A Preclinical Murine Model for the Detection of Circulating Human Tumor Cells

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Abstract. *Background/Aim: Circulating tumor cells (CTCs), cancer cells that disseminate from primary tumors and enter the bloodstream in the course of metastasis, may serve as an important indicator of metastatic disease and poor prognosis in patients with cancer. The aim of this study was to establish a preclinical animal model for detecting and studying human CTCs. Materials and Methods: We performed a renal subcapsular implantation of human cancer cells in immunodeficient mice and recorded primary tumor growth, CTCs, and metastatic tumor development. Results: Immunofluorescence, or immunohistochemical staining and whole-body imaging analysis revealed that the implanted cells developed primary renal tumors, CTCs were detected, and successfully established metastatic tumors in several organs, including the lung, colon, and lymph nodes, depending on the implanted cells. Conclusion: This model may be useful for detecting and characterizing CTCs and for investigating the mechanisms underlying the course of tumor metastasis.*

Despite advances in cancer therapy, the high mortality rate from various types of human cancer has not decreased, primarily due to the occurrence of metastases (1). Given the multi-step nature of metastasis, considerable attention has been given to the early detection of circulating tumor cells (CTCs) in the peripheral blood of patients with cancer. Although the nature of CTCs is not fully-understood, they are largely regarded as an indicator of metastatic tumors (1, 2). It is also widely accepted that CTCs are drug-resistant and that their presence or increase may result from failure of anticancer therapy (1, 2). In response, the development of a drug delivery system for targeting CTCs was reported in a recent study (3). Furthermore, an increasing number of reports have presented the utilization of CTCs as a prognostic marker for various cancer types, including advanced metastatic breast cancer and small cell lung cancer (4, 5). Various techniques have emerged for the enrichment and detection of CTCs (6). Indeed, mRNA and microRNA profiling of CTCs has been used for the discovery of biomarkers and for determination of drug responsiveness (7-9). These findings indicate the great potential of CTCs as non-invasive, surrogate markers for tumor progression and as measures of therapeutic efficacy in clinical applications.

Despite great technological advances in isolating CTCs, their utilization is still limited, primarily due to their infrequency in the blood (10). Therefore, while clinical studies paved the way to detect and isolate CTCs from human blood, researchers have attempted to establish experimental animal models of metastatic tumor formation for CTC detection (11-15). These models include the implantation of human cancer cells into immunodeficient mice, either subcutaneously or orthotopically, and the injection of syngeneic cancer cells under the renal capsule in rodents (11-15). However, spontaneous metastasis from subcutaneously-injected xenografted tumors is not frequent, and thus the appearance of CTCs arising from subcutaneous tumors is rare. Implanting cancer cells under the renal capsule was originally established for the rapid screening of chemotherapeutic agents. Orthotopic metastatic nude mouse models with renal subcapsular injection of human renal cell carcinomas and mammary fat-pad injection of breast cancer cells have shown potential as tools for investigating CTCs (12, 16). However, orthotopic injection of several types of cancers, including non-small cell lung cancer (NSCLC), is complex, and the development of experimental animal models for such cell types is necessary.

The goal of the present study was to develop an *in vivo* murine model for studying human CTCs. We performed renal capsule implantation of human H226Br NSCLC, and HCT116 colon cancer cells with acquired resistance to the chemotherapeutic agent 5-fluorouracil (5-FU) (HCT116/R). We observed aggressive primary tumor formation, an increased

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Table I. *Primer sequences used in this study.*

	Gene		Sequence
RT-PCR	*GFP*	Forward (5'→3')	GGTCCTTCTTGAGTTTGTAACAG
		Reverse (5'→3')	CATCACCATCTAATTCAACAAG
	Human *actin*	Forward (5'→3')	ACTACCTCATGAAGATC
		Reverse (5'→3')	GATCCACATCTGCTGGAA
	Mouse *GAPDH*	Forward (5'→3')	AGGCCGGTGCTGAGTATGTC
		Reverse (5'→3')	TGCCTGCTTCACCACCTTCT
Real-time PCR	*GFP*	Forward (5'→3')	CAACAGCCACAACGTCTATATCAT
		Reverse (5'→3')	ATGTTGTGGCGGATCTTGAAG
	Human *actin*	Forward (5'→3')	GCGAGAAGATGACCCAGATC
		Reverse (5'→3')	GGATAGCACAGCCTGGATAG
	Mouse *GAPDH*	Forward (5'→3')	GAGTTGCTGTTGAAGTCGCA
		Reverse (5'→3')	GGTGGTGAAGCAGGCATCTG

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; GFP: green fluorescence protein.

number of CTCs, and metastatic tumor development. Our findings suggest that this animal model may be useful for detecting CTCs, monitoring the effectiveness of treatment options, and investigating the mechanisms underlying the course of tumor metastasis and anticancer therapies.

Materials and Methods

Reagents. Reagents for cell culture were purchased from Welgene Inc. (Daegu, Republic of Korea). Bovine serum albumin (BSA) solution was purchased from Dako (Glostrup, Denmark). Lipofectamine 2000 was purchased from Invitrogen (Grand Island, NY, USA). G418 was purchased from Enzo Life Sciences (Farmingdale, NY, USA). Histopaque 1083 and other reagents, unless otherwise specified, were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Cell culture. HCT116 human colon cancer and H226Br non-small cell lung cancer cells were kindly provided by Dr. Sang Kook Lee (Seoul National University, Republic of Korea) and Dr. Jack A. Roth (MD Anderson Cancer Center, Houston, TX, USA), respectively, and maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS). Cells were validated by analysis of their short tandem repeat (STR) profile. HCT116/R cells carrying acquired resistance to 5-FU were generated by continuous exposure to 5-FU at up to 128 μM for six months. Green fluorescence protein (GFP)-expressing cells were generated by stable transfection with pEGFP-N1 vector (Clontech Laboratories Inc., Mountain View, CA, USA). After selection with G418, a single-cell clone was obtained.

Implantation of cells into the renal capsule. All animal procedures were performed according to a protocol approved by the Seoul National University Institutional Animal Care and Use Committees (approval No. SNU-120830-3). Mice were kept in a specific pathogen-free (SPF) environment in the animal facility at Seoul National University. A total of 1.5×10^6 HCT116 and H226Br cells were implanted under the capsule of the left kidney in 3-15 BALB/c nude mice (4 to 6 weeks old; Japan SLC, Inc., Hamamatsu, Shizuoka, Japan) using a Hamilton syringe and an extension tube. The tumor volume was calculated by the following formula: (large diameter) × (small diameter) ×0.5. Fluorescence images were captured using an IVIS 2000 imaging system (Caliper Life Sciences, Hopkinton, MA, USA) at 1.5 months after implantation.

Evaluation of CTCs. Blood (0.7 ml per mouse) was collected from the orbital sinus of the mice. The retention of mononuclear cells and the lysis of red blood cells were performed using Histopaque 1083 accoring to the manufacturer's recommended procedure. Mononuclear cells were assembled onto a glass slide using a CytoSpin 4 Cytocentrifuge (Thermo Fisher Scientific, Waltham, MA, USA). Fluorescently-labeled cells in the blood were counted under a fluorescence microscope.

Reverse transcription-polymerase chain reaction (RT-PCR). RNA extraction and RT-PCR and real time-RT-PCR analyses were performed as described elsewhere (17, 18) using specific primers (Table I).

Immunofluorescence staining. Immunofluorescence staining was performed as described elsewhere (17) using a phycoerythrin (PE)-conjugated epithelial cell adhesion molecule (EpCAM) antibody (Cell Signaling Technology, Danvers, MA, USA).

Results and Discussion

We attempted to develop an *in vivo* murine model for studying human CTCs by taking advantage of previously described rodent models, including the orthotopic injection of highly metastatic human cancer cells into immunodeficient mice and the renal subcapsular injection of syngeneic cancer cells (12, 15). We first performed renal capsule implantation of GFP-labeled H226Br cells, a cell line that originated from a brain metastasis of primary tumor from which H226B cells were derived (19). At 1.5 months after cell implantation, H226Br cells exhibited very aggressive growth in the renal subcapsule of nude mice (Figure 1A). All primary tumors disfigured the abdomen, obliterating most of the renal parenchyma, and a large

Table II. *The incidence of primary tumor formation and metastasis after implantation of HCT116/R cells under the renal capsule.*

Number of mice	Number of mice		
	Primary tumor	Colon metastasis	Lung metastasis
6	6	2	4

portion of the intestine adhered to the primary tumors. To confirm whether these tumors were indeed derived from the implanted human cells in the kidney, we analyzed the expression levels of human actin and GFP in the kidney tissues and tumors from the mice. The expression levels of human actin and GFP, indicators of the implanted human-derived cells, were remarkably higher in the cancer cell-injected kidney (Figure 1B) and tumor (Figure 1C) tissues than in normal kidney.

Close examination of blood samples revealed green fluorescence in the blood of tumor-bearing mice (Figure 1D, left). RT-PCR and real-time RT-PCR analyses of the cells collected from the blood further revealed the expression of GFP and human actin, confirming the presence of disseminated human CTCs in the blood of the tumor-bearing mice (Figure 1D, middle and right). Additionally, the expression of GFP and an epithelial cell surface marker, EpCAM (20), in the lungs of tumor-bearing mice further revealed lung metastases of the kidney-implanted cells (Figure 1E). These results suggest that NSCLC cells implanted under the renal capsule can appear as CTCs in the blood and induce metastatic tumor development in mice.

We next determined whether this animal model can be applied to the evaluation of the metastatic potential of cancer cells with anticancer drug resistance. To this end, we generated a subline of HCT116 cells with acquired resistance to 5-FU (HCT116/R) by the continuous exposure to increasing concentrations of 5-FU for more than six months. We confirmed that HCT116/R cells were significantly less sensitive to 5-FU treatment compared with their parental line (IC_{50} of 5-FU: HCT116=7 μM; HCT116/R=50 μM). After labeling with GFP, we implanted these cells under the renal capsule of nude mice. We monitored the establishment of primary tumors in the mice 1.5 months after implantation using an IVIS system. As shown by image analysis of two representative animals from each group, we detected fluorescence only in the mice bearing HCT116/R tumors (Figure 2A), suggesting the HCT116/R subline grew more rapidly than the parental cells *in vivo* in the renal capsule. Similarly, the primary renal tumors established by the H226Br cells, most of the renal parenchyma was demolished by the primary tumors, which exhibited aggressive local invasion (Figure 2B, left). We also observed polygonal, odd nuclei in the HCT116/R cells and extensive blood vessels in the primary tumors (Figure 2B, right).

We next monitored the presence of CTCs by conducting a time-course study. Among five mice bearing HCT116/R cells, only one mouse had CTCs at three weeks after implantation, but the number of mice possessing CTCs gradually increased at four weeks (2 mice/total 5 mice) and five weeks (5 mice/total 5 mice) (Figure 2C). The increases in the CTCs were well-correlated with growth of primary tumors in the renal capsule (Figure 2D). We then assessed metastatic tumor formation in these mice using an IVIS system. At 1.5 months after implantation, metastatic tumor formation in the distal small intestine was observed only in mice with HCT116/R implantation (Figure 2E). At two months after implantation, metastatic tumor development in the liver, right kidney, intestine, and colon were observed (Figure 2F, top). Gross evaluation of the mice revealed obvious tumor nodules in the intestine and colon (Figure 2F, bottom). These findings suggested a positive correlation between the presence of CTCs and the appearance of metastatic tumor. We were not able to detect fluorescent signals in the lung. However, EpCAM-positive cell masses were found in the lung sections obtained from mice with HCT116/R cell implantation (Figure 2G, white arrow), implying the formation of lung metastases in HCT116/R-implanted mice. This might be due to a relatively weak signal in the lung caused by a small proportion of metastatic tumors. We observed that two over six and four over six of mice had tumor nodules in the colon and lung, respectively (Table II). Taken together, these results suggest that an increase in the number of CTCs is an indicative biomarker for metastatic tumors derived from colon cancer cells with acquired resistance to 5-FU.

In summary, we have successfully demonstrated that i) the renal capsule model is applicable to detecting CTCs and primary and metastatic tumor formation derived from different human cancer cell types and that ii) resistance to anticancer drugs may result in increased metastatic potential in cancer cells, contributing to an increase in CTCs and metastatic tumor formation. Although this model requires for the use of immunodeficient mice and has thus limited relevance to host-tumor interaction during metastasis, this model might be useful for various preclinical studies, including detection and capture of CTCs and their genetic, epigenetic, and biochemical characterization. The expression profiling of CTCs would further provide with useful information about biomarkers involved in the highly selective metastatic process and mechanisms of anticancer drug resistance. Knowledge of the distinct pattern of metastatic tumor formation, depending on the implanted cells, may also aid different aspects of experimental research, such as the investigation of the target organs of specific cancer types in metastatic disease.

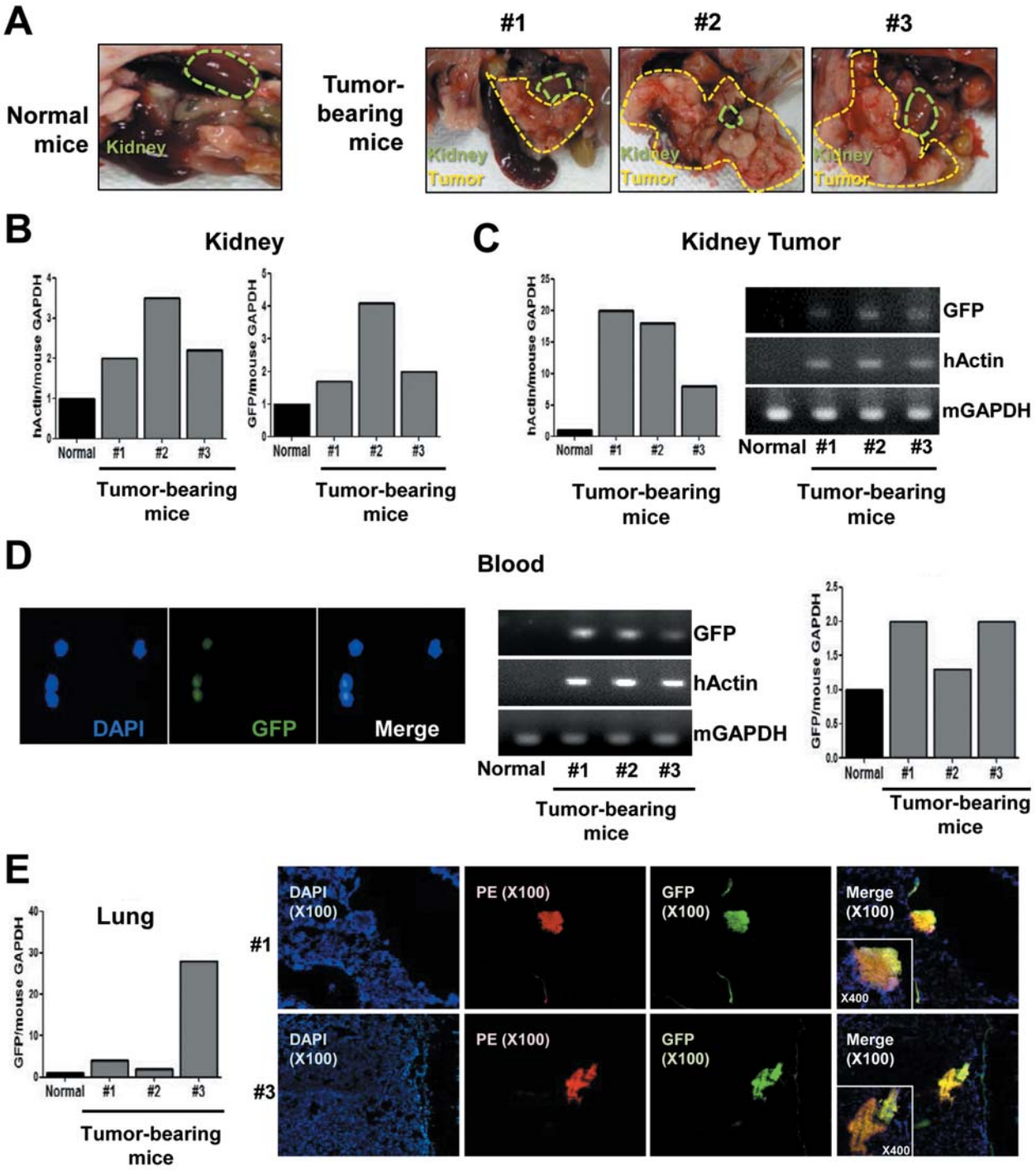

Figure 1. *Establishment of a murine model using H226Br cells. Green fluorescence protein (GFP)-labeled H226Br cells were implanted under the capsule of the left kidney in BALB/c nude mice. At 1.5 months after inoculation, the formation of primary tumors in the kidney was determined (A). The expression levels of human actin (hActin) and GFP in the kidney tissues that had been injected with cancer cells (B) and tumors surgically removed from the kidney tissues (C) of H226Br-implanted nude mice. The mRNA expression levels of these genes were evaluated by real-time polymerase chain reaction (PCR) (B, C) and reverse transcription-polymerase chain reaction (RT-PCR) (C). The presence of circulating tumor cells (CTCs) and metastatic tumor nodules in mice with H226Br cell implantation as determined by detection under a fluorescence microscope (D, left). RT-PCR (middle) and real-time PCR (right) analyses of the expression levels of GFP, hActin, and mouse glycerahdeyde 3-phosphate dehydrogenase (GAPDH in the blood of H226Br-implanted nude mice. Real-time PCR analysis of the expression levels of GFP and mGAPDH in the lung of H226Br-implanted nude mice (E, left). Secondary lung metastasis in H226Br-implanted mice (E, right). The expression of epithelial cell adhesion molecule (EpCAM) and GFP was observed under a fluorescence microscope after immunofluorescence staining.*

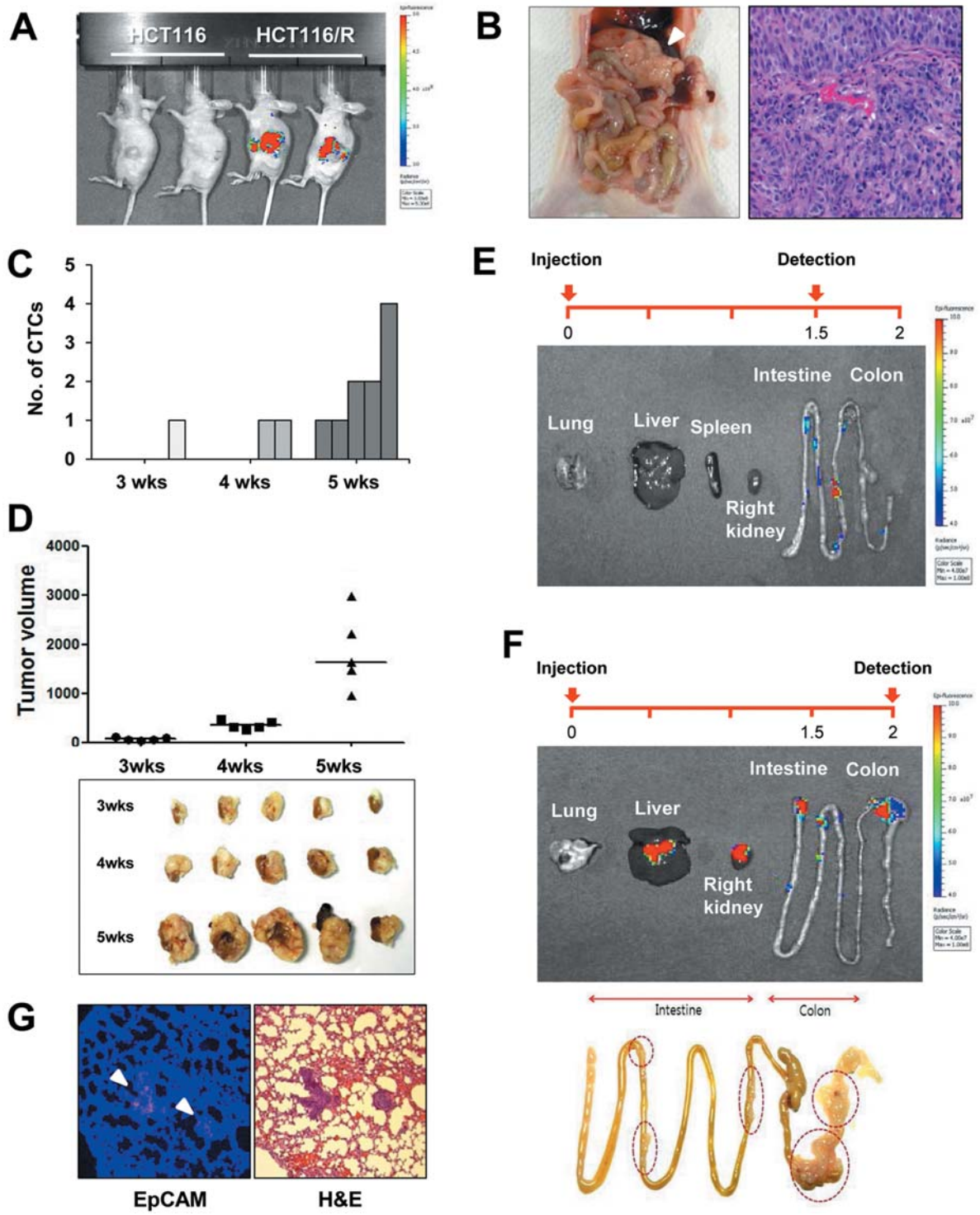

Figure 2. *Generation of primary tumors, secondary metastasis, and the presence of circulating tumor cells (CTCs) in mice with HCT116/R cells implanted in the renal subcapsule. Parental HCT116 and 5-fluorouracil (5-FU)-resistant HCT116/R cells were implanted under the capsule of the left kidney in BALB/c nude mice. A: After 1.5 months, the formation of primary tumors was evaluated using an IVIS system. B: Formation of primary tumors in the kidney and local invasion were photographed (left). Photomicrograph of the hematoxylin&eosin (H&E)-stained primary tumors (right). C: The time-dependent increase in the number of CTCs in the blood of mice implanted with HCT116/R cells. The GFP-positive cells in blood were counted. D: Primary tumor formation at each time point (3 to 5 weeks). At 1.5 (E) or 2 (F) months after inoculation, metastatic tumor formation was detected using an IVIS system. G: Metastatic tumor formation in the lung of HCT116/R-implanted nude mice. Epithelial cell adhesion molecule (EpCAM)-positive cell masses (indicated by a white arrow) were observed under a fluorescence microscope. H&E staining further confirmed secondary tumor formation in the lung.*

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References

1 Valastyan S and Weinberg RA: Tumor metastasis: molecular insights and evolving paradigms. Cell *147*: 275-292, 2011.
2 Parkinson DR, Dracopoli N, Petty BG, Compton C, Cristofanilli, Deisseroth MA, Hayes DF, Kapke G, Kumar P, Lee JSH, Liu MC, McCormack R, Mikulski S, Nagahara L, Pantel K, Pearson-White S, Punnoose EA, Roadcap LT, Schade AE, Scher HI, Sigman CC and Kelloff GJ: Considerations in the development of circulating tumor cell technology for clinical use. J Transl Med *10*: 138, 2012.
3 Mitchell MJ, Chen CS, Ponmudi V, Hughes AD and King MR: E-selectin liposomal and nanotube-targeted delivery of doxorubicin to circulating tumor cells. J Control Release *160*: 609-617, 2012.
4 Pierga JY, Hajage D, Bachelot T, Delaloge S, Brain E, Campone M, Diéras V, Rolland E, Mignot L, Mathiot C and Bidard FC: High independent prongnostic and predictive value of circulating tumor cells compared with serum tumor markers in a large prospective trial in first-line chemotherapy for metastatic breast cancer patients. Ann Oncol *23*: 618-624, 2012.
5 Naito T, Tanaka F, Ono A, Yoneda K, Takahashi T, Murakami H, Nakamura Y, Tsuya A, Kenmotsu H, Shukuya T, Kaira K, Koh Y, Endo M, Hasegawa S and Yamamoto N: Prognostic impact of circulating tumor cells in patients with small cell lung cancer. J Thorac Oncol *7*: 512-519, 2012.
6 Arya SK, Lim B and Rahman ABA: Enrichment, detection and clinical significance of circulating tumor cells. Lab Chip *13*: 1995-2027, 2013.
7 Zhou H, Xiao B, Zhou F, Deng H, Zhang X, Lou Y, Gong Z, Du C and Guo J: MiR-421 is a functional marker of circulating tumor cells in gastric cancer patients. Biomarkers *17*: 104-110, 2012.
8 Yu M, Ting DT, Stott SL, Wittner BS, Ozsolak F, Paul S, Cicilliano JC, Smas ME, Winokur D, Gilman AJ, Ulman MJ, Xega K, Contino G, Alagesan B, Brannigan BW, Milos PM, Ryan DP, Sequist LV, Bardeesy N, Ramaswamy S, Toner M, Maheswaran S and Haber DA: RNA sequencing of pancreatic circulating tumour cells implicates WNT signaling in metastasis. Nature *487*: 510-513, 2012.
9 Contractor K, Aboagye EO, Jacob J, Challapalli A, Coombes RC and Stebbing J: Monitoring early response to taxane therapy in advanced breast cancer with circulating tumor cells and ((18)F)3'-deoxy-3'-fluorothymidine PET: a pilot study. Biomark Med *6*: 231-233, 2012.
10 Nelson NJ: Circulating tumor cells: Will they be clinically useful? J Natl Cancer Inst *102*: 146-148, 2010.
11 Bonnomet A, Syne L, Brysse A, Feyereisen E, Thompson EW, Noël A, Foidart JM, Birembaut P, Polette M and Gilles C: A dynamic *in vivo* model of epithelial-to-mesenchymal transitions in circulating tumor cells and metastases of breast cancer. Oncogene *31*: 3741-3753, 2012.
12 Allan AL, Vantyghem SA, Tuck AB, Chambers AF, Chin-Yee IH and Keeney M: Detection and quantification of circulating tumor cells in mouse models of human breast cancer using immunomagnetic enrichment and multiparameter flow cytometry. Cytometry A *65A*: 4-14, 2005.
13 Helzer KT, Barnes HE, Day L, Harvey J, Billings PR and Forsyth A: Circulating tumor cells are transcriptionally similar to the primary tumor in a murine prostate model. Cancer Res *69*: 7860-7866, 2009.
14 Illert B, Otto C, Thiede A and Timmermann W: Detection of disseminated tumor cells in nude mice with gastric cancer. Clin Exp Metastasis *20*: 549-554, 2003.
15 Duyverman AMMJ, Kohno M, Roberge S, Fukumura D, Duda DG and Jain RK: An isolated tumor perfusion model in mice. Nat Protoc *7*: 749-755, 2012.
16 An Z, Jiang P, Wang X, Moossa AR and Hoffman RM: Development of a high metastatic orthotopic model of human renal cell carcinoma in nude mice: Benefits of fragment implantation compared to cell suspension injection. Clin Exp Metastasis *17*: 265-270, 1999.
17 Kim JH, Choi DS, Lee OH, Oh SH, Lippman SM and Lee HY: Antiangiogenic antitumor activities of IGFBP-3 are mediated by IGF-independent suppression of ERK1/2 activation and EGR-1-mediated transcriptional events. Blood *118*: 2622-2631, 2011.
18 Kim WY, Jin Q, Oh SH, Kim ES, Yang YJ, Lee DH, Feng L, Behrens C, Prudkin L, Miller YE, Lee JJ, Lippman SM, Hong WK, Wistuba II and Lee HY: Elevated epithelial insulin-like growth factor expression is a risk factor for lung cancer development. Cancer Res *69*: 7439-7448, 2009.
19 Mori J, Haisa M, Naomoto Y, Takaoka M, Kimura M, Yamatsuji T, Notohara K and Tanaka N: Suppression of tumor growth and down-regulation of platelet-derived endothelial cell growth factor/thymidine phosphorylase in tumor cells by angiogenesis inhibitor TNP-470. Jpn J Cancer Res *91*: 643-650, 2000.
20 Trzpis M, McLaughlin PMJ, de Leij LMFH and Harmsen MC: Epithelial cell adhesion molecule: more than a carcinoma marker and adhesion molecule. Am J Pathol *171*: 386-395, 2007.

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