Published in final edited form as:

Toxicol Appl Pharmacol. 2012 September 1; 263(2): 225–232. doi:10.1016/j.taap.2012.06.014.

A peroxisome proliferator-activated receptor ligand MCC-555 imparts anti-proliferative response in pancreatic cancer cells by PPARgamma-independent up-regulation of KLF4

Kyung-Won Min¹, Xiaobo Zhang^{1,2}, Temjenmongla Imchen¹, and Seung Joon Baek^{1,*}

¹Department of Biomedical and Diagnostic Sciences, College of Veterinary Medicine, University of Tennessee, Knoxville, TN, 37996, USA

²College of Animal Science and Technology, Northwest A&F University, Yangling, Shaanxi, 712100, China

Abstract

MCC-555 is a novel PPAR α/γ dual ligand of the thiazolidinedione class and was recently developed as an anti-diabetic drug with unique properties. MCC-555 also has anti-proliferative activity through growth inhibition and apoptosis induction in several cancer cell types. Our group has shown that MCC-555 targets several proteins in colorectal tumorigenesis including nonsteroidal anti-inflammatory drug (NSAID)-activated gene (NAG-1) which plays an important role in chemoprevention responsible for chemopreventive compounds. NAG-1 is a member of the TGF- β superfamily and is involved in tumor progression and development; however, NAG-1's roles in pancreatic cancer have not been studied. In this report, we found that MCC-555 alters not only NAG-1 expression, but also p21 and cyclin D1 expression. NAG-1 and p21 expression was not blocked by PPAR γ -specific antagonist GW9662, suggesting that MCC-555-induced NAG-1 and p21 expression is independent of PPAR γ activation. However, decreasing cyclin D1 by MCC-555 seems to be affected by PPAR γ activation. Further, we found that the GC box located in the NAG-1 promoter play an important role in NAG-1 transactivation by MCC-555. Subsequently, we screened several transcription factors that may bind to the GC box region in the NAG-1 promoter and found that KLF4 potentially binds to this region. Expression of KLF4 precedes NAG-1 and p21 expression in the presence of MCC-555, whereas blocking KLF4 expression using specific KLF4 siRNA showed that both NAG-1 and p21 expression by MCC-555 was blocked. In conclusion, MCC-555's actions on anti-proliferation involve both PPAR γ -dependent and -independent pathways, thereby enhancing anti-tumorigenesis in pancreatic cancer cells.

Keywords

MCC-555; KLF4; p21; NAG-1; cyclin D1; and PPAR

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*To whom correspondence should be addressed: Seung Joon Baek, Department of Biomedical and Diagnostic Sciences, College of Veterinary Medicine, University of Tennessee, 2407 River Drive, Knoxville, TN 37996-4542 Tel: 865 974 8216; fax: 865 974 5616. sbaek2@utk.edu.

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1. Introduction

Pancreatic cancer is a major cause of cancer-related deaths in developed countries and has the highest mortality rate among major cancers. Pancreatic cancers may cause only vague symptoms before being detected, and chemotherapeutic regimens for this disease have provided very limited improvements in tumor regression and overall survival rates after diagnosis (Bardeesy and DePinho, 2002). Although the precise pathogenesis of pancreatic cancer remains unclear, common mutations in several cell proliferation-related genes have been described: mutation of K-ras, p16, p53, and Smad4 genes has been identified in sporadic pancreatic tumors (Hezel *et al.*, 2006).

The peroxisome proliferator-activated receptors (PPARs) are members of the nuclear receptor superfamily and function as ligand-activated transcription factors. PPARs function in a variety of roles including cell differentiation, metabolism, immune function and cell growth. Thiazolidinediones, synthetic PPAR γ ligands, are used to treat patients with type 2 diabetes, and current studies have shown that thiazolidinediones also have anti-tumorigenic activity in a wide variety of cancer cells (Osawa *et al.*, 2003; Baek *et al.*, 2004b; Cekanova *et al.*, 2008; Wei *et al.*, 2010). Intense studies on the role of PPAR γ ligands in colorectal cancer cells have showed that PPAR γ activation may be implicated in the inhibition of cell proliferation genes and induction of apoptosis in colorectal cancer cells (Baek *et al.*, 2003; Osawa *et al.*, 2003; Yamaguchi *et al.*, 2008); however, effects and molecular targets of PPAR γ ligands in pancreatic cancer cells have not been studied in detail.

MCC-555 (RWJ-241947) is a novel class of thiazolidinediones and was recently established as an anti-diabetic drug in animal models of Type 2 diabetes, and is more effective in the anti-diabetic potency compared to other PPAR γ ligands. Like other thiazolidinediones, MCC-555 binds to PPAR γ and increases transcriptional activities, but its binding affinity for PPAR γ is relatively weak compared to other PPAR γ -specific agonists (Yamaguchi *et al.*, 2006). MCC-555 effects not only PPAR γ as effective anti-diabetic agents but also PPAR α as anti-hyperlipidemic agents (Lazarenko *et al.*, 2006). MCC-555 has been reported to be over 50-fold more potent than rosiglitazone in decreasing blood glucose levels in rodent models of type 2 diabetes and 5- to 10-fold less effective than rosiglitazone in inducing adipogenesis in mouse preadipocytes (Pickavance *et al.*, 1998; Upton *et al.*, 1998). These effects may be explained by the ability of MCC-555 to act as a PPAR γ agonist, partial agonist, or antagonist, depending on cell context (Reginato *et al.*, 1998). The same scenario may apply to the effect of MCC-555 on anti-proliferative activity. MCC-555 has an anti-tumorigenic activity against prostate cancer (Kumagai *et al.*, 2004) and colorectal cancer (Yamaguchi *et al.*, 2006) in both PPAR γ -dependent and -independent manners. It has been shown that dual ligands exhibit better activity with respect to cell growth inhibition, compared to the PPAR γ specific agonists (Liu *et al.*, 2004). Thus, it is expected that dual ligands for PPAR may provide a better outcome to prevent cancer. Since the anti-proliferative potency of MCC-555 has not been examined in pancreatic cancer cells, we extended our knowledge to pancreatic cancer to investigate whether MCC-555 affects anti-cancer activity along with several molecular targets affected in tumorigenesis.

MCC-555 and other PPAR γ ligands modulate the expression of various genes such as NAG-1, p21, cyclin D1, and KLF4 through a PPAR γ -dependent or -independent pathway (Chintharlapalli *et al.*, 2007b; Cho *et al.*, 2008; Yamaguchi *et al.*, 2008). We have reported that KLF4 is the key regulator that induces NAG-1 by MCC-555 in colorectal cancer cells (Cekanova *et al.*, 2010); however, in this study, we found a novel *cis*-acting element where KLF4 binds to the NAG-1 promoter in pancreatic cancer cells. KLF4 is a well-known transcription factor that induces p21 and suppresses cyclin D1 (Shie *et al.*, 2000; Rowland and Peeper, 2006); however, KLF4 seems to not be involved in cyclin D1 down-regulation

in the presence of MCC-555. Therefore, the mechanisms by which MCC-555 induces cell growth inhibition may be associated with both PPAR γ dependence and independence and affects multiple targets in pancreatic cancer cells.

2. Methods

2.1. Cell lines, reagents, antibodies, and DNA constructs

Human pancreatic cancer cell lines BxPC3 and AsPC-1 were purchased from American Type Culture Collection (Manassas, VA). Both cell types were maintained in RPMI1640 medium. The culture media contained 10% fetal bovine serum (Hyclone), 50 U/ml penicillin and 50 μ g/ml streptomycin. MCC-555, ciglitazone, rosiglitazone, troglitazone, 15-deoxy- $\Delta^{12,14}$ -prostaglandin J₂, and GW9662 were purchased from Cayman Chemical Co. (Ann Arbor, MI), and all chemicals were dissolved in dimethylsulfoxide (DMSO). Anti-human NAG-1 antibody was previously described (Baek *et al.*, 2001b). Antibodies against KLF4, Sp1, Sp3, ZF9, BTEB1, MAZ, cyclin D1 and actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), whereas p21 antibody was purchased from Cell Signaling Technology (Beverly, MA). The NAG-1 promoter constructs pNAG-1-3500/+41, pNAG-1-1086/+41, pNAG-1-474/+41, pNAG-1-133/+41 and the internal deletion mutant clones derived from pNAG-1-133/+41 were described previously (Baek *et al.*, 2001a; Lee *et al.*, 2010; Nualsanit *et al.*, 2011).

2.2. Cell proliferation analysis

Cell proliferation was investigated using the CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega, WI). Cells were plated in a 96-well plate at 1,000 cells/well in four replicates. Cells were then stimulated with different doses of MCC-555 in the presence of 1% serum. After two days treatment, 20 μ l of CellTiter 96 Aqueous One Solution was added to each well, and the plate was incubated for 1 h at 37°C. Absorbance at 490 nm was recorded in an enzyme-linked immunosorbent assay plate reader (Bio-Tek Instruments, Winooski, VT).

2.3. Caspase 3/7 enzyme activity

Enzyme activity of caspase 3/7 was analyzed using the Caspase-Glo 3/7 Assay kit (Promega) according to the manufacturer's protocol. Briefly, cells were seeded onto a 96-well plate at 5,000 cells/well in four replicates. The cells were then treated with 10 μ M MCC-555 in the absence of serum. After 24 hours, 100 μ l of caspase-Glo 3/7 Reagent was added into each well, and the plate was incubated for 1 hour at room temperature. Luminescence was measured using an FLX800 microplate reader (Bio-Tek).

2.4. Cell cycle analysis

Cells were plated at 4×10^5 cells/well in 6-well plates, and then treated with MCC-555 (10 μ M) for 24 hours. The cells (attached and floating cells) were then harvested, washed with phosphate-buffered saline (PBS), fixed by slow addition of 1 ml cold 70% ethanol and stored at -80°C. The fixed cells were pelleted, washed with 50% and 30% ethanol in PBS, and stained with 0.5 ml of 70 μ M propidium iodide containing 1 mg/ml RNase for 15 min at room temperature. Ten-thousand cells were examined by flow cytometry using a Becton Dickinson fluorescence-activated cell sorter equipped with CellQuest software, by gating on an area vs. width dot plot to exclude cell debris and cell aggregates.

2.5. TUNEL assay

Apoptosis in the BxPC-3 cells was determined by the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end labeling (TUNEL) staining method using an in situ

cell detection kit (TACS 2 TdT Blue kit, Trevigen, Gaithersburg, MD) according to the manufacturer's instructions. Briefly, the cells on coverslips were fixed with 3.7% formaldehyde and permeabilized with proteinase K solution. The slides were then immersed in quenching solution and 1 X terminal deoxynucleotidyl transferase (TdT) labeling buffer and incubated in labeling reaction mix containing TdT dNTP mix and TdT enzyme. After 1 hour, the cells were immersed in strep-HRP solution for 10 min and then were incubated in TACS-blue label solution. The slides were counterstained with Nuclear Fast Red solution, and the stained slides were observed and photographed using light microscopy.

2.6. Western blot analysis

Western blot analysis was performed as described previously (Cekanova *et al.*, 2010). Briefly, cells were starved overnight, and treated with MCC-555 at the indicated time and dose in serum-free media. Cell lysates were obtained using RIPA buffer containing protease inhibitor cocktail (Calbiochem, San Diego, CA). Protein concentration was determined by bicinchoninic acid protein assay (Pierce, Rockford, IL). Proteins were separated on SDS/polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Osmonics, Minnetonka, MN). The membranes were incubated with a specific primary anti-serum in tris-buffered saline containing 0.05% Tween 20 (TBS-T) and 5% non-fat dry milk at 4°C overnight. After three washes with TBS-T, the blots were incubated with peroxidase conjugated immunoglobulin G for 1 hour at room temperature, visualized using ECL (Amersham Biosciences, Piscataway, NJ) and quantified by Scion Image Software (Scion Corp., Frederick, MD).

2.7. Real-Time RT-PCR Analysis

Total RNA was isolated from BxPC3 and AsPC-1 cells using RNA isolation kit (OMEGA Bio-Tek, Norcross, GA), and 1 µg of RNA was reverse-transcribed with an iScript cDNA kit (BioRad, Hercules, CA) according to the manufacturer's instruction. PCR was carried out using Absolute qPCR SYBR Green Mix (Thermo Scientific, UK) with primers for human *NAG-1*, *p21*, *cyclinD1* and *GAPDH* as follows: *NAG-1*, forward 5'-ATGCCCCGGCAAGAACTC-3' and reverse 5'-CATATGCAGTGGCAGTC-3'; *p21*, forward 5'-GCGACTGTGATGCGCTAAT-3' and reverse 5'-TAGGGCTTCTCTTGGAGAA-3'; *cyclin D1*, forward 5'-ATGGAACACCAGCTCCTGTGCTGC-3' and reverse 5'-TCAGATGTCCACGTCCCGCACGT-3'; *GAPDH*, forward 5'-GGGCTGCTTTTA ACT CTGGT-3' and reverse 5'-TGGCAGGTTTTTCTAGACGG-3'. Gene expression levels were calculated and *GAPDH* was used as a control gene, using MyiQ thermal cycler (Bio-RAD). Vehicle-treated samples were set to 1 and fold change are represented as mean ± S.D.

2.8. Transient transfection and luciferase reporter assays

BxPC3 cells were plated in 12-well plates at 2×10^5 cells/well. The next day, plasmid mixtures containing 0.5 µg of *NAG-1* promoter linked to luciferase and 0.05 µg of *pRL-null* vector were transfected by PolyJet transfection reagent (Signagen, Rockville, MD) according to the manufacturer's protocol. After transfection, cells were treated with DMSO or MCC-555 (10 µM) in serum-free media for 24 hours. Cells were harvested in 1 × passive lysis buffer (Promega), and luciferase activity was measured using DualGlo Luciferase Assay Kit (Promega). The results were normalized to *pRL-null* luciferase activity.

2.9. RNA interference

Klf4 siRNA was purchased from Santa Cruz Biotechnology and control siRNA was purchased from Ambion. BxPC-3 cells were transfected with 10 µM of *KLF4* or control

siRNA using PepMute siRNA Transfection reagent (Signagen), according to the manufacturer's protocol. After transfection for 24 hours, cells were serum starved overnight and treated as indicated. Total protein was subjected to Western blot analysis as described.

2.10. Chromatin immunoprecipitation

Cells were fixed with 1% formaldehyde for 10 minutes at 37°C and sonicated four times for 10 seconds. Cell lysates (0.2 ml) were diluted with 0.8 ml of immunoprecipitation buffer (0.1 % SDS, 1 % Triton X-100, 0.1 % Na-deoxycholate and 140 mM NaCl) and immunoprecipitated with 10 µg specific antibodies for normal IgG or KLF4 at 4°C overnight. The chromatin-associated DNA was eluted, reverse cross-linked by heating at 65°C for 4 hours and treated with proteinase K at 45°C for 2 hours. DNA was purified by phenol/chloroform extraction, and precipitated DNA was amplified using the following primer pairs: forward 5'-CCAGAAATGTGCCCTAGCTT-3' and reverse 5'-GAGCTGGGACTGACCAGATG-3'. PCR products (202 bp) were resolved on 2% agarose gel and visualized under UV light.

2.11. Statistical analysis

SAS for windows (v9.2; SAS institute, Inc.) statistical analysis software was used. For multiple group comparisons, analysis of variance with Tukey's multiple comparison test was used to compare mean values. The Student *t* test was used to analyze differences between samples. Results were considered statistically significant at * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

3. Results

3.1. MCC-555 induces cell growth arrest and apoptosis in pancreatic cancer cells

The effects of PPAR γ agonists on cancer are mediated in PPAR γ -dependent and/or -independent manners, depending on cell types or ligand structures (Elrod and Sun, 2008). In this study, we have investigated the therapeutic properties of MCC-555 in human pancreatic adenocarcinoma cells. BxPC-3 cells were treated with MCC-555 for 2 days, and we observed the reduction of cell proliferation in MCC-555 treated cells in a dose dependent manner, compared to DMSO-treated cells (Fig. 1A). To investigate whether MCC-555 arrests cell cycle, we stained cells with propidium iodide and examined cell cycle status. Treatment with MCC-555 resulted in a significant increase in cells arrested at the G1 phase (Fig. 1B), consistent with previous reports that PPAR γ ligands arrest cell cycle at the G1 phase in other cells (Cerquetti *et al.*, 2011; Kim *et al.*, 2011). To examine whether MCC-555 affects apoptosis of human pancreatic cancer cells, we performed caspase 3/7 activity and TUNEL assay using BxPC-3 cells treated with either DMSO or MCC-555 (10 µM). As shown in Fig. 1C, MCC-555 treatment significantly increased caspase 3/7 activity, and addition of caspase inhibitor with MCC-555 resulted in decreasing caspase 3/7 activity, indicating that MCC-555-induced apoptosis is mediated by caspase 3/7 activity. We also performed TUNEL assay to detect apoptotic cells in the presence of MCC-555. As shown in Fig. 1D, MCC-555 enhanced TUNEL positive cells in MCC-555-treated cells, compared to vehicle-treated cells. Taken together, MCC-555 seems to increase cell cycle arrest and apoptosis in pancreatic cancer cells; these results appear to account for the cell growth arrest as seen in Fig. 1A.

3.2. MCC-555 induces NAG-1 and p21, and suppresses cyclin D1 expression

To elucidate the molecular mechanism(s) by which MCC-555 induces cell cycle arrest and apoptosis in human pancreatic cancer cells, we analyzed the expression of pro-apoptotic gene NAG-1, and cell cycle regulators p21 and cyclin D1. It has been reported that several PPAR γ agonists induce NAG-1 expression, which exerts anti- cancer activity in colorectal

cancer cells (Baek *et al.*, 2003; Chintharlapalli *et al.*, 2005). Likewise, expression of cyclin D1, and p21 are altered in the presence of PPAR γ ligands (Hong *et al.*, 2004; Nasrallah *et al.*, 2010). BxPC-3 cells were treated with several PPAR γ agonists, including MCC-555, rosiglitazone (RGZ), 15-deoxy- $\Delta^{12,14}$ -prostaglandin J₂ (PGJ₂), and troglitazone (TGZ). MCC-555 and TGZ dramatically induced NAG-1 expression compared to other PPAR γ agonists. MCC-555 and TGZ also induced p21 as well as suppressed cyclin D1 expression (Fig. 2A). In this report, we focus on MCC-555's effects since TGZ was withdrawn from the market due to the prevalence of adverse liver effects (Graham *et al.*, 2003). NAG-1 and p21 were induced by MCC-555 not only in BxPC-3 cells, but also in other pancreatic cancer cells AsPC-1 in a dose- and time-dependent manner, whereas cyclin D1 was suppressed by MCC-555 in both cells in a dose- and time-dependent manner (Fig. 2B and C). Long-term treatment (48 h) of MCC-555 exhibits the reduction of p21 protein in both cell lines, probably due to cytopathic effects.

3.3. Cyclin D1, but not NAG-1 and p21 expression is involved in PPAR γ activation

We examined whether NAG-1, p21 and cyclin D1 expression by MCC-555 is regulated by PPAR γ transcription factor activation. First, qRT-PCR results showed that these transcripts were dose-dependently altered by MCC-555 in BxPC-3 and AsPC-1 cells (Fig. 3A). It has been reported that BxPC-3 and AsPC-1 cells express PPAR γ and treatment with PPAR γ agonists induces apoptosis and differentiation in these cells (Eibl *et al.*, 2001; Ohta *et al.*, 2002). To determine whether NAG-1, p21 and cyclin D1 expression by MCC-555 is dependent on PPAR γ activation, PPAR γ antagonist GW9662 was used to treat the cells. As shown in Fig. 3B, NAG-1 and p21 induction by MCC-555 were not blocked by GW9662 treatment, but reduction of cyclin D1 by MCC-555 was inhibited by GW9662 treatment, indicating that NAG-1 and p21 induction by MCC-555 is independent of PPAR γ activation, whereas cyclin D1 suppression by MCC-555 is dependent on PPAR γ activation.

3.4. The GC box region in -133/+41 NAG-1 promoter is responsible for NAG-1 transactivation

Unlike cyclin D1 and p21 regulation by PPAR γ ligands, NAG-1 expression by PPAR γ ligands in pancreatic cancer cells has not been studied in detail. Therefore, we decided to further examine the transcriptional regulation of NAG-1 affected by MCC-555. BxPC-3 cells were transfected with the four serial deletion constructs of the human NAG-1 promoter, and then were treated with MCC-555. Treatment with MCC-555 resulted in increasing the promoter activity in all of the constructs tested (Fig. 4A). Therefore, the -133/+41 promoter region was further analyzed using the TFSEARCH site (<http://www.cbrc.jp/research/db/TFSEARCH.html>) and TESS (<http://www.cbil.upenn.edu/cgi-bin/teess/tess33?RQ=SEA-FR-Query>). There are several *cis*-acting elements in this region including activating transcription factor 3 (ATF3), MYC-associated zinc finger protein (MAZ), retinoic acid receptor α (RAR- α), xenobiotic response element binding factor (XF-1) and specificity protein 1 (Sp1) binding sites. To investigate the responsible site in MCC-555-mediated transcriptional regulation of NAG-1, we used internal deletion clones in which the aforementioned transcription binding sites were deleted. As shown in Fig. 4B, transfection with a double deletion clone lacking the two potential Sp1 binding sites (or GC box) significantly attenuated MCC-555-induced promoter activity, compared to wild-type promoter, suggesting that these two GC box sites are important in MCC-555-induced NAG-1 expression in pancreatic cancer cells.

3.5. KLF4 is a key molecule to regulate MCC-555-induced NAG-1 and p21 expression

To determine what *trans*-acting elements are involved in these GC boxes, we decided to examine the expression of proteins that could bind to these sites. Sp or several Sp-related transcriptional factors could bind to these GC boxes. The results indicate that expression of

Sp transcription factors (Sp1 and Sp3) was not altered; only KLF4 was increased by MCC-555 in a dose-dependent manner, implying that KLF4 expression by MCC-555 could be responsible for NAG-1 expression in human pancreatic cancer cells (Fig. 5A). This is consistent with the previous report, indicating that MCC-555 increases KLF4 expression in human colorectal cancer cells (Cekanova *et al.*, 2010). Other zinc-finger proteins including ZF9 and MYC-associated zinc finger protein (MAZ) were marginally expressed in pancreatic cancer cells, whereas basic transcription element-binding protein 1 (BTEB1) was highly expressed; however, MCC-555 does not affect their expression. If KLF4 regulates NAG-1 expression in responding to MCC-555, KLF4 expression should be an earlier event than NAG-1 expression. As shown in Fig. 5B, KLF4 expression preceded NAG-1 expression in the presence of MCC-555. To confirm KLF4's role in MCC-555-induced NAG-1 expression, specific KLF4 siRNA was utilized. The results clearly indicate that blocking KLF4 expression resulted in the reduction of NAG-1 and p21 expression (Fig. 5C). Interestingly, blocking KLF4 expression did not affect MCC-555-induced cyclin D1 suppression, suggesting that KLF4 expression may not be involved in MCC-555-induced cyclin D1 suppression. Finally, a ChIP assay was performed to confirm that KLF4 binds to the NAG-1 promoter in responding to MCC-555 (Fig. 5D).

4. Discussion

Pancreatic cancer is the fourth leading cause of cancer deaths, and the survival rate is very poor compared to other cancers. Since conventional therapeutic approaches do not decrease the incidence of mortality of this deadly cancer, we have paid more attention to alternative research including identification of molecular target approaches for an increasing survival rate.

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) cancers *in vitro* and *in vivo*. Recently, clinical success in cancer prevention research and diabetic treatment with PPAR γ agonists was overshadowed by reports of several side effects that include obesity, cardiotoxicity, and liver toxicity (Nissen and Wolski, 2007). However, Choi *et al.*, reported CDK5 is a new molecular target of the PPAR γ ligand (Choi *et al.*, 2010), and this finding could reverse the negative swing of the PPAR γ ligand in diabetic research. As a similar token, identification of novel targets induced by PPAR γ ligands in cancer research may help to promote their potential use. Dual ligands for PPAR α and PPAR γ , such as MCC-555, alternatively have been developed to improve treatment of metabolic syndrome, including hyperglycemia and hyperlipidemia. These dual ligands also possess anti-proliferative activities against a variety of cancer cell lines (Yamaguchi *et al.*, 2006; Yamaguchi *et al.*, 2008) with greater potency than conventional PPAR γ -specific ligands. In this report, we used MCC-555 as a PPAR dual ligand and investigated a new molecular target: p21, NAG-1, cyclin D1 and KLF4 in pancreatic cancer cells.

The biological activity of KLF4 in tumorigenesis is controversial. 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 (Zhao *et al.*, 2004) and is frequently lost in other human cancer types (Wei *et al.*, 2005). Consistent with a tumor suppressor function of KLF4 in colon cancer (Dang *et al.*, 2003), its overexpression reduces tumorigenesis in pancreatic cancer cells *in vivo* (Zammarchi *et al.*, 2011). Moreover, it has been shown that KLF4 inhibits

metastasis of several cancers, including pancreatic cancer (Wei *et al.*, 2008). Thus, KLF4 may play an important role in pancreas as an anti-tumorigenic protein, but its exact function may be dependent on cell context. Our data support KLF4's anti-proliferative role in this report, showing that KLF4 controls cell cycle inhibitor p21 and pro-apoptotic protein NAG-1.

A study showed that some PPAR γ or PPAR α/γ agonists regulate the *Klf4* mRNA expression in PPAR γ -dependent or independent manner, providing KLF4 as a novel anti-proliferative target of PPAR γ ligands in pancreas (Chintharlapalli *et al.*, 2007a). From our results, we identified that MCC-555 affects cyclin D1 expression in a PPAR γ -dependent manner; however, KLF4 seems to be regulated by MCC-555 in a PPAR γ independent manner (Supplemental Data). Our data also support the view that KLF4 is a key protein that controls cell cycle regulator p21 and pro-apoptotic protein NAG-1 in the presence of MCC-555. We showed that MCC-555 increased KLF4 expression, which results in an increased expression of p21 and NAG-1 expression. This view is in agreement with reports showing that p21 and NAG-1 are the targets of KLF4 in other cancer cells (Cekanova *et al.*, 2010; Chew *et al.*, 2011). Interestingly, it has been known that KLF4 regulates cyclin D1 expression, but our data clearly suggest that KLF4 is not involved in cyclin D1 regulation in the presence of MCC-555. It remains unclear, but MCC-555 may affect the modification of KLF4, thereby not binding to the cyclin D1 promoter. Alternatively, MCC-555 has been shown to activate ERK and other kinases (Yamaguchi *et al.*, 2008), and KLF4 could be phosphorylated by these kinases (Ohnesorge *et al.*, 2010), rendering KLF4 activity in cyclin D1 transcriptional regulation. Another possible explanation is KLF4 acetylation. Since KLF4 is acetylated by p300, thereby controlling its activity (Evans *et al.*, 2007), MCC-555 may affect KLF4 post-translational modification by acetylation, affecting cyclin D1 expression. Finally, PPAR γ activation may control more effectively than KLF4 activity in the presence of MCC-555; however, the detail mechanism needs to be elucidated.

We have shown that NAG-1 transgenic mice exhibit resistance to carcinogenic-induced colon and lung adenomas (Baek *et al.*, 2006; Cekanova *et al.*, 2009), and NAG-1 expression is induced by many cancer chemopreventive compounds (Baek *et al.*, 2004a; Lee *et al.*, 2010), indicating that NAG-1 possesses cancer preventive activity and its induction is beneficial. Therefore, NAG-1 expression induced by MCC-555 may explain the chemopreventive activity of MCC-555 in pancreatic cancer.

In summary, our data suggest that KLF4 is an upstream molecule that controls NAG-1 and p21 expression by MCC-555 at the transcriptional level, along with the suppression of cyclin D1 by MCC-555. KLF4/NAG-1 and KLF4/p21 affected by MCC-555 are mediated in a PPAR γ -independent manner, whereas cyclin D1 suppression by MCC-555 is mediated in a PPAR γ -dependent manner (Fig. 6).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Financial Support: This work was supported by grants from the National Institutes of Health (R01CA108975), and the University of Tennessee Center of Excellence in Livestock Diseases and Human Health to SJ Baek. Financial supports for XZ was partially provided by the Program in Organizational or Personal Cooperation with Foreign Counterparts (2010630161), China. TI is a recipient of National Overseas Scholarship Award [No. 11016/07/2010-Education] from Ministry of Tribal Affairs, New Delhi, India.

We thank Misty Bailey for her critical reading of this manuscript.

References

- Baek SJ, Horowitz JM, Eling TE. Molecular cloning and characterization of human nonsteroidal anti-inflammatory drug-activated gene promoter. Basal transcription is mediated by Sp1 and Sp3. *J Biol Chem*. 2001a; 276:33384–33392. [PubMed: 11445565]
- Baek SJ, Kim JS, Jackson FR, Eling TE, McEntee MF, Lee SH. Epicatechin gallate-induced expression of NAG-1 is associated with growth inhibition and apoptosis in colon cancer cells. *Carcinogenesis*. 2004a; 25:2425–2432. [PubMed: 15308587]
- Baek SJ, Kim JS, Nixon JB, DiAugustine RP, Eling TE. Expression of NAG-1, a transforming growth factor-beta superfamily member, by troglitazone requires the early growth response gene EGR-1. *J Biol Chem*. 2004b; 279:6883–6892. [PubMed: 14662774]
- Baek SJ, Kim KS, Nixon JB, Wilson LC, Eling TE. Cyclooxygenase inhibitors regulate the expression of a TGF-beta superfamily member that has proapoptotic and antitumorigenic activities. *Mol Pharmacol*. 2001b; 59:901–908. [PubMed: 11259636]
- Baek SJ, Okazaki R, Lee SH, Martinez J, Kim JS, Yamaguchi K, Mishina Y, Martin DW, Shoieb A, McEntee MF, Eling TE. Nonsteroidal anti-inflammatory drug-activated gene-1 over expression in transgenic mice suppresses intestinal neoplasia. *Gastroenterology*. 2006; 131:1553–1560. [PubMed: 17101328]
- Baek SJ, Wilson LC, Hsi LC, Eling TE. Troglitazone, a peroxisome proliferator-activated receptor gamma (PPAR gamma) ligand, selectively induces the early growth response-1 gene independently of PPAR gamma. A novel mechanism for its anti-tumorigenic activity. *J Biol Chem*. 2003; 278:5845–5853. [PubMed: 12475986]
- Bardeesy N, DePinho RA. Pancreatic cancer biology and genetics. *Nat Rev Cancer*. 2002; 2:897–909. [PubMed: 12459728]
- Bren-Mattison Y, Van Putten V, Chan D, Winn R, Geraci MW, Nemenoff RA. Peroxisome proliferator-activated receptor-gamma (PPAR(gamma)) inhibits tumorigenesis by reversing the undifferentiated phenotype of metastatic non-small-cell lung cancer cells (NSCLC). *Oncogene*. 2005; 24:1412–1422. [PubMed: 15608671]
- Cekanova M, Lee SH, McEntee MF, Baek SJ. MCC-555-induced NAG-1 expression is mediated in part by KLF4. *Eur J Pharmacol*. 2010; 637:30–37. [PubMed: 20385121]
- Cekanova M, Lee SH, Donnell RL, Sukhthankar M, Eling TE, Fischer SM, Baek SJ. Nonsteroidal anti-inflammatory drug-activated gene-1 expression inhibits urethane-induced pulmonary tumorigenesis in transgenic mice. *Cancer Prev Res (Phila Pa)*. 2009; 2:450–458.
- Cekanova M, Yuan JS, Li X, Kim K, Baek SJ. Gene alterations by peroxisome proliferator-activated receptor gamma agonists in human colorectal cancer cells. *Int J Oncol*. 2008; 32:809–819. [PubMed: 18360708]
- Cerquetti L, Sampaoli C, Amendola D, Bucci B, Masuelli L, Marchese R, Misiti S, De Venanzi A, Poggi M, Toscano V, Stigliano A. Rosiglitazone induces autophagy in H295R and cell cycle deregulation in SW13 adrenocortical cancer cells. *Exp Cell Res*. 2011; 317:1397–1410. [PubMed: 21376716]
- Chew YC, Adhikary G, Wilson GM, Reece EA, Eckert RL. Protein Kinase C (PKC) δ Suppresses Keratinocyte Proliferation by Increasing p21Cip1 Level by a KLF4 Transcription Factor-dependent Mechanism. *J Biol Chem*. 2011; 286:28772–28782. [PubMed: 21652709]
- Chintharlapalli S, Papineni S, Baek SJ, Liu S, Safe S. 1,1-Bis(3'-indolyl)-1-(p-substitutedphenyl)methanes are peroxisome proliferator-activated receptor gamma agonists but decrease HCT-116 colon cancer cell survival through receptor-independent activation of early growth response-1 and nonsteroidal anti-inflammatory drug-activated gene-1. *Mol Pharmacol*. 2005; 68:1782–1792. [PubMed: 16155208]
- Chintharlapalli S, Papineni S, Liu S, Jutooru I, Chadalapaka G, Cho SD, Murthy RS, You Y, Safe S. 2-cyano-lup-1-en-3-oxo-20-oic acid, a cyano derivative of betulinic acid, activates peroxisome proliferator-activated receptor gamma in colon and pancreatic cancer cells. *Carcinogenesis*. 2007a; 28:2337–2346. [PubMed: 17724373]
- Chintharlapalli S, Papineni S, Safe S. 1,1-bis(3'-indolyl)-1-(p-substitutedphenyl)methanes inhibit growth, induce apoptosis, and decrease the androgen receptor in LNCaP prostate cancer cells

- through peroxisome proliferator-activated receptor gamma-independent pathways. *Mol Pharmacol.* 2007b; 71:558–569. [PubMed: 17093136]
- Cho SD, Chintharlapalli S, Abdelrahim M, Papineni S, Liu S, Guo J, Lei P, Abudayyeh A, Safe S. 5,5'-Dibromo-bis(3'-indolyl)methane induces Kruppel-like factor 4 and p21 in colon cancer cells. *Mol Cancer Ther.* 2008; 7:2109–2120. [PubMed: 18645021]
- Choi JH, Banks AS, Estall JL, Kajimura S, Bostrom P, Laznik D, Ruas JL, Chalmers MJ, Kamenecka TM, Bluher M, Griffin PR, Spiegelman BM. Anti-diabetic drugs inhibit obesity-linked phosphorylation of PPARgamma by Cdk5. *Nature.* 2010; 466:451–456. [PubMed: 20651683]
- Dang DT, Chen X, Feng J, Torbenson M, Dang LH, Yang VW. Overexpression of Kruppel-like factor 4 in the human colon cancer cell line RKO leads to reduced tumorigenicity. *Oncogene.* 2003; 22:3424–3430. [PubMed: 12776194]
- Eibl G, Wente MN, Reber HA, Hines OJ. Peroxisome Proliferator-Activated Receptor γ Induces Pancreatic Cancer Cell Apoptosis. *Biochem Biophys Res Commun.* 2001; 287:522–529. [PubMed: 11554760]
- Elrod HA, Sun SY. PPAR γ and Apoptosis in Cancer. *PPAR Research.* 2008; 2008:1–12.
- Elstner E, Muller C, Koshizuka K, Williamson EA, Park D, Asou H, Shintaku P, Said JW, Heber D, Koeffler HP. Ligands for peroxisome proliferator-activated receptorgamma and retinoic acid receptor inhibit growth and induce apoptosis of human breast cancer cells in vitro and in BNX mice. *Proc Natl Acad Sci U S A.* 1998; 95:8806–8811. [PubMed: 9671760]
- Evans PM, Zhang W, Chen X, Yang J, Bhakat KK, Liu C. Krüppel-like Factor 4 Is Acetylated by p300 and Regulates Gene Transcription via Modulation of Histone Acetylation. *J Biol Chem.* 2007; 282:33994–34002. [PubMed: 17908689]
- Foster KW, Frost AR, McKie-Bell P, Lin CY, Engler JA, Grizzle WE, Ruppert JM. Increase of GKLf messenger RNA and protein expression during progression of breast cancer. *Cancer Res.* 2000; 60:6488–6495. [PubMed: 11103818]
- Foster KW, Ren S, Louro ID, Lobo-Ruppert SM, McKie-Bell P, Grizzle W, Hayes MR, Broker TR, Chow LT, Ruppert JM. Oncogene expression cloning by retroviral transduction of adenovirus E1A-immortalized rat kidney RK3E cells: transformation of a host with epithelial features by c-MYC and the zinc finger protein GKLf. *Cell Growth Differ.* 1999; 10:423–434. [PubMed: 10392904]
- Graham DJ, Drinkard CR, Shatin D. Incidence of idiopathic acute liver failure and hospitalized liver injury in patients treated with troglitazone. *Am J Gastroenterol.* 2003; 98:175–179. [PubMed: 12526954]
- Hezel AF, Kimmelman AC, Stanger BZ, Bardeesy N, Depinho RA. Genetics and biology of pancreatic ductal adenocarcinoma. *Genes Dev.* 2006; 20:1218–1249. [PubMed: 16702400]
- Hong J, Samudio I, Liu S, Abdelrahim M, Safe S. Peroxisome proliferator-activated receptor gamma-dependent activation of p21 in Panc-28 pancreatic cancer cells involves Sp1 and Sp4 proteins. *Endocrinology.* 2004; 145:5774–5785. [PubMed: 15345676]
- Kim S, Lee JJ, Heo DS. PPAR γ ligands induce growth inhibition and apoptosis through p63 and p73 in human ovarian cancer cells. *Biochem Biophys Res Commun.* 2011; 406:389–395. [PubMed: 21329655]
- Kumagai T, Ikezoe T, Gui D, O'Kelly J, Tong XJ, Cohen FJ, Said JW, Koeffler HP. RWJ-241947 (MCC-555), a unique peroxisome proliferator-activated receptor-gamma ligand with antitumor activity against human prostate cancer in vitro and in beige/nude/ X-linked immunodeficient mice and enhancement of apoptosis in myeloma cells induced by arsenic trioxide. *Clin Cancer Res.* 2004; 10:1508–1520. [PubMed: 14977855]
- Lazarenko OP, Rzonca SO, Suva LJ, Lecka-Czernik B. Netoglitazone is a PPAR-gamma ligand with selective effects on bone and fat. *Bone.* 2006; 38:74–84. [PubMed: 16137931]
- Lee SH, Krisanapun C, Baek SJ. NSAID-activated gene-1 as a molecular target for capsaicin-induced apoptosis through a novel molecular mechanism involving GSK3beta, C/EBPbeta and ATF3. *Carcinogenesis.* 2010; 31:719–728. [PubMed: 20110283]
- Liu DC, Zang CB, Liu HY, Possinger K, Fan SG, Elstner E. A novel PPAR alpha/gamma dual agonist inhibits cell growth and induces apoptosis in human glioblastoma T98G cells. *Acta Pharmacologica Sinica.* 2004; 25:1312–1319. [PubMed: 15456533]

- Nasrallah R, Clark J, Corinaldi J, Paris G, Miura P, Jasmin BJ, Hébert RL. Thiazolidinediones alter growth and epithelial cell integrity, independent of PPAR- γ and MAPK activation, in mouse M1 cortical collecting duct cells. *Am J Physiol Renal Physiol*. 2010; 298:F1105–F1112. [PubMed: 20164153]
- Nissen SE, Wolski K. Effect of Rosiglitazone on the Risk of Myocardial Infarction and Death from Cardiovascular Causes. *N Engl J Med*. 2007; 356:2457–2471. [PubMed: 17517853]
- Nualsanit T, Rojanapanthu P, Gritsanapan W, Lee SH, Lawson D, Baek SJ. Damnacanthal, a noni component, exhibits antitumorigenic activity in human colorectal cancer cells. *J Nutr Biochem*. 2011
- Ohnesorge N, Viemann D, Schmidt N, Czymai T, Spiering D, Schmolke M, Ludwig S, Roth J, Goebeler M, Schmidt M. Erk5 Activation Elicits a Vasoprotective Endothelial Phenotype via Induction of Krüppel-like Factor 4 (KLF4). *J Biol Chem*. 2010; 285:26199–26210. [PubMed: 20551324]
- Ohta T, Elnemr A, Yamamoto M, Ninomiya I, Fushida S, Nishimura G, Fujimura T, Kitagawa H, Kayahara M, Shimizu K, Yi S, Miwa K. Thiazolidinedione, a peroxisome proliferator-activated receptor- γ ligand, modulates the E-cadherin/beta-catenin system in a human pancreatic cancer cell line, BxPC-3. *Int J Oncol*. 2002; 21:37–42. [PubMed: 12063547]
- Osawa E, Nakajima A, Wada K, Ishimine S, Fujisawa N, Kawamori T, Matsuhashi N, Kadowaki T, Ochiai M, Sekihara H, Nakagama H. Peroxisome proliferator-activated receptor gamma ligands suppress colon carcinogenesis induced by azoxymethane in mice. *Gastroenterology*. 2003; 124:361–367. [PubMed: 12557142]
- Pickavance L, Widdowson PS, King P, Ishii S, Tanaka H, Williams G. The development of overt diabetes in young Zucker Diabetic Fatty (ZDF) rats and the effects of chronic MCC-555 treatment. *British Journal of Pharmacology*. 1998; 125:767–770. [PubMed: 9831913]
- Reginato MJ, Bailey ST, Krakow SL, Minami C, Ishii S, Tanaka H, Lazar MA. A Potent Antidiabetic Thiazolidinedione with Unique Peroxisome Proliferator-activated Receptor γ -activating Properties. *J Biol Chem*. 1998; 273:32679–32684. [PubMed: 9830009]
- Rowland BD, Peeper DS. KLF4, p21 and context-dependent opposing forces in cancer. *Nat Rev Cancer*. 2006; 6:11–23. [PubMed: 16372018]
- Shie JL, Chen ZY, Fu M, Pestell RG, Tseng CC. Gut-enriched Kruppel-like factor represses cyclin D1 promoter activity through Sp1 motif. *Nucleic Acids Res*. 2000; 28:2969–2976. [PubMed: 10908361]
- Upton R, Widdowson PS, Ishii S, Tanaka H, Williams G. Improved metabolic status and insulin sensitivity in obese fatty (fa/fa) Zucker rats and Zucker Diabetic Fatty (ZDF) rats treated with the thiazolidinedione, MCC-555. *British Journal of Pharmacology*. 1998; 125:1708–1714. [PubMed: 9886762]
- Vignati S, Albertini V, Rinaldi A, Kwee I, Riva C, Oldrini R, Capella C, Bertoni F, Carbone GM, Catapano CV. Cellular and molecular consequences of peroxisome proliferator-activated receptor- γ activation in ovarian cancer cells. *Neoplasia*. 2006; 8:851–861. [PubMed: 17032502]
- Wei D, Gong W, Kanai M, Schlunk C, Wang L, Yao JC, Wu TT, Huang S, Xie K. Drastic down-regulation of Kruppel-like factor 4 expression is critical in human gastric cancer development and progression. *Cancer Res*. 2005; 65:2746–2754. [PubMed: 15805274]
- Wei D, Kanai M, Jia Z, Le X, Xie K. Krüppel-like Factor 4 Induces p27Kip1 Expression in and Suppresses the Growth and Metastasis of Human Pancreatic Cancer Cells. *Cancer Res*. 2008; 68:4631–4639. [PubMed: 18559508]
- Wei S, Kulp SK, Chen CS. Energy restriction as an antitumor target of thiazolidinediones. *J Biol Chem*. 2010; 285:9780–9791. [PubMed: 20093366]
- Yamaguchi K, Cekanova M, McEntee MF, Yoon JH, Fischer SM, Renes IB, Van Seuning I, Baek SJ. Peroxisome proliferator-activated receptor ligand MCC-555 suppresses intestinal polyps in ApcMin/+ mice via extracellular signal-regulated kinase and peroxisome proliferator-activated receptor-dependent pathways. *Mol Cancer Ther*. 2008; 7:2779–2787. [PubMed: 18790758]
- Yamaguchi K, Lee SH, Eling TE, Baek SJ. A novel peroxisome proliferator-activated receptor gamma ligand, MCC-555, induces apoptosis via posttranscriptional regulation of NAG-1 in colorectal cancer cells. *Mol Cancer Ther*. 2006; 5:1352–1361. [PubMed: 16731769]

- Zammarchi F, Morelli M, Menicagli M, Di Cristofano C, Zavaglia K, Paolucci A, Campani D, Aretini P, Boggi U, Mosca F, Cavazzana A, Cartegni L, Bevilacqua G, Mazzanti CM. KLF4 is a Novel Candidate Tumor Suppressor Gene in Pancreatic Ductal Carcinoma. *Am J Pathol.* 2011; 178:361–372. [PubMed: 21224073]
- Zhao W, Hisamuddin IM, Nandan MO, Babbitt BA, Lamb NE, Yang VW. Identification of Kruppel-like factor 4 as a potential tumor suppressor gene in colorectal cancer. *Oncogene.* 2004; 23:395–402. [PubMed: 14724568]

Highlights

- PPAR α / γ ligand MCC-555 exhibits anti-proliferative activity in pancreatic cancer cells.
- MCC-555 affects KLF4 expression following by NAG-1 and p21 expression in a PPAR γ independent manner.
- MCC-555 also affects cyclin D1 down-regulation in a PPAR γ dependent manner.
- To our knowledge, this is the first report that MCC-555 affects anti-proliferative activity in pancreatic cancer cells, along with multiple targets.

Figure 1.

MCC-555 inhibits cell proliferation and induces apoptosis in human pancreatic cancer cells.

A, BxPC-3 cells were treated with vehicle or MCC-555 in a dose dependent manner. Cell growth was measured using the CellTiter 96 Aqueous One Solution Cell Proliferation Assay. Values are expressed as mean $\pm$ S.D. of four replicates. $P < 0.01$ ** and $P < 0.001$ ***, compared to vehicle-treated cells. **B**, Flow cytometric analysis of vehicle- or MCC-555-treated BxPC-3 cells. Cells were treated with vehicle or MCC-555 for 24 h and stained with propidium iodide as described in Materials and Methods. Percentage of G1 is expressed as mean $\pm$ S.D. of three replicates. $P < 0.01$ **. **C**, Caspase 3/7 enzymatic activity was measured as described in Materials and Methods. BxPC-3 cells were pretreated with either vehicle or caspase inhibitor (10 μ M, Z-VAD-FMK) for 30 min, and then were stimulated with MCC-555. Values are expressed as mean $\pm$ S.D. of three replicates. $P < 0.001$ ***. **D**, Apoptosis was analyzed by TUNEL assay using BxPC-3 cells treated with 10 μ M MCC-555 for 24 h (40X). The graph indicates the percentage of MCC-555-treated cells versus the vehicle-treated cells from the reading of three randomly defined areas. $P < 0.05$ *.

Figure 2.

MCC-555 induces NAG-1 and p21, and suppresses cyclin D1 expression. **A**, BxPC-3 cells were treated with DMSO or 10 μ M of various PPAR γ ligands for 24 h, and Western blot was performed with antibodies for NAG-1, p21, Cyclin D1 and Actin. **B**, **C**, BxPC-3 and AsPC-1 cells were treated with 10 μ M of MCC-555 at the indicated times and were treated with the indicated doses for 24 h. Western blot was performed with antibodies for NAG-1, p21, Cyclin D1 and Actin. N.S., non specific.

Figure 3. Cyclin D1, but not NAG-1 and p21, expression is involved in PPAR γ activation. **A**, Total RNAs were isolated from BxPC-3 and AsPC-1 cells after MCC-555 treatment. The expression of transcripts for NAG-1, p21 and Cyclin D1 was analyzed by qRT-PCR, normalized to GAPDH. Fold change compared to vehicle-treated cells are represented, vehicle-treated cells are set as 1. Error bars represent the mean $\pm$ S.D of three replicates. **B**, Effects of PPAR γ antagonist on MCC-555-induced NAG-1 and p21 up-regulation, and MCC-555-induced Cyclin D1 down-regulation. BxPC-3 cells were pretreated with GW9662 for 30 min at the indicated dose and then the cells were treated with 10 μ M of MCC-555 for 24 h. Total proteins were isolated for Western blot analysis. Equal loading was confirmed by Actin.

Figure 4.

Identification of the cis-acting elements responsible for MCC-555-induced *NAG-1* transactivation. **A**, Indicated constructs of the *NAG-1* promoter (0.5 μ g) and *pRL-null* (0.05 μ g) were transfected into BxPC-3 cells for overnight. The transfected cells were exposed to MCC-555 for 24 h. The promoter activity was measured as a ratio of firefly luciferase activity/Renilla luciferase activity. The results are the mean $\pm$ S.D of three replicates. $P < 0.05^*$ and $P < 0.01^{**}$, compared to DMSO treated sample. RLU, relative luciferase unit. **B**, BxPC-3 cells were transfected with each internal deletion construct of -133/+41 *NAG-1* and then treated with vehicle or 10 μ M MCC-555 for 24 h. The x-axis shows fold induction relative to vehicle-treated samples. Data were analyzed using Tukey's multiple comparison test; mean with same letters indicate no significance ($P < 0.05$).

Figure 5.

MCC-555-induced KLF4 is responsible for NAG-1 and p21 expression. **A, B**, BxPC-3 cells were treated with MCC-555 at indicated time and dose. Protein lysates were harvested and subjected to Western blot analysis using KLF4, Sp1, Sp3, Zf9, BTEB1, MAZ, NAG-1, and Actin antibodies. **C**, BxPC-3 cells were transfected with either control or KLF4 siRNA using PepMute siRNA Transfection reagent according to the manufacturer's protocol. Cells were serum starved overnight and treated with DMSO or MCC-555 for 24 h, followed by Western analysis. **D**, Chromatin immunoprecipitation assay was performed using a DNA-protein complex treated with 10 μM MCC-555 for 6 h as described in Materials and Methods. The Sp1 binding sites of the human *NAG-1* promoter (-133/+41) were amplified by PCR primer pairs (arrows). The input represents PCR products obtained from 1% aliquots of chromatin pellets before immunoprecipitation.

Figure 6.

Schematic diagram of cell growth inhibition induced by MCC-555 in human pancreatic cancer cells. MCC-555 inhibits cell growth via multiple pathways. In addition to Cyclin D1 down-regulation via the PPAR γ -dependent pathway, KLF4 is up-regulated via the PPAR γ -independent pathway that is responsible for the MCC-555-induced NAG-1 and p21 expression, leading to cell growth inhibition in human pancreatic cancer cells.