Vasculogenic mimicry *in vitro* in tumour cells derived from metastatic malignant pleural effusions

Sir,

Malignant pleural effusions (MPE) are most commonly caused by advanced metastatic malignancy, with an average survival time of 3-12 months post diagnosis.\(^1\) The pathogenesis of MPE is not fully understood, but impaired fluid drainage by blood or lymphatic vessels, inflammation and increased vascular permeability are known to contribute. More recently, angiogenesis has been recognised as playing role in the development of MPE, and vascular endothelial growth factor (VEGF), which drives vascular development, is a recognised prognostic factor. Anti-angiogenic therapies directed at VEGF may improve effusion control in some settings.\(^1,2\) However, anti-VEGF therapy has limited effects on vasculogenic mimicry (VM), the process by which tumour cells differentiate into vascular endothelial-like cells to form new vascular networks.\(^3\) VM has been identified in 36% of lung \(^4\) and 20-30% of primary breast carcinomas \(^5\)-- and lung and breast carcinomas are the most common causes of MPEs. In a meta-analysis of 3062 cases, Cao *et al.* associated VM with poor patient survival (31% five year overall survival in VM-positive patients, compared to 56% in VM-negative patients).\(^6\) Effusions are routinely drained for patient comfort and diagnosis, and provide a source of patient-derived primary tumour cells that could be used to characterise tumour cell behaviour to optimise therapy. We have previously shown that mesothelioma cell harvested from MPE display VM *in vitro*, whereas previous studies have focussed on the use of primary tumour cells to predict chemotherapy-sensitivity.\(^7\) Here, we demonstrate that effusion-derived tumour cells are a source of tumour cells that display VM and could be used to test therapy response.
A total of 18 metastatic MPEs (seven breast adenocarcinomas, six lung adenocarcinomas, one lung small cell carcinoma, one clear-cell renal cell carcinoma, one chronic lymphocytic leukaemia, one adenocarcinoma of unknown primary site and one squamous cell carcinoma of unknown primary site) were processed for cell culture either directly or after separation using a density gradient (approved by Southern Adelaide Clinical Human Research Ethics Committee). Cells were directly processed as previously described and Ficoll (GE Healthcare Biosciences AB, Uppsala, Sweden) was used as the density gradient to fractionate the cells. Ficoll processing was aimed at enriching the tumour cell population and involved centrifuging half of the effusion (250-750 ml) at 400 g for 10 min. The pooled cell pellets were resuspended in 4 ml complete DMEM (10% foetal calf serum, 50 units/ml penicillin and 50 μg/ml streptomycin), layered onto 4 ml Ficoll media and centrifuged at 400 g for 30 min at room temperature. The lowest density fraction of the cellular layer was cultured. Purity of the samples was assessed by immunohistochemistry on cell blocks prepared as for clinical samples using NATA-approved protocols. Cells labelling positive for tumour-related markers (e.g., RCC) and negative for calretinin were considered metastatic malignant cells.

Fifteen out of 18 (83%) MPEs collected could be cultured. Percentages of tumour cells (as of total cell population) at passage 0 ranged from 1-99%, with enrichment of tumour cell proportion at later passages in all three samples tested, ranging from 1.2-65 fold increase.

Proliferative ability at different passages was assessed using the MTS assay. The proliferative potential of cultured cells increased at later passages in four out of seven samples tested (Table 1 and Figure 1). This may indicate that later passages select for more ‘aggressive’ clones, and passage number should be considered when testing cells for therapy response.
A comparison of processing methods showed no increase in sample purity in Ficoll-processed samples, but directly processed cells reached confluency sooner, permitting faster completion of functional tests. The decreased handling may maintain intermolecular cross-talk and tumour microenvironment better and yield a more clinically relevant cell population.

However, both processing methods yielded cells suitable for in vitro tube formation assays, which are a surrogate for VM. Cells were seeded at $1.7 \times 10^5$ cells/well on an Ibidi µslide angiogenesis slide (Ibidi, Germany) coated with 10 μl of high concentrate, growth factor reduced matrigel (BD, Massachusetts, USA) and incubated for 6 h at 37 °C. Photos were taken at 6 h using an Olympus DP73 camera on an Olympus Fluorescence IX73 inverted microscope (Olympus, Tokyo, Japan).

A total of 10 out of 15 (67%) malignant samples tested formed tubes in vitro. Four of six breast adenocarcinomas (67%), three of four lung adenocarcinomas (75%), the lung small cell carcinoma, the adenocarcinoma of unknown primary origin and the squamous cell carcinoma demonstrated VM in vitro (Table 1, Figure 2). These findings confirm that VM is not specific to tumour types but is an independent trait in a variety of tumours. The incidence of VM was high in this study compared to previous work in primary tumours (36% of lung adenocarcinomas and 20-30% of breast adenocarcinomas). However, VM is associated with advanced tumour stage, of which MPE is a manifestation. Four samples that did not perform VM under normoxic conditions were incubated in a humidified hypoxic chamber (37°C, 5% CO₂, 0.1% O₂) (Coy Laboratory Hypoxic Workstation Glove Box, Michigan, USA), and one of these samples acquired VM under hypoxia. Hypoxia inducible factor alpha (HIF-1α) induces epithelial mesenchymal transition (stem-like properties) and stimulates the expression of VEGF and its receptors, which are currently thought to play a pivotal role in the mechanism of VM.
In conclusion, tumour cells isolated from MPE by either direct processing or Ficoll gradient are suitable for functional assays including VM, making it feasible to test tumour response to therapy in vitro, contributing to personalised therapy.

Conflicts of interest and sources of funding: Funding was provided by the Flinders Medical Centre Foundation and the Faculty of Health Sciences of Flinders University. The authors state there are no conflicts of interest to disclose.

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Bibliography


Legends
Table 1: Cytopathology of MPE and their *in vitro* behaviour. Other malignancies included one lung small cell carcinoma, one renal cell carcinoma, one chronic lymphocytic leukaemia and two carcinomas of unknown primary site.

<table>
<thead>
<tr>
<th>Type</th>
<th>Increased proliferation potential with increasing passage (n increase/n tested)</th>
<th>Positive for vasculogenic mimicry in normoxia at passage zero (n positive/n tested)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Breast adenocarcinomas</td>
<td>0/1</td>
<td>4/6</td>
</tr>
<tr>
<td>Lung adenocarcinomas</td>
<td>1/2</td>
<td>3/4</td>
</tr>
<tr>
<td>Other malignancies</td>
<td>2/3</td>
<td>3/5</td>
</tr>
<tr>
<td>Total</td>
<td>3/6</td>
<td>10/15</td>
</tr>
</tbody>
</table>
Figure 1: Proliferative potential increases with increasing passage number in a renal cell carcinoma cell culture. The slope of the line is representative of proliferation potential.

Slope of the line at passage zero, 0.041; passage one, 0.053; passage three, 0.095. Each point represents mean ± standard deviation of 490 nm absorbance of triplicate wells.
Figure 2: *In vitro* vasculogenic mimicry in breast and lung adenocarcinomas. Cells were seeded at $1.7 \times 10^5$ cells/well and incubated for 6 h. Images were taken using the 4x objective lens on the Olympus IX73 inverted fluorescence microscope. Scale bar = 200 μm.

Vasculogenic mimicry is an independent trait and not specific to tumour type. Cells undergoing vasculogenic mimicry *in vitro* in A and B compared to cells incapable of undergoing vasculogenic mimicry *in vitro* in C and D. A and C: individual breast adenocarcinoma samples. B and D: individual lung adenocarcinoma samples.