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TITLE:

Comparing Metastatic Clear Cell Renal Cell Carcinoma Model Established in Mouse Kidney and on Chicken Chorioallantoic Membrane

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KEYWORDS:
renal cell carcinoma, metastasis, animal model, renal implantation, CAM, VHL gene deletion, intratumoral heterogeneity

SUMMARY:

Metastatic clear cell renal cell carcinoma is a disease without a comprehensive animal model for thorough preclinical investigation. This protocol illustrates two novel animal models for the disease: the orthotopically implanted mouse model and the chicken chorioallantoic membrane model, both of which demonstrate lung metastasis resembling clinical cases.

ABSTRACT:
Metastatic clear cell renal cell carcinoma (ccRCC) is the most common subtype of kidney cancer. Localized ccRCC has a favorable surgical outcome. However, one third of ccRCC patients will develop metastases to the lung, which is related to a very poor outcome for patients. Unfortunately, no therapy is available for this deadly stage, because the molecular mechanism of metastasis remains unknown. It has been known for 25 years that the loss of function of the von Hippel-Lindau (VHL) tumor suppressor gene is pathognomonic of ccRCC. However, no clinically relevant transgenic mouse model of ccRCC has been generated. The purpose of this protocol is to introduce and compare two newly established animal models for metastatic ccRCC. The first is renal implantation in the mouse model. In our laboratory, the CRISPR gene editing system was utilized to knock out the VHL gene in several RCC cell lines. Orthotopic implantation of heterogeneous ccRCC populations to the renal capsule created novel ccRCC models that develop robust lung metastases in immunocompetent mice. The second model is the chicken chorioallantoic membrane (CAM) system. In comparison to the mouse model, this model is more time, labor, and cost-efficient. This model also supported robust tumor formation and intravasation. Due to the short 10 day period of tumor growth in CAM, no overt metastasis was observed by immunohistochemistry (IHC) in the collected embryo tissues. However, when tumor growth was extended by two weeks in the hatched chicken, micrometastatic ccRCC lesions were observed by IHC in the lungs. These two novel preclinical models will be useful to further study the molecular mechanism behind metastasis, as well as to establish new, patient-derived xenografts (PDXs) toward the development of novel treatments for metastatic ccRCC.

INTRODUCTION:
Renal cell carcinoma (RCC) is the 7th most common cancer in the United States. Annually, 74,000 Americans are estimated to be newly diagnosed, accounting for more than 14,000 deaths (http://seer.cancer.gov/statfacts/html/kidrp.html). Clear-cell histological subtype, or ccRCC, is the most common subtype, accounting for approximately 80% of RCC cases. Patients with localized malignancy are treated with nephrectomy and have a favorable 5-year survival rate of 73%[1]. However, 25%–30% of patients develop distant metastases to vital organs such as the lungs, resulting in a poor mean survival of 13 months and 5-year

survival rate of only 11%[1-3]. Further understanding of the metastatic mechanism is needed to improve the deadly outcome for metastatic ccRCC.

The loss of the VHL tumor suppressor gene is a hallmark genetic lesion observed in a majority of human ccRCC cases[4-7]. However, the precise oncogenic mechanism of VHL loss in ccRCC is unknown. Also, VHL expression status is not predictive of outcome in ccRCC[8]. Notably, despite numerous attempts at renal-epithelial-targeted VHL knockout, scientists have failed to generate renal abnormality beyond the preneoplastic cystic lesions observed in mice[9], even when combined with deletion of other tumor suppressors such as PTEN and p53[10]. These findings support the idea that VHL loss alone is insufficient for tumorigenesis or the subsequent spontaneous metastasis.

Recently, our laboratory created a new VHL knockout (VHL-KO) cell line using CRISPR/Cas9 mediated deletion of the VHL gene in the murine VHL+ ccRCC cell line (RENCA, or VHL-WT)[11,12]. We showed that VHL-KO is not only mesenchymal, but also promotes epithelial to mesenchymal transition (EMT) of VHL-WT cells[12]. EMT is known to play an important role in the metastatic process[13]. Our work further showed that distant lung metastasis occurs only with co-implantation of VHL-KO and VHL-WT cells in the kidney, supporting a cooperative mechanism of metastasis . Importantly, our orthotopically implanted VHL-KO and VHL-WT model leads to robust lung metastases, recapitulating the clinical ccRCC cases. This spontaneous metastatic ccRCC model compensates for the lack of a transgenic metastatic mouse model, especially in the development of novel anti-metastasis drugs. This protocol demonstrates the renal capsule implantation of the heterogeneous cell populations of genetic engineered RENCA cells.

Chicken CAM models have a long history in research for angiogenesis and tumor biology due to their numerous advantages, as summarized in **Table 1**[14-18]. Briefly, the time window for CAM tumor growth is short, allowing a maximum of 11 days until the CAM is destroyed upon hatching of the chicken[16]. Despite the short growth time, the rich nutrition supply and immunodeficient state of the chicken embryo enable very efficient tumor engraftment[16,19-21]. Finally, the cost of each fertilized egg is ~$1, compared to over $100 for a SCID mouse. Together, the CAM model can serve as a valuable alternative animal model in establishing new PDXs at a great saving in time and cost in comparison to the mouse. In this protocol, we assessed whether the model was able to recapitulate the biology of metastatic ccRCC observed in the mouse orthotopic model. [Place **Table 1** here]

PROTOCOL:

All methods described here have been approved by the Institutional Animal Care and Use Committee (IACUC), designated as UCLA Chancellor’s Animal Research Committee (ARC) (ARC 2002-049-53 and ARC 2017-102-01A). The 2002-049-53 protocol is optimized for the implantation of ccRCC tumor cells

137 into the kidney capsule of Nude or BALB/c mice. Tumor implantation
138 experiments in fertilized chicken eggs prior to hatching does not require
139 IACUC approval. To extend the time for establishment of lung metastasis, the
140 embryos with CAM tumor are allowed to hatch and grow into chickens. The
141 2017-102-01A protocol covers these animal experiments.
142
143 **1. Orthotopic tumor studies in mice**
144
145 NOTE: The timeline for this experiment is shown in **Figure 1A**. These
146 procedures were adapted from previous publications[11,12].
147
148 1.1. Preparing single cell suspension for grafting
149
150 1.1.1. Detach the RENCA VHL-WT and VHL-KO cells from the culture dishes
151 using trypsin/EDTA.
152
153 1.1.2. Count the cells with a hemocytometer and resuspend in a precooled
154 1:1 mixture of PBS and extracellular matrix solution at a concentration of 1 x
155 10^5 cells/µL.
156
157 NOTE: Use a 1:4 ratio of VHL-WT:VHL-KO cells for heterogeneous implants
158 and VHL-WT alone for homogeneous implants.
159
160 1.1.3. Transfer the resuspended cells into a 1.5 mL microcentrifuge tube and
161 keep on ice until implantation.
162
163 1.2. Implantation to renal capsule
164
165 1.2.1. Anesthesia: Preheat a warm pad to 37 °C and cover it with a piece of
166 thick sterile drape. Anesthetize the mouse by either isoflurane inhalation via
167 an induction chamber or intraperitoneal (IP) injection of 1% pentobarbital
168 sodium at the dosage of 10 mL/kg.
169
170 1.2.2. Shave the hair from the surgical site. This step can be skipped for
171 nude mice.
172
173 1.2.3. Disinfection and surgical draping: Disinfect the back of the mouse
174 entirely with povidone-iodine 3x followed by 70% ethanol 1x and wipe it dry
175 with sterile cotton swabs. Then apply three sterile medical dressings
176 sequentially, covering the whole back in order to create a surgical field as
177 well as to immobilize the mouse.
178
179 1.2.4. Incision and kidney exteriorization: Before the operation, disinfect the
180 operator's fingers with povidone-iodine or use a pair of sterile gloves. Place
181 the mouse in the prone position and use the fingers to locate the left kidney
182 right under the left flank. Use a pair of blunt forceps and scissors to cut the

183 skin open and the muscle layer under the specified location. Use sterile
184 cotton swabs and forceps to partially exteriorize the left kidney out of the
185 abdomen.
186
187 1.2.5. Tumor cell implantation: Load the cell suspension prepared in step 1.1
188 in either insulin syringes or customized Hamilton syringes (see **Table of**
189 **Materials** for specifications). Inject 20 µL of resuspended cells under the
190 kidney capsule.
191
192 **NOTE**: Successful injection is determined by the formation of a translucent
193 bulge on the surface of the kidney. Accidental injection into the renal
194 parenchyma results in bleeding and a post-operative mortality rate as high
195 as 90% due to fatal hemorrhage at the injection site.
196
197 1.2.6. Slowly pull out the needle in order to allow the extracellular matrix
198 solution to solidify and prevent hemorrhage or tumor cell leakage. Then,
199 using a sterile cotton swab, push the kidney back into the abdomen.
200
201 1.2.7. Wound stitching: Use a 5-0 coated VICTRYL suture to stitch the muscle
202 layer. Disinfect the skin with povidone-iodine once and close the skin with
203 wound autoclips.
204
205 1.2.8. Recovery: Place the mouse on the warm pad until it wakes up. If the
206 mouse was anesthetized by inhalation, withdraw the isoflurane and keep the
207 mouse on the warm pad until it is awake.
208
209 1.3. Bioluminescence imaging (BLI) and tissue collection
210
211 1.3.1. Six weeks after tumor implantation, take firefly-luciferase-based BLI
212 images. Then euthanize mice with isoflurane inhalation followed by cervical
213 dislocation.
214
215 1.3.2. Collect blood for circulating tumor cell (CTC) detection by flow
216 cytometry.
217
218 1.3.3. Harvest tumor and organs of interest (kidneys, lungs, liver, intestines,
219 and spleen) using a sterile tissue harvesting technique[22]. Fix them in 4%
220 paraformaldehyde overnight for paraffin-wax embedding.
221
222 **2. CAM tumor xenograft model**
223
224 NOTE: These procedures were adapted and modified from previously
225 published protocols[23,24]. The timeline for this procedure is shown in **Figure**
226 **1B**. This article presents only the streamlined protocol. For detailed
227 protocols, please refer to another JoVE article published by our group[25].
228

229 2.1. Preincubation: Incubate freshly laid, fertilized chicken eggs in a rotating
230 egg incubator at 37 °C and 55–65% humidity for 7 days.
231
232 2.2. Drop the CAM and open window (developmental day 7):
233
234 2.2.1. Locate and mark the air sac and veins.
235
236 NOTE: Usually 10–15% of the eggs are removed because they are either
237 unfertilized or die within 7 days of fertilization.
238
239 2.2.2. Create a new air sac on top of a marked vein.
240
241 2.2.3. Delineate the new air sac, apply packing tape, and put the eggs back
242 to the incubator.
243
244 NOTE: The procedure can be paused here. It is recommended to resume the
245 procedures within the same day because the air sac may move.
246
247 2.2.4. Open a window: Using a pair of curved microdissecting scissors and a
248 pair of needle-nose forceps, cut a 1.5 x 1.5 cm circular window in the shell.
249
250 NOTE: Disruption of CAM is indicated by the blood and a piece of CAM
251 present on the cut shell piece.
252
253 2.2.5. Seal the hole with transparent medical dressing and place the eggs in
254 a stationary incubator at 37 °C and 55%–65% humidity.
255
256 NOTE: Use the same egg incubator as in step 2.1. Turn off the rotator to
257 make it stationary.
258
259 2.3. Health check (developmental day 9): Remove dead eggs and then
260 randomly group the rest of the eggs for tumor cell implantation.
261
262 NOTE: Ideally, the survival rate at this point is approximately 80% of
263 developmental day 0.
264
265 2.4. Grafting the tumor cells onto the newly exposed CAM (developmental
266 day 10)
267
268 2.4.1. Dilute the extracellular matrix solution in double the volume of
269 precooled RPMI 1640 (with L-glutamine). Detach the RENCA cells and
270 resuspend in the above solution to reach a concentration of 2 x 10^4 cells/µL.
271
272 **NOTE:** Use a 1:1 ratio of VHL-WT:VHL-KO cells for heterogeneous implants
273 and VHL-WT alone for homogeneous implantation.
274

275 2.4.2. To presolidify the cell suspension, fill 100 µL of each cell mix in 200 µL
276 pipette tips and place them in a cell incubator for 15 mins.
277
278 2.4.3. Implant 100 µL of cell suspension for each egg on the CAM surface
279 through the window.
280
281 NOTE: Some protocols require scratching the CAM before implantation[23]. This
282 is not necessary for RENCA cells, because they grow very quickly.
283
284 2.5. Grow cells on the CAM for 10 days and photograph every 2 days.
285
286 2.6. Euthanize and harvest the tumor, blood, and organs.
287
288 2.6.1. On developmental day 20 (tumor day 10), collect blood via the
289 chorioallantoic vein with a heparinized 10 mL syringe.
290
291 2.6.2. Euthanize the embryos by putting them on ice for 15 min.
292
293 2.6.3. Harvest and weigh tumors. Dissect lungs and livers using a sterile
294 tissue harvesting technique similar to that used for mice[22].
295
296 NOTE: Chickens livers have two lobes, which are the first organs seen in the
297 abdominal cavity. Do not confuse these with the lungs. The chicken lungs are
298 located under the heart and septum[26]. The successful collection of the lungs
299 can be confirmed by exposed ribs.
300
301 2.6.4. Fix the tumors and the dissected organs in 4% paraformaldehyde
302 overnight for paraffin-wax embedding.
303
304 2.7. Hatch the chickens.
305
306 2.7.1. To allow an extended period of tumor growth, continue the incubation
307 through day 21 and let the chickens hatch at 37 °C and at least 60%
308 humidity.
309
310 **NOTE:** Chickens naturally hatch after day 21 over a 24 h time period but
311 occasionally have trouble hatching by themselves. In this case, cracking the
312 eggshells some helps. It is important for chickens to complete the hatching
313 process within 24 h because they will die from lack of nutrients after then.
314
315 2.7.2. Grow the chickens in an animal facility (2017-102-01A) for 2 weeks.
316
317 2.7.3. On developmental day 34 (tumor day 24), euthanize the chicks with
318 isoflurane inhalation followed by cervical dislocation.
319

2.7.4. Dissect the lungs using a sterile tissue harvesting technique similar to those used for mice[22]. Then, fix them in 4% paraformaldehyde overnight for paraffin-wax embedding.

3. Immunohistochemistry

NOTE: All tissue sectioning and H&E staining was done by the Translational Pathology Core Laboratory (TPCL) at the University of California, Los Angeles.

3.1. Bake slides at 65 °C for 20 min and deparaffinize 3x using xylene and rehydrate serially from 100% ethanol to water.

3.2. Retrieve the antigens in a citrate buffer boiled in a vegetable steamer for 25 min.

3.3. Apply 1% BSA for blocking. Then apply the primary antibodies (anti-VHL, anti-HA, anti-flag) prepared at a 1:200 dilution ratio in PBS. Incubate overnight at 4 °C.

3.4. After washing 3x with TBST (7 min each), incubate slides with the secondary antibody at 1:200 dilution. Wash 3x with TBST (7 min each) and apply DAB reagents followed by hematoxylin counterstaining.

4. Flow Cytometry

4.1. Process the mouse or chicken blood with red blood cell (RBC) lysis buffer according to the manufacturer’s protocol.

NOTE: Sufficient RBC lysis is especially important when analyzing CAM blood because chicken RBCs are nucleated and cannot be easily distinguished in flow cytometry using the forward and side scatter.

4.2. Run flow cytometry on the blood lysate and analyze the data for mStrawberry and EGFP expression.

4.3. Set the primary gates based on the forward and side scatter excluding debris, dead cells, and unlysed RBCs.

4.4. Set the fluorescence gates based on the unstained samples and single stained controls. Use blood lysate primed with VHL-WT, VHL-KO, or unlabeled RENCA cells as the single stained controls and unstained controls, respectively.

REPRESENTATIVE RESULTS:

Each experiment was performed at least 3x unless otherwise stated. Data are presented as mean ± standard deviation (SD). Significance was

366 determined by a paired, Student's T-test when there were two groups or by a
367 one-way ANOVA when there were three or more groups. A p-value cutoff of
368 0.05 was used to establish significance.
369
370 Orthotopically implanted RENCA cells successfully grew on the mice kidneys,
371 as confirmed by BLI and H&E staining (**Figure 2A–B**). Although there was no
372 difference in the primary growth, only the heterogeneous, metastatic tumor
373 had robust metastasis to the lung as indicated by the very strong BLI signal
374 and metastatic nodules in the H&E staining. On the other hand,
375 homogeneous, nonmetastatic tumors did not metastasize to distant organs.
376 CTC counts were higher in the mice bearing metastatic tumors than those
377 bearing nonmetastatic tumors (**Figure 2C**, **D**).
378
379 In concordance with the mouse model, the CAM system successfully retained
380 the growth and metastatic behavior of the RENCA tumors. While there was
381 no growth difference between metastatic and nonmetastatic tumors, the CTC
382 counts were significantly higher in the eggs with metastatic tumors than
383 those with nonmetastatic counterparts (**Figure 3A**–**C**). Hatching the eggs
384 with CAM tumors and allowing the chicks to grow an additional two weeks
385 extended the period for metastatic cancer cells to establish histologically
386 detectable metastases in the lung of chicks, as shown in the H&E and HA
387 stain of chicken lung tissue sections (**Figure 3D**). A majority of the
388 metastatic nodules consisted of HA-tagged VHL-WT cells, whereas flag-
389 tagged VHL-KO were rarely seen, as we have observed in mice.
390
391 **FIGURE AND TABLE LEGENDS:**
392 **Figure 1: Overview of the two animal models for metastatic ccRCC**
393 **xenografts**. (**A**) Schematic representation of the mouse orthotopic model.
394 3- or 4-week-old mice are orthotopically implanted with either nonmetastatic
395 or metastatic tumors. Six weeks after implantation, tumor growth and
396 metastasis were visualized with BLI. Then, tumor, blood, and organs were
397 collected for downstream analyses. (**B**) Schematic representation of the CAM
398 model showing the following steps: Preincubation, window opening, cell
399 implantation, and euthanasia before or after hatching. The same analyses as
400 the mouse model are conducted for the collected samples.
401
402 **Figure 2: Tumor growth and metastasis in orthotopically implanted**
403 **mice.** (**A**) BLI of mice and extracted organs (kidney, lung, liver, intestine,
404 and spleen) 6 weeks after orthotopic implantation of RENCA cells. Left:
405 nonmetastatic (non-met), right: metastatic. (**B**) Gross view and increasing
406 magnification of H&E staining for the kidney and lung (20x and 100x). Left:
407 nonmetastatic (non-met), right: metastatic. (**C**) Representative flow analysis
408 for detecting mStraw+ and EGFP+ cells circulating in the blood. (**D**) Percent
409 population graph of circulating mStraw+ and EGFP+ cells. Non-met:
410 nonmetastatic. Panel A was adapted from Hu et al.[27].
411

Figure 3: Tumor growth and metastasis in the CAM model. (**A**) RENCA tumors grown in CAM. There were 7 repeats for each group. Non-met: nonmetastatic. (**B**) Representative flow analysis for detecting mStraw+ and EGFP+ cells circulating in the blood. (**C**) Percent population graph of circulating mStraw+ and EGFP+ cells. **$p < 0.01$. (**D**) IHC staining of the lung from a 2-week-old chick bearing a metastatic tumor during its embryonic stage. From left, the sections show H&E, HA, and flag staining. #: chicken pulmonary artery; arrowhead: metastatic nodules. This figure was adapted from Hu J et al.[27].

Table 1: Advantages and limitations of the mouse and CAM models. This table compares the two models for their advantages and limitations in terms of required time, cost, labor, as well as the biology. The CAM model has advantages in efficiency, but it also has its own unique limitations due to the different morphology between birds and mammals. Therefore, it is important to confirm that the model can retain the biology of the xenografts.

DISCUSSION:

For many patients with epithelial malignancies, metastasis to vital organs is the primary cause of mortality. Therefore, it is essential to find the underlying mechanism and a new avenue of therapy for metastatic disease. Unfortunately, there is a lack of relevant metastatic ccRCC animal models. The challenge in large part is due to the inability to recreate ccRCC in mice despite the generation of numerous transgenic kidney epithelial-targeted VHL knockout mouse models[9,10]. Here, we demonstrate the methods to establish an implantable metastatic ccRCC tumor in two animal systems, the mouse and the chicken CAM. These findings validated the metastatic behavior of the tumors in two disparate environments, and thus provide unique opportunities to further investigate the molecular mechanism of metastasis. In the first model, the heterogeneous RENCA population was implanted to immunocompetent mice orthotopically to their renal capsule. After 6 weeks, these mice showed rampant lung metastasis. In concordance with the mouse model, implantation of heterogeneous RENCA cells on the CAM successfully grew and intravasated into the blood of the chick embryos. By extending the tumor growth period to 2 weeks after hatching, lung metastases resembling those seen in the mouse were observed in the chicks.

For both models, careful attention to the technical details of each step and practice to improve technical skills are essential to increase animal survival and successful tumor engraftment and metastasis. For the mouse model, careful choice of equipment and accurate injection of the tumor cells to the renal capsule maximizes the success rate by decreasing the post-operative mortality and increasing the chance for the tumor to get an adequate blood supply to grow and metastasize. The CAM model requires more optimization in the setup and the technique. In our studies, the embryo viability was

below 30% at the beginning. It is important to keep both the temperature and humidity to the desired level at all times by having good equipment, frequent monitoring, and faster completion of the procedures. Even after optimization, the survivability ranges from 50–75% depending on the experimenter and the individual batch of eggs. It is recommended to always order extra eggs for backup. In our experience, mastering the CAM techniques requires over 1 year. Dropping the CAM membrane and opening the window is the critical step where accidental, fatal damage to the embryo most often occurs. The viability of the chick embryo can be improved by preventing damage to the CAM.

There are several limitations to the CAM tumor model. First is the applicability of the model to all tumor cell types. We have had a 100% success rate engrafting different established tumor cell lines on CAM, including kidney, bladder, and prostate tumor cell lines (RENCA, ACHN, T24, HT1376, CWR22Rv1, C4-2, Myc-CaP) and ovarian cancer cell lines (ID8 and SKOV3). Two additional studies from our group provide further information on these CAM tumors[25,27]. However, the growth of some ovarian cancer cells on CAM is enhanced by the supplementation of growth factor or tumor-associated cells[25]. The optimization of cell number or essential growth factor(s) for each cell line or type is important. We also incorporate reporter or marker genes, such as luciferase, protein tags (e.g., HA or flag), or fluorescence tags (e.g., mStrawberry or EGFP), to facilitate the monitoring of the growth and metastasis of the tumor in the animals[25,27]. Based on our experience, a large majority of proliferating cancer cell lines can be established on CAM. A key limitation to engraftment might be the short 10 day window allowed for tumor growth, which could be especially challenging for a slow growing cell line to establish sufficient mass in such a short time frame.

Another shortcoming of the CAM model is the difference in physiology between the avian embryo and mammals. Metastasis from the CAM tumor to major organs such as the liver or lungs of the embryo has been detected predominantly by sensitive PCR techniques[28]. The short time period of growth in CAM would be insufficient to establish large metastatic lesions that can be verified by histological analyses. Furthermore, the reduced vascular perfusion of the uninflated embryo lung is not favorable for establishing or supporting the growth of lung metastases. To overcome these limitations, an approval from our institutional animal use committee (IACUC) was obtained to hatch chickens from the CAM tumor bearing embryos and house them an additional 2 weeks after hatching. Extending the time of tumor growth in this manner enabled us to detect distant lung metastases by IHC. Although the hatched chicken studies require the additional IACUC approval that CAM tumor studies do not, this approach provides a valuable opportunity to study the metastatic cascade in chickens as previously done in mice. The chicken immune system has been reported to develop starting on day 12 post

fertilization[20]. Given the high efficiency of engrafting murine derived RENCA tumors reported here and many other human cancer cell lines and PDXs in the CAM on day 10 after fertilization[24,25,27], we could deduce that the immune system in the embryo is not fully developed at this point. The interplay of the chicken's immune system and the CAM tumor clearly warrants further investigation.

Our work provides strong supportive evidence that the CAM tumor model could be a simple initial in vivo model to study cancer biology, including metastasis. Due to the limitations noted above, the CAM model should not replace the mouse model, but complement it. Our ongoing research suggests that signal crosstalk between heterogeneous cell populations in ccRCC is instrumental in governing metastatic progression[11,12]. The use of both the CAM and mouse models can be a valuable means to validate the metastatic crosstalk at play in ccRCC. We believe the numerous advantages of the CAM model presented here could accelerate the pace of discovery of novel metastatic mechanisms and effective treatments to remedy this deadly stage of cancer.

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DISCLOSURES:

The authors have nothing to disclose.

REFERENCES:

1. Cohen, H. T., McGovern, F. J. Renal-cell carcinoma. *The New England Journal of Medicine.* **353** (23), 2477–2490 (2005).
2. Bianchi, M. et al. Distribution of metastatic sites in renal cell carcinoma: a population-based analysis. *Annals of Oncology.* **23** (4), 973–980 (2012).
3. Hsieh, J. J. et al. Renal cell carcinoma. *Nature Reviews Disease Primers.* **3**, 17009 (2017).

549 4. Foster, K. et al. Somatic mutations of the von Hippel-Lindau disease
550 tumour suppressor gene in non-familial clear cell renal carcinoma. *Human*
551 *Molecular Genetics.* **3** (1994).
552 5. Young, A. C. et al. Analysis of VHL Gene Alterations and their
553 Relationship to Clinical Parameters in Sporadic Conventional Renal Cell
554 Carcinoma. *Clinical Cancer Research.* **15** (24), 7582–7592 (2009).
555 6. Gossage, L., Eisen, T., Maher, E. R. VHL, the story of a tumour
556 suppressor gene. *Nature Reviews Cancer.* **15** (1), 55–64 (2015).
557 7. Sato, Y. et al. Integrated molecular analysis of clear-cell renal cell
558 carcinoma. *Nature Genetics.* **45** (8), 860–867 (2013).
559 8. Choueiri, T. K. et al. The role of aberrant VHL/HIF pathway elements in
560 predicting clinical outcome to pazopanib therapy in patients with metastatic
561 clear-cell renal cell carcinoma. *Clinical Cancer Research.* **19** (18), 5218–5226
562 (2013).
563 9. Hsu, T. Complex cellular functions of the von Hippel-Lindau tumor
564 suppressor gene: insights from model organisms. *Oncogene.* **31** (18), 2247–
565 2257 (2012).
566 10. Albers, J. et al. Combined mutation of Vhl and Trp53 causes renal cysts
567 and tumours in mice. *EMBO Molecular Medicine.* **5** (6), 949–964 (2013).
568 11. Schokrpur, S. et al. CRISPR-Mediated VHL Knockout Generates an
569 Improved Model for Metastatic Renal Cell Carcinoma. *Scientific Reports.* **6**,
570 29032 (2016).
571 12. Hu, J. et al. A Non-integrating Lentiviral Approach Overcomes Cas9-
572 Induced Immune Rejection to Establish an Immunocompetent Metastatic
573 Renal Cancer Model. *Molecular Therapy Methods & Clinical Development.* **9**,
574 203–210 (2018).
575 13. Heerboth, S. et al. EMT and tumor metastasis. *Clinical and*
576 *Translational Medicine.* **4**, 6 (2015).
577 14. DeBord, L. C. et al. The chick chorioallantoic membrane (CAM) as a
578 versatile patient-derived xenograft (PDX) platform for precision medicine and
579 preclinical research. *American Journal of Cancer Research.* **8** (8), 1642–1660
580 (2018).
581 15. Hagedorn, M. et al. Accessing key steps of human tumor progression in
582 vivo by using an avian embryo model. *Proceedings of the National Academy*
583 *of Sciences of the United States of America.* **102** (5), 1643–1648 (2005).
584 16. Ribatti, D. The chick embryo chorioallantoic membrane as a model for
585 tumor biology. *Experimental Cell Research.* **328** (2), 314–324 (2014).
586 17. Ismail, M. S. et al. Photodynamic Therapy of Malignant Ovarian
587 Tumours Cultivated on CAM. *Lasers in Medical Science.* **14** (2), 91–96 (1999).
588 18. Kaufman, N., Kinney, T. D., Mason, E. J., Prieto, L. C., Jr. Maintenance of
589 human neoplasm on the chick chorioallantoic membrane. *The American*
590 *Journal of Pathology.* **32** (2), 271–285 (1956).
591 19. Janse, E. M., Jeurissen, S. H. Ontogeny and function of two non-
592 lymphoid cell populations in the chicken embryo. *Immunobiology.* **182** (5),
593 472–481 (1991).

594 20. Leene, W., Duyzings, M. J., van Steeg, C. Lymphoid stem cell
595 identification in the developing thymus and bursa of Fabricius of the chick.
596 *Zeitschrift fur Zellforschung und Mikroskopische Anatomie.* **136** (4), 521–533
597 (1973).
598 21. Solomon, J. B. in *Foetal and neonatal immunology Frontiers of biology,*
599 xv, 381 p. with illus. (North-Holland Pub. Co., 1971).
600 22. JoVE Science Education Database. *Lab Animal Research*. Sterile Tissue
601 Harvest. JoVE, Cambridge, MA. (2019).
602 23. Palmer, T. D., Lewis, J., Zijlstra, A. Quantitative analysis of cancer
603 metastasis using an avian embryo model. *Journal of Visualized Experiments*.
604 (51), e2815 (2011).
605 24. Fergelot, P. et al. The experimental renal cell carcinoma model in the
606 chick embryo. *Angiogenesis.* **16** (1), 181–194 (2013).
607 25. Sharrow, A. C., Ishihara, M., Hu, J., Kim, I. H., Wu, L. Using the Chicken
608 Chorioallantoic Membrane In Vivo Model to Study Gynecological and
609 Urological Cancers. *JoVE.* (In Press).
610 26. Lőw, P., Molnár, K., Kriska, G. in *Atlas of Animal Anatomy and*
611 *Histology*. pp. 265–324 (2016).
612 27. Hu, J., Ishihara, M., Chin, A. I., Wu, L. Establishment of xenografts of
613 urological cancers on chicken chorioallantoic membrane (CAM) to study
614 metastasis. *Precision Clinical Medicine.* **2** (3), 140–151 (2019).
615 28. Casar, B. et al. In vivo cleaved CDCP1 promotes early tumor
616 dissemination via complexing with activated beta1 integrin and induction of
617 FAK/PI3K/Akt motility signaling. *Oncogene.* **33** (2), 255–268 (2014).
618