Comparison of Key Signaling Pathways in Lung Cancer Cells Grown in 2D and 3D Formats

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BACKGROUND

Lung cancer is the **leading cancer killer in the United States**. It is also being increasingly diagnosed in canines and felines. Two oncogenic pathways implicated in lung cancer are:

- Wnt/β-catenin pathway (Fig. 1)
- Bone morphogenic protein (BMP) signaling cascade (Fig. 2)

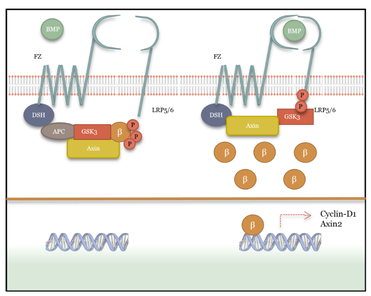


Figure 1: Wnt/B-catenin Pathway
In the absence of Wnt ligands, B-catenin is phosphorylated and subjected to degradation by the destruction complex (Axin, GSK-3, APC).
Upon binding of Wnt ligands, the destruction complex is destabilized and β-catenin is able to translocate to the nucleus where it can initiate transcription of Wnt targeting genes such as Cyclin-D1 and Axin.

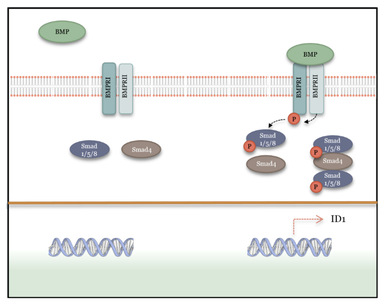


Figure 2: BMP Pathway
BMP ligands bind to BMP receptors leading to their dimerization and subsequent phosphorylation. Smad1/5/8 is further phosphorylated by the BMP receptors causing it to associate with Smad4. The complex then translocates into the nucleus for transcription of target genes such as ID1.

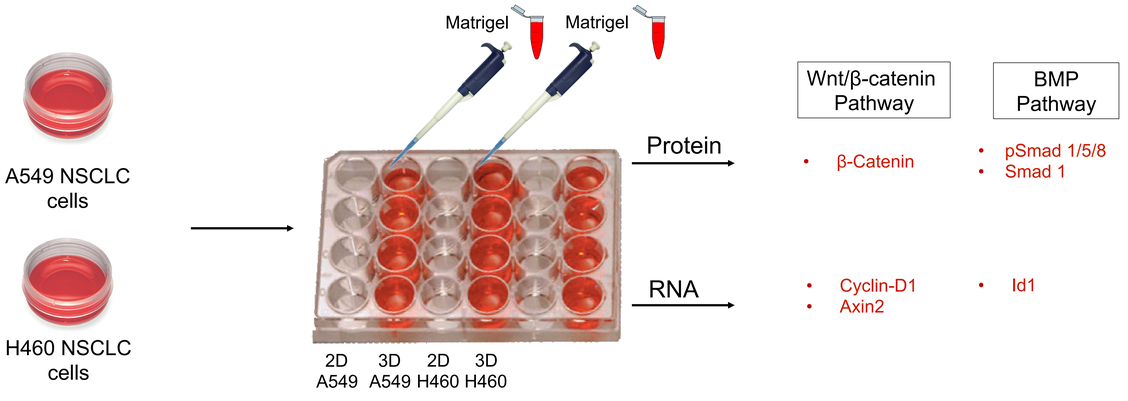
Increasing evidence shows that cancer cell cultures in the form of three-dimensional (3D) spheroids are more reflective of in-vivo studies than two-dimensional (2D) cell culture formats.

- 2D cell cultures are widely used but provide a poor model for in-vivo conditions and behaviors
- 3D cell cultures mimic the microenvironment of cells allowing for a more realistic cell behavior analysis

OBJECTIVE

To evaluate Wnt/β-catenin and BMP pathway protein and gene expression in NSCLC cell lines A549 and H460 in 2D and 3D cultures.

MATERIALS AND METHODS



A549 and H460 cells were cultured until confluence in 100mm conventional tissue culture plastic dishes in complete medium consisting of DMEM (Dulbecco's Modified Eagle Medium) supplemented with 10% FBS, 1% NEAA, and 1% penicillin/streptomycin antibiotics at 37°C in 5% CO2.
A549 and H460 cells were then seeded into 2D or 3D environments in a 24-well plate for 7 days in complete medium at 37°C in 5% CO2.

- 2D cells were seeded into uncoated wells at 40,000 cells/well.
- 3D cells were seeded into wells coated with a matrigel/media solution (1:1 ratio). Cells were seeded as a suspension in matrigel/media solution (1:50 ratio) at 40,000 cells/well.

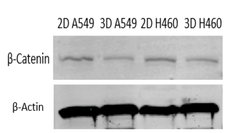
After 7 days protein and RNA were extracted

- Protein
  - Protein was prepared using RIPPA buffer supplemented with protease and phosphatase inhibitors.
  - Protein was analyzed using SDS-PAGE then transferred to PVDF membrane.
  - Blots were incubated overnight at 4°C with appropriate primary antibodies (β-catenin, pSmad 1/5/8, Smad1, α-tubulin, β-actin). Secondary antibodies were applied for 1 hour at room temperature
  - Bands were visualized using an Odyssey scanner and analyzed with Image Jay Ver.
- RNA:
  - Extracted via lysis buffer and purified by filtration following GeneJET RNA Purification Kit protocol.
  - cDNA was synthesized using SuperScript III kit.
  - RT-PCR reactions using cDNA as templates were carried out with Fast a SyberGreen Master Mix in triplicate.
  - Results between 2D and 3D gene expression was analyzed using double Delta Ct.

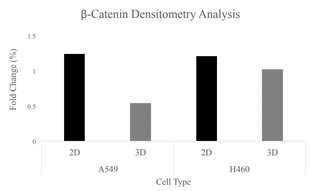
RESULTS

Wnt/β-Catenin Pathway

Cyclin-D1, a postulated target of the Wnt/ β-catenin pathway, had increased gene expression in 3D cultures (Fig.4) suggesting that our protein levels of β-catenin should also be increased. However our decreased β-catenin levels in 3D cultures (Fig.3) correlate with a recent finding by Sansom et al. that states that Cyclin-D1 may be important for tumor progression and not initiation. Our 3D cultures showed increased expression of Axin2 (Fig. 4) whose transcription is induced by β-catenin. These results do not correlate with our decreased β-catenin levels and should be investigated further.



(A) Protein expression of β-catenin in 2D and 3D cultures of A549 and H460 cells determined using western blot.



(B) Quantification of β-catenin was determined using ImageJ and normalized to β-actin.

Figure 3: Protein Expression

BMP Pathway

Despite several attempts, Smad1 and pSmad1/5/8 did not give consistent enough results to determine protein expression. BMP pathway gene expression showed increased expression of ID1 in 3D cultures (Fig. 5). This supports previous findings that increased levels of ID1 in NSCLC cultures correlates with tumor metastasis and adhesion.

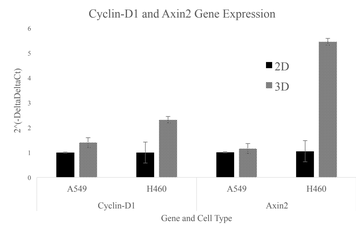


Figure 4: Gene Expression
2D and 3D culture results of Cyclin-D1 and Axin2 expression in A549 and H460 cells determined using RT-PCR.

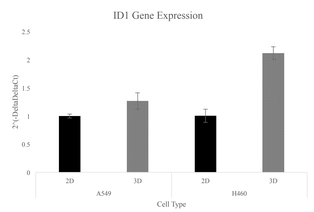
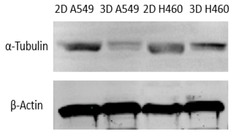


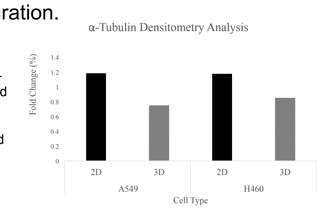
Figure 5: Gene Expression
2D and 3D culture results of ID1 expression in A549 and H460 cells determined using RT-PCR.

α-Tubulin

We had an unexpected finding that protein and gene expression of α-tubulin, a cytoskeleton protein that was used as our loading control, gave decreased levels in 3D cultures (Fig. 6 and Fig. 7). The decrease seen could potentially be due to acetylation of α-tubulin. Acetylated α-tubulin has been linked to adhesion and invasive migration.



(A) Protein expression of α-tubulin in 2D and 3D cultures of A549 and H460 cells determined using western blot.



(B) Quantification of α-tubulin was determined using ImageJ and normalized to β-actin.

Figure 6: Protein Expression

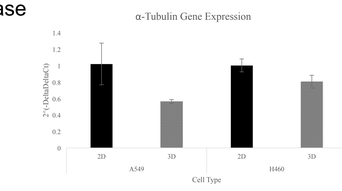


Figure 7: Gene Expression
2D and 3D culture results of a-tubulin expression in A549 and H460 cells determined using RT-PCR.

CONCLUSIONS

Overall our results based on protein and gene expression indicate that 3D cultures are more representative of adhesion and metastasis in NSCLC cells.

Gene expression of Axin2 did not correlate with expected results based on β-catenin levels. Further experimentation should be conducted to determine significance. We had an unexpected finding of α-tubulin having reduced protein and gene expression in 3D cultures. Further investigation should be conducted to determine if there was acetylation of α-tubulin.

Target	Protein/Gene	↑ or ↓ in 3D	Explanation
Wnt Pathway			
β-catenin	Protein	↓	Tumor initiation
Cyclin-D1	Gene	↑	Tumor progression
Axin2	Gene	↑	Further investigation needed
BMP Pathway			
ID1	Gene	↑	Tumor metastasis and adhesion in NSCLC
α-Tubulin			
α-tubulin	Gene and protein	↓	Acetylation leading to adhesion and invasive migration

BIBLIOGRAPHY AND ACKNOWLEDGEMENTS

Bibliography can be found at: **http://bit.ly/2akDGQ2**

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