Supplementary Materials and Methods

Human tissue specimens and patient information. For tissue microarray (TMA) construction and immunohistochemical analysis, we used human colon tumor specimens obtained from 203 patients with colon cancer that were preserved in the Colon Cancer Tissue Bank at Shanghai Jiaotong University Affiliated First People's Hospital (Shanghai, People's Republic of China). The primary colon cancer in these patients was diagnosed (and later confirmed by at least two pathologists) and the patients were accepted for colectomy at Affiliated First People's Hospital from 2001 to 2003. The 203 formalin-fixed, paraffin-embedded specimens were selected to represent all of the stages and histological types of colon cancer. Tumor staging for the specimens was carried out according to the American Joint Committee on Cancer staging criteria (40). The patients' disease-free survival (DFS) and overall survival (OS) durations were defined as the interval from initial surgery to clinically or radiologically proven recurrence or metastasis and from initial surgery to death, respectively. The follow-up period for this analysis concluded on June 29, 2008. The use of human specimens was approved by proper institutional review boards.

TMA construction and immunohistochemistry. After screening hematoxylin- and eosin-stained slides for optimal tumor tissue and tumor-adjacent tissue up to 2 cm from the tumor, TMA slides were constructed (in collaboration with Shanghai Biochip). Two cores were taken from each formalin-fixed, paraffin-embedded tumor and matched normal colon tissue specimen as well as at least one lymph node metastasis core using punch cores that measured 2 mm in greatest dimension from the nonnecrotic areas of tumor, lymph node metastasis, and matched normal colon tissue specimens. Among the 203 study patients were 66 from whom primary colon tumor and matched lymph node metastasis specimens were obtained. These specimens were included in the TMA. Sections (4 μm thick) of formalin-fixed, paraffin-embedded colon tumor specimens were prepared and processed for immunostaining using a

rabbit polyclonal antibody against human uPAR (American Diagnostic Inc) and a monoclonal antibody against FoxM1 (Santa Cruz Biotechnology).

Cell lines and culture conditions. The human colon cancer cell lines SW620 and SW480 were purchased from the American Type Culture Collection. All cells were maintained at 37°C in 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS).

Western blot analysis. Whole-cell lysates were prepared from the colon cells as described previously (39). Standard Western blot analysis of the lysates was performed with anrabbit polyclonal antibody against human uPAR (American Diagnostic Inc) and a monoclonal antibody against FoxM1 (Santa Cruz Biotechnology). The membranes were then stripped and blotted with an anti-β-actin antibody (Sigma Chemical Co.) and used as loading controls. The probe proteins were detected using an enhanced chemiluminescence system (Amersham Life Sciences) according to the manufacturer's instructions.

Transient transfection of colon cancer cells. To induce overexpression of FoxM1 in SW620 and SW480 cells, the cells were transfected with pcDNA3.1-FoxM1 or pcDNA3.1- as described previously (21). To inhibit FoxM1 expression in these cells, they were transfected with a pool of FoxM1 small interfering RNA (siRNA) oligonucleotides (Santa Cruz Biotechnology; 50 nmol/L) or control siRNA oligonucleotides (Santa Cruz Biotechnology; 50 nmol/L). Also, the cells treated with Oligofectamine reagent alone were included as mock controls.

Cell migration and invasion assay. Cell migration and invasion assays were conducted using a modified 24-well Boyden chamber with a membrane that was uncoated or coated with Matrigel (BD Biosciences), respectively. Briefly, 24 h after transfection of both SW620 and SW480 cells either with a control (mock or control siRNA-treated) or FoxM1 siRNA, or pcDNA3.1-FoxM1 or pcDNA3.1, the cells were harvested and suspended in DMEM at a concentration of 8×10^4/mL. Cells prepared in 500 μL of DMEM were loaded in the upper wells,

and a medium containing 20% FBS was placed in the lower wells as a chemoattractant stimulus. Migrated cells on the bottom surface of the filter were fixed, stained with H&E, and counted under a microscope in three randomly selected fields at a magnification of 200×.

Animals. Female athymic nude mice were purchased from The Jackson Laboratory. The mice were housed in laminar flow cabinets under specific pathogen-free conditions and used when they were 8 weeks old. The animals were maintained in facilities approved by the Association for Assessment and Accreditation of Laboratory Animal Care International in accordance with the current regulations and standards of the U.S. Department of Agriculture, Department of Health and Human Services, and National Institutes of Health.

Tumor growth and metastasis. To prepare tumor cells for inoculation into the mice, cells in the exponential growth phase were harvested by brief exposure to a 0.25% trypsin/0.02% ethylenediaminetetraacetic acid solution (w/v). Cell viability was determined using trypan blue exclusion, and only single-cell suspensions that were more than 95% viable were used. Tumor cells (1×10^6 per mouse) were then injected into the subcutis or cecum wall of nude mice in groups of five. The animals were killed 60 days after the tumor-cell injection or when they had become moribund. Next, the resulting primary CRC tumors were weighed. Also, each mouse's liver was removed and fixed in Bouin solution for 24 h to differentiate neoplastic lesions from the organ parenchyma; liver metastases were determined (double-blinded) as described previously (16).

Stable and transient transfection of CRC cells. Full-length human FoxM1 was released by *Eco*RI and *Xba*I digestion of the cytomegalovirus human FoxM1 cDNA expression vector (17) and subcloned into pcDNA3.1 (Invitrogen) to generate the pcDNA3.1-FoxM1 plasmid expression vector. Also, four FoxM1-small interfering RNAs (siRNAs) were designed and synthesized by Qiagen to generate a FoxM1-siRNA expression vector for gene-knockdown studies. An siRNA with the sequence CUCUUCUCCCUCAGAUAUAdTdT was determined to be

the most effective siRNA in inhibiting FoxM1 expression. The FoxM1-siRNA was further incorporated into the pSilencer plasmid (Ambion). A pSilencer neo vector expressing a hairpin siRNA with limited homology with any known sequences in the human, mouse, and rat genomes (Ambion) was used as a negative control. SW620 and SW480 cells were transfected with the FoxM1-siRNA expression vector or the control vector with the use of Lipofectamine 2000 Transfection Reagent (Invitrogen) . The cells stably transfected were isolated using neomycin (G418) selection after the cells were transfected with pcDNA3.1-FoxM1 or control plasmids.

Analysis of uPAR promoter activity. The activity of full-length puPAR1103 and its deletion mutant puPAR627 promoter constructs was analyzed as described previously (17). In brief, plasmids containing firefly luciferase reporters were co-transfected into CRC cells in triplicate with an internal control pMiniTK-RL using the Lipofectamine method (Invitrogen). The pMiniTK-RL contained a full-length Renilla luciferase gene under the control of a minimal thymidine kinase promoter (636-757 bp from pTK-RL; Invitrogen). In some of the experiments, the reporters were co-transfected with pcDNA3.1-FoxM1 and pcDNA3.1. After co-transfection, the cells were washed three times with phosphate-buffered saline and refed fresh medium. The activity of both the firefly and Renilla luciferase reporters was determined 48 h later using the Dual Luciferase Assay kit (Promega). The specific uPAR promoter activity was calculated as described previously (39).

Statistical analysis. The two-tailed χ^2 test was used to determine the significance of the difference among the covariates. Survival durations were calculated using the Kaplan-Meier method. The log-rank test was used to compare the cumulative survival rates in the patient groups. A Cox proportional hazards model was used to calculate univariate and multivariate hazard ratios for the study variables. The FoxM1 expression level, patient age, disease stage (American Joint Committee on Cancer system), and tumor differentiation and distant metastasis

were included in the model. The significance of the *in vitro* data was determined using the Student *t*-test (two-tailed). In all of the tests, *P* values less than 0.05 were considered statistically significant. The SPSS software program (version 12.0; SPSS Inc.) was used for statistical analyses.