A Biological Study of αv-integrin's Role in Tumorigenesis

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Intracellularly, integrins are in charge of mediating attachment between cells and their surrounding tissues, as well as performing both outside-in and inside-out cellular signaling. While regulation of these functions is essential to proper tissue development, dysregulation can lead to harmful abnormal growth and proliferation of cells. Studies suggest that the ablation of αv-integrin in basal epithelial cells has been shown to cause tumorigenesis similar to squamous cell carcinoma (SCC) (*McCarty et al.).* In other words, αv-integrin gene deletion leads to cellular transformation and often, metastasis. This insinuates that αv-integrin provides growth regulation and exhibits tumor repression-like functions, which further suggest that this specific integrin plays a role in the activation of Transforming Growth Factor-β (TGF-β), an essential part of the signaling cascade that contributes to the development of multiple diseases such as autoimmunity, heart disease, and most important to my research, cancer. This proposed signaling cascade begins with αv-integrin activation of TGF-β, which in turn phosphorylates SMAD, a protein that acts as a transcription factor for the regulation of certain genes. My research focused on studying this model by means of a transgene expressed knockout mouse model that studies the effects of a loss of αv-integrin and TGF-β by looking at mouse mammary tumors with and without the stated proteins. The cells used in proceeding experiments were grown from mammary specific MMTV-Polyoma middle T antigen transgenic mice crossed with α-v floxed mice and MMTV-cre. Control tumors were harvested and epithelial cells were grown *in vitro*. To generate av

null cells, control cells were treated with adenoviral C recombinase to remove the loxP sites that were on either end of the α-v integrin locus. A reporter construct was inserted into both cell genotypes, which provided a transcription response element that recognizes phosphorylated SMAD.

My main focus during my 9 weeks of research was studying the nature of αv-integrin and its affects on tumorigenisis by means of western blots. The western blot is an analytical technique that allows for the detection of certain proteins in a sample of tissue, and I used this method to detect phosphoSMAD, as well as Glyceraldehyde 3-phosphate dehydrogenase (GAPDH). While phosphoSMAD correlates to TGF-β levels in tissues, GAPDH is an enzyme that catalyses particular steps in glycolysis. Since glycolysis occurs in all cells, GAPDH should therefore be present in all cells, allowing for its use as an experimental control to quantify phosphoSMAD levels.

The western blot begins by loading equal amounts of sample onto a 15 well 4-15% tris glycine protein gel. These samples are divided into two categories: time-matched, where all samples arrived from tumors that were palpated after relatively the same number of days, and size-matched, where samples were from tumors of relatively the same size. Furthermore, each time-matched and size-matched sample grouping is further split down into αv-null and wildtype, providing two models in one experiment. Next, gel electrophoresis separates the proteins by polypeptide length, and the completed gel is transferred to a Polyvinylidene Fluoride (PVDF) membrane by an electrical current. The membrane is probed for detection of specific proteins – in this case, phosphoSMAD and GAPDH – by use of primary antibodies specific to that protein (anti-phosphoSMAD and anti-GAPDH) at concentrations of 1:10,000 for @SMAD

(Epitomics) and 1:8,000 for @GAPDH respectively in blocking solution (0.25g non-fat milk/5ml Phosphate Buffered Saline/50microliters Tween-20%/5microliters 10% SDS), and allowed to incubate overnight at 4 degrees. The next day, the membranes are probed once more with a light sensitive secondary antibody, either mouse anti-mouse, or mouse anti-rabbit at concentrations of 1:8,000 for both SMAD (mouse anti-rabbit) and GAPDH (mouse anti-mouse) in previously described blocking solution. These secondary antibodies result in the fluorescence of the proteins, allowing for colorimetric detection on a scan. We are looking for results that indicate a specific differentiation pattern between null and wildtype samples: the presence of phosphoSMAD on a scan means that αv-integrin downregulates TGF-β and tumor cell growth is repressed, while the opposite should be true for a lack of phosphoSMAD.

Additional experiments on which I worked include cell culture and maintenance, cell lysing, PCR, and immunohistochemistry. Cell culture and maintenance was carried out in order to provide samples for later Western Blot experiments. Cells were grown in a flask coated with collagen (BD Bioscience). To coat the flasks, a mixture consisting of collagen (1:40 with acetic/water mixture) is placed into plates and allowed to sit for 1 hour, after which it is aspirated and rinsed with Phosphate Buffered Saline (PBS). The cells are fed regularly with a media consisting of 40% MMTV media (Gibco), 10% fetal bovine serum, and 1% of L-glutamine and 100X PIS H/09 and washed regularly with PBS. Cells were split at 80% confluency. To split the cells, the media is aspirated and 1 mL of trypsin is added into the flask. The flask is then placed in the incubator for 5-10 minutes until the majority of the cells become detached. This is followed by resuspension of the cells with 4 mL of media and the flask is washed again with another 5 mL of

media. The cells and media are combined into a 15 mL falcon tube and then added to new collagen coated flasks along with fresh media.

To freeze the cells, the cells are grown to 80% confluency. The day before freezing, they are washed and fed. Cells are trypsinized as described. The cells/media are then placed into a falcon tube where they are centrifuged at 4°C at 1200 rpm for 5 minutes. The pellet is resuspended in freeze media (40% complete media, 50% fetal bovine serum, and 10% DMSO), aliquoted (1/10 T75 per cryovial) and frozen slowly, first being placed in the -20 °C for 30 minutes and then stored in -80 °C.

Cell lysing follows cell maintenance. Here, cells are plated in 6-well plates and monitored daily until 80% confluent. Once achieved, the cells are serum starved for 8 hours prior to lysing. After serum starvation, the cells are treated with TGF-B for 1 hour and 15 minutes. Each genotype is treated with a concentration of $ED_{50,}$ 1/10 $ED_{50,}$ and 10x ED_{50} of both active and latent TGF-B. After the incubation, TGF-B is aspirated and the cells are rinsed with PBS. Lysate Buffer was created (1:1000 concentration of RIPA Buffer to Halt Protease and Phosphatase Inhibitor) and placed on ice. After the cells are washed, they are placed on ice and lysate buffer is added to completely cover the bottom of the wells and incubated for 10 minutes. Afterwards, the cells are scraped with cell scrapers and the well's contents are pipetted into labeled microcentrifuge tubes. The tubes are then centrifuged at 4°C at 13000 rpm for 5 minutes. The spun out supernatent is pipetted into a new tube and then mixed with 2x sample reducing buffer with DTT (Bio-Rad) and stored in 20°C until used in a Western Blot.

Immunohistochemistry is a process that localizes antigens in tissue by means similar to that of western blots. Antibodies are exploited once more for their function of

targeting specific proteins. Here, paraffin-embedded tumor samples are mounted on slides. First, the samples are deparaffinized by use of xylene, and rehydrated by placement into an ethanol series of varying ethanol concentrations. This allows for the antigen retrieval step, a high-heat process used to expose antigens that have been concealed by the tissue fixation process. Next, the samples are incubated in primary and secondary antibodies to identify the desired proteins, and stained with hematoxcylin for contrast. Areas exhibiting the presence of phosphoSMAD will fluoresce brown, while untainted areas appear blue, the color of the hematoxcylin.

To examine the samples on a genetic level, polymerase chain reaction (PCR) was carried out to convert RNA into cDNA, and then to amplify the cDNA by several orders of magnitude. Selective amplification is achieved by use of primer sets, or short strands of DNA that are complementary to the target DNA section. The initially created DNA template is then replicated by use of thermal cycles, and the results are analyzed for the functions of specific genes. This fortifies our assumption that αv-integrin is present by showing it on a genetic level.

Some of the difficulties I encountered were mainly in correlation with the western blots. I had difficulty achieving the desired results due to picture quality issues: human error often resulted in uneven loading, unsuccessful transfer, or a miscalculation in the amount of antibody required to properly fluoresce the selected protein. Furthermore, the cells often became infected with yeast, a lethal infection for the cells, destroying the created cell cultures and eliminating the possibility for western blots to be run on those samples. Furthermore, it was interesting to see how a lab actually functions. Whereas research during the semester is slow and experiments are widely spaced out,

summer research is rather fast paced with a brutal learning curve. However, all the difficulties, trials, and tribulations lead to a long but steady learning process.