

Novel *in vitro* assays for the characterization of EMT in tumourigenesis

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Abstract. *Background:* Two novel assays quantifying Epithelial to Mesenchymal Transition (EMT) were compared to traditional motility and migration assays. TGF- β_1 treatment of AY-27 rat bladder cancer cells acted as a model of EMT in tumourigenesis.

Methods: AY-27 rat bladder cancer cells incubated with 3 ng/ml TGF- β_1 or control media for 24 or 48 h were assessed using novel and traditional assays. The Spindle Index, a novel measure of spindle phenotype, was derived from the ratio of maximum length to maximum width of cells. The area covered by cells which migrated from a fixed coverslip towards supplemented agarose was measured in a novel chemoattractant assay. Motility, migration and immunoreactivity for E-cadherin, Vimentin and cytokeratin were assessed.

Results: TGF- β_1 treated cells had increased "spindle" phenotype together with decreased E-cadherin, decreased Cytokeratin-18 and increased Vimentin immunoreactivity. After 48 h, the mean Spindle Index of TGF- β_1 treated cells was significantly higher than Mock ($p = 0.02$, Bonferroni test) and there were significant differences in migration across treatment groups measured using the novel chemoattractant assay ($p = 0.02$, Chi-square). TGF- β_1 significantly increased matrigel invasion.

Conclusion: The Spindle Index and the novel chemoattractant assay are valuable adjunctive assays for objective characterization of EMT changes during tumourigenesis.

Keywords: Matrigel, TGF- β_1 , chemoattractant, invasion, motility, migration, EMT, bladder cancer, AY-27, spindle phenotype

1. Introduction

Bladder cancer progression, metastasis and therapeutic resistance, which are associated with Epithelial to Mesenchymal Transition (EMT), signify a grave prognostic outlook [10,15]. The need to recognise, characterise and understand tumour progression and its response to therapy has led to the development of many diverse *in vitro* assays, but current techniques have limitations. In the scratch wound assay [6], cells that are damaged as a result of wound scratching release potent growth inhibitors and promoters [14]. In the Matrigel

assay, cells that migrate through the reconstituted basement membrane are enumerated [1]. Matrigel's three main limitations are: First, cells are not observed during the assay. Therefore those that have successfully invaded and migrated across the matrigel could themselves begin to divide and proliferate leading to potential bias resulting from mixed cell populations. Second, matrigel does not accurately mimic the constituents of basement membrane. Third, the matrigel assay does not facilitate the separation of aggressive and non aggressive sub-populations for subsequent comparative analyses.

EMT is associated with progression and a poor prognosis in many cancers. EMT can be induced by Transforming growth factor- β_1 (TGF- β_1). During EMT coordinated changes act together to induce a mesenchymal cell type. These include the modulation of

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epithelial tight and adherens junctions, re-organisation of the actin cytoskeleton, loss of apical-basal polarity, induction of a mesenchymal gene-expression programme and migration through basal membranes and tissues [4,5,13]. EMT is characterised by *de novo* expression of the intermediate filament protein, Vimentin [4] in tandem with the loss of Cytokeratin intermediate filaments, E-cadherin and proteins associated with a polarised epithelial phenotype [9]. The resultant 'fibroblastoid' or 'spindle' morphology confers invasive characteristics and may contribute to metastatic potential. Spindle phenotype can be categorised by microscopic assessment of morphological features such as shape and the incidence of lamellipodia, ruffles and spikes. Unfortunately, this subjective approach has intra- and inter-observational biases and reproducibility in pathology can be poor [7]. An objective assessment of spindle phenotype would be very valuable and would improve the accuracy in comparative studies.

TGF- β_1 is a cytokine that enhances characteristics associated with metastasis, e.g. motility [8,17,18], invasiveness and adherence [4]. TGF- β_1 over-expression has been associated with recurrent superficial bladder tumours [2]. High plasma levels of TGF- β_1 have been correlated with invasive bladder tumours [3,11].

The AY-27 rat bladder cancer cell line is an interesting candidate for manipulation with TGF- β_1 because it is invasive, but not metastatic. The AY-27 cell line was developed as a consequence of feeding the chemical carcinogen N-[4-(5-nitro-2-furyl)-2-thiazolyl]formamide (FANFT) to the diet of F344 Fisher rats [16]. Subsequent inoculation of AY-27 cells into the bladders of female F344 rats resulted in colonisation of the urothelium with locally invasive tumours [16].

In this study we demonstrate proof of concept of two novel assays. We present the Spindle Index as an objective measure of spindle phenotype and the novel chemoattractant assay as an objective assay of cell migration which has the potential to separate out non-migratory and migratory sub-populations for subsequent phenotypic, genetic or proteomic profiling.

2. Materials and methods

2.1. Cell culture

The rat bladder cancer cell line AY-27 (a gift from Professor R. Moore, University of Alberta, Canada) was grown in RPMI-1640 (Gibco, Invitrogen, UK) supplemented with 10% Fetal Calf Serum (FCS) (Labtech International, UK), 2% L-glutamine (Sigma-Aldrich Co. Ltd, UK) and 0.2% penicillin/streptomycin

at 37°C in a humidified CO₂ incubator (Shel Lab, USA) with 95% oxygen and 5% carbon dioxide.

2.2. TGF- β_1 and control treatments

TGF comprising recombinant human TGF- β_1 (R&D systems, Minneapolis, MN, USA) was made into a suspension according to the manufacturer's recommendations using Bovine Serum Albumin (BSA) and Hydrochloric acid (HCL). Two control treatments were run to control for the effects of the HCL and serum deprived medium (SdM), separately. SdM contained RPMI-1640 supplemented with 0.002% FCS and 2% L-Glutamine. Mock contained the appropriate concentrations of HCL diluted in SdM. Preliminary experiments with AY-27 cells demonstrated (1) that 3 ng/ml was the optimal dose of TGF- β_1 to induce morphological changes and cell migration in AY-27 cells and (2) that after 48 h, AY-27 cells died by apoptosis. Therefore assessments of the effects of 3 ng/ml TGF- β_1 were undertaken 24 and 48 h using the two novel assays and the two traditional assays.

2.3. Morphological assessment

Aliquots containing 1×10^5 AY-27 cells were seeded in triplicates on 18 mm coverslips in 6-well plates and incubated in supplemented RPMI-1640 under standard cell culture conditions until 20–30% confluent. Wells were washed twice with SdM before the media was replaced with SdM, Mock or 3 ng/ml TGF- β_1 for 24 h. For the 48 h experiment media was replaced with fresh media supplemented with FCS for 24 h prior to assessment. Stained coverslips were assessed by a pathologist (OS) who commented on the cell shape, the incidence of mitosis and cell to cell contact.

2.4. EMT immunocytochemistry

After treatment, cells on the coverslips were fixed in 99% alcohol for 48 h. Cells were incubated with 2 μ g/ml E-cadherin (Dako, England), 0.5 μ g/ml Vimentin Clone V9 (Sigma-Aldrich, UK), 1.0 μ g/ml Cytokeratin 18 (Abcam, UK) or 2 μ g/ml Cytokeratin 5/6 (Dako) for 1.5 h at RT. Controls were incubated with the equivalent concentrations of appropriate isotype control antibodies. Anti-mouse Polymer from DAKO Envision Kit (EnVision KitTM, Dako) was used for visualisation. Slides were blinded for assessment by two independent observers.

2.5. Novel assay I: Spindle Index

Coverslips were prepared as previously described under morphological assessment, mounted onto slides and anonymised. Four hundred images from 40 coverslips were captured using the Leica Microscope DM250 equipped with a high resolution DC300 Leica camera (Leica Microsystems AG, Germany) and the $\times 40$ objective. Ten randomly selected fields that contained cells at the periphery of cell clusters were captured from each coverslip. Cells were captured from the periphery because crowding of AY-27 cells had been noted to induce a spindle phenotype. The cell perimeter of individual cells was traced and a best fit rectangular boundary was computed using KS400 Imaging System, Release 3.0 (Carl Zeiss GmbH, Germany) software. The maximum linear length and maximum width of each cell was automatically derived by KS400. The Spindle Index was calculated as the ratio of the maximum length: maximum width of each cell (Fig. 1).



Fig. 1. Spindle Index. KS400 software was used to derive the Spindle Index which was the ratio of maximum length to the maximum width (measured normal to the length axis) of each cell. (The colors are visible in the online version of the article.)

2.6. Novel assay II: Novel chemoattractant assay

Optimisation

A series of optimisation experiments were carried out for the novel chemoattractant assay (Fig. 2). These demonstrated that agarose containing RPMI supplemented with FCS at concentrations of over 50% was essential for migration. Cells that moved onto the plastic did not survive when the agarose was supplemented with 10 or 30% FCS. No difference in terms of cell migration was observed following supplementation with FCS at concentrations of 50, 70 or 90%.

To determine the optimal migration gap, sterile 12, 14 or 16 mm diameter metal rings were used to remove the circle of agarose matrix from the centre of wells, resulting in gap widths of 1, 2 and 3 mm respectively, between the centrally-placed coverslip and the agarose matrix. Experiments showed that a 1 mm gap between the agarose and the central coverslip was optimal for cell migration. After removal of the central portion of agarose and washing twice in SdM the wells were scanned using light and phase-contrast microscopy. Any wells that had remnant agarose were discarded.

TGF- β_1 treatment

One millilitre of agarose (2%) containing RPMI-1640 supplemented with 50% FCS, 2% L-glutamine and antibiotics was pipetted into each well in a 12-well plate (Orange Scientific, Belgium) and allowed to cool and solidify. A sterile 12 mm diameter metal ring

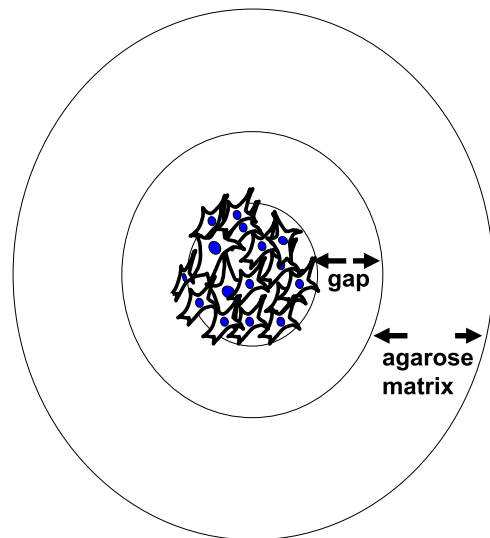


Fig. 2. Novel chemoattractant assay. Schematic of the novel chemoattractant assay showing a centrally placed 10 mm coverslip with confluent AY-27 cells surrounded by a 1 mm gap. An agarose matrix on the periphery of the 12 mm tissue culture well contained RPMI supplemented with 50% FCS.

was used to cut out the central portion of agarose in each well. A 10 mm coverslip, on which AY-27 cells were growing at 100% confluency, was washed twice in SdM before careful placement in the empty centre of the well. The coverslip was secured using 1 μ l xylene (Fig. 2).

Mock, SdM or TGF treatment solutions in 1 ml aliquots were added to six or more replicate wells for 24 or 48 h. The agarose was removed before cells were stained using Speedy-Diff Rapid Giemsa (Clin-Tech Ltd, Essex, UK). Representative digital images were captured from all wells. A 5 mm by 5 mm box grid was superimposed on top of each image and two independent blinded observers counted the numbers of 5 mm² boxes covered by cells.

2.7. Traditional assay I: Matrigel invasive assay

RPMI-1640 (0.75 ml) supplemented with 10% FCS was added as a chemo-attractant to the lower compartment of 6-well BioCoat Matrigel invasion chambers (Becton Dickinson Labware, NJ, USA) with 8 μ m pore polycarbonate filter coated with 0.5 mm Matrigel. To the upper compartment, 8×10^5 cells were added into Mock, SdM or TGF treatment solutions and incubated for 24 or 48 h at 37°C in a humidified incubator with 5% CO₂. Cells that passed through the filter into the lower wells were stained with Speedy-Diff Rapid Giemsa technique (Clin-Tech Ltd, UK). The number of cells in 10 random high power fields images on each filter was counted using a Leitz Labrolux K (Germany) and the $\times 40$ objective.

2.8. Traditional assay II: Scratch wound assay

AY-27 cells were seeded at a density of 1.0×10^6 cells in 6-well tissue culture plates and grown to 100% confluency. A gap of approximately 1 mm was created in each well by scraping the monolayer using a sterile pipette tip. The wells were washed twice with SdM before treatment with Mock, SdM or TGF solutions. Three fixed points were marked in each well and phase-contrast images were captured at these points, 24 and 48 h post treatment. The percentage area covered by motile cells, i.e. cells that had travelled into the 1 mm gap, was determined as described under the novel chemoattractant assay.

2.9. Comparisons between treatment groups

Statistical analysis was undertaken using SPSS version 16. The conservative Bonferroni test was used following ANOVA to compare the means of Spindle Indices and the means of the percentage coverage in the scratch wound assay across the treatment groups. The number of boxes covered by cells in the novel chemoattractant assay was categorized as below or above the median prior to Chi-square analysis to determine differences across the treatment groups. Mann-Whitney U was used to determine differences across groups with respect to matrigel invasion. Significance for all analyses was taken as $p < 0.05$. Visual comparisons were made between treatment groups for morphology and immunohistochemistry.

3. Results

3.1. TGF- β_1 induced morphological changes

Upon morphological assessment, untreated AY-27 cells were predominantly polygonal shape with high nuclear-to-cytoplasmic ratios and features of anaplasia. Mitotic cells were present. There were occasional spindle shaped cells exhibiting linear geometry. After TGF- β_1 treatment, there was an increase in the proportion of the spindle shaped cells and cells with cytoplasmic processes, but the differences were subtle highlighting the need for objective assessment (see Section 3.3). In tandem, there was a decrease in the number of polygonal shaped cells and cell-to-cell contact compared to control Mock or SdM treated cells. Mitotic cells were rare indicating that TGF- β_1 treatment had induced an anti proliferative effect (Fig. 3).

3.2. TGF- β_1 induced immunoreactivity changes associated with EMT

E-cadherin immunoreactivity was membranous in untreated (not shown) and in control treated AY-27 cells. After treatment with TGF- β_1 membranous E-cadherin immunoreactivity was reduced or lost with concomitant loss of cell-to cell contact and increased E-cadherin cytoplasmic positivity. There was increased expression and polarisation of the mesenchymal marker Vimentin and a corresponding decreased expression of the epithelial marker Cytokeratin 18 (Fig. 4). Cytokeratin 5/6 was not detected in untreated or TGF- β_1 treated AY-27 cells.

3.3. The mean Spindle Index increased 48 h following TGF- β_1 treatment

A total of 5365 cells growing immediately adjacent to the exterior of cell clusters were captured from 400 images for subsequent objective analysis using KS400 software. There were significant differences between the mean Spindle Indices across groups (ANOVA $p < 0.01$) and between the mean Spindle Index of the TGF- β_1 treated AY-27 cells (mean = 2.05; CI 2.00–2.10) and that of SdM (mean = 1.94; CI 1.89–1.98) or Mock (mean = 1.93; CI 1.89–1.98) 48 h after treatment (* $p = 0.03$ and ** $p = 0.02$ respectively, Bonferroni test). There was no significant difference between the means of TGF and control treated Mock or SdM cells at 24 h (Table 1). The percentage of cells with a Spindle Index greater than 3 progressively in-

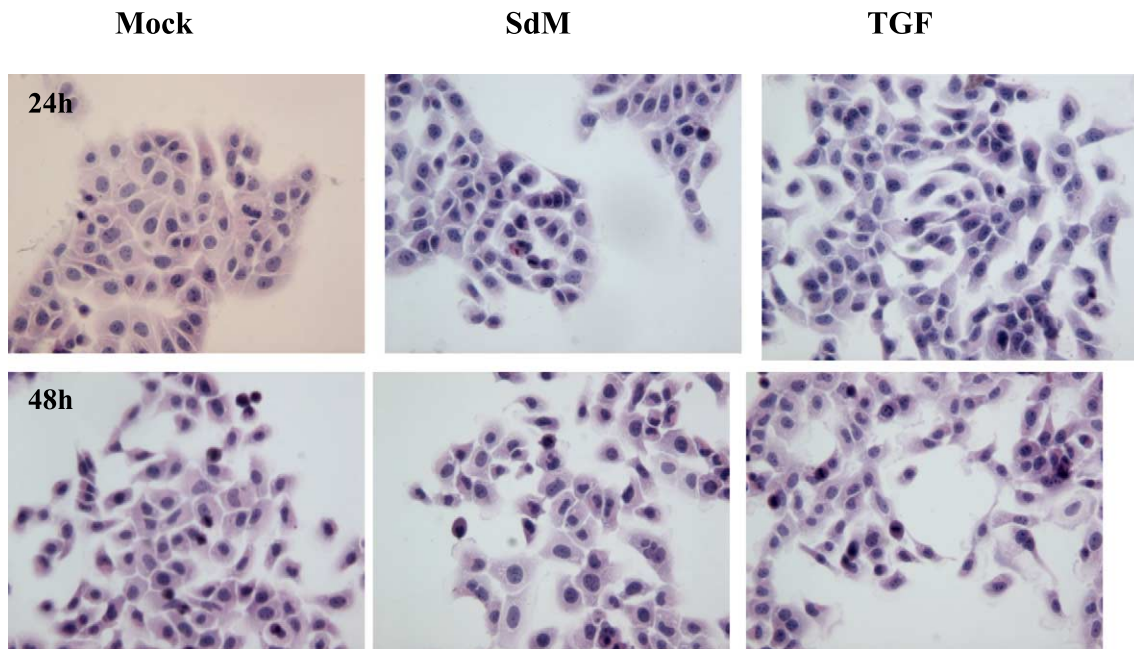


Fig. 3. TGF- β_1 treatment induced subtle changes to the spindle cell phenotype. Cells were treated with Mock, SdM or TGF for 24 h and then cultured in fresh media supplemented with FCS for another 24 h and assessed at 48 h. Cells with spindle morphology and cells with long cytoplasmic processes were more frequently observed following TGF- β_1 treatment ($\times 10$ objective, Leitz Laborlux K, Germany). (The colors are visible in the online version of the article.)

Table 1
Mean Spindle Index increased 48 h after treatment with TGF- β_1

Group	N	SI > 3	Mean	SD	F (df)	p-value
Untreated	765	5.9%	1.90	0.71	3.851 (6.5358)	$p < 0.001$
SdM24	639	6.9%	1.98	0.63		
Mock24	926	7.8%	1.98	0.71		
TGF24	683	8.2%	1.99	0.68		
SdM48	836	7.1%	1.94*	0.66		
Mock48	852	7.3%	1.93**	0.70		
TGF48	664	9.5%	2.05***	0.66		

Note: The percentage of cells in each group with a Spindle Index (SI) greater than 3 is included for comparison.

creased with treatment over time. It was observed that as the Spindle Index value increased the spindle phenotype became more pronounced. AY-27 cells were linear with no evidence of convolution (Fig. 5).

3.4. Trend for cell migration to increase following TGF- β_1 treatment in the novel chemoattractant assay

A significant association between the percentage of covered boxes and treatment was observed in the novel chemoattractant assay (Chi-square $p = 0.02$). The median number of boxes covered was 31. Twenty-three,

40 and 70% of the replicate measurements were above the median for SdM, Mock and TGF, respectively at 24 h ($n = 10$ for each group). After 48 h 17, 83 and 83% were above the median, respectively ($n = 6$ for each group) (Fig. 6). There was a significant correlation between the results from the two independent observers ($r = 0.98$, $p < 0.01$, Spearman Rho).

3.5. TGF- β_1 increased cell invasion in the matrigel assay and cell motility in the scratch wound assay

Significantly more TGF- β_1 treated cells migrated into the matrigel at both 24 and 48 h compared to

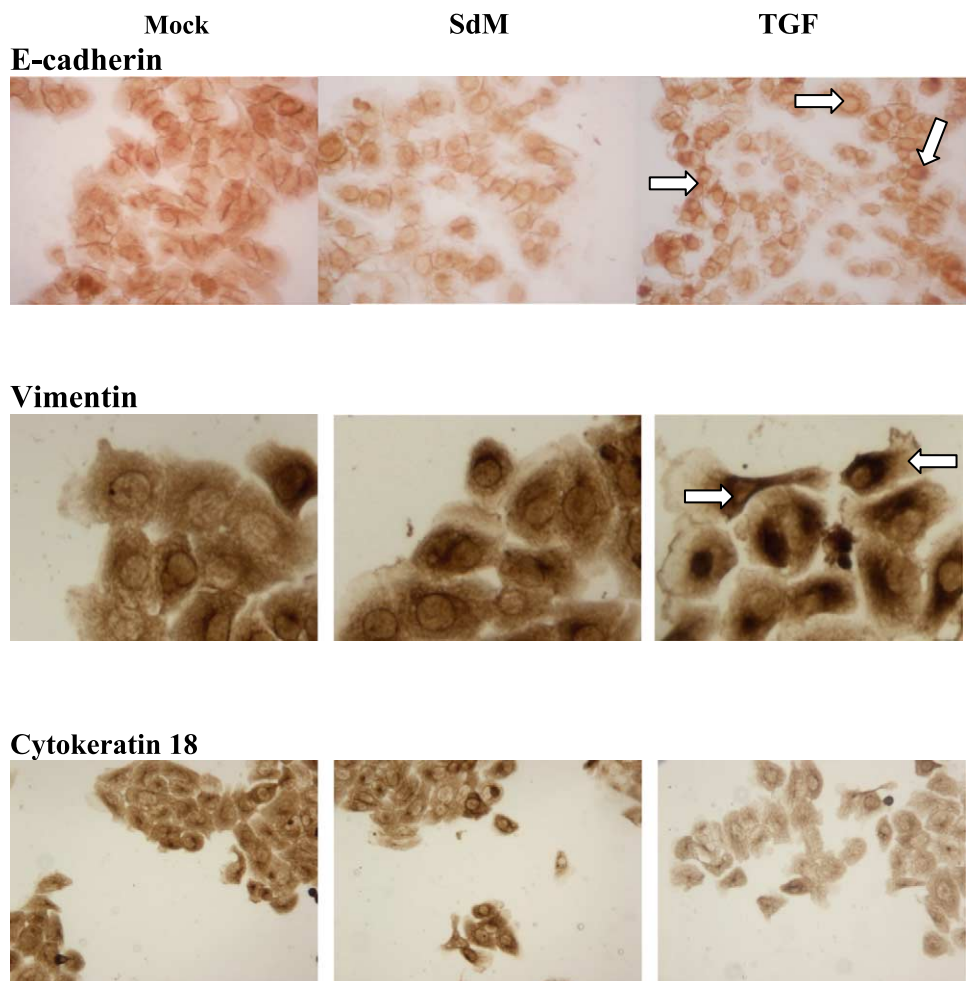


Fig. 4. Decreased E-cadherin, polarised Vimentin and decreased Cytokeratin 18 expression after TGF- β_1 treatment. Strong membranous E-cadherin immunoreactivity was observed after Mock treatment. Membranous E-cadherin immunoreactivity was retained in SdM treated cells, but was less intense when compared to Mock. After TGF- β_1 treatment, E-cadherin immunoreactivity was predominantly perinuclear and cytoplasmic (arrows). Cell to cell contact was reduced. Homogenous staining of Vimentin was observed following treatment with either SdM or Mock. In contrast, Vimentin had a polarised distribution (arrows) and increased staining intensity following TGF- β_1 treatment. Further evidence of EMT changes were evident in the reduction of Cytokeratin 18 immunoreactivity in AY-27 cells following TGF- β_1 treatment the immunophenotypes observed at 24 and 48 h were very similar. The images at 24 h are presented ($\times 10$ and $\times 40$ objectives, Leitz Laborlux K, Germany). (The colors are visible in the online version of the article.)

SdM ($p < 0.01$) or Mock treated ($p < 0.01$) (Mann-Whitney U) (Fig. 7).

There were significant differences between the mean percentage areas of the wound covered by motile cells across groups (ANOVA $p < 0.01$) and between the mean percentage area covered by TGF- β_1 treated cells (mean = 95.4%; CI 89.7–101.2), SdM (mean = 28.9%; CI 10.1–47.5) or Mock (mean = 70.4%; CI 60.8–80.0) 24 h after treatment ($*p < 0.01$ and $**p = 0.02$ respectively, Bonferroni test). Interestingly by 48 h a significantly higher percentage of the wound area was covered by Mock (mean = 76.2%; CI

66.3–86.0) when compared to TGF (mean = 53.3%; CI 40.2–66.3) treated cells ($***p = 0.04$, Bonferroni test) (Table 2). There was a significant correlation between the results from the two independent observers ($r = 0.95$, $p < 0.01$, Pearson correlation).

4. Discussion

TGF- β_1 induced increased invasive properties in tandem with characteristic EMT changes in AY-27 cells corroborating findings from previously published

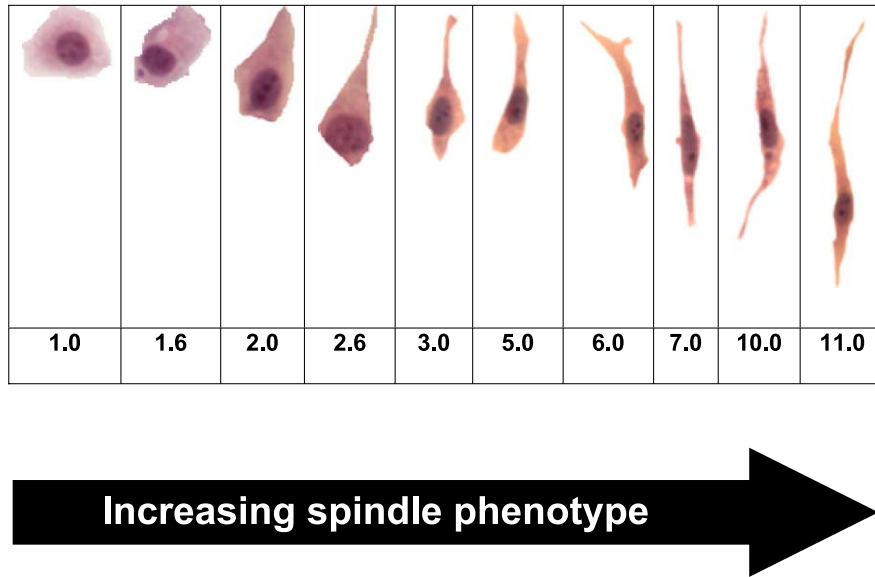


Fig. 5. Spindle Index was greatest in cells with the most pronounced spindle phenotype. The Spindle Index was derived from the ratio of maximum length to maximum width in individual AY-27 cells. As the Spindle Index increased the spindle morphology increased. This figure contains representative AY-27 cells which are predominantly of linear shape. (The colors are visible in the online version of the article.)

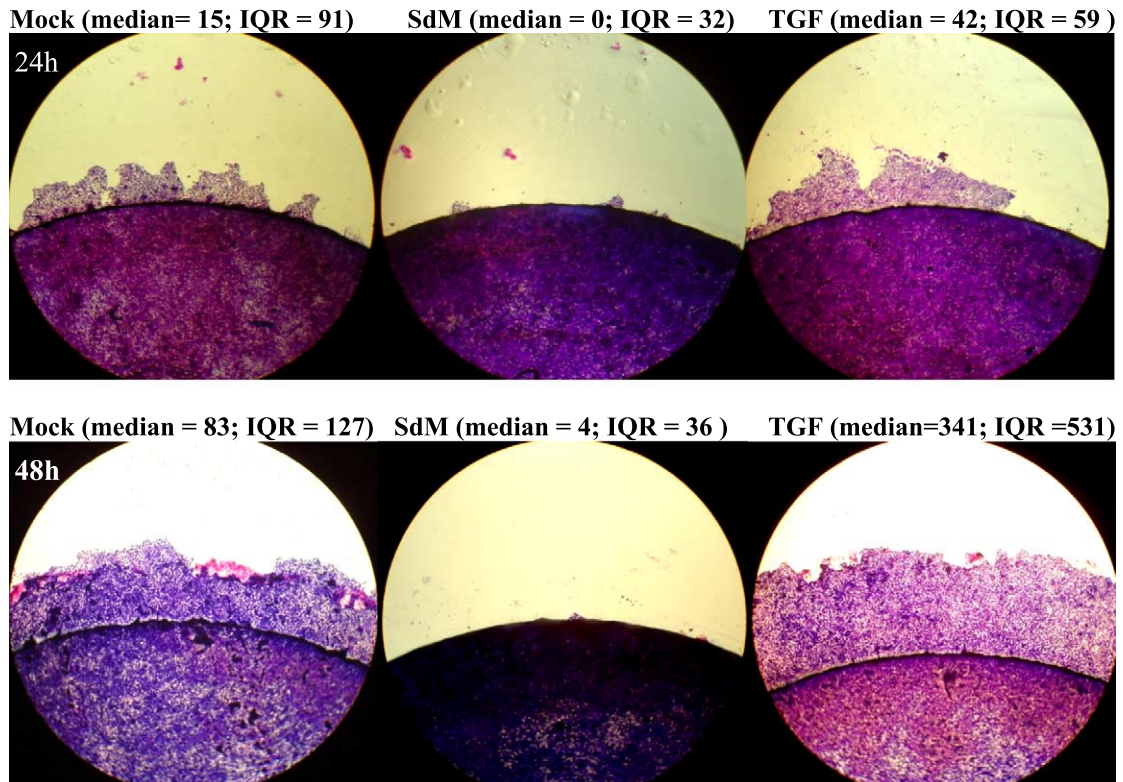


Fig. 6. Increased migration following TGF- β_1 treatment in the novel chemoattractant assay. A representative well from each treatment group is shown. The brackets contain the median number of boxes covered by AY-27 cells migrating across the gap for each treatment group and the Interquartile range (IQR). (The colors are visible in the online version of the article.)

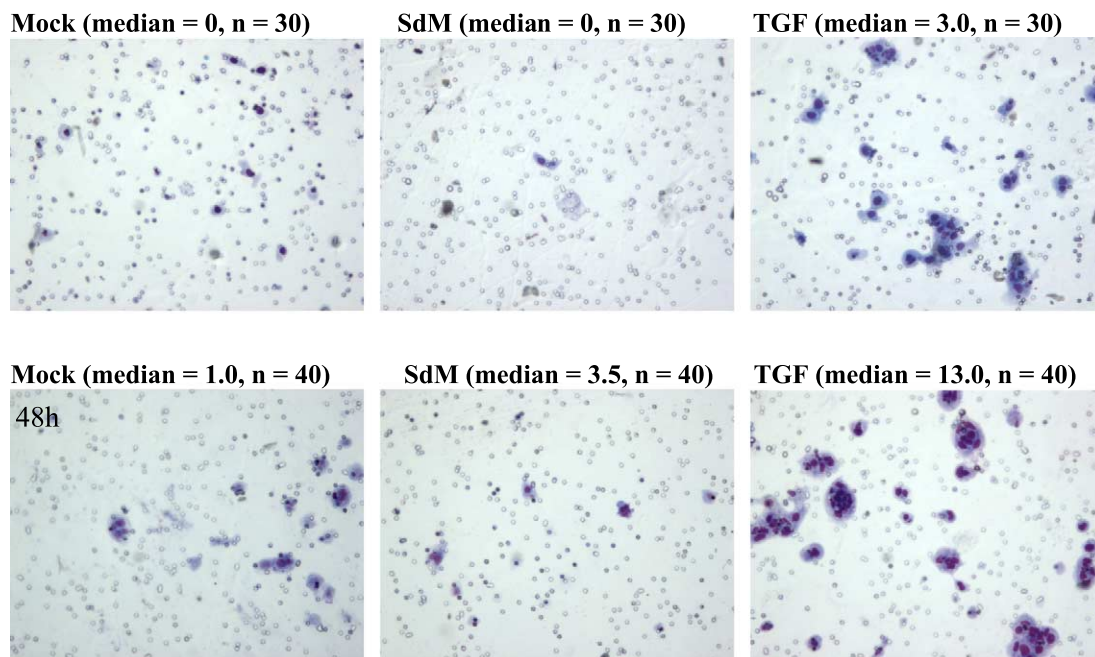


Fig. 7. Increased matrigel invasion at both 24 and 48 h following TGF- β_1 treatment. The number of cells in 10 random high power fields (HPF) images on each filter was counted using $\times 40$ objective on a Leitz Labrolux K (Germany) light microscope. The brackets contain the median cell counts/HPF and n – the number of HPF assessed for each group. Significantly more TGF- β_1 treated cells migrated into the matrigel at both 24 and 48 h compared to SdM ($p < 0.01$, Mann-Whitney U). (The colors are visible in the online version of the article.)

Table 2

TGF- β_1 enhanced wound closure at 24 h in the scratch wound assay

Group	N	Mean	SD	F (df)	p -value
SdM24	10	28.87*	26.12	21.115 (5.54)	$p < 0.0001$
Mock24	10	70.39**	13.43		
TGF24	10	95.44***	8.02		
SdM48	10	45.23	12.99		
Mock48	10	76.16***	13.72		
TGF48	10	53.29***	18.20		

studies on a wide spectrum of cancers [8,18]. We have demonstrated proof of concept of the Spindle Index and the novel chemoattractant assays as measures of spindle phenotype and migration, respectively.

It may not be appropriate to use the scratch wound assay to assess EMT which has been induced by TGF- β_1 . EMT is a morphogenic programme which is switched on during wound healing and TGF- β signaling has been shown to be essential for joint morphogenesis [12]. This may explain why Mock wound coverage was greater than that of TGF- β_1 treated cells at 48 h.

The Spindle Index will be a valuable adjunct for the objective measurement of spindle phenotype for investigation into the mechanisms involved in EMT across

all cancer cell lines. The KS400 software facilitated the derivation of the Spindle Index which was based on the assumption that the morphology of spindle shaped cells was linear. If cells are non-linear, the best fit rectangular boundary may underestimate the maximum length and overestimate the maximum width and introduce bias into the Spindle Index calculations. In this study non-linear spindle shaped AY-27 cells were rare and therefore the linear assumption was valid. Application of the Spindle Index to other cell lines would require a pre-screening validation to ensure that the spindle shaped cells were predominantly linear. If cell lines were non-linear computational/image processing methodologies could address the issue of calculating the length of cell along its central axis.

We observed that overcrowding in AY-27 cells *per se* altered morphological features adding further weight to the hypothesis that the AY-27 cell line represents a model on the threshold between local invasion and EMT. For this reason, only cells on the periphery of the cell clusters were measured and cells were seeded to ensure that the confluence at the time of assessment was 60% across all groups.

After 24 h mean Spindle Indices were similar across the groups. At 24 h all cells had been under stressful

environmental conditions because they were in serum deprived media. This factor, *per se*, may have been sufficient to shift AY-27 cells towards an aggressive phenotype. However, at 48 h when all cells had been incubated for 24 h in 10% FCS the mean Spindle Index was significantly greater in the TGF- β_1 treated group. This would suggest that the TGF- β_1 effects persisted. The novel Spindle Index assay utilises a standardised measure which is not influenced by intra- and inter-observer variability. Future work on other cancer cell lines using a substantive panel of EMT markers would be required to validate the relationship between the Spindle Index and EMT during tumourigenesis. Depending on the outcome of these experiments, there could be the potential for development of the Spindle Index into an automated software programme for user-friendly application.

We developed the novel migration assay in order to quantify cell migration and to facilitate characterisation of the aggressive motile sub-population of the cell population under investigation. The preliminary results were encouraging.

The main shortcomings in the novel chemoattractant assay are: the height of the coverslip, the accuracy of the 1 mm gap and difficulties associated with reproducibility of the well set-up. Commercial development would overcome these problems. All treatment groups demonstrated cell migration but, bias could have been introduced by the height of the coverslip. To overcome this problem we propose that there should be a precisely crafted indented receiving zone in each well to receive the coverslip. Commercialisation would also ensure reproducible well set-up and would remove the labour intensive preparation and therefore facilitate experiments with more replicates in each group.

A potential advantage of the novel chemoattractant assay is the ability to assess cellular movement and change in cellular phenotype in real-time with the use of time-lapse video-microscopy. Furthermore, cells that have successfully moved off the coverslip onto the gap region can be easily separated for differential profiling. This unique feature could contribute to the identification of proteins that play a key role in migration.

5. Conclusions

We have presented proof of concept for the two novel assays which will complement existing *in vitro* assessments of EMT characteristics.

The Spindle Index is the first objective measure of spindle cell phenotype. The novel chemoattractant assay offers the facility to separate migratory and non-migratory sub-populations for further analyses.

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