APPLICATION NOTE

AlphaLISA Technology

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Detection and Modulation of Extracellular Matrix Protein Deregulation in Human Prostate Carcinoma Cells Using AlphaLISA Technology

Introduction

Cancer still remains the second leading cause of the death in the United States despite recent advances in detection and treatment. The most deadly forms of cancer are metastatic, where cells from primary tumor sites migrate through the lymphatic system to secondary locations. While not all of the underlying mechanisms that cause cancer to metastasize are understood, one of the more crucial events that occurs is the epithelial to mesenchymal transition (EMT) which enables tumor cells to become motile and invade other tissues. EMT is molecularly characterized by rearrangement of the extracellular matrix (ECM) due to downregulation of the epithelial adhesion protein E-cadherin and upregulation of the mesenchymal adhesion proteins such as vimentin, N-cadherin, and fibronectin. This shift in protein expression accompanies further downstream modulation of cytokines, proteinases, and kinases as well as phenotypic changes in cell morphology. New drugs that are able to block the earliest stages of metastases, such as EMT, are crucial in slowing cancer progression and preventing fatalities. Here, we show that AlphaLISA® technology can be used to measure ECM-associated protein modulation caused by human transforming growth factor-beta (TGF-β) induction of EMT using a human prostate carcinoma cell model.

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. AlphaLISA assays can easily be automated and miniaturized to increase assay throughput.

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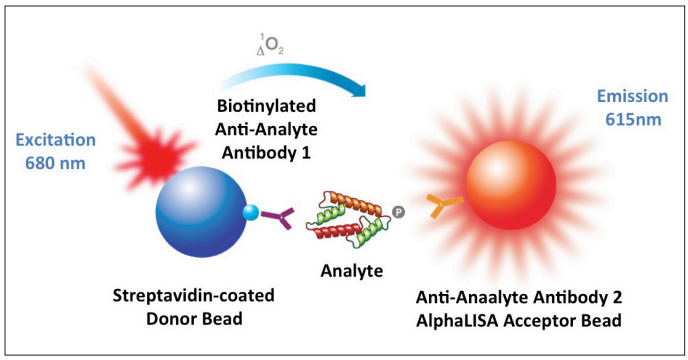

Figure 1. AlphaLISA assay schematic.

Materials and Methods

Cell-based Experiments

DU 145 cells (ATCC® HTB-81™) were cultured in EMEM (ATCC®30-2003™) supplemented with 10% FBS (10437-028, Thermo Fisher) and handled according to ATCC guidelines. For cell-based assays, 200 µL of cells were seeded at the stated cell density in a black, clear bottom PerkinElmer 96-well CellCarrier™ microplate (#6005550) in EMEM with 10% FBS and allowed to adhere for at least 18 hours overnight at 37°C in 5% CO^2. Media was then removed and replaced with 200 µL of EMEM lacking FBS for 24 hours. Treatment with simvastatin (Sigma, #S6196), SD 208 (Sigma, #S7071), LY294002 (Tocris, #1130) and/or TGF-β (BioLegend, 580702) was performed in 50 µL of EMEM lacking FBS. All inhibitor compounds were diluted in 100% DMSO before adding to EMEM lacking FBS. Inhibitor treatment (2.5 µM), duration was two hours followed by 48 hours with TGF-β (5 ng/mL) at 37°C, 5% CO_2 in a total volume of 100 µL. Cells were imaged 48 hours post-TGF-β treatment. After imaging, cell supernatant was transferred to a PerkinElmer 96-well StorPlate (#6008290) and either tested immediately in AlphaLISA assays or frozen at -80°C for further testing. Cells were then lysed with 50 µL of 1X lysis buffer (PerkinElmer, #AL003C) supplemented with protease inhibitors (ThermoFisher, #87786) and phosphatase inhibitors (Roche, #04906845001) for 10 minutes with gentle shaking. Lysates were either tested immediately in AlphaLISA assays or frozen at -80°C for later testing.

Detection of E-cadherin, Fibronectin, MMP-9, and IL-6

AlphaLISA immunoassays for E-cadherin (#AL370C), fibronectin (#AL351C), MMP-9 (#AL243C), and human IL-6 (#AL223C) were performed according to the recommended protocols provided in each kit manual. For MMP-9 and IL-6 assays 5 µL of each supernatant sample was added to a 384-well white OptiPlate followed by a mixture AlphaLISA acceptor beads and biotinylated antibody. After one hour of incubation, streptavidin donor beads were added for 30 minutes prior to reading Alpha signal. For fibronectin assays, 5 µL of each lysate or supernatant sample was added to a 384-well white OptiPlate followed by a mixture AlphaLISA acceptor beads and biotinylated antibody. After one hour of incubation, streptavidin donor beads were added for 30 minutes prior to reading Alpha signal. For E-cadherin detection, 5 µL of each lysate or supernatant sample was pre-incubated with AlphaLISA acceptor beads for 30 minutes prior to the addition of biotinylated antibody. After one hour of incubation, streptavidin donor beads were added for 30 minutes prior to reading Alpha signal. TopSeal-A (PerkinElmer, #6050185) was used to seal the assay plates and prevent evaporation during incubations.

Detection of Phosphorylated and Total AKT Proteins

The Alpha *SureFire® Ultra™* Multiplex assay kits provide the dual measurement of a phosphoprotein from cells, combined with the measurement of either the total amount of the same protein or of another phosphorylation event. A schematic of the Alpha *SureFire Ultra* Multiplex Phospho and Total assay format is shown in Figure 2. The Alpha 615 acceptor bead is directly-conjugated with an antibody to the phosphorylated site on the target protein. The Alpha 545 acceptor bead is coated with the Capt*Sure*™ agent, which binds the Capt*Sure*-tagged anti-total target protein antibody. The Alpha donor bead binds the biotinylated anti-total target protein antibody. Assay signal is measured in both the 615 nm and 545 nm channels.

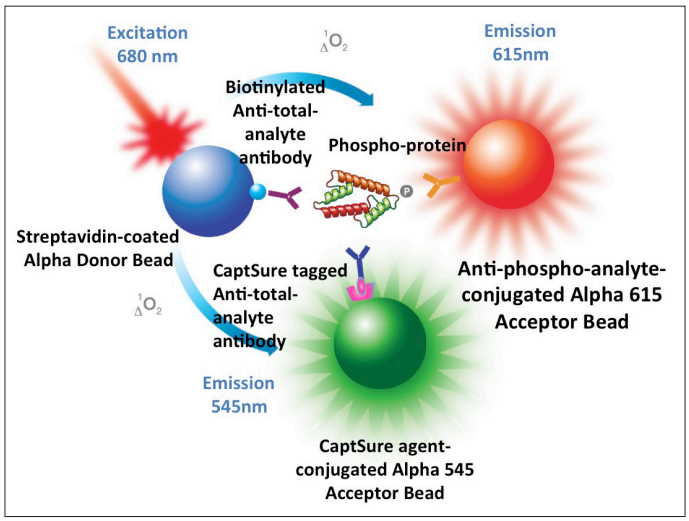

Figure 2. Alpha SureFire Ultra Multiplex assay schematic.

To measure phospho-AKT (Ser473) (pAKT (Ser473)) and total AKT protein, we used the Alpha *SureFire Ultra* Multiplex kit (#MPSU-PTAKT-K500). Cell lysate (10 µL) was transferred to a 384-well white OptiPlate followed by the addition of 5 µL of the acceptor mix (i.e., Alpha615 and Alpha545 acceptor beads together with Activation Buffer and the biotinylated and Capt*Sure*-labeled antibodies diluted as per the kit's protocol) for a 1-hour incubation. Donor mix (5 µL of streptavidin donor beads diluted 1:50 in Dilution Buffer as per the kit's protocol) was added to each well for an additional hour incubation.

Instrumentation

All Alpha assays were measured on a standard Alpha EnVision™- 2104 multimode plate reader equipped with two detectors. Alpha *SureFire Ultra* Multiplex assays were read in simultaneous mode with dual emission reads of terbium and europium. Brightfield imaging was performed on the EnSight™ multimode plate reader.

Standard Curve and Data Analysis

Standard curves for each AlphaLISA immunoassay were performed in the same diluent as the samples being tested (1X lysis buffer with supplements, or EMEM) using the recombinant standards provided in each kit. Curves were plotted in GraphPad Prism version 5.0 according to a nonlinear regression using the

4-parameter logistic equation (sigmoidal dose-response curve with variable slope) and $1/Y^2$ data weighting. Assay lower detection limits (LDL) were calculated by taking three times the standard deviation of the average of the background and interpolating off of the standard curve. Quantitation of protein levels in cellular assays were interpolated off their respective standard curves in this same manner. Interpolated analyte concentrations represent the amount of protein in 5 µL of sample. Alpha *SureFire Ultra* Multiplex data was corrected for spectral overlap before plotting. All data shown is the average of at least triplicate measurements.

Results

Inducing EMT with TGF-β

Human recombinant transforming growth factor-β (TGF-β) is a well-established inducer of EMT[1,2]. Brightfield images of DU 145 cells with or without TGF-β treatment are shown in Figure 3. Without treatment, the cells appear polygonal and epithelial-like (3A) while in the presence of 5 ng/mL TGF-β they appear elongated and fibroblastic (3B), indicating that the cells have undergone EMT.

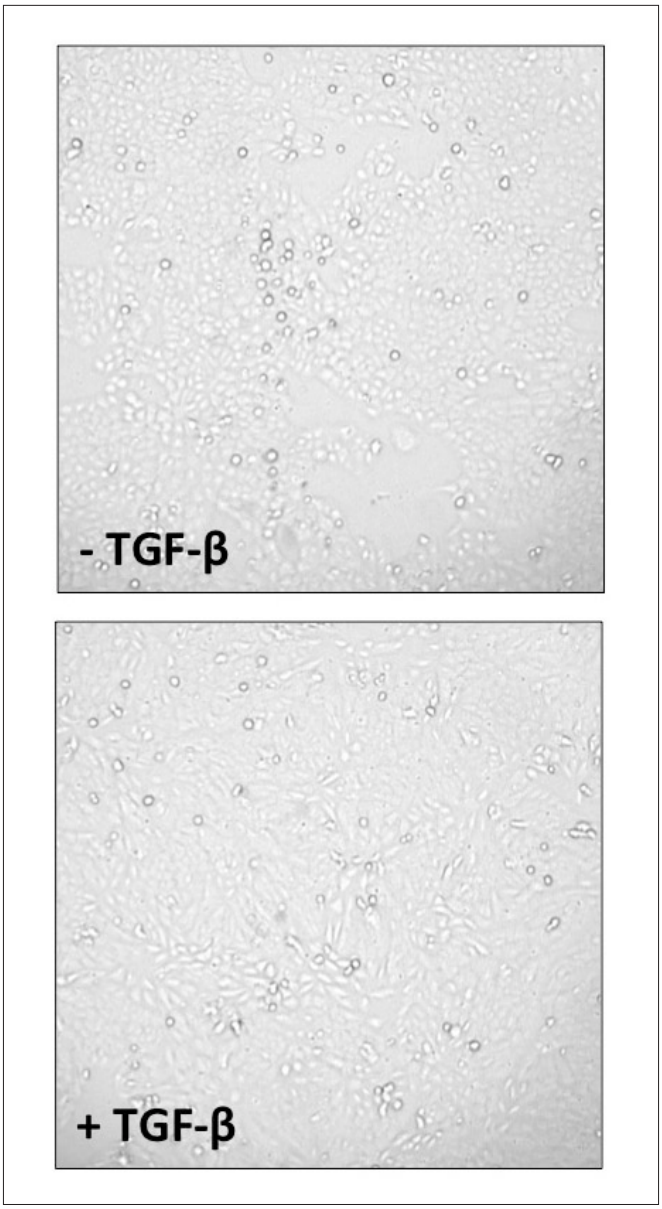

Figure 3. Brightfield images of DU 145 cells 48 hours post-treatment with or without TGF-β. Representative images for 6000 cells/well shown.

TGF-β Downregulates E-Cadherin and Upregulates Fibronectin

In order to determine the level of ECM protein modulation after EMT induction, AlphaLISA assays were used to quantitate E-cadherin and fibronectin levels in both cell lysates and cell supernatants. As shown in Figures 4 and 5, quantities of both E-cadherin and fibronectin increase with increasing cell number overall. In the presence of TGF-β, levels of E-cadherin are reduced, while levels of fibronectin are increased in both the lysates and supernatants tested, consistent with EMT progression.

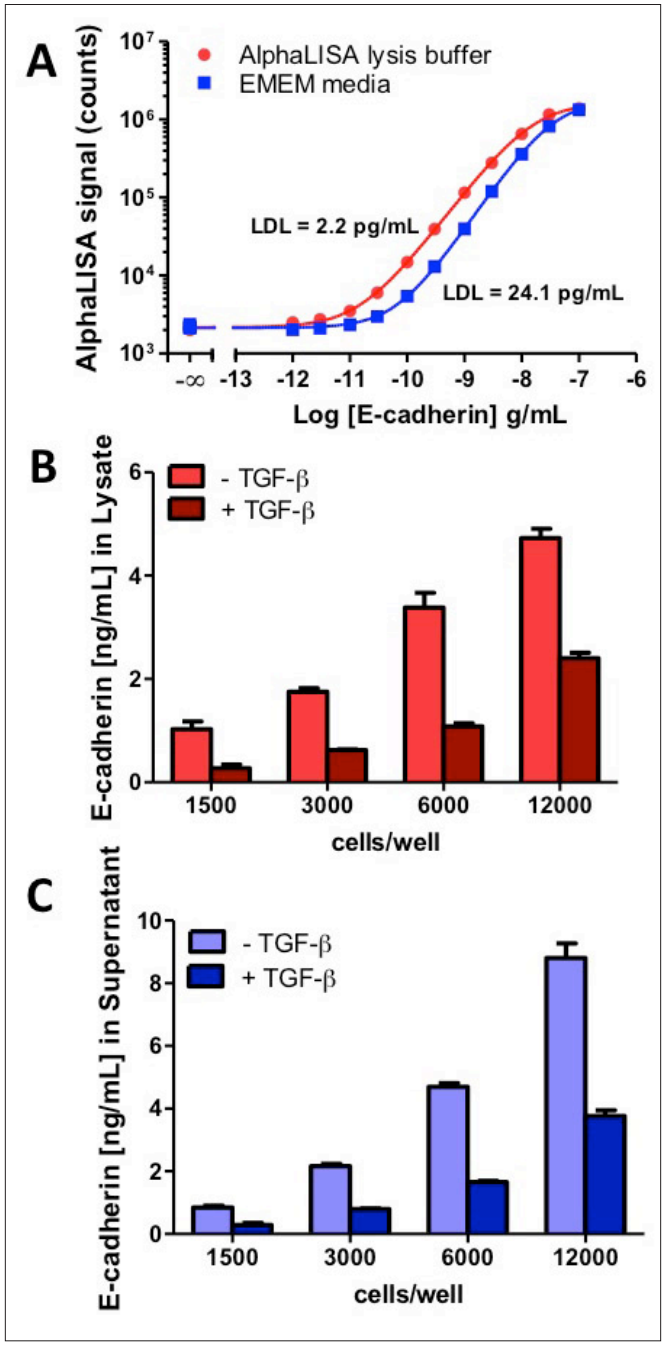

Figure 4. E-cadherin detection using AlphaLISA. A) Standard curves generated in lysis buffer and EMEM. B) Interpolated concentrations of E-cadherin in DU 145 lysates. C) Interpolated concentrations of E-cadherin in DU 145 supernatants.

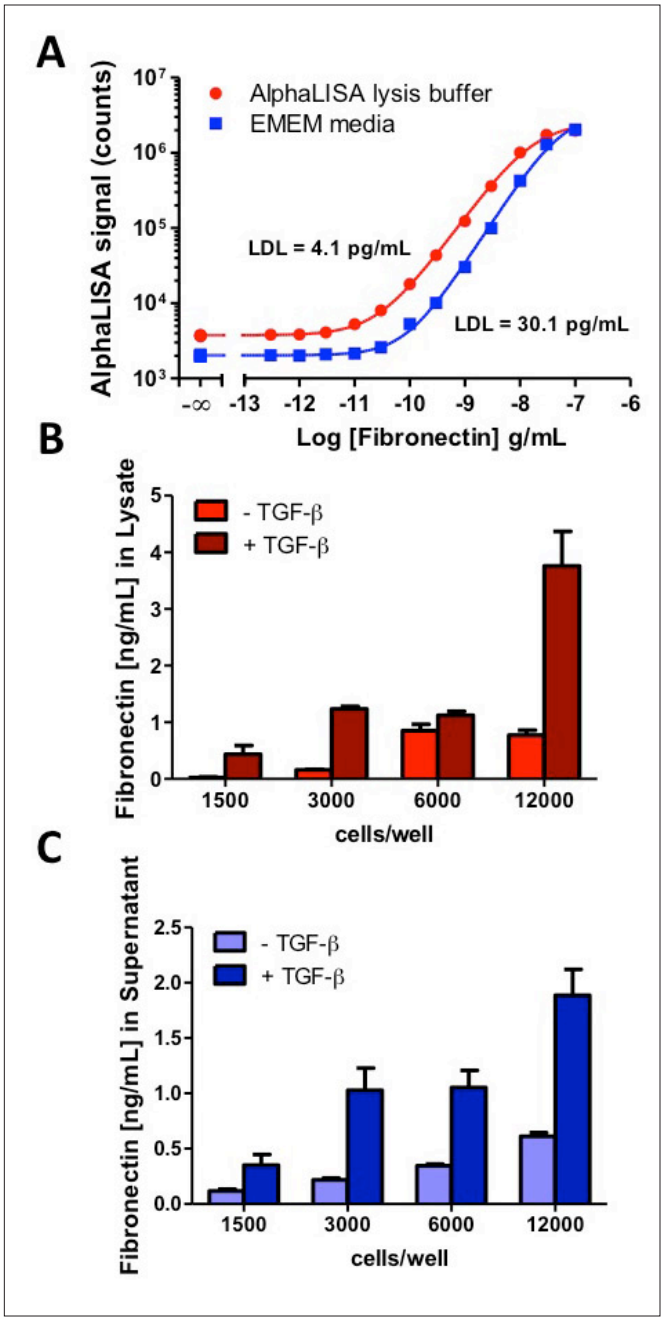

Figure 5. Fibronectin detection using AlphaLISA. A) Standard curves generated in lysis buffer and EMEM. B) Interpolated concentrations of Fibronectin in DU 145 lysates. C) Interpolated concentrations of fibronectin in DU 145 supernatants.

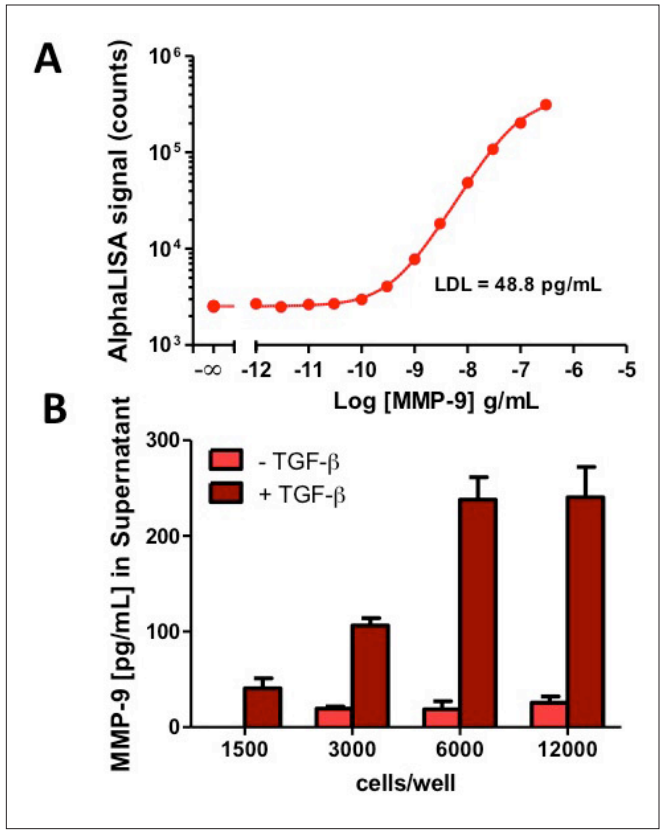

Figure 6. MMP-9 detection using AlphaLISA. A) Standard curve generated in EMEM. B) Interpolated concentrations of MMP-9 in DU 145 supernatants 48 hours post-TGF-β treatment.

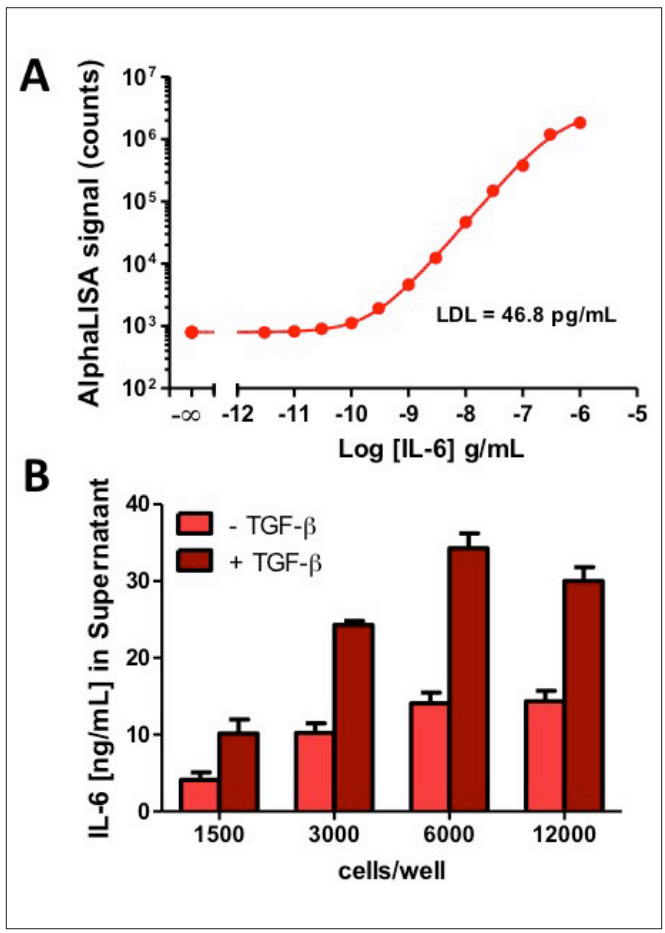

Figure 7. IL-6 using AlphaLISA. A) Standard curve generated in EMEM. B) Interpolated concentrations of IL-6 in DU 145 supernatants 48 hours post-TGF-β treatment.

TGF-β Upregulates MMP-9 and IL-6 Secretion

Matrix metalloproteinase-9 (MMP-9) plays an important role in ECM remodeling and has been implicated in tumor metastasis, while interleukein-6 (IL-6) is a pro-inflammatory cytokine associated with cancer progression. In order to confirm that these proteins are modulated during EMT, secretion levels of MMP-9 and IL-6 were measured in cell supernatants using AlphaLISA assays. Results are shown in Figures 6 and 7. The induction of EMT increased secretion of both MMP-9 and IL-6 in the TGF-β-treated cells.

TGF-β Upregulates Phosphorylation of AKT

TGF-β signaling is mediated through a vast array of signaling pathways, including the phosphoinositide 3-kinase-AKT-mTOR pathway[3]. Total AKT and pAkt (Ser473) levels were measured

using Alpha *SureFire Ultra* Multiplex assays. Multiplexing allows for the simultaneous detection of both phosphorylated and total AKT proteins in the same well and allows for normalization of the Alpha signal of pAkt (Ser473) to the signal of total AKT, which can reduce variability from well-to-well. Results demonstrate increasing levels of phosphorylated (Figure 8A) and total AKT (Figure 8B) with increasing cell number. Treatment with TGF-β increases levels of pAkt (Ser473), while levels of total AKT were not affected by TGF-β (Figure 8C).

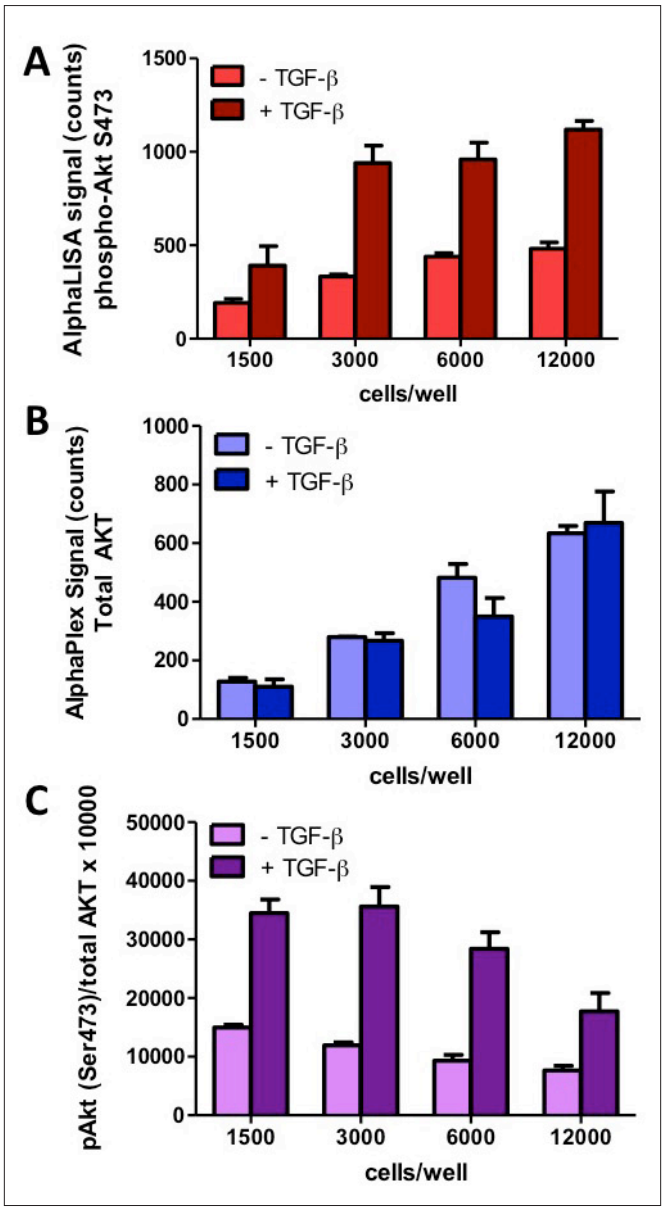

Figure 8. pAkt (Ser473) and total AKT detection using Alpha *SureFire Ultra* Multiplex. A) Alpha 615 signal (pAkt (Ser473)) of DU 145 cell lysates with or without TGF-β treatment. B) Alpha 545 signal (total AKT) of DU 145 cell lysates with or without TGF-β treatment. C) Normalized levels of pAkt (Ser473) (Alpha 615) to total AKT (Alpha 545).

Blocking TGF-β induced EMT

Three different compounds were tested for their ability to block TGF-β induced EMT. Simvastatin (HMG-CoA reductase inhibitor), SD 208 (TGF-βR1 inhibitor), and LY294002 (inhibitor of phosphoinositide 3-kinase, PI 3-kinase) were pre-incubated with cells prior to treatment with TGF-β. Brightfield images of the three different inhibitor treatments are shown in Figure 9. Strikingly, the SD 208-treated cells resemble untreated cells (-TGF-β, Figure 3) of the epithelial phenotype, while the simvastatin- and LY294002-treated cells more closely resemble the mesenchymal phenotype (+ TGF-β, Figure 3), though there are subtle differences in the morphology. It also appears that the simvastatin-treated cells have experienced some toxicity compared to the SD 208- and LY294002-treated cells.

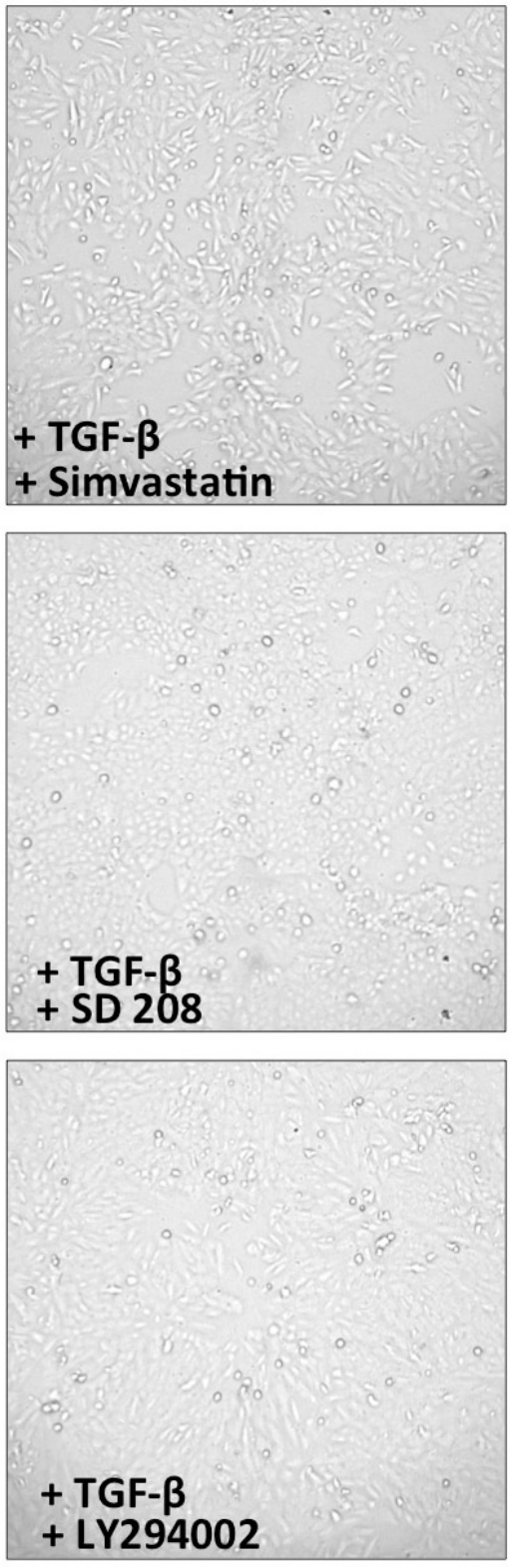

Figure 9. Brightfield images of DU 145 pre-incubated for 2 hours with simvastatin, SD 208, or LY294002 prior to TGF-β treatment. Representative images for 6000 cells/well shown.

Inhibiting TGF-β Modulation of E-cadherin and Fibronectin

E-cadherin and fibronectin concentrations were measured in cells co-treated with TGF-β and inhibitors (Figure 10). SD 208-treated cells had levels of E-cadherin and fibronectin that were similar to the untreated (-TGF-β) cells, indicating this compound blocks TGF-β induced changes in these extracellular matrix components. Conversely, simvastatin and LY294002 had levels of E-cadherin and fibronectin similar to TGF-β-treated cells, indicating these compounds did not block the action of TGF-β.

Inhibiting TGF-β Induced MMP-9 and IL-6 Secretion

Cells treated with SD 208 secreted levels of MMP-9 and IL-6 similar to cells untreated with TGF-β, further confirming that this compound blocks TGF-β induced EMT (Figure 11).

LY294002-treated cells also secreted lower levels of MMP-9 and IL-6 compared to the TGF-β-treated cells, indicating that this compound reduces the inflammatory response. Simvastatin did not reduce the levels of secreted MMP-9 or IL-6.

Inhibiting TGF-β Induced AKT Phosphorylation

Relative levels of pAkt (Ser473) and total AKT were measured on inhibitor-treated cells and compared to cells treated with or without TGF-β (Figure 12). While the amount of total AKT did vary between each of the three inhibitors and with TGF-β treatment, when phosphorylated levels were normalized to total AKT levels for each condition tested, both simvastatin and LY294002 reduced the levels of pAkt (Ser473) to the levels of the untreated

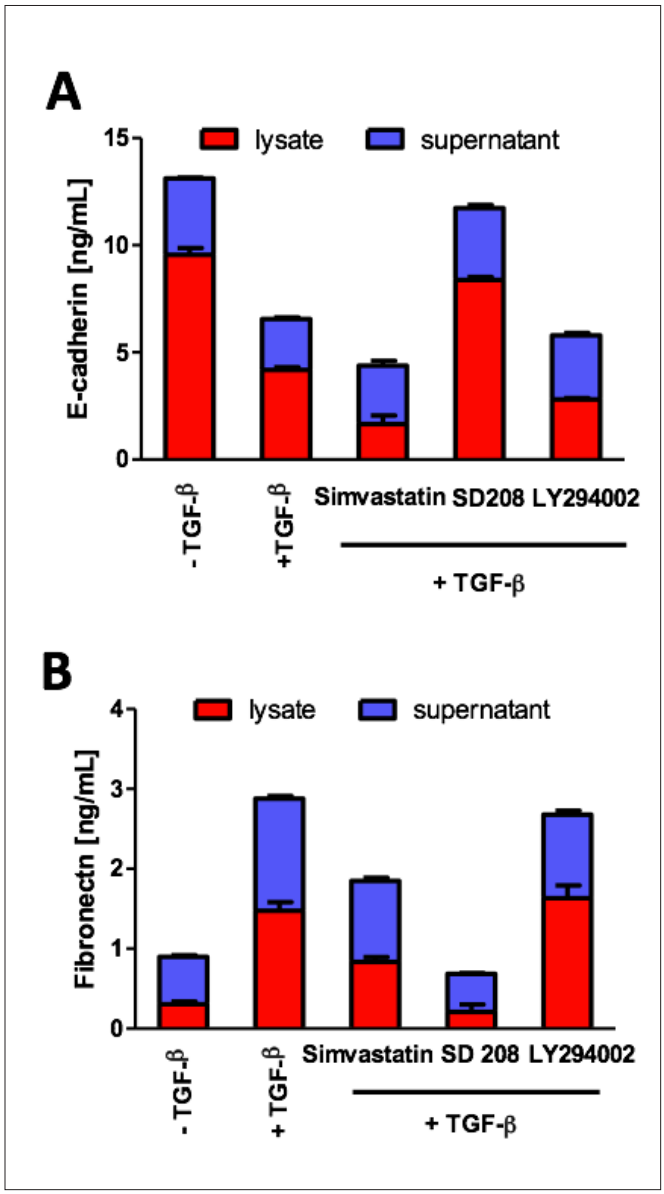

Figure 10. Interpolated concentrations of E-cadherin (A) and fibronectin (B) in lysates and supernatants of DU 145 cells (6000 cells/well) pre-treated with inhibitor prior to TGF-β treatment.

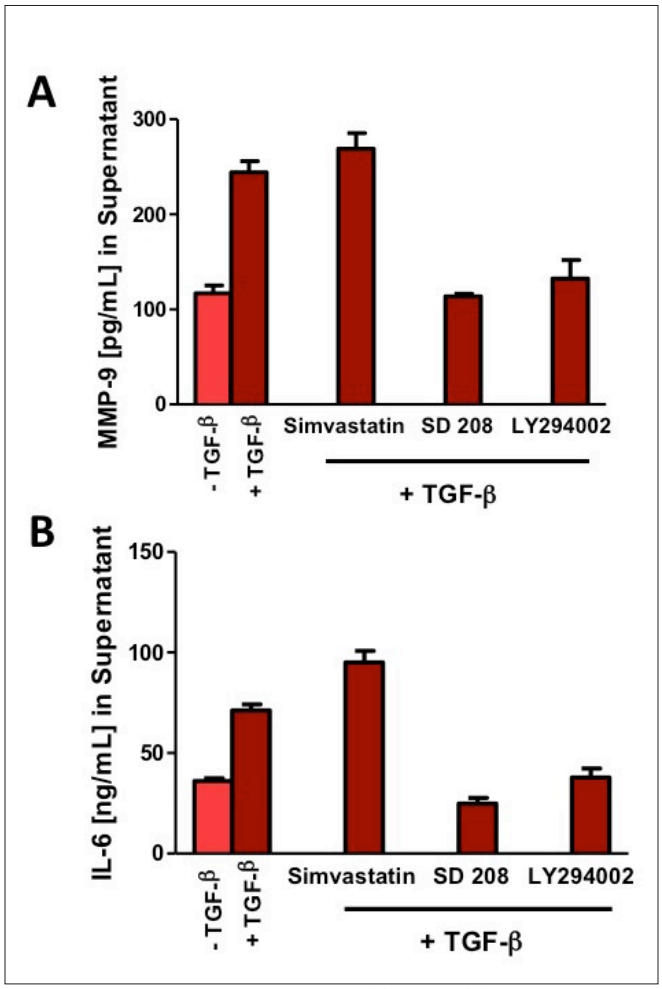

Figure 11. Interpolated concentrations of MMP-9 (A) and IL-6 (B) in supernatants of DU 145 cells (6000 cells/well) pre-treated with inhibitor prior to TGF-β treatment.

cells, while the SD 208-treated cells had levels similar to the TGF-β-treated cells. This indicates that although simvastatin and LY294002 do not block TGF-β induced EMT, they do inhibit TGF-β induced phosphorylation of AKT. While SD 208 does appear to block TGF-β induced EMT, it does not do so in a manner that affects PI 3-kinase activity.

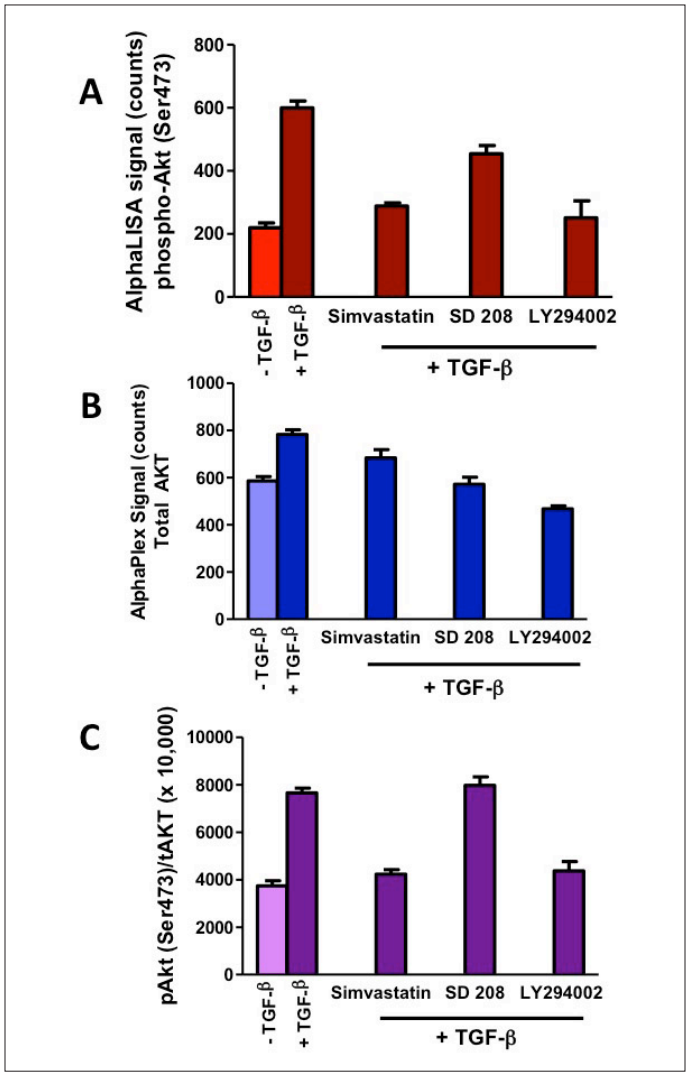

Figure 12. pAKT (Ser473) and total AKT measurement of lysates of DU 145 cells (6000 cells/well) treated with inhibitor A) Alpha 615 (phosphorylated AKT) B) Alpha 545 (total AKT), and C) Normalized levels of pAkt (Ser473) (Alpha 615) to total AKT (Alpha 545) for each condition tested.

Conclusions

In this application note we were able to confirm that TGF-β induced EMT in DU 145 cells by examining changes in both cell morphology using the EnSight multimode plate reader and protein expression using AlphaLISA. Alpha assays provide robust and reproducible data, as samples were tested across multiple days in multiple assays and generated correlating results. We were able to block EMT using SD 208, confirming published results[4]. Interestingly, we did not observe simvastatin-mediated inhibition of TGF-β induced EMT as measured by others[5], though in those studies higher concentrations of inhibitor were used. During the experiments conducted here, higher concentrations of simvastatin were tested but resulted in significant cell toxicity. Both simvastatin and LY294002 reduced PI 3-kinase activation caused by TGF-β and LY294002 also reduced TGF-β induced inflammation.

These data combined demonstrate how Alpha technology can be used to measure the modulation of extracellular matrix protein deregulation and can help elucidate inhibitory actions of potential therapeutic compounds on cancer biology models. PerkinElmer technologies allows for orthogonal measurements of compound modulation by providing the user with both a phenotypic assay readout using cellular imaging and a quantitative assay readout using AlphaLISA.

References

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