Investigation of Acute Hypoxia Effects on a 3D model of Renal Cell Carcinoma

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Introduction

- Eukaryotic systems precisely and efficiently cope to environmental stressors. Among these, adaptation to the variation of oxygen levels (Hypoxia/Hyperoxia) is essential for survival.
- Genetic alterations of the hypoxia response pathway are common drivers of clear cell renal cell carcinoma (ccRCC). On the other hand, non-clear cell renal cell carcinoma (nccRCC) usually display an intact response to the hypoxia stress pathway.
- Understanding how different genomic driven Renal Cancers survive under hypoxic stress is of extreme importance for the understanding of Renal Cancer evolution

Hypothesis: nccRCC tumor models possess unique abilities to survive and adapt to hypoxia and therefore will show phenotypic differences in terms of viability and stem cells characteristics.

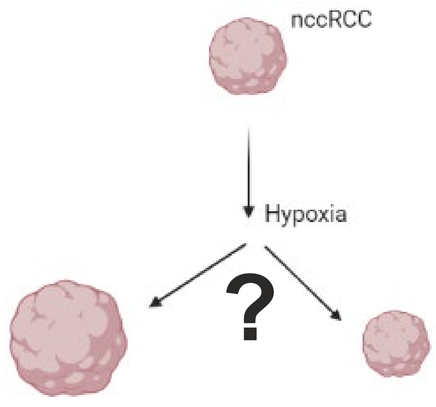

Figure 1. Schematic showing possible effects of hypoxia on growth of nccRCC models. Created by BioRender.com

Methods

GEM model of metastatic nccRCC

- Tumor explants were derived from a metastatic model of nccRCC. Briefly, 4-6 weeks old $Pax8^{Cre}$, $H11^{lsl\text{-}spCas9}$, $R26^{lsl\text{-}fsf\text{-}tdTomato}$ mice received renal subcapsular injection of 10^9 AAV viral particles, carrying sgRNA for the knockout of known genetic drivers of nccRCC (*Nf2, Setd2, Cdkn2a, Cdkn2b*) and a conditional FLEX-Flpo system for the tissue-specific activation of the Tdtomato fluorescent reporter. At terminal disease, mice were euthanized, and tumor cell lines were extracted from primary tumors and cultured in 3D as organoids

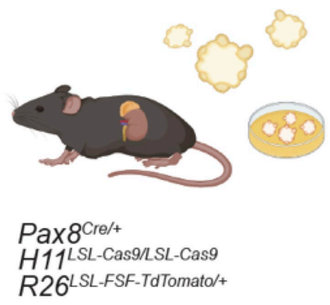

Fig. 2 Experimental design of the generation of GEMM derived tumor organoids. This system allows for the rapid generation of somatic mosaic alterations commonly observed in human nccRCC and, thus, a reliable experimental model mirroring patients' disease progression

Generation and propagation of tumor organoids *in vitro*

- Kidney tumor organoids were plated into a 12 well plate in 40ul of Cultrex per each well and supplemented with 1mL of DMEM/F12 Medium containing 10% FBS and 1% PS.
- Organoids were passaged by aspirating the medium from the wells and adding 1mL of ice-cold PBS to each well in order to disaggregate organoids from Cultrex. Organoids were, then, centrifuged for 5 minutes at 150g; the pelleted organoids were dissociated with Trypsin incubation of 5' at 37C 5%CO2. Single cell suspensions were plated as previously described with a concentration of 5,000 cells/40ul of Cultrex..

Data Quantification

- Three days after organoid plating, two different plates were incubated at 37C, 5% CO2 in normoxic conditions (21% oxygen) or severe hypoxic conditions (1% oxygen) for 72 hours.
- Images of tumor organoids were collected at time 0 (immediately before hypoxia or normoxia treatment) and 72 hours after hypoxia or normoxia treatment. Fields were randomly selected at 40X magnification.
- Data analysis was performed with ImageJ software FIJI and statistical analysis with GraphPad Prism 9

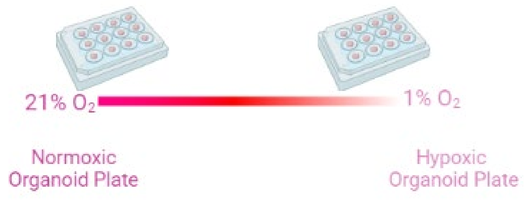

Figure 3. Experimental design for testing hypoxia effects on nccRCC organoids. One plate was incubated in normoxia (21% oxygen) and another plate was incubated in hypoxia (1% oxygen) for 72 hours each. Created by BioRender.

Results

- nccRCC tumor organoids were successfully generated and propagated *in vitro.*
- Organoids showed different morphology and sizes, suggesting phenotypical tumor heterogeneity.
- Analysis of organoid size and number showed a statistically significant difference between tumor organoids under the effect of normoxic but not under the effect hypoxic environment.

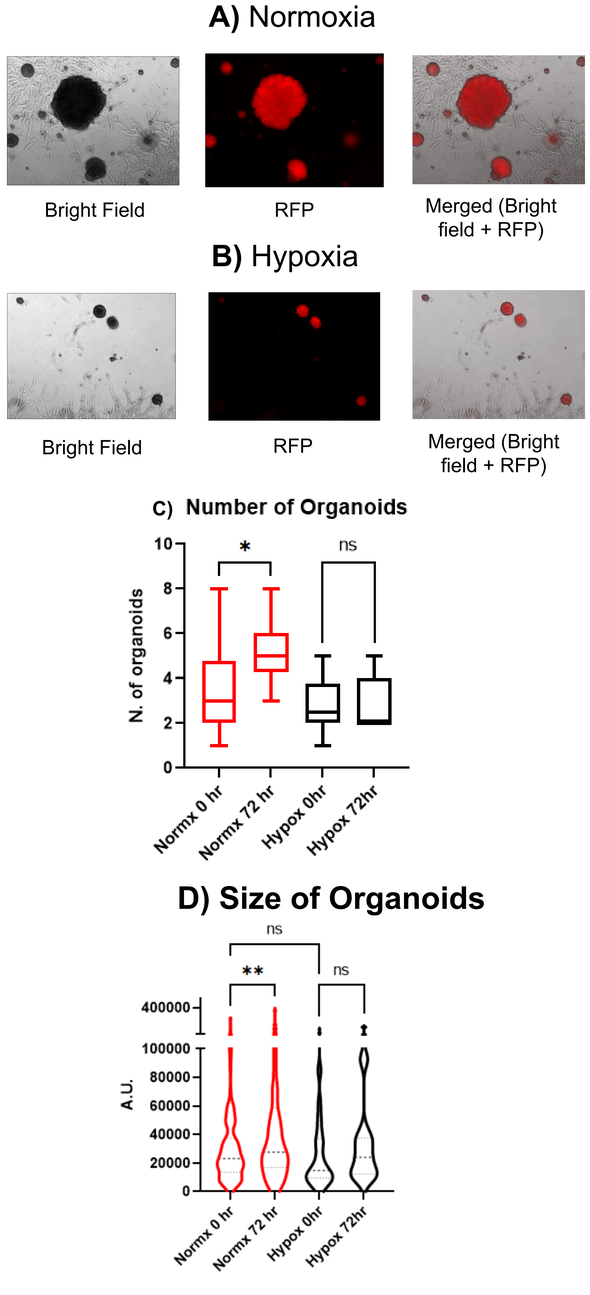

Figure 4. **A)** Three pictures showing organoids exposed to normoxia with bright field, tdTomato, and merged channels at 40x magnification. **B)** Three pictures depicting organoids after being exposed to hypoxia at 40x magnification on different channels. **C)** Box and whiskers plot comparing the number of organoids at the beginning and end of the experiment (Normx 0hr; N= 113, Normx 72hr; N= 178, Hypox 0hr; N = 81, Hypox 72h; N=130). **D)** Violin plot comparing the size of organoids before and after normoxia and hypoxia (N=33; N=56; N=33; N56) *, p value < 0.05; **, p value < 0.01

Discussion

- Following data analysis, we observed that there was a statistically significant ($p < 0.05$) difference in the number of organoids in the plate that went into normoxia at 0 hours compared to 72 hours while no differences have been observed in hypoxia, suggesting that hypoxic conditions have a negative impact on stemness capabilities of nccRCC.
- Likewise, the size of organoids in normoxia showed statistical significance ($p<0.01$) between the 0 hour and 72 hour time points, while no differences have been observed in hypoxic condition. These data suggest that hypoxia severely impact tumor organoids proliferation.

Conclusion

Given our data, it can be understood that nccRCC cells have a sensitivity to low oxygen levels which prevents cell proliferation from occurring therefore rely on a specific oxygen level to proliferate. This suggests that low oxygen levels are not always the cause for uncontrollable tumor growth in renal cell cancer.

Future Works

The vulnerability of nccRCC cells to hypoxia can be used to find new therapeutic methods to reduce the severity of this type of kidney cancer. A longer experiment (i.e 7 days) can be done in the future to further confirm the effects of hypoxia in nccRCC cells.

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