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MCC-555-induced NAG-1 expression is mediated in part by KLF4

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Abstract

Peroxisome proliferator-activated receptor gamma (PPAR γ) plays a central role in cell differentiation, metabolism and tumorigenesis. We have investigated the therapeutic properties of 5-[[6-[(2-fluorophenyl)-methoxy]-2-naphthalenyl]-methyl]-2,4-thiazolidinedione (MCC-555) a PPAR γ agonist in human colorectal cancer cells. To elucidate the molecular mechanism(s), by which MCC-555 exerts its effects on the human colorectal cancer cells, we have analyzed the expression of two proapoptotic proteins, Krüppel-like factor 4 (KLF4) and nonsteroidal anti-inflammatory drug (NSAID)-activated gene-1 (NAG-1). MCC-555-induced expression of the transcription factor, KLF-4 was blocked by a PPAR γ specific antagonist GW9662 in PPAR γ -dependent manner in HCT-116 cells. We further identified a new KLF4 target gene NAG-1, which shows a pro-apoptotic activity. We confirmed that PPAR γ agonists-induced NAG-1 expression was abolished using KLF4 siRNA in HCT-116 cells. Subsequently, KLF4 expression enhances the NAG-1 promoter activity in HCT-116 cells, and functional KLF4 binding sites in the NAG-1 promoter were also identified. MCC-555, a PPAR γ agonist induced the expression of Klf4 mRNA and protein in murine intestinal tumors from MCC-555-treated mice, as assessed by RT-PCR and immunohistochemistry. This study shows that PPAR γ agonists up-regulate KLF4 expression in receptor-dependent manner, and KLF4 was identified as a novel transcription factor that controls NAG-1 promoter activity in human and mouse colorectal cancers.

Keywords

MCC-555; KLF4; NAG-1; thiazolidinediones

1. Introduction

The PPAR γ agonists affect cell proliferation, differentiation, and apoptosis in a PPAR γ -dependent and/or -independent manner, and thereby represent a potentially important family of therapeutic compounds for cancer treatment. Many studies describe the beneficial effects of the PPAR γ agonists for treatment of lung (Bren-Mattison et al., 2005), ovarian (Vignati et al., 2006), breast (Elstner et al., 1998) and colorectal (Baek et al., 2003; Sarraf et al., 1999) cancers *in vitro* and *in vivo*. Subsequent studies from several groups suggest that PPAR γ agonists control many genes that are involved in cellular and physiological pathways in a

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PPAR γ -dependent manner (Cekanova et al., 2008; Chintharlapalli et al., 2007a). A similar result suggests that both PPAR γ -dependent and -independent pathways are important for PPAR γ agonists' action (Lei et al., 2006). Thus, PPAR γ agonists mediate their anti-cancer activities through PPAR γ -dependent and -independent pathways, and further studies of the detailed molecular mechanisms related to the identification of a unique PPAR γ agonist target gene are required.

The Krüppel-like family of zinc-finger transcription factors (KLFs) regulates a range of biological functions in cells, such as differentiation, development, and cell growth. It has been observed that KLF4 is increased in human mammary tumors (Foster et al., 2000) and oral squamous-cell carcinomas (Foster et al., 1999), suggesting that overexpression of KLF4 may contribute to tumorigenesis. Conversely, it has been shown that KLF4 is down-regulated during tumorigenesis of the gastrointestinal epithelium (Choi et al., 2006; Zhao et al., 2004) and is frequently lost in other human cancer types (Katz et al., 2005; Wei et al., 2005). Consistent with a tumor suppressor function of KLF4, its overexpression reduces tumorigenesis in colonic cancer cells *in vivo* (Dang et al., 2003). These studies suggest that KLF4 negatively regulates cell proliferation; however, the mechanisms of how KLF4 affects tumorigenesis are not completely clear in human colorectal cancer. Recent studies show that some PPAR γ agonists regulate the *KLF4* mRNA expression, but exact mechanisms of their action are still unknown (Chen and Tseng, 2005; Chintharlapalli et al., 2007b). The nonsteroidal anti-inflammatory drug (NSAID)-activated gene-1 (NAG-1) has been shown to have anti-tumorigenic activities in colorectal cancer *in vitro* (Baek et al., 2001b; Li et al., 2000) and *in vivo* (Baek et al., 2006). Transgenic mice (NAG-1^{Tg+/BL6}) expressing human NAG-1 are resistant to chemically- and genetically-induced intestinal polyp formation by 50% and 40%, respectively (Baek et al., 2006). These studies suggest that NAG-1 is a potential tumor suppressor in colorectal cancers.

Since both KLF4 and NAG-1 are pro-apoptotic and tumor suppressor proteins induced by PPAR γ agonists in colorectal cancer (Baek et al., 2003; Chintharlapalli et al., 2007a; Rageul et al., 2009; Yamaguchi et al., 2006), we have focussed on whether KLF4 is involved in the regulation of NAG-1 expression. In the present study, we report that KLF4 as a PPAR γ agonist target gene mediated anti-tumorigenic activity via NAG-1 expression. We described a novel mechanism of the regulation of tumor suppressor gene NAG-1 in human colorectal cancer via PPAR γ agonists-induced KLF4 expression.

2. Materials and Methods

2.1 Reagents, Antibodies, and Cells

5-[[6-[(2-fluorophenyl)-methoxy]-2-naphthalenyl]methyl]-2,4-thiazolidinedione (MCC-555) was obtained as a gift from Mitsubishi Pharma Corporation (Tokyo, Japan); troglitazone (TGZ), rosiglitazone (RGZ), ciglitazone (CGZ), pioglitazone (PGZ), 15-deoxy- $\Delta^{12,14}$ -prostaglandin J₂ (PGJ₂), and GW9662 were purchased from Cayman Chemical Co. (Ann Arbor, MI). NAG-1 antibody recognizing the specific human NAG-1 protein was previously described (Baek et al., 2001b). Antibodies for KLF4, cyclin D1 and actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and antibody for V5 was obtained from Invitrogen (Invitrogen Corporation, Carlsbad, CA). KLF4 siRNA was purchased from Santa Cruz Biotechnology, and control siRNA was purchased from Ambion (Austin, TX). All other chemicals and reagents were purchased from Thermo Fisher Scientific (Pittsburgh, PA), unless otherwise specified. Human colorectal cancer cell lines HCT-116, LoVo, SW480, HT-29, and Caco-2 were obtained from American Type Culture Collection (ATCC, Manassas, VA). The cells were maintained in McCoy's 5A, Ham's F12K, RPMI-1640, and DMEM medium, respectively, supplemented with 10% fetal bovine serum, 100 I.U. penicillin, and 100 μ g/ml streptomycin and grown in an atmosphere of 5% CO₂ at 37°C. As a vehicle control for the

various PPAR γ agonist treatments *in vitro*, we have used DMSO (max 1/1,000 of the total volume of media) to avoid any non-specific effects.

2.2 RT-PCR

Total RNA was isolated from HCT-116 cells using an RNeasy mini kit (Qiagen), and then cDNA was synthesized from 1 μ g of total RNA using an iScript cDNA Synthesis kit (Bio-Rad, Hercules, CA) according to the manufacturer's protocol. One microliter of synthesized cDNA was then added to a 20 μ l PCR reaction mixture (GoTaq Green Master Mix PCR Reaction Mixture, Promega, Madison, WI) with each set of gene-specific primers. The primer sequences used in our study were *h-KLF4* (F: 5'-cccacacaggtgagaacct-3', R: 5'-ttctggcagtggtgggtcata-3'); *h-NAG-1* (F: 5'-acgctacgaggacgtgctaa-3', R: 5'-agattctgccagcagttggt-3') human glyceraldehyde-3-phosphate dehydrogenase (*h-GAPDH*, F: 5'-gaccacagtcctgcatcact-3', R: 5'-tccaccaccctgtgtgctgtag-3'); *m-Klf4* (F: 5'-ctgaacagcaggagctgt-3', R: 5'-gtgtgggtggctgtctttt-3'); and *m-Gapdh* (F: 5'-caggagcagaccccactaacat-3', R: 5'-gtcagatccacgacggacacatt-3'). The final PCR products were loaded onto 1% agarose gels and photographed under UV light. The experiments were repeated in three independent replicates. The signal contour length on images was measured using Scion Image software (Scion Corp., Frederick, MD).

2.3. Western blotting

Cells were lysed in ice-cold RIPA buffer (1XPBS, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) supplemented with protease and phosphatase inhibitors (1 μ g/ml aprotinin, 1 μ g/ml leupeptin, 1 mM PMSF, 0.1 mM Na₃VO₄, and 10 mM NaF) for 30 min. Equal amounts of proteins were subjected to SDS-PAGE and transferred onto a nitrocellulose membrane (Osmonics, Minnetonka MN). After blocking (5% non-fat milk in TBST for 1 h at room temperature), the membranes were incubated with primary antibody at 4°C overnight and then with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. Immunoreactive bands were visualized with an enhanced chemiluminescence system (Pierce Biotechnology, Rockford, IL) and detected on X-ray film.

2.4. Plasmid constructs

Full-length KLF4 cDNA was amplified using ReadyMix Tag polymerase (Sigma) with the following primers F: 5'-ctgggccccacattaatgaggcag-3' and R: 5'-aaaatgcctcttcatgtgtaaggcagg-3'. PCR was performed for 30 cycles at 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min. The amplified product was subcloned into pcDNA3.1/V5-His-TOPO (Invitrogen). The NAG-1 promoters pNAG-1-3500/+41, pNAG-1-1086/+41, pNAG-1-474/+41, and pNAG-1-133/+41 were described previously (Baek et al., 2001a). Internal deletion mutant clones for KLF4-binding sites of the NAG-1 promoter were generated from the pNAG-1-1086/+41 wild-type clone using the QuickChange II site-direct Mutagenesis kit (Stratagene) according to the manufacturer's protocol. The primers used in this assay for pNAG-1-1086/+41 Δ -807/-803 (Δ KLF4-A) were F: 5'-tcaacaatcacctcggcctccaaagtgc-3' and R: 5'-aggccgaggtgattgttgagcccaggagt-3'; primers for pNAG-1-1086/+41 Δ -678/-671 (Δ KLF4-B) were F: 5'-gggttgggagcttagactggaagataat-3' and R: 5'-cagtctaagctccaaaccccatccttttg-3'; primers for pNAG-1-1086/+41 Δ -558/-554 (Δ KLF4-C) were F: 5'-actgcaataaagtttatcaagtgcctccta-3' and R: 5'-ttgataaactttattgcagtactatcctc-3'.

2.5. Luciferase construct transfection and assay

Transient transfection experiments were performed using Lipofectamine reagent (Invitrogen) according to the manufacturer's instructions. Briefly, HCT-116 cells were seeded at 3×10^5 cells per well in 12-well tissue culture plates in complete media overnight. Then HCT-116 cells were transfected with reporter plasmid containing human NAG-1 promoters and co-transfected with pRL-null vector (0.05 μ g), and with the expression plasmid containing human KLF4,

using Lipofectamine reagent in serum free media for 5 h and then replaced with fresh complete media. After 24 h, the cells were harvested in 1X luciferase lysis buffer, and luciferase activity was determined and normalized to the pRL-null luciferase activity using a dual luciferase assay kit (Promega, Madison, WI) according to the manufacturer's protocol.

2.6. siRNA Transfection

For RNA interference of KLF4, HCT-116 cells were transfected with KLF4 (100 nM), or control siRNAs (100 nM), using TransIT-TKO transfection reagent (Mirus, Madison, WI), according to the manufacturer's instructions. After 24 h transfection, the cells were treated with vehicle (DMSO), MCC-555 (5 μ M) or troglitazone (5 μ M) in serum free media for an additional 24 h.

2.7. EMSA assay

Electrophoretic mobility shift assay was performed using a Gel Shift Assay System (Pierce, Rockford, IL) according to the manufacturer's protocol. Wild-type (WT) biotin-labeled and unlabeled oligonucleotide probes contained three copies of the binding motif of KLF4, 5'-ccaccaccaccaccaccacca-3' and the mutant unlabeled probe contained a substitution of 3-bp (underlined) 5'-ccaaaaacacaaaacacaaa-3'. Briefly, 1x gel shift binding buffer was incubated with nuclear extracts (NE, 5 μ g), which were prepared from the HCT-116 cells treated with vehicle (DMSO), MCC-555 (5 μ M) or troglitazone (5 μ M) for 24 h using the Nuclear Extract kit (Active Motif, Carlsbad, CA) according to the manufacturer's instructions. Biotin-labeled probe (5 ng) was added and reaction was incubated for 20 min at room temperature. For competition assay, nuclear extract from 5 μ M MCC-555-treated HCT-116 cells was preincubated with unlabeled WT or mutant probe with concentrations of 5 ng, 50 ng, and 500 ng, per reaction for 10 min at room temperature. To prove specificity of the binding site of the probe, we used an in vitro translated KLF4 protein (0.01, 0.1, 1 μ l of IVT KLF4). DNA-protein complexes were resolved by 5% nondenaturing polyacrylamide gel and transferred to a nylon membrane followed by the detection of the biotin-labeled probe by the Chemiluminescent Nucleic Acid Detection Module (Thermo Scientific) according to the manufacturer's instructions. The membranes were exposed to X-ray films to visualize the signal.

2.8. In vitro translation of KLF4 protein

In vitro translation of KLF4 protein was performed using a TNT Quick Coupled Transcription/Translation Systems kit (Promega, WI) according to the manufacturer's instructions.

2.9. Immunohistochemistry

Small intestinal tissues from Apc^{Min/+} mice treated with MCC-555 were formalin-fixed, embedded in paraffin, and sectioned at 5 μ m. These samples were obtained during the course of a previously study (Yamaguchi et al., 2008). After deparaffinization and hydration, the sections were heated in Tris-EDTA buffer (pH 8.0) for 20 min for antigen unmasking. Endogenous peroxidase activity was blocked by 3% hydrogen peroxide, and nonspecific binding of secondary antibody was eliminated by incubation with protein block buffer (Biogenex USA, San Ramon, CA) for 30 min at room temperature. Slides were then incubated with primary antibodies overnight at 4°C followed by incubation with biotinylated anti-rabbit IgG and streptavidin/biotin-horseradish peroxidase complex, which was visualized by DAB staining. Sections were lightly counterstained with Mayer's hematoxylin. The images were captured by an Olympus DP71 camera attached to an Olympus microscope.

2.10. Statistical analysis

Statistical analyses of obtained data were performed using the Student *t* test. Results were considered statistically significant at **P*<0.05, ***P*<0.01, and ****P*<0.001.

3. Results

3.1. MCC-555 a PPAR γ agonist induces simultaneously KLF4 and NAG-1 mRNA and protein expressions in colorectal cancer cells

Many studies have shown that PPAR γ agonists such as thiazolidinedione have anti-tumorigenic properties in colorectal cancer by increasing the expression of tumor suppressor genes (Baek et al., 2003; Chintharlapalli et al., 2007a; Yamaguchi et al., 2006). In this study, we have investigated the therapeutic properties of MCC-555 in human colorectal cancer cells. To elucidate the molecular mechanism(s) by which MCC-555 exerts its effects on the human colorectal cancer cells, we have analyzed the expression of two proapoptotic proteins, KLF4 and NAG-1. As shown in Fig. 1A, a PPAR γ agonist MCC-555 significantly increased the expression of both *KLF4* and *NAG-1* mRNA in human HCT-116 colorectal cancer cells after 24 h treatment. We subsequently examined the expression of KLF4 and NAG-1 protein levels by different PPAR γ agonist treatments (5 μ M of MCC-555, rosiglitazone, PGJ₂, ciglitazone, troglitazone, pioglitazone) in HCT-116 cells for 24h (Fig. 1B). We observed that all tested PPAR γ agonists, except PGJ₂, highly increased protein expression of KLF4, with the most significant increase by MCC-555, troglitazone, and ciglitazone. The KLF4 antibodies recognized three bands in Western blots, out of which the middle band (55 kDa) is specific for KLF4, and upper and lower bands are non-specific bands (labeled as n.s.). Interestingly, similar pattern of the NAG-1 protein expression was observed after different PPAR γ agonist treatments. The most significant inductions of NAG-1 were detected by MCC-555 and troglitazone treatments (Fig. 1B). The expression of cyclin D1 was also investigated and we found that PPAR γ agonists MCC-555 and troglitazone significantly down-regulated cyclin D1 expression, consistently with the previous reports (Qin et al., 2003). These data suggest that MCC-555 and troglitazone can affect similar signaling pathways to regulate KLF4, NAG-1, and cyclin D1 expression in HCT-116 cells. The expression of KLF4 was detectably increased by MCC-555 treatment (5 μ M) in early time points starting from 0.5 h and highly increased after 8 h treatment, and the expression of NAG-1 protein was up-regulated after 1 h by MCC-555 treatment (5 μ M) with gradual increasing up to 24 h in HCT-116 cells (Fig. 1C). Moreover, we have tested the ability of MCC-555 (5 μ M) to induce the expression of KLF4 and NAG-1 proteins in other human colorectal cancer cells. As shown in Fig. 1D, the expression of KLF4 was up-regulated in HCT-116, LoVo, and HT-29 cells, which were also simultaneously observed NAG-1 induction after treatment with MCC-555 (5 μ M) for 24h. Interestingly, neither KLF4 nor NAG-1 proteins were upregulated in SW480 and CaCo-2 cells by MCC-555 treatment (5 μ M).

3.2. PPAR γ agonists induce KLF4 expression in a PPAR γ -dependent pathway

Since PPAR γ agonists can alter gene expression in a PPAR γ -dependent and -independent manner, we have evaluated the role of PPAR γ in KLF4 and NAG-1 expressions in HCT-116 cells. As shown in Fig. 2A, the expression of KLF4 was up-regulated by MCC-555 (5 and 10 μ M), but pretreatment with GW9662, a specific PPAR γ antagonist, for 30 min abolished MCC-555-induced KLF4 expression. These findings suggest that KLF4 expression is regulated through, at least in part, a PPAR γ -dependent manner. Conversely, MCC-555-induced NAG-1 expression was not affected by GW9662, which was consistent with the previous report showing that NAG-1 expression is not affected by PPAR γ antagonists (Yamaguchi et al., 2006). To determine a direct link between KLF4 and NAG-1 expression, we performed experiments using KLF4 siRNA to study NAG-1 expression in HCT-116 cells. As shown in Fig. 2B, PPAR γ agonist-induced NAG-1 expression was down-regulated in the absence of KLF4, suggesting partial regulation of NAG-1 through the transcription factor KLF4.

3.3. NAG-1 expression is controlled by the KLF4 transcription factor

To determine whether KLF4 can regulate NAG-1 promoter activity, we performed co-transfection experiments using a series of NAG-1 promoter-reporter constructs and an expression plasmid containing the full length of KLF4 tagged with V5 (pcDNA3.1/KLF4/V5/His). After 24h transient transfection, KLF4 was highly expressed in HCT-116 cells as assessed by Western blot analysis (Fig. 3A, right panel) as well as immunofluorescence staining (data not shown). Our data revealed that KLF4 activated two promoters constructs containing -3500, and -1086 bp of the NAG-1 promoter (Fig. 3A, left panel). However, the KLF4 was not able to transactivate two other NAG-1 promoter constructs containing -474 and -133 bp, suggesting that the possible binding sites of KLF4 are in the region between -1086 and -474 bp of the NAG-1 promoter. The sequence between -1086 and -474 of the NAG-1 promoter was examined, and possible KLF4 binding sites containing the CACCC sequence were identified in the NAG-1 promoter. To prove the importance of each cis-acting element in KLF4-induced NAG-1 expression, we made the internal deletion constructs for each KLF4 binding site as shown in the scheme of Fig. 3B (Δ KLF4-A, Δ KLF4-B, and Δ KLF4-C). The Δ KLF4-A and Δ KLF4-C constructs showed an approximately 50% reduction of NAG-1 promoter activity compared to parent (WT) construct (pNAG1-1086/+41) (Fig. 3B). However, the deletion of the KLF4-B binding site did not significantly affect NAG-1 promoter activity. It is clear that KLF4-A and -C sites were *cis*-acting elements that bind KLF4 *trans*-acting elements.

3.4. KLF4 transcription factor binds to the NAG-1 promoter

To determine whether KLF4 binds to the CACCC sequence identified above from the NAG-1 promoter, we performed a mobility shift assay with a biotin-labeled oligonucleotide containing three copies of the CACCC sequence. Fig. 4A shows the results of the EMSA assay with nuclear extract prepared from HCT-116 cells treated by vehicle (DMSO), MCC-555 (5 and 10 μ M) and troglitazone (5 μ M) for 24 h. PPAR γ agonist-induced KLF4 expression resulted in an increase in formation of the DNA-protein complex, compared to nuclear extract prepared from vehicle-treated HCT-116 cells. The resulting DNA-protein complexes were competed out by an increased amount of unlabeled wild-type oligonucleotide (1 $\times$, 10 $\times$, 100 $\times$ fold), but not by mutated unlabeled oligonucleotide (Fig. 4B). To further analyze the specificity of the protein/DNA complex, we performed the EMSA assay using the *in vitro* translated (IVT) KLF4 protein (Fig. 4C). The formation of the DNA-protein complex was increased by increasing the amount of IVT KLF4 in a dose-dependent manner, supporting the specificity of KLF4 binding to the KLF4-binding site of the NAG-1 promoter (Fig. 4C).

3.5. MCC-555 induced KLF4 expression in Apc^{Min/+} mice

We have previously published that MCC-555 (30 mg/kg/day) given for 4 weeks reduces polyp formation in the small intestine in Apc^{Min/+} mice by 54.8% compared to control mice (Yamaguchi et al., 2008). Using the same tissue samples, the effects of MCC-555 on *Klf4* expression were examined *in vivo*. We performed RT-PCR and immunohistochemistry analysis of normal and neoplastic small intestinal tissue from vehicle and MCC-555-treated Apc^{Min/+} mice. Our RT-PCR data revealed that MCC-555 significantly induced the expression of *Klf4* in tumors of the small intestine compared to those in untreated mice (Fig. 5A lower panel, $*P<0.05$). The expression of *Klf4* in normal intestinal tissue, however, was not affected by the MCC-555 treatment (Fig. 5A upper panel). Immunohistochemical analysis revealed strong expression of Klf4 in the nuclei of normal small intestinal epithelium (N, left panels) on the upper regions of villi (Fig. 5B) and epithelium in upper regions of the colonic crypts (data not shown), but no detectable expression of Klf4 in nuclei of polyps (T, left panels) of vehicle treated Apc^{Min/+} mice. In addition, MCC-555 appeared to slightly increase staining for Klf4 in the neoplastic epithelium (T, Fig. 5B right panels) compared to the vehicle-treated controls (T, Fig. 5B left panels). These results support the RT-PCR data, shown in Fig. 5A.

4. Discussion

In the present study, we demonstrated for first time that two PPAR γ agonists MCC-555 and troglitazone significantly induced the expression of two tumor suppressor genes KLF4 and NAG-1 on mRNA and protein levels in human colorectal cancer cells. We also propose molecular mechanism by which KLF4 directly regulates NAG-1 expression, leading to activation of a tumor suppressor network in colorectal cancer.

MCC-555 is a relatively new member of the thiazolidinediones family and has potent anti-proliferative effects against prostate and colorectal cancers *in vitro* and *in vivo* (Yamaguchi et al., 2008; Yamaguchi et al., 2006); it also enhances the anti-tumor activity of As2O3 against myeloma cells (Kumagai et al., 2004). MCC-555 seems to possess a unique property compared to other PPAR γ agonists, in that MCC-555 binds to PPAR γ as well as PPAR α (Yamaguchi et al., 2006). Interestingly, dual-acting agonists for PPAR α and PPAR γ have been shown to have clear therapeutic advantages over selective PPAR agonists in animals as well as humans (Balakumar et al., 2007). However, the molecular mechanisms that mediate anti-tumorigenic effects of dual PPAR agonists in either a PPAR-dependent or independent manner are not yet clear. In addition, receptor-mediated transactivation is dependent on several factors including tissue and cell-specific expression of co-regulatory proteins (co-activators and co-repressors), ligands structures, or promoter accessibility of target genes. We previously reported several molecular target genes induced by MCC-555, which showed anti-tumorigenic activity. These include Muc2, NAG-1, and others (Cekanova et al., 2008; Yamaguchi et al., 2008; Yamaguchi et al., 2006). In this report, we found a novel MCC-555 target gene, a transcription factor KLF4. Unlike NAG-1, KLF4 induction by PPAR γ agonists mediates in a PPAR γ -dependent manner. As shown in Fig. 2, MCC-555-induced KLF4 expression was abolished in the presence of the GW9662, a PPAR γ -specific antagonist, suggesting the PPAR γ -dependent manner of MCC-555-induced KLF4 expression. Our data are in agreement with the previously published data where glycyrrhetic acid derivatives as PPAR γ agonist induced KLF4 expression in a receptor-dependent manner in HT-29 and SW480 colorectal cancer cells (Chintharlapalli et al., 2007a). In contrast, PGJ₂ upregulated KLF4 expression independently of PPAR γ through activation of the MAPK signaling pathway in HT-29 colon cancer cells (Chen and Tseng, 2005). The differences we've confirmed in ligand-receptor activity may provide an explanation for differences of action even in the same colorectal cancer cells. Our data clearly demonstrated that KLF4 is induced by MCC-555 and troglitazone, PPAR γ agonists, in a PPAR γ -dependent manner, but we were not able to definitively identify the presence of a functional PPAR γ binding site (PPREs) in the KLF4 promoter region of 10 kb by ChIP assay (data not shown). In our study, we identified KLF4 as a new target gene of MCC-555, although there is still need to identify the functional PPAR γ binding site (PPREs) in the KLF4 promoter as well as roles of different receptor co-activators and co-repressors.

KLF4 has an inhibitory effect on cell proliferation by blocking the transition from the G1 to the S phase of cell cycle, probably mediated by the increased expression of cyclin-dependent kinase inhibitor p21^{WAF1/CIP1} (Zhang et al., 2000). KLF4 not only activates p21^{WAF1/CIP1} through a specific, Sp1-like *cis*-element in the p21^{WAF1/CIP1} proximal promoter, but also mediates the activating effects of p53 in response to DNA damage on the same promoter. In addition, KLF4 has the ability to inhibit cyclin D1 expression and contribute to the inhibition of the cell cycle in colorectal cancer cells (Shie et al., 2000). However, biological function of KLF4 depends on the tissue type, and many studies have shown that the expression of KLF4 is associated with both inhibition and induction of cell proliferation, leading to tumor suppression or activation (Dang et al., 2003; Foster et al., 2000; Rowland et al., 2005). However, recent data suggest that depletion of KLF4 in a genetic animal model enhances colorectal tumorigenesis, indicating that KLF4 acts as a tumor suppressor in colorectal cancer (Ghaleb et al., 2007). These findings are in agreement with our data, showing that Klf4 mRNA

and protein levels were down-regulated in polyps compared to normal small intestinal tissue of *Apc^{Min/+}* mice, and the expressions of *Klf4* mRNA and protein were up-regulated in polyps of the MCC-555-treated mice (Fig. 5). Although KLF4 may play a pro-tumorigenic role in other cancer types (Foster et al., 2000), our data provide evidence that KLF4 has a role as a tumor suppressor in colorectal cancer.

In this report, we focussed on the study of identifying the potential binding site of KLF4 in the NAG-1 promoter. NAG-1 is regulated by different drugs and chemicals at the transcription level, documented to prevent tumor formation and development (Baek et al., 2002b). Nonsteroidal anti-inflammatory drugs activate ESE-1/EGR-1 transcription factor and lead to an increase in NAG-1 expression (Lee et al., 2008), and some phytochemicals induce NAG-1 expression in either a p53-dependent or -independent manner (Baek et al., 2002a; Lee et al., 2005). For the PPAR γ agonist, troglitazone increased NAG-1 expression via the ERK/EGR-1 pathway, whereas PGJ₂ increases NAG-1 expression through a PPAR γ -dependent manner (Baek et al., 2003). MCC-555 also induces NAG-1 expression at the post-transcriptional level (Yamaguchi et al., 2006). Therefore, different PPAR γ agonists affect NAG-1 expression through a different pathway. In this report, we demonstrated that MCC-555 affects NAG-1 expression via KLF4, which is likely regulated by the PPAR γ transcription factor in HCT-116 cells. The current study also provides evidence that MCC-555 affects anti-tumorigenesis in PPAR γ -dependent and -independent manners. PPAR γ agonists regulate the post-transcriptional level as MCC-555 affects the 3'UTR of the NAG-1 gene, inducing NAG-1 expression (Yamaguchi et al., 2006). Our data shows for the first time that MCC-555, a unique PPAR γ agonist, induces the expression of NAG-1 by a new mechanism through the activation of KLF4 in colorectal cancer cells. Increased KLF4 expression leads to the alteration of NAG-1 expression at the transcriptional level in HCT-116 cells. We have identified two specific KLF4 binding sites located between positions -1086 and -474 in the NAG-1 promoter region, which are essential for the transactivation of KLF4-induced NAG-1 expression. Furthermore, these two KLF4 binding sites may work equally to transactivate NAG-1 expression, because the mutation of each site showed similar reduction of KLF4-induced NAG-1 promoter activity (Fig. 3). The specificity of the KLF4 binding sites were also confirmed by EMSA, as shown in Fig. 4. Summarized signaling pathways of MCC-555-induced NAG-1 expression are shown at Fig. 6. In this report, we provide evidence of new additional mechanism of NAG-1 regulation through KLF4 at the transcriptional level after stimulation by PPAR γ agonists.

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Abbreviations

APC	adenomatous polyposis coli
CGZ	ciglitazone
EGR-1	early growth response-1
EMSA	electrophoretic mobility shift assay
KLF4	Krüppel-like factor 4
MCC-555	5-[[6-[(2-fluorophenyl)-methoxy]-2-napthalenyl]methyl]-2,4-thiazolidinedione
NAG-1	non-steroidal anti-inflammatory drug (NSAID)-activated gene- 1

PGJ ₂	15-deoxy- $\Delta^{12,14}$ -prostaglandin J ₂
PGZ	pioglitazone
PPAR γ	peroxisome proliferator-activated receptor gamma
RGZ	rosiglitazone
RT-PCR	reverse- transcription polymerase chain reaction
TGZ	troglitazone

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Figure 1. PPAR γ agonists increased KLF4 and NAG-1 mRNA and protein expression in human colorectal cancer cells

(A) Total RNAs isolated from human HCT-116 colorectal cells treated by MCC-555 (MCC, 5 μ M) in serum-free media for 24 h were analyzed for the expression levels of *KLF4* and *NAG-1*, and representative images of three independent experiments are shown. The *GAPDH* was used as loading control. The bar graph shows the quantitative analysis of three independent experiments of fold induction of *KLF4* and *NAG-1* by MCC-555 treatment (grey bars) over vehicle treated (white bars). The band intensities of *KLF4* and *NAG-1* were first normalized to that of *GAPDH*, and then MCC-555 treatments were compared to vehicle treatments, and the Student *t* test was used to analyze the data, ** $P < 0.01$. (B) A representative image of three independent experiments of Western blot analysis for KLF4, NAG-1 and cyclin D1 protein levels in HCT-116 cells treated by 5 μ M PPAR γ agonists: MCC-555 (MCC), rosiglitazone (RGZ), 15-deoxy- $\Delta^{12,14}$ -prostaglandin J₂ (PGJ₂), ciglitazone (CGZ), troglitazone (TGZ), and pioglitazone (PGZ) for 24h. Actin was used as a loading control. Arrows indicate specific KLF4 band is shown on left site of the images; n.s., non-specific band. (C) Serum-starved HCT-116 cells were treated with 5 μ M MCC-555 and collected at the indicated time points. The KLF4 and NAG-1 protein expression was analyzed by Western blot and a representative image of three independent experiments is shown. (D) KLF4 and NAG-1 protein expression in different human colorectal cancer cell lines (HCT-116, SW480, LoVo, HT-29 and CaCo-2) treated by 5 μ M MCC-555 for 24h were analyzed by Western blot and a representative image of three independent experiments is shown.

Figure 2. KLF4 is induced by PPAR γ agonists in a PPAR γ -dependent manner

(A) HCT-116 cells were pretreated with GW9662 (10 μ M) for 30 min followed by treatment with the indicated concentrations of MCC-555 (5 and 10 μ M) for an additional 24 h. Total cell lysates were isolated and subjected to Western blots to detect KLF4 and NAG-1 protein expression. Actin was used as a loading control. A representative image of three independent experiments is shown. (B) HCT-116 cells were transiently transfected with either control or KLF4 siRNAs (100 nM) as described in the *Material and Methods* section. After 24 h transfection, the cells were treated with vehicle (DMSO), MCC-555 (5 μ M MCC), or troglitazone (5 μ M TGZ) for an additional 24 h. The KLF4 and NAG-1 protein expressions were analyzed by Western blots. Actin was used as a loading control. A representative image of three independent experiments is shown.

Figure 3. KFL4 regulates NAG-1 expression at the transcription level

(A) HCT-116 cells were transiently transfected with NAG-1 promoter with either empty vector (EV) or KLF4 expression vector (KLF4). The activities of the NAG-1 promoters were analyzed by luciferase assay as described in the *Material and Methods* section. The graph shows a mean $\pm$ S.D. of three replicates, and the Student *t* test was used to analyze the data, *** P <0.001. A representative blot of V5 (KLF4) expression is shown in the right panel. (B) At the top, the schematic diagram shows the identified potential KLF4 binding sites in the region of -1086/-474 of the NAG-1 promoter, KLF4-A at position -807/-803 (CACCC), KLF4-B at position -678/-671 (AGCACCCT) and KLF-C at position -558/-554 (CACCC). At the bottom, the fold change of deleted clones (Δ KLF4-A, Δ KLF4-B, and Δ KLF4-C) were compared to the wild-type -1086/+41 NAG-1 promoter (WT). The graph shows a mean $\pm$ S.D. of three replicates, and the Student *t* test was used to analyze the data, *** P <0.001.

Figure 4. KLF4 binds to the NAG-1 promoter

(A) Gel shift assay using HCT-116 nuclear extracts prepared from HCT-116 cells after treatment with vehicle (DMSO), MCC-555 (5 and 10 µM) or troglitazone (5 µM) for 24 h. Five g of nuclear extract was incubated with a biotin-labeled, double-stranded oligonucleotide corresponding to the KLF4-binding sites of the NAG-1 promoter. PPAR γ agonist-induced KLF4 expression resulted in the formation of a DNA/protein complex (*arrow*). At the bottom of the gels, the *arrow* points to a free biotin-labeled probe. (B) Competition experiments were done in the presence of a 1-, 10-, or 100-fold excess of non-biotin-labeled oligonucleotide corresponding to the wild type (WT) or mutant KLF4 oligonucleotide (Mut). The *arrow* indicates shift bands, and a free probe is shown with arrow at the bottom of the gel. (C) Specificity of KLF4 binding to the CAGCC sequence of the NAG-1 promoter. The EMSA assay was performed using the *in vitro* translated (IVT) KLF4 protein instead of nuclear extract. Formation of the DNA-KLF4 complex (*arrow*) was increased due to increasing the amount of IVT KLF4 (0.01, 0.1, 1 µl per reaction) in a dose-dependent manner.

Figure 5. MCC-555-induced *Klf4* expression in *Apc*^{Min/+} mice

(A) Small intestinal (SI) tissues sample preparation was previously described (Yamaguchi et al., 2008). RT-PCR was performed to detect the levels of *Klf4* and the representative data are shown. Averages of the intensities of *Klf4* over *Gapdh* from four representative mice from each treatment group are shown in the graph. Bars represent mean $\pm$ S.D. and the Student *t* test was used to analyze the data (ns, non-significant, $*P < 0.05$). (B) Immunohistochemistry revealed strong expression of *Klf4* in the nuclei of vehicle treated normal intestinal epithelium in villi (N, left panels), but no detectable expression of *Klf4* in nuclei of tumor cells (T, left panels) in small intestine (SI) of vehicle treated *Apc*^{Min/+} mice. MCC-555 induced the expression of *Klf4* in nuclei of SI adenomas (T, right panels) as compared to nuclei of tumors isolated from vehicle treated mice. Top panel images of SI taken at low (10 $\times$ objective) magnification and bottom panel images of SI taken at higher (40 $\times$) magnifications. The scale bars of the top panels represent 200 μ m and bottom panels represent 50 μ m, respectively.

Figure 6. Model for the regulation of NAG-1 protein by PPAR γ agonists

We propose three signaling pathways regulating the expression of NAG-1 by PPAR γ agonists leading to apoptosis in human colorectal cancer cells. The first pathway of NAG-1 regulation is mediated by the activation of PPAR γ receptor and binding to the PPAR γ responsive element (PPRE) of the KLF4 promoter to transactivate KLF4 expression. The KLF4, as a transcription factor, binds and transactivates NAG-1 expression. The silencing of PPAR γ and its antagonist, GW9662, inhibited the expression of KLF4, providing evidence of a PPAR γ -dependent pathway. The second pathway of NAG-1 regulation involves PPAR γ -independent regulation possibly through the transcription factor EGR-1 after PPAR γ treatment (troglitazone) (Baek et al., 2003). The third pathway illustrates posttranscriptional modifications of NAG-1 leading to increasing expression of NAG-1 by PPAR γ agonists (MCC-555) (Yamaguchi et al., 2006).