

Sunitinib induces early histomolecular changes in a subset of renal cancer cells that contribute to resistance

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ABSTRACT: Sunitinib is the standard-of-care, first-line treatment for advanced renal cell carcinoma (RCC). Characteristics of treatment-resistant RCC have been described; however, complex tumor adaptation mechanisms obstruct the identification of significant operators in resistance. We hypothesized that resistance is a late manifestation of early, treatment-induced histomolecular alterations; therefore, studying early drug response may identify drivers of resistance. We describe an epithelioid RCC growth pattern in RCC xenografts, which emerges in sunitinib-sensitive tumors and is augmented during resistance. This growth modality is molecularly and morphologically related to the RCC spheroids that advance during in vitro treatment. Based on time-lapse microscopy, mRNA and microRNA screening, and tumor behavior-related characteristics, we propose that the spheroid and adherent RCC growth patterns differentially respond to sunitinib. Gene expression analysis indicated that sunitinib promoted spheroid formation, which provided a selective survival advantage under treatment. Functional studies confirm that E-cadherin is a key contributor to the survival of RCC cells under sunitinib treatment. In summary, we suggest that sunitinib-resistant RCC cells exist in treatment-sensitive tumors and are histologically identifiable.

KEY WORDS: kidney cancer; differential drug response; receptor tyrosine kinase inhibitor

INTRODUCTION

Renal cell carcinoma (RCC) is among the 10 most common cancers in North America, and 85% of RCC cases fall within the clear cell renal cell carcinoma (ccRCC) subtype. The multitargeted receptor tyrosine kinase (RTK) inhibitor sunitinib is the standard, first-line treatment for the 30% of patients with RCC who develop distant metastases (1). Although 47% of patients with metastatic RCC (mRCC) initially respond to sunitinib (2), long-term response and complete remission are rare because of acquired resistance (3). Since the FDA approved sunitinib for the treatment of mRCC, much work has been done to uncover the molecular mechanisms that contribute to resistance (2, 4–9). The dynamics of the potential sunitinib-adaptation mechanisms, however, and therefore, the root of resistance, remain unexplored. With recent promising results of immune checkpoint therapies, there is an emerging interest in the combination of different immune therapies as well as the combination of an immunotherapy with RTKs. Teasing out the most potent combination treatment will be aided by preclinical models, and characterization of RTK resistance will be crucial in investigating the interaction between immunotherapy and RTK-resistant cancer cells. Previous studies aimed to capture the drivers of sunitinib resistance by comparing sunitinib-sensitive and sunitinib-resistant specimens. That approach, however, neglects drivers that may already operate at the sensitive stage. We hypothesized that the sensitive and resistant stages were at different time points of the single continuum of molecular events induced by sunitinib. Therefore, studying molecular and related histologic changes that occur early during treatment may identify the relevant alterations that invoke resistance. In this study, we used *in vitro* and *in vivo* models (both allogeneic and syngeneic xenograft models) to examine the effect of sunitinib treatment on kidney cancer cells during both sensitive and resistant stages. Our results show that sunitinib treatment provides selective advantage for RCC cells that are able to form tight epithelioid spheroids *in vitro* or the corresponding morphology *in vivo*. Furthermore, we show that the resistant morphology is induced by the treatment.

MATERIALS AND METHODS

Cell culturing and *in vitro* treatment

ACHN, 786-0, and Renca cells were obtained from American Type Culture Collection (Manassas, VA, USA) and were cultured per the distributor's description. Cells were treated with 1 mM sunitinib-malate (Selleckchem, Houston, TX, USA) dissolved in DMSO or with DMSO only (vehicle). When cells were passaged, floating cells were also collected from the medium by centrifugation and were trypsinized together with the adherent cells. RCC spheres were passaged as described below. Resistance index was measured and calculated as previously described (10, 11).

Secondary and tertiary sphere formation

RCC spheres were picked under a microscope and were trypsinized and further disrupted by pipetting through a 200- μ l pipette tip or a 30-gauge needle. Sphere disruption was monitored under a microscope. Cells (23104 cells/well) were plated on 6-well plates (Sarstedt, Nümbrecht, Germany) and coated with Matrigel (Thermo Fisher Scientific, Waltham, MA, USA). Cells were then stained with Hoechst-3422, and cell number/well was determined by ImageXpress Micro Acquisition System (Molecular Devices, Sunnyvale, CA, USA), using the IMX software (IMX Software Group, London, England). The minimum sphere size was 40 μ m, and the maximum spheroid size was 250 μ m.

Mouse tumor models Syngeneic xenograft model

BALB/c mice (6–8 wk old, female; Charles River Laboratories, Wilmington, MA, USA) were injected with 53105 cells, s.c. in the femur area. Injection contained a 1:1 ratio of Matrigel (Thermo Fisher Scientific) and single-cell suspension.

Allogenic xenograft model

NOD/SCIDg (NSG) mice (6–8 wk old, female; The Jackson Laboratory, Bar Harbor, ME, USA) were injected with 53105 cells subcutaneously in the femur area. Injection contained a 1:1 ratio of Matrigel (Thermo Fisher Scientific) and single-cell suspension.

In vivo sunitinib treatment

Mice were treated with 40 mg/kg sunitinib (Selleckchem) or with citrate buffer (vehicle-treated group) by gavage, 7 d/wk. Sunitinib treatment started once xenografts reached the estimated volume of 100 mm³ (for Renca xenografts) or 50 mm³ (for ACHN xenografts). Tumor size was monitored daily by manual caliper, and tumor volume was estimated with the following equation: Volume = $\frac{1}{2} \times \text{width}^2 \times \text{length} \times \frac{\pi}{6}$

Spontaneous metastasis model

Fifty ACHN or 103 Renca cells with an equal volume of Matrigel were subcutaneously injected into the neck area of 6–8-wk-old, female BALB/c or NSG mice. Tumor formation was monitored 3 d/wk. Mice were euthanized at 27 d (Renca-recipient mice) or 60 d (ACHN-recipient mice) after inoculation.

Tail-vein injection metastasis assay

Renca (53103) or ACHN cells were injected directly into the tail vein of 6–8-wk-old female BALB/c or NSG mice. Metastatic disease was monitored by observing changes in body condition (hunched back, loss of body weight, and dehydration). Renca recipient mice were euthanized 40 d after inoculation, and ACHN-recipient mice were euthanized 80 d after inoculation.

Histologic assessment

Tumors and organs (liver, kidneys, lungs, spleen, stomach, guts, and any areas suspicious for metastasis) were collected and snap-frozen for RNA analysis or fixed in 10% formalin. All specimens were paraffin embedded, and 5–8-mm sections were prepared and stained with hematoxylin and eosin. Two pathologists (S.R. and G.M.Y.) independently assessed the stained sections.

Immunohistochemistry

Specimens were fixed in PBS-buffered 10% formalin and embedded in paraffin. The following antibodies were used: Ki-67 [1:100, heat-induced epitope retrieval (HIER), cell conditioner 1 (CC1) for 32 min; Biocare Medical, Concord, CA, USA], b-catenin (1:400, clone 14, HIER, CC1 for 64 min; Cell Marque, Rocklin, CA, USA), E-cadherin (1:50, HIER, CC1 for 64 min; Inter Medico, Markham, ON, Canada), pancytokeratin (pan-CK; prediluted, clone ae1/ae3/pck26, protease 1–4, CC1 for 16 min; F. Hoffmann-La Roche, Basel, Switzerland), low-MWCK (1:20, CAM5.2, protease 1–4 min; Becton Dickinson, Franklin Lakes, NJ, USA). Antigen retrieval was performed on automated Ventana Discovery Ultra (F. Hoffmann-La Roche).

Immunocytochemistry

Spheroids were stained as described by Weiswald et al. (12). Anti-E-cadherin and anti-b-catenin antibodies were purchased from Santa Cruz Biotechnology (sc-7963; Dallas, TX, USA) and Cell Signaling

Technology (4627; Danvers, MA, USA) and were used as directed by the manufacturers. DAPI staining was performed to visualize the nuclei.

RNA isolation and quantitative RT-PCR analysis

Tumors were snap-frozen and stored at -80°C for RNA isolation. Total RNA was isolated with the microRNA (miRNA) kit (miRNeasy Isolation Kit; Qiagen, Hilden, Germany) following the manufacturer's recommendations. Reverse transcription for mRNA analysis was performed with the High Capacity RNA-to-cDNA Kit (Thermo Fisher Scientific), as recommended by the manufacturer. RNA quality and quantity were determined with a Bioanalyzer RNA Chip (Agilent Technologies, Santa Clara, CA, USA) and a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific). Quantitative PCR reactions were performed on Viia7 Real-Time PCR System (Thermo Fisher Scientific), using the SYBR Green Master Mix (Thermo Fisher Scientific). Cycle threshold values were normalized against the geometric mean of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), peptidylprolyl isomerase A (PPIA), ribosomal protein lateral stalk subunit PO (RPLPO), and hypoxanthine phosphoribosyltransferase 1 (HPRT1). The $\Delta\Delta\text{Ct}$ method was used to obtain the relative quantification values.

Imaging

Live imaging was performed with an Axio Observer Live Cell with Fluorescence Lifetime Imaging instrument (Carl Zeiss, Oberkochen, Germany), and images were analyzed with Zen Black (Carl Zeiss). Confocal microscopy was performed with a Carl Zeiss LSM700. For spheroid counting, cultures were stained with Hoechst 33342, and automated spheroid counting was performed by ImageXpress Micro Acquisition System. Minimal spheroid size was adjusted to 40 μm , and maximum size was 250 μm .

Proliferation and apoptosis assay

A proliferation assay was performed by WST-1 (Millipore Sigma, Billerica, MA, USA). Absorbance was measured at 650 and 450 nm with a SpectraMax M5 spectrophotometer (Molecular Devices). Because of the broad fluorescence spectrum of sunitinib, cytotoxicity was quantified with the Live/Dead Fixable Far Red Dead Cell Stain Kit (Thermo Fisher Scientific). Flow cytometry analysis was performed on a BD LSRFortessa X-20 system.

Statistics

Data were analyzed with the Prism 7 package (GraphPad Software, La Jolla, CA, USA). We used 1-way ANOVA to visualize the tumor-growth curves and to analyze mRNA expression values.

Microarray and nanostring expression analysis

Microarray gene expression was performed at Centre for Applied Genomics of The Hospital for Sick Children (Toronto, ON, Canada). A Nanostring Encounter Human miRNA Expression Assay Kit was used to profile the expression of 800 miRNAs. Data were processed with the R Bioconductor 3.2.3 software (R Foundation for Statistical Computing, Vienna, Austria). Affymetrix Mouse Gene 2.0 ST transcriptome array (Thermo Fisher Scientific) data were processed using R functions contained within affy package 1.48.0, and the background of the arrays was corrected by robust multiarray averaging. The HT-12 Expression BeadChip Kit (Illumina, San Diego, CA, USA) array data were processed using R functions contained within the Beadarray 2.20.1 package, whereas the NanoString data were processed

with functions within NanoString Norm package (R Foundation), and probes with 30 read count for all the samples were excluded. In all 3 studies—human arrays, mouse arrays, and Nanostring—between-sample/array quantile normalization was performed, followed by the gene expression changes between groups using a t test (for 2 groups) and subjected to correction for multiple testing with the Benjamini-Hochberg false-discovery rate, in the limma package 3.26.9. The data sets supporting the conclusions in this article are available in the Gene Expression Omnibus database (National Center for Biotechnology Information, Bethesda, MD, USA). Lentiviral transduction

Third-generation, self-inactivating lentiviral vectors (LVs) were produced by cotransfection of human embryonic kidney 293T cells with polyethylenimine and packaging plasmids pMDL-g/pRRE, pMD2-VSVg, and pRSV-Rev (Addgene, Cambridge, MA, USA) as well as the LV transfer vectors carrying the short hairpin RNA (shRNA) sequences against E-cadherin (TRCN0000039665, TRCN0000039664, and TRCN0000039667; Dharmacon, Lafayette, CO, USA). Oligonucleotide sequence and the position of the shRNAs were as follows

TRCN0000039664	forward,
59-CCGGCCAGTGAACAACGATGGCATTCTCGAGAATGCCATCGTTGTTCACTGGTTTTTG-39,	reverse oligo
sequence,	59-AATTCAAAAACCAAGTGAACAACGATGGCATTCTCGAGAATGCCATCGTTGTTCACTGG-39,
match	position,
1562;	TRCN0000039665
forward,	59-CCGGCCAAGCAGAATTGCTCACATTCTCGAGAATGTGAGCAATTCTGCTTGGTTTTTG-39,
reverse,	59-AATTCAAAAACCAAGCAGAATTGCTCACATTCTCGAGAATGTGAGCAATTCTGCTTGG-39,
match	position,
682;	and
TRCN0000039667	forward,
59-CCGGCCAACCAAGAATCTATCATTCTCGAGAATGATAGATTCTTGGGTTGGTTTTTG-39,	reverse,
5	9-AATTCAAAAACCAACCAAGAATCTATCATTCTCGAGAATGATAGATTCTTGGGTTGG-39,
match	position,
2210.	

Subsequently, LVs were concentrated by ultracentrifugation of the human embryonic kidney 293T cell medium at 20,000 rpm for 2 h at 4°C and stored at 280°C. Cells were transduced with lentivector with a multiplicity of infection of 10.

RESULTS

Sunitinib-treated tumor xenografts exhibit aggressive tumor behavior that starts early during response phase and is marked by unique histomorphologic changes

We used xenograft model to assess the effect of sunitinib on cancer cells in vivo. BALB/c mice were xenografted with Renca RCC cells and were used as the immunocompetent model (n = 20). The Renca cell line was established from a tumor that arose spontaneously as a renal cortical adenocarcinoma in BALB/cCr mice. This cell line gives a high number of spontaneous metastases to the lung and liver, accurately mimicking human adult renal cell carcinoma, thus making Renca the most used immunocompetent murine model for RCC. To assess the behavior of human RCC, 786-0 and ACHN cell lines were used in this study, with the 786-0 cell line derived from a primary RCC site, and showing canonical clear cell histology, depicting the most common RCC subtype. The 786-0 cells lack functional von Hippel-Lindau tumor suppressor protein expression, which is a widely accepted driver of ccRCC. The ACHN cell line was isolated from a malignant pleural RCC effusion. NSG mice were used as a host for human-derived tumor xenografts. In addition to the scid mutation, which renders the strain deficient in T and B cells, NSG mice are also deficient in functional NK cells, minimizing rejection. NSG mice were xenografted with 786-0 RCC cells and were used as an immunocompromised model (n = 12). Mice were randomized to vehicle-treated and sunitinib-treated groups. Treatment response was assessed based on the tumor growth curve. Most xenografts exhibited initial drug sensitivity (flat tumor growth curve), followed by resistance (steep increase in growth tumor curve). The tumors that were harvested at the time of rapid growth after an initial response were considered treatment defiant and were used as the sunitinib-resistant cohort (Fig. 1). Some xenografts, however, showed an extended drug-sensitive period and were used as the sunitinib-

sensitive cohort (Fig. 1A). Sunitinib-sensitive xenografts of both Renca and 7860 cell lines showed early signs of aggressive behavior, which manifested as irregular invasive borders, local invasion, and extensive metastatic deposits. Multinucleated giant cells, which are described as indicators of genomic instability (13), were also frequent in the sensitive xenografts. These features became even more prominent in the sunitinib-resistant xenografts (Supplemental Fig. S1 and Supplemental Table S1). Strikingly, sunitinib-treated tumors showed viable, compact tumor islands (tongues of tumors within necrotic spaces), which contrasted with the confluent necrosis observed in vehicle-treated tumors. These tumor tongues were epithelioid and expressed mesenchymal and epithelial markers (vimentin, CKs). PAX8 staining confirmed their renal origin (Fig. 1B). Budding of those epithelioid nests was observed at the tumor front. Such a phenomenon is associated with invasive potential and poor prognosis in other cancers and indicates the aggressive potential of the surviving RCC tumor tongues.

In vitro sunitinib treatment induced formation of RCC spheroids with increased colonization potential and expression of stem cell markers

To better understand sunitinib's early effect on cancer cells, ACHN and Renca cells were treated with sunitinib in vitro. Sunitinib sensitivity or resistance was assessed by the resistance index (10,11). Treated cultures showed increased expression of stem cell-related markers, such as OCT4, NANOG, Kruppel-like factor 4 (KLF4), and stem cell markers of kidney and other cancers, such as neuronal cell adhesion molecule (NRCAM), CD105, leucine-rich repeat containing G protein-coupled receptor 5 (LGR5), ABCC9, and GLIS2 (Supplemental Fig. S2) (14). These changes were detected early, within 1 wk of treatment and before the onset of resistance. Quantitative PCR analysis did not suggest epithelial-mesenchymal transition (EMT) changes. We previously described the formation of RCC spheroid structures with low efficiency under standard culturing conditions (15). Compared with the DMSO control, sunitinib increased the spheroid formation rate by 8-fold, starting as early as d 2 after treatment (Fig. 2A–C). To investigate whether sunitinib also affected the ability of spheroids to differentiate into adherent cells, we dissociated and propagated RCC spheroids in the presence of DMSO or sunitinib and quantified the number of spheroids that were formed and the ratio of cells that engaged in spheroid formation. Sunitinib did not significantly increase the number of secondary and tertiary spheroids. The rate of cells that participated in spheroid formation, however, significantly increased under sunitinib treatment, compared with the DMSO-treated control. This indicates that sunitinib created an imbalance between 2-dimensional (2D) and 3-dimensional (3D) growth patterns (Fig. 2D, E), either by inhibition of adherent growth or by selecting for the spheroid architecture. Sunitinib has previously been reported to enhance RCC metastasis in an eoadjuvant setting (16,17). In our xenograft model, ACHN and Renca cells retained their tumorigenic and metastatic ability, regardless of sunitinib treatment and growth modality. We thought to separately examine extravasation potential of RCC cells because it is a crucial step in metastasis. During tail-vein assay, cancer cells are directly injected into the host's circulatory system. This assay tests the potential of cancer cells to exit the vasculature and generate metastatic deposits. To explore the relation among the extravasation potential of RCC cells, sunitinib treatment, and spheroid formation, the following 4 subpopulations of ACHN and Renca cells were injected into mice tail-vein: 1) vehicle-treated, adherent; 2) vehicle-treated, RCC spheroids; 3) sunitinib-treated, adherent; and 4) sunitinib-treated, RCC spheroids. Mice injected with the ACHN spheroid-derived suspension formed 10 times more metastatic deposits in the lungs and liver compared with adherent cells. Additionally, spheroids metastasized to the kidney, which was rare in the case of the adherent RCC cells. In line with the more aggressive nature of the Renca cells, all mice developed metastases. However, spheroid-injected mice had significantly more deposits in

thelungsandhadshortersurvival(SupplementalFig.S3).

Taken together, sunitinib led to the accumulation of RCC spheroids with increased stem cell-related marker expression, and enhanced extravasation potential. Of note, the overall metastatic ability of RCC spheroids was similar to that of the adherent patterns, rather than the extravasation, and/or subsequent survival of metastases was increased. Increased extravasation potential of other cancer spheroids has been reported (18).

Sunitinib preferentially affects adherent RCC cells compared with spheroid-forming cells

We have shown that sunitinib treatment favored RCC spheroid growth *in vitro*. To verify the differential response of RCC spheroids and adherent cells to sunitinib, we monitored RCC growth dynamics under treatment by real-time microscopy for 55 h. We tested 4 conditions: 1) cells untreated for 1 wk that remained untreated during the time-lapse imaging; 2) cells pretreated with sunitinib for 1 wk with continued treatment during imaging; 3) cells untreated for 1 wk and then switched to sunitinib treatment during imaging; and 4) cells pretreated with sunitinib for 1 week and switched to no treatment during imaging (Fig. 3A). Untreated cells predominantly grew as an adherent, 2D monolayer, whereas sunitinib-treated cells showed significantly more spheroid formations (Fig. 3B, C). Untreated cells reached confluence at 25–32 h of imaging, indicating high proliferation and migration ability (condition 1). Conversely, sunitinib treatment enhanced spheroid formation, whereas adherent colonies were unable to advance (condition 2). Likewise, when cells were switched to sunitinib-treatment (condition 3), RCC spheroids grew in diameter, whereas adherent cell growth was inhibited. In condition 4, sunitinib withdrawal promoted 2D adherent growth, whereas the spheroids lost their compact morphology and were replaced by a monolayer growth pattern (Fig. 3). Quantification by ImageXpress confirmed that sunitinib had a contrasting effect on the different RCC growth patterns, favoring 3D spheroids and limiting 2D monolayer growth.

Overlapping molecular signatures mark spheroid formation and sunitinib treatment

To gain information about the molecular pathways associated with spheroid formation and with sunitinib treatment and to assess whether those were related, we compared global mRNA and miRNA expression between the aforementioned 4 subpopulations in ACHN and Renca cell lines. Comparison of DMSO-treated ACHN spheroids and DMSO-treated adherent cells with stringent criteria (fold change, ≥ 3 ; false discovery rate, $\leq 4\%$) indicated the up-regulation of cell adhesion and its auxiliary gene ontology (GO) categories, such as membrane trafficking, vesicular transport, and cell polarity in the spheroids. These results suggest that cell aggregation through the relocalization of adhesion factors is a primary contributor to spheroid formation (Fig. 4A). Adherent ACHN cells were treated with vehicle or sunitinib for 2 d to investigate early response. We noted a significant overlap between pathways that were induced in the adherent ACHN cells upon treatment and pathways that were induced during spheroid formation. Cell-adhesion-related GO categories, such as cytoskeletal dynamics, vesicular transport, and disruption of cell polarity were overrepresented in the treated adherent cells. Additionally, sunitinib induced the small GTPase-mediated signaling along with several developmental processes, such as tubule development, negative regulation of development, and anatomic structure morphogenesis. Down-regulated categories included kinetochore organization, regulation of microtubule cytoskeleton organization, and regulation of mitotic nuclear division. Increased presence of the multinucleated giant cells in the sunitinib-treated xenografts morphologically supported these results. The overlap between molecular events that occur in spheroids and during early sunitinib treatment of adherent cells indicate that sunitinib may generate a cell status that endorses spheroid formation and opposes the 2D adherent state. This suggestion is supported by the *in vitro* observation that sunitinib increased the spheroid number. Among the signaling pathways, PI3K

signaling/AKT activation, cell–cell interaction, and G protein-coupled receptors (GPCRs) activation were the most significantly upregulated under treatment. AKT is activated by RTKs, GPCRs, and cell–cell adhesion molecules, such as cadherins (19–21). Because sunitinib reportedly inhibits most RTKs (22), it is plausible that PI3K/AKT-based survival relies on alternative inputs under treatment. For example, GPCR and cadherin-mediated activation could compensate for the lost input, thus propelling survival (Fig. 4B). Additionally, sunitinib-treated spheroids overexpressed cytoskeletal/ microtubular proteins and small GTPases and also showed overrepresentation of developmental categories, such as anatomic structure and morphogenesis. Transcriptome analysis of the 4 Renca subpopulations showed similar results. Overall, transcriptome analyses revealed the operation of overlapping molecular mechanisms during early response to sunitinib and spheroid formation. Our results suggest that sunitinib treatment supports spheroid formation by promoting cell–cell contact and vesicle-mediated transport.

miRNA expression analysis reveals different treatment responses in adherent vs. spheroid-forming RCC cells

Vehicle-treated adherent, vehicle-treated spheroid, sunitinib-treated adherent, and sunitinib-treated spheroid ACHN cells were evaluated for miRNA expression. Of the 825 screened miRNAs, 246 miRNAs were significantly and differentially expressed in a comparison. Sunitinib treatment had a greater effect on adherent cells than on spheroid-forming cells. miR-1268b, miR-302b, miR-579, and miR-1185-2-3p were significantly greater in adherent cells, whereas miR-7-5p was up-regulated in the DMSO-treated adherent cells only. We have not identified miRNAs that were specific for the spheroids but were not related to sunitinib treatment, supporting our hypothesis that sunitinib treatment promoted spheroid formation and counterbalanced adherent growth. Our data indicated that miRNAs had a significant effect on ubiquitin-mediated proteolysis under sunitinib treatment, and miR-579 appeared to be a key regulator of that process. The BMP receptor- and activin receptor-mediated branches of TGF- β signaling appeared to be differentially regulated in adherent RCC cells vs. spheroids. Differentiation driven by the NODAL (nodal growth differentiation factor)/ACVR2 (activin A receptor type 2A)/SMAD2/SMAD4 axis appeared to be under miRNA inhibition in the RCC spheroids but not in adherent ACHN cells. Overall, mRNA and miRNA expression converged on the regulation of intracellular vesicular trafficking (Supplemental Fig. S4). Tumor islands that appear during early sunitinib treatment in vivo share characteristics with RCC spheroids that emerge under sunitinib treatment in vitro.

To establish the link between the in vitro spheroids and the surviving tumor islands in vivo, we performed immunohistochemistry (IHC) for E-cadherin and β -catenin expression and localization, based on our in vitro mRNA expression data showing cell–cell adhesion as the most significant difference between adherent and spheroid-forming cells. Both spheroids (in vitro) and epithelioid tongues (in vivo) showed membranous positivity for E-cadherin and stained positive for β -catenin, whereas DMSO-treated adherent cells (in vitro) and other xenograft tumor areas (in vivo) exhibited low levels of cytoplasmic (but not membranous) E-cadherin staining and were negative for β -catenin (Fig. 5A,B). Further, we injected either adherent cells or spheroids into mice to evaluate the histology of their xenografts. ACHN spheroid xenografts exhibited a spectrum of morphologic patterns, including epithelioid nests, cytoplasmic clearing (typical ccRCC morphology), and spindled pattern (23). Additionally, compact, epithelioid tumor tongues were frequently observed. Tumors were invasive to fat and had multiple small, distant metastases and vascular co-option in the lungs (Fig. 5). Overall, the growth pattern was similar to that of the sunitinib-sensitive xenografts. In contrast, adherent RCC-derived xenografts were mostly spindle shaped with no morphologic diversity and with minimal presence of tumor tongues (Fig. 5C–E and Supplemental Table S2). We concluded that the in vitro and

in vivo growth patterns were related, and E-cadherin that expressed in tumor tongues in sunitinib-treated xenografts reflected the in vitro RCC spheroids that emerged under sunitinib treatment.

E-cadherin-based cell–cell contact protects RCC spheres from sunitinib-mediated cell death with vehicle, and apoptosis and proliferation were quantified after 72 h. Sunitinib had a significant cytotoxic effect on adherent cells, whereas it did not increase apoptosis in the RCC spheroids (Fig. 6A–C). Consistent with our real-time microscopy data, spheroid cultures showed increased proliferation under sunitinib treatment, whereas the proliferation of adherent cells did not change significantly (Fig. 6D). Because

Sunitinib exerted a contrasting effect on the different growth modalities of RCC. To evaluate whether disparate response was reflected by alterations in commonly tested tumor characteristics, adherent and spheroid ACHN cultures were treated with sunitinib or cadherin-mediated cell–cell adhesion was a top upregulated GO category in sunitinib-treated cells and spheroids, we thought to assess whether cadherin-mediated cell–cell contact could promote survival of RCC spheres under treatment. Cells were switched to Ca²⁺-free condition to disrupt cadherin-mediated adhesions (24) and were treated with sunitinib for 72 h. Ca²⁺-free condition sensitized RCC spheroids to sunitinib treatment, which manifested as an increased ratio of apoptotic cells in addition to decreased proliferative capacity (Fig. 6B, D). To determine whether E-cadherin was specifically involved in protecting spheroids under sunitinib treatment, cultures were transduced by lentiviral particles carrying appropriate shRNAs. Adherent and spheroid cultures were subsequently treated with sunitinib for 72 h. Viability

and proliferation of RCC spheroids decreased significantly in response to E-cadherin inhibition. In contrast, sunitinib's cytotoxicity did not change when spheroids were transduced with control enhanced green fluorescent protein lentiviral particles, whereas a nonsignificant increase in proliferation was observed (Fig. 6C, E). In vivo, the proliferative marker Ki-67 showed markedly strong positivity within the epithelioid tumor tongues of sunitinib-sensitive xenografts, compared with the spindle morphology of the same tumor (Fig. 6F, G). Overall, our data indicated that spheroid formation via cadherin-mediated cell–cell contact enhanced the viability and proliferation of RCC spheres under sunitinib treatment. This observation is in agreement with the emergence of E-cadherin–positive tumor areas on sunitinib-treated xenografts.

DISCUSSION

The antiangiogenic sunitinib remains the standard of care for mRCC. We followed initial molecular and morphologic changes under sunitinib treatment to identify alterations that could be relevant to developing resistance.

Sunitinib resistance has been studied extensively using xenograft models. However, in most experimental designs the sunitinib-sensitive phase was represented by tumors that have been terminated very early, to avoid building up resistance. In that setup, drug-sensitive tumors were given far less time to grow in the host. Moreover, the time when each sunitinib-sensitive xenograft would enter the resistant phase cannot be foreseen, creating an essentially diverse pool of the drug-sensitive tumor cohort. We, therefore, believe that the comparison between sunitinib-resistant tumors and xenografts that grow at a slower pace under treatment (sunitinib sensitive in our experimental setup) is a more relevant representation of sunitinib sensitivity because it allows the study of the dynamics of treatment response. Additional limitations of the xenograft model are the use of immunodeficient mice and the preferential selection for aggressive tumors when cell lines are established. Sunitinib response was characterized based on the tumor growth curve, and treatment resistance was defined as a rapid growth phase after an initial response. Using 3 RCC model cell lines, we did not find truly sunitinib-sensitive xenografts that completely halted their growth under treatment. All tumors became larger over the duration of the experiment, mimicking

the clinical scenario, in which the median of progression-free survival in patients is 8–9 mo, and durable response is rare. The most prominent histologic change between the in vivo sunitinib- and vehicle-treated xenografts was the presence of epithelioid tumor tongues vs. the diffuse necrotic spaces observed in the control tumors. The extent of surviving epithelioid islands corresponded to the sensitive or resistant states. Morphologic and IHC-based similarities between the in vitro spheroids and in vivo tumor islands that survive sunitinib treatment provided initial evidence that these patterns were related. Additionally, among the different model cell lines, a close correlation was apparent among the spheroid-forming ability, the xenograft's histologic appearance, and the duration of in vivo drug response. DMSO-treated Renca cells formed many spheroids in vitro, which were reflected by the high frequency of spheroid-like islands in the vehicle-treated xenografts. Accordingly, Renca tumors quickly developed spectacular epithelioid tumor tongues upon sunitinib treatment, which was paired with a very short sunitinib-sensitive phase. ACHN and 786-O cell lines had a moderate or low capacity for spontaneous spheroid formation in vitro, which paralleled the rare epithelioid or spheroid-like morphology in the untreated xenografts and with an extended sunitinib-sensitive period. Our results are in agreement with previous reports that suggested a nongenetic base for sunitinib resistance (25). Time-lapse microscopy, mRNA analysis, and a metastatic colonization assay show that sunitinib has a preferential effect on the adherent RCC pattern, compared with the spheroids. All our results pointed in the same direction: the spheroid architecture is beneficial for cancer cell survival under treatment. The presence of a spheroid-associated signature in adherent sunitinib-treated cells suggests that sunitinib primes RCC cells for spheroid formation. Our data imply that cells that survive sunitinib treatment switch the RTK-based activation of PI3K/AKT to alternative inputs, such as GPCRs and cell-adhesion complexes, to ensure continued activity and survival. Indeed, GPCRs, such as the calcitonin receptor-like receptor and GPCR 162, are significantly up-regulated in RCC, their expression correlates with poor prognosis (26–28), and they affect the carbohydrate metabolism–AKT–mTOR axis, the quintessential RCC driver (29). More specifically, our results indicate the upregulation of the GPCR CDC42, a primary regulator of cell polarity and cytoskeletal organization in renal epithelial tubular cells during embryogenesis (30). Similarly, sunitinib-treated gastrointestinal tumors showed elevated phospho-AKT (31). RCC spheroids showed epithelioid characteristics and highly expressed Ca²⁺-dependent cell–cell adhesion proteins, such as E-cadherin. This result seemingly contradicts reports that stress the necessity of EMT and mesenchymal features to gain aggressive tumor features and drug resistance (6, 32–35). Recently, however, several laboratories reported a more-complex relation between EMT and cancer (36). Several studies suggest that EMT is not a prerequisite for metastasis. Genetic tracing of cells that underwent EMT and, in an independent study, the deletion of Snail and Twist revealed that EMT is not compulsory for the initiation of primary and metastatic luminal breast adenocarcinoma and pancreatic ductal adenocarcinoma (36, 37).

RCC cells likely reside in an intermediate gray zone of the EMT spectrum and are known to coexpress mesenchymal (vimentin) and epithelial (low-MW CK, panCK, and EMA) markers. Recent data show that in some cancers, including RCC, expression of the mesenchymal signature and lack of epithelial signature correlated with better disease-free and overall survival (38). Additionally, RCC cases with a more-mesenchymal gene signature responded better to compounds targeting microtubule dynamics, indicating that the epithelioid compartment has a differential response for various drugs (38). Lastly, it is possible that a balance exists between the less-proliferative, but more therapy-resistant epithelioid pattern, and the more-proliferative mesenchymal pattern. Despite membranous E-cadherin expression, RCC spheroid cells did not appear to be differentiated, polarized epithelia. RCC spheroid cells were tightly packed, which may indicate loss of polarity and deficiency of

microtubular structure and cytoskeletal network. Alternatively, these epithelioid islands and the budding phenotype on the tumor front may reflect a discrete form of invasion. For example, during renal embryonic development, epithelioid cell clusters collectively migrated during nephric duct elongation, highlighting the migratory potential of epithelioid cells (39–41). Our results show that sunitinib-resistance is induced in the treatment-sensitive stage, and nonresponding cells can be morphologically identified at this early time point. These findings may have future implications where sunitinib is used as part of a combination therapy. Several ongoing trials assess sunitinib or other antiangiogenic RTKs in combination with nivolumab or pembrolizumab (42). Reportedly, sunitinib itself has immune modulatory effect by suppressing regulatory T (Treg) cells and could, therefore, be used in sequential therapeutic strategies to prime antitumor immune response (43). Antitumor immune response can also be generated by oncolytic viruses, and that response is augmented by sunitinib (44). However, our data and that of others show that, in sunitinib resistance, the tumor acquires molecular signatures that promote tumor survival and may affect the tumor's immunologic properties and thus the effectiveness of the subsequent immunotherapy. During sunitinib resistance, the tumor acquires molecular signatures that promote tumor survival under treatment and may affect the tumor's immunologic properties and thus the effectiveness of the subsequent immunotherapy. Understanding which histologic and molecular characteristics dominate during early and late phases of sunitinib response may be exploited in the future to optimize interference between TK and immunotherapy (42).

ACKNOWLEDGMENTS

The authors are grateful for the dedicated work of Vidya Beharry (St. Michael's Hospital) regarding the immunohistochemistry stains; for the advice and support we received from the Animal Care Committee of the Keenan Research Centre; and for the animal care and handling expertise of Danielle Gifford and Alex Rivera (Keenan Research Centre for Biomedical Sciences, St. Michael's Hospital), which they have generously shared with us. The authors also thank Samantha Levin (Lunenfeld-Tanenbaum Research Institute, Toronto, ON, Canada) for proofreading the manuscript and for helpful comments. This work was supported by grants from the Canadian Institutes of Health Research (MOP-119606), the Kidney Foundation of Canada (KFOC130030), and Prostate Cancer Canada Movember Discovery Grants (D2013-39). Z.L. was supported by the Kidney Research Scientist Core Education and the National Training Program Postdoctoral Award from the Kidney Foundation of Canada. The authors declare no conflicts of interest.

REFERENCES

1. Motzer, R. J., Hutson, T. E., Tomczak, P., Michaelson, M. D., Bukowski, R. M., Rixe, O., Oudard, S., Negrier, S., Szczylik, C., Kim, S. T., Chen, I., Bycott, P. W., Baum, C. M., and Figlin, R. A. (2007) Sunitinib versus interferon- α in metastatic renal-cell carcinoma. *N. Engl. J. Med.* 356, 115–124
2. Larkin, J. M., Chowdhury, S., and Gore, M. E. (2007) Drug insight: advances in renal cell carcinoma and the role of targeted therapies. *Nat. Clin. Pract. Oncol.* 4, 470–479
3. Grunwald, V., McKay, R. R., Krajewski, K. M., Kalanovic, D., Lin, X., Perkins, J. J., Simantov, R., and Choueiri, T. K. (2015) Depth of remission is a prognostic factor for survival in patients with metastatic renal cell carcinoma. *Eur. Urol.* 67, 952–958
4. Chen, G., Xu, X., Zhang, L., Fu, Y., Wang, M., Gu, H., and Xie, X. (2014) Blocking autocrine VEGF signaling by sunitinib, an anticancer drug, promotes embryonic stem cell self-renewal and somatic cell reprogramming. *Cell Res.* 24, 1121–1136

5. Giuliano, S., Cormerais, Y., Dufies, M., Grépin, R., Colosetti, P., Belaid, A., Parola, J., Martin, A., Lacas-Gervais, S., Mazure, N. M., Benhida, R., Auberger, P., Mograbi, B., and Pagès, G. (2015) Resistance to sunitinib in renal clear cell carcinoma results from sequestration in lysosomes and inhibition of the autophagic flux. *Autophagy* 11, 1891–1904
6. Hammers, H. J., Verheul, H. M., Salumbides, B., Sharma, R., Rudek, M., Jaspers, J., Shah, P., Ellis, L., Shen, L., Paesante, S., Dykema, K., Furge, K., Teh, B. T., Netto, G., and Pili, R. (2010) Reversible epithelial to mesenchymal transition and acquired resistance to sunitinib in patients with renal cell carcinoma: evidence from a xenograft study. *Mol. Cancer Ther.* 9, 1525–1535
7. Qian, C. N. (2013) Hijacking the vasculature in ccRCC—co-option, remodelling and angiogenesis. *Nat. Rev. Urol.* 10, 300–304
8. Shojaei, F., Lee, J. H., Simmons, B. H., Wong, A., Esparza, C. O., Plumlee, P. A., Feng, J., Stewart, A. E., Hu-Lowe, D. D., and Christensen, J. G. (2010) HGF/c-Met acts as an alternative angiogenic pathway in sunitinib-resistant tumors. *Cancer Res.* 70, 10090–10100
9. Varna, M., Gapihan, G., Feugeas, J. P., Ratajczak, P., Tan, S., Ferreira, I., Leboeuf, C., Setterblad, N., Duval, A., Verine, J., Germain, S., Mongiat-Artus, P., Janin, A., and Bousquet, G. (2015) Stem cells increase in numbers in perinecrotic areas in human renal cancer. *Clin. Cancer Res.* 21, 916–924
10. Butz, H., Ding, Q., Nofech-Mozes, R., Lichner, Z., Ni, H., and Yousef, G. M. (2017) Elucidating mechanisms of sunitinib resistance in renal cancer: an integrated pathological-molecular analysis. *Oncotarget* 9, 4661–4674
11. Pénzváltó, Z., Tegze, B., Szász, A. M., Sztupinszki, Z., Likó, I., Szendrői, A., Schöfer, R., and Györfy, B. (2013) Identifying resistance mechanisms against five tyrosine kinase inhibitors targeting the ERBB/RAS pathway in 45 cancer cell lines. *PLoS One* 8, e59503
12. Weiswald, L. B., Guinebreière, J. M., Richon, S., Bellet, D., Saubaméa, B., and Dangles-Marie, V. (2010) In situ protein expression in tumour spheres: development of an immunostaining protocol for confocal microscopy. *BMJ Cancer* 10, 106
13. Zhang, S., Zhang, D., Yang, Z., and Zhang, X. (2016) Tumor budding, micropapillary pattern, and polyploidy giant cancer cells in colorectal cancer: current status and future prospects. *Stem Cells Int.* 2016, 4810734
14. Zhang, Y., Sun, B., Zhao, X., Sun, H., Cui, W., Liu, Z., Yao, X., and Dong, X. (2016) Spheres derived from the human SN12C renal cell carcinoma cell line are enriched in tumor initiating cells. *J. Exp. Clin. Cancer Res.* 35, 163
15. Lichner, Z., Saleh, C., Subramaniam, V., Seivwright, A., Prud'homme, G. J., and Yousef, G. M. (2015) miR-17 inhibition enhances the formation of kidney cancer spheres with stem cell/tumor initiating cell properties. *Oncotarget* 6, 5567–5581
16. Dufies, M., Giuliano, S., Ambrosetti, D., Claren, A., Ndiaye, P. D., Mastri, M., Mograbi, W., Cooley, L. S., Ettaiche, M., Chamorey, E., Parola, J., Vial, V., Lupu-Plesu, M., Bernhard, J. C., Ravaud, A., Borchellini, D., Ferrero, J. M., Bikfalvi, A., Ebos, J. M., Khabar, K. S., Grépin, R., and Pagès, G. (2017) Sunitinib stimulates expression of VEGFC by tumor cells and promotes lymphangiogenesis in clear cell renal cell carcinomas. *Cancer Res.* 77, 1212–1226
17. Ebos, J. M., Mastri, M., Lee, C. R., Tracz, A., Hudson, J. M., Attwood, K., Cruz-Munoz, W. R., Jedeszko, C., Burns, P., and Kerbel, R. S. (2014) Neoadjuvant antiangiogenic therapy reveals contrasts in primary and metastatic tumor efficacy. *EMBO Mol. Med.* 6, 1561–1576

18. Tomlinson, J. S., Alpaugh, M. L., and Barsky, S. H. (2001) An intact overexpressed E-cadherin/a,b-catenin axis characterizes the lymphovascular embolus of inflammatory breast carcinoma. *Cancer Res.* 61, 5231–5241
19. Fukata, M., and Kaibuchi, K. (2001) Rho-family GTPases in cadherin-mediated cell–cell adhesion. *Nat. Rev. Mol. Cell Biol.* 2, 887–897
20. Neves, S. R., Ram, P. T., and Iyengar, R. (2002) G protein pathways. *Science* 296, 1636–1639
21. New, D. C., Wu, K., Kwok, A. W., and Wong, Y. H. (2007) G protein-coupled receptor-induced Akt activity in cellular proliferation and apoptosis. *FEBS J.* 274, 6025–6036
22. Patyna, S., Arrigoni, C., Terron, A., Kim, T. W., Heward, J. K., Vonderfecht, S. L., Denlinger, R., Turnquist, S. E., and Evering, W. (2008) Nonclinical safety evaluation of sunitinib: a potent inhibitor of VEGF, PDGF, KIT, FLT3, and RET receptors. *Toxicol. Pathol.* 36, 905–916
23. Stewart, G. D., O'Mahony, F. C., Laird, A., Eory, L., Lubbock, A. L., Mackay, A., Nanda, J., O'Donnell, M., Mullen, P., McNeill, S. A., Riddick, A. C., Berney, D., Bex, A., Aitchison, M., Overton, I. M., Harrison, D. J., and Powles, T. (2015) Sunitinib treatment exacerbates intratumoral heterogeneity in metastatic renal cancer. *Clin. Cancer Res.* 21, 4212–4223
24. Nagar, B., Overduin, M., Ikura, M., and Rini, J. M. (1996) Structural basis of calcium-induced E-cadherin rigidification and dimerization. *Nature* 380, 360–364
25. Adelaiye, R., Ciamporcerio, E., Miles, K. M., Sotomayor, P., Bard, J., Tsompana, M., Conroy, D., Shen, L., Ramakrishnan, S., Ku, S. Y., Orillion, A., Prey, J., Fetterly, G., Buck, M., Chintala, S., Bjarnason, G. A., and Pili, R. (2015) Sunitinib dose escalation overcomes transient resistance in clear cell renal cell carcinoma and is associated with epigenetic modifications. *Mol. Cancer Ther.* 14, 513–522
26. Chinello, C., Cazzaniga, M., De Sio, G., Smith, A. J., Gianazza, E., Grasso, A., Rocco, F., Signorini, S., Grasso, M., Bosari, S., Zoppis, I., Dakna, M., van der Burgt, Y. E., Mauri, G., and Magni, F. (2014) Urinary signatures of renal cell carcinoma investigated by peptidomic approaches. *PLoS One* 9, e106684
27. Ibragimova, I., Slifker, M. J., Maradeo, M. E., Banumathy, G., Dulaimi, E., Uzzo, R. G., and Cairns, P. (2013) Genome-wide promoter methylome of small renal masses. *PLoS One* 8, e77309
28. Nikitenko, L. L., Leek, R., Henderson, S., Pillay, N., Turley, H., Generali, D., Gunningham, S., Morrin, H. R., Pellagatti, A., Rees, M. C., Harris, A. L., and Fox, S. B. (2013) The G-protein-coupled receptor CLR is upregulated in an autocrine loop with adrenomedullin in clear cell renal cell carcinoma and associated with poor prognosis. *Clin. Cancer Res.* 19, 5740–5748
29. O'Hayre, M., Degese, M. S., and Gutkind, J. S. (2014) Novel insights into G protein and G protein-coupled receptor signaling in cancer. *Curr. Opin. Cell Biol.* 27, 126–135
30. Elias, B. C., Das, A., Parekh, D. V., Mernaugh, G., Adams, R., Yang, Z., Brakebusch, C., Pozzi, A., Marciano, D. K., Carroll, T. J., and Zent, R. (2015) Cdc42 regulates epithelial cell polarity and cytoskeletal function during kidney tubule development. *J. Cell Sci.* 128, 4293–4305
31. Demetri, G. D., Heinrich, M. C., Fletcher, J. A., Fletcher, C. D., Van den Abbeele, A. D., Corless, C. L., Antonescu, C. R., George, S., Morgan, J. A., Chen, M. H., Bello, C. L., Huang, X., Cohen, D. P., Baum, C. M., and Maki, R. G. (2009) Molecular target modulation, imaging, and clinical evaluation of gastrointestinal stromal tumor patients treated with sunitinib malate after imatinib failure. *Clin. Cancer Res.* 15, 5902–5909
32. Ruan, H., Yang, H., Wei, H., Xiao, W., Lou, N., Qiu, B., Xu, G., Song, Z., Xiao, H., Liu, L., Zhou, Y., Hu, W., Chen, K., Chen, X., and Zhang, X. (2017) Overexpression of SOX4 promotes cell migration and invasion of renal cell carcinoma by inducing epithelial-mesenchymal transition. *Int. J. Oncol.* 51, 336–346

33. Berkers, J., Govaere, O., Wolter, P., Beuselinck, B., Schöffski, P., van Kempen, L. C., Albersen, M., Van den Oord, J., Roskams, T., Swinnen, J., Joniau, S., Van Poppel, H., and Lerut, E. (2013) A possible role for microRNA-141 down-regulation in sunitinib resistant metastatic clear cell renal cell carcinoma through induction of epithelial-to-mesenchymal transition and hypoxia resistance. *J. Urol.* 189, 1930–1938
34. Ni, D., Ma, X., Li, H. Z., Gao, Y., Li, X. T., Zhang, Y., Ai, Q., Zhang, P., Song, E. L., Huang, Q. B., Fan, Y., and Zhang, X. (2014) Downregulation of FOXO3a promotes tumor metastasis and is associated with metastasis-free survival of patients with clear cell renal cell carcinoma. *Clin Cancer Res.* 20, 1779–1790
35. Xiong, J., Liu, Y., Luo, S., Jiang, L., Zeng, Y., Chen, Z., Shi, X., Lv, B., and Tang, W. (2017) High expression of the long non-coding RNA HEIRCC promotes renal cell carcinoma metastasis by inducing epithelial-mesenchymal transition. *Oncotarget* 8, 6555–6563
36. Huang, R. Y., Wong, M. K., Tan, T. Z., Kuay, K. T., Ng, A. H., Chung, V. Y., Chu, Y. S., Matsumura, N., Lai, H. C., Lee, Y. F., Sim, W. J., Chai, C., Pietschmann, E., Mori, S., Low, J. J., Choolani, M., and Thiery, J. P. (2013) An EMT spectrum defines an anoikis-resistant and spheroidogenic intermediate mesenchymal state that is sensitive to E-cadherin restoration by a Src-kinase inhibitor, saracatinib (AZD0530). *Cell Death Dis.* 4, e915
37. Fischer, K. R., Durrans, A., Lee, S., Sheng, J., Li, F., Wong, S. T., Choi, H., El Rayes, T., Ryu, S., Troeger, J., Schwabe, R. F., Vahdat, L. T., Altorki, N. K., Mittal, V., and Gao, D. (2015) Epithelial-to-mesenchymal transition is not required for lung metastasis but contributes to chemoresistance. *Nature* 527, 472–476
38. Tan, T. Z., Miow, Q. H., Miki, Y., Noda, T., Mori, S., Huang, R. Y., and Thiery, J. P. (2014) Epithelial-mesenchymal transition spectrum quantification and its efficacy in deciphering survival and drug responses of cancer patients. *EMBO Mol. Med.* 6, 1279–1293
39. Stewart, K., and Bouchard, M. (2014) Coordinated cell behaviours in early urogenital system morphogenesis. *Semin. Cell Dev. Biol.* 36, 13–20
40. Vasilyev, A., Liu, Y., Mudumana, S., Mangos, S., Lam, P. Y., Majumdar, A., Zhao, J., Poon, K. L., Kondrychyn, I., Korzh, V., and Drummond, I. A. (2009) Collective cell migration drives morphogenesis of the kidney nephron. *PLoS Biol.* 7, e9
41. Xi, W., Sonam, S., Beng Saw, T., Ladoux, B., and Teck Lim, C. (2017) Emergent patterns of collective cell migration under tubular confinement. *Nat. Commun.* 8, 1517
42. Carlo, M. I., Voss, M. H., and Motzer, R. J. (2016) Checkpoint inhibitors and other novel immunotherapies for advanced renal cell carcinoma. *Nat. Rev. Urol.* 13, 420–431
43. Liu, D., Li, G., Avella, D. M., Kimchi, E. T., Kai, J. T., Rubinstein, M. P., Camp, E. R., Rokey, D. C., Schell, T. D., and Staveley-O'Carroll, K. F. (2017) Sunitinib represses regulatory T cells to overcome immunotolerance in a murine model of hepatocellular cancer. *Oncol Immunology* 7, e1372079
44. Lawson, K. A., Mostafa, A. A., Shi, Z. Q., Spurrell, J., Chen, W., Kawakami, J., Gratton, K., Thakur, S., and Morris, D. G. (2016) Repurposing sunitinib with oncolytic reovirus as a novel immunotherapeutic strategy for renal cell carcinoma. *Clin. Cancer Res.* 22, 5839–5850

Figure Legends

Figure 1. Common histologic changes are present in sunitinib-sensitive and resistant xenografts compared with vehicle-treated tumors. A) Tumor growth curves of vehicle-treated, sunitinib (SU)-sensitive, and SU-resistant Renca and 786-0 xenografts. Lines depict the fitted slope of tumor growth curves. B) Compact cancer tongues interrupted necrotic spaces in SU-sensitive and resistant xenografts. Arrows indicate live tumor islands. Surviving tumor areas within the necrotic spaces expressed epithelioid markers, such as PanCK and low-MW CK (LMWCK). Tumors also stained positively for the mesenchymal marker vimentin (VIM) and the renal marker PAX8.

Figure 2. Sunitinib increases tumor spheroid formation in vitro. Renca and ACHN cells were treated with sunitinib (SU) or with vehicle. A, B) Treated cells showed significant increase in tumor spheroid formation. Number of spheroids, adherent cells, and spheroid-forming cells were quantified by ImageXpress, on Hoescht-3422-stained cultures. A representative picture of vehicle and SU-treated cultures. Cell nuclei were stained by Hoechst 3342 to enable automated spheroid and cell counting by ImageXpress (B). Spheroids are highlighted as light-blue areas. C) Number of tertiary spheres formed under vehicle- or SU treatment. D) Percentage of cells that participate in spheroid formation under vehicle- or SU treatment.

Figure 3. Sunitinib has a distinct effect on adherent and spheroid growth patterns of RCC in vitro. The effect of sunitinib on adherent and spheroid growth patterns of ACHN cells was followed by time-lapse microscopy. A) Four conditions were evaluated. Condition 1 was vehicle-treated cells; condition 2, sunitinib treatment; condition 3, vehicle-treated cells were switched to sunitinib treatment; and condition 4, sunitinib-treated cells were switched to vehicle-treatment. Time-lapse microscopy start point was at 0 h, and cultures were followed for 55 h. B) Representative photographs of the time-lapse microscopy. Yellow dotted line outlines the border of the adherent RCC areas. Spheroids are shown with orange arrows, whereas adherent growth is denoted by white arrows. C) Quantitative representation of changes in the percentage of area covered by adherent and spheroid patterns under the 4 conditions.

Figure 4. Overlapping cellular processes are predicted to operate under sunitinib treatment and spheroid formation. Transcriptome analysis indicates that cell–cell adhesion, cytoskeletal organization, vesicular transport, and cellular polarity are similarly altered during spheroid formation (A) and sunitinib treatment (B). Additionally, sunitinib treatment is predicted to induce the PI3K/AKT survival pathway through GPCRs.

Figure 5. In vitro RCC spheroids and in vivo tumor islands surviving sunitinib (SU) treatment are related. A) 786-0 spheroids (786) or adherent cells were assessed for E-cadherin (E-CAD) and b-catenin expression by immunocytochemistry. Nuclei were visualized with DAPI staining. B) Vehicle-treated, SU-sensitive, and SU-resistant xenografts were assessed for E-CAD and b-catenin expression by IHC. Arrowheads indicate membranous positive staining. C–M) Adherent (adh) RCC cells or spheroids were xenografted. Tumor morphology was compared on hematoxylin and eosin-stained sections. Adh ACHN xenografts appear to be high-grade tumors with diffuse necrotic cores and necrotic cores with mainly spindled morphology (C–E). Spheroid (sph)-initiated tumors show large, compact tumor tongues, similar to the SU-treated xenografts (F–H). The border of sph-based xenografts shows epithelial nests invading adjacent tissue (H). Black arrows indicate epithelial tumor nests (G, H). Sph-derived xenografts exhibit

different histologic patterns, such as cytoplasmic clearing and areas with giant multinucleated cells (I, J). Black arrows indicate giant cells. Sph-derived and SU-treated Renca xenografts show multifocal tumors with compact sph-like patterns (K–M). In contrast, adherent Renca cell xenografts have uninterrupted necrotic space.