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A 3D Human Renal Cell Carcinoma-on-a-Chip for the Study of Tumor Angiogenesis[1]

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Abstract

Tractable human tissue-engineered 3D models of cancer that enable fine control of tumor growth, metabolism, and reciprocal interactions between different cell types in the tumor microenvironment promise to accelerate cancer research and pharmacologic testing. Progress to date mostly reflects the use of immortalized cancer cell lines, and progression to primary patient-derived tumor cells is needed to realize the full potential of these platforms. For the first time, we report endothelial sprouting induced by primary patient tumor cells in a 3D microfluidic system. Specifically, we have combined primary human clear cell renal cell carcinoma (ccRCC) cells from six independent donors with human endothelial cells in a vascularized, flow-directed, 3D culture system ("ccRCC-on-a-chip"). The upregulation of key angiogenic factors in primary human ccRCC cells, which exhibited unique patterns of donor variation, was further enhanced when they were cultured in 3D clusters. When embedded in the matrix surrounding engineered human vessels, these ccRCC tumor clusters drove potent endothelial cell sprouting under continuous flow, thus recapitulating the critical angiogenic signaling axis between human ccRCC cells and endothelial cells. Importantly, this phenotype was driven by a primary tumor cell–derived biochemical gradient of angiogenic growth factor accumulation that was subject to pharmacological blockade. Our novel 3D system represents a vascularized tumor model that is easy to image and quantify and is fully tunable in terms of input cells, perfusate, and matrices. We envision that this ccRCC-on-a-chip will be valuable for mechanistic studies, for studying tumor-vascular cell interactions, and for developing novel and personalized antitumor therapies.

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Abbreviations: ccRCC, clear cell renal cell carcinoma; Ct, cycle threshold; EGM, endothelial growth medium.

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Introduction

Cancer of the kidney and renal pelvis is the eighth most common malignancy in the United States, with a 5-year survival of less than 12% when diagnosed as metastatic disease [1]. Approximately 85% to 90% of kidney cancers are derived from the proximal tubular epithelial cells of the renal cortex and are classified as renal cell carcinoma (RCC) [2,3]. Of these, RCC of the clear cell type (ccRCC) accounts for approximately 75% of cases. Most sporadic ccRCCs in humans carry inactivating mutations in the *VHL* tumor suppressor gene, which lead to constitutive stabilization of the hypoxia inducible transcription factors HIF-1α and HIF-2α [4,5]. These transcription factors in turn activate a canonical cellular response which includes metabolic reprogramming towards aerobic glycolysis and secretion of numerous factors, including VEGFA, that promote angiogenesis [6,7]. If caught at an early stage, ccRCC is potentially curable, whereas advanced disease remains difficult to treat. Therapy relies in part on inhibition of the potent VEGFA-driven angiogenic signaling axis, but the development of resistance is common, owing in part to the upregulation of alternate angiogenic factors [8–10]. While there is a need to identify more effective therapeutic strategies, current research tools to study ccRCC have limitations, and patient-specific human models are needed to develop precision therapies.

Most kidney cancer research to date has utilized primary cultures or long-standing tumor cell lines grown in 2D monolayers, or animal tumor models. The major drawback of conventional 2D tumor cultures *in vitro* is the failure to maintain the primary tumor phenotype and complexity over time [11]. A recent study created primary human ccRCC 3D organoids within decellularized renal extracellular matrix or synthetic polysaccharide scaffolds and demonstrated that the tumor cells sustained the original tumor immunohistochemical and gene expression features longer than conventional 2D monolayer cultures [12]. However, this system did not incorporate endothelial cells or study the effects of directional flow/perfusion on tumor phenotypes.

Though rodent models have much improved our understanding of ccRCC, they still fail to completely capture key features of the human disease. For example, patients with von Hippel–Lindau disease inherit one defective copy of the *VHL* gene and develop ccRCC at very high rates following sporadic inactivation of the remaining allele in kidney tubular epithelial cells [13]. In contrast, the *Vhl*-null mouse develops tubular hyperplasia but not frank carcinoma [14–16]. Mice transgenic for a constitutively active form of HIF-1α appear to develop cystic ccRCC *in situ* but only at low frequency [17]. In fact, only after inactivation of the tumor suppressor genes *Trp53* and *Rb* together with *Vhl* do mice spontaneously develop ccRCC [18]. Even with the long latency associated with spontaneous tumor development in these systems, spontaneous animal ccRCC tumor models still do not capture the complete molecular and phenotypic complexity of the human disease.

Recent studies using primary ccRCC xenografts ("tumorgrafts") in mice [19–21] or on chicken chorioallantoic membranes [22] have permitted the examination of patient-to-patient variability in drug sensitivity. However, the use of mice for serial transplantation of xenografts is expensive, time consuming, and labor intensive. Furthermore, not all patients' tumors will engraft successfully into mice [20,21]. Lastly, it is difficult to mechanistically dissect human-specific tumor-vascular and other cell-cell reciprocal interactions in these xenograft systems.

Thus, 3D tissue-engineered, patient-specific culture systems that efficiently recapitulate the *in vivo* tumor phenotype would overcome many of these challenges and provide a tractable tool for mechanistic and pharmacological studies. In this regard, the well-defined cell types in the ccRCC microenvironment (tumor, vascular endothelium, and circulating immune cells) and the typical lack of a prominent stromal response make this an ideal tumor type for modeling in microphysiological culture systems. Here, we aimed to reconstruct the 3D vascular microenvironment of ccRCC by incorporating primary tumor cell clusters into the matrix surrounding engineered human vessels subjected to continuous flow. We have previously used this framework to develop a microphysiological system for studies of human renal proximal tubule function [23]. We demonstrate that this platform reproduces the ability of ccRCC to stimulate angiogenic sprouting and provides a basis for studies of pharmacological blockade. We envision that this approach will also prove useful for studying tumor-endothelial cell interactions, testing the efficacy of novel antitumor agents on a patient-specific basis, and exploring the mechanisms of transformation.

Materials and Methods

Unless otherwise stated, all media and reagents were purchased from Thermo Fisher Scientific (Waltham, MA).

Primary Culture of ccRCC and Normal-Adjacent Renal Cortex

The research protocol to access tumor and normal-adjacent tissue from patients undergoing nephrectomies for renal masses at the University of Washington Medical Center was approved by the Institutional Review Board (File 44773). Informed consent to use leftover tissue was obtained. A final pathologist's diagnosis of ccRCC was confirmed for all cases included in this study (Supplementary Table S1). Tissues were procured by NWBioTrust (Seattle, WA) immediately following surgery in HBSS containing penicillin-streptomycin and stored at 4°C prior to transfer and processing within 24 hours. Tissues were minced to a slurry with a sterile blade and disrupted in RPMI containing 0.2 mg/ml Liberase-Thermolysin and 100 U/ml DNase I (Roche, Mannheim, Germany) with shaking at 37°C for 30 minutes and vortexing every 10 minutes. Digests were stopped with RPMI and 10% heat-inactivated FBS; strained through a 40-μm strainer; diluted with an equal volume of Dulbecco's PBS (Corning Life Sciences, Tewksbury, MA); collected at 370×*g* for 3 minutes; resuspended in primary culture media consisting of RPMI, 10% heat-inactivated FBS, 1× antibiotic-antimycotic supplemented with insulin, transferrin, sodium selenite, BSA, and linoleic acid (ITS+ media supplement, Sigma Aldrich, St. Louis, MO); and cultured at 37°C 5% CO_2. Initial PBS wash and media change were performed within 1 to 2 days and thereafter every 3 to 4 days.

Gene Expression

Gene expression values from RNA sequencing data from primary ccRCC and normal renal cortex tissues were obtained from The Cancer Genome Atlas expression browser [24]. For real-time gene expression assays, RNA was extracted using RNeasy Plus kits (Qiagen, Valencia, CA). The cDNA was synthesized using random hexamers and the Superscript III First-Strand Synthesis Kit and was used to seed real-time SYBR Green or Taqman PCR reactions for each gene (Supplementary Table S2) using standard cycling conditions for the Applied Biosystems 7900HT thermocycler. The presence of a single peak in the dissociation curve analysis was confirmed for all SYBR Green assays. Cycle threshold (Ct) values were determined using the Applied Biosystems Sequence Detection