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## A reciprocal relationship exists between non-steroidal anti-inflammatory drug-activated gene-1 (NAG-1) and cyclooxygenase-2

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### Abstract

Non-steroidal anti-inflammatory drug (NSAID)-activated gene-1 (NAG-1) and COX-2 are involved in cellular processes such as inflammation, apoptosis, and tumorigenesis. To address the relationship between COX-2 and NAG-1 expression, we investigated the expression of NAG-1 and COX-2 in normal and tumor tissue from human patients, *Apc*<sup>Min/+</sup> mice, and COX-2(–/–) mice. While COX-2 expression is highly induced in tumor tissue, NAG-1 expression is reduced. Furthermore, PGE<sub>2</sub> reduces NAG-1 while celebrex induces NAG-1 expression. The results suggest that a possible inverse relationship exists between the expression of NAG-1 and COX-2 in tumor formation of colon tissue.

### Keywords

GDF-15; NAG-1; COX-2; Colorectal cancer

### 1. Introduction

Colorectal cancer (CRC) represents the third leading cause of cancer-related deaths in both men and women in the United States [1]. Epidemiological studies have shown that patients who use non-steroidal anti-inflammatory drugs (NSAIDs) for 10–15 years have a reduced risk of developing colorectal cancer [2]. NSAIDs inhibit two enzymes involved in prostaglandin biosynthesis: cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2), reduce the number and size of adenomas in patients with familial adenomatous polyposis (FAP), and prevent the development of colon cancer in *Apc*<sup>Min/+</sup> mice [3]. The chemopreventive and anti-tumorigenic activities of NSAIDs against CRC and other cancers

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are well established. Emerging clinical and experimental evidence now supports an anti-tumorigenic efficacy of NSAIDs in CRC [4,5] and implicates the contribution of COX-2 and one of its metabolites, prostaglandin E<sub>2</sub> (PGE<sub>2</sub>), in CRC development [6,7]. Celecoxib, a COX-2 specific inhibitor, reduces polyp burden in FAP patients [8]. However, other evidence suggests that COX-2 may not be the only target for chemoprevention by NSAIDs as NSAIDs also inhibit tumor growth through COX-independent pathways [2].

Previously, we showed that NSAIDs modulate the expression of several genes, including non-steroidal anti-inflammatory drug-activated gene (NAG-1), also known as MIC-1, GDF-15, PTGFB, PLAB, and PDF [9]. NAG-1 is a member of the transforming growth factor (TGF- $\beta$ ) super-family and is involved in cellular processes such as inflammation, apoptosis, and anti-tumorigenesis [9]. NAG-1 expression is up-regulated by several NSAIDs independently of prostaglandin formation in human colorectal cancer cells [9]. In addition, anti-tumorigenic compounds, such as resveratrol, genistein, and a peroxisome proliferator-activated receptor (PPAR- $\gamma$ ) ligand, troglitazone, induce NAG-1 expression [10]. NAG-1 is regulated by p53, Egr-1, and AKT, and may have tumor suppressor activity [11]. Indeed, NAG-1 transgenic mice that ubiquitously express the human protein are less susceptible to both chemical and genetically induced intestinal cancer [12].

NAG-1 is expressed in mature intestinal epithelial cells; however its expression is significantly reduced in human colorectal carcinoma samples [13]. A similar finding of reduced NAG-1 expression was observed in neoplastic intestinal polyps of Apc<sup>Min/+</sup> mice [13]. However, COX-2 is up-regulated in human colorectal tumors and is also expressed at higher levels in the neoplastic intestinal polyps of Apc<sup>Min/+</sup> mice [14–16]. These results suggest a possible inverse relationship between the expression of NAG-1 and COX-2 in intestinal tumor tissue, implying that the expression of one protein could cause the suppression of the other. In this study, we demonstrate that NAG-1 expression is greater in COX-2(-/-) mice compared to wild type littermates, but its expression is decreased in COX-2(-/-) mice treated with PGE<sub>2</sub>. Furthermore, treatment of COX-2 mice with doses of celecoxib that inhibit tumor growth induce NAG-1 expression.

## 2. Materials and methods

### 2.1. Animals

The development of COX-1 and COX-2 deficient mice has been previously reported [17,18]. These mice carry mutations on the genes encoding COX-1 and COX-2 and have been maintained on the 129/Ola/C57BL/6 background for more than 40 generations. COX-2(-/-) mice were given PBS (vehicle) or 0,150, or 300  $\mu$ g/mouse of PGE<sub>2</sub> twice daily by oral gavage for 7 days. Celecoxib was administered in the food for 4 weeks at 0, 1000, and 1500 ppm. The mice were then euthanized and the intestines removed for analysis.

Apc<sup>Min/+</sup> mice were purchased from Jackson laboratories (West grove, PA). The mice were euthanized by CO<sub>2</sub> inhalation after 4 h of fasting and the entire intestinal tract was removed, flushed with cold saline, and opened longitudinally. Both polyps and normal adjacent normal tissue were stored in RNAlater at 4 °C until RT-PCR analysis. Human NAG-1

transgenic (NAG-1<sup>Tg/Lox</sup>) mice were crossed with Apc<sup>Min/+</sup> mice to yield mice (Apc<sup>Min/+</sup>/NAG-1<sup>Tg/Lox</sup>) that have mutant Apc and express human NAG-1 in the intestinal tract [12].

## 2.2. Human colorectal tissue samples

Human colorectal tumor specimens were obtained from surgical resections by the Southern Division of the Cooperative Human Tissue Network. For each tumor sample, matched adjacent normal mucosa was collected for comparison. All samples were snap-frozen and stored in liquid nitrogen.

## 2.3. RNA isolation and reverse transcription

RNA was isolated from tissue using the RNeasy Mini Kit from Qiagen (Valencia, CA) according to the manufacturer's protocol. RNA was stored in a  $-80^{\circ}$  freezer until use. RNA was treated with 1 unit of amplification grade Deoxyribonuclease I (Life Technologies) per  $\mu$ g of RNA at room temperature for 15 min to remove genomic DNA. The addition of 2.5 mM EDTA and incubation at  $65^{\circ}$  C inactivated the Deoxyribonuclease I reaction. Reverse transcription (RT) was performed using Qiagen's Omniscript reverse transcription kit according to the manufacturer's protocol. A negative control containing all of the RT reagents in the absence of RT enzyme was routinely performed. After RT, cDNA was treated with 1 unit RNase H (Life Technologies) per  $\mu$ g RNA at  $37^{\circ}$  C for 20 min.

## 2.4. Real-time PCR

Real-time RT-PCR was performed in triplicate two or more times for each gene tested. Primer design (Table 1), deoxyribonuclease treatment, reverse transcription, and real-time RT-PCR assay using ABI Prism 7700 (Applied Bio-systems, Foster City, CA) were performed as previously described.

## 2.5. Western blotting

Proteins from mouse tissue and tissue culture cells were isolated as previously described [13], at 50  $\mu$ g and 20  $\mu$ g protein, respectively, separated by SDS-PAGE, and transferred onto nitrocellulose membranes. Blots were blocked overnight with 10% skim milk in TBS containing 0.1% Tween-20 (TBS-T), probed for 1 h at room temperature in 5% milk with TBS-T containing anti-NAG-1 (generated in this laboratory), anti-COX-2 (Cayman), or anti-actin (Santa Cruz Biotechnologies, Santa Cruz, CA), and washed in TBS-T. Anti-mouse NAG-1 was generated in this laboratory in rabbit from a specific peptide of the mouse NAG-1 C-terminus (DSGVSLQTYDDLVARG). The antibody recognized both the precursor and secreted forms of NAG-1. The membranes were treated with the appropriate horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnologies) in 5% milk with TBS-T for 1 h at room temperature, and then washed several times with TBS-T and the signal was detected by enhanced chemiluminescence (Amersham Biosciences) followed by autoradiography. When necessary, membranes were stripped by sealing in a plastic bag containing 62.5 mM Tris-HCl (pH 6.8), 2% (w/v) SDS, and 100 mM  $\beta$ -mercaptoethanol for 30 min with constant agitation at  $50^{\circ}$  C and washed with TBS-T prior to reprobing.

## 2.6. Statistical analysis

Statistical significance was determined according to a one-sided *t* test with a 0.05 level of significance on  $C_t$  values following adjustment for  $\beta$ -actin.

## 3. Results

### 3.1. NAG-1 expression is inversely associated with COX-2 expression in human colorectal cancer tissue and $Apc^{Min/+}$ polyps

We previously reported that the expression of NAG-1 on the surface epithelium of intestinal villi overlaps with regions that are undergoing apoptosis and is down-regulated in colon tumors as compared to normal adjacent tissue [13]. A comparison of human colorectal cancer and adjacent normal tissue revealed a reduction in NAG-1 expression in malignant tissue compared to normal tissue (Fig. 1). Real-time PCR analysis indicated that NAG-1 expression was reduced by approximately 65% in the colon cancer tissue as compared to the corresponding adjacent normal tissue (Fig. 1A). Because COX-2 expression is frequently up-regulated in colon cancer tissue [19,20], we measured COX-2 expression in colon cancer tissue and compared its expression to the adjacent normal tissue. RT-PCR analysis indicated that COX-2 expression was induced about eightfold in cancer tissue compared to normal tissue (Fig. 1B).

The findings of a possible reciprocal relationship between COX-2 and NAG-1 in human samples led us to examine if this relationship also existed in the  $Apc^{Min/+}$  mouse, a mouse model for intestinal cancer. Human colorectal polyps contain inactivating mutations in the *Apc* gene and  $Apc^{Min/+}$  mice, which have a mutation in the *Apc* gene, show increased formation of polyps in the small intestine. As shown in Fig. 1C, the expression of the NAG-1 pro-form and secreted form are reduced in  $Apc^{Min/+}$  polyps compared to normal tissue, whereas  $Apc^{Min/+}$  polyps had increased expression of COX-2 compared to normal tissue. Thus, there appears to be an inverse relationship between COX-2 and NAG-1 expression in both normal murine intestinal tissue and in tumors.

### 3.2. COX-2 expression was reduced in the intestinal polyps from $Apc^{Min/+}/NAG-1^{Tg/Lox}$ mice

To further investigate the expression of COX-2 and NAG-1 in normal and adenoma tissue,  $Apc^{Min/+}$  mice were mated with transgenic mice ( $NAG-1^{Tg/Lox}$ ) expressing human NAG-1. Previously we reported that  $Apc^{Min/+}/NAG-1^{Tg/Lox}$  mice had about a 40% reduction in both small intestine polyp numbers and tumor load [12] compared to littermate  $Apc^{Min/+}$  mice. These data indicate a role for NAG-1 in suppression of polyp formation in  $Apc^{Min/+}$  mice, and the effect of hNAG-1 expression on the expression of COX-2 was examined in the polyps of  $Apc^{Min/+}/NAG-1^{Tg/Lox}$  mice. Fig. 1D shows a low level of COX-2 expression in the normal intestinal tissue from both  $Apc^{Min/+}/NAG-1^{Lox/Lox}$  and  $Apc^{Min/+}/NAG-1^{Tg/Lox}$  mice. In contrast, the intestinal polyps from  $Apc^{Min/+}/NAG-1^{Lox/Lox}$  mice had higher expression of COX-2 compared to  $Apc^{Min/+}/NAG-1^{Tg/Lox}$  mouse polyps. These findings suggest that hNAG-1 may suppress polyp formation in  $Apc^{Min/+}$  mice, in part, by suppressing COX-2 expression, or that the reduced expression of COX-2 results in tumor growth inhibition, both suggesting an inverse relationship.

### 3.3. NAG-1 expression was up-regulated in COX-2(-/-) mice, but not in COX-1(-/-) mice

COX-1(-/-) and COX-2(-/-) mice, when crossed with *Apc*<sup>Min/+</sup> mice, have reduced intestinal polyps [14] and therefore, mNAG-1 expression in COX-1(-/-) and COX-2(-/-) was measured. As shown in Fig. 2, mNAG-1 mRNA expression was higher in COX-2(-/-) mice as compared to COX-2(+/+) littermate mice. However, there was no difference in the expression of mNAG-1 in COX-1(+/+) and COX-1(-/-) mice. These results suggest that COX-2 may act to suppress NAG-1 expression.

### 3.4. PGE<sub>2</sub> treatment suppressed NAG-1 expression in COX-2(-/-) mice

NSAIDs inhibit cyclooxygenase activity and lower the level of prostaglandins such as PGE<sub>2</sub>. Both COX-2 and PGE<sub>2</sub> are increased in intestinal tumor tissue, compared to normal tissue [21]. Furthermore, treatment of *Apc*<sup>Min/+</sup> mice with PGE<sub>2</sub> leads to enhanced development of intestinal adenoma growth [22]. To determine the effect of PGE<sub>2</sub> treatment on NAG-1 expression, COX-2(-/-) mice were treated with 0, 150, or 300 µg/mouse of PGE<sub>2</sub> twice daily for 7 days. These doses are reported to enhance polyp formation in mice [22]. PGE<sub>2</sub> treatment of the COX-2(-/-) mice resulted in a dose dependent suppression of NAG-1 mRNA and protein expression (Fig. 3). These results indicate that PGE<sub>2</sub> causes NAG-1 suppression and also further supports an inverse association between NAG-1 and COX-2.

### 3.5. NAG-1 expression in celecoxib-treated wild type and COX-2(-/-) mice

Celecoxib, a selective COX-2 inhibitor, has been used extensively to inhibit tumor growth [23–25] and induces NAG-1 expression in cancer cells. NAG-1 expression induced by celebrex appears to be mediated by blocking the activation of the AKT pathway [26], a pathway activated by PGE<sub>2</sub>. In addition, ATF-3 is also induced by NSAIDs and its expression is repressed in colorectal cancer tissue compared to normal tissue [27]. NAG-1 and ATF-3 are known downstream targets of GSK-3β [26,28] and an increase in ATF-3 expression causes apoptosis and altered growth of cells in a xenograft mouse model [27]. However, there are no reports in the literature investigating the effect of celecoxib on NAG-1 or ATF-3 expression *in vivo*. Therefore, we administered celecoxib to mice to determine its effects on NAG-1 and ATF-3 expression to access if prostaglandin formation altered the expression and if celebrex increased expression *in vivo*. COX-2(+/+) and COX-2(-/-) littermate mice were treated with 0, 1000, and 1500 ppm celecoxib for 4 weeks, at doses reported to suppress tumor growth [29]. Celecoxib treatment increased NAG-1 expression in a dose dependent manner in COX-2(-/-) mice and ATF-3 in wild type mice (Fig. 4). Thus, celebrex not only inhibits COX-2 and lowers PGE<sub>2</sub> levels, but also induces the expression of NAG-1 and ATF-3, suggesting both targets may play a role in the suppression tumor growth by this NSAID.

## 4. Discussion

NAG-1 was identified as a divergent member of the TGF-β superfamily from an NSAID-induced library in COX-2 negative colorectal cancer cells by a PCR-based subtractive hybridization [30] Many chemopreventive drugs, including COX inhibitors, up-regulate NAG-1 expression in human colorectal cells in culture and the increased NAG-1 expression

appears to mediate NSAID-induced increases in apoptosis. The up-regulation of mNAG-1 was observed in the intestinal tract and liver of mice fed tumor suppressive doses of sulindac [13]. Recently, we generated NAG-1 transgenic mice (Cre/NAG-1<sup>Tg/Lox</sup>) which express human NAG-1, and the anti-tumorigenic activity in mice expressing hNAG-1 was evaluated with the known colon carcinogen azoxymethane (AOM). NAG-1<sup>Tg/Lox</sup> mice had smaller numbers of aberrant crypt foci (ACF) in their colons compared to littermate controls. Furthermore, Apc<sup>Min/+</sup>/NAG-1<sup>Tg/Lox</sup> mice showed ~40% reduction in small intestine polyp numbers and in tumor load [12]. These findings suggest that NAG-1 may play a role in the development of intestinal polyps in mice and CRC in humans.

COX-2 expression in mouse models of intestinal cancer and in human CRC is well documented. Higher COX-2 expression is noted in human CRC and in several mouse models for intestinal cancer. Deletion of the COX-2 gene results in decreased tumor formation in the small intestine and colon of Apc<sup>Min/+</sup> and Apc<sup>716</sup> mice [14,31]. Hansen-Petrik et al. [21] reported that PGE<sub>2</sub> reversed NSAID-induced adenoma repression in Apc<sup>Min/+</sup> mice. Wang et al. [22] also reported that PGE<sub>2</sub> accelerated intestinal adenoma growth in Apc<sup>Min/+</sup> mice. PGE<sub>2</sub> exerts its effects by binding to its cognate receptors, EP<sub>1-4</sub>, which belong to the seven transmembrane G-protein coupled receptor family. PGE<sub>2</sub>, via the EP<sub>2/4</sub> receptors, activates the PI3 Kinase/AKT pathway and interacts with the EGFR pathway [32,33]. COX-2 expression causes transactivation of EGFR by enhancing phosphorylation of EGFR, leading to tumor growth. The evidence clearly indicates the importance of COX-2 and the EP receptors in the development of intestinal cancer.

Because COXs and NAG-1/ATF3 are targets for COX inhibitors and COX-2 is reported to be highly expressed in intestinal tumors, we decided to further investigate a possible relationship between the expressions of these two proteins. We first confirmed in human colorectal tissue samples the high expression of COX-2 and a corresponding lower expression of NAG-1. A similar pattern of expression was observed in normal tissue and polyps obtained from Apc<sup>Min/+</sup> mice, suggesting an inverse relationship between these two proteins in intestinal cancer. The expression of hNAG-1 in the Apc<sup>Min/+</sup> not only inhibited polyp formation [12], but also reduced COX-2 expression was observed in the polyps. NAG-1 could directly inhibit the expression of COX-2, but more likely the decrease in COX-2 is the result of suppression of tumor development by NAG-1. However, we do not know if the expression of COX-2 in normal cells would suppress NAG-1 expression. Higher mNAG-1 expression was observed in the intestinal tract of COX-2(-/-), but not in the COX-1(-/-) as compared to littermate controls. Furthermore, treatment of COX-2(-/-) mice with PGE<sub>2</sub> at doses that increase polyp formation and activate the PI3 K/AKT/GSK-3 $\beta$  pathway caused a dose dependent decrease in the expression of mNAG-1. Thus, based on these data, we proposed that the increase in COX-2 expression in the tumor suppresses the expression of NAG-1.

NAG-1 is an important downstream target of the tumor suppressor gene p53 and Egr-1 [11] and is up-regulated following inhibition of PI3 K/AKT/GSK-3 $\beta$  pathway [26]. Activation of the PI3 K/AKT/GSK-3 $\beta$  pathway by PGE<sub>2</sub> formed by COX-2 would result in lower expression of NAG-1 in tumors expressing COX-2. We have previously shown that some NSAIDs increase NAG-1 expression *in vitro* [9]. Sulindac, administered in drinking water at



doses that reduce tumor formation in mice, induces mNAG-1 expression in the liver and colon [12]. Celebrex increases the expression of NAG-1 in a number of cells in culture by inhibiting the phosphorylation, and hence activation, of AKT [24]. Treatment of COX-2(+/+) and COX-2(-/-) mice with celebrex caused a dose dependent increase in the expression of NAG-1 and another downstream target of AKT, ATF-3, in intestinal tissue. Modulation of AKT activity either by inhibition with celebrex or other chemicals, or by activation via the EP2/4 receptors via increases in COX-2, can increase or decrease, respectively, the expression of NAG-1 in intestinal tissue, thereby altering the development of intestinal tumors.

We suspect that many COX-2 inhibitors have two cellular targets that contribute to the inhibition of tumor growth. One is COX-2, the inhibition of which reduces the levels of prostaglandins. A second is NAG-1, whose induced expression is mediated by inhibition of AKT activation. Thus we suspect both of these targets may contribute to the inhibition of intestinal cancer by the long term use of COX inhibitor, celebrex.

COX-2 selective inhibitors have been touted for their possible use as chemoprevention agents. However, recent concerns over the safety of COX-2 inhibitors have prompted researchers to develop more effective agents with minimal toxicity. Understanding the molecular mechanism for the inhibition of tumor development will help to identify specific safer targets. NAG-1 may play an important role as a potential candidate.

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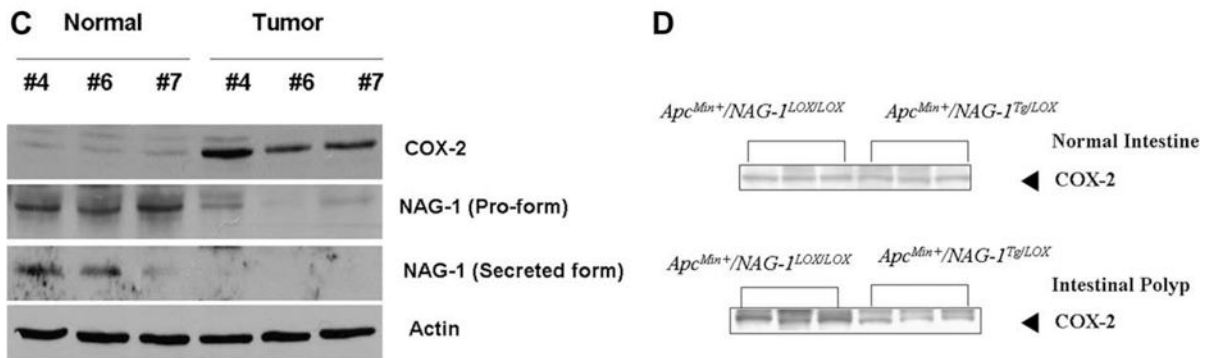
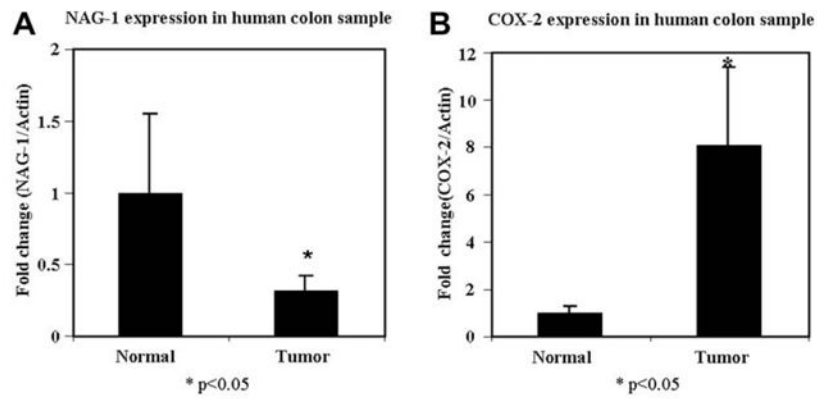
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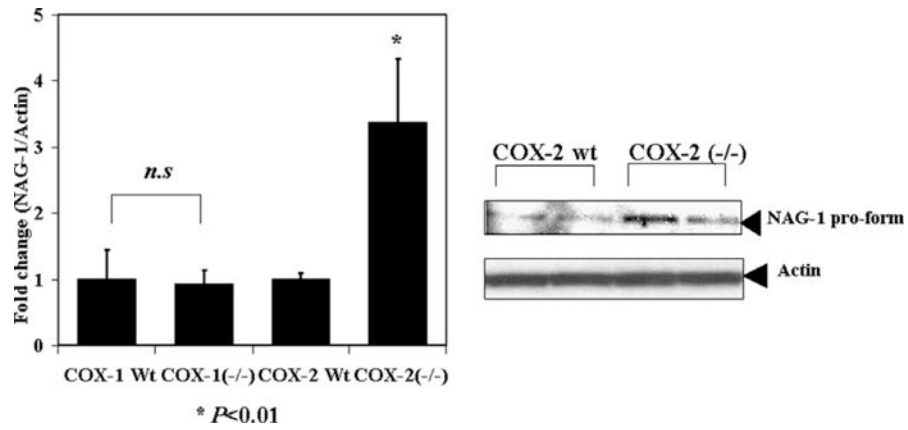
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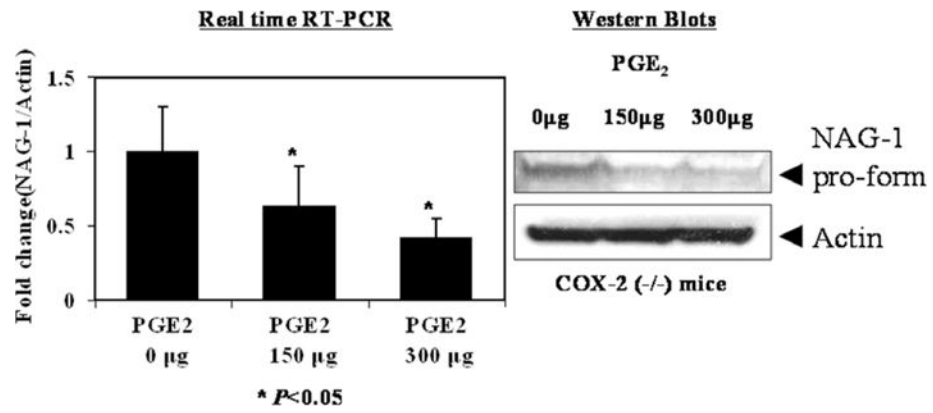
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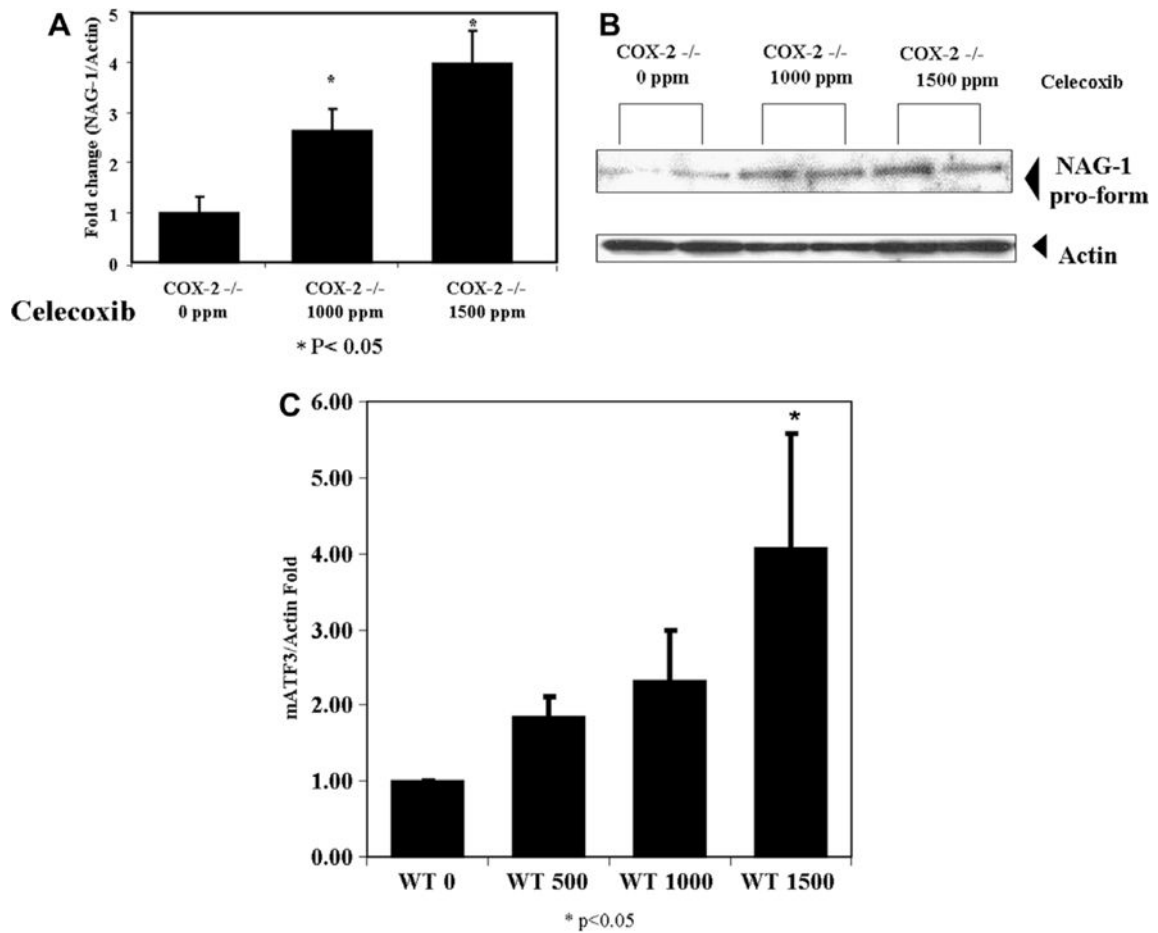
**Fig. 1.** The protein and mRNA level of NAG-1 was up-regulated in normal tissue while COX-2 expression was up-regulated in neoplastic tissue. (A) and (B) NAG-1 and COX-2 mRNA expressions in normal and neoplastic tissue from human colon samples. Values are means ( $\pm$ SEM) and are expressed as fold induction relative to control. Five pairs of samples were analyzed in five replicates and differed at  $P < 0.05$  compared with control group. (C) Western blot of COX-2 and NAG-1 proteins in normal and tumor tissue of *Apc<sup>Min/+</sup>* mouse. (D) Expression of COX-2 in normal and tumor tissue of *Apc<sup>Min/+</sup>/NAG-1<sup>Tg/Lox</sup>*.



**Fig. 2.** The protein and mRNA levels of NAG-1 were up-regulated in COX-2(-/-) mice, but no change was observed in COX-1 (-/-) mice. NAG-1 expression in COX-1/COX-2(+/+) and (-/-) mice as measured by Western blot and real-time RT-PCR. Values are means ( $\pm$ SEM) and are expressed as fold induction relative to control. Each group consists of at least five mice. (*n.s.* not significant, \* $P < 0.01$  compared with control group).



**Fig. 3.** PGE<sub>2</sub> suppressed NAG-1 expression in COX-2(-/-) mice. NAG-1 mRNA expression was quantified by real time RT-PCR in COX-2(-/-) mice treated with vehicle or 0,150, and 300 μg of PGE<sub>2</sub> in 100 μl sterile PBS by twice daily gavage for 7 days. Values are means (±SEM) for four mice in each group and are expressed as fold induction relative to control. \*  $P < 0.05$  compared with control group.



**Fig. 4.** The expression of NAG-1 in Celecoxib treated COX-2(-/-) mice. (A) and (B) NAG-1 mRNA and protein expression in COX-2 knockout mice and (C) ATF-3 mRNA expression in COX-2 wild type mice treated with 0,1000, and 1500 ppm celecoxib for 4 weeks. Western blot and real time RT-PCR analyzes are relative to 0 ppm treated mice and adjusted for  $\beta$ -actin. Values are means ( $\pm$ SEM) for three mice in each group and are expressed as fold induction relative to control (\*  $P < 0.05$  compared with control group).

**Table 1**

The list of primers.

<b>Primer</b>	<b>Forward Sequence</b>	<b>Reverse Sequence</b>
Mouse NAG-1	5' TTCTGTGGGACGGTCAG 3'	5' CGGGTGACCAGGCTAATC 3'
Mouse Actin	5' TGACAGGATGCAGAAGGAGA 3'	5' CGCTCAGGAGGAGCAATG 3'
Human NAG-1	5' TGCCCGCCAGCTACAATC 3'	5' TCTTTGGCTAACAAAGTCATCATAGGT 3'
Human Actin	5' CCTGGCACCCAGCACAAAT 3'	5' GCGGATCCACACGGAGTACT 3'
Mouse COX-1	5' CCTCTTTCCAGGAGCTCACA 3'	5' TCGATGTCACCCGTACAGCTC 3'
Mouse COX-2	5' GCTCTTCCGAGCTGTGCT 3'	5' GATTGGAACAGCAAGGATTG 3'

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