PROJECT SUMMARY: FULL PROJECT 2

Even though the growth of prostate cancer (PCa) is largely driven by androgens, a subset usually develops that is refractory to androgen ablation (also known as castration resistant PCa; CRPC) with potential for metastasis. Preliminary data from our laboratory has implicated fetuin-A, also known as alpha 2-Heremans-Schmid glycoprotein (AHSG), in the growth of PCa cells and in the production of "uptake-competent" exosomes. The objective of the proposed studies is to define the role and significance of fetuin-A in prostate cancer progression. We hypothesize that PCa cells express ectopic fetuin-A which is secreted and taken up by the cells via TLR4 to mediate the biogenesis of 'uptake-competent' exosomes that promote PCa growth via activation of pAKT/pERK; moreover, we postulate that elevated fetuin-A expression serves as a prognostic biomarker for PCa. Three specific aims are proposed: **Aim 1. To determine if fetuin-A expression is higher in AA PCa tissues relative to Caucasian American (CA) PCa tissues and whether high fetuin-A expression is associated with high Gleason Scores (>6) and enhanced pAKT and pERK.** We will analyze mRNA expression of fetuin-A using NanoString as well as pAKT/pERK protein levels using immunohistochemistry (IHC) analysis of human PCa tissues. Multivariable linear regression analysis will be used to determine the correlation between fetuin-A, pAKT, pERK and Gleason scores in PCa tissues of AA and CA patients, as well as other progression parameters such as positive margins and spread of PCa. It is expected that fetuin-A, pAKT and pERK will be expressed at high levels in PCa tissues of AA patients particularly those with high Gleason scores (>6). **Aim 2. To determine the role of ectopic fetuin-A in exosome biogenesis, promotion of 2-D and 3-D growth, motility and invasive capacity of PCa cells.** In this aim, we will overexpress and knockout fetuin-A in two PCa cell lines to determine whether fetuin-A plays a causal role in the biogenesis of 'uptake competent' exosomes that transmit growth signals in recipient cells. We expect to demonstrate that exosomes from fetuin-A overexpressing cells will promote 2-D, 3-D growth and motility and invasion of PCa cells while exosomes from fetuin-A null cells will not. **Aim 3. To investigate the efficacy of targeting fetuin-A mediated signaling on the suppression of prostate tumor initiation and growth in mice.** In this aim, we will utilize the *Pten*-null mouse model for PCa to determine whether Pten loss requires intact fetuin-A gene to mediate its tumorigenic role and whether loss of fetuin-A in *Pten*$^{-/-}$*/fetuin-A*$^{-/-}$ double mutant mice attenuates the tumorigenic role of Pten-null. We expect reduced tumor growth in the double mutant mice compared to *Pten*-null fetuin-A$^{+/+}$ mice. Significance: There is an urgent need to identify biomarkers that can differentiate CRPC from indolent PCa and this proposal addresses that need and evaluates the process by which fetuin-A enhances PCa tumor growth.

SPECIFIC AIMS: FULL PROJECT 2

Prostate cancer (PCa) is a leading cause of cancer related death in American men. African American (AA) men are disproportionately affected by this disease, where their death rate is almost twice that of Caucasian American (CA) men. The molecular signaling pathways that drive PCa progression, in particular castration resistant PCa (CRPC), have yet to be fully elucidated. Data from our laboratory and others have implicated fetuin-A synthesized by PCa to promote their growth. More importantly, we show that fetuin-A, also known as alpha 2-Heremans-Schmid glycoprotein (AHSG), is expressed at a higher level in prostatic cancer tissues of AA compared to CA men. The objective of the proposed studies is to dissect the role and significance of ectopic fetuin-A in localized PCa and to determine if it contributes to poor disease outcomes such as high Gleason Grades (GG) and CRPC. Fetuin-A is a multi-functional glycoprotein with roles ranging from inhibition of ectopic calcification to tumor growth promotion. We hypothesize that PCa cells express ectopic fetuin-A, which is secreted and taken up by the cells via Toll Like Receptor-4 (TLR4), to mediate the biogenesis of 'uptake competent' exosomes that promote PCa growth via activation of pAKT/pERK. Moreover, we postulate that fetuin-A expression levels-serve as a prognostic biomarker for PCa. Three specific aims are proposed.

Aim 1: To determine if fetuin-A expression is higher in AA PCa tissues relative to CA PCa tissues and whether high fetuin-A expression is associated with high Gleason score (>6) and enhanced pAKT and pERK. Expression of fetuin-A mRNA will be analyzed from a pool of over 1,000 frozen PCa tissues by NanoString and will be correlated to Gleason scores. We will also analyze TMAs representing over 1,000 patients for fetuin-A, pAKT and pERK expression by IHC in reference to Gleason scores and patient outcomes. We expect elevated levels of fetuin-A, pAKT and pERK in AA PCa tissues with high Gleason scores (>6) relative to CA patients.

Aim 2: To determine the role of ectopic fetuin-A in exosome biogenesis, promotion of 2-D and 3-D growth, motility and invasive capacity of PCa cells. The working hypothesis is that fetuin-A mediates the biogenesis of 'uptake competent' exosomes that promote not only 2-D and 3-D growth of PCa cells but also their invasive capacity. We will overexpress and knockout fetuin-A in two PCa cell lines; AA-MDA-PCa 2b and CA-LNCaP. The cells used in the experiments will be divided into three groups: 1) transfection controls (TC), 2) fetuin-A overexpressing cells (Fet-A-OE) and 3) fetuin-A knockout cells (Fet-A-KO). The growth of these cells in 2-D, 3-D as well as *in vivo* as xenografts, motility and invasion will be evaluated. Exosomes will be isolated from TC (Exoso-1); Fet-A-OE (Exoso-2) and Fet-A-KO (Exoso-3) cells and used to stimulate the 2-D and 3-D growth, as well as invasive potential of naïve MDA-PCa 2b and LNCaP cells in serum free medium (SFM). The levels of pAKT and pERK in the stimulated cells will be analyzed by western blotting. It is expected that Fet-A-OE cells will have the most robust growth/invasion in SFM and similarly Exoso-2 will show the most growth promoting potential in 2-D, 3-D, motility and invasive potential. The corollary is that Fet-A-KO cells as well as Exoso-3 will show the least growth/invasion and promotion potential respectively.

Aim 3: To investigate the efficacy of targeting fetuin-A mediated signaling on the suppression of prostate tumor initiation and growth in mice. The working hypothesis is that fetuin-A potentiates the sustained pAKT activation in *PTEN*-null tumors via the synthesis of bioactive exosomes. The approach involves use of a Pten-null mouse model for prostate cancer initiation and progression. This model was developed by Zhenbang Chen, PhD, Meharry co-Investigator. We will generate Pten-null/fetuin-A null mouse model to specifically determine how fetuin-A affects PCa progression in the established Pten-null mice. To generate the *Pten*-null/*Fet-A*-null double mutant, we will cross female Fet-A knockout mice with male $Pten^{LoxP/+}$; *PB-Cre4* (*Probasin-Cre4*) mice. We will obtain $Pten^{LoxP/LoxP}$, $Fet\text{-}A^{-/-}$ and PB-Cre4 (prostate specific) compound mutant mice referred to as $Pten^{pc-/-}$ - $FetA^{-/-}$ which we have designated *Pten/Fet-A* among the third-generation progenies. The Cre-recombinase is only activated by the androgen-AR signaling, therefore the inactivation of Pten and Fet-A in these progenies is prostate specific after the male mice reach puberty. Moreover, mice negative for PB-Cre4 are like wild-type (WT). It is expected that loss of fetuin-A will reduce the tumor volume and attenuate the PI3 kinase/AKT signaling pathway in *Pten* mutant mice.

Impact: The proposed studies will determine if fetuin-A expression is associated with high Gleason scores (>6) and positively associated with PI3 kinase/AKT-MAP kinase signaling in PCa tissues and poor prognosis. We will also determine if fetuin-A-OE promotes *in vitro* and *in vivo* growth of these cells under different conditions including absence of androgens. Knockout of fetuin-A in these cells will determine if growth is attenuated particularly in the absence of androgens. The studies have the potential to lead to the discovery of new classes of PCa treatment and prevention drugs for CRPC.

RESEARCH STRATEGY: FULL PROJECT 2

A. BACKGROUND AND SIGNIFICANCE

Prostate cancer (PCa) is the second most life-threatening malignancy in American men.[1] In 2020, about 190,000 new PCa cases have been confirmed in the US, with 33,000 deaths projected.[2] Surgery, radiation and hormone deprivation therapy are common strategies for treating PCa. However, PCa patients often die of relapse due to the recurrent growth of PCa cells that result in castration-resistant prostate cancer (CRPC). The molecular signaling pathways that contribute to PCa progression, particularly those leading to CRPC, remain elusive. Moreover, the incidence of and mortality rate for PCa are disproportionally higher in African American (AA) males compared to other ethnic groups. Specifically, the PCa death rate for AA men is 2.5-fold higher than that for Caucasian American (CA) men.[3] Therefore, novel and effective biomarkers for diagnosis and prognosis in addition to therapies to cure/control or manage PCa are urgently needed. More importantly, detailed understanding of the mechanisms involved in PCa progression, such as the development of CRPC, will go a long way to introduce more effective targeted therapies likely to eliminate the current disparities in mortality and PCa disease sequelae in the two racial groups. Previous studies have demonstrated that PCa growth, including progression to CRPC, results from the activation of oncogenic pathways.[4-7] These pathways include aberrant elevation of pAKT, PI3K, SKP2, EZH2, c-Myc, ERG, NF-kB and TGF-β detected in various human cancers, including PCa.[8-14] Several *in vitro* and *in vivo* studies have shown that overexpression of these oncogenes leads to the initiation and development of malignancy.[11,15-17] In addition, tumor suppressor genes such as PTEN, TP53, p27, RB, Nkx3.1 and p57 are frequently deleted or mutated in PCa.[18-25] PTEN loss is detected in up to 50% of PCa cases, as well as in breast, lung cancers, glioblastoma and endometrial carcinoma.[26-28] Reduction of PTEN protein levels to various extents – hyper (25% loss), het (50% loss), hypo (75% loss) and biallelic (100% loss) – is associated with the initiation and progression of PCa.[29-30] PTEN protein levels are also regulated through post-translational modifications such as ubiquitination and sumoylation.[31-34] Interestingly, PTEN protein loss results in the hyper-activation of AKT, which in turn activates many downstream pathways to drive PCa progression and CRPC growth.[6,33,35,36] In addition to the oncogenic and tumor suppressor genes whose roles in PCa progression have been extensively investigated, the proposed studies focus on fetuin-A, a glycoprotein whose increased synthesis by PCa tissues was recently shown to correlate with propensity of PCa cells to progress to metastasis and CRPC.[37] Interestingly, our preliminary data suggest that fetuin-A is expressed at a higher level in prostatic tissues of AA PCa patients than in CA patients at the same stage of disease.

A.1 Fetuin-A. We previously demonstrated that fetuin-A, also known as alpha 2-Heremans-Schmid glycoprotein (AHSG) is a significant growth driver of some of the most invasive tumors such as glioblastoma[38] and head and neck squamous cancer cells.[39] Fetuin-A is mainly synthesized by the liver but it can also be synthesized ectopically by tumor cells.[37,38] The ectopic fetuin-A is more glycosylated than the liver derived form.[40] In tumor cells, data implicate fetuin-A activates PI3K/AKT pathway.[41,42] Recently, Mintz et al.[37] demonstrated, using phage display fingerprinting approach, that a unique peptide appeared to be elevated in the serum of an index PCa patient as his disease progressed. It turned out that fetuin-A (AHSG) was the putative protein corresponding to the peptide mimic. They also demonstrated increased serum antibody reactivity to fetuin-A as disease progressed in the index patient, suggesting that the ectopic fetuin-A synthesized by tumor cells was modified relative to the liver derived form. Studies in our laboratory suggest that fetuin-A mediates its proliferative signals in tumor cells via bioactive exosomes.[44,45] Fetuin-A is synthesized, secreted and then internalized (autocrine or paracrine) via TLR4[46] to mediate the biogenesis of 'uptake competent' exosomes that can be taken up by endocytosis and stimulate the same PCa cell (autocrine) or neighboring cells (paracrine).

A.2 Exosomes. These nano-vesicles (30-100 nm in diameter) originate from the inward budding of an endosome's limiting membrane into its lumen. The inward budding produces endosomes that contain multiple intraluminal vesicles also known as multi-vesicular bodies (MVBs). These endosomes can fuse their outer membranes with the plasma membrane and release their intraluminal vesicles into the extracellular space as exosomes.[47,48] Our data suggest that fetuin-A is responsible for the assembly and secretion of bioactive exosomes that are competent in transmitting growth signals to recipient cells.[47] Our data show that exosomes isolated from PCa cells do indeed contain fetuin-A and an assortment of histones, an observation corroborated with data from ExoCarta and other published work on PCa-derived exosomes.[49] Bioactive exosomes mediate PI3K/AKT as well as MAP kinase signaling networks in recipient cells.[50-52] They have also been implicated in anchorage independent or 3-D growth in tumor cells.[52]

A.3 Scientific Premise. Given that PCa largely depends on androgens for growth and survival, androgen ablation therapy (castration) is still regarded as the gold standard of advanced PCa treatment. A subset of PCa in patients, particularly in AA men, progress into CRPC. Other pathways can potentiate the PI3K/AKT signaling

driven by androgens in CRPC, forming the scientific premise for the proposed studies. Interestingly, Mintz et al., demonstrated that fetuin-A is highly expressed by prostatic tumor tissues of patients suffering from CRPC.[37]

B. INNOVATION

The proposed role of fetuin-A in PCa initiation and progression is a new concept in the field. The postulated role of fetuin-A in the biogenesis of uptake competent (bioactive exosomes) (Exoso-1 and Exoso-2) that drives the 3-D growth of PCa cells is a key innovative aspect of this proposal. We hypothesize that during the development of Prostatic Intraepithelial Neoplasia (PIN), tumor cells begin to synthesize fetuin-A to maintain the 3-D growth of the cells by organizing and mediating the biogenesis of fetuin-A loaded exosomes. As tumor grows rapidly and becomes full blown prostate carcinoma, more fetuin-A is synthesized and hence more exosomes, transmitting growth as well as metastatic signals (PI3K/AKT-MAP kinase signaling) as depicted in **Figure 1**. We have reported that in addition to fetuin-A, these exosomes also contain histones that promote their uptake via syndecan-4.[43,45] Analysis of exosomal protein expression in the public database (EXOCARTA) show that indeed fetuin-A and histones are very prominent in the micro-vesicles (exosomes) released from prostate cancer cells.

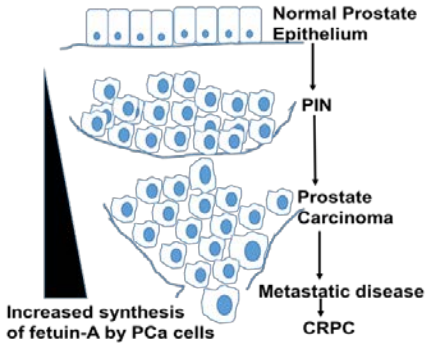

Figure 1. Fetuin-A expression increases as normal prostate cells transform to prostate carcinoma. Further increases are observed through development of metastatic disease and castration-resistant prostate cancer (CRPC)

C. PRELIMINARY STUDIES

C.1 Aim 1: To determine if fetuin-A expression is higher in AA PCa tissues relative to CA PCa tissues and whether high fetuin-A expression is associated with high Gleason scores (>6) and enhanced pAKT and pERK. We have examined the expression pattern of fetuin-A in a cohort of PCa tissue samples available in our Meharry Tissue Procurement Core facility. This particular cohort has samples from AA and CA PCa patients (N=26 for AA and N=8 for CA). Significantly (**Fig. 2E**: P<0.05) higher expression of fetuin-A was observed in the PCa tissues of AA patients (**Fig. 2A and 2B**) compared to their CA counterparts (**Fig. 2C and 2D**). Studies by Mintz et al.[37] looked at expression of fetuin-A in prostate tissues and demonstrated high expression of fetuin-A in metastatic prostate cancer cells (bone metastasis) while no expression was detected in normal prostate tissue. These investigators also demonstrated by Western blot analysis that prostate cancer cell lines, MDA-PCa 2b (derived from AA patient), DU145, PC3 and LNCaP all expressed fetuin-A whereas normal prostate epithelium did not.[37] Based on this higher expression of fetuin-A in prostate cancer tissues of AA patients relative to CA patients (**Fig. 2**), we are proposing to interrogate a much larger pool of samples to test our hypotheses that

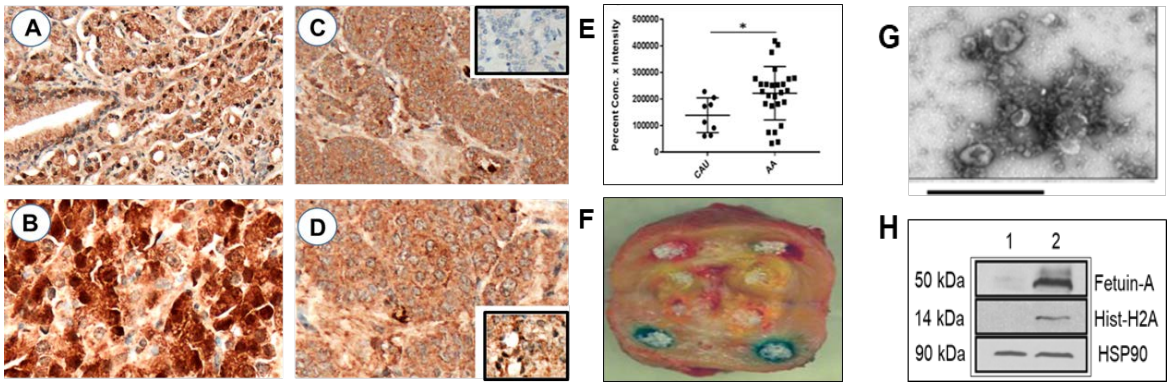

Figure 2. Immuno-histochemical staining (IHC) for fetuin-A in prostate tumors from African American (AA) and Caucasian (CA) patients. A, Gleason grade-3 (GG-3) PCa tumors from AA. **B**, GG-4 PCa tumors from AA. **C,** GG-3 of CA PCa**. D**, GG-4 of CA PCa. **E**, quantification of staining (*P<0.05; N=8 and 26 for CA and AA patients, respectively). Fetuin-A was not detected in normal prostate by the antibody-negative control (inset, upper corner of C). Antibody detected fetuin-A in kidney epithelial cells-Positive staining control (inset, lower corner of D). **F**, Vanderbilt snap frozen prostate tissue procurement; eight 6 mm core biopsies are taken in each case and color coded. PZ, mid-base red; TZ, mid-base yellow; PZ, mid-apex green, TZ mid-apex orange. For further information, see Facilities and Other Resources. **G**, transmission electron micrograph of exosomes purified from BT-549 breast carcinoma cells (scale bar=500 nm). **H**, western blot analysis of the exosomal proteins from BT-549 transfected with (lane 1), empty vector or fetuin-A expression vector (lane 2). The same human fetuin-A antibody (AF1184, R&D Systems) that was used for IHC (A-D) was used for the Western blot. HSP90 was used as loading control.

fetuin-A is a major driver of prostate tumor growth in patients and that higher levels of fetuin-A in AA patients could account for PCa disease disparity. In our preliminary studies using LNCaP PCa cells, we have demon-strated that fetuin-A supports the 3-D growth of these cells in culture (**Fig. 3A and 3B**). Given that 3-D growth of PCa cells mimics the *in vivo* growth pattern of these cells, the data provide compelling rationale for evaluating fetuin-A expression in patient samples. Our studies suggest that fetuin-A is taken up by tumor cells via TLR4[46] and that inhibition of TLR4 internalization by resatorvid (CLI-095) abrogates the 3-D growth of these cells as shown in **Figure 3C**.

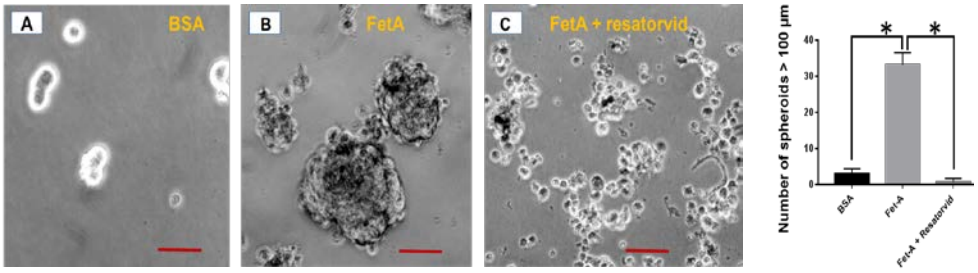

Figure 3. Fetuin-A promotes the 3-D growth of LNCaP cells. The cells were harvested, viability determined using trypan blue and 2,000 single cells/well added to 24-well ultra-low attachment plates in **A**, serum free medium containing BSA (2 mg/ml); **B**, purified FetA (2 mg/ml) and **C**, FetA (2 mg/ml) plus resatorvid (5 µg/ml) dissolved in DMSO. Both the BSA and FetA wells also contained the same amount of DMSO (vehicle controls). The plates were allowed to incubate in humidified CO_2 incubator at 37ºC for 21 days and the cells photographed. Number of spheroids > 100 µm/20X field were counted (6 fields per panel). *P<0.05; N=6; Scale bar = 100 µm.

C.2 Aim 2: To determine the role of ectopic fetuin-A in exosome biogenesis, promotion of 2-D and 3-D growth, motility and invasive capacity of PCa cells. PCa cells rely on fetuin-A for their proliferative potential on plastic in serum free (2-dimensional growth). As shown in **Figure 4A**, the prostate cancer cell line DU145 proliferate vigorously in SFM in the presence of added purified fetuin-A.[42] Even though prostate cancer cell lines such as DU145 and PC3 cells can synthesize their own fetuin-A[37] that would enable them to proliferate in SFM, the cell culture adaptation means they rely mostly on fetuin-A that is present in fetal bovine serum. A culture medium that is supplemented with 10% fetal bovine serum contains approximately 2 mg/ml of fetuin-A. As such in complete medium, the synthesis of ectopic fetuin-A (AHSG) is depressed.[38] We have observed consistently that DU145 cells become larger in size and undergo senescence in SFM, revealed by the beta-GAL staining in the senescence assay, due to very low concentrations of endogenous fetuin-A as shown in **Figure 4B**. The tendency for cells to enlarge and undergo senescence in the absence or presence of very low levels of fetuin-A

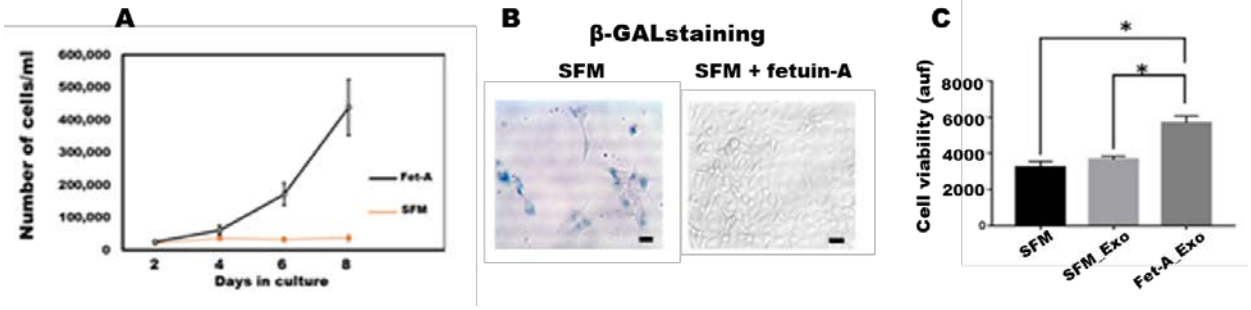

Figure 4. Fetuin-A promotes the growth DU145 PCa cells via exosomes. A, DU145 cells were seeded in 96-well culture plates (20,000 cells/well) and cultured for eight days in serum free medium (SFM) and SFM containing fetuin-A (1mg/ml). In **B**, DU145 cells in SFM exhibited cellular senescence (large blue cells) which was abrogated by fetuin-A. Scale bar = 20 µm. In **C**, DU 145 cells were seeded as in A in the presence of SFM, SFM containing exosomes secreted in the presence of SFM (SFM_Exo) and exosomes secreted in the presence of fetuin-A (Fet-A_Exo). *P<0.05.

has been observed in other types of tumor cells such as the glioblastoma LN229 cells.[38]

Interestingly, DU145 cells proliferate and remain viable in the presence of either purified fetuin-A as shown in **Figure 4B** or in the presence of exosomes isolated from CU145 in the presence of purified fetuin-A (Fet-A Exo) as shown in **Figure 4C**. These exosomes, in the concentration range of 20-100 µg/ml are sufficient to drive the proliferation of these cells. On the other hand, exosomes isolated from DU145 cells in SFM, failed to support 2-D growth of DU145 cells on plastic, suggesting that fetuin-A is required to assemble bioactive exosomes to support the 2-D growth of prostate cancer cells. We have repeatedly shown that the prostate cancer cell line PC3 can proliferate in SFM albeit less vigorously compared to growth in complete medium supplemented with 10% SFM. To determine whether the ability of PC3 cells to proliferate on plastic in SFM is due to endogenous

fetuin-A, we knocked down fetuin-A expression in PC3 cells using shRNA (**Fig. 5A**). Fetuin-A KD significantly reduced the proliferation of PC3 cells in SFM consistent with our hypothesis (**Fig. 5B**). PCa cells rely on fetuin-A for their proliferative potential in 3-dimensional growth or anchorage independent growth in vitro. Different approaches including growth in soft agar and Matrigel, have been used to demonstrate 3-D or spheroid growth assays of tumor cells without a clear mechanistic underpinning. A number of laboratories, including ours, have demonstrated that 3-D growth of tumor cells in soft agar or Matrigel is mediated by serum exosomes[52], but the nature of these exosomes, their purity, protein composition, miRNA content and so on have yet to be clearly defined. Furthermore, growth in soft agar and even Matrigel do not exactly mimic or recapitulate the *in vivo* growth of tumor cells. The ability of tumor cells, particularly cancer stem cells to grow as spheroids on ultra-low attachment plates appear to be much closer to the *in vivo* growth conditions without the supporting cells such as tumor associated fibroblasts. As shown in **Figure 6**, which is a representative of three separate experiments, using ultra-low attachment plates, we have demonstrated (unpublished preliminary data) that exosomes (50 µg/ml) isolated from LNCaP cells in the presence of fetuin-A in SFM supported the growth of LNCaP cells. Within ten days of plating the cells, spheroids containing more than 10 cells (>100 µm) can be seen forming (**Fig. 6B, E**). Interestingly, within the same period, exosomes (50 µg/ml) isolated from the same LNCaP cells in the presence of bovine serum albumin (BSA; negative controls) failed to transmit 3-D growth signals and mostly single cells are observed (**Fig. 6A, E**). As expected, fetuin-A at concentrations similar to those in complete medium containing 10% FBS, also mediated robust 3-D growth (**Fig. 6C, E**) to the same extent as complete medium (**Fig 6D, E**). Even though pAKT(S473) remained activated 1h after serum starvation (24h) with slightly higher levels in the presence of fetuin-A or complete medium, pAKT(S473) disappeared after 24h when the cells became 100% confluent (**Fig. 6F**). Interestingly pERK remained activated even in confluent cells (24h incubation) (**Fig. 6F**). Given these results, it would be interesting to investigate if knockout of endogenous fetuin-A would attenuate activation of both pAKT and pERK. The fetuin-A used is a highly purified form of Pedersen fetuin-A.[43] It has been reported that MAP kinase signaling can maintain growth and survival in LNCaP cells while MDA PCa 2b cells rely mostly on PI3K/AKT signaling in androgen depleted microenvironments.[53] Since 3-D growth is more closely linked to *in vivo* growth, it is not far-fetched to imagine that increased ectopic fetuin-A synthesis by PCa cells would promote growth of these cells in any hostile microenvironments such as absence of androgens.

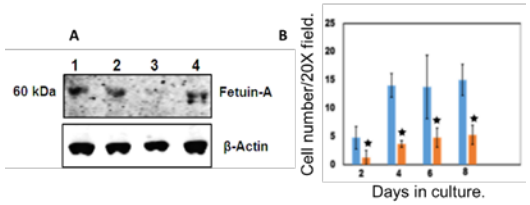

Figure 5. Fetuin-A knockdown reduces the proliferation of PC3 cells. A, Western blotting analysis of PCa cell lysates. Lanes 1, LNCaP; 2, DU145; 3, PC3-sh-fetuin-A; 4, PC3-NSC. B, PC3-non-silencing control (NSC) (blue bars) and PC3-sh-fetuin-A cells (orange bars) were seeded in 24-well plates (500 cells/well) in serum free medium and incubated in humidified CO_2 incubator at 37ºC for eight days. Every other day, the cells were fixed in 4% formalin. At the end of the 8 days, the fixed cells were stained with crystal violet and the number of cells/20X field counted. **B.** A total of six fields/well and each well representing 2-days's growth, were counted. *P<0.05; N=6

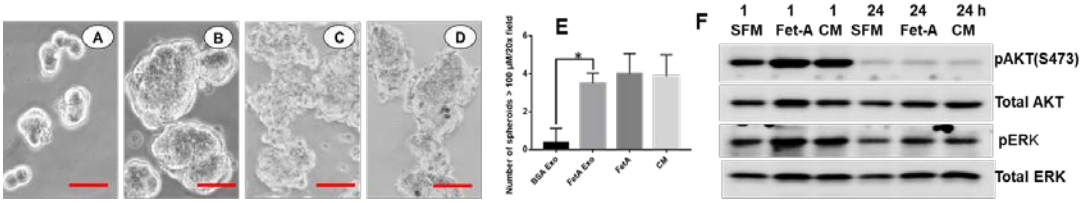

Figure 6. **Fetuin-A drives 3-D growth of LNCaP cells via FetA-Exosomes**. LNCaP cells were added to ultra-low attachment 24-well plates (2,000 cells/well) and incubated with exosomes isolated and purified from the same cells in the presence of; **A**, BSA in serum-free medium (SFM) (BSA exosomes, 50 µg/ml); or **B**, exosomes isolated and purified in the presence of fetuin-A (2 mg/ml of SFM) (Fet-A exosomes, 50 µg/ml). As controls, the cells were also grown in the presence of; **C**, fetuin-A in SFM (2 mg/ml) or **D**, complete medium (CM) containing 10% FBS. After ten days of growth, spheroids >100 µm (20X field) were counted and quantified in **E** (*P<0.05; N=6; scale bar = 100 µm). In **F**, LNCaP cells were grown in 6-well plates until ~75% confluent. The cells were then serum starved for 24h. The SFM was removed and replaced with fresh SFM, fetuin-A (2 mg/ml in SFM) (Fet-A), or complete medium (CM) and incubated at 37°C in humidified CO_2 incubator for 1h or 24h. At the end of the incubation periods the cells were lysed in RIPA buffer, resolved in SDS/PAGE, transferred to nitrocellulose paper and probed for pAKT, Total AKT, pERK and total ERK.

C.3 Aim 3: Investigate the efficacy of targeting fetuin-A mediated signaling on the suppression prostate tumor initiation and growth in mice. Aberrant elevation of fetuin-A correlates with tumor growth in mice. Data from Dr. Chen's laboratory (co-Investigator) demonstrated that *Pten* loss results in the development of invasive

PCa in mice.[29] IHC staining of fetuin-A (anti-mouse fetuin-A-MA5-29650, ThermoFisher) showed that protein levels were strikingly elevated in prostate tumors of *Pten* mice compared to those in normal prostates of wild type (*WT)* mice; pAKT levels detected by anti-mouse pAKT (mAB#4051, Cell Signaling) were also elevated in prostate tumors of *Pten* mice (**Fig. 7**). Fetuin-A was detected mainly in the cytosol of tumor cells. Furthermore, prostate tumors from *Pten/Trp53* mice recapitulated the features of recurrent PCa growth and other biological alterations in human CRPC.[7,54]

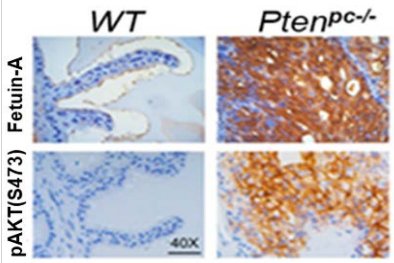

Figure 7. Increased expression of fetuin-A and pAKT (S473) in prostate tumors of Pten-null mice. Fetuin-A and activated AKT are elevated in prostate tumor cells of *Pten*-null mice as compared to normal prostate cells in wild type mice.

C.4 Leadership Team. Josiah Ochieng, PhD, (Meharry Medical College (MMC)) brings expertise in the role of fetuin-A and exosomes in tumor progression; Robert Matusik, PhD, (Vanderbilt) and Zhenbang Chen, PhD, (MMC) bring strong expertise in mechanisms of prostate cancer progression; Billy Ballard, MD, (MMC) brings expertise in tissue pathology; Andries Ziljlstra, PhD, (Genentech/Vanderbilt) provides expertise in KNIME methods of analysis of TMA tissue staining and also provides prostate TMA tissue; and Justin Balko, PhD, PharmD, brings expertise in nanostring and RNA seq analysis.

D. APPROACH

D.1 Aim 1. *D.1.1 Introduction*. The objective of this aim is to determine if fetuin-A is elevated in AA PCa versus CA PCa patient tissues and to determine if fetuin-A levels correlate with tumor progression and patient outcome. The working hypothesis is that fetuin-A potentiates pAKT/pERK activation resulting in enhanced tumor growth and poor prognosis. Our approach to testing this working hypothesis is to examine the expression pattern of fetuin-A mRNA in snap frozen prostatic tissue specimens and protein level/cell types by immunohistochemistry (IHC) on a Tissue MicroArray (TMA). The frozen (-80^{o}C) patient tissue specimens (both AA and CA) are stored in the laboratory of our Vanderbilt co-PI, Dr. Matusik. The TMA was developed by Dr. Ziljlstra while at Vanderbilt (Genentech collaborator, see attached letter) and will be provided by the Vanderbilt Interdisciplinary Prostate Research (VIPR) group (see attached letter from Paula Hurley, PhD). We will be able to measure fetuin-A mRNA expression in AA with low (Gleason ≤6: n=43) versus high grade (Gleason >6: n=48). We will correlate this information to invasion and patient outcome among AA to marching numbers of CA PCa patients from snap frozen tissue. IHC studies using the TMA will reveal fetuin-A expression as well as its relationship to pAKT (Ser473 and Thr308) and pERK, which we postulate are downstream consequences of elevated fetuin-A availability. *In vivo* tumor growth or 3-D growth depends on the activation of PI3K/AKT as well as MAP kinase signaling.[55,56]

D.1.2 Snap Frozen Tissue mRNA Analysis. Dr. Matusik's PCa biorepository contains 1,510 patient samples (91 AA) where each patient sample had eight cores including at least two PCa cores from the peripheral zone (PZ: the major site of PCa) and benign (control) tissue (**Fig. 2F**) resulting in a repository of 12,080 snap frozen samples (see Facilities and Resource Page). From the snap frozen PCa, Dr. Matusik's lab will isolate RNA from 91 AA and 91 CA PCa patients. To increase rigor, two separate cores per patient will be analyzed to provide 182 data points per AA and 182 per CA. PCa from the AA patients will be age/grade matched to CA patients. The snap frozen tissue was collected on all patients having surgery on PCa between 2000-2006. Many of these patients returned to their urologist/community for follow-up and so patient outcome data is limited on these samples. The primary endpoints using the snap frozen tissue for this Aim is to determine fetuin-A mRNA levels as measured by Nanostring analysis. This is divided into three sub-aims: *1.1*) Is fetuin-A mRNA significantly elevated in AA (n=91) vs CA (n=91) PCa patient samples? *1.2*) Does fetuin-A mRNA correlate with low-grade (Gleason ≤6: n=43 each in AA and CA) versus high-grade (Gleason >6: n=48 each in AA and CA); *1.3*) Does fetuin-A mRNA correlate with positive margins and spread of PCa (low grade n=8 and high grade n=19 positive margins per AA and CA)? As secondary endpoints for each sub-aim, mRNA will be analyzed by NanoString to measure 12 mRNAs (12 gene set): including fetuin-A and its receptor TLR4, as well as mRNA for proteins that are found in uptake competent exosomes (ANXA2, LGALS3BP, HIST2H4A)[57]; PTEN/PI3K signaling (PTEN, ERK1/2, PIK3CA, mTOR1/2); and PCa markers (adenocarcinoma: AR; Neuroendocrine prostate cancer, NEPC: FOXA2, SPY).[58] We recognize that proteins regulated by phosphorylation may not show changes in mRNA levels hence the TMA. Dr. Balko will be our consultant on Nano-String (see attached letter).

D.1.3 TMA Analysis. While at Vanderbilt, Dr. Zijlstra constructed a TMA of 504 PCa patient samples (59 AA) with at least six cores per patient (four PCa cores and two adjacent benign cores).[59] Dr. Zijlstra's TMA remains at Vanderbilt under the supervision of Dr. Hurley (see attached letter). The TMA was created by Dr. Zijlstra by

selecting patients from 2000-2012 at Vanderbilt that remained as patients for a median follow-up of nine years; therefore, progression and outcome data are available on a limited number of patients. Since the AA (n=59) and CA (n=445) samples are randomly distributed over the TMA, all slides/patients are required to be analyzed. Since the TMA include four PCa cores, two adjacent benign cores and lymph nodes when available per AA and CA patient, this increases our rigor and analysis for statistical significance. To further increase rigor for IHC studies, the specificity of the fetuin-A antibody (Goat anti-human fetuin-A, AF1184, R&D Systems) is shown by Western blot analysis (**Fig. 2H)** and a second antibody validated by the Human Protein Atlas (HPA00152) will be used. The TMA will measure changes in pAKT (Ser473 and Thr308) and pERK that we postulate are a downstream consequence of elevated levels of fetuin-A. All antibodies will be validated by Western blot analysis and/or confirmation of validation that appears on the Human Protein Atlas website (www.proteinatlas.org). TMA sections provided by Dr. Hurley and the necessary antibodies and reagents will be shipped to Dr. Zijlstra at Genentech. Primary antibodies to fetuin-A, TLR4, pAKT1, pERK1/2, AR and SYP (either rabbit, goat, mouse, etc.) are visualized by using Alexa Fluor® conjugated to secondary antibodies that are species specific for the primary antibody. The Alexa Fluor® conjugated dye are brightly fluorescent at the specific excited wavelength (Thermo-Fisher Scientific). The complete immune-fluorescent (IF) stained TMA slides are digitized by whole-slide scanning. Computer-assisted image analysis will be used with workflow developed by the Konstanz Information Miner (KNIME).[59] The image of each core is extracted, annotated and integrated by KNIME into a data table. Images are processed to map and quantify IF signals onto masks that identifies epithelial (luminal by KRT 8/18 and basal by KRT5) or stromal (fibronectin**)** cells. Nuclei are identified by DAPI.[59] The KNIME image analysis quantifies the IF signal strength in prostate cancer and adjacent benign tissue in AA and CA patients. Since pathology can change as the TMA is sectioned, an H&E of the serial section cores will be reviewed by our resident pathologist, Dr. Ballard, to determine if the TMA core is low or high grade cancer. The primary endpoints using the TMA will be to determine fetuin-A cellular levels and invasive cancer. When possible, we will match TMA data to sub-aims 1.1-1.3 from the frozen cores. Here the TMA will be analyzed with the following sub-aims: *1.4*) Is fetuin-A significantly elevated in AA (n=59) vs CA (n=59) PCa patient samples?; *1.5*) Does fetuin-A correlate with low (Gleason ≤6; n=13 each in AA vs CA) versus high grade (Gleason >6; n=46 each in AA vs CA)?; *1.6*) Does fetuin-A correlate with positive margins and spread of PCa (low grade n=4 and high grade n=22 positive margins per AA and CA)?. Since some AA samples from the snap frozen (collected 2000-2006) and the TMA (2000-2012) will overlap, we will correlate these for mRNA and IF levels.

D.1.4 Power and sample size calculation. Snap frozen PCa and TMA information is entered into the REDCap database including Gleason score, tumor volume, PSA level and invasion (margins, seminal vesicle, lymph node, bladder neck). Overall survival, disease free survival and biochemical recurrence (PSA elevation) may be limited on the snap frozen tissue since not all of these patients were followed at Vanderbilt but follow-up exists for all the patients selected for the TMA. The sample size estimation is completed using two-sample Student's t-test. For sub-aims 1.1-1.3, the endpoints are fetuin-A gene expression by Nanostring. For aim 1.1, with 91 AA and 91 CA patients, this aim provides at least 80% power to detect an effect size of 0.5xSD (standard deviations). For aim 1.2, with 86 high grade (n=43 each for AA and CA) and 96 low grade (n=48 each for AA and CA) PCa patients, this aim provides at least 80% power to detect an effect size of 0.5xSD. For aim 1.3, with 54 invasion (AA n=27 and CA n=27) and 128 non-invasion PCa patients, this aim provides at least 80% power to detect an effect size of 0.55xSD. For aim 1.4, with 59 AA and 59 CA patients, this aim provides at least 80% power to detect an effect size of 0.65xSD**.** For aim 1.5, with 92 (n=46 each in AA and CA) high grade and 26 (n=13 each in AA and CA) low grade PCa patients, this aim provides at least 80% power to detect an effect size of 0.79xSD. For aim 1.6, with 26 (total low and high grade) invasion and 92 non-invasive PCa patients, this aim provides at least 80% power to detect and effect size of 0.79xSD. Two-sided significance level was set to 0.0083 to control for multiple testing. The effect size is defined as the mean difference of each gene expression to the standard deviation.

D.1.5 Analysis plan. NanoString data processing will be conducted using the R package *NanoStringNorm*, including data quality control and normalization. Technical variations will be reduced by adjusting each sample's counts based on their relative value to the geometric mean across all samples. Background count levels, which will be calculated using the mean and standard deviation of negative controls, will be subtracted from all samples. Normalization factor will be calculated from geometric means of housekeeping genes. Normalized counts will be calculated by dividing sample RNA content data by the normalization factor. For aim 1.1, we will use t-test to compare fetuin-A gene between AA and CA prostate cancer patients in univariate analysis. Transformations will be performed to improve distributional assumptions as needed. Nonparametric Wilcoxon rank-sum test will be used when the normality assumption is violated. Multivariable linear regression will be used to model the

association between gene expression and race with our covariate set, including demographic and clinic variables. For aim 1.2, in addition to analysis performed in aim 1.1, an interaction term of grade by race will be included in the model to assess potential racial disparities. If there is a significant grade-race interaction, stratified analyses will be performed to separately evaluate gene-grade associations in AA and CA prostate cancer patients. Similar data analysis plan as described in aim 1.2 will be applied to aims 1.3-1.4. Same method as in aims 1.1-1.3 will also be applied to other genes in NanoString as exploratory analysis. Overall gene expression patterns will be summarized and visualized in the three PCa related pathways mentioned above, as well as function categories in GENE ONTOLOGY[60] and KEGG[61] database by methods including hypergeometric testing, principal component analysis and heat-map, to detect if there is any racial disparity related expression pattern changes or functional enrichment.

D.1.6 Expected Results. We expect that fetuin-A and TLR4 will be highly expressed in prostate tissues of patients with poor disease outcomes such as CRPC and metastasis and high Gleason grades. We can increase the analysis to do additional pathways both by NanoString and the TMA. The expression levels of the genes at the mRNA level will not determine the phosphorylation state. To overcome this limitation, phosphorylation of pAKT1 and pERK1/2 protein will be measured by IF based IHC and quantified. Presently, we are planning only to compare mRNA of AA vs CA PCa in duplicate samples but the biorepository contains eight cores from each patient including benign (control) prostatic tissue. We can increase the NanoString analysis to include additional cores. We do not propose performing RNA-Seq due to the high cost to do 91 AA and 91 CA PCa samples (estimated at $91,000). Since the TMA has some outcome data on all patients, we may be able to determine the correlation between fetuin-A and failure to therapy. In the unlikely event that our preliminary studies (**Fig. 2**) on significantly increased fetuin-A levels in AA vs CA is not confirmed, we can nonetheless proceed with the mechanistic studies in Aims 2 and 3. This is because even positive correlations in patient data will not test the causal role of fetuin-A in disease initiation and progression to metastasis or CRPC.

D.2 Aim 2. *D.2.1 Introduction*. The objective here is to determine whether or not tumor expressed ectopic fetuin-A plays a causal role in the growth of PCa cells both *in vitro* and *in vivo*. Ectopic fetuin-A elevation is associated with the malignancy of various human cancers, including PCa.[37] To explore this causal role, we will both overexpress and eliminate fetuin-A expression to determine whether fetuin-A loss suppresses PCa growth and whether fetuin-A overexpression enhances PCa growth. We previously reported that fetuin-A knockdown (KD) using short-hairpin RNA (shRNA) decreased cell growth and motility through the induction of cellular senescence in glioblastoma cells, and the addition of fetuin-A protein to the culture media restored cell proliferation.[38] However, the mechanism for these effects has not been explored in depth. Our working hypothesis is that fetuin-A mediates the biogenesis of 'uptake competent' or bioactive exosomes that promote 2-D and 3-D growth of PCa cells as well invasive capacity. Our approach will involve the overexpression (OE) and knockout (KO) of fetuin-A in two PCa cell lines: 1) MDA-PCa 2b, derived from an AA and 2) LNCaP derived from a CA. Each of these cell lines will be studied under three conditions: Transfection controls (TC) of each of two cell lines: fetuin-A overexpressing cells (Fet-A-OE) and fetuin-A knockout cells (CRISPR-cas9) (Fet-A-KO cells). The growth and invasive potentials of the cells will be evaluated in serum free medium (SFM). Exosomes will be isolated from TC cells (Exoso-1), Fet-A-OE cells (Exoso-2) and Fet-A-KO cells (Exoso-3). These exosomes will be used to stimulate the growth and invasive capacity of naïve MDA-PCa 2b and LNCaP in SFM. The levels of pAKT and MAP kinases in the exosome-exposed cells will be analyzed by Western blotting. It is expected that Fet-A-OE cells will have the most robust growth and invasive capacity in SFM and similarly, Exoso-2 will show the most growth and invasion promoting potential both in 2-D and 3-D. The corollary is that Fet-A-KO cells as well as Exoso-3 will show the least growth and invasion stimulating potential, respectively.

D.2.2 Approach. To determine whether ectopic fetuin-A promotes growth and invasive capacity of PCa cells in the absence or presence of intact active PTEN via exosome-mediated signaling.

D.2.3 Effects of fetuin-A overexpression, knockout and fetuin-A loaded exosomes on cell proliferation, motility and invasion. We have demonstrated that even though the prostate cancer cell lines such as LNCaP synthesize ectopic fetuin-A[37], it may not be enough to promote their growth in SFM. We will overexpress fetuin-A in MDA PCa 2b and LNCaP, both of which express androgen receptors. MDA PCa 2b has intact PTEN while PTEN is inactivated in LNCaP.[30,62] Both cell lines express low levels of fetuin-A.[37] We will clone fetuin-A (AHSG-bio-His, #52175, Addgene) into the pMSCV viral vector to generate the pMSCV-fetuin-A OE PCa cells as previously described[63] to generate Fet-A OE cells. These will be compared to their transfection controls (TC-cells). The cells will be maintained in RPMI or F-12K medium supplemented with 10% or 20% fetal bovine serum respectively. To determine the impact of fetuin-A in the proliferative potential, TC and Fet-A-OE cells will be seeded at 20,000 cells/well in 24-well plates in SFM and cell numbers on days 0, 2, 4 and 6 determined using

Alamar blue.[42] Boyden chambers will be used for motility and invasion assays.[37] In these assays, epidermal growth factor in SFM in the lower chambers will be used as chemo-attractant.[64] To increase the rigor of our experiments, we will also knockout fetuin-A in MDA-PCa 2b and LNCaP both of which synthesize fetuin-A[37] and assess their growth in SFM. The fetuin-A knockout cells (Fet-A-KO) will be generated using CRISPR/cas-9 approach optimized by the laboratory of Zhenbang Chen, PhD (Meharry collaborator)[65] and compared to their transfection controls (TC cells). In this regard, we will use three sets of gRNA to obtain several positive clones. To determine the impact of fetuin-A loss in PCa cell growth and invasion, TC and Fet-A-KO cells will be assayed as described above. TC, Fet-A-OE and Fet-A-KO cells will be grown in large 150 cm^2 flasks until ~70% confluence. The complete medium will be replaced with SFM and exosomes harvested from conditioned medium for up to three days as previously described.[66] The abilities of the exosomes isolated from TC cells (Exoso-1), Fet-A-OE cells (Exoso-2) and Fet-A-KO (Exoso-3) to drive the growth, motility and invasion of naïve LNCaP or MDA PCa 2b cells in SFM will be determined in 24-well plates and Boyden chambers as described above. In motility and invasion assays, increasing concentrations of the exosomes will be added to the upper chambers. Lastly, TC and Fet-A-OE cells will be plated in 75 cm^2 flasks and allowed to grow until ~70% confluence then serum starved for 24-48 hours. Exoso-1 and Exoso-2 isolated from the serum starved cells will then be added to the flasks containing naïve cells in concentrations ranging from 20 µg/ml to 400 µg/ml for 0-24 h. The cells will be lysed in cold RIPA buffer containing protease inhibitors and the levels of pAKT1 and MAP kinases determined by Western blotting.[43,52]

D.2.4 Effects of fetuin-A- evoked exosomes on PI3K/AKT and MAP kinase signaling in PCa cells. Fetuin-A knockdown PCa cells (Fet-A-KO), transfection controls (TC) and fetuin-A OE (Fet-A-OE) cells will be grown in large 150 cm^2 culture flasks until ~80-90% confluence. The cells (~1 x 10^8 cells/flask) will be dislodged with 2 mM EDTA, washed in PBS and re-suspended in 1 ml SFM and incubated with rotation at 37^{o}C for 1h. The cells will be pelleted and the supernatants subjected to a modification of differential centrifugation steps as described.[67] Briefly, crude pellets of exosomes or small extracellular vesicles (SEVs) will be suspended in ice-cold PBS mixed with ice-cold iodixanol/PBS at a concentration of 30% iodoxanol/PBS solution. This will be added to the bottom of the centrifuge tube and on top of this will be layered (step gradient) 6-30% of iodoxanol/PBS. The tubes will be subjected to ultracentrifugation at 120,000 x g for 15h at 4^{o}C using a SW41 T1 Swinging bucket rotor. Fractions (1 ml) will be carefully collected from the top of the gradient and subjected to mass spec analysis (Meharry Proteomics Core). The major proteins identified in the fractions will be validated by Western blotting. The purity of exosomes will be ascertained by Transmission Electron Microscopy. We will look for exosomal marker proteins such as CD63, annexins, CD81 and histones.[43] The small micro-vesicles or exosomes will be subjected to NanoString (Vanderbilt Vantage Core) to identify microRNA (miR) and/or mRNA found in fetuin-A exosomes. We hope to identify unique proteins or miRNA/mRNA that promote growth of PCa in exosomes from Fetuin-A-OE cells compared to Fet-A-KO cells. Our working hypothesis here is that fetuin-A mediates the biogenesis of bioactive exosomes that are easily taken up by recipient cells and transmit growth signaling to them. Purified exosomes usually at a concentration of 100 µg/ml will be added to naïve PCa cells in SFM in 10 cm dishes for various time points. The cells will be lysed and the levels of key signaling proteins as well as their phosphorylation status determined by Western blotting assays. A number of studies have reported that exosomes evoke PI3K/AKT and MAP kinase signaling in recipient tumor cells.[50-52] Interestingly, miRs 182,183, 141, 375, 107 and 573-3p delivered to PCa cells by exosomes have been shown to evoke oncogenic signals in these cells.[68-71] We will therefore determine whether all or some of these miRs are found in exosomes from Fetuin-A-OE cells and not found in Fetuin-A-KO cells.

D.2.5 Effects of fetuin-A overexpression and knockout on the 3-D growth of PCa cells. To test our hypothesis that fetuin-A synthesized by the tumor cells promotes biogenesis of bioactive exosomes that drive the 3-D growth as spheroids, fetuin-A will be overexpressed in these cells (Fet-A-OE) and also knocked out (Fet-A-KO) as described above. Together with their transfection controls (TC), the PCa cells will be allowed to grow in ultra-low attachment plates in SFM for up to 20 days with weekly media change. Spheroids with diameters >100 µm will be counted after ~20 days of growth. In parallel, exosomes isolated and purified from TC, Fet-A-OE and Fet-A-KO cells will be added to naïve MDA-PCa 2b and LNCaP cells in a concentration dependent manner and 3-D growth in SFM will be monitored for up to 20 days with weekly media changes and addition of fresh exosomes. We expect to demonstrate that fetuin-A synthesized by prostate tumor cells as well as exosomes purified from these cells, play a causal role in 3-D growth of these cancer cells and by extension prostate tumor growth *in vivo*. We previously reported that fetuin-A containing serum exosomes promote anchorage independent or 3-D growth of tumor cells.[52]

D.2.6 Examine the oncogenic role of fetuin-A in tumor growth of PCa cells in mice. **(I)** The impact of Fetuin-A

over-expression (Fet-A-OE) on tumor growth. To evaluate the impact of *fetuin-A* OE in PCa tumor growth of both AA and CA derived cell lines, we will use a humanized xenograft mouse model (immuno-compromised mice). We will inject either human MDA-PCa 2b-*Fetuin-A* cells (AA) or human LNCaP-*Fetuin-A cells* (CA)(1 x 10^6 cells) (Fet-A-OE cells) orthotopically into the prostate tissues of athymic male mice (13 mice/group for 81% power) at 8 weeks (wks.) of age. Mice injected with MDA-PCa 2b-vec ctrl cells (or LNCaP-vec ctrl cells) (TC-cells) will act as controls (**Table 1**). We will measure the tumor sizes 28 days after injection. The experiment will reveal the impact of *fetuin-A* OE on PCa growth, as well as how AA and CA PCa cells respond to *fetuin-A* OE *in vivo*. **(II)** Impact of fetuin-A knock-out (KO) on PCa tumor growth in mice. To evaluate the impact of fetuin-A-KO on tumor growth, cells in which fetuin-A is knocked out using CRISPR-cas9, as well as transfection controls, will be utilized. We will inject either MDA-PCa 2b-*KOFetuin-A* or LNCaP-*KOFetuin-A* (1 x 10^6 cells) (Fet-A-KO cells) orthotopically into the prostates of athymic male mice (13 mice/group). After 28 days, mice will be euthanized and their prostates collected and weights and sizes determined. Lastly, the animal experiments will be repeated exactly as above except that exosomes isolated from Groups 1 (Exoso-1), 2 (Exoso-2) and 3 (Exoso-3) cells will be injected together with the PCa cells to determine whether they enhance or decrease tumor growth. The expression of pAKT, pERK and Ki-67 in the prostatic tissues will be determined by IHC as readouts.

D.2.7 Statistical Plan. A power analysis was conducted to determine the minimum number of animals needed to compare all the groups. Using an effect size of 0.2xSD and Type I error probability of 0.05, the MDA-PCa 2b-Fetuin-A, MDA-PCa 2b-vec ctrl, LNCaP-Fetuin-A and LNCaP-vec ctrl cell groups will require a total of 52 mice to achieve a power of approximately 81%. The same number of animals will be used in the fetuin-A knockout and vector control groups. We will perform descriptive statistical analysis for all study variables. We will summarize continuous variables with statistical values such as mean, median, standard deviation, range, coefficient of variation and confidence intervals.

D.2.8 Expected results. It is expected that overexpression of fetuin-A in PCa cells will significantly promote the growth (both 2-D and 3-D) and motility and invasion of the PCa cells in SFM *in vitro* and in mice relative to their transfection controls. Similarly, exosomes from these cells (Exoso-2) will have the most impact in growth and promotion of motility and invasion (*in vitro* and in mice) as well as activation of AKT and MAP kinases relative to Exoso-1. The corollary viewpoint is that fetuin-A KO cells will grow poorly in SFM and in mice relative to their transfection controls and exosomes from these cells (Exoso-3) will not be able to stimulate growth (in SFM and in mice) nor activate AKT and MAP kinases in naïve PCa cells. We also expect to show that exosomes from fetuin-A OE cells (Exoso-2) and to a lesser extent from transfection controls (Exoso-1) will be decorated with unique proteins, such as fetuin-A, histones and miRs, that promote their uptake and signaling activities in recipient cells. We do not expect to see these proteins in Exoso-3.

Table 1. Impact of fetuin-A on PCa cell growth in mice

Ethnicity	Athymic mice with xenografts (8 wks old)		Tumorigenesis
AA	Fet-A OE	MDA-PCa 2b-*Fetuin-A cells (13 mice)* MDA-PCa 2b-vec ctrl cells (13 mice)	Tumor sizes will be determined 28 days later.
CA	Fet-A OE	LNCaP-*Fetuin-A cells (13 mice)* LNCaP-vec ctrl cells (13 mice)	
AA	Fet-A KO	MDA-PCa 2b-*KOFetuin-A cells (13 mice)* MDA-PCa 2b-vec ctrl cells (13 mice)	
CA	Fet-A KO	LNCaP-*KOFetuin-A cells (13 mice)* LNCaP-vec ctrl cells (13 mice)	

D.2.9 Problems/Proposed Solutions. It is quite possible that knocking out fetuin-A may result in Exoso-3 missing key proteins involved in mediation of growth due to off-target issues. If this is the case, then exosomal isolation and purification will be repeated in the absence or presence of purified fetuin-A. If the addition of purified fetuin-A can offset these deficiencies, then it will be clear that indeed fetuin-A is essential in the biogenesis of bioactive exosomes. Since athymic nude mice will still express fetuin-A in the liver and other organs that may contribute to tumor growth, we can breed the fetuin-A KO mouse into the athymic nude mouse background for grafting of fetuin-A OE or KO human cell lines (**Table 1**). We have extensive experience working with humanized xenografts. If we do not observe any suppressive effect of *fetuin-A* KO on tumor growth after 28 days, we will extend the observation period to 56 days. We will perform a standard histopathology examination on all tissues to detect distant metastases if any.

D.2.10 Leadership Team. Drs. Chen, Ochieng, Matusik, Ballard, a TBN postdoc, and a TSU student will work together to carry out the approaches described for this aim. The animal studies will occur at MMC led by Drs. Ochieng and Chen, in collaboration with the postdoctoral fellow and TSU undergraduate student, with Dr.

Matusik providing collaboration. Dr. Ballard and the Translational Pathology Shared Resource Core will be involved in the mouse tumor tissue analysis.

D.3 AIM 3

D.3.1 Introduction. If, as we postulate, that fetuin-A overexpression and release of fetuin-A loaded exosomes plays a causal role in PCa, then fetuin-A should have demonstrable effects *in vivo*, just as reducing fetuin-A availability should suppress tumor formation *in vivo*. The cancer model that we will use is the *Pten* mouse model for prostate cancer. Our studies serve as a precedent for this hypothesis and the feasibility of performing the studies. Thus, we have shown that fetuin-A deficiency significantly decreases mammary tumorigenesis in polyoma middle T antigen (PyMT) mice, as well as lung tumorigenesis in C57/B6 mice.[41] However, the *in vivo* oncogenic function of fetuin-A and its associated signaling pathways in PCa remains unknown. Studies on the role of fetuin-A in exosome function *in vivo* and prostate tumorigenesis will provide novel and valuable information on PCa malignancy. Our preliminary data reveal that *Pten* inactivation resulted in an aberrant elevation of fetuin-A in prostate tumors of *Pten* mice. Both fetuin-A elevation and AKT activation occurred only in *Pten*-null cells, suggesting that fetuin-A may be a key factor that regulates AKT activation and promotes malignancy *in vivo* upon *Pten* loss. The working hypothesis is that fetuin-A potentiates the sustained pAKT activation in *PTEN*-null tumors via the biogenesis of bioactive exosomes; hence, fetuin-A inhibition is a novel strategy for treating PCa. Our approach to testing the working hypothesis is to generate a *Pten/fetuin-A (Fet-A)* mouse model to analyze prostate tumorigenesis, and to determine how fetuin-A affects PCa progression in *Pten*-null mice. Investigation on the impact of *fetuin-A* deficiency on *Pten*-null mouse prostate tumors will provide mechanistic insights into PCa progression and may lead to novel therapies for PCa.

D.3.2 To investigate the effects of fetuin-A loss on prostate tumorigenesis in Pten-null mice. *Pten/Fet-A* mice: Mice lacking the fetuin-A protein are phenotypically normal and fertile; however, they exhibit calcification in the kidney, testis, skin, heart and vasculature.[72,73] We will obtain *Fet-A* KO mice from Willi Jahnen-Dechent, PhD of RWTH Aachen University in Germany (see attached collaboration letter). To generate the *Pten/Fet-A* double mutant mice, we will cross female *Fet-A* KO mice with male $Pten^{LoxP/+}$ *PB-Cre4* (*Probasin-Cre4*) mice (**Fig. 8**). We will obtain $Pten^{LoxP/LoxP}$, $Fet\text{-}A^{-/-}$ and *PB-Cre4* compound mutant mice (referred to as $Pten^{pc-/-}$– $Fet\text{-}A^{-/-}$ or *Pten/Fet-A*). Since the Cre-recombinase is only activated by the androgen-AR signaling, the inactivation of *Pten* and *Fet-A* in these progenies is prostate-specific after the male mice reach puberty. Mice negative for *PB-Cre4* are like wild-type (*WT*); therefore, we consider them as such. We will perform genotyping by polymerase chain reaction (PCR) for the *Pten*, *Fet-A* and *Cre* alleles as previously described.[29]

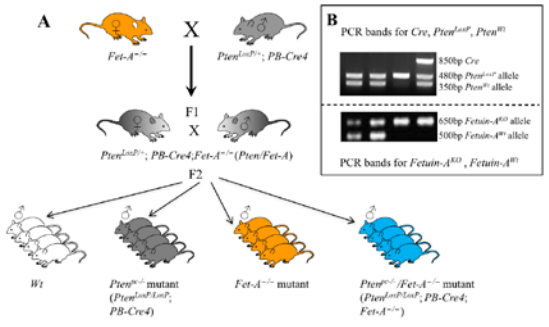

Figure 8. Generation of *Pten/Fet-A* double mutant mice. A, Using *Fet-A* KO ($Fet\text{-}A^{-/-}$) mice, we can obtain *Pten/Fet-A* double mutant mice by crossing *Fet-A* female mice with $Pten^{pc+/-}$ ($Pten^{LoxP/+}$; *PB-Cre4*) male mice. We will analyze prostate tumorigenesis and compare the genotypes of age-matched mice. **B**, we will perform genotyping for the *Cre*, $Pten^{LoxP}$, $Pten^{Wt}$, $Fet\text{-}A^{KO}$ and $Fet\text{-}A^{Wt}$ alleles as previously described.[1]

D.3.3 Statistical Power calculation. With an effect size of 0.35 and a Type 1 error probability of 0.05, a total of 96 animals divided equally among the four groups (WT, $Pten^{pc\text{-}/\text{-}}$, $Fet\text{-}A^{-/-}$ and Pten/FetA double mutant), will achieve a power of approximately 81%.

D.3.4 Analysis of prostate tumorigenesis. We will euthanize mice from four cohorts (N=24 mice/group; (*WT*, $Fet\text{-}A^{-/-}$, $Pten^{pc-/-}$ and $Pten^{pc-/-}$– $Fet\text{-}A^{-/-}$) when they are three and six months old as previously described.[74] Briefly, we will collect prostatic lobes from the anterior prostate (AP), ventral prostate (VP) and dorsolateral prostate (DLP) in each mouse immediately after euthanasia. We will determine the mass of each lobe and image the lobes to compare their actual sizes after biopsy. We will use portions of all three prostatic lobes from each mouse for standard histopathology analyses. Lastly, we will freeze the remaining portions on dry ice and store them at -80ºC for subsequent mRNA/protein extraction and *in vivo* senescence assay. For the *Pten/Fet-A* double mutant mice, we will assess their prostate tumors for incidence of prostatic intraepithelial neoplasia (PIN) and invasive cancer based on their histology. Dr. Ballard (Meharry Medical College) will perform the histopathological analysis to obtain representative images for each phenotype. Chen *et al.* previously observed that additional oncogenic insults dramatically enlarged the AP lobes in *Pten* mice, while only slightly enlarging the VP and DLP lobes.[74] Therefore, to determine the incidence of PIN and invasive cancer accurately, Dr. Ballard will examine at least ten sections with 15 µm intervals between each section for each lobe. Taken together, these data will reveal: (a) whether *Fet-A* loss (loss of one or both alleles)

prevents or delays the onset of PIN and invasive cancer in *Pten*-null mice; and (b) whether *Fet-A* deficiency alters or modulates the pattern of cancer progression in each prostatic lobe.

D.3.5 The impact of fetuin-A on AKT signaling in prostates. PTEN inactivation leads to prostate tumorigenesis and PCa progression through the hyper-activation of AKT, whereas AKT loss (or inhibition) impairs but does not block prostate tumorigenesis of *Pten*-null mice.[75] We will examine whether *fetuin-A* deficiency suppresses AKT activation in *Pten*-null prostate tumors. We will determine the protein levels of pAKT (Ser473 and Thr308), PI3K (p85 and p110), mTOR1/Rictor and SK6. Using western blotting and IHC analysis, we will compare the levels of these proteins in the prostates of *Fet-A null* and *WT* mice at one, three and six months of age, respectively. We will perform the same comparison for *Pten/Fet-A* and *Pten* mice. *Fet-A* KO male mice show calcification in their kidneys and testes (fetuin-A inhibits ectopic calcification). This phenomenon suggests that fetuin-A loss may affect the function of prostates in mice.

D.3.6 The impact of fetuin-A on the composition of exosomal proteins and miRNA. Currently various laboratories are investigating the nature of protein or proteins that mediate the uptake of exosomes by recipient cells so that they can offload their cargo inside the cells. Whereas other laboratories have suggested that fibronectin on exosomes is essential for exosomal uptake[76], we have demonstrated that histones on exosomes mediate their uptake via syndecan-4.[45] Our data further suggest that fetuin-A is involved in the recruitment or loading of histones on to exosomes.[43] There are still gaps in our knowledge of exosomal uptake and more studies are needed. A number of labs have identified tumorigenic miRs that can be delivered to cells by exosomes in prostate cancer, including miRs 182, 183, 141, 375, 107 and 573-3p.[68-71] We will collect blood (orbital bleeds) from *Pten/Fet-A* double mutant and *Pten* mutant mice, isolate and purify exosomes from the blood using differential centrifugation and glycerol step gradient as described herein.[67] We will then determine the protein composition of exosomes including presence of Argonautes (proteins that interact with miRs) and miRs from *Pten/Fet-A* and *Pten mice* (Proteomics Core Facility as well as VICC VANTAGE sequencing CORE).[43] Venn diagrams will be constructed to reveal those proteins and miRs that are shared between the two sets of exosomes and those that are exclusively present only in exosomes from *Pten* mice, as well as those that are exclusively found only in exosomes from *Pten/Fet-A* mice. Fetuin-A will be found only on exosomes from fetuin-A wild type *Pten* mice. If, as we postulate, that fetuin-A is responsible for loading histones on exosomes, then histones will be found exclusively on exosomes from *Pten* mice.

D.3.7 PTEN knockout in MDA-PCa-2b. PTEN deletion is one of the genetic changes associated with prostate cancer disease progression.[26,28,29] Based on the preliminary data presented above, where fetuin-A expression was enhanced in the prostate tissues of PTEN null relative to wild-type mice, we hypothesize that knocking out *Pten* in a prostate cancer cell line with intact Pten, like MDA-PCa 2b[30,62], will upregulate fetuin-A gene expression in these cells. Using the CRISPR-cas9 approach, PTEN will be knocked out in the prostate cancer cell line, MDA-PCa-2b, derived from an AA patient. Upon generation of the PTEN knockout clone, we will determine using proteomics or deep sequencing of RNA the proteins that are upregulated and those that are down regulated wherein 'a volcano plot' of the proteins will be plotted from the experimental data. Growth of these cells will be assayed in 2-D and 3-D spheroid growth assays. It is expected that the cells will grow more rapidly in SFM in 2-D as well as 3-D spheroid assays compared to transfection controls. According to our working hypothesis, fetuin-A synthesized by the tumor cells potentiates PI3K/AKT and MAP kinase signaling via 'uptake' competent exosomes.[43,44] Results will be compared to the LNCaP-Androgen independent cell line (LNCaP-AI) established from a Caucasian male that expresses the androgen receptor as well as the AR-V7 that is androgen independent. This line expresses an inactivated form of PTEN and will be compared to LNCaP-AI cells transduced with an active form of PTEN.

D.3.8 Leadership Team. Drs. Chen, Ochieng, Matusik, Ballard, a TBN postdoc, and a TSU student will work together to carry out the approaches described for this aim. The animal studies will occur at MMC led by Drs. Ochieng and Chen, in collaboration with the postdoc and TSU student, with Dr. Matusik providing collaboration. Dr. Ballard and the Translational Pathology Shared Resource Core will be involved in the mouse tumor tissue analysis.

D.3.9 Statistical Analysis Plan. One-way ANOVA will be used to compare WT, $Pten^{pc-/-}$, $Fet\text{-}A^{-/-}$ and Pten/FetA double mutant groups followed by a Tukey or Bonferroni post-hoc test to determine significant pairwise differences.

D.3.10 Expected results. We expect to obtain $Pten^{pc-/-}$– $Fet\text{-}A^{-/-}$ mutant mice from our breeding scheme described in **Figure 8**. We expect to see large tumors in *Pten* null mice and significantly smaller tumors in *Pten/Fet-A* double mutant mice. We expect that exosomes from *Pten* mice will show presence of fetuin-A, histones and unique miRs while exosomes from *Pten/Fet-A* double mutant mice will lack these proteins and

growth promoting miRs. We expect that exosomes from *Pten* mice will be able to activate the signaling pathways in naïve PCa cells. Exosomes from *Pten/Fet-A* will lack this ability because they will be uptake incompetent if our working hypothesis is correct. This information is currently not available. We have previously worked with *Fet-A*$^{-/-}$ mice and are aware of the fact that these mice exhibit abnormal bone development. We expect that we will be able to generate stable PTEN knockout sub-clone of MDA-PCa 2b, the only widely accepted cell line from PCa tissues of an AA patient. We expect that it will express higher levels of fetuin-A and perhaps other oncoproteins and perhaps grow better *in vitro* and *in vivo* than the parental line. We expect that overexpression of an active form of PTEN in LNCaP-AI cells will reduce Fet-A expression and alter expression of oncoproteins.

D.3.11 Problems/Proposed Solutions. We have previously worked with *Fet-A*$^{-/-}$ mice and are aware of the fact that these mice exhibit abnormal bone development. If the *Fet-A*$^{-/-}$ mice are problematic, we will use *Fet-A*$^{+/-}$ mice to generate progenies with the desired genotypes. Therefore, we do not foresee significant difficulties in obtaining enough *Pten/Fet-A* mutant mice for our experiments. If the tumor sizes are not distinguishable between *Pten* and *Pten/Fet-A* mice at six months of age, we will compare them when they are one year old. Alternatively, we will analyze the mouse prostate tumors in order to uncover the effect of fetuin-A inactivation on tumor formation at the molecular level. In this case, we will examine prostate tumors from *Pten/Fet-A* mutant mice (*Pten*$^{pc+/-}$*– Fet-A*$^{-/-}$, *Pten*$^{pc-/-}$*– Fet-A*$^{+/-}$, *Pten*$^{pc+/-}$*– Fet-A*$^{+/-}$ and *Pten*$^{pc-/-}$*– Fet-A*$^{-/-}$) and compare them with tumors from age-matched *Pten*$^{pc-/-}$ and *Pten*$^{pc+/-}$ mice, respectively. If levels of pAKT and AR in the prostates of *Pten/Fet-A* mice are comparable to those in the prostates of *Pten* mice, we will examine their target genes by qRT-PCR. If fetuin-A inhibition does not significantly affect the prostate tumor size in *Pten* mice, we will conduct the same experiment using *Pten/Trp53* mutant mice.

D.4 Integration. This project will rely heavily upon the Biostatistics and Bioinformatics Shared Resource Core for data analysis. We will utilize the VANTAGE Core at VICC for RNAseq, if required, and we will utilize the Cancer Outreach Core to encourage men with prostate cancer in the AA and Hispanic communities in the Metropolitan Statistical Area (MCA) to provide biopsy tissue for expansion of the number of specimen for analysis of the correlation between fetuin-A expression and prognosis. We will work with the Planning and Evaluation Core and the Administrative Core to ensure we meet the administrative and evaluation goals of this application. We will work with the Research Education Core to bring trainees into this project to work with us to meet the goals of this project. We will integrate the studies in Aim 2 with the Administration Core, VANTAGE (Vanderbilt Genomics Core), and the consolidated research instrumentation, informatics, statistics and Learning Integration Suite (CRISALIS) Core facility-Meharry. Aim 3 will be integrated with the Administration Core and will use the CRISALIS Core facility, and the VANTAGE Core affiliated with VICC.

D.5 Future Directions and Timeline. Data obtained will be used to apply for R21/R01/DOD applications aimed at both improving PCa diagnosis and prognosis (particularly in minority populations) and the development of liquid biopsies for prostate cancer. A future goal is to identify unique miR's in the uptake competent exosomes whose biogenesis is influenced by fetuin-A only in blood of PCa patients, particularly AA PCa patients. Data will inform additional examination of normal and benign prostatic hyperplasia (BPH) for fetuin-A expression. Fetuin-A expression in BPH may be a strong indicator of future malignant transformation. Overall, the studies will provide reasonable explanation as to why AA PCa patients are more likely to develop the more aggressive forms of the disease, if it becomes clear that their PCa cells synthesize and secrete more fetuin-A that drives the oncogenic and motility signals. The next steps will involve methods to mitigate tumor growth-promoting abilities of fetuin-A (**Table 2**). For example, in our preliminary studies, we have shown that a specific inhibitor of TLR4 abrogates fetuin-A uptake. Interestingly, statins which are regularly consumed by men over 40 years, and which also inhibit the activities of TLR4, have shown epidemiological evidence that they can ameliorate the aggressive forms of prostate cancer.

Table 2. Timeline of Activities

	Year 1	Year 2	Year 3
Aim 1	NanoString/IHC analysis of frozen PCa tissues, data acquisition and analysis	NanoString/IHC analysis cont.; Manuscripts, data sharing, grant application	NanoString /IHC analyses cont.; Manuscripts, community engagement
Aim2	Complete FetA-OE and KO experiments; Start animal experiments	Complete animal experiments; Grant applications	Revise grant applications if necessary; Data sharing
Aim3	Animal breeding, genotyping, data collection	Complete data collection, grant applications, manuscripts	Manuscripts, data sharing, grant applications

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Vertebrate Animals

1. Description: Mice will be used in aims 2 and 3. In aim 2, Athymic mice will be injected with PCa cells according to the Table below.

Table 1. Effect of fetuin-A on tumor growth from AA PCa cells in mice

Ethnicity		**Athymic mice with xenografts (4–8 weeks)**	**Tumorigenesis**
AA	Fet- A KD*	MDA PCa 2b-shFetuin-A cells (12 mice)	Tumor sizes will be determined weekly, and tissues will be harvested for WB and IHC analysis.
		MDA PCa 2b-vec ctrl cells (12 mice)	
CA		LNCaP-shFetuin-A cells (12 mice)	
		LNCaP-ven ctrl cells (12 mice)	
AA	Fet- A OE	MDA PCa 2b-Fet-A cells (12 mice)	Distant metastasis will be examined in mouse lung after 6 wks.
		MDA PCa 2b-vec ctrl cells (12 mice)	
CA		LNCaP-Fet-A cells (12 mice)	
		LNCaP-vec ctrl cells (12 mice)	
*KD-knockdown, OE-overexpression			

PCa cells (1 x 106 cells) will be injected subcutaneously into the flanks of athymic male mice (12 mice/group for 80% power) at 8 weeks (wks) of age. Mice injected with vector control cells will act as control cells according to **Table 1**. We will measure the tumor sizes weekly for 4-8 wks after injection using a digital caliper. In aim 3, studies are designed to investigate the effects of fetuin-A loss on prostate tumorigenesis in Pten-null mice. For this analysis we will generate Pten/FetA compound mutant mice according to **Table 2**, below.

Table 2. Generation and tumor analysis of Pten/Fet-A compound mutant mice

Genotype	**Total mice**	**At 3m**	**At 6m**	**Analysis of prostate tumorigenesis**
Wt	24	12	12	All three prostate lobes of each mouse will be collected separately, and their actual sizes, masses and histology of individual prostate lobes will be analyzed and compared.
Pten	24	12	12	
Fet-A	24	12	12	
Pten/Fet-A	24	12	12	

2. Justification of animal use: There is a state of the art AAALAC approved animal care facility at Meharry, supervised by a highly qualified veterinarian and associates. The facility has about 20,000 sq. ft. of area. One separate room with 200 sq. ft. is available for this project. The objective of this study is to define the role of fetuin-A in the growth of PCa cells as xenografts in mice and more importantly the initiation of PCa using mouse model for PCa that has been perfected in Dr. Chen's laboratory. The minimal number of mice necessary to produce scientifically valid results will be used, and every effort will be made to minimize pain and discomfort when procedures are performed, as outlined below.

3. Minimization of pain and distress: The proper guidelines of animal care as stipulated in the standard operating procedures will be followed exactly. Animals will be monitored daily for any signs of discomfort and examined more frequently once tumors develop. Tumor growth in the brain of the mice will be imaged by bioluminescent techniques. The animals which develop heavy tumor loads before the end-point of the experiments will be given analgesics to relieve their pain. Animals will be sacrificed by CO2 asphyxiation at the end of the experiments or whenever they show signs of moribund.