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Research Plan 2019

A. Rational:

Prostate cancer is one of the most widespread cancers affecting the male population in the United States causing over 30,000 deaths each year. A large disparity in survival rate exists between people diagnosed with metastatic prostate cancer (30%) and those diagnosed with non-metastatic prostate cancer (99%), but the cause of this disparity is unknown (ASCO, 2019). This may be due to effects non-tumor cells have on prostate cancer cells; it has been demonstrated that cultured cancer cells develop differently when exposed to normal body cells (Zhau et al., 1997). However, more research is necessary to reveal a connection between non-prostate cells and cancer cells. Organoids have the potential to save researchers time and money if they can model all of prostate cancer's phenotypes based on the set of mutations they express.

Organoids are a valuable tool in cancer research as they model internal conditions more accurately than traditional two dimensional cultures and allow for cell evolution. They can also transplanted into a mouse model to confirm results and link the *in vitro* results to *in vivo* results (Lee et al, 2018). This allows researchers to assess the role of factors, such as metabolism and genomics, on cancer development or predict the effectiveness of a new drug (Walsh et al, 2016). Organoids have successfully been used to model multiple forms of cancer in the past. Breast cancer cells in human-based and xenograft-based organoids will be able to model disease progression, drug response, and drug resistance (Walsh et al, 2014). Organoids are extremely useful for research, but they need to contain all of the cells located in the organ that is being recreated to be an effective model. Small changes in the type of cell present or concentration of various cells can have a large impact on organoid development (Campbell et al, 2011). However, human-derived prostate organoids are difficult to make, and the accuracy of mouse-derived prostate organoids in modeling aspects of prostate cancer is unknown (Risbridger, Toivanen, & Taylor, 2018).

Researchers have successfully used mouse models to gain tremendous insight into the metabolic and genetic aspects of cancer. This research has shown great variation within prostate cancer due to the activation of different genes and expression of different proteins. For example, AKT1 production mainly increases glycolysis and metabolism outside the mitochondria. Myc overexpression, on the other hand, leads to increased fatty acid metabolism and mitochondrial metabolism (Fan, Dickman & Zong, 2010; Priolo et al, 2010; Gouw et al, 2015). Various genes have been associated with these proteins and the development of prostate cancer. The Phlpp2 gene is an important suppressor of AKT production, but it also stabilizes the Myc protein. Phlpp2 inhibition in cancer cells has been shown to reduce cell division and metastasis (Nowak et al, 2019). These traits are enhanced when both the Pten and P53 genes are deleted because this dual deletion increases Phlpp2 expression (Nowak et al, 2015). Further, both Pten and P53 are tumor suppressors, so their loss will contribute to the formation of tumors (Ho et al, 2005;

Kluth et al, 2014). The Pten gene is a less important tumor suppressor as its deletion may not always cause the development of cancer (Radziszewska et al, 2008; Kim et al, 2009).

B. Hypothesis:

This study looks to create a line of organoids to gain insight into the viability of organoids in modeling prostate cancer progression and phenotype. It is predicted that $Pten^{-/-}$ organoids will demonstrate minimal cancer formation compared to the wild type (no deletions) organoid. The $Pten^{-/-}$ and $P53^{-/-}$ organoids will have the most aggressive cancer phenotype and metastases, and the $Pten^{-/-}$, $P53^{-/-}$ and $Phlpp2^{-/-}$ organoids will develop non-metastatic cancer. This will be determined through analysis of Ki67, c-Myc, and Akt protein levels in the organoids.

C. Methodology:

Procedures: A previously harvested prostate organ, with cells containing Lox-P sites around their genome, from an adult male mouse will be used to create organoids. First, the prostate tissue will be minced with scalpels for four minutes to begin breaking it down. Then, the tissue will be suspended in 20 mL of TrypLE buffer and will sit for 90 minutes in the OHAUS shaker (150 rpm at 37°C) to separate cells. The solution will then be centrifuged (1000g for 1 minute) to form a pellet of cells. After discarding the supernatant, 10 mL of TrypLE will be added. The pellet will be resuspended and passed through a syringe two times to prevent clumps of cells from passing through. The remaining solution will be centrifuged again (1000g for 5 minutes), and the resulting pellet will be resuspended in 2 mL of full media. This will be passed through a cell strainer cap. This solution will be re-centrifuged (1000g for 5 minutes) and the supernatant will be removed. The cell pellet will be resuspended in 88 µL of full media and 264 µL of matrigel (1:3 ratio between media and matrigel). This solution will be plated and incubated at 37°C in a six well plate to form four groups of organoids. The plate will be flipped upside down after one minute to prevent sinkage and collapse into two dimensional culture. It will be flipped again after one hour, and 2 mL of media will be added to each well.

The organoids will be allowed to grow for three days before they are passaged (re-plated) and exposed to adenovirus containing the Cre. Organoids will first be detached from the well using TrypLE. They will incubate in the TrypLE for five minutes to break apart the matrigel and separate cells. Medium will then be added and the solution will be centrifuged (1200 rpm for three minutes). The TrypLE-medium solution will be aspirated and the remaining cell pellet will be resuspended in 1.5 mL of DMEM full medium. Each resuspended cell pellet will be split into two tubes; one will be a control and will not receive virus while the other one will be the experimental group and receive the virus. 2.4 uL of virus will be added to each experimental tube, while 2.4 uL of PBS will be added to the control tubes. The virus will be removed by centrifuging the tubes to pellet the cells. Viral medium will be aspirated and the cells will be resuspended in 264 uL of medium and 792 uL of matrigel. After being passaged, the organoids

will be allowed to continue growing. A second set of organoids will be created using the same procedures, but cells will be passed through a filter before the virus is added.

Proteins will then be extracted from cells in the organoid to run a western blot analysis. This will check the expression of Pten, P53, and Phlpp2 to ensure the Cre-loxP system was successfully injected into cells by the virus; it will also ensure that the Cre-loxP system worked. The western blot will analyze concentrations of the AKT and Myc oncoproteins, as well as the three proteins of interest (Pten, P53, and Phlpp2), to ensure the organoids retain *in vivo* genetic relationships and the knockouts are successful. Cells will first be lysed using RIPA lysis buffer to release proteins. Lysates will be sonicated for ten minutes at the high bioruptor level in 30 second intervals separated by 30 second rest periods. A protein concentration assay will be conducted to determine the volume of protein needed in each well to ensure equal mass. To determine concentration, 7uL of lysate from organoids will be combined with 693 uL of assay dye. Lysate samples with dye will be plated alongside a set of standards on a 96 well plate. Standards (ranging from 2,000 ug/mL protein concentration to 0 ug/mL) will be created by serial dilution. 28 uL of BSA will be added to one test tube, and 14 uL of NFW (Nuclease free water) will be added to seven other tubes. Half of the BSA will be moved from the first tube (2,000 ug/mL) and mixed into the second tube(1,000 ug/mL). This process will be repeated until there is BSA in all tubes except the last one (0 ug/mL). Instead of being mixed into the zero tube, half of the BSA-NFW solution in the seventh tube (31.25 ug/mL) will be disposed of. 686 uL of assay dye will be added to each standard before planting. The Plate Reader will determine protein concentration of all samples. After obtaining these results, the western blot gel will be run for 1.5 hours at 100 volts. The resolving gel will be created using 4.6 mL water, 2.7 mL 30% acrylamide, 2.5 mL of 1.5 M Tris base, 100 uL of 10% SDS, 100 uL of 10% ammonium persulfate, and 6 uL of TEMED. The stacking portion of the gel consisted of 2.1 mL of water, 0.5 mL of 30% acrylamide, 380 uL of 1 M Tris, 30 uL of 10% SDS, 30 uL of 10% APS, and 3 uL of TEMED. To prepare samples for gel running, 58 uL of 4x Laemmli buffer and 12 uL of β-mercaptoethanol will be added to 160 uL of remaining sonicated lysate. The iBlot machine will be used to transfer proteins from the gel to a membrane which will allow them to be exposed to antibodies. Membranes will sit in blocking solution for one hour before rocking in primary antibodies overnight. They will be washed the next day allowing the addition of secondary antibodies. Secondary antibodies will be removed and membranes will be washed after one hour; BioRad detection reagents will then be added allowing the results to be developed. A film will be exposed to the membrane in a dark room to make the results visible. Hyperfilm will be exposed to the membrane for 15 second, 30 second, one minute, two minute, four minute, eight minute, and one hour intervals.

To prepare organoids for immunofluorescence, the medium in the slide well will first be replaced with 300 uL of 4% paraformaldehyde. Organoids will then be washed in IF buffer (solution of 0.2% Triton X-10 and 0.05% tween in PBS) to remove the paraformaldehyde. Permeabilization solution will be added to the wells to allow antibodies to enter the cell, and it

will be washed out with IF buffer after 30 minutes. Organoids will then sit in blocking solution containing primary antibodies overnight. Primary antibodies stained for CK5, CK8, Pten, androgen receptor, Myc, and Akt. Before secondary antibodies are added, the primary antibody-containing blocking solution will be removed by washing organoids three times in IF buffer. DAPI will be added at the same time as secondary antibodies. Both DAPI and secondary antibodies will be removed by washing the organoids in 300 uL of IF buffer for 15 minutes. IF buffer will then be drained from the wells, and the chambers will be detached leaving just the microscope slide with organoids. Each organoid dome will be coated in 25 uL of ProLong Gold antifade mounting medium before the slide is sealed with the cover slip. Imaging will be conducted using the Zeiss 880 confocal microscope.

Risk and Safety: Potential risks involved in this project include exposure to various hazardous chemicals or viral agents used in the experiment. However, these risks will be limited by safety precautions used. Dangerous chemicals will be used in diluted concentrations, and all work with such substances occurred under a chemical fume hood or biological safety cabinet. Work with viral agents (such as adenovirus) took place in a biosafety cabinet. This prevented release of infectious agents into the environment. All items that potentially came into contact with viral agents will be soaked in 10% bleach. The biosafety cabinet was also cleaned with 70% ethanol after use, and the UV light was turned on when the cabinet was not in use. Additionally, exposure to these chemicals and viral agents was further limited by wearing a lab coat, gloves, and goggles at all times. Personal Protective Equipment ensured that no skin was exposed.

Data Analysis: Western blots will be conducted on organoid cultures to gain insight into their protein expression. The presence/absence of bands will be compared, and the darkness of bands will also be compared to qualitatively compare protein expression. Immunofluorescence imaging will also be conducted on organoid cultures to further compare their expression of various proteins.

3. POTENTIALLY HAZARDOUS BIOLOGICAL AGENTS RESEARCH:

- Adenovirus:
    - Source: University of Iowa (Lot#Ad4160)
    - Biosafety Level: Two
    - Safety Precautions: Gloves, a lab coat, and safety goggles will be worn at all times when handling adenovirus, and all work will be done in a biosafety cabinet

to minimize risk of exposure. All items that come into contact with adenovirus will be decontaminated using bleach and an autoclave machine. Liquid waste that may have been contaminated with adenovirus, such as viral medium, will also sit in bleach overnight to kill the virus. The biosafety cabinet will be cleaned using 70% ethanol after adenovirus is used, and UV radiation, which will be on overnight, will further sterilize the cabinet. Gloves will also be sprayed with 70% ethanol when entering and leaving the cabinet, and they will be disposed of in biohazardous waste containers for proper disposal.

- Mouse cells in organoid
    - Source: Nowak Lab, Weill Cornell Medicine
    - IACUC Approval Number: 2018-0017
    - Biosafety Level: Two
    - Safety Precautions: Prostate tissue will only be handled under a biosafety cabinet while wearing gloves and a lab coat. Items used to hold or handle tissue/cells will be autoclaved or disposed of in biohazardous waste containers for proper disposal.

4. HAZARDOUS CHEMICALS, ACTIVITIES & DEVICES:

- Paraformaldehyde (4%)
    - Risk Assessment: PFA has a high risk due to potential flammability and toxicity to humans. It can irritate the skin and damage the respiratory system if inhaled.
    - Safety Precautions: PFA will only be used under a fume hood to minimize exposure. A lab coat and gloves will also be worn at all times when handling it to prevent harm if there is a spill. Risks of using PFA is further limited by using a low concentration of it and storing it at 4℃ to prevent it from catching fire. It will be disposed of in on-site biohazardous waste containers to prevent contamination. Additionally, items, such as test tubes and pipette tips, that are used in handling it will be disposed of in biohazardous waste containers as well. PFA will be disposed of in a special waste container, not in biohazardous waste containers.
- 1x β-mercaptoethanol
    - Risk Assessment: β-mercaptoethanol has a high associated risk due to flammability and toxicity. It is harmful if ingested and can irritate the skin.
    - Safety Precautions: β-mercaptoethanol will only be used under a fume hood to minimize exposure. A lab coat and gloves will also be worn at all times when handling it to prevent harm if there is a spill. It will be disposed of in on-site biohazardous waste containers to prevent contamination. Additionally, items, such as test tubes and pipette tips, that are used in handling it will be disposed of

in biohazardous waste containers as well. β-mercaptoethanol will also be stored at 4℃ to minimize the risk of the chemical catching fire.

- 10% Bleach
    - Risk Assessment: Bleach is moderately reactive and can be an irritant if it comes into contact with the skin. It is not a fire hazard.
    - Safety Precautions: Beakers containing bleach for sterilization will be covered in aluminum foil to prevent the release of hazardous fumes. Additionally, gloves, a lab coat, and goggles will be worn when handling bleach to ensure no skin can be exposed to the bleach.
- 70% Ethanol
    - Risk Assessment: Ethanol is flammable and can irritate the skin on contact. It is also hazardous if ingested as it can damage a variety of organs.
    - Safety Precautions: Gloves and a lab coat will be worn when handling ethanol to prevent contact with the skin. It will also be kept in sealed containers and kept away from heat to prevent a fire from starting.
- 1 x TrypLE
    - Risk Assessment: There is a low risk associated with TrypLe. It is not flammable, highly reactive, or toxic to humans, but it can break apart intercellular connections.
    - Safety Precautions: It will be used in a biosafety cabinet when creating/passaging organoids. Gloves and a lab coat will be worn at all times when dealing with it, so no skin will be exposed. This will prevent TrypLe from affecting body cells. Finally, all pipette tips used to handle TrypLe will be discarded into a biohazardous bag on site for proper disposal.
- 4 x Laemmli buffer
    - Risk Assessment: Laemmli buffer is not a flammable chemical and it is stable. It is not an irritant either, so it has a low associated risk.
    - Safety Precautions: It will be used under a fume hood and only handled while wearing gloves and a lab coat. Finally, all pipette tips used to handle Laemmli buffer will be discarded into a biohazardous waste bag on site for proper disposal.
- TEMED (Tetramethylethylenediamine)
    - Risk Assessment: TEMED is flammable chemical that can irritate or burn the skin, but it has a low reactivity rating.
    - Safety Precautions: Gloves and a lab coat will be worn at all times when handling TEMED to prevent exposure to the skin. All work will be done in a chemical fume hood to prevent exposure to harmful vapors. Finally, all pipette tips used to handle TEMED will be discarded into a biohazard bag on site for proper disposal.
- 30% Acrylamide
    - Risk Assessment: Acrylamide can cause irritation if it comes into contact with

skin, and it is a potent toxin when inhaled or ingested. It also has a minor associated risk due to its flammability and reactivity.
    - Safety Precautions: Acrylamide will be stored in diluted concentrations and kept in a sealed container to prevent exposure to light and water. Additionally, it will only be handled under a fume hood while gloves and a lab coat are being worn.
- 10% Sodium Dodecyl Sulfate
    - Risk Assessment: SDS is a potentially toxic irritant to the skin, but it is not a flammable or highly reactive chemical.
    - Safety Precautions: Gloves and a lab coat will be worn at all times when handling this substance to prevent it from touching the skin. The stock of SDS is also at a low concentration to minimize risk and ensure safety. All objected used in handling SDS will be disposed of in biohazardous waste containers.
- Tris Base (1 M and 1.5 M)
    - Risk Assessment: Skin irritant that can cause damage to the eyes and respiratory system if inhaled
    - Safety Precautions: Tris will be handled under a chemical fume hood to minimize exposure to vapors, and a lab coat and gloves will be worn at all times when handling it to prevent it from touching the skin. Additionally, pipette tips and other items used in handling Tris will be disposed of in biohazardous waste bags for proper disposal.
- 10% Ammonium persulfate
    - Risk Assessment: APS can irritate the skin and respiratory system, if inhaled. It is not flammable, but it is a reactive oxidizing compound
    - Safety Precautions: APS will only be used under a chemical fume hood when in liquid form to minimize exposure through vapors. Gloves and a lab coat will be worn at all times when using APS. The low concentration of the chemical further limits the risk associated with using it. All objected used in handling APS will be disposed of in biohazardous waste bags. Additionally, it will be stored in a dark closed container kept at a low temperature (4℃).
- Biorad detection reagent
    - Risk Assessment: The reagent is a mild irritant. There is little danger in its reactions, and it is not flammable.
    - Safety Precautions: These reagents will be used according to procedures provided by the manufacturer to ensure safety. Gloves and a lab coat will be worn at all times when working with the buffers.All objected used in handling the detection reagents will be disposed of in biohazardous waste canisters.
- 10 x RIPA lysis buffer
    - Risk Assessment: 10 x RIPA lysis buffer can corrode or irritate the skin, and it can also have damaging effects on ecosystems due to bioaccumulation. However,

it is not a flammable chemical.
    - Safety Precautions: This chemical will only be handled while gloves and a lab coat are worn to protect the body, and work will occur under a hood to further ensure safety. Additionally, all items that come into contact with the buffer will be placed in biohazardous waste containers for proper disposal.
- Triton X-100
    - Risk Assessment: Triton x 100 is a toxic chemical if ingested, and it can also damage the eyes. It is a stable and non-flammable chemical.
    - Safety Precautions: Gloves and a lab coat will be worn at all times when handling Triton x 100. Additionally, work will be completed in a chemical fume hood to prevent exposure to fumes. All items that come into contact with Triton x 100 will be placed in biohazardous waste containers for proper disposal. It will also be diluted to Triton x 10 for regular use.
- Tween 20
    - Risk Assessment: Tween 20 has the potential to cause minor irritation if it comes into contact with skin, but it has no other health related effects. It has a low risk of flammability. There is no risk associated with its reactivity.
    - Safety Precautions: Tween 20 will only be handled while wearing gloves and a lab coat. All items that come into contact with it will be placed in biohazardous waste containers for proper disposal.
- 2,000 ug/mL BSA (bovine serum albumin)
    - Risk Assessment:
    - Safety Precautions: A lab coat and gloves will be worn when handling BSA to prevent the skin's exposure to the chemical. All objects used in handling BSA will be disposed of in biohazardous waste bags. It will also be stored at 4℃
- FBS (fetal bovine serum)
    - Risk Assessment: FBS can potentially cause irritation if it comes into contact with skin or the gastrointestinal tract. It is not flammable, and there is no danger involved in its reactions.
    - Safety Precautions: Gloves and a lab coat will be worn at all times when handling FBS for protection. All objects used in handling FBS will be disposed of in biohazardous waste containers for proper disposal. It will also be stored at 4℃
- Ampicillin (100 mg/mL)
    - Risk Assessment: Ampicillin can cause an allergic reaction for some people, but there are no other health related risks for humans. There are no risks associated with reactivity when using ampicillin. It is not a flammable chemical.
    - Safety Precautions: Ampicillin will be stored at -20℃. Gloves and a lab coat will be worn when handling it, and all objects used in handling ampicillin will be disposed of in biohazardous waste canisters to be disposed of properly.

- 1 x Matrigel
    - Risk Assessment: Matrigel is not flammable or reactive, and there are no health risks associated with using the chemical.
    - Safety Precautions: Matrigel will be handled while using gloves and a lab coat. All pipette tips and other objects that come into contact with matrigel will be placed in biohazardous waste bags for proper disposal.
- 1 x DMEM full medium
    - Risk Assessment: There are no health risks associated with using 1 x DMEM medium as it has no effects on humans. It is not a flammable substance, and it does not cause dangerous reactions.
    - Safety Precautions: Proper protective equipment, gloves and a lab coat, will be worn at all times when using the medium. All pipette tips used to handle the medium will be discarded in biohazardous waste containers. Additionally, it will be aspirated into 10% bleach when it contains viral particles or organoid cells.
- 1 x Phosphate Buffered Saline (PBS)
    - Risk Assessment: There are no health, reactivity, or fire risks involved in using 1 x PBS. It is a stable, non-flammable compound that does not affect human health or cause irritation.
    - Safety Precautions: PBS will only be handled when wearing gloves and a lab coat. All objected used in handling PBS will be placed in biohazardous waste containers for proper disposal.
- 50 x TAE (Tris Acetate EDTA buffer)
    - Risk Assessment: TAE buffer can irritate the skin or eyes if it comes into contact with them, and it can irritate the respiratory system as well. However, it is a stable and non-flammable compound.
    - Safety Precautions: TAE buffer will only be handled when gloves and a lab coat are being worn. The 50 x TAE buffer will also be diluted to 1 x TAE for general lab use. Containers used to hold TAE buffer will be washed and autoclaved, while pipettes and other objects used to handle the buffer will be placed in biohazardous waste containers.
- Agarose
    - Risk Assessment: Agarose can cause irritation if it comes into contact with the skin or eyes. It can also cause irritation of the gastrointestinal tract if ingested. It is a stable compound that is not flammable.
    - Safety Precautions: Agarose will only be handled when wearing gloves and a lab coat to prevent the chemical from coming in contact with the skin. All objects used in handling agarose will be disposed of in biohazardous waste bags so they can be properly disposed.

All information on chemicals came from the manufacturer's material safety data sheet (MSDS)

D. Bibliography

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THERE ARE NO ADDENDUMS TO THIS RESEARCH PLAN