Perfused 3D model of the aberrant clear cell renal cell carcinoma vasculature to decipher its specific cellular and functional features

Co-direction of the project

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I. Scientific context, positioning and objectives of the proposal

• Aggressiveness of the ccRCC associated with hypervascularization causes aberrant architectures

Clear cell renal cell carcinoma (ccRCC) is a highly aggressive, metastatic cancer usually associated with a poor prognosis [1]. Caused, in most cases, by the alteration of the von Hippel-Lindau gene inducing high protein levels of the hypoxia-inducible factors 1 and 2, ccRCC is associated with a pseudo hypoxia that triggers an hypervascularization through angiogenesis, accompanied by epithelial-to-mesenchymal transition (EMT) and extracellular matrix (ECM) remodeling favoring tumor cell invasiveness. The tumor microenvironment (TME) is also populated by cancer-associated fibroblasts (CAFs) with a high secretory activity. The vascular endothelial growth factor (VEGF) pathway was identified at the origin of the hypervascularization of the ccRCC [2]. For this reason, anti-angiogenic treatments using tyrosine kinase inhibitors (TKI) that target this pathway, such as sunitinib, are used as approved first-line therapy for metastatic ccRCC [1]. Unfortunately, less than 30% of the patients respond favorably and 14% even present a progression of the disease, generally linked to the higher degree of complexity of the microenvironment of ccRCC solid tumors, partly explaining its resistance, either intrinsic or acquired.

• Past work: achievements, limitations and current challenges

Aiming to elucidate the role of the capillaries in ccRCC resistance to treatment, Team 2 has characterized and analyzed the aberrant tridimensional architecture of the ccRCC microvasculature both clinically and experimentally (*in vivo* and *in vitro*). Their results showed a combination of peculiar lumenized dilated ponds and long, flat sheets structures, composed of endothelial cells, co-existing in ccRCC vasculature, as reported by N. Brassard-Jollive in her PhD Thesis defended in 2022 (article in preparation). Differing strongly from other TME capillaries, these structures were recapitulated and studied inside a static vascularized microtumor model (VMT) with ccRCC cells and human umbilical vein endothelial cells (HUVEC) inside a 3D gel of collagen I. They managed to restore the specific architecture consisting of interconnected ponds and sheets around a TME. This important preliminary step now calls for an improved, perfused VMT model to perform a relevant characterization of the ccRCC microvasculature at both cellular and molecular levels, and especially during the emergence of this specific 3D architecture and in its response to anti-angiogenic treatments.

• State of the art of perfusing 3D tumors and spheroid inside microfluidic channels

To solve this and respond to current challenges, Team 1 and Team 2 have proposed to construct a microfluidic chip integrating the cell-laden hydrogel with ECM-like properties for long-term cell culture and perfusion. Some solutions compatible with Team 2's model requirements exist in Team 1 or can be developed readily using their current techniques. They consist of soft, fibrous hydrogel scaffolds made of collagen type I or fibrin, that encapsulate scattered endothelial cells within their network and are crosslinked inside a specific chamber placed between microchannels (see Figure). These engineered microchannels are used to control an interstitial flow within the crosslinked matrix and promote the emergence of a capillary bed architecture that opens its lumen and connected with the microchannels for perfusion, thereby enabling exogenous perfusion of tissue-like structures that mimic capillary functions with high fidelity [3]. Recently, Kamm's group, a pioneering team in this field, has shown that the seeding sequence and the support cells for co-culture is vital to guarantee a correct vascularization and a more efficient, *in vivo*-like perfusion of the tumor spheroid by the microcapillary bed (central channel of the chips of the Figure) that is itself interconnected with engineered microfluidic channel for perfusion (the two outer channels) [4].

• Working hypothesis and objectives

We believe that the joint experience of Team 1 and Team 2 can ideally merge synergistically to construct a perfused 3D VMT model of ccRCC by engineering a constant, controllable perfusion of the tissue of interest inside the chip. The addition of an interstitial flow has been proven to guide the emergence of an externally perfusable capillary architecture inside a 3D ECM hydrogel (see Figure). Team 1 has a proven expertise in developing microfluidic chips for cell culture integrating hydrogels, as well as endothelial cells mechanobiology [5-7], while Team 2 has a track record in studying the role of the ECM in capillary morphogenesis and homeostasis [8-10].

Objectives: to develop, test and optimize a perfused, hence dynamic vascularized microtumor (dynVMT) that offers long

co-culture duration of analysis of the tumor-capillaries cross-talks and that also provides a relevant model by adding the native features of flow inside the ccRCC vascularization for analysis of the responses to TKI. A multi-channel, hydrogel-based microfluidic device will be designed and fabricated to co-culture a perfusable blood vessel network (in the form of a microcapillary bed "µCB") together with cancer cell spheroids. This native-like microenvironment will enable the study of aberrant microvasculature observed in human renal cancer.

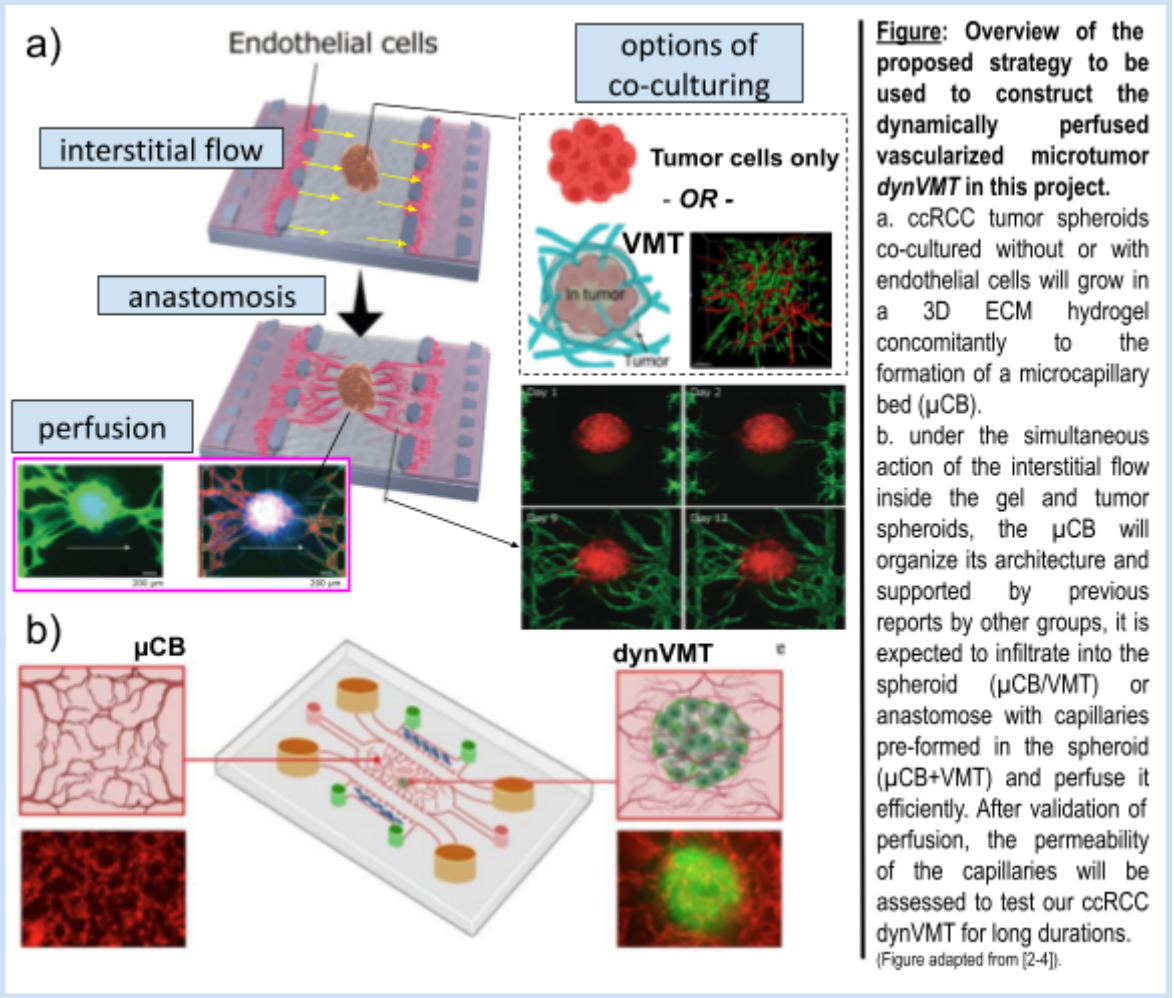

Figure: Overview of the proposed strategy to be used to construct the dynamically perfused vascularized microtumor *dynVMT* in this project.
a. ccRCC tumor spheroids co-cultured without or with endothelial cells will grow in a 3D ECM hydrogel concomitantly to the formation of a microcapillary bed (µCB).
b. under the simultaneous action of the interstitial flow inside the gel and tumor spheroids, the µCB will organize its architecture and supported by previous reports by other groups, it is expected to infiltrate into the spheroid (µCB/VMT) or anastomose with capillaries pre-formed in the spheroid (µCB+VMT) and perfuse it efficiently. After validation of perfusion, the permeability of the capillaries will be assessed to test our ccRCC dynVMT for long durations.
(Figure adapted from [2-4]).

• Organization and implementation of the project

The first aim is to successfully assemble and interconnect microvessels formed by endothelial cells inside an ECM-based hydrogel, together with the existing 3D model of ccRCC+HUVEc vascularized microtumor ("VMT") that was developed by Team 2. Two approaches that were successfully reported for other tissues and tumors will be tested here. One, called µCB/VMT, consists in assembling the µCB inside the chip but the VMT independently and individually, before placing it inside the angiogenesis chip to interconnect both sides' vessels via anastomosis, similarly to [2-4]. The second option that will be tested is called µCB+VMT and will consist in assembling both µCB and VMT simultaneously from the start, to directly vascularize the tumor with perfusable capillaries. The validation of this stage of the project will be reached when the aberrant vascular architectures that are found *in vivo* and were restored by Team 2 in their static VMT model are conserved (the posterior connection of the tumor spheroid to µCB will not modify the existing architecture of the static VMT model) or recapitulated (the architecture is obtained in the µCB+VMT). This second option may even offer an additional viewpoint, as it may provide important information regarding the dynamic of formation of these architectures when interacting with tumor cells (a control with healthy epithelial cells will be performed).
The second aim of this project is to precisely tune the onset and maintenance of both interstitial flows and exogenous VEGF gradients, known to greatly influence and guide the µCB and dynVMT inside the chip. It is indeed possible that the expected architectures will be partially restored or modified, due to the addition of flow modifying the interconnections or redirecting the VEGF secreted by the tumor spheroid or chemoattractants inside the gels. This has been observed in previous models and mitigated by controlling the flows of media and gradients of growth factors [11]. This will be achieved by the microfluidic control, but also via the tuning of ECM-based gel density and/or adding secreted factors from CAFs cultured in a permeable distinct compartment. The stiffness, ligands density and fibrous organization of the hydrogel will be assessed, as well as the cell density. The long-term preservation of the aberrant architecture will be key for a proper characterization of the involvement of cell-cell and cell-ECM interactions.

The third aim is to use the dynVMT model for analyzing the resistance mechanism of the ccRCC vasculature to TKI treatments. Team 2 demonstrated using the static VMT model that the ccRCC aberrant structures are resistant to sunitinib. The viability and integrity of the dynVMT will be assessed using relevant long-term perfusion of TKI.
Thanks to this project, both a luminal (internal) and an interstitial (external) fluid flow will be performed inside the chip: (a) the microcapillaries architecture that will be perfused by external reservoirs or pumps on the one hand; and (b) a specialized 3D chamber for the tumor cells to be ideally perfused by the dynVMT.

- **Innovative nature of dynVMT, ambitiousness and originality**

This innovative project is **based on the proof-of-concept (POC) of the static VMT**, which recapitulates the architectural and molecular features of the ccRCC-specific vascularization observed in patient samples and that will now be perfused. The high originality of the project consists in the double complex features of the TME, considering the 3D architecture and the (interstitial and intracapillary) flows. Building the dynVMT will provide an ambitious tool for recapitulating many cellular mechanisms involved in the ccRCC biology. Furthermore, the TKI delivery, submitted to flow variations imposed by the ccRCC-specific endothelial structures, will contribute to a relevant assessment of the resistance mechanism.

- ***Positioning of the project in relation to the research challenges of the IPV programme***

This interdisciplinary research project involving teams that are expert in microfluidic chips, biophysics and biomaterials (Team 1) and in the cell biology in physiopathology (Team 2), is perfectly in line with the IPV programme, since it applies original microengineering methods and cytocompatible biomaterials to create a new in vitro device useful to study mechanisms associated with health issues. A strength of this project lies in the **complementary expertise and convergent interests of the research teams**: mature technological capacities of Team 1 to conceive a microfluidic chip offering controlled hydrogel capillarization and external perfusion of endothelial cells and great expertise in ccRCC tumor vascularization and in their pathophysiology by Team 2.

II. Scientific consortium: two interdisciplinary and complementary teams

Team 1 is coordinating this project. It is led by Mathieu Hautefeuille, Professor at the Laboratoire de Biologie du Développement, Institut de Biologie Paris-Seine at Sorbonne Université, Paris (UMR7622). Starting at UNAM (Mexico), he now directs a new research team at IBPS. In the past 12 years, he has developed a wide expertise in the microfabrication of biomaterials and microfluidic systems for liver models. His team is focusing on reincorporating and studying the primordial role of the vasculature inside in vitro models with co-culture, in order to recapitulate the physiopathology of tissues and organs with external perfusion.
Team 2 is led by Catherine Monnot, CR-INSERM researcher. Her research expertise consists in analyzing the capillary network, its morphogenesis and functions in hypoxic microenvironments and the current main focus is on studying the remarkable features of the vasculature in the ccRCC tumor microenvironment. This triggered her laboratory to move to Pr. Isabelle Cremer's team at the CRC, where she is opening a new research line consisting in deciphering the vasculature architecture of tumor niches, bringing new *in vitro* approaches of 3D models, hence this collaboration with Team 1.

III. Bibliography: references related to the project (references from Team 1 and Team 2)

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