Up-regulation of GPR48 Induced by Down-regulation of p27<sup>Kip1</sup> Enhances Carcinoma Cell Invasiveness and Metastasis

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Abstract

A reduced expression level of the cyclin-dependent kinase inhibitor p27<sup>Kip1</sup> is associated with increased tumor malignancy and poor prognosis in individuals with various types of cancer. To investigate the basis for this relation, we applied microarray analysis to screen for genes differentially expressed between p27<sup>−/−</sup> and parental (p27<sup>+/+</sup>) HCT116 human colon carcinoma cells. Expression of the gene for G protein–coupled receptor 48 (GPR48) was increased in the p27<sup>−/−</sup> cells. Forced expression of GPR48 increased both in vitro invasive activity and lung metastasis potency of HCT116 cells. In contrast, depletion of endogenous GPR48 by RNA interference reduced the invasive potential of HCT116 cells. Moreover, GPR48 expression was significantly associated with lymph node metastasis and inversely correlated with p27 expression in human colon carcinomas. GPR48 may thus play an important role in invasiveness and metastasis of carcinoma and might therefore represent a potential prognostic marker or therapeutic target. (Cancer Res 2006; 66(24): 11623-31)

Introduction

Metastasis is a major clinical determinant of the poor prognosis of various human cancers. There are numerous studies indicating biological markers associated with distant metastasis, including lymph node metastasis. The expression of a cyclin-dependent kinase (CDK) inhibitor p27<sup>Kip1</sup> (hereafter p27) is reduced in many types of human cancer, including breast (1, 2) and colorectal (3) carcinomas, and it is inversely related to tumor aggressiveness and directly associated with prognosis in individuals with such cancers (4). p27 functions as an important negative regulator of the cell cycle by inhibiting the activity of G1 cyclin-CDK complexes during G<sub>0</sub> and G<sub>1</sub> phases (5). Degradation of p27 results in the activation of G<sub>1</sub> cyclin-CDK complexes and consequent promotion of cell cycle progression from G<sub>1</sub> to S phase. Elimination of p27 is regulated by SCF<sup>Skp2</sup>, an SCF-type ubiquitin ligase that catalyzes the ubiquitylation of p27 and thereby targets it for degradation by the 26S proteasome (6). Kip1 ubiquitylation-promoting complex was also recently identified as a ubiquitin ligase for p27 that promotes G<sub>1</sub> progression (7). The reduced abundance of p27 in human tumors results from enhanced degradation of this protein by the ubiquitin-proteasome pathway (8, 9). Expression of Skp2 is increased in certain transformed cell lines and various types of human cancer (10). Such increased expression of Skp2 may enhance the degradation of p27 and thereby promote cell cycle progression. However, accelerated cell growth alone cannot fully explain the highly aggressive nature of, and poor prognosis associated with, tumors with a low level of p27. To identify other mechanisms by which down-regulation of p27 expression might promote malignancy, we established a human cell line with a reduced level of p27 expression through targeted disruption of the p27 gene in HCT116 colon carcinoma cells and then examined the effects of p27 deficiency in these cells on gene expression. We found that expression of the gene for G protein–coupled receptor 48 (GPR48) was increased in the p27<sup>−/−</sup> cells.

GPR48, also known as leucine-rich repeat (LRR)–containing G protein–coupled receptor 4 (LGR4), is a member of the G protein–coupled receptor (GPCR) family of proteins, but its biological function is unclear (11, 12). The GPCR family comprises proteins with seven transmembrane domains that function as the receptors for various classes of ligand, including peptide hormones and chemokines (13), and which are major targets for pharmaceutical development (14). Several GPCRs, including thyroid-stimulating hormone receptor and chemokine (C-X-C) receptor 4, have also been found to contribute to carcinogenesis (15). GPR48 is composed of a large NH<sub>2</sub>-terminal extracellular domain containing 18 LRRs, the seven membrane-spanning segments, and a COOH-terminal intracellular domain (11, 12). Although it is structurally similar to receptors for gonadotropins and thyroid-stimulating hormone, GPR48 is currently classified as an orphan receptor because its ligand has not been identified. However, GPR48 knockout mice were shown to manifest intrauterine growth retardation and renal development (16, 17).

We have now identified GPR48 as a gene whose expression is up-regulated in p27-deficient HCT116 cells, and forced expression of GPR48 was found to increase the invasive and metastasis potential of HCT116 cells. Furthermore, GPR48 expression was inversely correlated with the expression of p27 in human colon carcinomas and was significantly associated with lymph node metastasis of these tumors. Our results suggest that GPR48 contributes to tumor invasiveness and metastasis.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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Materials and Methods

Cell culture and establishment of stable transformants. Primary mouse embryonic fibroblasts (MEF) were obtained from embryos at 13.5 days after coitus by standard procedures (18–20). MEFs and all cell lines were cultured under a humidified atmosphere of 5% CO₂ at 37 °C in DMEM supplemented with 10% fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 μg/ml). GPR48 and Skp2 cDNAs were generated by reverse transcription-PCR (RT-PCR) from HeLa cell mRNA and subcloned into pcDNA4 (Invitrogen, San Diego, CA), and the resulting plasmids were introduced into HCT116 cells by transfection with the FuGene reagent (Roche, Indianapolis, IN). Stable transformants were selected by culture in the presence of zeocin (400 μg/ml).

Targeted disruption of the p27 gene in HCT116 cells. The targeting vector contained about 9 kb of p27 intron sequences and polyadenylate [poly(A)]−less G418-resistant gene substituted for p27 coding region. Translation of neo mRNA was thus dependent on integration of the vector upstream of the poly(A) sequence of the p27 gene in HCT116 cell line that has also been used previously to generate somatic knockout cells (21). HCT116 cells were transfected with the targeting vector with the use of the Lipofectamine reagent (Invitrogen), and stable transformants were selected with G418 (600 μg/ml). The resulting clones were screened for homologous recombinants by PCR analysis of genomic DNA. Only one clone among 464 G418-resistant colonies screened proved to be heterozygous for the p27 gene. This finding was confirmed by Southern blot analysis (data not shown).

Microarray analysis. Gene expression was compared between p27−/− and parental HCT116 cells by DNA microarray analysis with Motorola CodeLink Bioarrays (Kurabo, Osaka, Japan).

Quantitative RT-PCR analysis. Total RNA was isolated from frozen tissue specimens or cultured cells with the use of an Isogen kit (Wako, Osaka, Japan), and was subjected to reverse transcription with random hexanucleotide primers and SuperScript Reverse Transcriptase II (Invitrogen). The resulting cDNA was subjected to real-time PCR with the LightCycler System and a QuantiTect SYBR Green PCR kit (Qiagen, Chatsworth, CA). The primer sequences were TGGGAATTCCTTTCCGTGCTG and GAAACTCAGACAGATGATGCC for Skp2, CAGTACCCAGTGAAGCCATT and GTTGTGCATCCACCCAGCA for GPR48, AAGCTAGGACTCGAGTACAT and GCCGTGTCCTCAGAGTTAGCC for p57, and ACTGCTGCGGCCAAGACAGTATGCC for Skp2, CAGTACCCAGTGAAGCCATT and GCCGTGTCCTCAGAGTTAGCC for p57, and ACTGCTGCGGCCAAGACAGTATGCC for Skp2, CAGTACCCAGTGAAGCCATT and GCCGTGTCCTCAGAGTTAGCC for p57. The abundance of glyceraldehyde-3-phosphate dehydrogenase mRNA or 18S rRNA as an internal standard. At least three independent analyses were done for each sample and for each gene.

Generation of antibodies to GPR48. A peptide (CQEIKQMRVMLDL) based on the large NH₂-terminal ectodomain of human GPR48 was chemically synthesized, conjugated with keyhole limpet hemocyanin, and injected into rabbits. Specific antibodies to GPR48 were purified from the resulting antisera by affinity chromatography with the peptide antigen as described in Gao et al. (22).

Immunoblot analysis. Cell lysates were prepared with lysis buffer (23), fractionated by SDS-PAGE on an 8% gel, and subjected to immunoblot analysis with antibodies to p27 (Transduction Laboratories, Lexington, KY), to Skp2 (Zymed, South San Francisco, CA), to β-actin (Sigma, St. Louis, MO), to CDK4 (Transduction Laboratories), to tubulin (Sigma), or to X-press (Invitrogen). Immune complexes were detected with horseradish peroxidase–conjugated secondary antibodies (Promega, Madison, WI) and an enhanced chemiluminescence system (NEN, Boston, MA).

RNA interference. Knockdown vectors for GPR48 or p27 small interfering RNAs (siRNA) were constructed by introduction of the 345-CAGTACCCAGTGAAGCCATT-369 (NM_018490) and 217-GTACGAGTGGTGTTGTCATCCAGCCACAGA for GPR48, AACGTGCGAGTGTCTAACGGGAGACAGTATGCC for Skp2, CAGTACCCAGTGAAGCCATT and GCCGTGTCCTCAGAGTTAGCC for p57, and ACTGCTGCGGCCAAGACAGTATGCC for Skp2, CAGTACCCAGTGAAGCCATT and GCCGTGTCCTCAGAGTTAGCC for p57. The abundance of glyceraldehyde-3-phosphate dehydrogenase mRNA or 18S rRNA as an internal standard. At least three independent analyses were done for each sample and for each gene.

Luciferase reporter assay. Cells (200,000 per well) cultured in six-well plates were transfected with the use of the GT-1 reagent (Dojindo, Kumamoto, Japan) with 1 μg of a luciferase reporter plasmid based on PGV-B (Wako) and with 250 ng of the CMV-β-gal plasmid, with or without 40 ng (or the indicated amount) of an expression vector for human E2F1 (a gift from Dr. E. Hara, Tokushima University, Tokushima, Japan). Cells were lysed 48 hours after transfection and assayed for luciferase and β-galactosidase activities, with the former being normalized by the latter.

Chromatin immunoprecipitation assay. HEK293 cells grown overnight in 100-mm dishes to 60% to 70% confluence were transfected with 1 μg of the promoter construct using the FuGene reagent (Roche). The untransfected 293 cells were used as a control vector were introduced into HeLa or HCT116 cells by transfection with the FuGene reagent (Roche, Indianapolis, IN). Stable transformants were selected by culture in the presence of zeocin (400 μg/ml).

In vitro cell invasion assay. Cells (5 × 10⁵ per well) were placed in the upper chamber of a 24-well Transwell apparatus containing Matrigel membranes (BD Biosciences, San Jose, CA), and the lower chamber was filled with 750 μl DMEM supplemented with 0.1% bovine serum albumin and fibronectin (10 μg/ml, Roche) as a chemoattractant. After incubation for 36 hours at 37 °C, cells that had migrated to the lower surface of each membrane were stained with the use of a Diff-Quik kit (International Reagents, Kobe, Japan) and counted. Each experiment was done with triplicate wells and repeated three times.

In vivo tumor metastasis. To estimate the metastatic ability of GPR48, we used two lung colonization models. Briefly, HCT116 cells were prepared as single-cell suspensions in sterile PBS at a concentration of 2 × 10⁶ per ml, and a volume of 250 μl (5 × 10⁶ cells) was s.c. injected into 6-week-old male athymic nude mice (BALB/c nu/nu; purchased from Seiken Co. Ltd., Tokyo, Japan). HeLa cells were prepared as mentioned above at a concentration of 5 × 10⁵ per ml, and a volume of 200 μl (1 × 10⁶ cells) was injected via the tail vein (i.v.) into 6-week-old male athymic nude mice. Animals were sacrificed on a days between 35 and 42. The lungs were excised, fixed in formalin overnight, and sectioned discontinuously into six portions. The 5-μm sections were immunostained with mouse anti-human cytokeratin monoclonal antibody (Santa Cruz Biotechnology). Metastatic deposits <0.2 mm were considered micrometastases.

Patient characteristics and tissue specimens. Specimens of colon carcinoma and paired normal mucosal tissue were obtained from 29 Japanese patients (19 men and 10 women) who received no therapy before surgery. The histopathologic grade of tumor differentiation was defined as described (24). The median age of the patients was 64.9 years (range, 42–88 years).

Immunostaining. For immunohistochemical analysis, formalin-fixed, paraffin-embedded sections (thickness = 5 μm) were deparaffinized, rehydrated, and irradiated in a microwave oven for 40 minutes in 10 mmol/L sodium citrate buffer (pH 6). The sections were then incubated with 0.3% H₂O₂ in absolute methanol for 30 minutes to quench endogenous peroxidase activity before exposure to antibodies to p27 (Transduction Laboratories) or to GPR48 (Hokkaido System Science generated in the present study). Immune complexes were detected with biotin-conjugated secondary antibodies, streptavidin-conjugated horseradish peroxidase, and diaminobenzidine with the use of a kit (Histofine SAB kit, Nichirei, Tokyo, Japan). The sections were lightly counterstained with hematoxylin and mounted with a permanent mounting medium. For the evaluation of p27 expression, the number of stained nuclei was counted (25); at least 10 high-power fields were chosen randomly for scoring of the percentage of cells positive for p27 staining among 1,000 cells examined per section.

Statistical analysis. Data are presented as means ± SE, and the statistical significance of differences was assessed with Fisher’s exact test. P < 0.05 was considered statistically significant.

Results

GPR48 expression is inversely related to p27 expression in HCT116 and MEF cells. To examine the mechanism by which p27 down-regulation increases tumor malignancy, we studied the human colon carcinoma cell line HCT116, which possesses wild-type p53
and retinoblastoma protein (pRB) genes and in which p27 degradation seems to occur normally during the cell cycle. This cell line has also been used previously to generate somatic knockout cells (21). We subjected the p27 gene in HCT116 cells to targeted disruption (Fig. IA), resulting in the isolation of one heterozygous recombinant clone (p27+/−) as revealed by PCR analysis (Fig. 1B, lane 1). Immunoblot analysis showed that the abundance of p27 was markedly reduced in the p27+/− cell clone compared with that in the parental HCT116 cells (wild type), and in Skp2-15 cell clone compared with that of the clone transfected with the corresponding empty vector (Fig. 1C, Vec.). The stable transformants and heterozygous recombinant clone above were subjected to an in vitro assay of cell invasion. The invasive activity of p27+/− was increased compared with parental cells (Fig. 1D, left). Therefore, down-regulation of p27 enhanced carcinoma cell invasiveness. With the use of DNA microarray analysis, we then screened for genes that are expressed differentially in the p27+/− and parental HCT116 cells. We found 26 differentially expressed genes between p27+/− and their parental cells by the microarray analysis (Fig. 1D, right).

We focused on GPR48. A, mRNA levels of both p27 and GPR48 in HCT116 clones stably transfected with a vector for p27 shRNA (p27-KD25 and p27-KD45) or with control siRNA plasmid vector (Cont-13 and Cont-16) were analyzed by quantitative RT-PCR. *P < 0.05. B, up-regulation of GPR48 expression in p27−/− MEFs and its down-regulation in Skp2−/− MEFs. RT-PCR analysis of p57Kip2 (female) and Skp2 mRNA and immunoblot analysis of p27 in MEFs of the indicated genotypes. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA and β-actin were analyzed as respective controls. Levels of GPR48 mRNA were measured by quantitative RT-PCR analysis.

Inverse correlation of expression levels between GPR48 and p27. A, mRNA levels of both p27 and GPR48 in HCT116 clones stably transfected with a vector for p27 shRNA (p27-KD25 and p27-KD45) or with control siRNA plasmid vector (Cont-13 and Cont-16) were analyzed by quantitative RT-PCR. *P < 0.05. B, up-regulation of GPR48 expression in p27−/− MEFs and its down-regulation in Skp2−/− MEFs. RT-PCR analysis of p57Kip2 (female) and Skp2 mRNA and immunoblot analysis of p27 in MEFs of the indicated genotypes. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA and β-actin were analyzed as respective controls. Levels of GPR48 mRNA were measured by quantitative RT-PCR analysis.

Analysis of these genes, 22 were down-regulated, and 4 were up-regulated in p27+/− cells compared with the parental ones. We selected several genes that could have potential to modulate signal transduction or cell motility or adhesion. We then did quantitative RT-PCR analyses of these genes to confirm the results of microarray analysis. Thus, we found that expression of the gene for GPR48 was reproducibly increased in the p27+/− HCT116 (Fig. 1D, right). We confirmed that the abundance of GPR48 mRNA in HCT116 cell lines stably expressing p27-shRNA was increased compared with that in control lines transfected with control short hairpin RNA (shRNA) plasmid vector (Fig. 2A). Moreover, Skp2-overexpressing cell lines with low p27 also showed an increased abundance of GPR48 mRNA (data not shown). The ligand and cellular functions of GPR48 are unknown. Because GPR48 knockout mice were shown to manifest intrauterine growth retardation and renal development (16, 17), we presumed that GPR48 might be involved in cell motility or differentiation as well as developmental events. Therefore, we focused on GPR48.

The inverse relation apparent between the expression of p27 and that of GPR48 in HCT116 cells was confirmed by experiments with MEF derived from p27 knockout (p27−/−) mice (18), heterozygous p57Kip2 knockout (p57+/−) mice (19), and Skp2 knockout (Skp2−/−) mice (ref. 20; Fig. 2B). The amount of GPR48 mRNA was increased in p27−/− MEFs compared with that in p27+/− MEFs, did not differ between p57+/− and p57+/− MEFs, and was reduced in Skp2−/−.
MEFs relative to that in Skp2+/+ MEFs. Together, these results suggested that p27 negatively affects expression of the GPR48 gene.

Activation of the GPR48 promoter by E2F1. To investigate the mechanism by which p27 deficiency results in increased GPR48 expression, we first examined the promoter of GPR48 for transcription factor binding sites with the use of the Tfsitescan database.7 We identified four putative E2F-binding sites (E2FA, E2FB, E2FC, and E2FD) in the GPR48 promoter (Fig. 3A, top). We speculated that the transactivation activity of E2F might be increased as a result of down-regulation of p27 expression, given that an increase in CDK activity has been shown to activate E2F in the pRB pathway (26). To determine whether E2F1 activates the GPR48 promoter, we did a luciferase reporter assay with the plasmid PGVB-GPR48-1671, which contains nucleotides -1671 to +444 (NM_018490; relative to the transcription start site) of human GPR48 and with various mutants thereof (Fig. 3A). The GPR48 promoter was activated by E2F1 in a concentration-dependent manner in transfected HCT116 cells (Supplementary Fig. S1). The luciferase activity of cells transfected with the plasmid PGVB-GPR48-239 in the absence or presence of the E2F1 plasmid was greatly reduced compared with the corresponding values obtained with PGVB-GPR48-1671 (Fig. 3A, left). Moreover, mutation of the E2FB site (mtE2FB) or of E2FB, E2FC, and E2FD sites (mtE2FBCD) resulted in a marked reduction in both basal and E2F1-dependent transcriptional activity of the GPR48 promoter (Fig. 3A, right). These results suggested that the E2FB site is indispensable for basal and E2F1-dependent activity of the GPR48 promoter. Next, we examined whether GPR48 promoter activity was enhanced in p27+/- cells using the reporter assay. The luciferase activity derived from PGVB-GPR48-1671 was substantially greater in p27+/- HCT116 cells than in the wild-type cells, whereas that derived from PGVB-GPR48-mtE2FB was low and similar in cells of both genotypes (Fig. 3B). Moreover, to address the mechanisms of activation of GPR48 promoter in p27+/- cells, E2F and RB protein were analyzed by Western blotting. We found that phosphorylation of RB protein was increased in p27+/- HCT116 cells (Fig. 3C). Moreover, we examined whether E2F1 protein actually bound to E2FB site using chromatin immunoprecipitation assay. As shown in Fig. 3D, immunoprecipitates with anti-E2F1 from cells with or without transfection of PGVB-GPR48-1671 reporter plasmid were contained with E2FB site on the GPR48 promoter. The result suggests that E2F1 actually bound to endogenous as well as exogenous the GPR48 promoter. These findings thus suggested that the up-regulation of GPR48 expression induced by deficiency of p27 is mediated by an increase in the transactivation activity of E2F1, which may be free from RB protein.

Figure 3. Activation of the GPR48 promoter by E2F1. A, schematic representation of the human GPR48 promoter–containing luciferase (Luc) reporter plasmids, showing the positions of putative E2F-binding sites. Arrow, transcription initiation site. The reporter plasmid pGVB-GPR48-1671 (WT), its deletion mutant plasmids (left), and its E2F site mutant plasmids (right) as indicated were cotransfected to HCT116 cells with (closed column) or without (open column) an expression vector for E2F1. Cells were subsequently lysed and assayed for luciferase activity. *, P < 0.01 versus values for pGVB-GPR48-1671 (WT). B, wild-type (WT) or p27+/- HCT116 cells were transfected with equimolar amounts of pGVB-GPR48-1671 or pGVB-GPR48-mtE2FB (right). E2F site–dependent activation of GPR48 promoter was observed in p27+/- HCT116 cells. *, P < 0.01 versus values for pGVB-GPR48-1671 (WT). C, phosphorylation of pRB in the wild-type (WT) and p27+/- HCT116 cells were examined for immunoblotting with anti–phospho-pRB antibody (phos-pRB) or indicated antibodies. D, binding of E2F1 to E2FB site was analyzed by chromatin immunoprecipitation assay using anti-E2F1 or a control IgG antibody in cells transfected without (left, for analysis of the endogenous GPR48 gene) or with PGVB-GPR48-1671 (right). PCR amplification products of immunoprecipitates using GPR48 promoter primers were analyzed by 5% acrylamide gel electrophoresis.

7 http://www.ifti.org/Tfsitescan/.
GPR48 increases the invasive activity of HCT116 cells. As described above, the invasive activity of p27<sup>+/−</sup> was increased compared with parental cells (Fig. 1D). Our findings suggested that GPR48 might be important in metastasis of human tumors. To determine whether GPR48 affects the invasive activity of HCT116 cells, we generated lines stably transfected with expression vectors for wild-type GPR48. There was no significant difference in growth rate between clones expressing wild-type GPR48 and control clones (see Supplementary Fig. S2). Comparing the structures of the constitutively active mutants of the luteinizing hormone and thyrotropin receptors with that of the related protein GPR48, we have successfully constructed GPR48-T755I, a constitutively active mutant of human GPR48 recently described above, the invasive activity of p27<sup>−/−</sup> was increased compared with parental HCT116 cells (Fig. 1A and D). Ectopic expression of GPR48 in HCT116 cells was suitable to evaluate the effects of GPR48 overexpression on invasiveness and metastasis. To confirm the relation between GPR48 expression and metastasis, we injected HCT116 clones into nude mice s.c. and compared the occurrence of pulmonary colonization in GPR48-overexpressing clones (GPR48-19 and GPR48-M6; see Fig. 4A) versus corresponding vector-transfected HCT116 clones (Vector2) after a period of 6 weeks. To confirm that these metastatic lesions had originated from injected cells, we check theses samples by immunostaining with human-specific cytokeratin (Fig. 4B). We defined that metastatic deposits <0.2 mm were considered micrometastases, and >0.2 mm were considered macrometastases. We used eight mice for each lung metastasis assay and did statistical analyses. Interestingly, overexpression of wild-type GPR48 significantly increased the number of macrometastasis and micrometastasis in lung compared with vector-transfected cells (Fig. 4B and C), whereas there was no significant difference in primary s.c. tumor weight between clones overexpressing wild-type or mutant GPR48 and control clones (Supplementary Fig. S4). Moreover, constitutive active mutant of GPR48 (GPR48-M6) was more effective on metastasis than wild-type GPR48 (GPR48-19). The results suggest that enhanced expression of GPR48 promotes not only invasiveness but also metastasis.

Depletion of endogenous GPR48 reduces the invasion potential of HeLa cells. Quantitative RT-PCR analysis showed that GPR48 expression was quite low in parental HCT116 cells, as shown in Supplementary Fig. S5. Because the invasive activity of HCT116 was also low (Fig. 1D and Fig. 4A), we thought that HCT116 was suitable to evaluate the effects of GPR48 expression on invasiveness and metastasis. To confirm the relation...
between GPR48 expression and cell invasion potential, we used RNAi to deplete endogenous GPR48 of HeLa and Lewis lung carcinoma (mouse LLC) cells (Fig. 5A). Expressions of GPR48 in HeLa and LLC cells were 17- and 3.7-fold, respectively, higher than that in HCT116 cells (Supplementary Fig. S5). Moreover, these two cells showed high invasive potential as shown in Fig. 5B and C, respectively. Therefore, we thought that HeLa and LLC cells were suitable to evaluate the effects of down-regulation of endogenous GPR48 on invasiveness and metastasis. We found that siRNA against GPR48 (GPR48-si450) remarkably inhibited invasion of HeLa cells. Moreover, we established HeLa cell clones stably expressing GPR48 shRNA (GPR48-KD1 and GPR48-KD4). The depletion of GPR48 was confirmed by immunofluorescence (Supplementary Fig. S6A) and quantitative RT-PCR (Fig. 5B, open column) analyses; the amount of GPR48 mRNA was reduced by >80% in these clones compared with that in control clones transfected with control shRNA plasmid vector (Cont-1 and Cont-3). The invasive activity of both GPR48-KD1 and GPR48-KD4 clones was significantly reduced compared with that of the control clones (Fig. 5B, closed column; Supplementary Fig. S6A). The depletion of endogenous GPR48 in LLC cell clones stably expressing GPR48 shRNA also diminished invasive activities of both GPR48-KD3 and GPR48-KD24 clones (Fig. 5C). These results thus confirmed that endogenous GPR48 is involved in the invasive potential of cancer cells.

Depletion of endogenous GPR48 suppresses lung metastasis. Next, we injected GPR48-depleted HeLa cells (GPR48-KD4; see Fig. 5B) or the control clone (Cont-1) via the tail vein of nude mice. After 5 weeks, we found that GPR48-depleted HeLa cells have significantly reduced pulmonary colonization compared with control cells (Supplementary Fig. S6B and C). Moreover, we s.c. injected GPR48-depleted LLC cells (GPR48-KD3 and GPR48-KD4; see Fig. 5C) or the control clone (Cont-5 and Cont-6) into mice. Lung metastasis potencies of LLC cells were significantly decreased by stably expressing GPR48 shRNA (Fig. 5D), whereas there was no significant difference in primary tumor weight between GPR48-depleted and control clones (data not shown). These data suggest that GPR48 contributes to metastasis of carcinoma in vivo as well as in vitro. Because metastasis is a multi-step process, we investigated whether GPR48 affected angiogenesis using immunohistochemical analyses with anti-CD34 antibodies. Although further experiments are required, there was no significant effect of GPR48 expression on angiogenesis in the primary tumors (data not shown). Moreover, we did terminal deoxynucleotidyl transferase–mediated nick-end labeling assay with the primary tumors and found no significant effect of GPR48 expression on apoptosis in the primary tumors (data not shown).

Clinical relevance of GPR48 expression in colon carcinoma. To evaluate clinical relevance between GPR48 expression and metastasis, we next measured the amount of GPR48 mRNA in...
surgical specimens of human colon carcinoma and paired adjacent normal mucosal tissue. The abundance of GPR48 mRNA in the colon carcinoma tissue was more than twice that in the corresponding normal tissue.

Relation among p27, Skp2, and GPR48 expression in colon carcinoma. We investigated the relation between the abundance of p27 and GPR48 in the 29 specimens of colon carcinoma. As shown in Fig. 6C, as a representative data using immunohistochemical analysis, the amounts of the two proteins seemed to be inversely related. Quantitation of the proportion of tumor cells expressing p27 in each specimen indeed revealed an inverse correlation with the abundance of GPR48 mRNA (Fig. 6D, top). In contrast, the amount of Skp2 mRNA was directly correlated with that of GPR48 mRNA in the tumor samples (Fig. 6D, bottom). These results indicated that GPR48 expression was significantly associated with lymph node metastasis and inversely correlated with p27 expression in human colon carcinomas.
Discussion

Given that p27 is an important negative regulator of the cell cycle, the major effect of a decrease in the expression of p27 has been thought to be an increase in the rate of cell growth. Pathologic studies have revealed that the abundance of p27 in tumors is inversely correlated with malignancy and is associated with clinical prognosis of human cancers (9). However, the biological mechanism underlying the increased malignancy of p27-deficient tumors has not been shown. The expression of Skp2, a component of an SCF-type ubiquitin ligase that targets p27 for degradation, is associated with poor prognosis in several human cancers (9, 10). Skp2 regulates the degradation not only of p27 but also of p130 (9), c-Myc (27), p57 (19), and Cdt1 (9). We therefore subjected the p27 gene of the HCT116 human colon carcinoma cell line to targeted disruption to provide a model for carcinoma cells with a low level of p27 expression. We found that expression of the GPR48 gene was increased not only in p27+/− HCT116 cells but also in HCT116 cells depleted of p27 by RNAi and in p27−/− MEFs, and that the expression of p27 and the abundance of GPR48 mRNA were inversely correlated in clinical specimens of colon carcinoma. Moreover, we showed that GPR48 increases the invasive or potential of cancer cells in vitro, promotes metastasis in vivo and GPR48 expression, was associated with lymphatic metastasis of colon carcinoma. There was no difference in in vitro cell growth (Supplementary Fig. S2; data not shown), in vivo primary tumor growth (Supplementary Fig. S4), and soft agar colony formation (data not shown) regardless of GPR48 expression. These data strongly suggest that GPR48 enhances carcinoma cell invasiveness and metastasis but not cell proliferation and cell transformation. GPR48 is an orphan receptor of which function and signal transduction have not been clear. Although further study is required, our study shows that up-regulation of GPR48 induced by p27-down-regulation promotes tumor cell invasion.

We identified four putative E2F-binding sites in the upstream regulatory region of GPR48 and showed that these sites mediate transcriptional induction of GPR48 in response to down-regulation of p27 expression. A decrease in the abundance of p27 is thought to result in activation of G1 cyclin-CDK complexes, phosphorylation and inactivation of pRB, and abrogation of inhibition of E2F by pRB (26). The resulting increase in E2F activity might thus underlie the transcriptional activation of GPR48 apparent in p27-deficient cells. Previous studies have suggested that p27 inhibits the motility of various cell types (29–31). Cytoplasmic p27 has also been shown to inhibit stathmin-mediated microtubule destabilization by binding to stathmin and thereby inhibits sarcoma cell migration (32). Skp2 also promotes the formation of filopodia and cell motility (33). On the other hand, p27 and other members of the Cip-Kip family have been shown to inhibit the Rho signaling pathway that underlies reorganization of the actin cytoskeleton and cell migration (34, 35). The increase in cancer cell invasiveness induced by degradation of p27 may thus be mediated by CDK-independent effects on microtubules and actin filaments as well as by CDK-dependent transcriptional activation of GPR48 via unknown mechanisms. It has been reported that some GPCR, such as endothelin receptor, linked Rho family via Go12 and RabGEF to promote tumor metastasis (36, 37). GPR48 is an orphan GPCR of which function has not been elucidated except for stimulation of CAMP production by its activation as recently reported by us (22). We will investigate whether GPR48 also links Rho family GTPase and regulates actin filament organization promoting cell motility.

Whereas expression of GPR48 in normal colon tissue was low, it was markedly up-regulated in colon carcinoma. Moreover, a high level of GPR48 expression was associated with lymph node metastasis and lymphatic involvement. GPR48 is thus a potential marker for prediction of metastasis or poor prognosis in colorectal cancer. Depletion of endogenous GPR48 by RNAi resulted in a marked decrease lung metastasis in mouse models as well as in the invasive activity of HeLa and LLC cells. A similar down-regulation of GPR48 expression induced by a specific siRNA or antisense oligonucleotides in vivo might thus prevent metastasis of colorectal carcinoma or other tumors. Human type neutralizing monoclonal antibody against GPR48 may be clinically applicable for prevention of metastasis. Identification of the native ligand for GPR48 should also facilitate the development of GPR48-based therapeutics.

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