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

#5793

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1 Introduction

Epithelial-mesenchymal transition (EMT) is characterized by rearrangement of the extracellular matrix (ECM) and differential regulation of ECM proteins. We induced EMT in DU 145 cells using TGF-β and phorbol-12-myristate-13-acetate (PMA) and compared expression levels of specific biomarkers, including E-cadherin, fibronectin, and IL-6, using AlphaLISA® and LANCE® (TR-FRET) assay technologies. 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 TGFβ 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 observe 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 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.

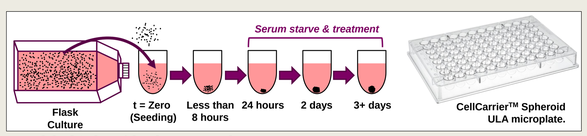
2 Materials and Methods

Cell culture and treatment: DU 145 cells (ATCC® HTB-81™) were seeded (100 µL/well) into PerkinElmer 96-well CellCarrier™ (6005550) or CellCarrier Spheroid ULA 96-well microplate (3D) (6055330) and grown for at least 18 hours (see spheroid growth illustration below). Cells were serum starved for 24 hours prior to treatment with recombinant human TGF-β1 (BioLegend, 580702) or PMA (Sigma cat# P1585) 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.

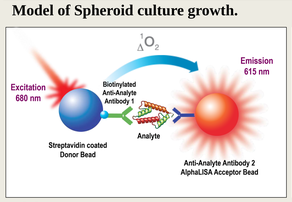
Biomarker quantification assays: For biomarker detection assays, 50 µL of media was removed (for testing supernatants). Cells were then lysed with 50 µL of 2X Alpha *SureFire® Ultra* lysis buffer (ALSU-LB-10mL) for 10 minutes. Lysates and supernatants were frozen at -80°C and later thawed for testing in AlphaLISA and LANCE assays (see models for each assay type below). 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 Fibronectin **LANCE *Ultra* TR-FRET assay** (TRF1351C), 15 µl of lysate was added to a 384-well white OptiPlate and assays were performed according to the manual. AlphaLISA and LANCE assays were measured on a standard EnVision® multilabel plate reader using standard Alpha Settings and reading TR-FRET with the Laser excitation.

ATPlite™ 1step and ATPlite™ 1step 3D assays: Cellular proliferation, growth & vitality were measured by assessing the concentration of ATP using ATPlite 1step and ATPlite 1step 3D luminescence-based assays following kit protocols. Luminescence was measured using the EnSight™ multimode plate reader.

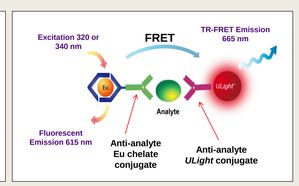
Cellular imaging: For cellular imaging, cultures were first labeled with Hoechst 33342 (Life Technologies, #H3570), Tetramethylrhodamine (TMRM; Life Technologies, #T-668) and CellTox Green (Promega, #G8742). Monolayer cultures were imaged using the cellular imaging module of the EnSight plate reader using Brightfield and UV fluorescence filters and cell numbers were quantified using the Count Nuclei function. 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).



Model of Spheroid culture growth.

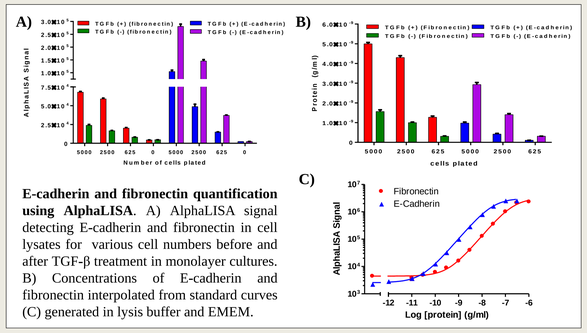


AlphaLISA assay



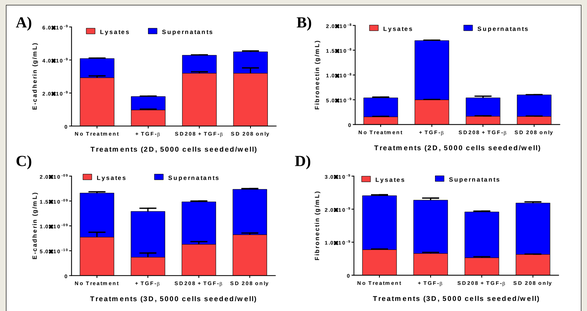
LANCE TR-FRET assay

3 E-cadherin and Fibronectin modulated by TGF-β measured with AlphaLISA in monolayer cultures



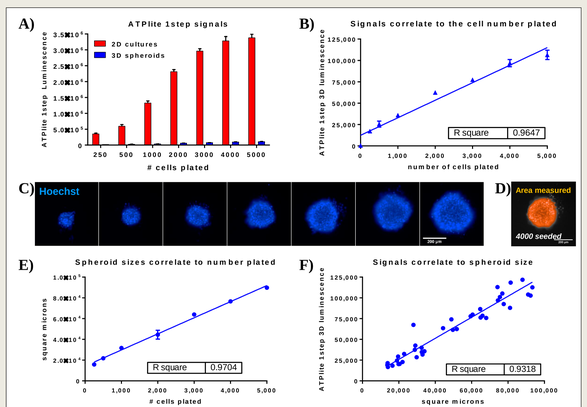
E-cadherin and fibronectin quantification using AlphaLISA. A) AlphaLISA signal detecting E-cadherin and fibronectin in cell lysates for various cell numbers before and after TGF-β treatment in monolayer cultures. B) Concentrations of E-cadherin and fibronectin interpolated from standard curves (C) generated in lysis buffer and EMEM.

4 Modulation of E-cadherin & Fibronectin Levels in 2D and 3D cultures



E-cadherin (A, C) and fibronectin (B, D) levels in supernatants and lysates from 2D (A, B) and 3D (C, D) cultures before and after treatment with TGF-β and/or TGF-β inhibitor SD 208.

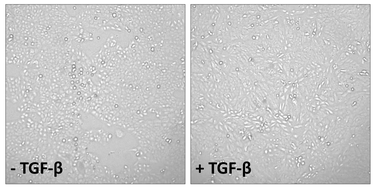
5 Assessing cell proliferation in 2D & 3D

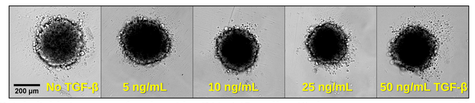


Monolayer (2D) cultures proliferate much more than spheroid (3D) cultures. 2D and 3D cultures were plated at 7 concentrations, grown for 4 days and assessed with ATPlite 1step and ATPlite 1step 3D assays (with the final step being to transfer 50 µL of the reaction to a gray OptiPlate for reading luminescence on the EnSight (n=3). 3D cultures were labeled with Hoechst, spheres were imaged on the Operetta (C), and cross-sectional spheroid area measured using an intensity cutoff (D). Spheroid area correlates to number plated (E) and luminescence signals (F).

6 TGF-β effects on morphology

Brightfield images of DU 145 cells 48 hours post-treatment with or without 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 shown (right).

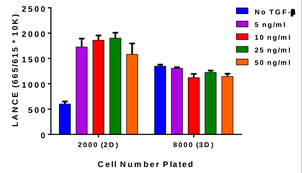




Brightfield images of spheroids DU 145 cells 48 hours post-treatment with increasing concentrations of TGF-β on the Operetta High Content Imaging system.

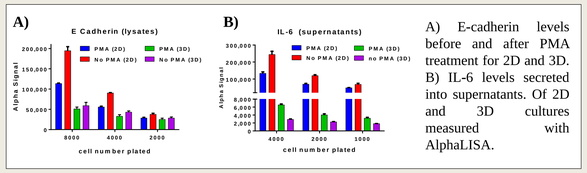
7 Examining TGF-β effects on Fibronectin expression with LANCE TR-FRET

To see if 3D spheroids and more cells require more TGF-β and larger cell numbers to illicit a response, 10, 25, and 50 ng/mL were tested. Higher concentrations did not increase fibronectin levels further in 2D and did not affect levels at all in 3D. To account for differences in proliferation over time between 2D and 3D, we plated 4X higher number of cells for 3D (8000/well) than for 2D (2000/well).



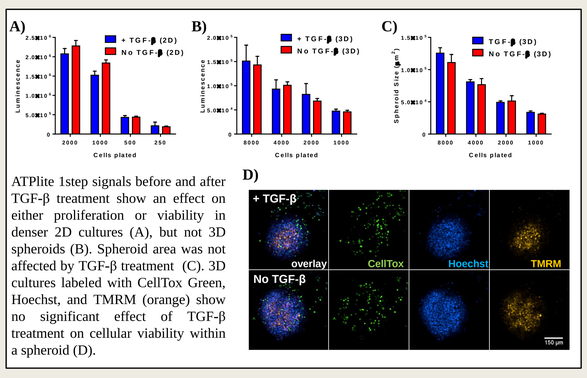
8 PMA -induced changes in E-cadherin and IL-6 expression

PMA has been shown to induce EMT also. Here, we show E-cadherin levels are decreased in 2D and less so in 3D after treatment with 150 nM PMA. Interestingly, the IL-6 secretion levels increase for 2D and decrease for 3D after PMA treatment.



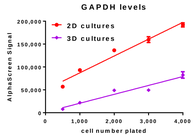
A) E-cadherin levels before and after PMA treatment for 2D and 3D. B) IL-6 levels secreted into supernatants. Of 2D and 3D cultures measured with AlphaLISA.

9 Affects of TGF-β on cell viability



ATPlite 1step signals before and after TGF-β treatment show an effect on either proliferation or viability in denser 2D cultures (A), but not 3D spheroids (B). Spheroid area was not affected by TGF-β treatment (C). 3D cultures labeled with CellTox Green, Hoechst, and TMRM (orange) show no significant effect of TGF-β treatment on cellular viability within a spheroid (D).

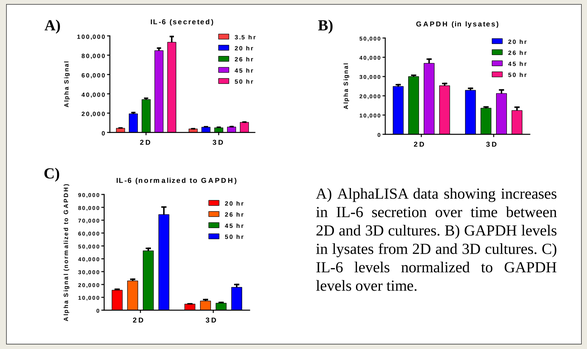
10 GAPDH levels may be used to correlate cell number differences between 2D and 3D over time



DU 145 cells were plated in 2D and 3D cultures at 5 concentrations and GAPDH levels were assessed in lysates using the AlphaScreen *SureFire* GAPDH kit (cat# TGRGDS500). As shown here, the amount of GAPDH is ~4-fold lower in 3D cultures.

11 Changes in IL-6 levels over time between 2D and 3D

To assess the changes in IL-6 secretion over time, 4000 cells were plated in multiple wells in 2D and 3D. At each time point, supernatants were removed and cells lysed and aliquots were frozen at -20°C. AlphaLISA was used to measure IL-6 levels in the samples and GAPDH was used to normalize to potential differences in cell number due to differences in growth rates. The dramatic difference in IL-6 secretion over time in 2D compared to 3D cannot simply be explained by differences in cell numbers.



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.

12 Summary

- **We confirmed that treatment of DU 145 cells with TGF-β is sufficient for inducing changes in both EMT biomarker expression and cellular morphology in monolayer cultures.**
- **AlphaLISA and LANCE (TR-FRET) biomarker assays can be used to measure ECM-associated protein modulation caused by human transforming growth factor-beta (TGF-β) induction of EMT in a 3D Spheroid model of human prostate carcinoma.**
- **E-Cadherin is downregulated by TGF-β in both 2D and 3D cultures, wherease Fibronectin is increased significantly only in 2D monolayer cultures.**
- **Monolayer cultures proliferate considerably more than cells in spheroid cultures.**
- **PMA and TGF-β treatment induced significant differences in IL-6 secretion levels between 2D and 3D cultures.**
- **TGF-β affects proliferation or viability of cells in 2D cultures differently than in 3D cultures.**
- **These data 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.**

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