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OXIDATIVE STRESS-INDUCED TUMORIGENESIS IN THE SMALL INTESTINES OF DNA REPAIR-DEFICIENT MICE

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Oxygen radicals are produced through normal cellular metabolism, and the formation of such radicals is further enhanced by radiation and by various chemicals. Oxygen radicals attack DNA and its precursor nucleotides, and consequently bases with various modifications are introduced into the DNA of normally growing cells. One such modified base, 8-oxo-7, 8-dihydroguanine (8-oxoG) is highly mutagenic because of its ambiguous pairing property. Three enzymes, MTH1, OGG1, and MUTYH, play important roles in avoiding 8-oxoG-related mutagenesis in mammalian cells (Figure 1)[1-4].

We have established an experimental system for oxidative DNA damage-induced mutagenesis and tumorigenesis in the gastrointestinal tracts of mice[5]. Oral administration of an oxidizing reagent, potassium bromate ($KBrO_3$), effectively induced G:C to T:A transversions and epithelial tumors in the small intestines of *Mutyh*-deficient mice, implying the significance of Mutyh in the suppression of mutagenesis and tumorigenesis induced by oxidative stress (Figure 2, 3). We performed mutation analysis of the tumor-associated genes amplified from the intestinal tumors developed in four mutant mice that had been treated with $KBrO_3$. Many tumors had G:C to T:A transversions in either *Apc* or *Ctnnb1*. No mutations were found in either *K-ras* (exon 2) or *Trp53* (exon 5-8). Our findings confirm the association between MUTYH-deficiency and the recessive form of hereditary multiple colorectal adenoma/carcinoma in humans, known as MUTYH-associated familial adenomatous polyposis[6], with the characteristic feature; G:C to T:A transversions in the GAA sequence context. Also, our results suggest that the abnormality in Wnt signal transduction pathway is causatively associated with oxidative stress induced tumorigenesis in the small intestines of *Mutyh*-deficient mice (Figure 4). In addition, the

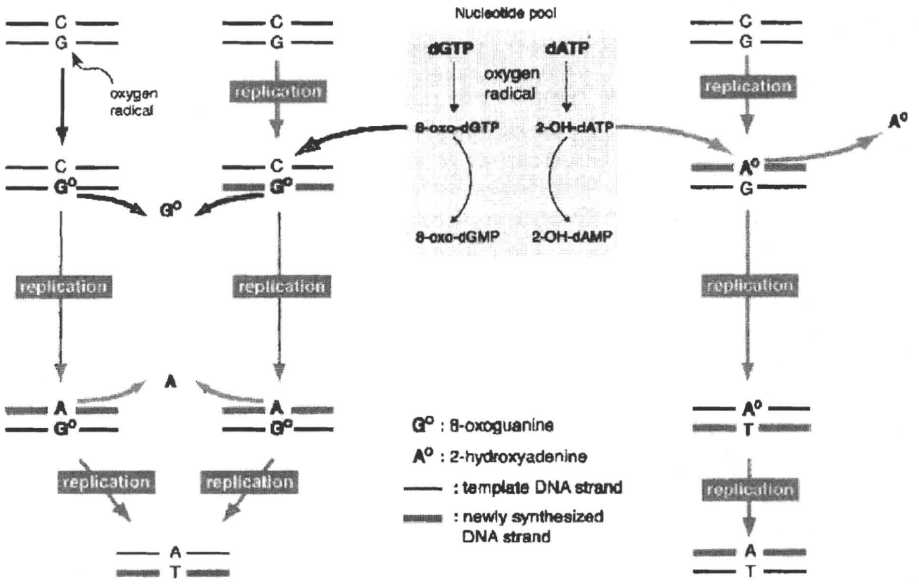
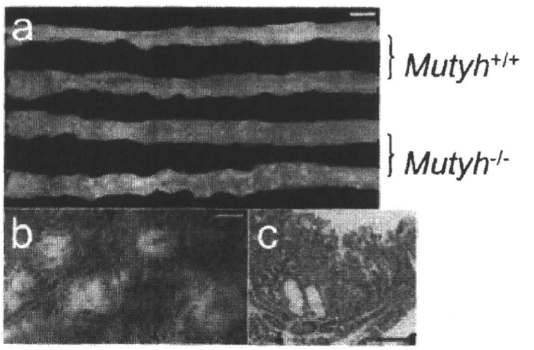


Figure 1 Mutagenesis induced by oxidative DNA and its avoiding mechanisms in mammals. Among the various types of oxidative damage in DNA, the oxidized forms of guanine and adenine, 8-oxo-7,8-dihydroguanine and 1,2-dihydro-2-oxoadenine, can form relatively stable base pairs with either adenine or guanine in DNA, respectively. During DNA replication, they are thought to induce spontaneous mutagenesis, such as A: T to C: G and G: C to T: A transversions. The direct oxidation of DNA by reactive oxygen species has been reported to generate a substantial amount of 8-oxo-7,8-dihydroguanine but little 1,2-dihydro-2-oxoadenine. In contrast, 1,2-dihydro-2-oxoadenine is generated exclusively by the oxidation of dATP in the nucleotide pool. Studies on mutator mutants have revealed that *Escherichia coli* has several error-avoiding mechanisms that minimize the deleterious effects of 8-oxo-7,8-dihydroguanine, and in which MutT, MutM and MutY proteins play important roles. MutT protein hydrolyzes 8-oxo-dGTP to 8-oxo-dGMT and pyrophosphate, thus avoiding the occurrence of A: T to C: G transversion mutations during DNA replication. MutM and MutY proteins are DNA glycosylases, the former excises 8-oxoG paired with cytosine whereas the latter removes adenine paired with 8-oxo-7,8-dihydroguanine. Mammalian cells are also equipped with elaborate error-preventing mechanisms similar to those found in prokaryotes; MTH1as a MutT homolog, OGG1 as a functional homolog for MutM, and MUTYH (MYH) as a MutY homolog. Recent studies showed that MTH1 effectively hydrolyzes 2-OH-dATP as well as 8-oxo-dGTP, while MUTYH has the ability to excise 1,2-dihydro-2-oxoadenine inserted opposite guanine in the template strand as well as the ability to remove adenine incorporated opposite 8-oxo-7,8-dihydroguanine in the template. As a result of the cooperative action among MTH1/OGG1/MUTYH and other repair pathways, mammalian cells effectively protect the occurrence of spontaneous mutations such as A: T to C: G and G: C to T: A transversions, which are caused by 8-oxo-7,8-dihydroguanine and1,2-dihydro-2-oxoadenine.

multiple formation of tumor in the small intestines of *Mutyh*-deficient mice provides a suitable model system to investigate the processes of intestinal tumorigenesis.

Xie *et al.* showed that *Mutyh/Ogg1* double-deficient mice predominantly developed lung and ovarian tumors as well as lymphomas. They also showed that 8.6% of *Mutyh/Ogg1* double-deficient mice exhibited adenomas/carcinomas in their gastrointestinal tracts, which were not observed in wild-type mice[7]. We and other groups have previously reported that there was little difference in the number of intestinal tumors in wild-type and Ogg1-null mice, although an Ogg1 deficiency resulted in 8-oxoG buildup in genomic DNA and an elevated mutation frequency in the latter[4,8,9]. Thus, the development of intestinal tumors in *Mutyh/Ogg1* double-deficient mice supports the notion that having a Mutyh deficiency does indeed increase susceptibility to intestinal tumorigenesis regardless of the genetic background or environmental factors.

I. $KBrO_3$-induced intestinal tumors in wild-type and *Mutyh*-deficient mice



II. Frequency of $KBrO_3$-induced intestinal tumors in wild-type and *Mutyh*-deficient mice (tumors/mouse)

Genotype	Sex	Mean ± S.D.
Mutyh $^{+/+}$	♂	0.50 ± 0.55
	♀	1.00 ± 0.71
Mutyh $^{-/-}$	♂	72.75 ± 24.24
	♀	51.00 ± 28.35

Figure 2 $KBrO_3$-induced tumors in the small intestine of *Mutyh*-deficient mice.
I. a, The upper part of the small intestines (duodenum and a part of jejunum) from $KBrO_3$-treated mice are shown. Multiple polyp formations are observed in the $KBrO_3$-treated *Mutyh*-deficient mice (-/-), but not in the treated wild-type mice (+/+). Upper: female, lower: male. **b,** A high-power view of the polyps in the $KBrO_3$-treated *Mutyh*-deficient female mouse. **c,** A section of the $KBrO_3$-induced polyp stained with haematoxylin and eosin. Scale bars; **a**: 1 cm, **b:** 1 mm, **c**: 100 μm. **II.** $KBrO_3$ was administered to wild-type and *Mutyh*-deficient mice in their drinking water for 16 weeks. Body weight and water consumption were monitored weekly. The animals were sacrificed at the age of 20 weeks, and their intestines were removed and fixed in 4% paraformaldehyde fixative. Microscopic inspection for tumor formation was performed (adopted from reference 5).

1) Familial adenomatous polyposis with a recessive trait

- Patients have germ-line mutations in the *MUTYH* gene (About 80% of the mutations were identified as Y165C or G382D in the patients of Europe and USA)
- Onset: 50 years of age (median, range:16~59 years of age) *
- No. of tumors: 40/colon (median, range: 18~100 tumors) *

*United Kingdom patients (156) with multiple (5~100) adenomas

2) Tumors from the patients all showed G:C to T:A mutations in the *APC* gene.

3) Almost all the G:C to T:A mutations occur in the GAA sequence context of the *APC* gene

Figure 3 MutYH-associated polyposis (MAP).

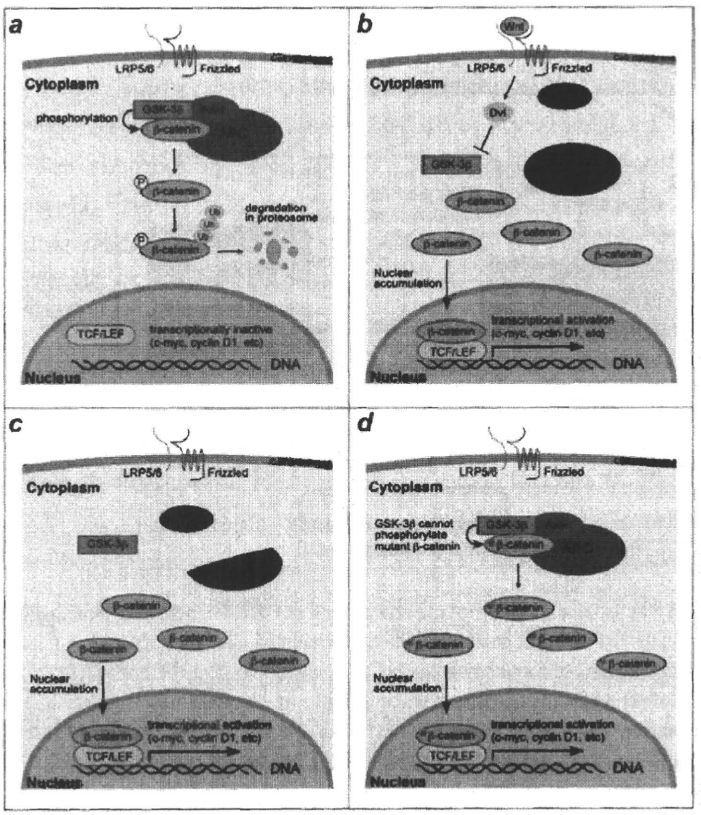


Figure 4 Wnt signaling pathway (canonical).
a) In the absence of Wnt signaling, β-catenin is phosphorylated by GSK3β in a complex with axin and Apc, and is subsequently ubiqutinated and degradated in the proteasome. *b*) In the presence of Wnt signaling, activated Dishevelled (Dvl) inhibits phoshorylation of β-catenin, consquently leading to stabilization of β-catenin. Nuclear accumulated β-catenin activates transcription of the target genes. Mutations in either APC (*c*) or in the phosphorylation sites of β-catenin (*d*) abolish the phoshorylation of β-catenin, which in turn leads to an accumulation of nuclear β-catenin, thereby up-regulating the expression of the target genes without Wnt signaling, and resulting in carcinogenic cell growth.

It is of interest that the deficiency of Mutyh but not Ogg1 makes mice susceptible to intestinal tumorigenesis, although the deficiency of either Mutyh or Ogg1 increases G:C to T:A transversion at almost equal frequency in the small intestines of mice. It is possible that this difference may be attributed to the additional substrate; MUTYH excises 2-hydroxyadenine, an oxidized adenine, paired with guanine, beside adenine paired with 8-oxoguanine, from DNA. However, we recently reported the involvement of Mutyh in cell death caused by oxidative DNA damage[10]. Thus, the defect in Mutyh would simultaneously compromise both DNA repair and cell-death induced by oxidative DNA damage (Figure 5). This may explain why among the factors involved in suppressing oxidative damage-induced mutagenesis, only MUTYH is, so far, identified to be associated with hereditary colorectal cancers in humans.

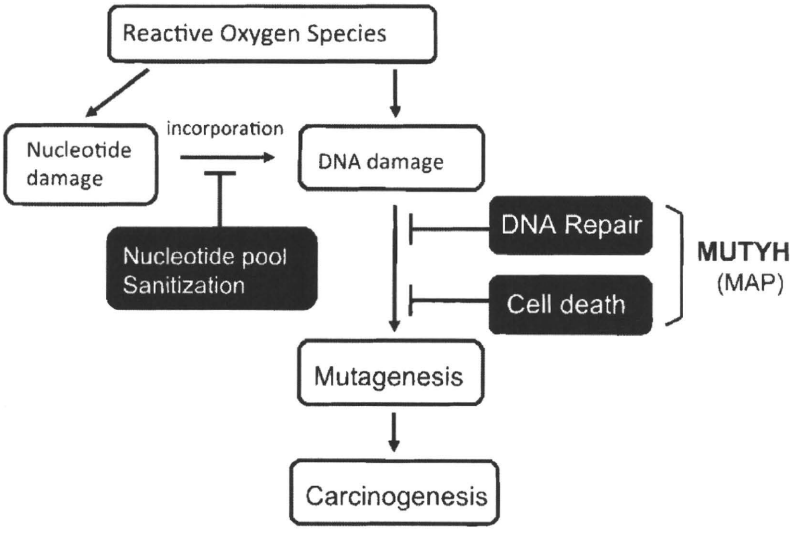


Figure 5 The roles of MUTYH in the avoiding mechanisms for ROS-induced mutagenesis and carcinogenesis.
The defect in Mutyh simultaneously compromise both DNA repair and cell-death induced by oxidative DNA damage. Thus, the defect in Mutyh makes mice highly susceptible to oxidative stress-induced tumorigenesis. This may provide molecular bases for explaining why among the factors involved in suppressing oxidative damage-induced mutagenesis, only MUTYH is, so far, identified to be associated with hereditary colorectal cancers in humans.

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Mini review

MicroRNA, SND1, and alterations in translational regulation in colon carcinogenesis

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ABSTRACT

Post-transcriptional regulation of gene expression by microRNA (miRNA) has recently attracted major interest in relation to its involvement in cancer development. miRNA is a member of small non-coding RNA, consists of 22–24 nucleotides and regulates expression of target mRNA species in a post-transcriptional manner by being incorporated with RNA-induced silencing complex (RISC). *Staphylococcal* nuclease homology domain containing 1 (SND1), a component of RISC, is frequently up-regulated in human colon cancers and also chemically induced colon cancers in animals. We here showed that SDN1 is involved in miRNA-mediated gene suppression and overexpression of SND1 in colon cancer cells causes down-regulation of APC without altering APC mRNA levels. As for the miRNA expression profile in human colon cancer, *miR-34a* was among the list of down-regulated miRNA. Expression of *miR-34a* is tightly regulated by p53, and ectopic expression of *miR-34a* in colon cancer cells causes remarkable reduction of cell proliferation and induces senescence-like phenotypes. *MiR-34a* also participates in the positive feedback loop of the p53 tumor suppressor network. This circuitry mechanism for p53 activation is of interest in understanding the tumor suppressive function of *miR-34a* in colon carcinogenesis. miRNA should also be considered as novel anti-cancer agents in tumor suppressive therapeutic applications.

1. Introduction

Colon cancers have been demonstrated to develop after multistep accumulation of genetic and epigenetic alterations, accompanying substantial changes in global gene expression profiles as a consequence [1–3]. Genetic alterations, such as chromosomal translocation, gene amplification, copy number aberrations, and point mutations, result in activation or inactivation of genes involved in cancer development [1]. Epigenetic alterations, such as hyper- or hypo-methylation of CpG sites in promoter regions of genes and modification of histone also contribute to the substantial changes of gene expression profiles in cancer cells [2,3].

Recently, post-transcriptional regulation of gene expression by microRNA (miRNA) has attracted major attention among cancer researchers in relation to its involvement in cancer development [4]. Indeed, altered expression of miRNA has been found in almost all human cancers [5–8]. miRNA, which is a member of small non-coding RNA, consists of 22–24 nucleotides, and pairs with complementary sequences located mainly in the 3′ untranslated regions of target mRNAs and regulates gene expression in a post-transcriptional manner by being incorporated with RNA-induced silencing complex (RISC) [9], and contributes significantly to the development of human carcinogenesis [5–8]. Interestingly, we frequently observe upregulation of *Staphylococcal* nuclease homology domain containing 1 (SND1) in colon carcinogenesis, even at early stages [10,11]. SND1 is one of the components of RISC and its disruption was shown to cause perturbation of small interfering RNA-induced gene silencing [12]. In this article, we describe the possible involvement of miRNA and its effector complex SND1 in colon carcinogenesis *via* post-transcriptional regulation of gene expression.

2. SND1, a component of RISC, is a regulator of gene expression

SND1, also known as Tudor-SN and p100, is a highly conserved protein from yeast to humans and is suggested to be associated with multiple steps involved in the regulation of gene expression, includ-

Abbreviations: APC, adenomatous polyposis coli; AT1R, angiotensin II type I receptor; HA, hemagglutinin; Luc, luciferase; miRNA, microRNA; RISC, RNA-induced silencing complex; SA-β-gal, senescence-associated β-galactosidase; SIRT1, silent information regulator 1; SND1, staphyloccal nuclease homology domain containing 1; 3′-UTR, 3′-untranslated region.

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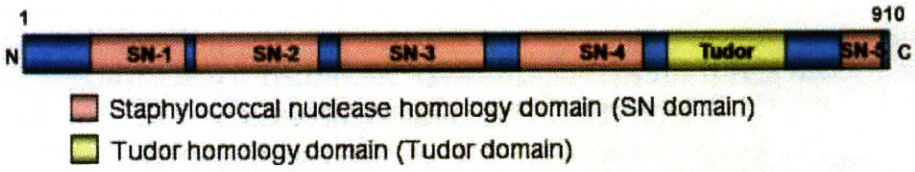

Fig. 1. Structural feature of SND1 protein. SND1 is composed of 910 amino acid residues, has 4 complete (SN-1–4) and one incomplete (SN-5) *Staphylococcal* nuclease homology domains and a Tudor homology domain.

ing transcription and pre-mRNA splicing, as well as translation and RNA interference, as detailed later. SND1 has four complete and one incomplete sets of repeats of *Staphylococcal* nuclease homology domains (SN domain), and a Tudor homology domain (Tudor domain) in its C-terminal half (Fig. 1). Whereas the biological function of these structural domains remains to be elucidated, it was recently reported that the Tudor domain is a binding platform for the peptide containing dimethylated lysines [13] with high affinity and the repeats of SN domains are required for binding to RNA [14].

SND1 was originally identified as a protein interacting with the Epstein-Barr virus nuclear antigen 2 (EBNA2), and promotes EBNA2-dependent transcription [15,16]. The protein was also reported to interact with some transcriptional regulators, including c-Myb, STAT5 and STAT6, suggesting its biological role as a transcriptional co-activator [17–19]. SND1 was also demonstrated to be involved in pre-mRNA splicing through the enhancement of spliceosome formation by interacting with snRNP proteins [20,21]. It was also reported that SND1 regulates translation of angiotensin II type I receptor (AT1R) by binding to the 3′ UTR of its mRNA [22].

Recently, Caudy et al. showed that SND1 is one of the components of RISC, and disruption of SND1 in *Caenorhabditis elegance* was demonstrated to cause defects in siRNA mediated gene silencing [12]. More recently, SND1 was further shown to be a component of *let-7* directed RISC [23]. Although the biological function of SND1 is not fully determined, these intriguing findings suggested the possible involvement of SND1 as a key regulator for gene expression at both transcriptional and post-transcriptional levels [24].

3. Possible involvement of SND1 in activation of the Wnt signaling pathway in colon carcinogenesis

We recently reported up-regulation of SND1 in human colon cancers and also in colon cancers induced in rat by chemical carcinogens, such as a food-borne carcinogen, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine, and an alkylating agent, azoxymethane [11]. The expression of *SND1* mRNA in colon cancer tissues was remarkably high, being 5-fold or even greater, compared with their normal counterparts. Interestingly, cytoplasmic accumulation of SND1/Snd1 protein was also observed in aberrant crypt foci, putative pre-cancerous lesions of the colon [11].

Stable over-expression of recombinant Snd1 in rat intestinal epithelial cells, IEC6, lead to the enhancement of cell proliferation and anchorage-independent growth activity in a soft agar colony formation assay, accompanying the subcellular translocation of E-cadherin to the cytoplasm. This suggests that up-regulation of SND1 may induce loss of contact inhibition and thereby promotes cell proliferation. Interestingly, in Snd1-transfected cells, considerable reduction of Apc protein by 25–75% was observed without significant changes in its mRNA level [11]. Exogenous and transient introduction of SND1 into human cancer cell lines, HCT 116, SW48 and HeLa cells, harboring wild type APC, induced down-regulation of the APC protein without altering its mRNA level (data not shown), and also caused up-regulation of the β-catenin protein (Fig. 2A). Furthermore, knockdown of SND1 in SW48 cells caused restoration of APC protein levels (Fig. 2B). Taking all these results together, over-expression of SND1 may induce activation of the Wnt-β-catenin signaling pathway through down-regulation of the APC protein, even at early stages of colon carcinogenesis.

4. SND1 serves as a regulator for miRNA-mediated gene silencing

As for the mechanism for the post-transcriptional regulation of gene expression by SND1, the fact that SND1 binds to APC mRNA [11] leads to the intriguing scenario that down-regulation

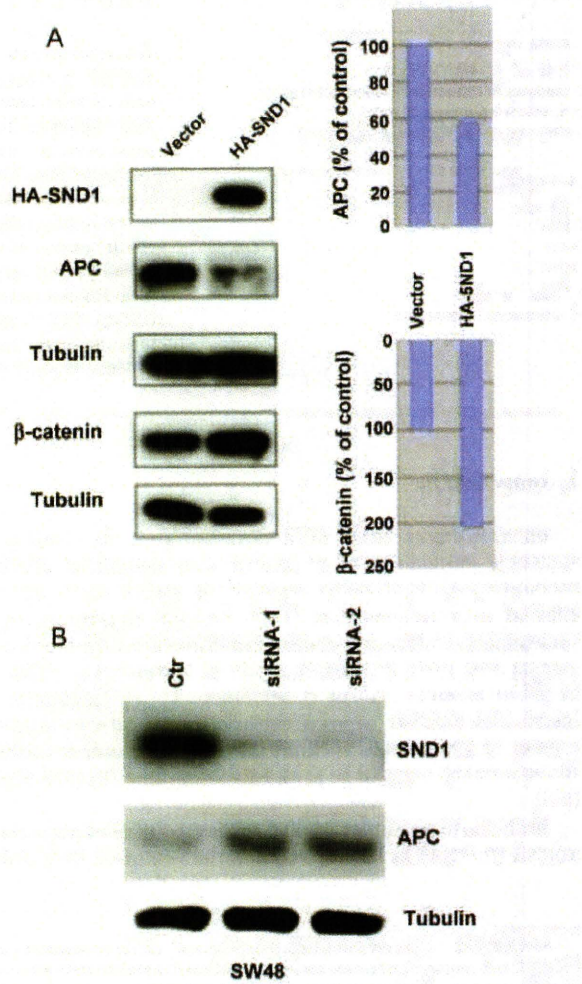

Fig. 2. SND1 is involved in the regulation of APC expression. (A) HeLa cells were transfected with either a mammalian expression plasmid containing HA-tagged SND1 (HA-SND1) cDNA or control vector [pCDA-HA] and propagated for 48 h. The protein levels of APC and β-catenin were analyzed by immunoblotting following the standard protocol. Graphs indicate the relative values of signal intensities of the bands for APC or β-catenin normalized to that of tubulin. (B) SW48 colon cancer cells were transfected with SND1 siRNAs (Qiagen) or control siRNA (ctr; Qiagen) for 48 h. After incubation, the protein levels of SND1 and APC were determined by immunoblot analysis.

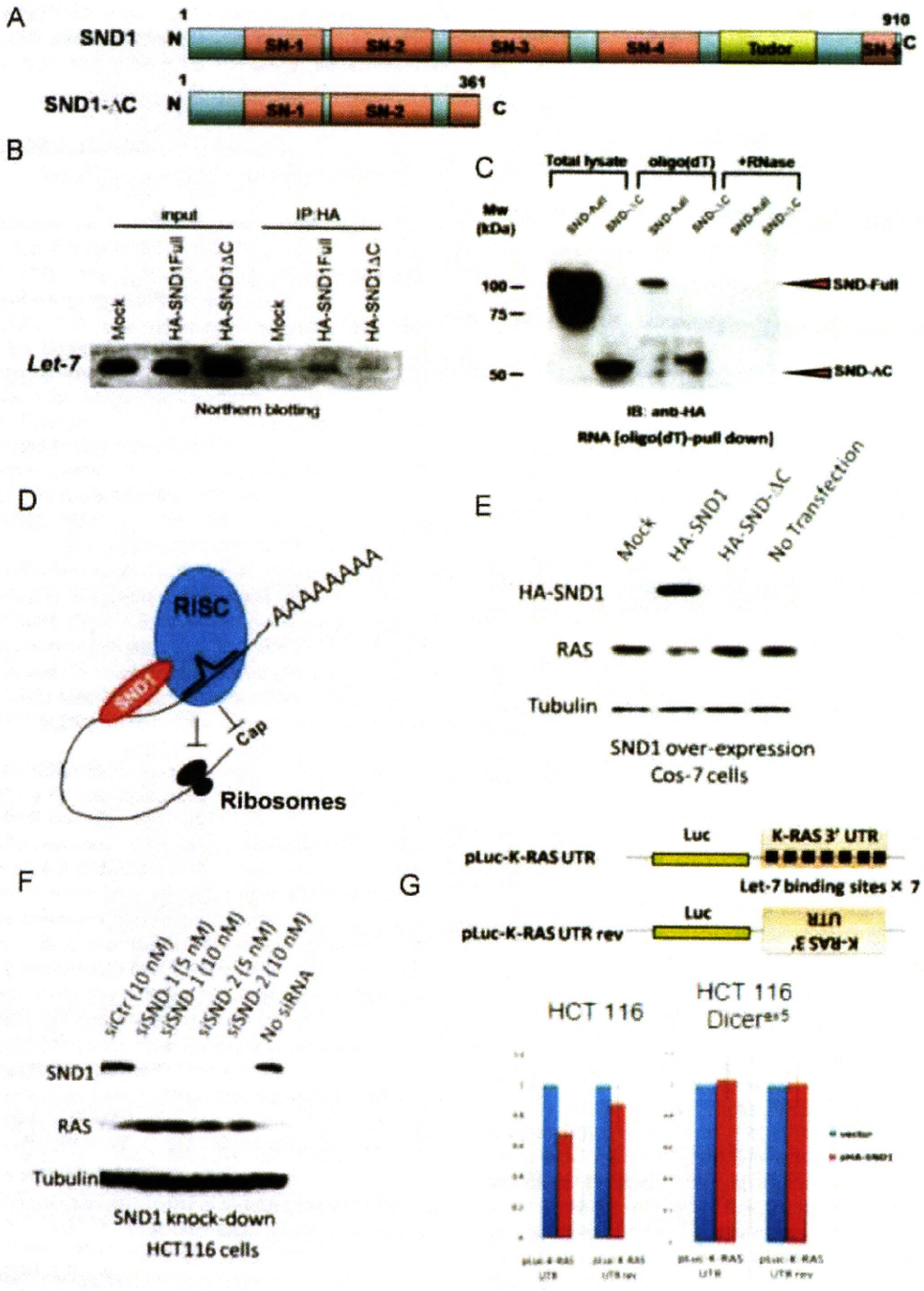

Fig. 3. Association of SND1 with miRNA-mediated gene silencing. (A) Structural features of the full length and ΔC form of SND1 deleting the C-terminal half. (B) Interaction of SND1 with *let-7* miRNA. Cell extracts of Tet-on HeLa cells (Clontech), expressing the full length or ΔC form of HA-SND1, were subjected to immunoprecipitation using anti-HA antibody. RNAs bound to either the full length or ΔC form of HA-SND1 were extracted by Trizol, and subjected to Northern blot analysis after size fractionation in polyacrylamide gel. *Let-7* miRNA sequence was hybridized with a labeled *let-7* anti-sense probe. (C) Physical interaction of SND1 with mRNA. Cytoplasmic lysates extracted from HeLa cells expressing the full length or ΔC form of HA-SND1 were mixed with oligo(dT) cellulose beads, and poly(A)$^+$ RNA–protein complexes were collected. After several washes of the oligo(dT) precipitants, pulled-down proteins were eluted from the beads by adding Laemmli's SDS-sample buffer, and HA-SND1 (SND-Full and SDN-ΔC) was determined by immunoblot analysis using an anti-HA antibody. Arrow heads indicate the full length and ΔC form of HA-SND1, respectively. (D) A proposed model of the ternary complex with SND1, RISC and mRNA in gene silencing. SND1 interacts directly with mRNA through its N-terminal region, and the association with RISC is mediated by the C-terminal half of the protein. The ternary complex of SND1 is considered to participate in repressing the expression of target genes *via* inhibition of either translation initiation complex or loading of ribosomes. (E) COS-7 cells were transiently transfected either with pHA-SND1 (full length), pHA-SND-ΔC (ΔC form), or control vector (pCDNA-HA). Protein levels of RAS and HA-SND1 were analyzed by immunoblot analysis, and tubulin was used as a loading control. Substantial reduction of RAS protein was observed by exogenous induction of HA-SND1, but not either by HA-SND ΔC or Mock transfection. (F) HCT 116 cells were transfected with siRNA targeting SND1 (siSND-1 and siSND-2) or control siRNA. Cell lysates were collected 48 h after transfection, and subjected to immunoblot analysis. (G) Effect of SND1 and the K-RAS 3′-UTR on enzymatic activity of *firefly* luciferase reporter protein. 3′ UTR of RAS mRNA or its reverse direction (negative control) was fused to the 3′ end of *firefly* luciferase reporter gene. HCT 116 and HCT 116 Dicer^{ex5} were co-transfected with the reporter plasmid and HA-SND1. After incubation for 48 h, cell extracts were prepared and luciferase activity was measured by Dual Luciferase Assay Kit (Promega). The activity of *firefly* luciferase for each sample was normalized by *Renilla* luciferase activity as detailed elsewhere.

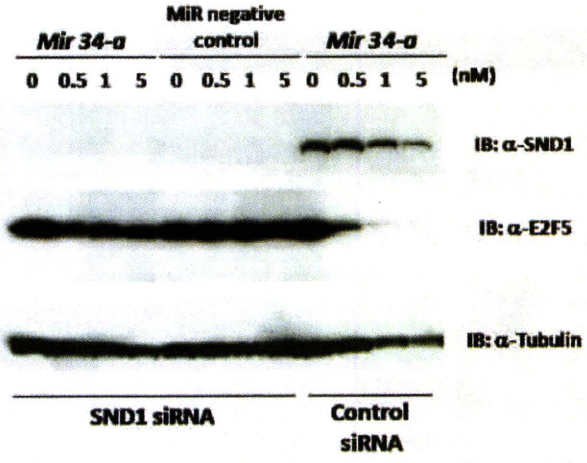

Fig. 4. Reduction of *miR-34a*-mediated repression of E2F-5 by SND1 knockdown. HCT 116 cells were introduced with either SND1 siRNA (left 8 lanes) or control siRNA (right 4 lanes) in combination with *miR-34a* (left 4 lanes and right 4 lanes) or miR-negative control (miR-NC; middle 4 lanes), incubated for 48 h, and cell extracts were subjected to immunoblot analysis.

of APC could be mediated by miRNAs. To support this idea, SND1 was indeed demonstrated to interact with endogenous miRNA by Northern blot analysis using a probe against *let-7* miRNA (Fig. 3A and B). The interaction of the ΔC form of SND1 deleting the C-terminal half of SND1 with *let-7* was not detectable (Fig. 3A and B), indicating that the C-terminal region of SND1 is required for the interaction with miRNA and the RISC complex. Furthermore, an oligo(dT) pull-down experiment using HeLa cell lysates demonstrated the interaction of SND1 with poly(A)$^+$ RNA is mediated through the N-terminal region of SND1 (Fig. 3C). Based on these observations, SND1 may serve as a platform for a molecular bridge between mRNA and RISC by incorporating the mRNA-specific miRNA species as depicted in Fig. 3D. In fact, over-expression of SND1 in COS-7 cells reduced the expression of RAS protein, the transcript of which is known to be targeted by *let-7* [23]. On the other hand, knockdown of SND1 by siRNA induced the substantial increase of RAS protein (Fig. 3E and F). In order to confirm the suppressive effect of SND1 on gene expression occurs in a *let-7*-dependent manner, we generated *firefly* luciferase reporter plasmids fused either with the 3′ UTR of RAS mRNA (pLuc-K-RAS UTR), containing several recognition sites of *let-7*, or with its reverse direction (negative control; pLuc-K-RAS UTRrev), and co-introduced with pHA-SND1 into HCT 116 and Dicer hypomorphic cells derived from HCT 116 (HCT 116 Dicer^{ex5}) [25]. As demonstrated in Fig. 3G, SND1 substantially suppresses expression of luciferase reporter gene containing 3′-UTR of *RAS* mRNA, but not of that with its opposite direction. Furthermore, not even a little change was detected in HCT 116 Dicer^{ex5} cells, even with Luc-K-RAS UTR (Fig. 3G), strongly suggesting the requirement of mature miRNA for this effect. Taking all these results together, SND1 was suggested to regulate gene expression in a *let-7*-dependent manner.

Further to this, SND1-mediated gene suppression was also observed in another gene, E2F5. The expression of E2F5, one of the target genes for *miR-34a*, is considerably repressed in the colon cancer cell line RKO by ectopic expression of *miR-34a* in a dose-dependent manner as depicted in Fig. 4 (see right-handed 4 lanes). Concomitant knock-down of SND1 by siRNA substantially suppressed the gene silencing activity of *miR-34a* against E2F5 (Fig. 4, left-handed 4 lanes). Although these observations suggested the contribution of miRNAs for the regulation of gene expression, it remains to be elucidated whether SND1 is specifically involved in the regulation of colon cancer-related genes, including APC, and how SND1 is associated with colon cancer development. Identification of mRNAs and miRNAs, being preferentially interacted with SND1, will provide new molecular insights into abnormalities of translational regulation of gene expression at early stages of colon carcinogenesis.

5. *MiR-34a*, a p53-regulated tumor suppressive miRNA, controls proliferation of colon cancers cells

Comprehensive analysis of miRNA expression in human colon cancers has revealed that a substantial fraction of miRNAs were either down-regulated or up-regulated in colon cancer tissues compared to their normal counterparts. Among the list of dys-regulated miRNA species in colon cancers, *miR-34a*, which we previously demonstrated to work as a strong repressor for cell proliferation [26], is significantly down-regulated in colon cancers. *miR-34a* is a member of the *miR-34* gene family, located at chromosome 1p36, and is composed of two exons. A nucleotide sequence for the mature form of the *miR-34a* is entirely embedded within the second exon [27]. The expression of *miR-34* family, including *miR-34a*, is tightly regulated by p53 [26–31], and the *miR-34* family negatively regulates cell proliferation *via* induction of apoptosis [27–29], cell cycle arrest [29,30], or senescence [26,31].

We previously demonstrated that introduction of *miR-34a* into colon cancer cells showed a remarkable reduction of cell numbers when compared to negative control miRNA. Cells that were transfected with *miR-34a* manifested characteristic morphological changes, namely large cellular/nuclear sizes and positive staining for senescence-associated β-galactosidase (SA-β-gal), suggesting the induction of senescence-like phenotypes in colon cancer cells (Fig. 5A).

To gain further insight into the biological role of *miR-34a*, we conducted global gene expression analysis using two colon cancer cell lines, HCT 116 (wild-type p53) and RKO (wild-type p53), transfected with either *miR-34a* or negative control miRNA. mRNAs, whose expression was commonly altered 2-fold or greater by *miR-34a* in both HCT 116 and RKO cells were extracted as detailed elsewhere [26]. *miR-34a* positively regulates the expression of some p53 target genes, including p21 and sestrin 1 [26,32]. In contrast, E2F family members and their target genes were down-regulated. This result suggested that one of the key roles of *miR-34a* in the anti-proliferation of cancer cells is the activation of the p53 network with the concomitant reduction of positive regulators for the cell cycle under the p53 wild type situation. In this regard, the direct repression of CDK4/6 and E2F3 by *miR-34a* was clearly demonstrated [26,30,31,33]. This result also suggested the positive feedback regulation between p53 and *miR-34a* [26].

6. SIRT1 participates in the positive feedback loop of the p53 tumor suppressor network

Recently, silent information regulator 1 (SIRT1) was identified as another target for *miR-34a* and demonstrated to regulate the induction of p53-dependent apoptosis [34]. SIRT1, a class III histone deacetylase, is known to be a negative regulator of p53 through the modulation of acetylation at K382 of p53 and thereby associated with multiple cellular processes, including apoptosis and cellular senescence [35–38]. We also observed the physical interaction of *miR-34a* and SIRT1 mRNA in HCT 116 cells (unpublished data), and the acetylated form of p53 accumulated in the nucleus by the exogenous introduction of *miR-34a*. Restoration of SIRT1 expression by introducing an SIRT1 open-reading frame (ORF) into *miR-34a*-transfeted cells abolished p53 activation caused by *miR-34a* (unpublished data). MDM2/MDMX, negative regulators of p53, were also demonstrated to be targets of *miR-34a* [39], and serve as key molecules for the negative regulatory circuitry of p53 in either

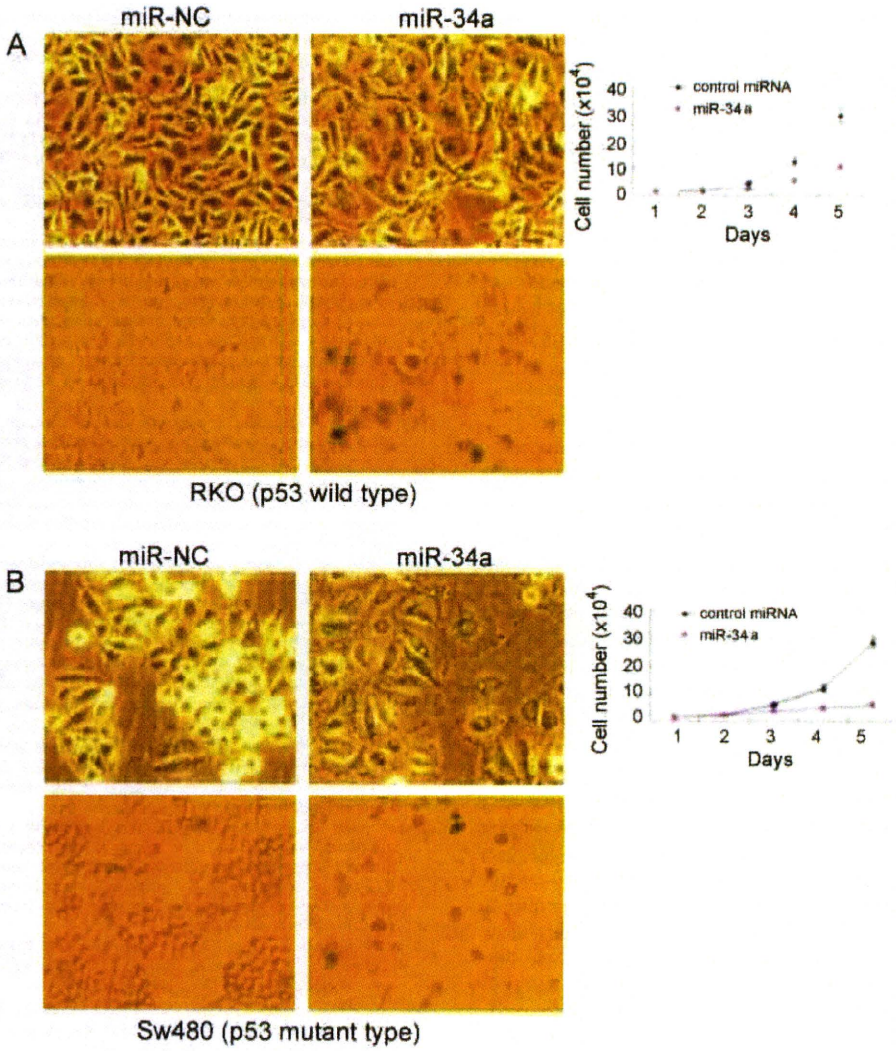

Fig. 5. Induction of senescence-like growth arrest by *miR-34a*. (A) RKO cells (wild-type p53) were transfected with *miR-34a* or miR-NC, and propagated for 4 days, and cells were subjected to SA-β-gal staining. The right upper panel indicates growth suppression of RKO cells by *miR-34a*. (B) Induction of senescence-like growth arrest was also observed in p53-mutant SW480 cells. Exogenous introduction of *miR-34a* was carried out as described above.

a direct or indirect fashion. These circuitry mechanisms for p53 activation are important for understanding the biological role of *miR-34a* in carcinogenesis. Importantly, the anti-proliferative activity of *miR-34a*, accompanying the senescence-like growth arrest, was also observed in SW480 colon cancer cells with the mutant p53 (Fig. 5B). This suggests that *miR-34a* may also participate in the p53-independent tumor-suppressor networks. Since the suppression of SIRT1 by *miR-34a* was observed even in the p53 mutant cell lines (unpublished data), it would be of interest to address whether SIRT1 could be involved in the induction of senescence-like growth arrest under the p53 mutant situation. Alternatively, other p53-independent tumor suppressor network activated by *miR-34a* should be involved, and the mechanism be clarified in the future.

7. Therapeutic application of tumor suppressive miRNA as a novel anti-cancer tool

Currently, development of RNAi drugs against various types of human disorders is being extensively investigated. For instance, systemic administration of *miR-26a* in a mouse model of Myc-induced hepatocellular carcinoma using adeno-associated virus (AAV) causes inhibition of cancer cell proliferation, induction of tumor-specific apoptosis, and drastic protection from disease progression without measurably manifestation of liver toxicity in normal hepatocytes [40]. Minimal adverse effect on normal hepatocytes by *miR-26a* could be based on the specific pattern of *miR-26a* expression, namely expression of *miR-26a* being suppressed specifically in liver cancers [40]. Further to this, inhibition of liver specific *miR-122* using an LNA-modified anti-miRNA oligonucleotide was recently shown to be implicated in cholesterol and lipid metabolism [41] and in hepatitis virus replication [42–44]. Systemic delivery of miR-16 is also reported to inhibit the growth of metastatic prostate tumors by suppressing multiple cell-cycle genes [45].

As for *miR-34a*, subcutaneous administration of *miR-34a*/atelocollagen complexes in mice caused significant suppression of xenograft tumor growth of both HCT 1116 and RKO colon cancer cells compared with the administration of a control miRNA/atelocollagen complex [26]. Tumor tissues treated with *miR-34a* showed a considerable degree of necrosis. In addition, ectopic expression of *miR-34a* in glioma and medulloblastoma cells clearly inhibits cell proliferation, G1/S cell cycle progression, and cell survival and cell migration [46]. Furthermore *miR-34a* expres-

sion was also demonstrated to inhibit *in vivo* glioma xenograft growth [46].

Although the tumor suppressive effect of *miR-34a in vivo* is still currently somewhat limited, repeated injection of *miR-34a*/atelocollagen complexes may achieve more efficient and significant regression of xenograft tumors and could also be applicable for *in vivo* tumors. Alternatively, identification of miRNA species that possess much higher tumor suppressive activity *in vivo* should be performed. Functional screening system *in vivo* should be of great help to achieve this goal. Toward this end, we recently succeeded to construct a drop-out functional screening system, as detailed in our recent publication [47], for identification of tumor suppressive miRNAs, which could be applicable for tumor treatment *in vivo*.

8. Conclusion

Translational regulation of gene expression, especially that by miRNA, has recently attracted major interest among cancer researchers. Because of the nature of miRNA regulating multiple target genes *via* the diverse manner of recognition of target sequences at 3′-UTR of genes, phenotypes caused by the exogenous introduction of miRNA are sometimes drastic even an *in vivo* setting in the cases of, for example, *miR-26a*, *miR-34a* and *miR-122*. Although the efficient delivery of miRNA to the target tissues/organs is a crucial subject to be solved, possible application of miRNA not only for therapeutic agents, but also for diagnostic biomarkers, is of great interest to be explored.

In addition to the translational researches' point of view, the biological nature of miRNA is quite intriguing, and it opens up a wide new field in cancer research. Although each miRNA targets and regulates functions of multiple genes in a post-transcriptional manner, it seems to suppress a set of genes involved in certain signaling pathways, such as cell cycle, apoptosis, cell proliferation, differentiation, and so on [48,49]. The p53 tumor suppressor network is, for example, regulated by several miRNAs, including *miR-34a* [50]. *Let-7* regulates the Ras signaling pathway [51,52], and the PI3K/AKT/PTEN regulatory circuit is regulated by several miRNAs, such as, *miR-21* [53,54], miR-17-92 cluster [55], *miR-126* [56], and *miR-214* [57]. The feature of an oncogene-tumor suppressor gene network as one of the hallmarks of cancer [58] now requires incorporation of miRNA as an essential component to explain the integrated signal circuit of the cell.

Conflicts of interest

None declared.

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Induction of Glandular Stomach Cancers in *Helicobacter pylori*-infected Mongolian Gerbils by 1-Nitrosoindole-3-acetonitrile

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Key words: gastric cancer, *Helicobacter pylori*, Mongolian gerbil, 1-nitrosoindole-3-acetonitrile, indole-3-acetonitrile

Abbreviations: DMSO, dimethyl sulfoxide; *H. pylori*, *Helicobacter pylori*; H&E, hematoxylin and eosin; MG, Mongolian gerbil; MNNG, *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine; MNU, *N*-methyl-*N*-nitrosourea; NIAN, 1-nitrosoindole-3-acetonitrile.

Appropriate category: Carcinogenesis

Abstract

Helicobacter pylori (*H. pylori*) infection and high intake of various traditional salt-preserved foods are regarded as risk factors for human gastric cancer. We previously reported that Chinese cabbage contains indole compounds, such as indole-3-acetonitrile, a mutagen precursor. 1-Nitrosoindole-3-acetonitrile (NIAN), formed by the treatment of indole-3-acetonitrile with nitrite under acidic conditions, shows direct-acting mutagenicity. In the present study, NIAN administration by gavage to Mongolian gerbils (MGs) at the dose of 100 mg/kg two times a week resulted in three adduct spots (1.6 adducts/10^8 nucleotides in total), detected in DNA samples from the glandular stomach by ^{32}P-postlabelling methods. Treatment with six consecutive doses of 100 mg/kg of NIAN, two times a week for three weeks, induced well- and moderately-differentiated glandular stomach adenocarcinomas in the MGs at the incidence of 31% under *H. pylori* infection at 54 - 104 weeks. Such lesions were not induced in MGs given broth alone, broth + NIAN or infection with *H. pylori* alone. Thus, endogenous carcinogens formed from nitrosation of indole compounds could

be critical risk factors for human gastric cancer development under the influence of *H. pylori* infection.

Introduction

Gastric cancer is the second most frequent cause of cancer death worldwide.[1] Although gastric cancer has become a relatively rare cancer in North America and most Northern and Western European countries, it remains common in East Asia, Eastern Europe, Russia and selected areas of Central and South America.[2] *Helicobacter pylori* (*H. pylori*) is a well-established major risk factor for gastric cancer,[3-5] and the prevalence of *H. pylori* infection in East Asia countries, including Japan and Korea is reported to be relatively high.[6, 7] In addition, the risk of gastric cancer is increased with a high intake of various traditional salt-preserved foods.[3] In fact, pickled vegetable consumption is reported to increase gastric cancer risk in Japan and Korea.[8-10] In Korea, kimchi, commonly prepared with Chinese cabbage or radish, is a traditional and popular food, which contains high levels of nitrate (median 1550 mg/kg).[11] Furthermore, Chinese cabbage is well known as a pickled

vegetable commonly consumed in Japan. Moreover, ingestion of nitrate, mainly from food, is suggested to correlate with mortality from gastric cancer.[12-14] Ingested nitrate is mainly converted to nitrite by bacteria in the oral cavity after secretion into saliva.[15] Carcinogenic *N*-nitroso compounds can be formed from nitrite and secondary amines under acidic conditions. Furthermore, direct-acting *N*-nitroso compounds, such as *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine (MNNG)[16] and *N*-methyl-*N*-nitrosourea (MNU),[17] are known to induce cancer in the glandular stomach of experimental animals. Thus, it is suggested that *N*-nitroso compounds that are formed in the stomach under acidic conditions could be positively associated with the risk of gastric cancer. Nitric oxide, formed by nitric oxide synthase, is also reported to contribute to production of *N*-nitroso compounds.[18]

We have previously reported that treatments of various foodstuffs with nitrite under acidic conditions produce direct-acting mutagens towards *Salmonella* tester strains.[19, 20] Among those foodstuffs, Chinese cabbage is shown to contain three indole compounds, indole-3-acetonitrile,