Author's response to reviews

Title: Rapid chemokinetic movement correlates with the invasive potential of lung cancer cells; a functional molecular approach.

Authors: Sandra YY Fok (sfok5959@mail.usyd.edu.au) Jeffrey S Rubin (rubinj@mail.nih.gov) Fiona Pixley (pixley@aecom.yu.edu) John Condeelis (condeeli@aecom.yu.edu) Filip Braet (filip.braet@emu.usyd.edu.au) Lilian L Soon (lillian.soon@emu.usyd.edu.au)

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Author's response to reviews: see over
Dear Editor,

Thank you very much for your earlier correspondence and reports from the reviewers of our manuscript, “MS: 637877248751053 - Rapid chemokinetic movement correlates with the invasive potential of lung cancer cells; a functional molecular approach.” We have since addressed the queries raised by each reviewer as detailed below and resubmitted the revised manuscript as instructed. We look forward to hearing from you.

Yours sincerely,

Lilian.

**REVIEWER 1**

**Item 1.** *In P6, the invasion assay was carried out for 48 hours; it is difficult to exclude the different cell proliferation effect between KINE and CON cells. It will be better to reduce in experiment time period to 12 or 24 hours.*

Using a visualization assay we determined that while the cancer cells are invading across a collagen matrix, there is negligible cell proliferation. Apoptosis was also found to be negligible using an MTT assay. The Boyden Assay (used in the present manuscript) incorporates both a matrix layer and perforations simulating intravasation that impose significant challenges to cell proliferation. Most proliferation will occur when the cells have spread over the two-dimensional surface of the filter on the other side. The time period for the cells to reach the other side of the filter lies in the last 6-12 hr of the assay. Within this period of time there isn’t any difference in the proliferation rates of the two cell populations. We have found that the 48 h period was better suited for invasion assays using Boyden chambers.

**Item 2.** *How many arrays were performed? Did the author biologically duplicate the array*
Only one array experiment was performed. Robust experimental and chip design as well as statistical approaches allowed analysis or comparison of arrays to be made with reasonable confidence. Other approaches such as real-time PCR was used to verify the data. **Experimental design:** In our experience when using cell lines, RT-PCR will typically reproduce array data for 80% or more of the targets. RT-PCR has been performed for this particular experiment (see answer to Reviewer 1 (Items 3 and 4)). **Primers were designed** using the Primer Premier v.3 program where they were generated to span an intron and the 3’ non-coding region to avoid amplification of genomic contaminants and closely related sequences. **The cell lines provide ample RNA,** obviating the need for amplification, therefore avoiding errors due to non-linearity issues in amplifying small amounts of material. **Chip design and analysis:** In our study, we have used normalization procedures and ranked-based statistical methods for data comparison between chips. The Affymetrix Microarray Suite Software (MAS) was used to perform **Single Chip Analysis** to determine whether a gene is present or absent for each sample. The basis for this analysis is as follows: Each target has 10 probe sequences distributed on the chip. Each probe sequence, in turn, is paired; a perfect (P) and a mismatch (M) sequence. Intensity measurements followed by calculations using the equation, $R = (PM - MM) / (PM + MM)$, gives a discrimination score (R) for each probe set. The p-values are then determined using the Wilcoxon Signed Rank test to statistically assign a present or absent call for a particular target. The single chip analysis data was used to check that a present call was found at least one of the two datasets for a particular gene of interest. **For Between Chip Analysis** the same principle applies in utilizing the ten probes derived from the same gene to produce a population of probe intensity measurements for the statistical analyses to determine change calls and fold change. In this experiment, CON microarray data were designated the Baseline set and the KINE complement constituted the Experimental array. Both arrays were normalized using control probe sets present in the array and then scaled to reach a defined intensity. A further normalization step was carried out
called Robust Normalization, which accounted for unique probe set features such as affinity and linearity. This step was taken to reduce error stemming from using the average intensity of certain probe sets in the earlier normalization step. The Wilcoxon signed-ranked analysis tested significant difference between probe intensities found in the arrays and to assign p-values that ranged from 0 to 1. The p-values indicated both the likelihood and direction of change in expression levels. Estimation of the degree of change was performed using a Log Ratio Algorithm. Probe intensities were compared with corresponding partners in the arrays (baseline v. experimental) by using a Tukey’s Biweight method to obtain a mean of the log ratios of probe intensities across the two arrays. The Signal Log Ratio was then computed using a base of two, and therefore, the interpretation of SLR would be in multiples of two.

**Item 3** At least some of the differentially expressed genes obtained by microarray should be verified by RT-PCR or Western. If the authors can perform functional verification, the manuscript will be more informative. For example, knock down the over expressed genes in KINE cells can suppress chemokinesis as well as cancer cell invasion.

We have since verified genes from the same microarray experiment by RT-PCR; one of them was found to be downregulated by approximately 4-fold in the KINE population compared to CON. This gene has putative functions in protein transport to the cell membrane, cytoskeletal interactions and possibly cell adhesion. The gene was found to be overexpressed in four lung cancer cell lines compared to normal tissue. This data have been added to the manuscript.

**REVIEWER 2**

Major Compulsory Revisions (that the author must respond to before a decision on publication can be reached)

**Item 1.** The authors use genome-wide microarrays. However, only 7 up- or down-regulated genes
are shown. A number of differentially expressed genes should be presented. Moreover, function of those genes should be discussed. A scatter plot between two expression profiles can provide the overall difference.

**Item 2.** A subtitle “Microarray analysis and real-time PCR” is found on line 12 on page 8 (P8L12) in the
Methods, but data of real-time PCR and its protocol are not described. At least, data of real-time PCR of the above 7 genes should be added.

A purpose of this study was to identify genes that may have an effect on chemokinesis with consequences to cellular invasion. The use of microarrays was a means to achieve this goal, however, the authors have since refrained from making broad claims that genes are differentially expressed without verification. In the revised manuscript we have verified by RT-PCR the downregulation of a gene and tested its expression in a panel of cancer cells (please also refer to Items 2 and 3 from Reviewer 1).

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**Minor Essential Revisions (such as missing labels on figures, or the wrong use of a term, which the author can be trusted to correct)**

All Minor Essential Revisions have addressed for example p9 L17 now reads:
Chemokinetic conditions in the presence of serum were chosen over non-serum conditions to avoid stress induced by serum-starvation. A concentration of 1.5% was chosen because this serum level was sufficient to maintain cell survival and was shown to be effective for the assay.

Figure 6 has been removed and discussed in the text instead. Mesenchymal-like tumor cells is commonly used and refer to the stellate, fibroblast morphology adopted by epithelial cell that have undergone EMT. These cells tend not to be as invasive as cancer cells with amoeboïd-type morphology (Condeelis & Segall; Nat Rev Cancer. 2003 Dec;3(12):921-30).
REVIEWER 3

Major Compulsory Revisions (that the author must respond to before a decision on publication can be reached)

**Item 1. Microarray data:**

Please refer to Items 2 and 3 in Reviewer 1 response section.

**Item 2. Statistics:**

Statistics performed for the adhesion assay are reflected in the text (legend) and Figure 4.

![Figure 4 - KINE and CON cells were tested for strength of adhesion to substrate.](image)

Adhesion assays were conducted by plating cells on uncoated tissue culture dishes followed by trypsinization for 10 min using various concentrations of trypsin. T-test at 5% confidence levels showed significant difference at lower concentrations of trypsin used (asterisks), indicating greater
sensitivity to trypsinization in KINE compared to CON cells (n=3). Conversely, the resistance of CON cells to trypsinization suggests stronger adhesion to substrate.

Statistics have been incorporated for the analysis of cell motility in the Results section p11 and in Table 2 as follows:

Student’s t-test results reject the null hypothesis that there was no difference in the total path length, directionality, speed and area but not the net path length between KINE and CON cells. KINE cells achieved significantly greater total path length compared to CON cells, 

Table 2 - Parameter measurements of chemokinesis.

Student’s T-tests comparing parameters of motility between KINE and CON cells rejecting the Ho at P less than 0.05. Mean, Standard Deviation and P values are tabulated ((*) indicate significant difference; NS = non-significant difference; n=5).

<table>
<thead>
<tr>
<th>H460 Cells</th>
<th>Total Path Length (µm)</th>
<th>Net Path Length (µm)</th>
<th>Directionality</th>
<th>Speed (µm/min)</th>
<th>Area (sq.µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CON SE</td>
<td>24.56 +/-2.83</td>
<td>11.87 +/-3.75</td>
<td>0.48 +/-0.11</td>
<td>0.33 +/-0.02</td>
<td>661.42 +/-159.37</td>
</tr>
<tr>
<td>KINE SE</td>
<td>33.11 +/-4.71</td>
<td>7.81 +/-4.09</td>
<td>0.23 +/-0.11</td>
<td>0.43 +/-0.06</td>
<td>413.60 +/-83.37</td>
</tr>
<tr>
<td>T-Test</td>
<td>*P=0.014</td>
<td>P=0.181 NS</td>
<td>*P=0.018</td>
<td>*P=0.014</td>
<td>*P=0.025</td>
</tr>
</tbody>
</table>

Item 3. Many sentences are excessives:

The authors have modified excessive sentences in the Discussion to read as follows:
P17 L13. This study shows that cells demonstrating efficiency at both chemotaxis and chemokinesis performed significantly better in invasion assays than cells that are efficient in chemotaxis alone. This suggests that while chemotaxis remains an important mechanism of homing for cancer cells, chemokinesis maybe a better correlate for invasion in some cases.

P16 L7. This study shows that cancer cells capable of both chemotaxis and chemokinesis represent the highly motile cohort (faster speeds) that correlate well with invasiveness. We speculate chemokinesis in these cells may operate through an efficient stochastic process under isotropic conditions but in the presence of a strong gradient, their motility is convertible into chemotaxis.

P17 L23. A microarray analysis was conducted comparing gene expression of chemotactic-only cells and chemokinetic+chemotactic cells. The results indicate that some genes are expressed at different levels between the two cell populations.

Figure 6 has been removed.

Invasion assays showed that highly chemokinetic KINE cells were significantly more invasive than CON cells. The chemokinetic nature of KINE cells was demonstrated as movement at significantly greater speeds than CON cells - rapidity is a relative term used within the context of the experiments conducted. The adhesion assay and the localization of adhesion sites were used to illustrate the effect of cell adhesion on cell polarity, with inference on the type of motility the cells exhibited. Motility has long been associated with cell invasion and data in this paper showed that chemokinetic cells were highly invasive compared to cells that are less chemokinetic. Figure 5 showed that KINE cells are twice as invasive as CON cells. Therefore, the data is supportive of now modified inferences made in the discussion.