APPLICATION NOTE

Comparison of EMT biomarker expression in 2D monolayer and 3D spheroid cultures in a prostate cancer cell model.

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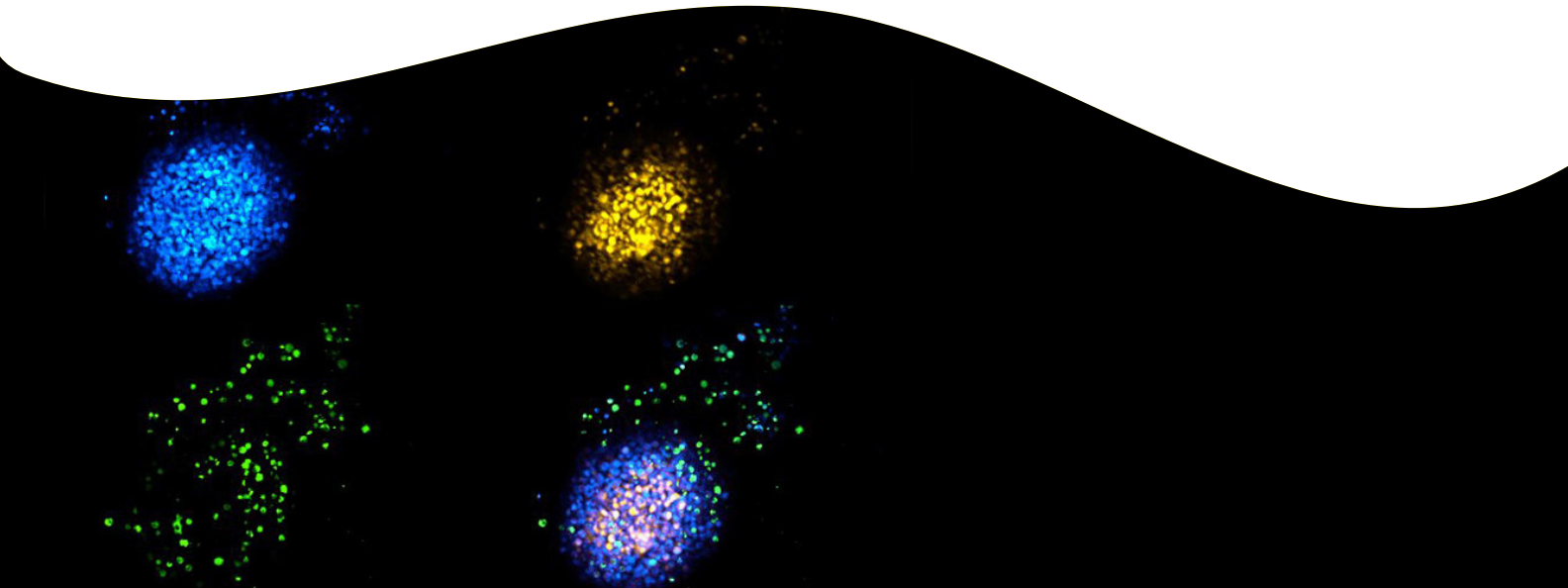
Introduction

The initiation of metastasis requires invasion, a process that is enabled by epithelial-mesenchymal transition (EMT). EMT is characterized by rearrangement of the extracellular matrix (ECM) and differential regulation of ECM proteins such as the epithelial adhesion protein E-cadherin and mesenchymal adhesion proteins such as fibronectin. This shift in protein expression accompanies further downstream modulation of cytokines, proteinases, and kinases as well as phenotypic changes in cell morphology. Previous studies have shown that human transforming growth factor-beta (TGF-β) can induce EMT in DU145 cells, a prostate cancer cell model.[1,2] Here we show the effects of TGF-β treatment on DU145 cells grown in monolayer (2D) and in spheroid cultures (3D). We compared expression levels of specific biomarkers, such as E-cadherin, fibronectin, and IL-6, using AlphaLISA™ assay technology. We confirmed that treatment with TGF-β is sufficient for inducing changes in both EMT biomarker expression and in promoting development of characteristic mesenchymal stromal cell morphology in monolayer cultures. However, in 3D spheroid cultures, we only observed a partial EMT response to the same TGF-β treatment as demonstrated by changes in the expected biomarker expression pattern. Cellular proliferation, growth and vitality were assessed using ATPlite™ luminescence assays and confocal microscopy of live-stained cells with a high content imaging system. Though we observed increased proliferation in monolayer cultures compared to 3D spheroids, the changes observed in protein expression patterns cannot be sufficiently explained by differences in cell number or viability. These data illustrate that AlphaLISA can be used to compare the differences in protein expression levels and in cellular tolerance for compound treatment between a human prostate cancer cell line grown in monolayers and those same cells grown in 3D spheroids.



AlphaLISA technology allows for the detection of molecules of interest in a homogeneous, no-wash format. As shown in Figure 1, a biotinylated anti-analyte antibody binds to streptavidin-coated Donor Beads, while another anti-analyte antibody is conjugated directly to AlphaLISA Acceptor beads. Both antibodies bind to the analyte, when present, bringing the Donor and Acceptor beads in close proximity of each other. Upon excitation at 680 nm, the Donor beads emit singlet oxygen molecules that travel in solution to activate the Acceptor beads, which then emit a sharp peak of light at 615 nm. This light emission can then be detected on an Alpha-enabled reader.

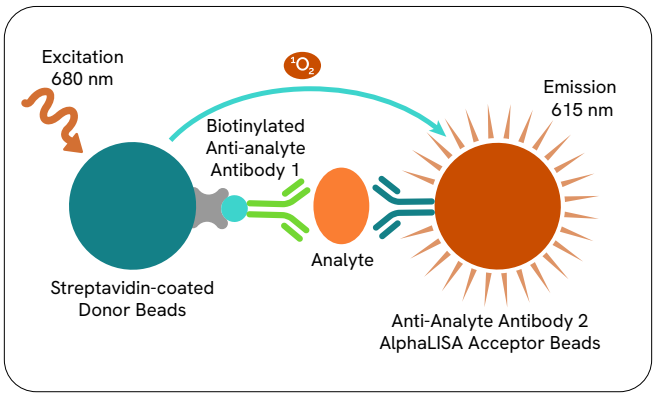


Figure 1: AlphaLISA schematic.

Materials and methods

Instrumentation

All AlphaLISA measurements were performed on the Revvity EnVision™ Multimode Plate Reader. Luminescence was measured using the EnSight™ multimode plate reader. 3D spheroid cultures were imaged with the 10X long WD objectives on the Operetta® and Opera Phenix™ High Content Imaging systems using Brightfield and appropriate fluorescence optics. Cross-sectional spheroid area was measured with Harmony® software using an intensity cutoff in the UV channel (Hoechst).

Cell culture and treatment

DU145 cells (ATCC® HTB-81™) were seeded (100 µL/well) into Revvity 96-well CellCarrier™ (6005550) or CellCarrier Spheroid ULA 96-well microplates (3D) (6055330) and grown for at least 18 hours. Cells were serum starved for 24 hours prior to treatment with recombinant human TGF-β1 (BioLegend, 580702) to induce EMT. In some experiments, cells were treated with 2.5 µM of TGF-β inhibitor SD 208 (Sigma, S7071) for two hours followed by 48 hours with TGF-β (5 ng/mL) in a total volume of 100 µL.

Biomarker quantification assays

For biomarker detection assays, 50 µL of media was removed (for testing supernatants). Cells were then lysed with 50 µL of 2X Alpha *SureFire® Ultra* lysis buffer (ALSU-LB-10mL) for 10 minutes with shaking and pipetting up and down vigorously. This lysis buffer was used as it gave the best signal for the AlphaLISA *SureFire Ultra* Total GAPDH (ALSU-TGAPD-A500) assay and was therefore, used for all experiments. AlphaLISA lysis buffer can also be used as samples from the same wells were used for both *SureFire Ultra* and other assays. Lysates and supernatants were frozen at -80 °C and later thawed for testing. For AlphaLISA assays detecting E-cadherin (AL370C), fibronectin (AL351C), and human IL-6 (AL223C), 5 µL of each lysate or supernatant sample was added to a 384-well white OptiPlate (6007290) and assays were performed according to the manual. For the AlphaLISA *SureFire Ultra* Total GAPDH Assay, 5 µL of lysis buffer was added to the 5 µL of lysate for a total of 10 µL of sample. Then the assay was performed according to the manual.

ATPlite™ 1step and ATPlite 1step 3D assays

Cellular proliferation, growth and vitality were measured by assessing the concentration of ATP using ATPlite 1step (6016731) and ATPlite 1step 3D (6066943) luminescence-based assays following kit protocols.

Cellular imaging

For cellular imaging, cultures were first stained with Hoechst 33342 (5 µg/mL; Life Technologies, #H3570), Tetramethylrhodamine (50 nM; TMRM; Life Technologies, #T-668) and CellTox™ Green (1:1000; Promega, #G8742) by diluting in media and adding directly to cultures. Monolayer cultures were imaged using the cellular imaging module of the EnSight plate reader using Brightfield optics. 3D Spheroid cultures were imaged with the 10X long WD objective on the Operetta and Opera Phenix High Content Imaging systems using Brightfield and appropriate fluorescence optics. Cross-sectional spheroid area was measured with Harmony software using an intensity cutoff in the UV channel (Hoechst).

Data analysis

The data were analyzed using GraphPad software. The standard curves were generated using nonlinear regression with a four-parameter logistic equation (sigmoidal dose-response curve with variable slope) and a $1/Y^2$ data weighting (minimize relative distances square). The Lower Detection Limit (LDL) is calculated using three standard deviations from the average of the background signal.

Results and discussion

Imaging DU145 cells treated with TGF-β in 2D and 3D

Human recombinant transforming growth factor-β (TGF-β) is known to be an inducer of EMT.[1,3,4] Brightfield images of cells plated in monolayer with and without TGF-β treatment (5 ng/mL) are shown in Figure 2. Without treatment (left panel), the cells appear polygonal and epithelial-like while in the presence of TGF-β they appear elongated and fibroblastic (right panel). This change in morphology indicates that the cells have undergone EMT.

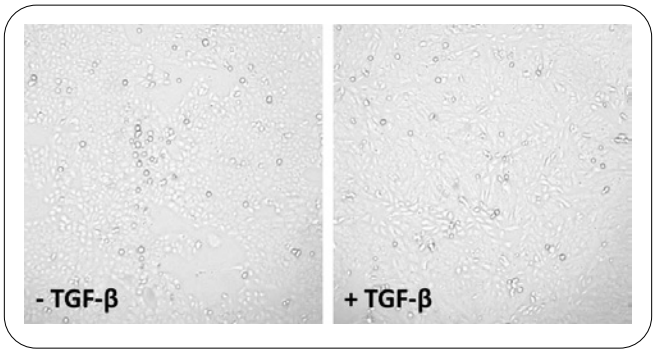


Figure 2: Brightfield images of DU145 monolayer cultures 48 hours post-treatment without (left) or with (right) TGF-β captured on the EnSight. TGF-β treatment induces development of the characteristic mesenchymal stromal cell morphology indicative of EMT. Representative images for 6000 cells/well are shown.

Figure 3 shows DU145 cells plated as 3D spheroids treated with increasing concentrations of TGF-β. In the presence of low amounts of TGF-β (5 ng/mL), the spheroids appear darker and slightly more compact than without treatment. Cells treated with higher amounts of TGF-β look similar to the 5 ng/mL treatment.

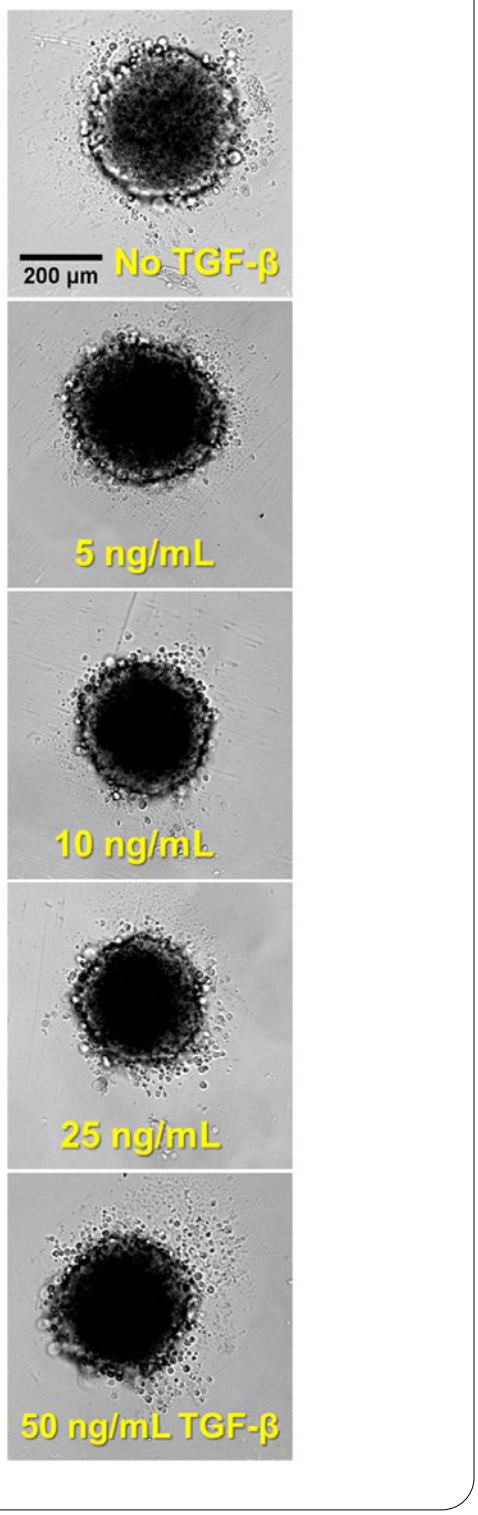


Figure 3: Brightfield images of spheroids DU145 cells 48 hours post-treatment with increasing concentrations of TGF-β on the Operetta High Content Imaging system.

Assessing cell viability after TGF-β treatment in 2D and 3D

In order to accurately assess the effect of TGF-β treatment on various biomarker protein expression levels, the viability of the DU145 cells before and after TGF-β treatment was measured using ATPlite 1step and ATPlite 1step 3D. These luminescent-based assays are based on the production of light caused by the reaction of ATP generated by cells in culture with added firefly (Photinus pyralis) luciferase and D-luciferin. As shown in Figure 4A and B, similar levels of luminescence (and therefore ATP output) are seen with and without TGF-β treatment, indicating that TGF-β treatment does not affect the cell viability. Therefore it can be assumed that differences in protein expression levels are due to upregulation or downregulation of the biomarker and not due to cell death.

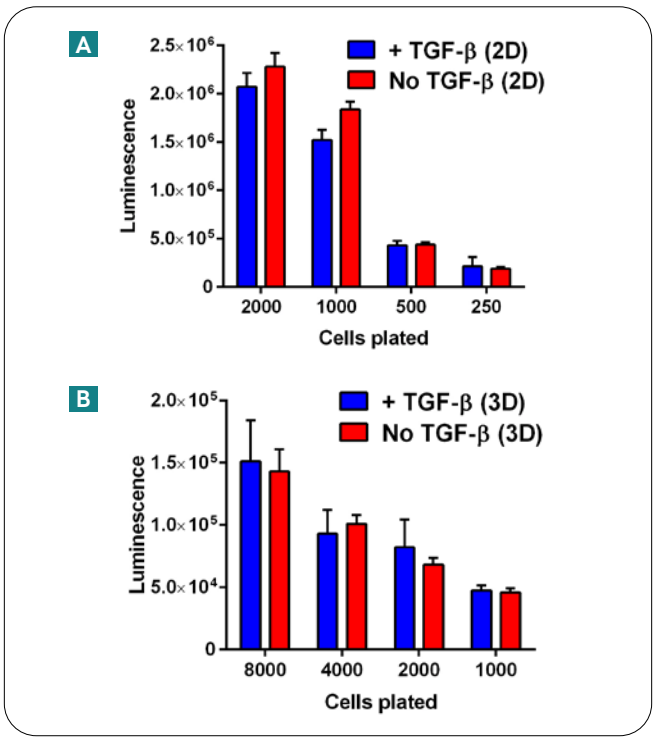


Figure 4: ATPlite 1step signals before and after TGF-β treatment show no significant effect on either proliferation or viability in 2D cultures (A), or 3D spheroids (B).

One alternative way of assessing growth and cell viability within spheroids is to use live cell fluorescent stains and high content imaging to measure spheroid size. Cross-sectional spheroid sizes of various cell numbers were measured with and without TGF-β treatment by imaging Hoechst stained cultures and using intensity cutoff on the Operetta. No significant differences in spheroid size were seen with treatment (Figure 5A). In addition, CellTox™ Green shows minimal differences in the number of dead cells and TMRM, a marker of healthy cells, shows minimal differences in live cells before and after TGF-β treatment (Figure 5B). These observations further indicate that the changes in EMT biomarkers observed in 3D are not due to cellular proliferation or viability.

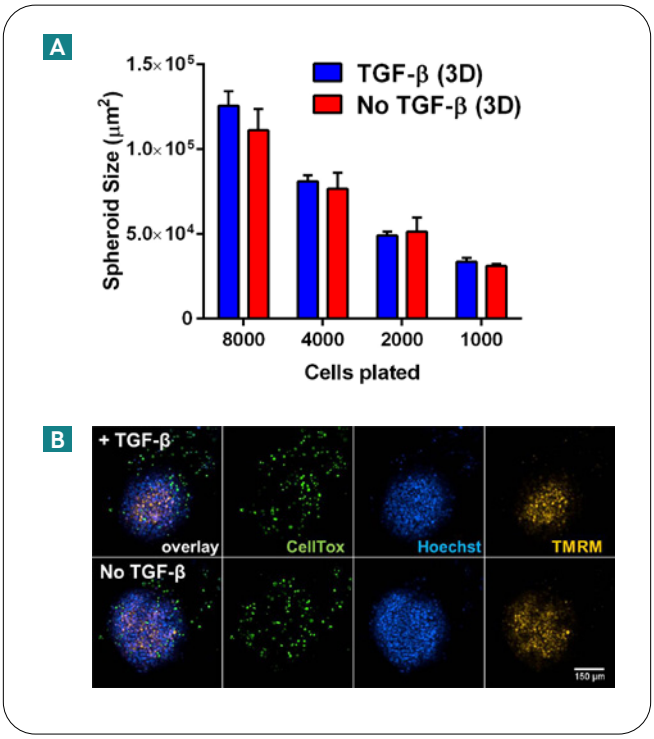


Figure 5: Spheroid size measurements of 3D cultures with and without TGF-β (A). 3D cultures labeled with CellTox™ Green, Hoechst, and TMRM (orange) show no significant effect of TGF-β treatment on cellular viability within a spheroid (B).

TGF-β downregulates E-cadherin in 2D and 3D and can be reversed by SD 208

In order to determine the level of ECM protein modulation after EMT induction, an AlphaLISA assay was used to quantify E-cadherin levels in both cell lysates and cell supernatants. E-cadherin is a transmembrane protein that plays an important role in cell adhesion and is downregulated after EMT. Figure 6A shows standard curves using increasing concentrations of a recombinant E-cadherin protein diluted in either media (EMEM) or a 1:1 mixture of media (EMEM) and lysis buffer (Alpha *SureFire Ultra* lysis buffer). The standard curves were used to convert the AlphaLISA signal determined from lysates (media + lysis buffer) or supernatants (media) into expression levels of E-cadherin. As shown in Figure 6B, in the presence of TGF-β, levels of E-cadherin are reduced in the lysates for cells grown in both 2D and 3D, consistent with EMT progression. The small molecule SD 208 blocks ATP binding to TGF-β and inhibits its kinase activity[5] and has been shown to reverse TGF-β-induced EMT.[2] When cells were co-treated with TGF-β and SD 208, the levels of

E-cadherin were similar to the untreated cells. This verifies that the effects seen are specifically due to TGF-β in both 2D and 3D cultures. Interestingly, the E-cadherin levels in the supernatants from 2D cultures decrease upon TGF-β treatment, but the levels in supernatants from cells grown in 3D do not change (Figure 6C).

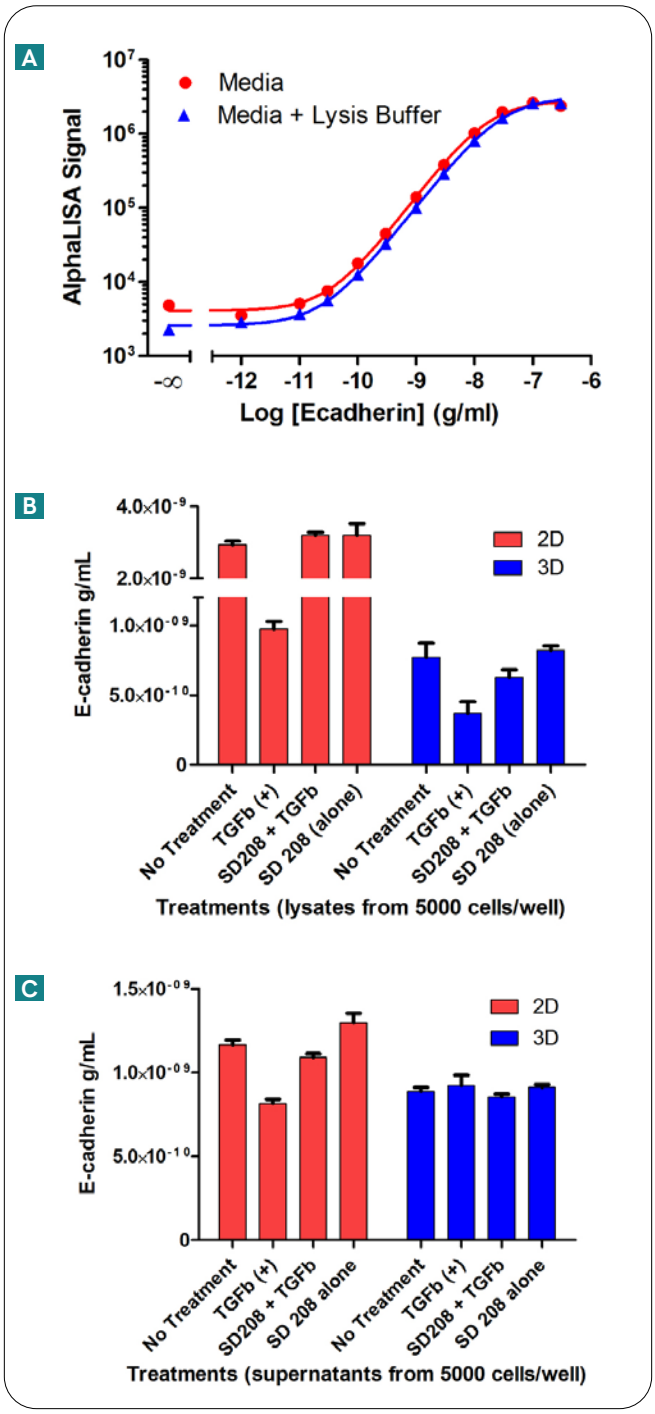


Figure 6: A) Standard curve of E-cadherin. B) E-cadherin levels in lysates and C) supernatants of DU145 cells grown in 2D (red) or 3D (blue). Expression levels are shown with and without 5 ng/mL TGF-β treatment or with a co-treatment of the TGF-β inhibitor SD 208.

Since there was no change in E-cadherin expression in the supernatants of cells grown in 3D, it may be that more TGF-β is needed to fully induce EMT in 3D spheroids. To determine whether or not 5 ng/mL TGF-β is sufficient for fully inducing EMT in 3D cultures, we tested higher concentrations of TGF-β (Figure 7). As expected, 5 ng/mL TGF-β induces a decrease in E-cadherin levels in the lysates but not in the supernatants. However, higher TGF-β concentrations did not further decrease the E-cadherin levels in the 3D lysates and had no effect on levels in the 3D supernatants. The increase in TGF-β concentration did not affect the levels of E-cadherin in 2D cultures either (data not shown). All measurements were well above the lower detection limit of the assay shown on the graph as reference.

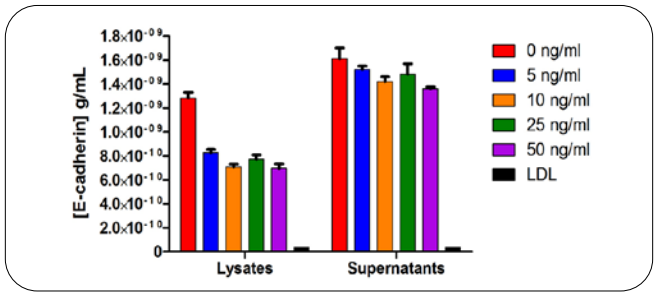


Figure 7: E-cadherin levels in lysates and supernatants of DU145 cells plated in 3D with increasing TGF-β concentration treatment. 8000 cells/well were plated. The lower detection limit for the assay (LDL) is shown on the graph.

TGF-β induces increased expression of fibronectin in 2D but not in 3D cultures

Another marker indicative of EMT induction is fibronectin. Fibronectin is a glycoprotein found in the extracellular matrix that binds to membrane-spanning receptor proteins and is expected to be upregulated after EMT. Fibronectin expression levels were measured from lysates and supernatants of cells grown in both 2D and 3D using AlphaLISA. Figure 8A shows standard curves of recombinant fibronectin diluted in either media (EMEM) or a 1:1 mixture of media (EMEM) and lysis buffer (Alpha *SureFire Ultra* lysis buffer). These curves were used to quantify the fibronectin expression levels in the samples. Upon treatment with TGF-β, the level of fibronectin from cells grown in 2D increased in both the lysates (8B) and the supernatants (8C). However, the levels of fibronectin from cells grown in 3D are detectable but do not change in either the lysates (8B) or supernatants (8C). This suggests that the 3D spheroids may only be going through a partial EMT whereas the cells grown in monolayer go through a full transition.
It is unclear whether these differences are due to the inability of TGF-β to fully penetrate the spheroid or if it is due to inherent differences in cellular interactions between cells grown in monolayer and as a spheroid.

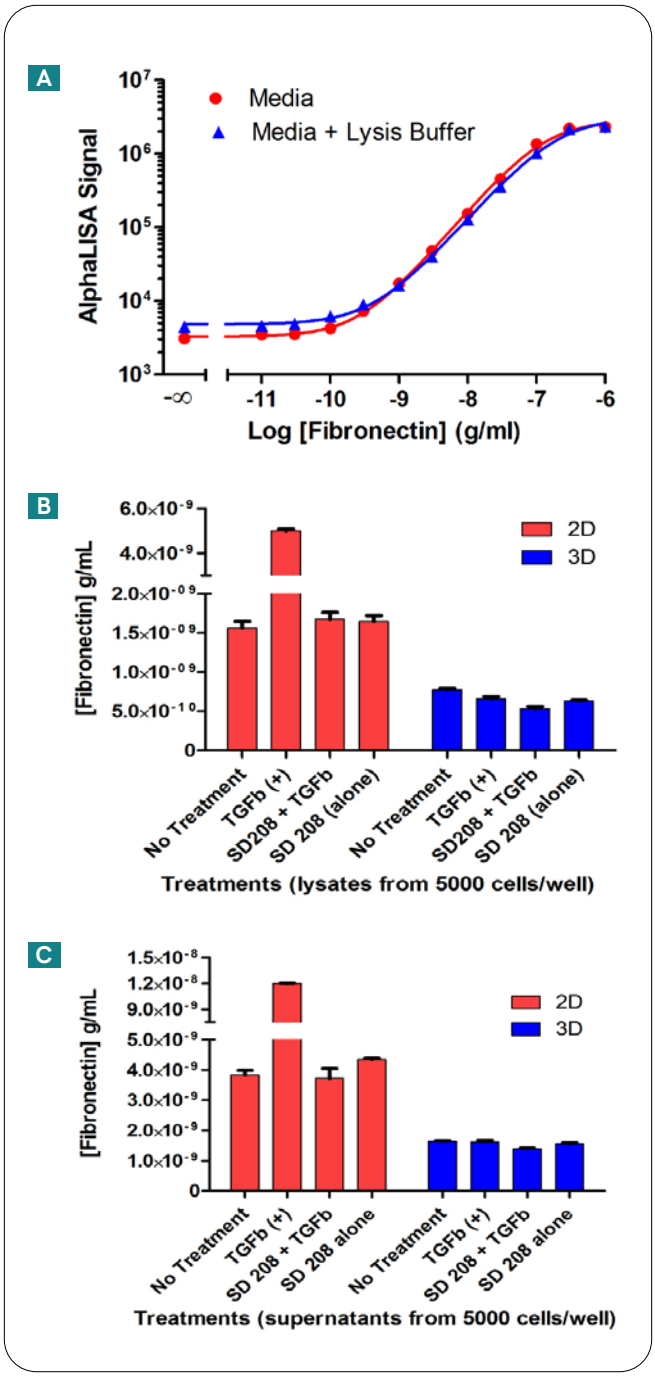


Figure 8: A) Standard curves of fibronectin. B) Fibronectin levels in lysates and C) supernatants of DU145 cells grown in 2D (red) or 3D (blue). Expression levels are shown with and without 5 ng/mL TGF-β treatment or with a co-treatment of the TGF-β inhibitor SD 208.

Modulation of IL-6 expression levels in 2D and 3D

Interleukin-6 (IL-6) is a pro-inflammatory cytokine associated with cancer progression. Previous data show that IL-6 expression levels increase in DU145 cell supernatants after treatment with TGF-β.[1] To determine if IL-6 is modulated and secreted in DU145 cells treated with TGF-β grown in 3D cultures, IL-6 levels were measured in cell supernatants from both 2D and 3D cultures using AlphaLISA (Figure 9). The induction of EMT increased secretion of IL-6 in the TGF-β-treated cells for both 2D and 3D and was reversed when co-treated with SD 208. Interestingly, the quantity of IL-6 secreted into the supernatant was ~2 orders of magnitude different for cells grown in 2D and 3D. Further experiments would need to be performed to fully understand why the amount of IL-6 secreted is so different.

Previous studies have shown that IL-6 can increase TGF-β signaling.[6] Therefore, the increased amount of IL-6 secreted into the supernatant in the 2D cultures may help to exaggerate the TGF-β induced responses in monolayer compared with the responses seen in the spheroids. Further experiments would need to be performed, such as co-treating the spheroid with recombinant IL-6, to see if this could be contributing to the differences in the other biomarker responses to TGF-β treatment.

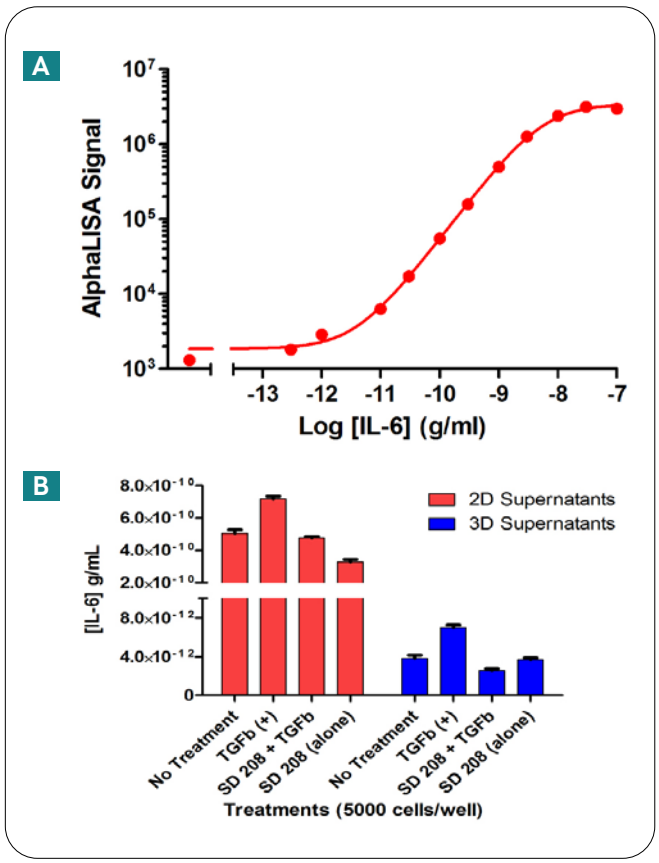


Figure 9: A) Standard curve of IL-6 performed in media (EMEM). B) IL-6 levels in supernatants of DU145 cells grown in 2D (red) or 3D (blue). Expression levels are shown with and without 5 ng/mL TGF-β treatment or with a co-treatment of the TGF-β inhibitor SD 208.

Using GAPDH expression levels to compare 2D and 3D IL-6 protein expression levels

We were interested in whether the large differences seen in IL-6 secretion between 2D and 3D cultures was due to a difference in the number of cells or a real difference in the expression levels. Directly comparing protein expression levels between cultures grown in 2D and 3D can be challenging. The main reason is that although the same numbers of cells are plated initially for an assay, the growth rates may be very different, and can lead to a large difference in the number of cells used in the final assay. Therefore, if the goal is to directly compare expression levels between cells grown in 2D and 3D, the data will need to be normalized to cell number. This can be done using trypan blue staining to count the number of cells; however, accurately counting a low number of cells in a spheroid using this method is not trivial. Therefore, we developed a normalization method using an AlphaLISA *SureFire Ultra* Total GAPDH Assay (ALSU-TGAPD-A500). Figure 10 shows the difference in GAPDH levels in cells grown in 2D and 3D for various numbers of DU145 cells plated and then measured after 72 hours. The DU145 cells grown in 3D have approximately four-fold less GAPDH than those grown in 2D, suggesting that the DU145 cells grow slower in 3D than 2D.

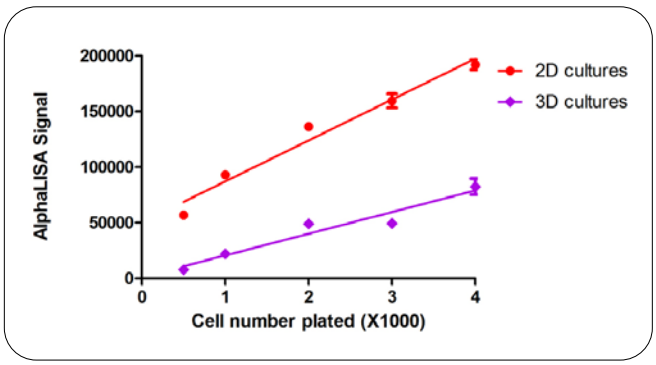


Figure 10: DU145 cells were plated in 2D and 3D cultures at 5 concentrations and GAPDH levels were assessed in lysates using the AlphaLISA *SureFire Ultra* Total GAPDH Assay Kit.

The IL-6 protein levels secreted into the supernatant were measured at various time points after cells were plated in either 2D or 3D cultures. Since GAPDH is not secreted, the GAPDH levels were measured from lysates and used to normalize the data over time. Figure 11A shows the AlphaLISA signal from IL-6 protein in the supernatants over time.

At 3.5 hours, the levels are similar between the 2D and 3D cultures. However, over time the levels of IL-6 secreted into the supernatant from 2D cultures increase rapidly while the levels from 3D cultures remain low. Figure 11B shows the GAPDH levels measured in the lysates between 20 and 50 hours, which do not change significantly. Figure 11C shows the normalized IL-6 expression levels over time. The data show that there are real, large differences in the amount of IL-6 secreted in the supernatants that cannot be explained just by differences in the cell numbers.

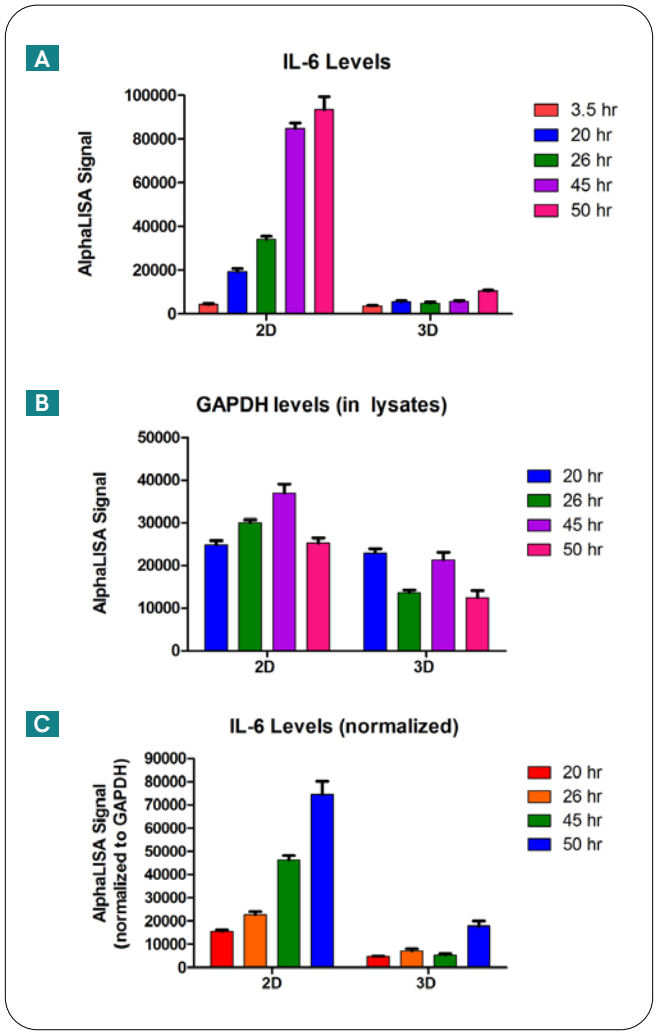


Figure 11: 4000 cells/well of DU145 cells were plated in either 2D or 3D cultures and aliquots of supernatants were taken over time. A) AlphaLISA data showing increases in IL-6 secretion over time between 2D and 3D cultures. B) GAPDH levels in lysates from 2D and 3D cultures. C) IL-6 levels normalized to GAPDH levels over time.

Conclusions

In this application note, we confirmed that treatment of DU145 cells with TGF-β is sufficient for inducing changes in both EMT biomarker expression and cellular morphology in monolayer cultures. AlphaLISA biomarker assays were also used to measure ECM-associated protein modulation caused by human transforming growth factor-beta (TGF-β) in a 3D spheroid model of human prostate carcinoma. E-cadherin is downregulated by TGF-β in both 2D and 3D lysates, whereas fibronectin is increased significantly only in 2D monolayer cultures. Monolayer cultures proliferate considerably more than cells in spheroid cultures as evidenced by the amount of GAPDH measured. TGF-β treatment induced increases in IL-6 secretion levels in both 2D and 3D cultures. However, the basal levels of IL-6 are significantly different between 2D and 3D cultures. We showed that AlphaLISA can be used to quantify and compare levels of protein expression from cultures grown in 2D and 3D. We used an AlphaLISA *SureFire Ultra* Total GAPDH Assay to normalize IL-6 protein expression levels from the same cells grown in monolayer or in a spheroid. In addition, the data presented here further illustrate the differences in protein expression levels and in cellular tolerance for treatment between a human prostate cell line grown in monolayers versus 3D spheroids as measured by AlphaLISA.

References

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