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Abstract:
FOXF2 is a transcription factor that plays a crucial role in organ development, and recent studies have shown that it suppresses tumor growth and progression in mouse prostate models by attenuating the cancer-associated fibroblasts (CAF) phenotype and transcriptionally downregulating *Cxcl5*. However, the effects of FOXF2 overexpression in prostate cancer cells have not been extensively studied. Here, we investigate the impact of FOXF2 overexpression in prostate cancer cells and demonstrate that it leads to a significant increase in cell apoptosis and a decrease in proliferation. These findings suggest that FOXF2 may have potential as an immunotherapy drug target for prostate cancer treatment.

Introduction:
Prostate cancer is the most common cancer among men in the United States[1], aside from melanoma. The disease is characterized by uncontrolled cell growth in the prostate gland, which is a male reproductive system located just below the bladder and in front of the rectum. According to the National Cancer Institute, an estimated 288,300 new cases of prostate cancer will be diagnosed in the United States in 2023, with approximately 34,700 deaths expected[2].

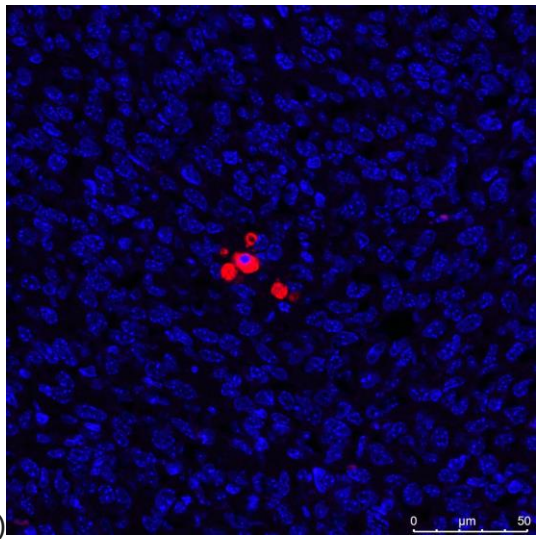
There are various treatment options for prostate cancer, including, but not limited to radiation, cryotherapy, hormone therapy, chemotherapy, and immunotherapy[3]. One of the promising therapeutic strategies to treat prostate cancer is targeting cancer-associated fibroblasts (CAFs). The generation, maintenance, and activity of CAFs are regulated by growth factors, cytokines and chemokines, reactive oxygen species, and mechanical signaling via several transcription factors such as SMADs, STAT3, NF-κB, HSF1, RBP-J, and YAP, etc[4]. It is crucial to uncover additional vital transcriptional regulatory mechanisms that drive tumor progression through CAFs. Recent research by Jia et al have demonstrated that suppressing the stromal forkhead box F2 (FOXF2) gene can reduce the progression and metastasis of prostate cancer by enhancing antitumor immunity[5].

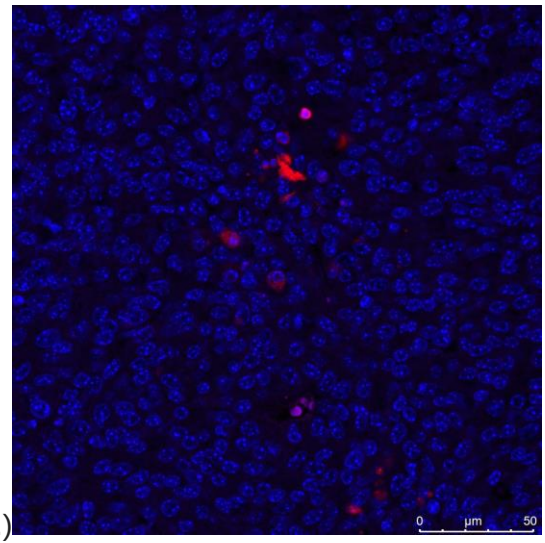
Foxf2 is a transcription factor that plays an important role in the development of many different organs, such as the brain, intestine, secondary palate, etc[6]. Multiple studies showed that the abnormal expression of Foxf2 is linked to several tumorigenic processes, including proliferation, invasion, and metastasis[7]. In this study, we use a combination of molecular, cellular, and genetic approaches to study the outcome of overexpressing the FOXF2 gene in prostate cancer cells, which opens the possibility for the development of different therapeutic treatments for prostate cancer.

Results:
In this experiment, foxf2 was overexpressed in cancerous prostate cells using lentivirus in cultured mice cancer prostate cells. Gene overexpression was measured and shown to be significantly higher than control-treated cancer cells. To determine whether increasing stromal cell Foxf2 expression affects prostate cancer cell growth and apoptosis in vitro, the control and Foxf2-expressing mPrSC were cocultured with mouse prostate cancer cells. The prostate cancer cell lines that we tested included two C57BL/6 syngeneic mouse prostate cancer cells.

The apoptosis index, or the rate of programmed cell death, is an important indicator of the efficacy of cancer treatments. In our study, we found that overexpression of the foxf2 gene significantly increased the rate of apoptosis in cancerous prostate cells (Figure 1). This finding is significant because it suggests that targeting the FOXF2 gene could be a potential strategy for inducing apoptosis in cancer cells.

On the other hand, we did not observe a significant decrease in the rate of proliferation in cancer cells overexpressing *Foxf2*. This was determined by comparing the percentages of cells marked with the BrdU immunostaining, which indicated the cells that were actively proliferating (Figure 2). The lack of effect on proliferation suggests that the FOXF2 gene may specifically target the apoptotic pathway, without affecting the cell cycle progression.

a) 

b.) 

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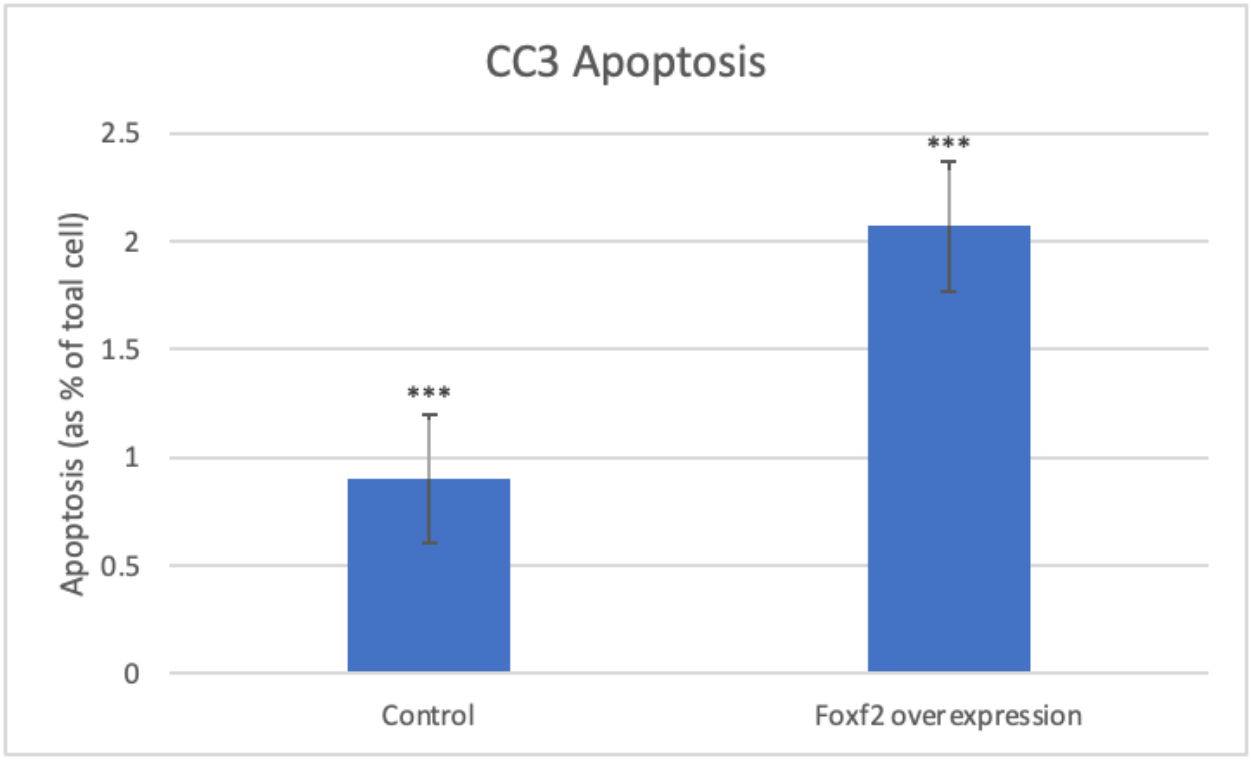
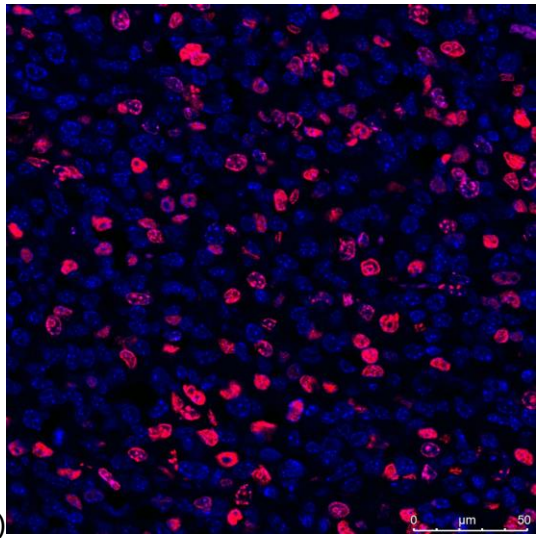
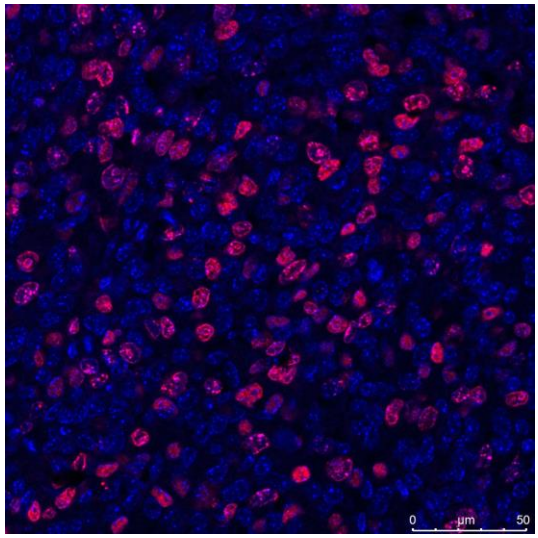


Fig 1. *Cancer FOXF2 overexpression does significantly increase cancerous stromal cell apoptosis a) Control immunostaining with DAPI(blue) and CC3(red) of prostate cancer cells as compared to b) overexpressed FOXF2. After c) analyzing the percent of cells that apoptosed, there is a significant difference in apoptotic index.*

a) 

b) 

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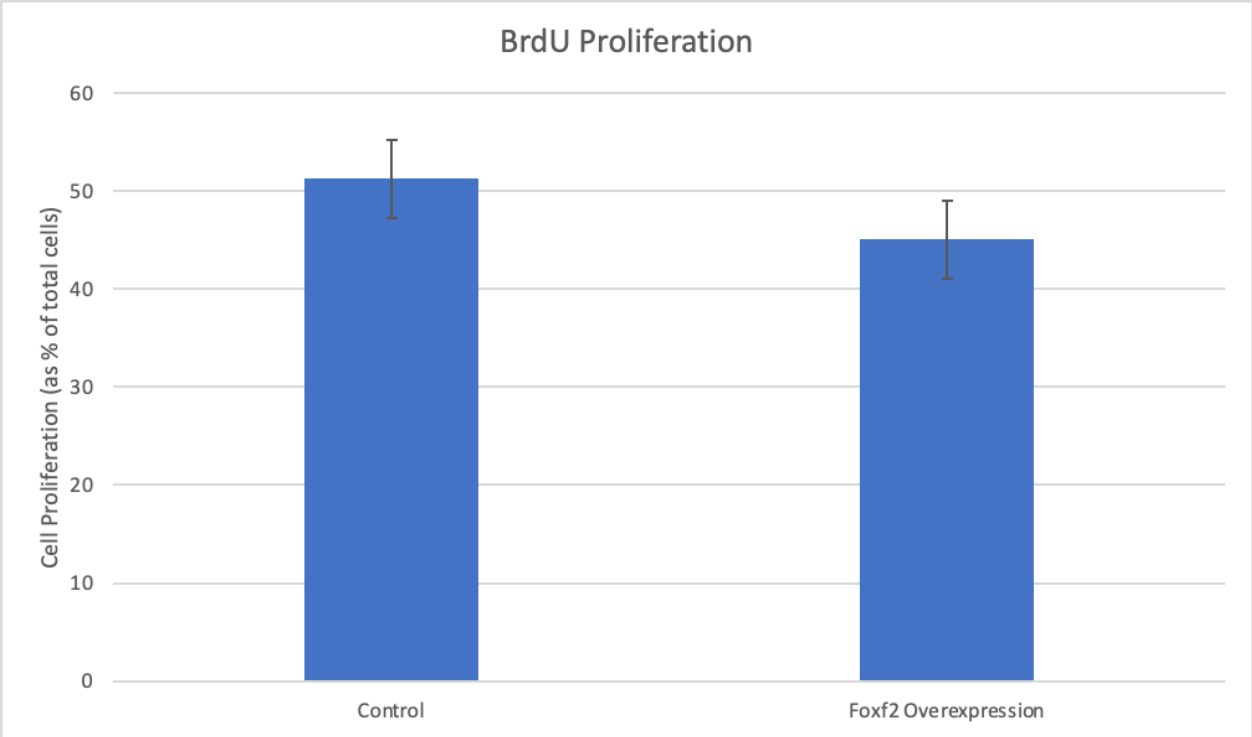

Fig 2. *Cancer FOXF2 overexpression does not significantly reduce cancerous stromal cell proliferation a) Control immunostaining with DAPI(blue) and BRDU(red) of prostate cancer cells as compared to b) overexpressed FOXF2. After c) analyzing the percent of cells that are proliferating, there is not a significant difference in proliferation.*

Discussion:

This study shows that overexpression of FOXF2 in cancerous prostate cells leads to a significant increase in cell proliferation rate. Since stromal *Foxf2* plays an important role to suppress tumor progression and metastasis in TRAMP mice (Jia et al) and Foxf2 is nearly not expressed in cancerous prostate cells, reintroducing Foxf2 into cancerous cells inhibits cancer progress by inducing more cell apoptosis.

Previously, Jia et al showed that *Foxf2* transcriptionally represses Cxcl5, which is an angiogenic factor that promotes cancer formation by recruiting immune cells to the tumor site. This is intriguing as our results show no significant difference in the cell proliferation rate between cancerous tumor cells with *Foxf2* overexpression versus the control cells. However, we did find a decrease in the proliferation rate in cells that have a high expression of Foxf2.

In future experiments, we plan to investigate the molecular mechanisms underlying *Foxf2*-mediated apoptosis by comparing the expression profile between the control cells and cancerous cells with Foxf2 overexpression via RNA-seq.

Methods:

All animals used in this study received humane care in compliance with the principles stated in the Guide for the Care and Use of Laboratory Animals, NIH Publication, 1996 edition, and the protocol was approved by the Institutional Animal Care Committees of Baylor College of Medicine and the University of Washington. In all the studies, the maximal allowable tumor size/burden (diameter less than 1.5 cm) was not exceeded. All human specimens were collected with informed consent according to the guidelines of the Declaration of Helsinki and approved by the Institutional Review Board (or Ethics Committee) of University of Washington (protocol code 2341).

Mice:
Mouse embryos were provided by the Jackson Laboratory (Bar Harbor, ME) and Baylor College of Medicine, and were kept at a 12 h/12 h dark/light cycle. The animals were kept at 22 ± 3 °C and the humidity at 55 ± 15%. All mice used in this study were on the C57BL/6 background. Polymerase chain reaction (PCR) was performed using mouse genomic DNA obtained from tail biopsy samples. The genotyping primers and the expected band sizes for PCR can be found in Supplementary Table 1. Gel electrophoresis on 1% agarose gel was used to visualize the genotyped products.

Tumor xenograft
In this experiment, the RM-1 syngeneic prostate cancer model in C57BL/6 mice was used. To produce subcutaneous tumor xenografts, 1.5×10^5 mouse prostate stromal cells were combined with 5×10^4 RM-1 or Pten/Kras cells in 100 µl of PBS and were then administered subcutaneously into male C57BL/6 hosts aged between 8 to 10 weeks. The experiment was terminated once the tumor cells reach 1.5cm or larger in diameter.

Tissue culture
The RM-1 and WPMY-1 cell lines were obtained from the University of Texas MD Anderson Cancer Center and Dr. Chawnshang Chang at the University of Rochester Medical Center, respectively. The RM-1 cells were cultured in DMEM with 10% FBS at 37°C/5% CO2, and the WPMY-1 cells were cultured in DMEM with 10% FBS at 37°C/5% CO2. The Pten-Kras cell line was established from the primary prostate tumor tissues of Pb-Cre;$Pten^{fl/fl}$; LSL-$Kras^{G12D}$ mice. To obtain Single cells, the tumor tissues were digested in DMEM/Collagenase/Hyaluronidase/FBS for 1 hour at 37°C, followed by an additional 1-hour digestion in 0.25% Trypsin-EDTA on ice. The dissociated single cells were cultured in DMEM with 10% FBS, 6 ng/mL EGF, and 5 µg/mL insulin. The tumor cells were then separated from the Sca-1-expressing stromal cells by flow cytometry, and all cells were cultured at 37°C/5% CO_2.

To obtain single cell culture, prostate tissue from C57BL/6 mice from 8 to 12 weeks old was extracted. The dissociated cells were then Biocoat Collage I-coated plates (Corning, Corning, NY) in Bfs medium (5% Nu-Serum, 5% FBS, 1×Insulin/Selenium, 1x L-Glutamine, 1× Penicillin/Streptomycin, and 1×10^{-10} M DHT in DMEM medium) at 37 °C with 5% CO2. Upon reaching 90% confluency, cells were trypsinized and replated in Biocoat Collagen I-coated plates for 30 minutes at 37°C/5% CO2, and then the unattached cells were discarded, and the remaining cells were cultured in Bfs until 80-90% confluency was reached. Only fresh primary cancerous stromal cells within 3 passages after single-cell dissociation from prostates were used for all

experiments in this study. One 8-week-old mouse prostate can provide 6-10 × 10^5 cancerous stromal cells after a 3-passage culture.

Overexpression of FOXF2 gene

We used a lentivirus to overexpress Foxf2 in primarily cultured mouse prostate stromal cells. These results were checked using qPCR with SYBR green dye. Gapdh was used as a control level to test against

Immunostaining

Cultured cells were processed according to a previously described method by Choi et al[5], and were then incubated with primary antibodies in 3% normal goat serum overnight. The cells were then incubated with secondary antibodies labeled with Alexa Fluor 488 and 594 (Invitrogen/Molecular Probes, Eugene, OR), which were diluted 1:250 in PBST. Hematoxylin or 4,6-diamidino-2-phenylindole (DAPI) was used for counterstaining.

Immunofluorescence staining was visualized with a Leica SP8 fluorescence microscope. Fiji image-processing package was used to process IHC images. DAPI staining was used to define stromal regions and enumerate cell numbers. BrdU staining was used to mark proliferation cells and CC3 staining indicated apoptotic cells. The BrdU cells were counted using Fiji software. CC3 stains were counted manually due to the program not being able to accurately count lysed cells. Student t-test was used to identify significant differences between groups and significance was determined as $p \leq 0.05$.

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Appendix:

Cancer is a disease that can cause significant suffering for individuals and their families. The physical and emotional toll of cancer can be immense, and can include pain, fatigue, nausea, and depression. One of the unique aspects of cancer is that it often involves not just physical suffering, but also a profound sense of loss of control and uncertainty. Individuals with cancer may experience changes in their bodies that they find difficult to understand or accept, and feel a sense of helplessness in the face of the disease.

Furthermore, cancer can impact not just the physical body, but also the sense of self and identity. Individuals with cancer may struggle with changes in their appearance or physical abilities, and feel that their sense of self has been fundamentally altered by the disease. It's important to recognize that suffering is not just a physical experience, but also a psychological and social one. Individuals with cancer may struggle with feelings of isolation, fear, and anxiety, and benefit from support and counseling services.

There is also a growing recognition of the importance of addressing the spiritual dimension of suffering in cancer care. For some individuals, cancer may prompt questions about the meaning of life, the nature of suffering, and the role of faith or spirituality in their lives.

Overall, the experience of cancer can be one of profound suffering, both for the individual with the disease and for their loved ones. It's important to approach cancer care with compassion, empathy, and a recognition of the complex and multifaceted nature of suffering.

Appendix 2:

My experiment examines the effect of the overexpression of the fox F2 gene in prostate cancer cells and how it causes an increase in cancer cell apoptosis and a decrease in proliferation. Prostate cancer is the most common cancer among men in the United States[1], aside from melanoma. The disease is characterized by uncontrolled cell growth in the prostate gland, which is a male reproductive system located just below the bladder and in front of the rectum. According to the National Cancer Institute, an estimated 288,300 new cases of prostate cancer will be diagnosed in the United States in 2023, with approximately 34,700 deaths expected[2].

There are various treatment options for prostate cancer, including, but not limited to radiation, cryotherapy, hormone therapy, chemotherapy, and immunotherapy[3]. One of the promising therapeutic strategies to treat prostate cancer is targeting cancer-associated fibroblasts (CAFs). The generation, maintenance, and activity of CAFs are regulated by growth factors, cytokines and chemokines, reactive oxygen species, and mechanical signaling via several transcription factors such as SMADs, STAT3, NF-κB, HSF1, RBP-J, and YAP, etc[4]. It is crucial to uncover additional vital transcriptional regulatory mechanisms that drive tumor progression through CAFs. Recent research by Jia et al have demonstrated that suppressing the stromal forkhead box F2 (FOXF2) gene can reduce the progression and metastasis of prostate cancer by enhancing antitumor immunity[5].

Foxf2 is a transcription factor that plays an important role in the development of many different organs, such as the brain, intestine, secondary palate, etc[6]. Multiple studies showed that the abnormal expression of Foxf2 is linked to several tumorigenic processes, including proliferation, invasion, and metastasis[7]. In this study, we use a combination of molecular, cellular, and genetic approaches to study the outcome of overexpressing the FOXF2 gene in prostate cancer cells, which opens the possibility for the development of different therapeutic treatments for prostate cancer.

This first figure in my slide is the experimental workflow of my study. The first step was breeding the mice. The mouse embryos were provided by the Jackson laboratory and Baylor college of medicine. In order to tell what the genotype of the mouse was, I used PCR and gel electrophoresis to examine the DNA. Several mice were needed so several generations were bred. After obtaining the amount of mice that I needed, I used a lentivirus to overexpress that gene foxf2 in cultured prostate stromal cancer cells. After overexpressing the FOXF2, The prostate stromal cells were combined and administered into male mice. Once the tumors wished and appropriate size, the mice were sacrificed and the tumors were extracted. Listen was were processed and then incubated with antibodies to prepare them eventually for the immunostaining. Domino's standing allowed me to be quantify the rate of apoptosis and the rate of proliferation which I was interested in studying to examine how overexpression of this gene affected cancer growth. in this study, I found that the order expression of the fox of two genes significantly increased the rate of the apoptosis In the cancerous cells. As you'll see from the graph on the slide, there's a significant difference between the rate of apoptosis in the control as compared to the FOXF2 overexpressed condition. On the other hand, I did not observe a significant decrease in the right of proliferation and cancer cells overexpressing methoxy after gene. As you can see here on the second graph while there is a small decrease, this decrease was not large enough to be considered significant. The lack of effect on proliferation suggested

that the fox F2 gene they specifically target the apoptotic pathway, without affecting the cell cycle progression.

Since stromal *Foxf2* plays an important role to suppress tumor progression and metastasis in TRAMP mice (Jia et al) and Foxf2 is nearly not expressed in cancerous prostate cells, reintroducing Foxf2 into cancerous cells inhibits cancer progress by inducing more cell apoptosis.

Previously, Jia et al showed that *Foxf2* transcriptionally represses Cxcl5, which is an angiogenic factor that promotes cancer formation by recruiting immune cells to the tumor site. This is intriguing as our results show no significant difference in the cell proliferation rate between cancerous tumor cells with *Foxf2* overexpression versus the control cells. However, we did find a decrease in the proliferation rate in cells that have a high expression of Foxf2.

In future experiments, I plan to investigate the molecular mechanisms underlying *Foxf2*-mediated apoptosis by comparing the expression profile between the control cells and cancerous cells with Foxf2 overexpression via RNA-seq.

Research on genes such as fox F2 allow scientists to better understand how cancer develops in humans. This allows them to design drugs that target specific pathways that will help reduce cancers. However, even after developing these drugs, it is still extremely difficult for patients to be able to access these drugs. For example, several years ago, a drug called ipilimumab It was approved by the Food and Drug administration otherwise called the FDA for the treatment of metastatic Melanoma. The benefit and survival compared to standard treatment was 3.7 months in previously treated patients and 2.1 months in previously untreated patients. However, the cost was around 120,000 for four doses. These extremely high prices cause it to be very difficult for patients even with insurance to be able to have access to these drugs that have been shown to be highly effective in treating certain cancers.

In a healthcare system in which third party payers whether it be private or governmental, cover the cost of cancer treatment and the insured public has presumed and legal right to access to all these approved drugs, the soaring price of cancer drugs poses at least three major problems. First, when every drug regardless of clinical importance is approved come on it costs a lot of money to be able to maintain that and in the long run it is an unsustainable practice. Second it becomes problematic when insurance companies are not able to price their policy premiums accurately because the approval, the clinical acceptance, and the incorporation of these new drugs are often unpredictable and vary based on location. Thus, many insurance premiums are kept high to be able to keep up with the cost of care. 3rd, almost all approved cancer drugs are eventually used for conditions in settings not approved by the FDA.

A study conducted in 2014 showed that cancer patients paid nearly $4 billion out-of-pocket for cancer treatments. And with the rising costs of healthcare, this number would only be larger as the years go on.

The high cost of cancer drugs are related to a number of factors. First of all it's very expensive to move findings from the bench like what I did to bedside and to perform all the regulatory studies to gain approval. The FDA process is very long and complicated and requires several years of testing on patients to be able to determine whether a drug is safe. Second, since most cancers are incurable, patients are treated with each approved drug treatment creates a virtual monopoly because the use of one drug does not automatically mean that there is no need for anything else. Even when this monopoly is broken with the arrival of a new or

approved version of the drug, the older drug tends to be viewed as a substandard treatment, and doctors and patients are not willing to use it. Finally, inherent in this system is the incentive to administer more chemotherapy and legal barriers that prevent agencies such as the FDA from taking economic and cost-effective considerations into account when approving new drugs.

So some of you may wonder why drug development costs are so high. This is often because many years and millions of dollars are often spent in Preclinical Research to identify a compound or design a drug, describe its mechanism of action, and generate preclinical data. Pharmaceutical companies spend $50 billion on research and development in 2008. Once ready for clinical testing, careful administrative trials, large patient sample sizes, and long follow-ups are required aspects of good clinical research period this costs all add up. Each drug is estimated to cost around 1.2 to $1.3 billion in research and development. Even though the patent is valid for around 20 years it takes around eight years to go from clinical testing to regulatory approval. This means that the actual patent life of a drug is often less than 10 years which is very limited time. The retail prices of drugs are a function of the cost of development, the addressable patient population, the patent life, and the projected returns on investment.

I think that cancer drugs represent a monopoly. This is partly due to the complex nature of cancer and also partially due to how our Healthcare system is set up. even with all this Cancer Research going on, little is known about cancer as compared to many other diseases. a search, most patients currently receiving chemotherapy will likely succumb to their disease. Because of this, there is no competition among truly effective cancer drugs to lower their cost. As compared to other less complex and curable conditions, commercial companies have been successful in developing multiple effective drugs. Since there are many substitutes, competition among the pharmaceutical companies keeps prices reasonable. For example in treating pneumonia there are different types of antibiotics that can be given to a patient. If a patient starts antibiotics it is cured, the patient will not need to use any other antibiotics. thus, this causes a genuine competition between pharmaceutical companies to provide the most effective product with the least adverse effects at the best value. however, most cancers are not curable and many approved drugs work only for a limited time. When one treatment fails, the patient will move on to other drugs until there are no more options. For example, say there are three approved drugs like in treat a particular incurable cancer. The viability of these three options does not produce the necessary competition to keep the prices down. This is because unlike the curable pneumonia that I talked about earlier, One drug does not preclude the concurrent or subsequent need for the other drugs. Rather, it is likely that the patient will use all of these drugs over the course of their treatment. Thus, each drug is an effective monopoly because each one will be used at some point during the course of a patient's illness. as in a monopoly, drugs that extend the survival of patients with incurable diseases, can be priced at whatever price patients are willing to pay.

This fact is only exacerbated by the lack of a true generic price check. Typically when a drug is first developed, the original developer enjoys a couple years where their patent is valid and they can charge whatever price they want for the drug. When the patent runs out, other companies are able to swoop in and make a more cost-effective drug. Those companies are not able to create new and improved treatments that simply offers incremental benefits over established treatments but costs considerably more than the generic versions because the

generic version works fine. However in the case of cancer which is life threatening and carries a much heavier weight, older drugs are often viewed as a substandard treatment option.

So in light of all these issues with the current system, what policies or interventions can we use to lower the cost of cancer drugs? I think some solutions include value-based reimbursement and pricing, improved national guidelines, and the creation of monopoly rules. To better understand the link between improved population outcomes and cost savings, value-based pricing pilot programs are currently under way for example in Maryland they launched the primary care medical home program in 2010 with the goal to improve quality care and slow the rise of health care costs. If a primary care physician elects to participate in the program, the physician receives a 12% increase to the current reimbursement fee schedule, $300 for creating and following a care plan for selected patients with chronic diseases, and shared savings from decreased healthcare expenditures over three years. Value based pricing is extremely important and it requires an economic analysis of the benefit provided to the patient. Quality adjusted life years or QALYs are the number of years of life that will be added by an intervention adjusted for quality of life. This is a key metric that is often used when assessing the value for money of a drug or device. Once the QALY is determined, the next step is to calculate the incremental cost effectiveness ratio or the ICER. The ICER per QALY calculation is an important metric but it does have certain limitations. Thus this cannot be considered in isolation. The calculation needs to be compared with a threshold that has been determined to be acceptable by some entity, whether it be society, patient, a physician, or a payer.

Current cancer guidelines often present a list of possible or acceptable treatments but they do not provide a comparative analysis to how patients and physicians choose the most cost effective option based on risks and benefits. There's a great need for national evidence-based guidelines that critically examine quality of life and mortality data, the S benefit and light not only risks but also cost and that provide transparency on the most cost-effective option. This approach will highlight the cost utility of a proposed treatment and help patients and physicians make better informed treatment decisions.

In the United States, where there is an emphasis on market forces to regulate prices, value-based pricing for all drugs may not be necessary; we need price controls only in “monopoly” situations that I mentioned before. First, drugs that are considered to be in such a niche will need to be subjected to price controls or more competition. the given monopoly drug should be subject to some legally mandated price control after the drug is approved. This process is best overseen by a panel of health care professionals who have no financial or nonfinancial conflicts of interest in the success of the drug. If a mandatory system is impossible to set up, at least a voluntary system could be set up in which pharmaceutical companies agree to such adjudication and imposition of price controls in exchange, for example, for expedited review or accelerated approval based on phase 2 studies and surrogate end points. Second, an important option for reducing monopoly situations is to approve additional drugs for the same indication based on equivalence and the same strength of data without a requirement to show superiority in safety or efficacy over the older approved drug (s).

Ultimately, we as a society must find a balance between health care affordability and profits that will provide the necessary incentive for continued innovation. Not doing so risks creating a health care system in which all participants lose.