transendothelial penetration and higher tumorigenicity in vivo relative to the parental UKF NB2 cells (8). Previous studies in this laboratory showed that selection of a non-invasive cell line, RPMI 2650, with paclitaxel and melphalan, resulted in multiple drug-resistant phenotypes and, in the case of melphalan but not paclitaxel, a highly invasive phenotype (3).

The murine mammary adenocarcinoma cell line 4T1-GFP exhibited some degree of superivasion. It was then selected with doxorubicin and paclitaxel and the invasive, superinvasive and adhesive status of the cells were determined. In order to determine the in vivo relevance of the “superinvasive” phenotype, parental 4T1-GFP cells and drug-selected variants were implanted into the mammary fat pad of BALB/c mice and spontaneous metastasis to the lungs was examined.

Materials and Methods

Chemicals. Paclitaxel was obtained from Bristol-Myers Squibb (Dublin, Ireland) and doxorubicin from Farmitalia Carlo Erba (Milton Keynes, UK). All media and serum used in the maintenance of the cell lines were obtained from Sigma Aldrich (Dublin, Ireland), as were all other chemicals.

Cell lines. 4T1-GFP (green fluorescent protein), mouse mammary adenocarcinoma cells transfected with pEGFP-C1 vector (Clontech, Palo Alto, CA, USA) were maintained in culture at 37°C using Dulbecco’s modified media (DMEM) supplemented with 2 mM L-glutamine (Gibco, Paisley, UK) and 5% foetal calf serum (FCS). 4T1-GFP/Adr-1 was derived by pulse selection of 4T1-GFP with IC₅₀ doxorubicin (200 ng/ml) for 4 h, once a week for 10 weeks. 4T1-GFP/Tax-1 and 4T1-GFP/Tax-4 were derived by pulse selection of 4T1-GFP with IC₅₀ paclitaxel (60 ng/ml) for 4 h, once a week for 10 weeks. Antibiotics were not used in the growth media. All cell lines were free from mycoplasma, as tested with the indirect Hoechst DNA staining method.

Cytotoxicity assays. Doxorubicin and paclitaxel toxicity against 4T1-GFP cells and the drug-resistant variants was determined by the acid phosphatase method which measures viable cell number after 5 days of continuous exposure to drugs, as previously described (9). The toxicity assays were carried out at least twice in triplicate.

Adhesion assays. Adhesion assays were performed using the method of Torimura et al. (10). Twenty-four-well plates were coated with 250 µl of 25 µg/ml collagen type IV, fibronectin and laminin (Sigma) and 250 µl of 1 mg/ml Matrigel and incubated overnight at 4°C; 0.5 ml of a sterile 0.1% w/v BSA/PBS (bovine serum albumen/phosphate-buffered saline) solution was dispensed into each well to reduce non-specific binding. The plates were incubated at 37°C for 20 min and then rinsed twice with PBS. Cells were added 2.5x10⁴ per well in triplicate and incubated at 37°C for 60 min. The control wells were coated but contained no cells. After 60 min, the medium was removed from the wells and rinsed gently with PBS. This was then removed and 200 µl of freshly prepared phosphatase substrate (10 mM p-nitrophenol phosphate (Sigma) in 0.1 M sodium acetate (Sigma), 0.1% (v/v) Triton X-100 (Sigma), pH 5.5) were added to each well. The plates were then incubated in the dark at 37°C for 2 h. The enzymatic reaction was stopped by the addition of 100 µl of 1 N NaOH and the absorbance at 405 nm was determined using a Bio-Tek Synergy HT Multi-Detection Microplate Reader with a reference wavelength of 620 nm.

Invasion, chemotaxis and motility assays. The invasion and chemotaxis assays were performed by a modification of the method described by Albini et al. (11). Matrigel (Sigma) was diluted to 1 mg/ml in serum-free DMEM medium. One hundred microlitres of 1 mg/ml Matrigel was placed into each insert (Falcon) (8.0 mm pore size), which were placed in wells of a 24-well plate (Costar). The inserts and the plate were incubated at 37°C for 1 h to allow gel polymerisation. The cells were harvested and suspended in DMEM containing 5% FCS (containing 5% FCS, 5% FCS (chemotaxis assays - serum gradient)) at a density of 1x10⁴ cells/ml. One hundred microlitres of cell suspension were added to each upper chamber and 500 µl of DMEM containing 5% FCS were added to the lower chamber. The cells were incubated at 37°C for 24 h. After this time-period, the upper surface of the insert membrane was wiped with a wet swab to remove the cells, while the underside of the membrane was gently rinsed with PBS and stained with 0.25% (w/v) crystal violet for 10 min, rinsed again and then allowed to dry. The inserts were subsequently viewed under the microscope. The procedure for carrying out motility assays was identical to that used for invasion assays, with the exception that the inserts were not coated with Matrigel. The levels of motility, chemotaxis and invasion were quantified by elution of crystal violet using 33% acetic acid and absorbance was read at 570/620 nm. The absorbance was proportional to the number of cells present.

Growth on extracellular matrix proteins. The growth assays were performed on CytoMatrix™ screening kits (Chemicon, UK). Ninety-six-well plates, individually pre-coated with fibronectin, vitronectin, laminin, collagen I or collagen IV, were rehydrated with 200 µl of PBS per well for 15 min at room temperature, after which PBS was removed. A control non-coated plate was included. The cells were plated at a density of 1x10⁵ cells per well in triplicate in DMEM containing 5% FCS and were incubated at 37°C for 7 days. After 7 days, the medium was removed from the wells and rinsed gently with PBS. This was then removed and 100 ml of freshly prepared phosphatase substrate (10 mM p-nitrophenol phosphate (Sigma) in 0.1 M sodium acetate (Sigma), 0.1% Triton X-100 (Sigma), pH 5.5) were added to each well. The plates were then incubated in the dark at 37°C for 2 h. The enzymatic reaction was stopped by the addition of 50 µl of 1 N NaOH and the plate read in an ELISA reader at 405 nm with a reference wavelength of 620 nm.

Anoikis assay. The cells were resuspended in 50-ml universals at a density of 6x10⁵ cells in 20 ml DMEM media supplemented with
5% FCS and were shaken at 3.5 rpm for 24 h to ensure minimal cell adhesion/contact. The cell viability was assessed based on Trypan Blue exclusion and % survival calculated. Each assay was performed in triplicate.

**Animals.** Female 10 to 12-week-old BALB/c mice (Charles River Institute, Margate, Kent, UK) were used. The animals were acclimatised for 1 week and caged in groups of 5 or less in an air-conditioned room at ambient temperature of 21-22°C and 50% humidity under a 12-h light-dark cycle (lights at 08.00). The animals were housed in a licensed biomedical facility (RSCI Department of Surgery, Beaumont Hospital, Ireland) and all procedures were carried out under animal licence guidelines of the Department of Health, Ireland and in accordance with the UK Coordinating Committee on Cancer Research (UKCCR) Guidelines for the Welfare of Animals in Experimental Neoplasia (12). The animals had ad libitum access to animal chow (WM Connelly & Sons Ltd., Kilkenny, Ireland) and water.

**In vivo experimental design.** The cells (5x10^6 (100 µl)) were injected into the mammary fat pad adjacent to the left foot after anaesthesia had been induced and maintained with inhalational halothane (10 mice per group). The primary tumours were measured on alternate days from 12 days post-injection of tumours using Vernier callipers. The tumour diameter (TD) was calculated as the square root of the product of 2 perpendicular diameters (13). The animals were euthanased when the mean TD for each group reached 16.5 mm. The primary tumours were excised and fixed in Bouins solution and the number of lung lesions determined with the aid of a dissecting microscope (14).

**Statistical analysis.** The ANOVA test was used to assess differences in the mean number of lung nodules, differences in the means of final tumour burdens and differences in time to reach specific tumour diameters between the drug-treatment groups. Bartlett’s test was used to assess the assumption of equal variances. The Kruskall-Wallis test was used to assess the significance of differences in the median number of lung nodules between the drug-treatment groups. A paired, two-sided Student’s t-test was used to assess differences between rates of motility, chemotaxis, invasion, adhesion, growth and resistance to anoikis in *vitro*.

**Results**

**TAX and ADR variants display altered adhesion in vitro invasion, motility and chemotaxis.** The levels of motility, chemotaxis and invasion in the selected variants compared to the parent cell line are depicted in Figure 1. The levels of cells which reached the underside of the insert and the levels of relocation from the underside of the insert to the bottom of the 24-well plate, described previously as supermotility and superinvansion (4), are described in Figure 1A and B, respectively. 4T1-GFP/Adr-1 and 4T1-GFP/Tax-4 displayed increased motility (*p<0.05* and *p<0.005*, respectively), chemotaxis (*p<0.005* and *p<0.005*, respectively) and invasion (*p<0.005* and *p<0.05*, respectively), compared to 4T1-GFP, while no significant changes in 4T1-GFP/Tax-1 motility, chemotaxis or invasion were observed (Figure 1A). Super motility, chemotaxis and invasion were greatly increased in 4T1-GFP/Adr-1 (*p<0.005*, *p<0.005* and *p<0.005*, respectively) and 4T1-GFP/Tax-4 (*p<0.005*, *p<0.05* and *p<0.005*, respectively) and were decreased in 4T1-GFP/Tax-1 (*p<0.005*, *p<0.05* and *p<0.005*, respectively) (Figure 1B). These results indicated that treatment of carcinoma cells with the chemotherapeutic agents paclitaxel and doxorubicin can result in the alteration of the motile and invasive potential of the cells. It is, however, unclear whether chemotherapy induced a phenotypic change or simply selected for pre-existing populations of cells with that phenotype. The resistance levels to paclitaxel and doxorubicin were modest in the selected variants (Table I).

**TAX and ADR variants display altered adhesion and growth on extracellular matrix (ECM) proteins.** The ability of 4T1-GFP and its resistant variants to adhere and grow on a variety of extracellular matrix proteins is shown in Figure 2. Adhesion assays were performed on matrigel, laminin, fibronectin and collagen type IV. Growth assays were performed on laminin, fibronectin, vitronectin, collagen type I and collagen type IV. The levels of cell adhesion after 60 min in the resistant variants normalised to adhesion levels of the parental cell line 4T1-GFP are shown in Figure 2A. The paclitaxel-selected variants 4T1-GFP/Tax-1 and 4T1-GFP/Tax-4 displayed increased adhesion to some of the ECM proteins tested. 4T1-GFP/Tax-1 showed significantly increased adhesion to collagen type IV and matrigel (*p<0.05*), but not to fibronectin or laminin. 4T1-GFP/Tax-4 showed significantly increased adhesion to collagen type IV, fibronectin, laminin (*p<0.005*) and matrigel (*p<0.05*). The doxorubicin-selected cell line 4T1-GFP/Adr-1 showed decreased adhesion to fibronectin and laminin (*p<0.05*), but not to collagen type IV or matrigel (*p>0.05*, not significant). The growth of the variants on various ECM proteins relative to the parental cell line 4T1-GFP is illustrated in Figure 2B. Similar to the changes in adhesion patterns, the paclitaxel-selected variants, 4T1-GFP/Tax-1 and 4T1-GFP/Tax-4, exhibited greatly increased

### Table I. IC₅₀ values for 4T1-GFP and its variants.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>4T1-GFP</th>
<th>4T1-GFP/Tax-1</th>
<th>4T1-GFP/Tax-4</th>
</tr>
</thead>
<tbody>
<tr>
<td>IC₅₀ (ng/ml)</td>
<td>10.6±0.40</td>
<td>11.7±0.83</td>
<td></td>
</tr>
<tr>
<td>Doxorubicin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IC₅₀ (ng/ml)</td>
<td>1.13±0.05</td>
<td>1.43±0.05*</td>
<td>2.16±0.15*</td>
</tr>
<tr>
<td>Taxol</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*The IC₅₀ values are the mean of a minimum of 3 repeat experiments. *p<0.05, Student’s t-test.
growth on fibronectin ($p<0.005; p<0.05$, respectively), collagen type IV ($p<0.05; p<0.05$, respectively) and tissue culture grade plastic ($p<0.005$). The growth on collagen type I was not altered, while growth on laminin was increased in 4T1-GFP/Tax-4 ($p<0.005$). Adhesion to vitronectin was decreased for 4T1-GFP/Tax-1 ($p<0.05$) and increased for 4T1-GFP/Tax-4 ($p<0.005$) relative to the parental cell line. The doxorubicin-selected cell line 4T1-GFP/Adr-1 displayed decreased growth on all ECM proteins (collagen type IV, fibronectin, vitronectin, $p<0.05$; collagen type I, laminin, $p<0.005$) and tissue culture grade plastic ($p<0.005$) and failed completely to grow on collagen type IV.
**TAX and ADR variants display altered resistance to anoikis.**

The ability of the 4T1-GFP variants to resist induction of a form of apoptosis, known as anoikis, induced by removal of cell-cell and cell-matrix adhesions, is shown in Figure 3. 4T1-GFP/Adr-1 and 4T1-GFP/Tax-4 displayed significantly increased resistance ($p<0.05$) to the induction of anoikis, compared to 4T1-GFP, while the resistance of 4T1-GFP/Tax-1 was similar to that of the parental cell line.

**In vivo tumour growth and lung metastasis.** In order to determine whether the changes in invasiveness displayed in Figure 1 could predict for metastasis *in vivo*, lung metastasis of...
the variant cell lines was examined in the mouse models. 4T1 mammary adenocarcinoma cells spontaneously metastasise to the lungs from the mouse mammary pad (13). Each of the 4 cell lines were implanted into the mouse mammary fat pad of BALB/c mice. The TD was measured every second day from day 12 using vernier callipers. The mice were sacrificed when the tumours had reached a mean TD of 16.5 mm. The tumour growth curve for each group is provided in Figure 4A. The growth rate of all variants was similar. However, the more in vitro superinvasive cells (4T1-GFP/Adr1 and 4T1-GFP/Tax 4) displayed a longer time delay in reaching 16 mm, as shown in Figure 4B. There were significant differences in the time taken to reach each TD of 12, 14 and 16 mm for the 3 variants relative to the parent cell line (ANOVA, \( p < 0.001 \)). After day 21, it can also been seen in Figure 4A that the primary tumour of the in vitro superinvasive groups reached a growth plateau.

In 2 of the variants, in vitro superinvasion predicted lung metastasis. It can been seen in Figure 4B that the 4T1-GFP/Adr-1 group, which displayed greater in vitro superinvasiveness than the parent, had more lung nodules (Kruskal Wallis test, \( p < 0.001 \)). Likewise, the 4T1-GFP/Tax-1 group, which displayed less in vitro superinvasiveness than the parent, had fewer lung nodules than the parental group (Kruskal Wallis test, \( p < 0.001 \)). Unexpectedly, the 4T1-GFP/Tax-4 group, which displayed greater in vitro superinvasiveness than the parent, had fewer lung nodules than the parental cell line (Kruskal Wallis test, \( p < 0.001 \)). This cell line was, however, far more adhesive than the parent cell line (Figure 2A), which would limit its ability to shed cells from the primary tumour site.

Discussion

Chemotherapy may influence the metastatic potential such that cells not eradicated by treatment are highly metastatic, either because chemotherapy induces phenotypic changes or selects for populations with highly metastatic potential, i.e., chemotherapy inadvertently increases metastasis of any cells not killed by the therapy. We have previously shown that selection of cancer cell lines with chemotherapeutic agents can alter the invasive potential of the cells (1, 3, 4). Metastasis is, by nature, an inefficient process (15). Millions of cancer cells can be injected into an experimental mouse, but only give rise to a few metastases that are detectable in ‘end-point’ assays. Using high-resolution in vivo video microscopy and quantitative cell-fate analyses, that monitor the loss of cells over time during metastasis, it was shown that early steps in the haematogenous metastatic process are completed remarkably efficiently (16). B16F1 murine melanoma cells injected through the mesenteric vein to target the cells to the liver were followed over time using quantitative cell-fate analysis. Over 87% of the injected cells were arrested in the liver and were present there 90 min after injection. Three days after injection, 83% of the cells that were originally injected had extravasated into the liver parenchyma and remained there, but only 2% had formed micrometastases. The persistence of micrometastases was poor with only 0.02% of total injected cells capable of forming macroscopic metastases, showing that the subsequent steps in the metastatic process are completed inefficiently. Additional studies have achieved similar results (17-19).
The development of an \textit{in vitro} model that predicts the \textit{in vivo} metastatic behaviour of carcinoma cells is of great importance, as it would provide us with an accurate tool to study the mechanism of invasion and metastasis in greater detail and also to give us the means to predict the effectiveness of novel, potential anti-metastatic agents. Here, an \textit{in vitro} model is described, in which the combination of two \textit{in vitro} properties, adhesiveness and superinvasiveness, predicts \textit{in vivo} metastasis of mouse mammary adenocarcinoma cells.

We previously reported a novel "superinvasive" phenotype (4), induced by pulse selection of an \textit{in vitro} invasive clonal population of the human breast cancer cell line, MDA-MB-435S, with doxorubicin and paclitaxel. The superinvasive phenotype is defined by the ability of a cell line to invade through the matrigel and squeeze through the membrane pores to the underside of the membrane. The cells must then be able to detach from the underside of the membrane. After detachment, the cell must survive in single cell suspension until it reaches the bottom of the 24-well
Further analysis of their capacity of these variants coincided with resistance to survive in the bloodstream (25, 26). The superinvasive of a tumour as metastasising tumour cells must be able to induction of anoikis (24). The acquisition of anoikis cells, loss of adhesion to the ECM contacts results in the form of apoptosis induced by cell detachment. In normal displayed a less superinvasive phenotype second paclitaxel-selected variant, 4T1-GFP/Tax-1, phenotype displayed a more superinvasive variant, 4T1-GFP/Tax-4, displayed a more superinvasive in vivo potential of these variants was subsequently assessed in an in vivo model of spontaneous metastasis. The doxorubicin-selected variant would have levels of metastatic behaviour similar to the parent cell line, 4T1-GFP. The doxorubicin-selected variant displayed decreased adhesion to laminin and fibronectin, with little change in adhesion to collagen type IV or matrigel relative to the parental cells. This cell line also displayed greatly decreased growth on laminin, collagen type I, fibronectin and vitronectin relative to the parental cells. It failed to grow on collagen type IV, despite little change in its ability to adhere to this substrate. Both paclitaxel-selected variants displayed increased adhesion to collagen type I and matrigel relative to the parental cells, while 4T1-GFP/Tax-4 also displayed increased adhesion to fibronectin and laminin. Collagen type IV appears to be their preferred attachment substrate. The growth assays performed revealed that growth was elevated on fibronectin and collagen type IV.

To investigate the in vivo relevance of this in vitro phenotype, pulse selection with doxorubicin and paclitaxel was used to generate variants of 4T1-GFP with differing levels of superinvasiveness in vitro and the metastatic potential of these variants was subsequently assessed in an in vivo model of spontaneous metastasis. The doxorubicin-selected variant, 4T1-GFP/Adr-1, and the paclitaxel-selected variant, 4T1-GFP/Tax-4, displayed a more superinvasive phenotype in vitro than the parental cell line, whereas the second paclitaxel-selected variant, 4T1-GFP/Tax-1, displayed a less superinvasive phenotype in vitro. Anoikis is a form of apoptosis induced by cell detachment. In normal cells, loss of adhesion to the ECM contacts results in the induction of anoikis (24). The acquisition of anoikis resistance is a critical step during the metastatic progression of a tumour as metastasising tumour cells must be able to survive in the bloodstream (25, 26). The superinvasive capacity of these variants coincided with resistance to anoikis. Further analysis of their in vitro characteristics revealed that the ability of the variants to superinvade mimicked their ability to resist induction of anoikis while in suspension.

The doxorubicin-selected variant displayed decreased adhesion to laminin and fibronectin, with little change in adhesion to collagen type IV or matrigel relative to the parental cells. This cell line also displayed greatly decreased growth on laminin, collagen type I, fibronectin and vitronectin relative to the parental cells. It failed to grow on collagen type IV, despite little change in its ability to adhere to this substrate. Both paclitaxel-selected variants displayed increased adhesion to collagen type I and matrigel relative to the parental cells, while 4T1-GFP/Tax-4 also displayed increased adhesion to fibronectin and laminin. Collagen type IV appears to be their preferred attachment substrate. The growth assays performed revealed that growth was elevated on fibronectin and collagen type IV.

In order to determine whether superinvasion could predict metastasis in vivo, 4T1-GFP and the drug-selected variants were implanted into the mouse mammary fat pad of BALB/c mice and metastasis to the lungs was assessed. The 4T1-GFP/Adr-1 cells, which were highly superinvasive, anoikis-resistant and poorly-adhesive in vitro, were highly metastatic in vivo. The 4T1-GFP/Tax-1 cells were weakly superinvasive, poorly resistant to anoikis, highly adhesive and were weakly metastatic in vivo. The 4T1-GFP/Tax-4 cells, although highly superinvasive in vitro, anoikis-resistant and highly adhesive, were less metastatic than the parental cell line.

These results suggest that the superinvasive phenotype, coupled with decreased adhesion and growth rates on various ECM proteins in vitro, predicts metastatic ability in vivo. If adhesive status alone could predict for metastatic behaviour in vivo, it would be expected that the paclitaxel-selected variants would have fewer metastases (with 4T1-GFP/Tax-4 being the least metastatic), while the doxorubicin-selected variant would have levels of metastasis similar to the parent cell line, 4T1-GFP. The in vitro adhesiveness of the paclitaxel-selected variants coincided with decreased metastasis to the lungs in vivo.

### Table II. Steps in the metastatic process reflected in the in vitro assays.

<table>
<thead>
<tr>
<th>Steps</th>
<th>Steps in metastatic process</th>
<th>In vitro model</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Detachment from primary tumour.</td>
<td>Reduced adhesion to ECM proteins.</td>
</tr>
<tr>
<td>2</td>
<td>Local invasion of ECM.</td>
<td>Invasion into the matrigel.</td>
</tr>
<tr>
<td>3</td>
<td>Intravasation of tumour cells into lymphatics or vasculature.</td>
<td>Movement through membrane pores to membrane underside.</td>
</tr>
<tr>
<td>4</td>
<td>Survival of tumour cells in circulation and avoidance of immunological attack.</td>
<td>Superinvasive cells in suspension are resistant to anoikis.</td>
</tr>
<tr>
<td>5</td>
<td>Extravasation of tumour cells from vasculature into secondary organ tissue.</td>
<td>Attachment to plate surface.</td>
</tr>
<tr>
<td>6</td>
<td>Survival and proliferation within organ parenchyma.</td>
<td>Growth.</td>
</tr>
</tbody>
</table>

Plate, reattaches to the surface and recommences growth. We suggest that the in vitro phenomenon of superinvasiveness and decreased adhesion reflects the process of metastasis in vivo, whereby a tumour cell which has detached from the primary tumour must make its way through the ECM (invasion through matrigel), intravasate through the capillary basement membrane (squeeze through pores), survive the blood stream (survival in single cell suspension) to the potential site of metastasis, attach to the capillaries, extravasate through the basement membrane and grow (re-attachment to the bottom of the plate and growth) (20). The adhesive properties displayed by the cell line in vitro may reflect the level of cells detaching from the primary mass in vivo. Following the initial transforming event in vivo, neoplastic cells proliferate to form the primary tumour mass, from which metastatic cells can detach. There is increasing evidence that, in epithelial malignancies, loss or down-regulation of adhesion and scaffolding proteins, such as with E-cadherin and caveolin 1 (21-23), correlates or down-regulation of adhesion and scaffolding proteins, such as with E-cadherin and caveolin 1 (21-23), correlates with metastatic spread.

To investigate the in vivo relevance of this in vitro phenomenon, pulse selection with doxorubicin and paclitaxel was used to generate variants of 4T1-GFP with differing levels of superinvasiveness in vitro and the metastatic potential of these variants was subsequently assessed in an in vivo model of spontaneous metastasis. The doxorubicin-selected variant, 4T1-GFP/Adr-1, and the paclitaxel-selected variant, 4T1-GFP/Tax-4, displayed a more superinvasive phenotype in vitro than the parental cell line, whereas the second paclitaxel-selected variant, 4T1-GFP/Tax-1, displayed a less superinvasive phenotype in vitro. Anoikis is a form of apoptosis induced by cell detachment. In normal cells, loss of adhesion to the ECM contacts results in the induction of anoikis (24). The acquisition of anoikis resistance is a critical step during the metastatic progression of a tumour as metastasising tumour cells must be able to survive in the bloodstream (25, 26). The superinvasive capacity of these variants coincided with resistance to anoikis. Further analysis of their in vitro characteristics revealed that the ability of the variants to superinvade mimicked their ability to resist induction of anoikis while in suspension.

The doxorubicin-selected variant displayed decreased adhesion to laminin and fibronectin, with little change in adhesion to collagen type IV or matrigel relative to the parental cells. This cell line also displayed greatly decreased growth on laminin, collagen type I, fibronectin and vitronectin relative to the parental cells. It failed to grow on collagen type IV, despite little change in its ability to adhere to this substrate. Both paclitaxel-selected variants displayed increased adhesion to collagen type I and matrigel relative to the parental cells, while 4T1-GFP/Tax-4 also displayed increased adhesion to fibronectin and laminin. Collagen type IV appears to be their preferred attachment substrate. The growth assays performed revealed that growth was elevated on fibronectin and collagen type IV.

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These results suggest that the superinvasive phenotype, coupled with decreased adhesion and growth rates on various ECM proteins in vitro, predicts metastatic ability in vivo. If adhesive status alone could predict for metastatic behaviour in vivo, it would be expected that the paclitaxel-selected variants would have fewer metastases (with 4T1-GFP/Tax-4 being the least metastatic), while the doxorubicin-selected variant would have levels of metastasis similar to the parent cell line, 4T1-GFP. The in vitro adhesiveness of the paclitaxel-selected variants coincided with decreased metastasis to the lungs in vivo.
whereas the weakly adhesive doxorubicin-selected cells displayed significantly increased metastasis to the lungs compared to the parent cell line. This indicated that adhesive status alone is less accurate in predicting metastatic potential than a combination of adhesive and superinvasive status. Compared to the parental line, the doxorubicin-selected variants displayed increased motility, invasiveness and superinvasiveness, accompanied by resistance to anoikis. Resistance to anoikis should increase the survival rate of the tumour cells in the bloodstream and thereby metastasis, as reflected by the in vivo results in this study.

The highly in vitro superinvasive cell lines, 4T1-GFP/Adr-1 and 4T1-GFP/Tax-4, showed substantially higher survival in suspension than the parental line, suggesting they would survive well in the circulation, thus increasing the likelihood of increased metastasis. 4T1-GFP/Adr-1 exhibited significantly higher rates of invasion than the parental cell line. 4T1-GFP/Tax-4, which would appear to have some of the necessary traits for metastasis, namely superinvasive and anoikis-resistant but not decreased adhesion, failed to show increased metastasis to the lungs, and was significantly less metastatic than the parental cell line. 4T1-GFP/Tax-1 was highly adhesive, had low anoikis resistance and displayed much lower rates of invasion and superinvasion and was also much less metastatic in vivo than the parental cell line.

These results appear to confirm the need for reduced adhesion, increased superinvasion and increased survival in suspension, in order for these cells to metastasise – cells must have ALL properties to be metastatic. Further development of this type of cell system may lead to better in vitro models in which to develop a better understanding of, and better therapies for, metastatic cancer.

Acknowledgements

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References


22 Bender FC, Reymond MA, Bron C and Quest AF: Caveolin-1 levels are down-regulated in human colon tumors, and ectopic expression of caveolin-1 in colon carcinoma cell lines reduces cell tumorigenicity. Cancer Res 60: 5870-5878, 2000.


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