mTOR driven metabolic shifts in cancers and in 3D bioprinted tissue-mimetic structures

Ph.D. thesis
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Budapest, 2025

1. Introduction

Changes in metabolism and energy use are important for cancers to survive and grow. Although tumors within the same (sub)type may share certain metabolic characteristics, each tumor is unique. This heterogeneity presents a major challenge for experimental cancer modeling, and therefore high-quality experimental models are essential. As the central regulator of cellular metabolism, the mechanistic target of rapamycin (mTOR) signaling pathway plays a pivotal role in coordinating these metabolic processes.

During our studies, we addressed two main research areas:

1. Establishment and characterization of 3D bioprinted cancer models: Over the past decades, 3D cell cultures gained increasing interest, because they better mimic human tissues compared to 2D monolayer cultures. More recently, 3D bioprinted models have emerged as an innovative approach, enabling the controlled reproduction of complex tissue microenvironments (TME) and heterogeneity – both of which play key roles in tumor behavior. In this work, we developed and characterized 3D bioprinted breast and renal carcinoma models.

2. Tumorigenic role of tacrolimus in post-transplant renal cell carcinoma: Following kidney transplantation, the risk of developing post-transplant (post-tx) malignancies is well established. This elevated risk is often attributed to impaired immune system caused by lifelong immunosuppressive (IS) therapy. However, as different IS agents are associated with varying incidences of malignancy, the possibility of a direct tumor-promoting effect must also be considered. In this study, we investigated the direct oncogenic effects of tacrolimus (TAC) – the most widely used IS drug in clinical transplantation – and compared its molecular effects with mTOR inhibitors.

2. Objectives

2.1. Establishment and characterization of 3D bioprinted cancer models

Our objectives aim to develop a standardized, physiologically relevant 3D tumor model that enhances the predictive value of preclinical testing and supports the development of more effective cancer therapies:

1) To establish and optimize a reproducible 3D bioprinting workflow.
2) To generate 3D bioprinted RCC and breast cancer models from various cell lines and tumor-derived cells.

3) To validate the usability of *in vitro* growth-monitoring assays in 3D bioprinted tissue-mimetic structures (TMSs).
4) To investigate tissue morphogenesis and spatial organization within the 3D bioprinted breast cancer TMSs.
5) To characterize the activity of the mTOR signaling pathway within the 3D bioprinted breast carcinoma TMSs.
6) To assess the therapeutic sensitivity of the 3D bioprinted breast carcinoma TMSs to mTOR inhibitors and chemotherapeutic agents.
7) To establish a 3D bioprinted "patient-derived" breast cancer model and compare its drug sensitivity profile with other *in vitro* and *in vivo* models.

2.2. Tumorigenic role of tacrolimus in post-transplant renal cell carcinoma

Our objectives aim to elucidate the differential effects of IS agents on oncogenesis and to investigate the molecular pathways underlying post-tx malignancies, with a particular focus on mTOR signaling:

1) To collect a comprehensive RCC cohort.
2) To examine the *in situ* effect of IS on mTOR pathway activity in ESRD.

3) To examine the *in situ* effect of IS on mTOR pathway activity in RCCs.
4) To evaluate the *in vitro* impact of IS on proliferation and mTOR pathway activation.
5) To compare the long-term effects of IS agents on tumor growth and mTOR pathway using in *in vivo* RCC xenograft mouse model and *in vitro* 3D bioprinted TMSs.

3. Methods

3.1. *In vitro* methods (cell cultures, proliferation assays, 3D bioprinting)

In vitro experiments were conducted using RCC and breast cancer cell lines. For drug sensitivity testing, cells were exposed for 72 hours or 21 days to the treatments (tacrolimus – TAC, rapamycin – RAPA, PP242, ipatasertib – Ipa, Cisplatin – Cis, doxorubicin – Doxo), then cell viability was measured using Alamar Blue (AB) and Sulforhodamine B (SRB) assays. For 3D bioprinting, two bioinks were formulated: a "cellular gel" and a "scaffold gel". The 3D bioprinted TMSs were produced with an extrusion-based bioprinter. The constructs were fabricated by alternating layers of the two gels, then crosslinked with $CaCl_2$.

3.2. *In vivo* methods (renal ischemia reperfusion model, xenograft model, syngeneic tumor model)

C57BL/6 mice were used to establish a renal ischemia-reperfusion (IR) model. IR was induced by clamping the left renal artery and vein, with the removal of the contralateral kidney. Treatments (TAC, Rapa) were given for three days. Xenograft models were established and used by subcutaneously injecting tumor cells into the flanks/breast region of SCID mice, while for syngeneic tumor model, tumor cells were injected into the breast region of BALB/c mice. When palpable tumors developed, treatments were administered for 21 days. For tumor-derived 3D bioprinting, solid tumors were excised from BALB/c mice and digested enzymatically (PE/EA/801-7/2020; 16 September 2020, PEI/001/1733-2/2015; 14 October 2015).

3.3. Patient samples (renal cell carcinoma cohort)

The study included post-tx RCCs (n = 44) and non-tx RCCs (n = 46). Kidney tissues from patients with ESRD (n = 10) and from donor kidneys (n = 3) were analyzed. All samples were obtained at Semmelweis University (2000-2015), with approval (No. 7/2006, SE-RKEB – 216/2020).

3.4. Statistical analysis

Statistical analyses were carried out using GraphPad Prism version 10.4.1. All results are based on data from at least of three independent experiments.

3.5. Protein analyzes (immunohistochemistry, western blot, WES™ Simple)

For immunohistochemistry, sections were deparaffinized, followed by endogenous peroxidase activity block, antigen retrieval, primary antibodies, then detection. For western blot, proteins were separated by SDS-PAGE, transferred to membranes and visualized by using ECL substrate. WES™ analysis was carried out on a 12–230 kDa Separation Module as indicated by protocol. Protein expression levels were normalized to β-actin.

4. Results

4.1. Establishment and characterization of 3D bioprinted cancer models

4.1.1. Establishment of 3D bioprinted cancer models via Gesim BioScaffolder

Rheological analysis of the gels revealed desirable shear-thinning behavior. For generating 3D bioprinted cancer models, various cell lines were successfully used. The 3D bioprinted TMSs showed continuous growth for 3 weeks, with cell line-specific differences in growth kinetics.

4.1.2. Validation of growth and cellular dynamics in 3D bioprinted tissue-mimetic structures

Tissue growth of the 3D bioprinted breast cancer TMSs was monitored via mCherry fluorescence (AB SRB). In the 3D TMSs, apoptotic cells were localized to central regions, while proliferating cells were fewer than in 2D monolayers.

4.1.3. Increase and redistribution of cell–cell and cell-matrix adhesion proteins within 3D bioprinted tissue-mimetic structures

In the 3D bioprinted TMSs, intense membrane-associated staining of β-catenin, E-cadherin and N-cadherin was detected. Fibronectin was predominantly detected perinuclear, while syndecan homogenously in the cytoplasm, indicating a role in transcriptional regulation or signaling functions.

4.1.4. Reduced activation and increased heterogeneity of mTOR-pathway components in 3D bioprinted tissue-mimetic structures

The 3D bioprinted TMSs displayed a decrease in mTOR pathway protein expression. Although the total levels of core proteins did not differ significantly between 2D and 3D models, a marked decline was detected in the expression of the phosphorylated forms in the 3D environment. Phospho-proteins were predominantly localized at the outer regions of the 3D bioprinted TMSs.

4.1.5. Quantitative analysis of mTOR signaling in 3D-bioprinted tissue-mimetic structures

Elevated levels of S6, pan-Akt, and Rictor, while reduced p-S6 and p-Akt were detected in the 3D bioprinted TMSs. A pronounced decrease in p-S6/S6 and p-Akt/Akt was observed in the 3D bioprinted TMSs, implicating diminished mTOR kinase activity under 3D culture conditions. Baseline comparisons revealed that TSC1 and p-SAPK/JNK expression levels were inherently higher in the 3D bioprinted TMSs than in the 2D monolayer cultures.

4.1.6. Altered response to mTOR-targeted therapies in 3D bioprinted tissue-mimetic structures

Treatments were administered *in vitro* (Rapa, Cis, Cis + Rapa; Cis + Ipa). The 3D bioprinted TMSs exhibited significantly decreased sensitivity to treatments.

4.1.7. 3D bioprinted “patient-derived” breast cancer model for drug testing

A 3D bioprinting-based “patient-derived” cancer model was applied, using 4T1 cell line growing in BALB/c mice. The majority of the isolated cells were tumor cells (~50%). These tumor-driven cell mixtures were applied for drug tests in 2D monolayer cultures, 3D bioprinted TMSs, BALB/c allograft and SCID mice xenograft models. The drug responses observed in

the BALB/c allograft model were consistent with the sensitivity pattern seen in the 3D bioprinted TMSs.

4.2. Tumorigenic role of tacrolimus in post-transplant renal cell carcinoma

4.3. Clinicopathological features of the renal cell carcinoma cohort

The post-tx RCC cohort included 44, while the non-tx RCC cohort included 46 cases. The subtype distribution among cohorts does not reflect of the actual incidence in general (*de novo*) population.

4.4. Elevated mTOR signaling is detected in end-stage renal disease following immunosuppression compared to healthy kidney tissue

ESRD kidneys exhibited a marked reduction in p-mTOR, p-S6, and Rictor, which suggests that the loss of functional nephrons in ESRD is associated with a downregulation of mTOR signaling components. CNI-based immunosuppression exhibited a pronounced upregulation of mTOR pathway markers, which indicates that CNI-mediated immunosuppression drives a reactivation of mTOR signaling.

4.5. Tacrolimus enhances the mTOR activity in the ischemic kidney of mice

To model ESRD *in vivo*, a murine renal ischemic model was established. Ischemia alone, slightly increased the expression of all examined mTOR markers. The effect was markedly enhanced by TAC treatment. In contrast, slight attenuation of both mTORC1 and mTORC2 signaling was noted in Rapa-treated ischemic kidneys.

4.6. Pronounced activation of the mTORC2 signaling axis in post-transplant renal cell carcinomas compared to non-transplant, de novo renal cell carcinomas

In ccRCC, p-mTOR expression was higher in post-tx cases, while in pRCC, both p-mTOR and Rictor expression levels were significantly elevated in post-tx patients. Post-tx RCCs of both subtypes exhibited increased mTORC2 activity. These findings indicate that mTORC2 activity is preferentially enhanced in RCCs occurring after transplantation.

4.7. mTOR signal activating effects of tacrolimus on normal tubular epithelial cell line *in vitro*

Proliferation of HK-2 was not significantly altered by TAC following a 72–hour and 21–day treatment. Protein analysis revealed an acute and persistent activation of mTORC1 following TAC treatment, indicating that long-term TAC treatment may activate mTORC1 signaling over time.

4.8. *In vitro* effects of tacrolimus on renal cell carcinoma cell lines: activation of mTOR signaling and increasing proliferation

Treatment with TAC for 72 hours and 21-days was associated with increased proliferation in A498 RCC cell line, and next to this, both mTORC1 and mTORC2 complexes were activated.

4.9. 3D effects of tacrolimus on renal cell carcinoma cell lines: increasing proliferation and tumor growth

72-hour and 21-day TAC treatment resulted in a significant increase in proliferation in 3D bioprinted A498 TMSs. TAC induced tumor growth was also confirmed in *in vivo* human xenograft mouse model of A498. A 21-day regimen of TAC was found to dramatically accelerate tumor growth.

4.10. Tacrolimus-mediated activation of mTOR signaling in 3D bioprinted renal cell carcinoma model and in tumors of xenograft mice

3D bioprinted A498 renal carcinoma TMSs were treated for 21 days. TAC treatment revealed a significant increase in p-S6/S6 and p-Akt/Akt ratios. Furthermore, increased activity of the downstream mTORC1 and mTORC2 targets was also demonstrated. Xenograft tumors showed increased mTORC1

activity and modest rises in Rictor and p-Akt expression after TAC treatment.

5. Conclusions

5.1. Establishment and characterization of 3D bioprinted cancer models

In this part of the work, we developed 3D bioprinted *in vitro* breast and renal cancer models, with characterization performed using the T47D breast cancer cell line:

1) Established a 3D bioprinting protocol.
2) Generated 3D bioprinted RCC and breast cancer models from various cell lines and tumor-derived cells.
3) Validated *in vitro* proliferation assays, confirming their applicability in the 3D bioprinted context.
4) Characterized tissue morphogenesis in the 3D bioprinted breast cancer model.
5) Identified an adaptive response to 3D stressors alongside reduced mTOR pathway activity in 3D bioprinted TMSs.
6) Observed reduced drug sensitivity of 3D bioprinted breast cancer structures to mTOR inhibitors.
7) Demonstrated that 3D bioprinted tumor-derived TMSs, more accurately mimic *in situ* drug responses than other preclinical models.

5.1. Establishment and characterization of 3D bioprinted cancer models

In this part of the work, we investigated the tumorigenic role of tacrolimus in post-transplant renal cell carcinoma using patient samples and experimental models:

1) Described for the first time that CNI-based immunosuppression increases mTOR activity in ESRD.

2) Confirmed *in vivo*, that TAC enhances mTOR activity in *in vivo* ESRD model.

3) Detected, that mTORC2 activity is elevated in post-tx RCCs under CNI-based immunosuppression.

4) Demonstrated that TAC enhances mTOR activity or proliferation *in vitro* in a cell line-dependent manner.

5) Found that TAC promotes tumor growth in both 3D bioprinted and *in vivo* A498 renal cell carcinoma models, through mTOR activation.

6) Showed that that short-term drug exposure may underestimate drug efficacy in 3D environment.

6. Bibliography of the candidate's publications

Publications related to the thesis:

1st author publications

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∑IF: 51,008