Study of the H-Ras-Rb Axis in Oncogene Induced Senescence

Honors Research Thesis

Presented in partial fulfillment of the requirements for graduation

with honors research distinction in Biology in the

undergraduate colleges of The Ohio State University

by

Ryan Orlosky

The Ohio State University

June 2012

Project Advisors: Dr. Kamal S. Pohar, Comprehensive Cancer Center, Department of Urology and Hongtao Jia, PhD Comprehensive Cancer Center

ABSTRACT –

Dysregulation of the oncogene H-Ras pathway is widely found in human superficial bladder cancer, while a Rb mutation co-exists with H-Ras mutation in the majority of invasive tumors. Preliminary data on mice with bladder-specific H-Ras activation and other previous reports on *in vitro* cell line assays have indicated that cellular senescence, a defense response that leads to irreversible cell cycle arrest and provides a barrier for tumor progression, can be induced by H-Ras activation. Extra mutations in tumor suppressors such as Rb could contribute to the breaking of senescence and tumor progression.

Investigation into senescence began with immunohistochemistry on human bladder samples for phosphoERK and Rb, which found that phosphoERK was more commonly involved with the initiation of bladder cancer while Rb was more involved with its progression. With this data, a double transgenic mouse model was created with constitutively activated H-Ras and knocked out Rb, which was hypothesized to break the senescence response. The mice were bred and euthanized, with their bladders' senescent activity bladders analyzed using beta-galactosidase and p15. Interestingly, we found a significant correlation between the H&E/beta-galactosidase of the double transgenic and H-Ras mice as compared to the normal mice, showing that an extra mutation was not sufficient to break senescence at the age of 6-months. Additionally, p15 was not found to not be an effective senescent indicator, with no patterns seen between the three genotypes of mice. Further analysis includes assessment of our 1-year-old mice and investigation towards other tumor suppressors that may drive tumor progression.

INTRODUCTION –

Bladder Cancer Background - Bladder cancer stands as the 4th most common cancer in males and the 8th most common in females.[9] Furthermore, bladder cancer is known as the most expensive type of cancer to treat due to its high recurrence rate and the costly methods of its treatment.[9] Extensive efforts are being put toward reducing the treatment costs and recurrence of this disease through the creation of a transgenic mouse model that ideally mimics human cancer conditions. This novel medium allows scientists to test and ensure the safety of anti-cancer therapies on mice, leading to their integration into human bladder cancer treatment. Since body structure, development, and gene regulation are very similar between mice and humans, using mice to examine this disease will provide a relatively accurate model with results that are applicable to human cancer.

Cancer is a disease that is often modeled in vitro, through the creation of cell lines, or in vivo, through the creation of animal models. Mouse models are often preferred in order to provide the most accurate model of bladder cancer development. To better understand and treat human bladder cancer, animal models are vital, especially those that include genetic changes identified in human tumors. Yet, there has been a lack of these types of models that are representative of the human disease in the field of bladder cancer research. The lab's objective has been to create a relevant mouse model that mimics the human form of bladder cancer in both phenotype and disease progression.

Two of the most common defense responses studied in cancer are apoptosis and senescence. While apoptosis involves programmed cell death, senescence leads to

cell cycle arrest and the cell entering a state of irreversible quiescence.[4] Senescence is a key cellular defense in bladder cancer that can halt the formation and spread of tumors.[4] Experimentally, senescence is often activated in response to an oncogenic signal, such as H-Ras.[3] Considerable cell line evidence exists to support the concept of oncogene induced senescence.[3] The Rb and p53 pathways may be key regulators of the senescence response.[1] A recent study in fibroblast cell lines suggested Rb as the key member of the RB family that has independent and non-overlapping functions that contribute to senescence in the context of Ras activation.[5] This information leads to the hypothesis that cellular senescence is the reason why a low rate of cellular proliferation and lack of a more aggressive tumor is seen in the H-Ras transgenic mouse model of bladder cancer. Additionally, this information advances the notion that the knockout of the tumor suppressor Rb will promote further tumor progression by releasing the senescence barrier.

Ras Pathway - Human bladder cancer is classified in two different forms: superficial, the tumor is confined to the epithelium; and invasive, the tumor invades through the epithelium and in to the underlying muscular layers (see Figure 1). Both pathways have unique mutation events that characterize their representative cancer progression pathways. Ultimately, each incidence of bladder cancer can be labeled by increasing severity, from Ta to T1 to T2 to T3 to T4 (see Figure 2). These stages of bladder cancer represent the tumor progression from the epithelium through the basement membrane and into the muscle layer. The superficial form is far more common, but the invasive form is more dangerous as it causes more deaths.[6]

The lab's efforts have focused on the specific pathway from low-grade superficial tumor to high-grade invasive tumor with the creation of a transgenic mouse model. 70-80% of superficial tumors are caused by dysregulation of the H-Ras pathway, but additional mutations such as p53 and Rb are necessary for the formation of the invasive cancer.[6] The model started with a mutated, activated H-Ras +/- mouse strain driven by Uroplakin II (UPII), a bladder-specific promoter, created by Dr. Wu at New York University.[6] The tumor that the mouse develops has a long latency, grows slowly, and does not progress to the invasive form. This preliminary information combined with lab mice staining shows that no matter how high the H-Ras gene dosage in the mouse bladder, the tumor will not become invasive and the rate of cellular proliferation remains low (see Figure 3).

These hematoxylin and eosin (H&E) stains show the epithelial (purple) vs. muscular (pink) layers in the mouse tissue, highlighting the difference between the normal epithelium (around 3 cell layers) and the Ras +/- mice which show hyperplasia (can exceed 10 cell layers thick). In addition, the urothelium in H-Ras mutated mice contains more senescent cells when compared to the bladder of wild-type mice (see Figure 4). Beta-galactosidase staining was completed on both tissue samples and the senescent cells are indicated through the formation of a blue stain. Therefore, the Ras signaling pathway in the bladder is associated with senescence, but this mutation in the pathway is not sufficient to induce invasive tumor progression.

METHODS –

Human Bladder Samples – Human bladder samples were obtained from the OSU tissue bank and were approved for use by the Ohio State Institutional Review Board.

Immunohistochemistry for Rb and phosphoERK - Human bladder sections were sliced to 4µm thick from paraffin blocks onto slides. After deparaffinization and rehydration, the tissue sections were blocked with hydrogen peroxide for 20 minutes, steamed in 10mM sodium citrate buffer (pH 6.0) for 12 minutes, and cooled to RT. The slides were blocked for 1 hour with goat serum and stained against phosphoERK (Cell Signaling, 1:100) and Rb (Cell Signaling, 1:100) overnight. Then, the slides were incubated with a secondary antibody (phoshpoERK – anti-rabbit 1:500, Rb - anti-mouse 1:500) and a strep label (1:250 peroxidase) for 1 hour each at RT. Lastly, the slides were incubated for 3 minutes in DAB solution, counter stained with hematoxylin, dehydrated, and mounted. For phosphoERK, the slides were rated “intensified” as based on comparison to WT (wild type, normal) human tissue samples. For Rb, the slides were rated “inactivated” if staining was seen completely negative due to the loss of the Rb gene entirely or if all of the Rb had been phosphorylated to become its inactivated version.

Mouse Model - Over the past two years, a double transgenic mouse was created that carries the activating mutation in H-Ras and knockout of the tumor suppressor Rb specifically in the urothelium, in order to determine whether Rb contributes to the senescence response and if the knockout of Rb changes the phenotype of the tumor (see Figure 5). An H-Ras mutated mouse driven by UPII (UPII-

H-Ras) was kindly provided by Dr. Wu's lab from New York University, and was then crossed with an $Rb^{f/f}$ mouse in order to knockout Rb in only the bladder epithelium.[6] Then, a Cre recombinase mouse with UPII (UPII-Cre), which was also kindly provided by Dr. Wu's lab, was crossed also with an $Rb^{f/f}$ mouse.[6] The transgenic UPII-Cre; $Rb^{F/+}$ mouse was crossed with the UPII-H-Ras; $Rb^{f/+}$ mouse in order to create a transgenic with the ultimate genotype of UPII-Cre; $Rb^{f/f}$; UPII-H-Ras. The wild type, UPII-H-Ras, and UPII-Cre; $Rb^{f/f}$ control groups were also maintained in order to have an experimental baseline. The mouse colonies were managed until the ages of 6 months and one year (the remaining mice will begin to reach one year of age in June). At these time points, the mice were euthanized and their bladders were harvested and preserved in paraffin as well as for fresh frozen samples.

Immunohistochemistry for p15 – Mouse bladder sections were sliced to 4µm thick from paraffin blocks onto slides. After deparaffinization/rehydration, the tissue sections were blocked with hydrogen peroxide, steamed in 10mM sodium citrate buffer (pH 6.0), and treated with protein-blocking solution for 10 minutes each (EXPOSE Rabbit Specific HRP/DAB detection IHC Kit, Abcam). The slides were stained against $p15^{INK4b}$ (antibodies-online, 1:200) for 1.5 hours and then were incubated with Rabbit HRP secondary antibody for 15 minutes at RT. Lastly, the slides were incubated for 3 minutes in DAB solution, counter stained with hematoxylin, dehydrated, and mounted. The slides were viewed under 10x magnification and were rated based on brown color intensity in the epithelial cytoplasm from + being the least intense to +++ being the most intense.

Beta-galactosidase staining – Mouse bladder sections were sliced to 10mm thick sections from frozen and plated on slides. Then, the slides were stained with fixing solution for 15 minutes, washed twice in PBS, then covered with staining solution mix and incubated at 37°C overnight according to manufacturer's instructions (Calbiochem). The slides were viewed under 10x magnification and significant progression towards senescence was defined as more than 20% of the slide's epithelial layer staining blue (occurring in the nucleus of the epithelial cells). All of the slides were rated using + to indicate 0-20% epithelial staining (normal), ++ to indicate 20-60% epithelial staining (significant senescent activity), and +++ to indicate 60-100% epithelial staining (substantial senescent activity).

RESULTS –

PhosphoERK Analysis – In order to provide future directions for the mouse model, the lab worked to extend the H-Ras mutation information by focusing on phosphoERK, a downstream component of the Ras signaling pathway. Ras is involved in the normal maintenance of the bladder through its control over the G1 to S checkpoint in the cell cycle (see Figure 6).[10] To test the role of Ras and the defense mechanisms involved in the body's protection against mutations that cause these tumors, phosphoERK and Rb were stained for in human bladder tumor tissue samples. ERK is a mitogen-activated protein kinase and phosphoERK is ERK's activated (phosphorylated) form. Rb is a key tumor suppressor involved in the last step of this cell cycle checkpoint pathway that releases E2F1-3 upon phosphorylation, which leads to the activation of the S phase.[10]

H&E staining of both of these downstream components of the Ras pathway has demonstrated that the Ras-MAPK pathway is activated in most human bladder tumors of different grades and stages (see Figure 7). PhosphoERK is more commonly activated in the early stages of bladder cancer (Ta through T2), while Rb is more commonly activated in the later stages of bladder cancer (T1 through T4). These results suggest that ERK plays a greater role in tumor initiation and Rb plays a greater role in tumor progression. As the lab's objective has been to create a mouse model that mimics the progression of bladder cancer from superficial to invasive, these findings substantiate our focus towards the mutation of the tumor suppressor Rb in the mouse model.

Additionally, H&E staining for phosphoERK was completed on WT (normal) and Ras mutated mice, both homozygous Ras +/+ and heterozygous Ras +/- (see Figure 8).

Although significant hyperplasia was seen in the UPII-H-Ras mutated mice, the high Ras concentration was not sufficient to push the bladder towards an invasive tumor as seen by the intact muscle layer. This further backs ERK's non-involvement in bladder cancer tumor progression.

Mouse Model – Bladders from the ultimate genotype (UPII-Cre; $Rb^{f/f}$; UPII-H-Ras) mice and the control group mice (wild type, UPII-H-Ras, and UPII-Cre; $Rb^{f/f}$) were collected (see figure 9). Phenotypically, minor bulging was seen in the bladder area with general confusion (running in circles, flipping), possibly caused by the "leaky" nature of the mutated H-Ras gene. Hyperplasia is seen in the epithelium of both the UPII-H-Ras mouse and the double transgenic (UPII-Cre; UPII-H-Ras; Rb f/f), as compared to the WT mouse.

Senescence Assay - A significant correlation was seen between the senescence in the Ras and double transgenic mice as compared to the WT mice, based on the beta-galactosidase staining (see figure 10 & 11). Intense blue coloration in both of these transgenic mice groups correlates to a significant amount of the senescence defense response being activated. The H&E stains of both animals also act to connect the Ras and double transgenic based on their similarities in hyperplasia as compared to the WT mice. An intact muscle layer on both mutated mice shows that they have only formed the low-grade superficial bladder cancer. However, the cytoplasmic p15 staining seen in the epithelium was across the board in intensity, with no considerable patterns seen between the different genotypes. With this in mind, p15 was found to be an ineffective senescence marker.

DISCUSSION AND FUTURE DIRECTIONS –

Preliminary data has shown that the mutation of H-Ras was not sufficient to drive bladder cancer from superficial (hyperplasia) to invasive (tumor). Staining for phospho-ERK, a downstream component of the Ras signaling pathway showed that it was mainly a player in the initiation, but not the progression of bladder cancer. Immunohistochemistry of human bladder tumor samples showed that Rb, another downstream player of the signaling pathway, was significant in the progression of tumor development. Thus, a double transgenic mouse model was created to mutate H-Ras and Rb, in order to accelerate tumor development.

Upon thorough analysis of the H&E, p15, and beta-galactosidase stains of the 6-month old mutated mice, it has been initially seen that inactivation of the tumor suppressor Rb is not sufficient to accelerate the development of bladder cancer from superficial to invasive. Based on a comparison to WT mice, great similarities were found between the H-Ras and our ultimate H-Ras; Cre; $Rb^{f/f}$ mice. The lab's initial hypothesis was that an additional mutation in the Ras signaling pathway with tumor suppressor Rb would be significant enough to break the cell's senescence defense response, but this was not seen in our 6-month mice. Therefore, another component in the biological signaling pathway must compensate for the loss of Rb.

One explanation for these results could be that another tumor suppressor, p53, is able to compensate for the loss of Rb.[8] Another possible explanation could be that other members of Rb's pocket protein family, such as p130 or p107, compensate for Rb's loss. A final explanation could be that a significant amount of time has not yet passed in

our 6-month mice in order to see the full transformation from superficial to invasive cancer and for specific biological modifications to occur.

Future project expansion includes further senescence staining with p16, Dec1, and DcR2 markers on our current mice bladder tissue. These markers have been documented as being useful in measuring senescence in the bladder and these results will work to corroborate with the lab's current results.[2] At the age of one year (starting in June), the remainder of our mice will be euthanized in order to provide a further benchmark for tumor development. Beta-galactosidase staining and immunohistochemical staining will again be completed for p15, p16, Dec1, and DcR2.

FIGURES -

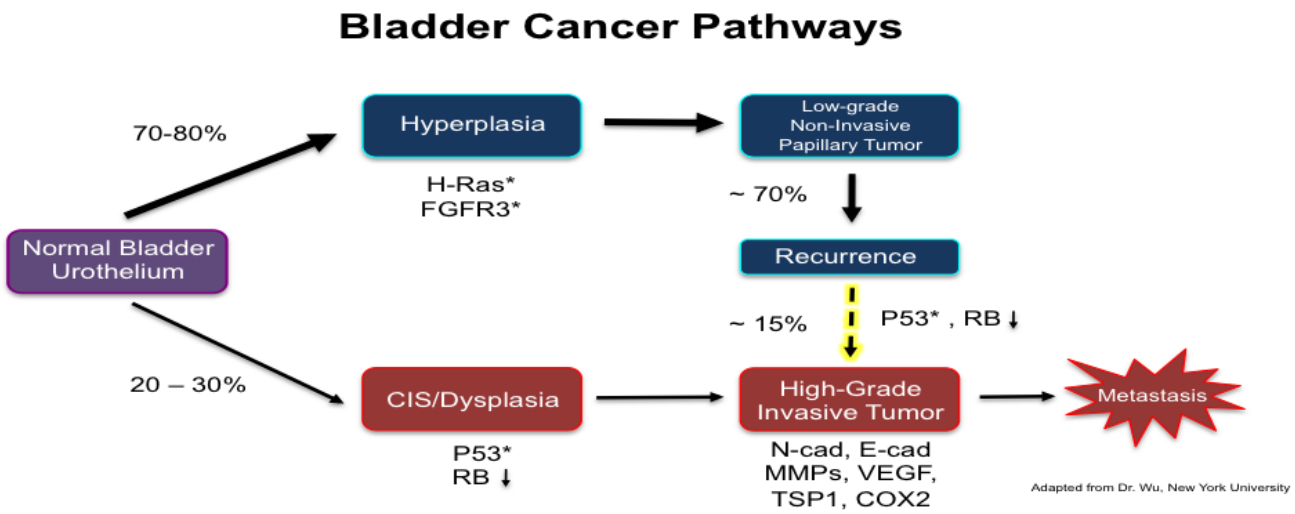

Figure 1

This figure represents the breakdown of bladder cancer into superficial (hyperplasia) and invasive (dysplasia) strands, highlighting some of the common biological pathways mutated in the progression of papillary to invasive tumors. The asterisks represent a mutated version of the original gene present in the correlating forms of bladder cancer.

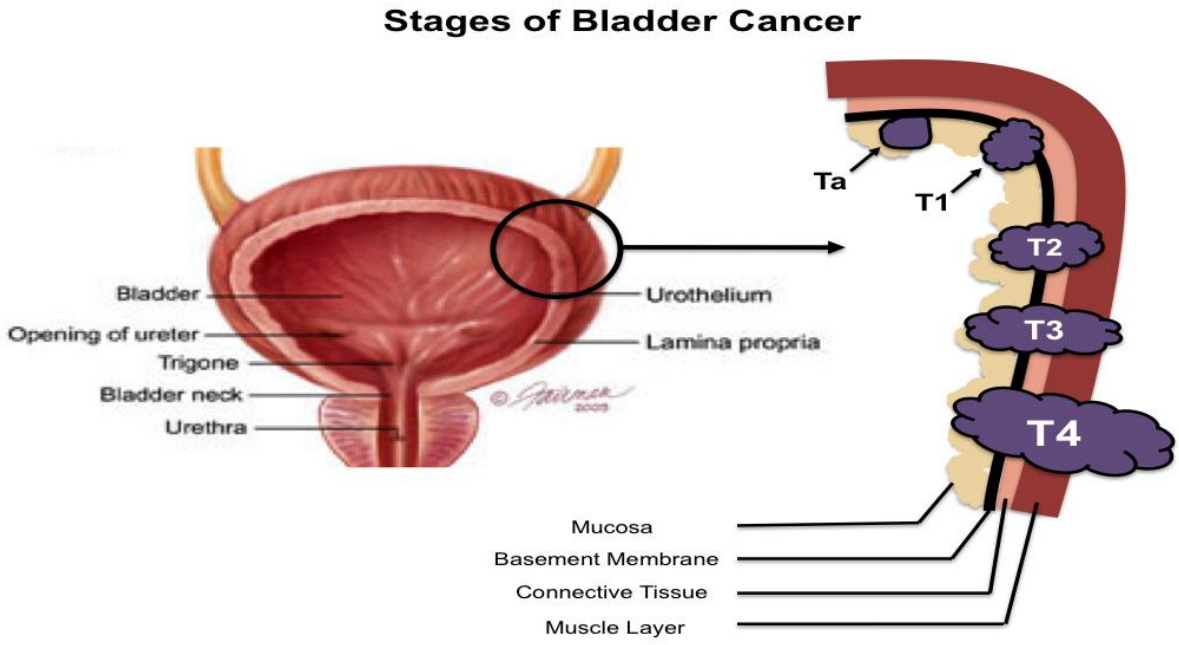

Figure 2

A visual representation of the bladder, including the various stages of bladder cancer progression and their invasion into the bladder muscle layer.

Figure 3

These H&E stains show the epithelial (purple) vs. muscular (pink) layers in the mouse tissue. Note the difference between the normal epithelium (around 3 cell layers) and the Ras +/- mice which show hyperplasia and near tumor progression (can exceed 10 cell layers thick).

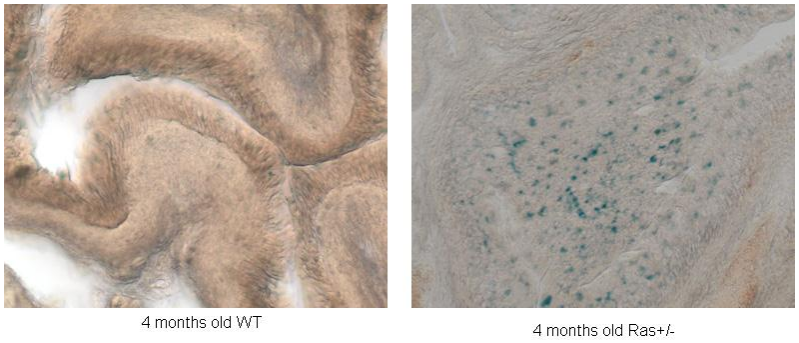

Figure 4

These beta-galactosidase stains indicate senescence levels through the appearance of a blue color in the nucleus of the cells. As seen here, the Ras +/- mouse has significantly more senescence than the WT mouse.

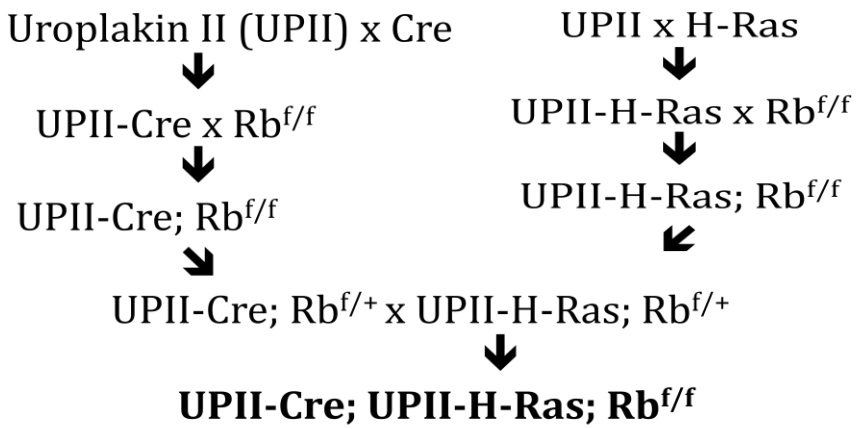

Figure 5

This breeding scheme shows the strategy used to create our ultimate double transgenic mouse model with bolded genotype above. We used the UPII (uroplakin II, bladder epithelial specific protein) promoter to drive the mutated H-Ras (constitutively activated form) to induce cancer expression. $Rb^{f/f}$ represents the floxed version of the normal tumor suppressor that can be knocked out by the expression of Cre inside their cells. A Cre-floxed system is powerful when used in animal models to knock out a gene, which is Rb in our mice.

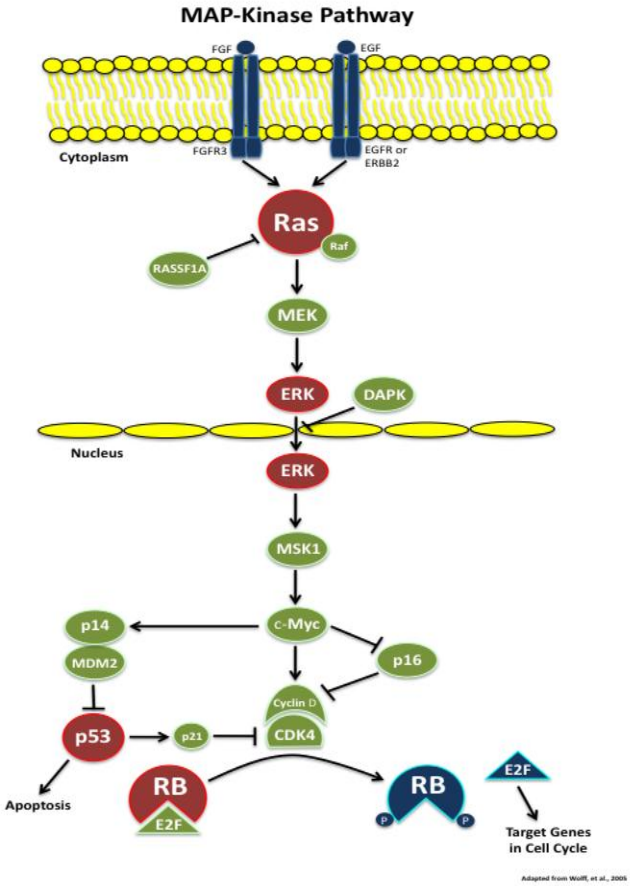

Figure 6

A vital biological signaling pathway involved in normal maintenance of the bladder's G1 to S checkpoint, highlighting the downstream roles of ERK (a mitogen-activated protein kinase) and Rb from Ras.

Human Bladder Tumors

	Intensified phospho-ERK	Rb inactivation
Low Ta	5/9 (55.6%)	5/9 (55.6%)
High Ta	3/6 (50%)	4/7 (51.7%)
High T1	11/13 (84.6%)	15/20 (75%)
High T2	11/23 (47.8%)	31/36 (86.1%)
High T3	2/27 (7.4%)	30/42 (71.5%)
High T4	0/9 (0%)	11/18 (61.1%)

Figure 7

This chart shows immunohistochemical staining for phospho-ERK (phosphorylated ERK, activated form of ERK) and Rb of human bladder tissue during the different stages of bladder cancer development. For phosphoERK, the slides were rated "intensified" as based on comparison to WT (normal) human tissue samples. For Rb, the slides were rated "inactivated" if staining was seen completely negative due to the loss of the Rb gene entirely or if all of the Rb had been phosphorylated to become its inactivated version. This most importantly notes the role of Rb in tumor progression and the role of phospho-ERK in tumor initiation, indicated by the yellow shading.

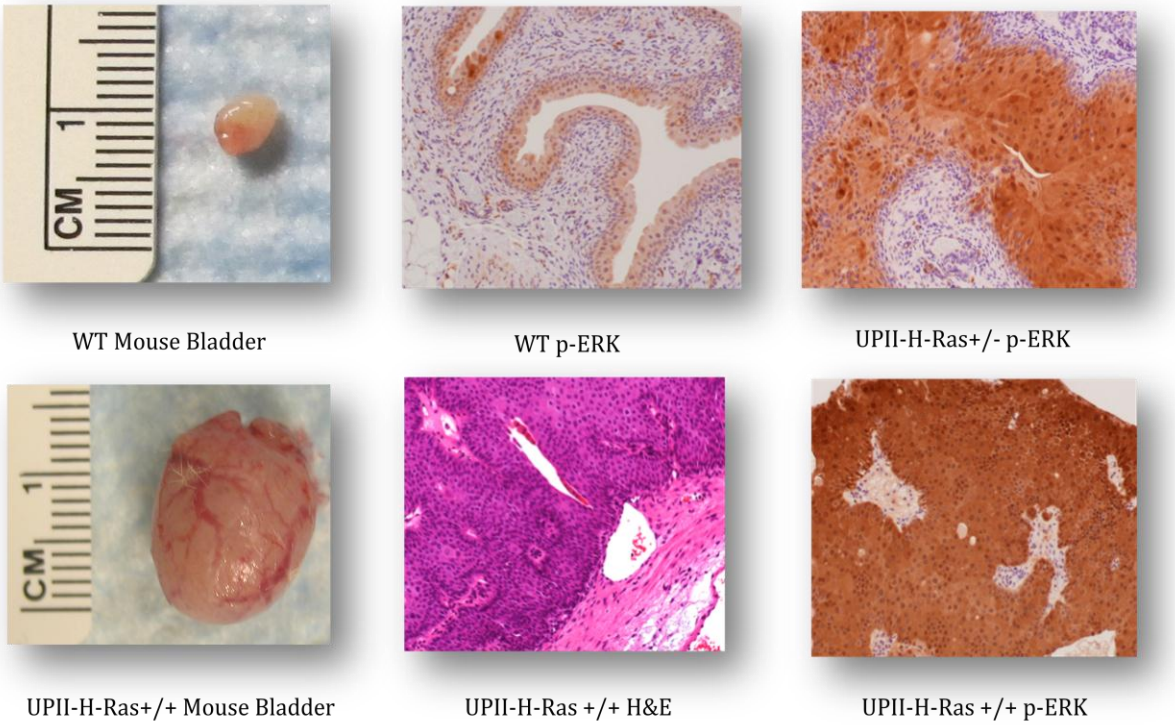

Figure 8

The first photos show the size of a WT mouse bladder (~0.5 cm) as well as immunohistochemical staining for phospho-ERK. Following those is phospho-ERK staining of a heterozygous Ras mouse displaying mild hyperplasia. The second row shows a homozygous Ras mouse bladder (~1.5 cm) with its H&E and phospho-ERK staining. Although extreme hyperplasia is seen in this homozygous mouse, the high Ras concentration is not sufficient to push the bladder towards an invasive tumor as seen by the intact muscle layer.

Mouse Colony Information

	Mice Euthanized at age of 6-months	Mice to be Euthanized at age of 1 year
Double Transgenic	5	26
Ras	6	29
WT	5	22

Figure 9

This chart shows the number of mice we have bred and the controls we have used to evaluate our hypothesis. Double transgenic refers to the mice with the ultimate genotype of UPII-Cre; $Rb^{f/f}$; UPII-H-Ras, while Ras refers to a genotype of UPII-H-Ras, and WT refers to a normal (non-mutated) mouse. The first group highlighted in green represent the mice already euthanized and analyzed, while the second column represents the group of mice we will sacrifice once they reach the age of 1 year old.

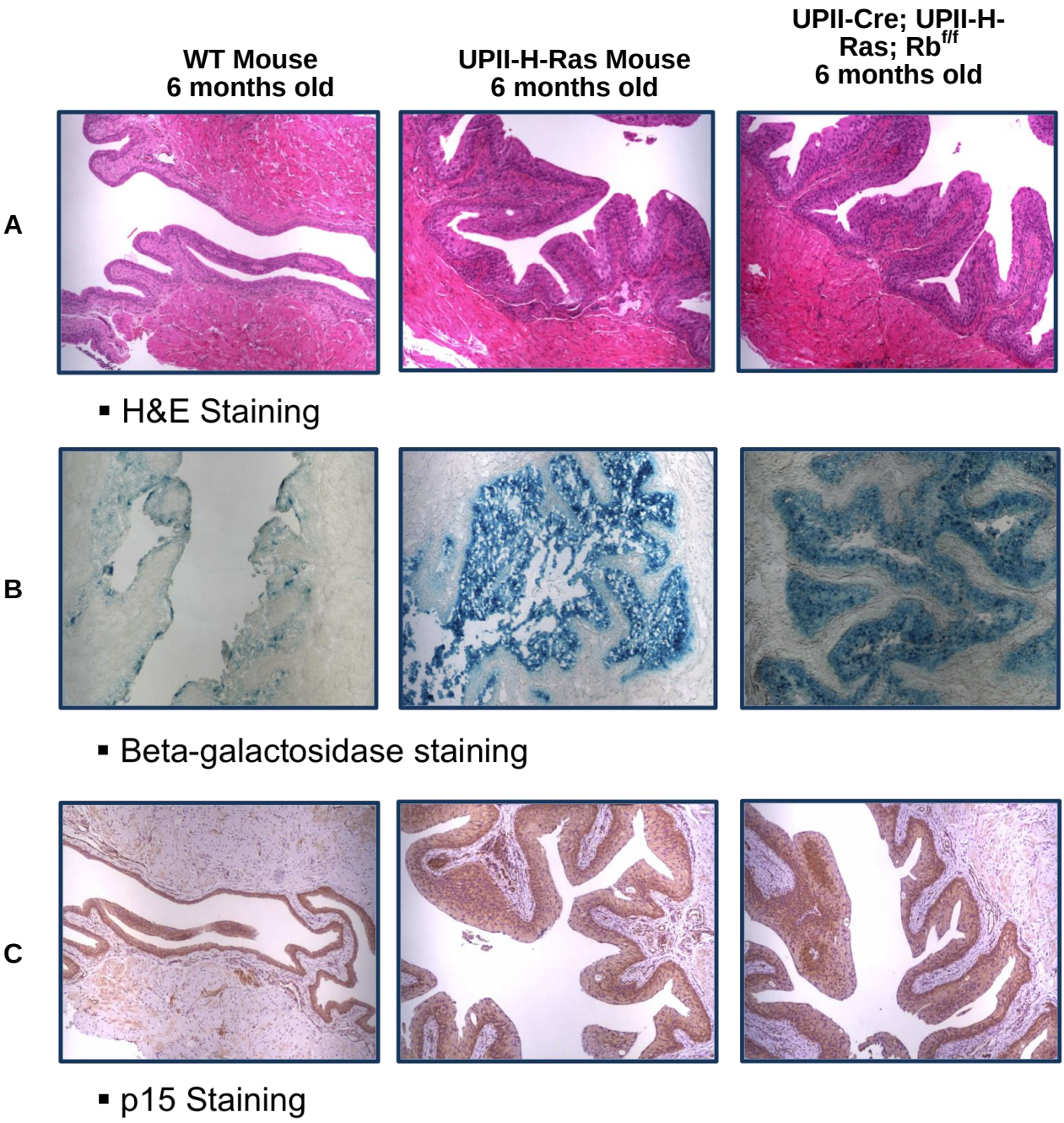

Figure 10

These mouse bladder sections represent a sample of the 16 total mice euthanized and harvested at the age of 6-months old. All 3 columns represent sections from the same animal, while each row represents a different type of staining analysis. All sections were viewed under the microscope at a magnification of 10x.

Row A represents the H&E stains of the 3 mice genotypes. With H&E staining, a lighter pink color represents the muscle layer of the bladder, while the darker purple layer represents the epithelial layer. The WT mouse represents a normal epithelium, around 3 layers thick. The Ras and double transgenic mice represent a bladder displaying hyperplasia or an increase in cell number, which can be 10 or more cell layers thick.

Row B represents beta-galactosidase staining of frozen bladder sections on the 3 different genotypes of mice. Senescent positive cells are stained blue in the nucleus, which is seen in the epithelium of the bladder. The stains were evaluated based on their proliferation in the epithelium (see figure 11). There is a significant correlation between the Ras and double transgenic mice as compared to the WT.

Row C represents immunohistochemical staining for p15 in the epithelium of the bladder, seen with the formation of a brown color. While it appears the transgenic mice have greater p15 staining, ultimately the assessment of these slides falls on the intensity of the p15 stains (darker brown color). The transgenic mice are hyperplastic and have p15 staining throughout the epithelium, but the intensity of the staining closely matches what was seen in the WT mouse.

A

Beta-Galactosidase

	+	++	+++
Double Transgenic	0/5	4/5	1/5
Ras	0/6	5/6	1/6
WT	5/5	0/5	0/5

B

p15

	+	++	+++
Double Transgenic	1/5	3/5	1/5
Ras	2/6	2/6	2/6
WT	3/5	1/5	1/5

Figure 11

Chart A - Double transgenic refers to the mice with the ultimate genotype of UPII-Cre; $Rb^{f/f}$; UPII-H-Ras, while Ras refers to a genotype of UPII-H-Ras, and WT refers to a normal (non-mutated) mouse. All beta-galactosidase slides were viewed under 10x magnification and significant progression towards senescence was defined as more than 20% of the slide's epithelial layer staining blue (occurring in the nucleus of the epithelial cells). All of the slides were rated using + to indicate 0-20% epithelial staining (normal), ++ to indicate 20-60% epithelial staining (significant senescent activity), and +++ to indicate 60-100% epithelial staining (substantial senescent activity). The results indicate the amount of mice out of the total that were found to have that correlating amount of senescence activity out of the total amount of that genotype of mice. In the chart, the yellow shading indicates those slides with significant senescence activity Notice here the similarities between the double transgenic and Ras mice.

Chart B – All p15 slides were viewed under 10x magnification and were rated based on brown color intensity in the epithelial cytoplasm from + being the least intense to +++ being the most intense.

ACKNOWLEDGEMENTS –

A tremendous thank you goes out to the Undergraduate Student Pelotonia Fellowship Program for their support over the past year in my research project and undergraduate education. Pelotonia has been an outstanding influence in my life over the past year and their support has greatly improved my research and undergraduate experience. I would also like to thank the OSUCCC Solid Tumor Biology Program for their support and for providing human bladder tumor samples.

None of this work would be possible without the tremendous guidance from Dr. Kamal Pohar and Hongtao Jia, PhD. These two fantastic mentors and advisors have taken me from day 1 to today and I can't thank them enough for all of their support in my undergraduate research experience. Together, Dr. Pohar and Hongtao have helped me learn so much and have made the last two years fly by.

BIBLIOGRAPHY –

1. Chen Z, Trotman LC, Shaffer D, Lin HK, Dotan ZA, Niki M, Koutcher JA, Scher HI, Ludwig T, Gerald W, Cordon-Cardo C, Pandolfi PP. Crucial role of p53-dependent cellular senescence in suppression of pten-deficient tumorigenesis. Nature 2005 Aug 4;436(7051):725-30.

2. Collado M, Gil J, Efeyan A, Guerra C, Schuhmacher AJ, Barradas M, Benguria A, Zaballos A, Flores JM, Barbacid M, Beach D, Serrano M. Tumour biology: Senescence in premalignant tumours. Nature 2005 Aug 4;436(7051):642.

3. DeNicola GM, Tuveson DA. RAS in cellular transformation and senescence. Eur J Cancer 2009 Sep;45 Suppl 1:211-6.

4. Schmitt CA. Cellular senescence and cancer treatment. Biochim Biophys Acta 2007 Jan;1775(1):5-20.

5. Wei W, Herbig U, Wei S, Dutriaux A, Sedivy JM. Loss of retinoblastoma but not p16 function allows bypass of replicative senescence in human fibroblasts. EMBO Rep 2003 Nov;4(11):1061-6.

6. Wu XR. Urothelial tumorigenesis: A tale of divergent pathways. Nat Rev Cancer 2005 Sep;5(9):713-25.

7. Zhang Z, Pak J, Huang H, Shapiro E, Sun T, Pellicer A, Wu X. Role of ha-ras activation in superficial papillary pathway of urothelial tumor formation. Oncogene 2001 04/12;20(16):1973.

8. Xue W, Zender L, Miething C, Dickins RA, Hernando E, Krizhanovsky V, Cordon-Cardo C, Lowe SW. Senescence and tumour clearance is triggered by p53 restoration in murine liver carcinomas. Nature 2007 Feb 8;445(7128):656-60.

9. Jemal A, Siegel R, Ward E, Hao Y, Xu J, Thun MJ. Cancer statistics, 2009. CA Cancer J Clin 2009 Jul-Aug;59(4):225-49.

10. Wolff EM, Liang G, Jones PA. Mechanisms of disease: Genetic and epigenetic alterations that drive bladder cancer. Nat Clin Pract Urol 2005 Oct;2(10):502-10.