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14. ABSTRACT We have successfully achieved the goals of the first aim, to optimize conditions for maintaining human prostate tissue slices (TSCs) in an NMR- compatible, 3-D tissue culture bioreactor and to verify the metabolic integrity of TSCs over time. Of particular importance, the results of the first year of funded studies demonstrate the feasibility of maintaining both the pathologic and metabolic integrity of benign and malignant human tissue cultures in the NMR compatible bioreactor for 32 hours. Moreover, non-hyperpolarized and hyperpolarized ¹³ C MR data acquired from human tissue slices demonstrated that the metabolism of the human tissue slices accurately reflects the metabolism of the <i>in vivo</i> human prostate and that [1- ¹³ C]hyperpolarized lactate will be an accurate biomarker of prostate cancer in patients.					
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INTRODUCTION

The overall objectives of this synergistic research is to optimize an MRS-compatible, 3D Tissue Culture Bioreactor for use with primary human prostate tissue cultures (TSCs) and use it to identify hyperpolarized molecular imaging biomarkers for improved prostate cancer patient-specific treatment planning and early assessment of response to hormone and chemotherapy. The hypotheses that will be tested in this project are that fresh human prostate tissue slices can be maintained without loss of structure, function or metabolism within a NMR compatible 3-D tissue culture bioreactor for up to three days, and that magnetic resonance spectroscopy studies of these human tissue slices can be used to identify hyperpolarized metabolic biomarkers of prostate cancer presence and aggressiveness and early response to therapy. The goals of this study will be achieved through the following specific aims. The first aim is to optimize conditions for maintaining human prostate tissue slices (TSCs) in an NMR- compatible, 3-D tissue culture bioreactor and to verify the metabolic integrity of TSCs over time. The second aim is to use this new experimental model to compare the metabolism of normal and malignant prostate tissues, and importantly, determine whether hyperpolarized metabolites correlate with pathologic grade (i.e., Gleason grade 3 versus grade 4/5). The third aim is to use NMR to evaluate the response of TSCs to anti-androgen therapy and chemotherapy (Triptolide). Our goal was to achieve Aim 1 in Year 1 and we have made significant progress toward this goal.

BODY

There has been no change in the tasks specified in Aim 1 from those proposed in the original Statement of Work. Specifically the goal of aim 1 was to optimize and validate conditions for maintaining the structure and function of human prostate tissue cultures in an MR-compatible, 3-D tissue culture bioreactor. As described in Dr. Peehl's progress report, she was involved in the optimizing the harvesting and storage of the prostate tissue slices at the time of surgery (TSCs), and in optimizing the culture media for the best maintenance of the structure and function of the TSCs for the subsequent MR spectroscopy studies (**task 1**). Her first task (**task 1a**) was to vary basal medium, growth and differentiation factors, hormones, tissue perfusion and oxygen levels that result in the best maintenance of structure and function of the TSC's (**task 1b**). She also worked with Drs. Kurhanewicz and Ronen to evaluate the metabolic integrity of both benign and malignant TSC's over time using NMR compatible TSC bioreactors and a combination of ^{31}P and hyperpolarized ^{13}C spectroscopy studies (**task 1c, Figure 1**).

In the first year of funding, my research in close collaboration with Dr. Ronen (**Task 2**) focused on the development and implementation of improved NMR compatible 3D tissue culture bioreactors and utilized these NMR compatible bioreactors to evaluate the metabolic, functional and pathologic integrity of benign and malignant human prostate tissue slices. Specifically, we demonstrated using 1 and 2-D HR-MAS studies that metabolism of the human tissue slices recapitulated the pathology and metabolism observed in patients (**Figures 1-4**). Additionally, we demonstrated that the metabolism of non-hyperpolarized $[3-^{13}\text{C}]$ pyruvate by the human tissue slices was consistent with the previously published literature and that metabolism of pyruvate to lactate was a biomarker of prostate cancer. Subsequently, we utilized a combination of 14T MRI, ^{31}P spectroscopy and fast dynamic HP ^{13}C MR after injection of hyperpolarized $[1-^{13}\text{C}]$ pyruvate (**task 3, figure 5**) in 3D human prostate tissue cultures in our first prototype 10mm NMR compatible bioreactor to determine how long the tissues could be kept metabolically viable and functioning using the optimized media developed by Dr. Peehl (**task 1a, Dr. Peehl's progress report**). The hyperpolarized MR data obtained from these studies suggested that hyperpolarized $[1-^{13}\text{C}]$ lactate was a sensitive and specific biomarker of prostate cancer (**Figure 5**). This work is currently being written up for publication.

In the first year of funding Dr. Peehl supplied Dr. Ronen and myself with 10 paired benign and malignant human prostate tissue slices that were optimally harvested, sectioned and transferred to UCSF in ice cold optimized media for *ex vivo* metabolic analyses and NMR compatible bioreactor studies. The optimized prostate-specific medium, PFMR-4A (**task 1**) used for culturing the tissues slices, was based on DMEM supplemented with growth factors and hormones as described by Dr. Ronen in her progress report. We determined that the antibiotic gentamicin could not be included in the medium because its peak interferes with the ^{31}P spectral analysis. Additionally, it was determined that for prostate tissue slicing HEPES-buffered saline (HBS) could not be used since it interfered with the 1-D and 2-D ^1H HR-MAS spectra acquired to demonstrate that the prostate tissue slices recapitulated *in vivo* prostate metabolism (**Figure 1**).

1-D and 2-D HR-MAS Prostate Tissue Culture Studies: Metabolic and Pathologic Accuracy of the NMR compatible Tissue Culture Model: Dr. Kurhanewicz's group has developed quantitative 1- and 2-D high resolution magic angle spinning (HR-MAS) spectroscopic techniques having the sensitivity to quantify not only the more sensitive proton metabolites (fig. 3) but also ^{13}C labeled metabolites (fig. 4) in human prostate tissues (1, 2). HR-MAS spectroscopy is a technique that allows the acquisition of high spectral resolution, solution like spectra from intact tissue samples (fig. 5) (1, 2). Since the technique is non-destructive, the sample can go on for pathologic, immunohistochemical, genomic and proteomic analysis. For quantification of metabolite concentrations, a synthetic quantitative signal (ERETIC) was transmitted during the acquisition of the NMR (3). Using this electronic reference the peak areas

of each of the prostate metabolites can be quantified. HR-MAS spectroscopy of TSCs yielded the same spectroscopic profile as normal and cancer tissues *in vivo* (**Figures 1- 4**).

1-D and 2-D HR-MAS HR-MAS Methods: A 35- μ L leakproof zirconium HR-MAS rotor (Varian Inc, Palo Alto, CA) was weighed using a balance capable of 0.01 mg accuracy. Three microliters of D₂O+TSP was pipetted into the rotor and provided a frequency reference. Three microliters of 101.24 mM ¹³C-2 glycine was then added to the rotor to enable calibration of the ¹³C pulses and provide a quantification reference for the HSQC spectra. The sample tube was removed from the dry ice, and its two rubber caps were cut off. The frozen tissue slice sample was transferred to the HR-MAS rotor by holding the sample tube (described in Dr. Ronen's report) above the rotor and pushing the tissues out the sample tube into the rotor using a small plastic rod. Following the insertion of each solution or cell sample, the rotor was weighed in order to determine the mass of the most recently added substance. The HR-MAS rotor was then assembled and closed as previously described by Swanson et al. (1).

The prostate tissue slice samples were analyzed using Varian INOVA 11.7 T (500 MHz for ¹H) spectrometer equipped with a 4 mm gHX nanoprobe, previously cooled to 1°C. Once the rotor was loaded into the HR-MAS nanoprobe which held the sample at the magic angle, the sample was spun at 2,250 Hz and maintained at 1°C. A pulse-acquire sequence was used to obtain a 1H[13C] spectrum with a 90° flip angle, 30,000 complex points, 20 kHz bandwidth, TR = 7 s, 256 averages, and 10 dead scans for a total scan time of 16.1 minutes. Carbon decoupling was implemented using the GARP scheme with pulse power of 43dB. The result spectrum was zero-filled to 131,072 points and Fourier transformed.

To obtain information from ¹³C labeled metabolites, a HSQC experiment was performed, allowing detection of signal only from those protons that were attached to ¹³C nuclei as previously described for primary human prostate cell studies (4). The evolution times were set to a single bond scalar coupling (J_{CH}) of 143 Hz, which corresponds to the J -coupling constant for the C-2 labeled glycine reference. This choice necessarily resulted in incomplete polarization transfer for ¹³C labeled metabolites of the pyruvate substrate, including glutamate C-4 (J_{CH} = 127 Hz), glutamate C-3 (J_{CH} = 130 Hz), aspartate C-3 (J_{CH} = 129 Hz) (J_{CH} = 130 Hz), alanine C-3 (J_{CH} = 130 Hz), and lactate C-3 (J_{CH} = 128 Hz). However, correction was made for the incomplete transfer, as described below. HSQC spectra were acquired with 4096 points and a 10 kHz spectral width along the proton dimension, 256 complex increments and a 15 kHz spectral width along the carbon dimension, GARP ¹³C decoupling during the data acquisition, a TR of 2.41 s, 32 dead scans, and 16 averages for each increment leading to a total scan time of 2.78 hrs. Linear prediction was used along the ¹³C dimension to produce an additional 512 points resulting in a matrix size of 4,096 X 768 points. This matrix was then zero-filled to 16,384 X 4,096 points and multiplied by Gaussian apodizations in both dimensions before Fourier transformation. Following the HSQC experiment, a second ¹H[¹³C] spectrum was recorded to determine whether the metabolite concentrations had changed during the course of the HR-MAS experiment.

1-D and 2-D HR-MAS Tissue Slice Biopsy Comparison: The representative ¹H[¹³C] HR-MAS spectrum presented in **Figures 1, 2 and 5** demonstrate that healthy prostate tissue slice cultures exhibited the characteristic metabolic profile of human prostate tissue with high levels of citrate and polyamines(5). In addition, the choline containing compounds were similar to *in-vivo* spectra with moderate levels of choline, PC, and GPC(5, 6). Whereas, the normal primary cultured prostate epithelial cells have exceptionally high levels of PC because they are actively multiplying in the culture environment. The representative ¹H[¹³C] HR-MAS spectrum from a histologically confirmed prostate cancer tissue culture sample also exhibited the same metabolic changes observed in *in-vivo* spectra. Namely, PC was higher in the cancer sample and citrate and polyamines were almost eliminated. For comparison, a representative snap frozen ¹H HR-MAS spectrum obtained from a biopsy of healthy prostate tissue and prostate cancer are shown in **Figure 2**. Reviewing the histology obtained from the prostate tissue cultures, the H&E stained

sections in **Figure 3** clearly demonstrate that high quality histology can be obtained from the prostate tissue cultures and that loss of the hallmark los glandular architecture was readily detected in the sections from the Gleason 4+3 cancer sample. Conducting a comprehensive metabolic comparison across multiple prostate biopsy samples (10 benign and malignant biopsies) and (10 benign and malignant biopsies) prostate tissue culture samples, the plots in **Figure 4** show that there were no significant differences between the metabolic profile of snap frozen biopsy and tissue culture samples. Although the amounts of alanine, lactate glutamate were higher in tissue cultures relative to biopsy samples due to the 4 mM [3-¹³C]pyruvate in the media, there was a significant decrease in citrate and an increase in lactate and total choline levels in cancer relative to benign tissues in both biopsy samples and tissue slice cultures.

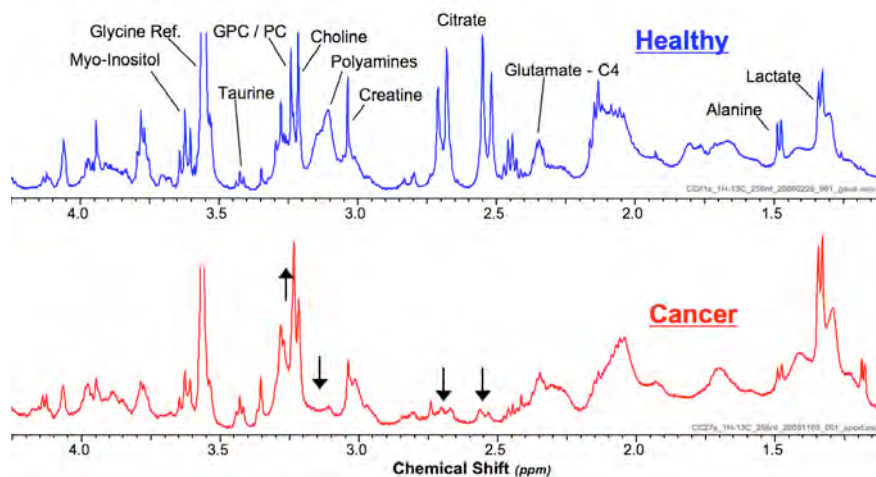


Figure 1. ¹H[¹³C] spectra acquired from ~25 mg of healthy (TOP) and cancerous (BOTTOM) prostate tissue slices after being cultured for 2 hours. Similar to *in-vivo* ¹H spectra, the healthy sample had high levels of citrate and polyamines and moderate levels of choline. Compared to the healthy sample, the cancer sample had higher levels of PC and lactate as well as almost no citrate nor polyamines.

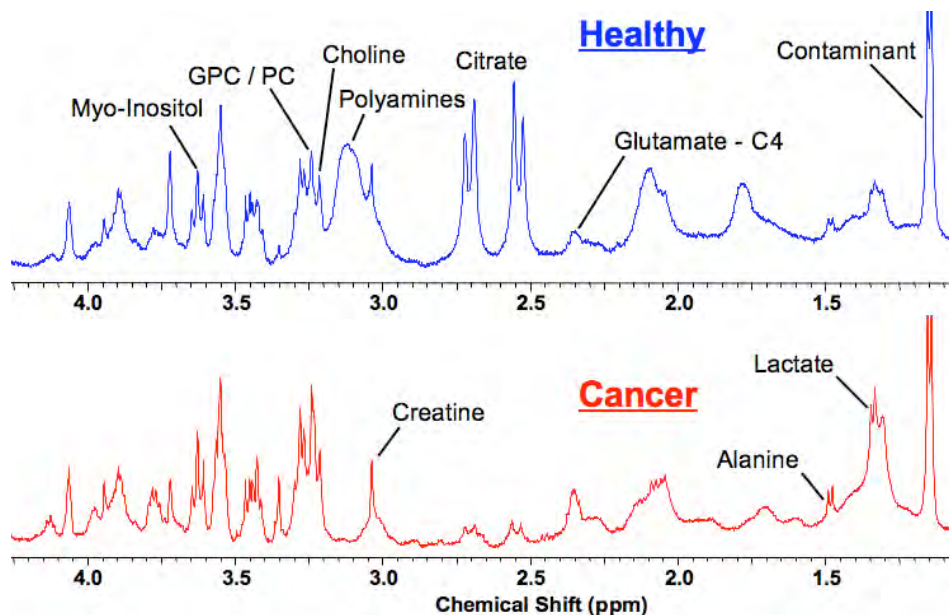


Figure 2. Proton spectra acquired from ~5mg of healthy (TOP) and cancerous (BOTTOM) prostate biopsies. The cancer shows a significant reduction in citrate, polyamines, and an increase in GPC / PC and lactate.

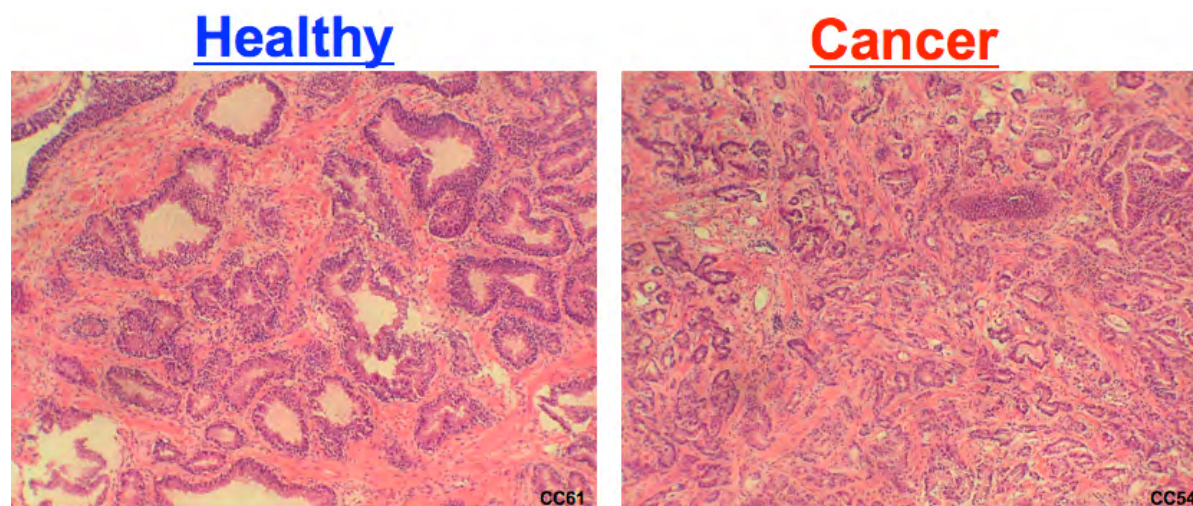


Figure 3. Prostate tissue slices viewed under 10X magnification after they were cut into 10 μ m thick sections and stained with Hematoxylin & Eosin. Based on the histology, the cancer sample was graded as a Gleason 3+4 cancer.

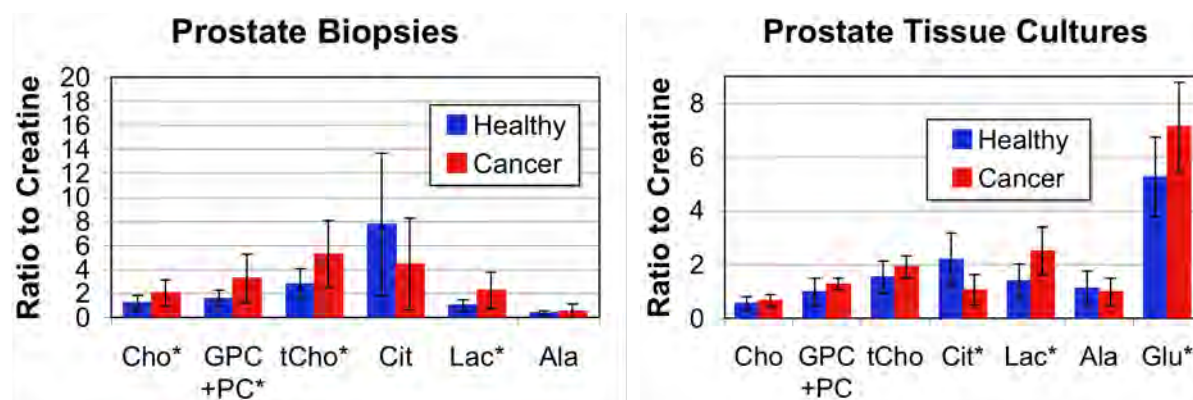


Figure 4 Metabolite averages and standard deviations for 10 healthy and 10 cancerous prostate biopsies (LEFT). Averages and standard deviations for 10 healthy and 10 cancerous prostate tissue cultures (RIGHT). Total choline (tCho) was computed by summing choline, PC, and GPC. Asterisks indicate statistical differences.

1-D and 2-D HR-MAS of Prostate Tissue Cultures Labeled with [3-¹³C]pyruvate : Fresh tissue cores (8 mm diameter) from radical prostatectomy specimens were embedded in agarose and rapidly sectioned (30 - 250 μ m sections) while immersed in chilled physiologic fluid as described in Drs. Peehl and Ronen's progress report. Tissue slices were then transferred to a specialized 2D culture apparatus that cycled the slices through a period of submersion in the medium containing 4mM [3-¹³C]pyruvate followed by exposure to air as described in Dr. Ronen's progress report. The amount of ¹³C labeling in the metabolic products of pyruvate was measured using an HSQC experiment described above.

Results: A representative HSQC obtained from a healthy and a cancerous prostate tissue culture are shown in Figure . Since the fractional enrichments in the healthy sample in figure 5 for glutamate, lactate, and alanine were 6%, 17%, and 38%, respectively, it is clear that the prostate tissue cultures incorporated the ¹³C-3 pyruvate added in medium into the metabolic pathways of interest. Furthermore, a qualitative comparison of the HSQC spectra from cancer (N=5) and benign samples (N=5) indicates that there was increase in the conversion of [3-¹³C]

pyruvate to [4-¹³C]glutamate and [3-¹³C]lactate in the cancerous tissue cultures relative to the healthy tissue cultures.

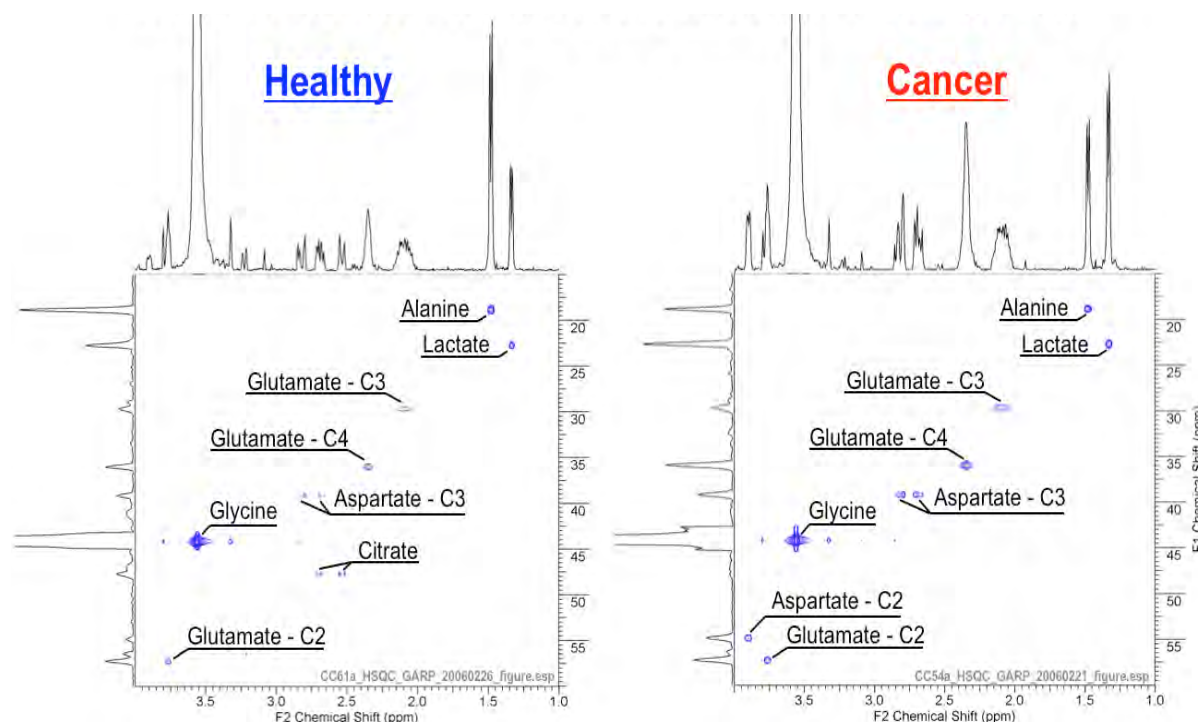


Figure 5 – 2D HR-MAS studies - ¹³C HSQC spectra acquired from healthy (*LEFT*) and cancerous (*RIGHT*) prostate tissue slices after being cultured for 2 hours with ¹³C-3 pyruvate. The cancer sample was graded as a Gleason 3+4 cancer.

Conclusions: As was previously observed for primary cultured prostate cancer cells (4), the increase in conversion of [3-¹³C]pyruvate to [4-¹³C]glutamate strongly supports the hypothesis that citrate oxidation by the citric acid cycle is up-regulated in prostate cancer, referred to as the Bioenergetic Theory of prostate cancer by Costello and Franklin (7). While the primary culture data demonstrated that [3-¹³C]pyruvate was converted to glutamate more than any of the other reporter metabolites, the preliminary tissue culture data suggests that the pyruvate conversion to glutamate is also much higher in prostate cancer than normal secretory prostate tissue. In addition, the preliminary tissue culture results suggest that there is an increase in the glycolytic lactate production with prostate cancer since there was more [3-¹³C]lactate produced in the cancer tissue culture relative to the normal tissue culture. These data suggest that ¹³C labeling of glutamate and lactate may provide new clinically useful biomarkers of prostate cancer presence and aggressiveness.

Hyperpolarized ¹³C NMR Compatible Tissue Slice Cultures: Fresh tissue cores (8 mm diameter) from radical prostatectomy specimens were embedded in agarose and rapidly sectioned (30 - 250 µm sections) while immersed in chilled physiologic fluid as described in Drs. Peehl and Ronen's progress report. Tissue slices were then transferred to a NMR-compatible bioreactor using a technique described in Dr. Ronen's progress report. The bioreactor is a completely contained 3D culture system with a continuous flow of 35°C media (containing a custom DMEM based hormone defined formulation, 10% FBS, and Penn/Strep). The 10 mm tissue culture NMR compatible bioreactor used for these initial studies represents a modification of the 10 mm cell culture bioreactor that we used to study the metabolic kinetics of hyperpolarized [1-¹³C]pyruvate in liver tumor cells(8). [1-¹³C] pyruvate was hyperpolarized using the Hypersense (Oxford Instruments) as described previously (1) and 1mL of 10 mM

pyruvate was injected into a custom designed 10mm flow system at 5 rpm(9). ^{13}C NMR spectra were acquired in intervals of 3 sec using a 13° pulse for 300 secs on a narrow-bore 11.7T Varian INOVA (125MHz ^{13}C , Varian Instruments) equipped with a 10mm triple tune direct detect broadband probe. Prior to and after injection of the hyperpolarized compounds, a 4 hour time course of ^{31}P spectra (202MHz ^{31}P) were acquired with a 60° pulse, nt=2048, and at=1s to assess the β -NTP resonance as a function of time and infer cell health. ^{31}P spectra were acquired from one benign sample for 32 hours. After perfusion in the bioreactor, samples were processed for pathology and LDH enzyme activity. Two pathologists used a five-point scale (1= excellent, 5 = poor) to quantify the quality of the pathology of the TSC's after the bioreactor study. Hyperpolarized metabolic data was processed using ACD 1-D NMR processor (ACD labs, Ontario, CA), and lactate (182 ppm) and pyruvate (171 ppm) peaks areas were integrated over time and normalized to tissue mass. The area under the metabolic time course curve (AUC), time to maximum hyperpolarized lactate, maximum hyperpolarized lactate peak, and LDH activity were statistically compared between benign and malignant tissue cultures using a Student T-test.

Results: NMR-compatible prostate TSC bioreactor studies were performed on 3 cancer TSCs (Gleason 3+3, 3+4 and 4+4), and 3 benign TSC's. The malignant and benign ^{31}P TSC spectra (**Figure 1A and B** respectively) were identical to what has been previously published for *in vivo* ^{31}P spectra from the human prostate (5), and the ^{31}P spectra remained constant over a 32 hour time period (**Figure 1C**). Additionally, pathology at the end of the bioreactor study demonstrated preservation of *in vivo* tissue structure (**Figure 6**), with pathologist's giving an average pathologic score of good (3.3 ± 0.2). This tissue culture platform provided a unique opportunity to investigate the metabolism of hyperpolarized [$1\text{-}^{13}\text{C}$]pyruvate in the human prostate prior to actual patient studies. As shown in **Figure 2A** prostate cancer TSCs demonstrated a significantly higher production of labeled hyperpolarized [$1\text{-}^{13}\text{C}$] lactate than benign TSCs, as evidenced by a significantly larger peak hyperpolarized lactate (3.0 ± 0.1 versus 1.3 ± 0.5 nmol/mg, $p=0.03$) and hyperpolarized lactate AUC (262 ± 17 versus 80 ± 47 , nmol/mg total, $p=0.02$, **Figure 2C**). The significant increase in hyperpolarized lactate signal is consistent with a significant increase in LDH activity in cancer (0.0027 ± 0.0003 versus 0.0018 ± 0.0003 nmol/mg/min of protein, $p=0.05$). There was no significant difference in the time to maximum hyperpolarized lactate (58 ± 5 sec versus 49 ± 3 sec, $p=0.2$) between benign and malignant TSCs.

Discussion: In these prostate TSC bioreactor studies, we have shown for the first time that we can maintain 300 micron thick discs (≈ 8 mm in diameter) of benign and malignant human prostatectomy tissue alive in a NMR compatible 3-D tissue culture bioreactor for 32 hours (Fig. 6). We obtained ^{31}P spectra from these tissues that were identical to what has been previously published for *in vivo* ^{31}P spectra from the human prostate, and the ^{31}P spectra remained relatively constant over the 32 hour time course(10). Additionally, pathology at the end of the bioreactor study demonstrated excellent maintenance of *in vivo* tissue structure. This tissue culture platform provided a unique opportunity to investigate the metabolism of hyperpolarized [$1\text{-}^{13}\text{C}$]pyruvate in the human prostate prior to actual patient studies. Benign tissues showed very low levels of hyperpolarized [$1\text{-}^{13}\text{C}$] lactate consistent with the major utilization of pyruvate being citrate production (11), while cancer showed high lactate, similar to the prior observation of increased lactate concentrations in human biopsy samples (12)and increased LDH activity. Moreover, there was minimal overlap of the labeled hyperpolarized lactate signal in individual cancer and benign tissues, suggesting that hyperpolarized lactate will be an accurate biomarker of prostate cancer in patients.

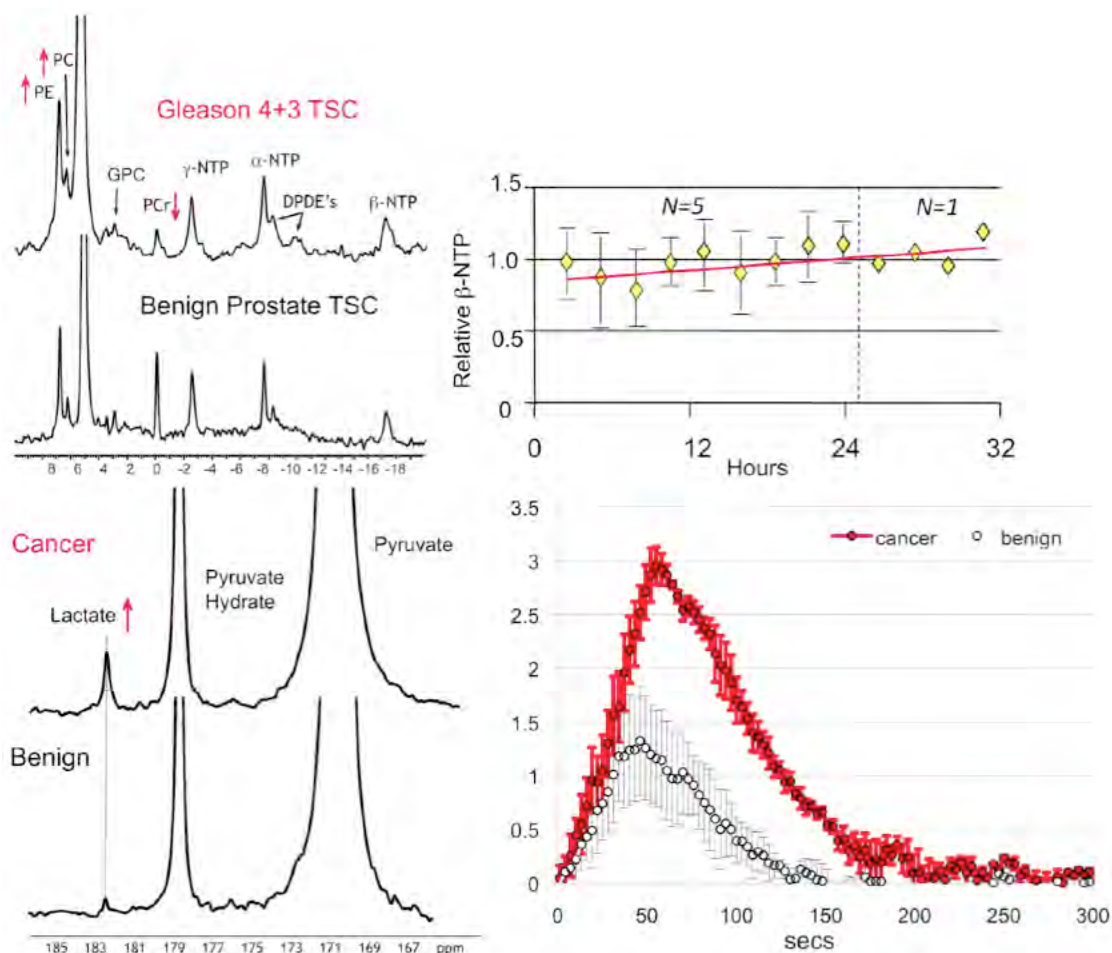


Figure 6: (left, top) ^{31}P spectra 4+3 prostate cancer and benign prostate tissue demonstrating metabolic profiles similar to what is observed in prostate cancer patients, (left, bottom) corresponding ^{13}C spectra after injection of hyperpolarized $[1-^{13}\text{C}]$ pyruvate demonstrating elevated lactate in prostate cancer relative to benign prostate tissue, (right, top) Plot of β -NTP versus time of prostate tissue in the NMR compatible bioreactor demonstrating, constant viable metabolism, (right, bottom) time-course of lactate metabolism after injection of hyperpolarized $[1-^{13}\text{C}]$ pyruvate demonstrating significantly higher levels of lactate in cancer with no overlap of individual benign and malignant levels.

KEY RESEARCH ACCOMPLISHMENTS:

- We demonstrated that the metabolism of prostate tissue slice cultures reflects *in vivo* metabolism by comparing of the metabolic profiles of cultured human tissue cultures with the metabolic profiles of snap frozen TRUS guided biopsies of healthy and malignant prostate tissues.
- We demonstrated that the metabolism of non-hyperpolarized $[3-^{13}\text{C}]$ pyruvate by the human tissue slices was consistent with the previously published literature and that metabolism of pyruvate to lactate was a biomarker of prostate cancer.
- We demonstrated for the first time that the pathologic and metabolic integrity of benign and malignant human prostate tissues can be maintained in a NMR compatible 3-D tissue culture

bioreactor for 32 hours.

- After administration of hyperpolarized [1-¹³C]pyruvate, the generation of labeled hyperpolarized lactate and LDH activity were significantly higher in malignant tissues (N=3) relative to benign human prostate tissues (N=3).
- There was minimal overlap of the labeled hyperpolarized lactate signal between individual cancer and benign tissues suggesting that hyperpolarized lactate will be an accurate biomarker of prostate cancer in patients.

REPORTABLE OUTCOMES: The data reported in all three progress reports has been written up and will be submitted to the journal *Cancer Research* by August 2011.

CONCLUSION: We have successfully achieved the goals of the first aim, to optimize conditions for maintaining human prostate tissue slices (TSCs) in an NMR- compatible, 3-D tissue culture bioreactor and to verify the metabolic integrity of TSCs over time. Of particular importance, the results of the first year of funded studies demonstrate the feasibility of maintaining both the pathologic and metabolic integrity of benign and malignant human tissue cultures in the NMR compatible bioreactor for 32 hours. Moreover, non-hyperpolarized and hyperpolarized ¹³C MR data acquired from human tissue slices demonstrated that the metabolism of the human tissue slices accurately reflects the metabolism of the *in vivo* human prostate and that [1-¹³C]hyperpolarized lactate will be an accurate biomarker of prostate cancer in patients.

Based on successfully accomplishing the goals of aim 1, studies have started determine whether hyperpolarized metabolites correlate with pathologic grade (i.e., Gleason grade 3 versus grade 4/5).

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APPENDICES: N/A