Peer Review File

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Reviewer A

1. There are many linguistic deficiencies, especially in the introduction and methods section; accordingly, the manuscript should be checked by a professional English language editing service before resubmission.

Response: *Thank you for your comment. This revised manuscript has been edited and proofread by Medjaden Bioscience Ltd.*

2. Abstract first sentence in the Section "Methods": reword this sentence, it does not make sense.

Response: *Thank you for your comment. In the revised manuscript, the Methods in the Abstract have been amended to state, "Tumor microcirculation and perfusion in subcutaneous and orthotopic LNCaP xenograft Balb/c athymic nude mice models were compared by investigating microbubble wash-in with CEUS" (Page 2, lines 23-25).*

3. Line 59: Androgen deprivation is not the treatment of choice for localized prostate cancer, but radical prostatectomy or radiotherapy.

Response: *Thank you for your comment. In the revised manuscript, this text has been amended to state, "Androgen deprivation therapy is widely used in the treatment of localized prostate cancer; however, tumors eventually progress, becoming metastatic and fatal (2)" (Page 4, lines 49-50).*

4. Line 69: LNCaP cells are typically implanted in immunodeficient mice, not in immunocompetent mice.

Response: *Thank you for your comment. In the revised manuscript, this text has been amended to state, "The LNCaP cell line can be implanted in subcutaneous and*

orthotopic sites in immunodeficient mice" (Page 4, lines 58-59).

5. Did the mice receive testosterone supplementation in parallel to tumor cell injection. If not, this could be used in future studies to reach higher engraftment rates.

Response: *Thank you for your comment. Mice were injected with tumor cells without testosterone supplementation. The effects of testosterone supplementation are a topic of future research. In the revised manuscript, text in the Conclusions has been amended to state, "Further research should verify the effects and mechanisms of testosterone supplementation on engraftment rates" (Page 15, lines 309-310).*

Reviewer B

1. As referenced by the author, prior studies have shown high vascularization of orthotopic prostate cancer in rat and PC-3 models. LNCaP cells are androgen-sensitive human prostatic adenocarcinoma cells.

Response: *Thank you for your comment. In the revised manuscript, text has been amended to state, "Prior studies have shown rich vascularization of orthotopic prostate tumors in rat and PC-3 models. LNCaP cells are androgen-sensitive human prostatic adenocarcinoma cells" (Page 4, lines 54-56).*

2. Results: Why were Atm, TtoPk, and WiAuC not significantly different between orthotopic prostate tumors and kidney (Atm: P=0.062; TtoPk: P=0.031; WiAuC: P=0.789)? Seems Atm and TtoPk approached or were different by P-value. What is expected in this comparison and why?

Response: *Thank you for this insightful comment. There are no significant differences in Atm, TtoPk, and WiAuC between orthotopic prostate tumors and kidney, because both have a rich blood supply. We speculate that the blood supply of orthotopic prostate tumors could promote tumor growth, and the microenvironment of the prostate is suitable for the growth of LNCaP cells. In the revised manuscript, text has been amended to state, "Atm, TtoPk, and WiAuC were not significantly different*

between orthotopic prostate tumors and kidney (Atm: P=0.062; TtoPk: P=0.031; WiAuC: P=0.789) (Figure 2), indicating both orthotopic prostate tumors and kidney have a rich blood supply, and the blood supply of orthotopic prostate tumors could promote tumor growth"(Page 11, lines 207-211).

3. Statement: Although the TICs showed no significant difference in mean Atm between the orthotropic prostate tumors and kidney, parametric imaging showed the orthotopic prostate tumor enhanced slower than kidney (Figure 3). – What explains this difference compared to parametric imaging.

Response: *Thank you for your interesting comment. The TICs can reflect the overall time intensity curve of the course of wash-in and wash-out. With a high blood flow velocity, it can reach peak enhancement in a short time for orthotropic prostate tumors and kidney, where minor differences in the tumor enhancement could be difficult to discern. Parametric imaging can capture more details about the progress of the tumor enhancement of orthotropic prostate tumors and kidney. From parametric imaging, it could be observed that it took 1.5s to completely contrast the kidney, by which time only the periphery of the orthotopic prostate tumor was enhanced. It can thus be concluded that the orthotopic prostate tumor enhanced slower than kidney. To avoid confusing, in the revised manuscript, text has been amended to state, "The TICs showed no significant difference in mean Atm between the orthotropic prostate tumors and kidney (Figure 2). With parametric imaging, which can capture more detailed information about the progress of the tumor enhancement, it can be observed that the orthotopic prostate tumor enhanced slower than kidney (Figure 3)." (Page 11, lines 214-218).*

4. Statement: Subcutaneous tumors were characterized by massive necrosis and hemorrhage. The morphology of the tumor vasculature was irregular, and vessels had thin walls. Orthotopic prostate tumors were characterized by gross hemorrhage and multifocal necrosis. - How do you think that the results that you saw were influenced by the described differences in necrosis and hemorrhage. Should discuss if using

smaller tumors would have altered results?

Response: *Thank you for your interesting comment. Both subcutaneous and orthotopic prostate tumors were associated with necrosis and hemorrhage; therefore, we consider that our comparisons are appropriate. Smaller tumors may have altered our findings. This will be investigated in future experiments.*

5. There is a statement that the prostatic glands were compressed by the tumor. - This suggests large tumors whereas most prostate cancers are found within the gland.

Response: *Thank you for your comment. In the revised manuscript, text has been amended to state, "Orthotopic prostate tumors compressed the prostate gland" (Page 12, line 223):*

6. Discussion: Statement: Primary advantages of subcutaneous xenograft models include the ease of implantation and monitoring of tumor growth, while orthotopic xenografts metastasize in a similar manner and to similar locations in rodents as in humans. – Please reference.

Response: *Thank you for your comment. This text has been referenced in the revised manuscript. We have cited: Delitto D, Pham K, Vlada AC, Sarosi GA, et al. Patient-derived xenograft models for pancreatic adenocarcinoma demonstrate retention of tumor morphology through incorporation of murine stromal elements - sciencedirect. Am J Pathol 2015;185(5):1297-1303 (Page 13, lines 249).*

7. Statement: Orthotopic LNCaP mouse models closely mimic the natural history of prostate cancer in humans; however, induction of orthotopic tumors in the prostate of nude mice is challenging due to the difficulty of operating on small animals, and because the tumorigenicity and metastatic potential of LNCaP cells is low. – Please reference or remove. The tumor was not followed longitudinally to show local progression or to show metastasis in the current paper.

Response: *Thank you for your comment. This text has been removed from the revised manuscript.*

8. Statement: The advantages of ultrasound compared to MRI and bioluminescence include cost, convenience and real time imaging, and ultrasound allows the clear delineation of orthotopic tumors from the surrounding tissues (12). – Reference PMID Biotechniques. 2020 Jul;69(1):395-403. PMID: 32363906, which shows MR imaging of prostate cancer with clear delineation from other structures.

Response: *Thank you for your comment. An additional reference has been added to the text in the revised manuscript. We have cited: Ravoori MK, Singh S, Yang P, Wei W, Kundra V. In vivo magnetic resonance imaging of orthotopic prostate cancer. BioTechniques, 2020; 69(1): 37-45 (Page 13, lines 253).*

9. Statement: Findings established the orthotopic LNCaP xenograft Balb/c athymic nude mouse model as a clinically relevant model of prostate cancer that could be used for the in vivo testing of novel anti-angiogenic strategies. The tumor take rate (68.2% vs. 58.3%) was higher, the first tumors were visualized earlier, tumor growth was faster, and tumor volume was greater at 8 weeks in the orthotopic model compared to the subcutaneous model, indicating that the dorsal prostate provided a more suitable microenvironment to support tumor engraftment and growth. No difference was statistically shown. Please adjust or remove this statement.

Response: *Thank you for your comment. This text has been removed from the revised manuscript.*

10. Conclusion: Statement: the orthotopic LNCaP xenograft Balb/c athymic nude mouse model closely mimics the natural history of prostate cancer in humans – Please remove this clause as longitudinal assessment for local spread and metastastic pattern was not assessed as mentioned above.

Response: *Thank you for your comment. This text has been removed from the revised manuscript.*

11. Statement: Applying CEUS to the assessment of angiogenesis in a mouse model of

prostate cancer may have clinical utility for monitoring tumor responses to antiangiogenic therapies and assessing tumor vascularity. – please remove the word clinical from this sentence. Data presented was in a mouse model and this sentence refers to a mouse model.

Response: *Thank you for your comment. The word 'clinical' has been removed from the revised manuscript.*