Sur8 mediates tumorigenesis and metastasis in colorectal cancer

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Sur8, a scaffold protein of the Ras pathway, interacts with Ras and Raf and modulates the Ras-extracellular signal-regulated kinase (ERK) pathway. Here we show that Sur8 is overexpressed in established human colorectal cancer (CRC) cell lines and CRC patient tissues. Moreover, Sur8 expression is increased during liver metastasis in CRC patients. Sur8 knockdown decreases ERK and Akt activities in CRC cell lines, regardless of their K-Ras, B-Raf or PI3K mutation status. Overexpression or knockdown of Sur8 increases or decreases, respectively, the proliferation or transformation of CRC cell lines. Sur8 knockdown increases or decreases, respectively, the proliferation or transformation of CRC cell lines. Sur8 knockdown attenuates the migration and invasion of HCT116 CRC cells. Subcutaneous or orthotopic injection of HCT116 cells harboring a doxycycline (Dox)-mediated Sur8 knockdown system in nude mice resulted in decreased tumorigenic potential and inhibited the liver metastatic potential of HCT116 cells. Taken together, our data support the role of Sur8 as a promoter of tumorigenesis and liver metastasis in CRC through its modulation of the Ras-ERK and PI3K-Akt signaling pathways.

INTRODUCTION
Colorectal cancer (CRC) is the third most common cancer worldwide and the second leading cause of cancer-related deaths in developed countries.1 Oncogenic activation of the epidermal growth factor receptor-Ras pathway has a major role in the progression and metastasis of CRC.2,3 The activating mutations that lock K-Ras into a GTP-bound active form occur at early and intermediate stages of human colorectal tumorigenesis at a frequency of 32–57%.4,5 In addition to K-Ras mutations, activating mutations in B-Raf and PI3K (the gene encoding the p110α subunit of phosphoinositide 3-kinase (PI3K)) are found in 10–15% and 10–20% of colorectal tumors, respectively.3,6

Approximately 40–50% of CRC patients develop liver metastases, and 80–90% of these metastases are unresectable, which contributes to a 5-year patient survival rate of less than 10%.7,8 Common sites of metastasis in patients with CRC include the liver, peritoneum and lungs. Thus, identification of new targets involved in colorectal tumorigenesis and metastasis is a critical step toward the development of novel therapeutics that can significantly increase the survival rate of CRC patients.

Oncogenic Ras is involved in tumorigenesis and metastasis via the activation of both the extracellular signal-regulated kinase (ERK) and PI3K signaling pathways.9–12 Regulation of the pathways downstream of Ras is coordinated by scaffold proteins such as KSR and Sur8 (also known as Shoc2).13 Sur8 was initially identified in a screening test for molecules that regulate Ras-mediated multivulva production in C. elegans as a Ras-binding protein with leucine rich repeats (LRR).14 The human homolog of Sur8 was subsequently characterized and implicated in fibroblast growth factor receptor signaling.15 Previous studies showed that Sur8 positively regulates Ras-ERK signaling either by forming a complex with Ras and Raf16 or by dephosphorylating the Ser-259 inhibitory site of Raf when Sur8 is in a complex with the catalytic subunit of protein phosphatase 1 (PP1c) and M-Ras.17,18 To date, investigations of Sur8 have mainly focused on the pathogenesis of Noonan-like syndrome,19–21 and its role in cancer is poorly understood despite its identification as a Ras-Raf scaffold protein. In a recent study, we found that Sur8 interacts with the p110α subunit of PI3K as well as with Ras and Raf and that it positively regulates not only the Ras-ERK signaling pathway but also the PI3K-Akt signaling pathway, suggesting a role for Sur8 in cell motility and tumor metastasis.22

In this study, we show, for the first time, that the Sur8 protein is significantly overexpressed in tissues obtained from
human CRC patients as well as in established CRC cell lines. We also investigated the involvement of Sur8 in colorectal tumorigenesis and metastasis. Using doxycycline (Dox)-mediated Sur8 knockdown and overexpression lentiviral systems, we found that Sur8 modulates both of the ERK and PI3K-Akt signaling pathways, promoting growth, transformation, migration, and invasion of CRC cells. Using Dox-mediated Sur8 knockdown, we further showed the involvement of Sur8 in colorectal tumorigenesis and metastasis in mouse models. Overall, our study has identified a novel role for the scaffold protein Sur8 in regulating colorectal tumor growth and liver metastasis and has identified Sur8 as a new therapeutic target in CRC treatment.

MATERIALS AND METHODS

Cell culture and transfection
Human embryonic kidney 293 T (HEK293T) cells, human normal colon cells (CCD18Co), and human CRC cells (Colo205, WiDr, HT29, RKO, Caco2, LoVo, SW480, DLD-1, HCT15 and HCT116) were obtained from the American Type Culture Collection (ATCC; Manassas, VA). DLD-1, Colo205, HT29, RKO, Caco2, HCT15 and SW480 cells were maintained in RPMI 1640 medium (Gibco BRL, Carlsbad, CA, USA). HEK293T, LoVo, and WiDr cells were maintained in Dulbecco’s modified Eagle’s medium (Gibco BRL, Carlsbad, CA, USA). HEK293T, LoVo, and WiDr cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 20% fetal bovine serum (Gibco BRL) and 1 x non-essential amino acids (Sigma-Aldrich, St Louis, MO, USA). All other media used were supplemented with 10% fetal bovine serum, 100 units per ml penicillin, and 100 µg ml\(^{-1}\) streptomycin (Gibco BRL). Cells were transfected using Lipofectamine transfection reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer’s instructions.

Patient tissues and tissue microarray array
Human normal and tumor colon samples used for immunoblotting were obtained from the National Cancer Hospital, Seoul, Korea. The use of human samples was approved by the Institutional Review Board of the National Cancer Hospital. Tissue microarray array (Catalog #BC05002) consisting of human normal colon and tumor samples was purchased from US Biomax. Paraffin-embedded CRC tissues from different tumor stages were obtained from the Severance Hospital, Yonsei University (Seoul, Korea) as previously described.12

Plasmids, lentivirus production and establishment of Sur8 knockdown or overexpression cell lines
The pGTS-PAK-CD (pGST-PAK-CRIB-Domain) plasmid was provided by Dr John G. Collard, The Netherlands Cancer Institute, The Netherlands.23 For the Dox-mediated knockdown of Sur8, the lentiviral shRNA construct is pLVX-Tight-Puro (Catalog #21915, Addgene, Cambridge, MA, USA) and was used, and the following plasmids with respective target sequences were constructed: InshCon (5’-CTCTAGGTGTTAAGTCCCGCCTGATGCGAGGGCCCACTGAACTCTTAG G-3’), InshSur8#1 (5’-GCACCGGCTGCGAGATGATTATTCTCGGA GAAATCAAGCATCAGGACCTTGAGTGTTTACCTCGAG TAAATAACGATCATCCGACCGCTTGTGTTAATTACTCGGAG TAAATTCCAGGATCCGCCAGGTCAGGTTTATGGTGCAGTGCAGG TATGCAAGGATCGATAGTTCGAGGATTGATTGTTT-3’) and InshSur8#2 (5’-CCG GAACCTGGATTTAGGGCACTGCAATCCTGCAATTGCAGTGCAGTGC AAGGTTGTCTCCGAG-3’). For the production of the Dox-mediated Con (InConOE) or Sur8 (InSur8OE)-overexpressing lentiviral plasmids, human Sur8 complementary DNA (cDNA) was amplified by reverse transcription (RT-PCR) using F-primer 5’-GCACCGGCTGCGAGATGATTATTCTCGGAG TAAATTCCAGGATCCGCCAGGTCAGGTTTATGGTGCAGTGCAGG TATGCAAGGATCGATAGTTCGAGGATTGATTGTTT-3’ and R-primer 5’-ACCACACTGCGGATCTCC ATGAGTGGTTAGGAAAAA-3’ and was introduced into the pLVX-Tight-Puro vector (Catalog #632162, Clontech, Palo Alto, CA, USA). For virus production, HEK293T cells were transfected with lentiviral DNA constructs combined with pMD2G and pAX2G at a ratio of 2:1:1, respectively. Immunoblotting analysis of HCT116 cells infected with either InshSur8 #1 or #2 lentivirus showed that both constructs effectively knocked down endogenous Sur8 (Supplementary Figure S1). InshSur8 #2 viral supernatant was used for Sur8 knockdown in all subsequent experiments. To construct the shRNA-resistant form of Sur8 (shR-Sur8), the following silent mutations shown in bold were introduced: AAGCTTGATCTCAGCAAACT.
Rac activation assay
Rac activity assays were performed as previously described by incubating the cell lysates with recombinant GST-PAK-CD fusion protein bound to glutathione-coupled agarose beads. Protein complexes were analyzed by immunoblotting with a Rac1 antibody.

Wound-healing assay
Cells were grown until they were confluent, and a scratch was introduced. After 36 h, cells were fixed with 4% paraformaldehyde and stained with crystal violet. The total distance travelled by the cells was quantified using NIS-Elements AR 3.1 software (Nikon).

Invasion assay
InshCon or InshSur8 HCT116 cells were treated with Dox for 72 h and seeded at a density of 5 × 10⁴ cells on Matrigel-coated chambers (BD Bioscience, Bedford, MA, USA). After 24 h, cells on the outer surface were fixed with 4% paraformaldehyde and stained with crystal violet.

Animals, xenografts and metastasis assays
All animal experiments were performed in accordance with the Korean Food and Drug Administration guidelines. Animal studies were approved by the Institutional Review Board of Yonsei University. Approximately 4- to 6-week-old male athymic Crl: Foxn1nu nude mice were purchased from Orient Biotech (Seoul, Korea). Stable InshCon or InshSur8 HCT116 cells (1 × 10⁶ cells per site) were mixed with 40% Matrigel and were subcutaneously injected into both flanks of athymic nude mice for the xenograft assay. For the metastasis assay, stable InshCon or InshSur8 HCT116 cells (5 × 10⁵ cells) were orthotopically implanted into the spleens of nude mice.

Immunohistochemistry
The sectioned tissues were autoclaved in 10 mM sodium citrate buffer (pH 6.0) for antigen retrieval. Sections were blocked in 5% bovine serum albumin and 1% goat serum in PBS and incubated with primary antibodies overnight at 4 °C. For diaminobenzidine (DAB) staining, immunohistochemistry (IHC) was performed with the UltraTek HRP kit (ScyTek Laboratories, Logan, UT, USA). For fluorescent IHC, the sections were incubated with the anti-rabbit Alexa Fluor 555 (Invitrogen) secondary antibody and counterstained.

Figure 1 The expression of Sur8 in established CRC cell lines and patients tissues. (a, b) WCLs of cell lines (a) and human CRC tissues (tumor and paired normal) (b) were analyzed by immunoblotting using anti-Sur8, α-tubulin or -ERK antibodies. (c) TMA containing normal colon (n = 5) and tumor tissues (n = 7) was subjected to IHC analysis using an anti-Sur8 antibody, and cell nuclei were visualized by counterstaining with DAPI. Scale bar = 250 μm. (d) The mean intensities of Sur8 expression in c were quantified using the NIS-Element AR image program. ***P < 0.0005. (e) Liver metastatic (n = 10) or non-metastatic (n = 39) tumor tissues of CRC were stained with DAB using an anti-Sur8 antibody. Scale bar = 250 μm. (f) The mean intensities of Sur8 expression in e were quantified using HistoQuest software. *P < 0.05. CRC, colorectal cancer; ERK, extracellular signal-regulated kinase; IHC, immunohistochemistry; TMA, tissue microarray array; WCLs, whole-cell lysates.
with 4′,6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich). For quantitative analysis, the intensity of staining was determined using NIS-Elements AR 3.1 software (Nikon) or HistoQuest software (TissueGnostics, Vienna, Austria).

### Statistical analysis

The data are presented as the mean ± s.d. Student’s t-test was used for statistical analysis using GraphPad Prism (La Jolla, CA, USA) and the results are indicated in the figures as follows: *P<0.05, **P<0.005 and ***P<0.0005. P-values <0.05 were considered statistically significant.

### RESULTS

**Sur8 is overexpressed in the established CRC cell lines and human CRC patient tissues**

To assess the involvement of Sur8 in human CRC, we analyzed Sur8 expression levels in 10 different human CRC cell lines and an immortalized normal colon cell line, CCD18Co. Compared to the levels in CCD18Co, Sur8 was highly overexpressed in 9 CRC cell lines, with the exception of SW480 cells (Figure 1a).

Sur8 was also highly increased in various human CRC tissues compared to the paired normal colon tissues (Figure 1b). Elevated Sur8 expression levels, compared with those in the normal colon tissues, were also detected in the colorectal tumor tissues as assessed by IHC using CRC tissue microarray array (Figures 1c and d). In human CRC tissues, Sur8 expression was significantly increased in liver metastases, compared with that in non-metastatic tumor tissues (Figures 1e and f).

**Sur8 has a role in the activation of ERK and Akt and in the proliferation of CRC cells**

When Sur8 was knocked down in various CRC cell lines, we observed that both ERK and Akt activities were decreased, irrespective of the mutational status of K-Ras, B-Raf or PI3K (Figure 2a). These decrements were rescued by expressing a shRNA-resistant form of Sur8 (Supplementary Figures S2a and S2b). As Sur8 was overexpressed not only in CRC cell lines but also in malignant CRC tissues, we examined the role of Sur8 in the proliferation of CRC cells. When Sur8 was knocked down in HCT116 and LoVo CRC cells, cell proliferation was reduced by 50% and 46%, respectively, after 5 days of cell culture (Figure 2b). Consistent with these results, immunoblotting analysis confirmed the reduction of proliferation-related markers such as proliferating cell nuclear antigen, c-myc, and cyclin D1 upon knockdown of Sur8 in HCT116 and LoVo CRC cells (Supplementary Figures S3a and S3b). In contrast, proliferation of the CCD18Co normal colon and SW480 CRC cells, which expressed low levels of endogenous Sur8 (Figure 1a), was increased by 54% and 50%, respectively, when Sur8 was overexpressed (Figure 2c and Supplementary...
Figure 3  Effect of Sur8 knockdown or overexpression on the transforming potential of CRC cells. (a, b) Stable HCT116 or LoVo cells infected with either InshCon or InshSur8 lentivirus were seeded in six-well plates and treated with fresh Dox every 24 h for 14 days to induce Sur8 knockdown. Foci were stained with crystal violet, and images were captured. (c, d) Stable CCD18Co and SW480 cells infected with either InConOE or InSur8OE lentivirus were seeded in 24-well plates for performing the anchorage-independent soft agar assay. Cells were treated with fresh Dox every 24 h to induce Sur8 overexpression, and images of the colonies were captured after 3 weeks. The data shown are the mean±s.d. of three independent experiments. ***P<0.0005. Dox, doxycycline.

Figure 4  Effect of Sur8 knockdown on the growth of xenograft tumors derived from HCT116 cells. (a–d) HCT116 cells (1×10^6) infected with either InshCon or InshSur8 lentivirus were subcutaneously injected into both flanks of nude mice (n=5), and the mice were fed with Dox-water (2 mg ml⁻¹ Dox) for 11 days after the tumor volume reached 200 mm³. (a) The gross images of isolated xenograft tumors. Scale bar=30 mm. (b) Quantitative analysis of tumor volumes on the indicated days. (c) The paraffin-embedded tumor tissues generated in the xenograft model (a) were subjected to H&E staining and DAB analyses using the indicated antibodies. Scale bar=25 μm. (d) Representative images of liver and peritoneal metastases observed in the nude mice injected with InshCon lentivirus-infected HCT116 cells. White arrows represent metastatic tumors. Two-sided Student’s t-test was used to determine the statistical significance. **P<0.005. Dox, doxycycline.
Figure S4a). The Dox-induced overexpression of Sur8 in SW480 cells activated both ERK and Akt, as revealed by the increase in phosphorylation of both proteins (Supplementary Figure S4b).

Sur8 plays a role in the transformation of CRC cells
When Sur8 was knocked down in HCT116 and LoVo CRC cells, their transforming abilities were significantly reduced (Figures 3a and b). However, when Sur8 was overexpressed in normal colon (CCD18Co) and SW480 CRC cells, which showed low levels of endogenous Sur8 (Figure 1a), anchorage-independent growth was increased in both cell lines (Figures 3c and d).

Sur8 knockdown suppresses the growth of xenograft tumors derived from HCT116 CRC cells
To explore the role of Sur8 in CRC tumorigenesis in vivo, we used the mouse xenograft model in which HCT116 cells harboring K-Ras and PI3K mutations were implanted into nude mice. We subcutaneously injected stable InshCon or InshSur8 HCT116 cells harboring the Dox-mediated Sur8 knockdown lentiviral system. When Dox was added to the feeding water to induce acute shutdown of endogenous Sur8 in the tumor cells implanted in vivo, the xenograft tumors from InshSur8-injected HCT116 cells were significantly decreased within 11 days of Dox treatment, compared to those from the InshCon-injected cells (Figures 4a and b). The anti-tumor effect of Sur8 knockdown on the xenograft tumors appeared to be caused by the inhibition of both the Ras-ERK and PI3K-Akt pathways, as revealed by the decrease in the positive signals of both p-ERK and p-Akt in the IHC analysis (Figure 4c). GTP-Rac was also found to be decreased on the xenograft tumors upon knockdown of Sur8. Both the staining intensity and the nuclear localization of the proliferation marker Ki67 were also decreased upon Sur8 knockdown. Interestingly, the liver and peritoneal metastases were observed only in the mice injected with InshCon HCT116 cells (Figure 4d), suggesting that Sur8 may have a role in driving liver/peritoneal metastases in CRC.

Sur8 knockdown reduces the migration and invasion potential of HCT116 CRC cells
Consistent with the changes in the xenograft tumor model in vivo, Rac activity was reduced by Sur8 knockdown in HCT116 cells in vitro (Figure 5a). In addition, expression of both MMP-2 and MMP-9 were decreased upon Sur8 knockdown (Figure 5b). As Sur8 knockdown decreased Rac and MMP activation, two key factors involved in Sur8-mediated cell migration and invasion, we next examined the involvement of Sur8 in the migration and invasion of HCT116 cells. We
found that the migration ability and invasive properties of HCT116 cells were decreased when Sur8 was knocked down (Figures 5c–f).

The liver metastatic potential of HCT116 CRC cells is reduced by Sur8 knockdown

To investigate the role of Sur8 in mediating CRC metastasis, stable InshCon or InshSur8 HCT116 cells harboring the Dox-mediated Sur8 knockdown system were orthotopically implanted into the spleens of nude mice. One day after the splenic injection of cells, mice were fed Dox-water for 3 weeks (Figure 6a). The acute shutdown of endogenous Sur8 significantly decreased the liver metastatic capacities of HCT116 cells from the spleen (Figures 6b and c). In the metastasized liver tumors, Sur8 expression was significantly decreased, concomitant with decreased ERK, Akt, and Rac activities, when Sur8 was knocked down (Figure 6d). These data support a role for Sur8 in driving liver metastasis in CRC.

DISCUSSION

In this study, we provide evidence for the involvement of Sur8, initially identified as a scaffold protein of the Ras-ERK pathway, in regulating tumorigenesis and liver metastasis of CRC. The role of Sur8 in colorectal tumorigenesis is indicated by overexpression of Sur8 in both established CRC cell lines and in CRC patient-derived tissues. The elevated expression of Sur8 in metastatic tumor tissues compared to that in non-metastatic tissues suggests that Sur8 may have a role in metastasis of CRC. ERK and Akt activities were attenuated upon Sur8 knockdown, indicating that Sur8 mediates tumorigenesis of CRC via activation of both the Ras-ERK and PI3K pathways. The inactivation of Akt and ERK by Sur8 knockdown correlates with our recent study showing that Sur8 interacts with PI3K and Raf and has a role in tumor malignancy via regulation of both the PI3K-Akt and Ras-Raf-ERK pathways.22

Our results from the Dox-mediated Sur8 knockdown/overexpression CRC cellular model also showed the involvement of Sur8 in regulating proliferation and transformation of CRC cells in vitro. Consistent with these observations, the tumor size was critically reduced when Sur8 was knocked down in the xenograft tumors of HCT116 cells in vivo. Sur8 knockdown decreased the activation of Rac and the expression of MMPs, thereby reducing the migration and invasion potential of HCT116 cells. In addition, shutdown of endogenous Sur8 in HCT116 cells injected into the spleens of nude mice decreased the liver metastatic abilities of these cells. These results indicate that Sur8 may have a critical role in colorectal tumorigenesis and metastasis, and especially in promoting liver metastases.

CRC is one of the most common cancer types harboring mutations in K-Ras, B-Raf or PI3K. Although chemotherapeutic agents have been used for the treatment of CRC, various adverse effects have been observed in CRC patients. Epidermal growth factor receptor-targeted monoclonal antibodies, which were considered the most promising new therapeutic option...
for CRC, are of limited utility, especially due to restricted responses in patients, especially in patients carrying K-Ras, B-Raf or PI3K mutations.\(^2,6,27,28\) As Sur8 modulates both the Ras-ERK and PI3K-Akt signaling pathways in CRC cells, despite the mutational status of K-Ras, B-Raf or PI3K, we suggest that the scaffold protein Sur8 may be a potential therapeutic target for colorectal tumorigenesis and metastasis.

**CONFLICT OF INTEREST**
The authors declare no conflict of interest.

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