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# Generation of Knockout Rats with X-Linked Severe Combined Immunodeficiency (X-SCID) Using Zinc-Finger Nucleases

**Tomoji Mashimo[1]*, Akiko Takizawa[1], Birger Voigt[1], Kazuto Yoshimi[1], Hiroshi Hiai[2], Takashi Kuramoto[1], Tadao Serikawa[1]**

1 Institute of Laboratory Animals, Graduate School of Medicine, Kyoto University, Kyoto, Japan, 2 Shiga Medical Center for Adult Disease, Moriyama, Japan

## Abstract

***Background:*** Although the rat is extensively used as a laboratory model, the inability to utilize germ line-competent rat embryonic stem (ES) cells has been a major drawback for studies that aim to elucidate gene functions. Recently, zinc-finger nucleases (ZFNs) were successfully used to create genome-specific double-stranded breaks and thereby induce targeted gene mutations in a wide variety of organisms including plants, drosophila, zebrafish, etc.

***Methodology/Principal Findings:*** We report here on ZFN-induced gene targeting of the rat interleukin 2 receptor gamma (*Il2rg*) locus, where orthologous human and mouse mutations cause X-linked severe combined immune deficiency (X-SCID). Co-injection of mRNAs encoding custom-designed ZFNs into the pronucleus of fertilized oocytes yielded genetically modified offspring at rates greater than 20%, which possessed a wide variety of deletion/insertion mutations. ZFN-modified founders faithfully transmitted their genetic changes to the next generation along with the severe combined immune deficiency phenotype.

***Conclusions and Significance:*** The efficient and rapid generation of gene knockout rats shows that using ZFN technology is a new strategy for creating gene-targeted rat models of human diseases. In addition, the X-SCID rats that were established in this study will be valuable *in vivo* tools for evaluating drug treatment or gene therapy as well as model systems for examining the treatment of xenotransplanted malignancies.

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* E-mail: tmashimo@anim.med.kyoto-u.ac.jp

## Introduction

Although several strategies are available for producing a wide variety of genomic alterations in the mouse, the same cannot be said of the rat. Rat ES cells [1,2] and induced pluripotent stem cells (iPS) [3,4] are available, but the culture conditions for these cells and the methodology for inducing homologous recombination are imperfect [5]. Rat spermatogonial stem cells (SSC) have also been isolated and cultivated *in vitro* but their yield proved unsatisfactory in terms of their ability to undergo homologous recombination [6,7]. Besides these methods which are based on the *in vitro* genetic engineering of pluripotent stem cells, transposon-mediated mutagenesis [8] and N-ethyl-N-nitrosourea (ENU) mutagenesis [9,10] have been used with some success for producing mutations in the rat genome. We recently reported on a high-throughput gene-driven strategy which uses the mutagen ENU and the Mu-transposition reaction (MuT-POWER) to rapidly detect induced mutations. This was in addition to our investigation of intracytoplasmic sperm injection (ICSI) for recovering heterozygous genotypes of interest out of a large sperm cell repository [11,12]. However, even if a large number of mutant strains already exists or may potentially be available, targeted modification or disruption of specific DNA regions is difficult to achieve. Even in the case of our gene-driven strategy, X-linked mutations are impossible to obtain because of the breeding protocol which is used [11].

Recently, a novel gene-targeting technology which employs zinc-finger nucleases (ZFNs) has been proven to work successfully in plants, *Caenorhabditis elegans*, frogs, drosophila, zebrafish, and human ESCs and iPSCs [13,14,15]. ZFNs are chimeric proteins that consist of a specific DNA-binding domain which is made of tandem zinc finger-binding motifs that are fused to a non-specific cleavage domain of the restriction endonuclease *Fok*I. ZFNs can create site-specific double-stranded breaks which are repaired via non-homologous end joining (NHEJ), a process that results in the arbitrary addition or deletion of base pairs. Consequently, repair by NHEJ is mutagenic and results in a knockout. Recently, it was reported that a single injection of DNA or messenger RNA that encodes specific ZFNs into one-cell transgenic rat embryos that express GFP could lead to a high frequency of animals that do not express the transgenic marker as a consequence of homologous recombination at the GFP site [16]. Here, we report on an experiment that involved using ZFN technology. The aim of the experiment was to inactivate the gene that encodes the interleukin 2 receptor gamma (*Il2rg*), which is essential for signaling by interleukins such as IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21. In

addition, the gene is involved in the X-linked form of severe combined immunodeficiency (X-SCID), one of the most common forms of human SCID [17,18]. A major motivation for performing this experiment was the observation that although SCID mouse animal models are the most commonly used in research on drug development, an X-SCID immunodeficient rat model would complement mouse models through the additional advantage of being employed for testing the pharmacodynamics and toxicity of potential therapeutic compounds. Following the results of research involving *Prkdc* SCID [19,20] and *Il2rg* X-SCID mice [21,22,23], *Il2rg* X-SCID rats should have a very low level of NK cell activity and thereby make xenotransplantation more successful.

## Results

### Injection of *Il2rg* ZFN-encoding mRNA into rat embryos

Of 443 ZFN-injected embryos, 230 (51.9%) were transferred into the oviducts of pseudopregnant female rats, and 54 (24.3%) of these embryos were successfully carried to term as shown in Figure 1a, b and Table 1. Sequence analysis of the ZFN target site of these 54 founder animals revealed that 5 males and 8 females (24.1%) carried a variety of mutations including from 3 to1,097 bp deletions and a 1 bp insertion in the region which overlapped the ZFN target site as seen in Figure 1c and Figure S1. Four out of five of the males carried different biallelic mutations at the *Il2rg* locus despite them only having one X chromosome. This suggests that mosaicism was induced by the ZFN treatment, a situation which is frequently observed in the DNA of transgenic founders. Three of the affected females had a monoallelic homozygous mutation, four had heterologous or mosaicism biallelic mutations, and the remainder had three different mosaic mutations. The normal F344-allele was not found in the affected founder animals. Most of these mutations were expressed as frameshifts or splicing errors and resulted in no or very little IL2RG mRNA being expressed as shown in Figure 1d probably due to nonsense-mediated decay. Western blotting with antibodies against the C-terminal domain of

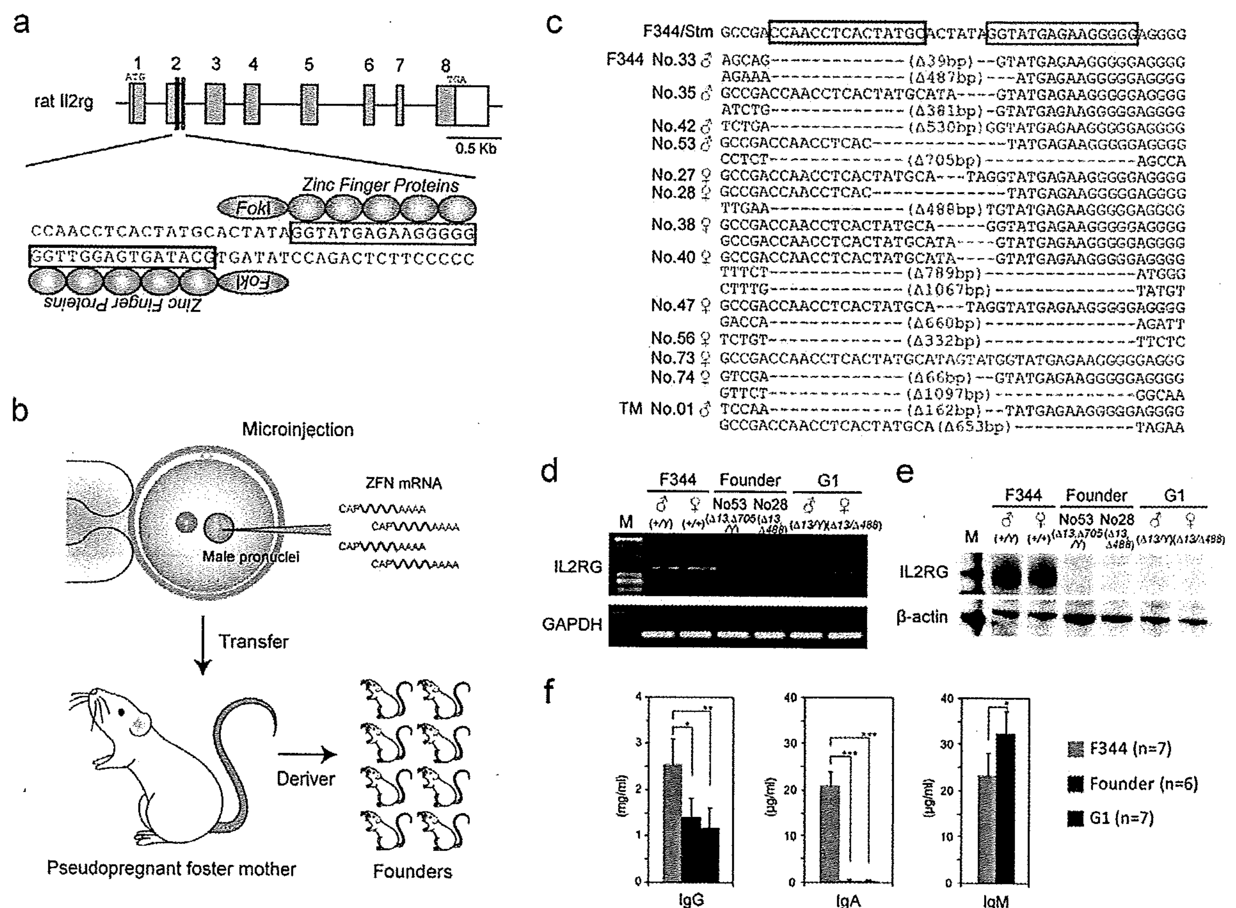


**Figure 1 Injection of *Il2rg* ZFN-encoding mRNA into rat embryos induced targeted loss-of-function mutations.** (a) Schematic representation of the rat *Il2rg* gene. Exons are represented as blue boxes. Regions used to design the ZFN templates are printed in red for the left ZFN and green for the right ZFN. The magnified views illustrate the binding sites for the ZFN pairs. Please see Figure S4 for further details. (b) Schematic representation of the method used for ZFN-targeted mutagenesis in rat embryos. (c) Sequencing assay for ZFN-induced mutations in the *Il2rg*-targeted region. Multiple deletions or insertions depicted using red dashes or letters, respectively, are aligned along the wild-type sequences shown on the top line. (d) RT-PCR analysis of IL2RG mRNA expression in the spleen of control F344, founder (G0), and G1 rats. GAPDH expression was used as an internal control. (e) Western blotting for IL2RG protein in the spleen of control F344, founder (G0), and G1 rats. β-actin was used as a loading control. (f) ELISA for serum IgG, IgA, and IgM levels in control F344, founder (G0), and G1 rats. *$P<0.01$, **$P<0.001$, and ***$P<0.0001$, indicated for each group in comparison with control F344 for independent sample Student t-tests.
doi:10.1371/journal.pone.0008870.g001

**Table 1.** Injection of ZFN-encoding mRNA into fertilized oocytes.

| Strain | Oocyte state | Injected oocytes | Transferred oocytes (%) | Born (%) | Mutants (%) |
|---|---|---|---|---|---|
| F344/Stm | Fresh | 234 | 32 (-) | ♂2,♀5 (21.9) | ♀2 (28.6) |
| | Cryopreserved[a] | | 57 (-) | ♂8,♀10 (31.6) | ♀2 (11.1) |
| | Fresh | 182 | 129 (68.3) | ♂16,♀11 (20.9) | ♂4,♀4 (29.6) |
| TM/Kyo | Fresh | 27 | 12 (44.4) | ♂1,♀1 (16.7) | ♂1 (50.0) |
| **Total** | | **443** | **230 (51.9)** | **♂27,♀27 (24.3)** | **♂5,♀8 (24.1)** |

[a]Injected oocytes were cultured in KRB overnight and cryopreserved at the two-cell stage.
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IL2RG did not reveal any protein in the founder animals as seen in Figure 1e.

To clarify whether the ZFNs only induced mutations in the targeted region, we checked 16 sites that showed a high rate of similarity with the targeted site at the sequence level with no more than 6 to 7 bp mismatches as illustrated in Table S1. Insertions or deletions were not observed at any of these off-target sites among the 13 ZFN-modified founders. This confirms that ZFNs can be reliably and efficiently used to produce mutant alleles at loci of interest. Although we cannot exclude the possibility that the ZFNs may have cleaved unknown off-target sites, such undesired mutations can subsequently be easily excluded from the genome of the carrier animals by backcrossing to the parental strain or another background strain.

## Germ line transmission of ZFN-modified genetic changes

To assess the transmission of ZFN-modified genetic changes to the next generation, we crossed the founder animals with the background strain F344/Stm as depicted in Table S2. All 38 offspring consisting of 18 males and 20 females that were obtained from the founder females mated with the F344 males had one of the maternal mutations. This indicates that ZFN-induced mutations were faithfully transmitted through the germ line. In the offspring that were obtained from the founder males, there were two cases where only one of the paternal bialleles was transmitted or both alleles were transmitted. This suggests that mosaicism occurred not only in somatic cells but also in the germ line of the founder animals. PCR analysis of genomic DNA isolated from several types of tissues indicated that somatic mosaicism occurred in the progenitors but not in their offspring as shown in Figure S2.

We intercrossed the G0 founders to produce hemizygous males (*Il2rg*$^{-}$/*Y*) and homozygous females (*Il2rg*$^{-}$/*Il2rg*$^{-}$) for the ZFN-induced mutation listed in Table S3 to characterize the immunodeficient phenotypes of the X-SCID rats. The hemizygous males and homozygous females appeared normal at birth and developed well as shown in Figure 2a. RT-PCR and Western blot assays was performed on these G1 rats and the results showed a complete loss of expression of the *Il2rg* gene as detailed in Figures 1d, e. ELISA for serum immunoglobulin (Ig) levels revealed reduced IgG, diminished IgA, and increased IgM levels in the G1 rats as noted in Figure 1f.

## Characterization of *Il2rg*-deficient X-SCID rats

Gross and microscopic analyses at five weeks of age showed that the X-SCID rats underwent abnormal lymphoid development as depicted in Figure 2. The thymus of X-SCID rats was extremely hypoplastic as seen in Figure 2b and consisted of an epithelial rudiment without any lymphocytes as seen in Figure 2d. The spleen was moderately decreased in size as noted in Figure 2c, and the white pulp was severely hypoplastic and the red pulp contained myeloid cells as shown in Figure 2f. Peripheral lymph nodes and Peyer's patches were not identified by necropscopy. In the peripheral blood (PB) profiles, the numbers of white blood cells (WBCs) was reduced compared to those of control rats as detailed in Table S4. Differential counts of WBCs showed a dramatic decrease in leukocytes in the X-SCID rats (Table S5). Flow cytometry analysis of cell populations isolated from PB, bone marrow (BM), and the spleen also revealed a dramatic decrease in the number of the lymphocytes as seen in Figure 2h and Figure S3. The number of CD4$^{-}$CD8$^{+}$ T-cells was markedly diminished and the number of CD4$^{+}$CD8$^{-}$ T-cells was decreased although some cells were present in PB, BM and the spleen. The numbers of CD3$^{-}$CD45RA$^{+}$ B-cells and CD3$^{-}$CD161a$^{+}$ NK cells were markedly diminished in PB and BM, but some cells were present in the spleen. Heterozygous females exhibited normal lymphoid development and were indistinguishable from normal control females (data not shown).

## Xenotransplantation of human tumor cells

These immunodeficient phenotypes of the X-SCID rats were very similar to those of the previously reported X-SCID mice and were characterized by a nearly complete lack of T-cells, B-cells and NK cells [21,22,23]. Since X-SCID mice cannot reject transplanted tissues from other species including humans, we tested *Il2rg*-deficient rats as a host for xenotransplantation of human ovarian cancer tumor cells. All X-SCID rats developed tumors within 14 days after injection of the cells (6/6, 100%), while control F344 rats showed no tumor growth (0/6, 0%) as seen in Figure 3a, b. The tumors were confirmed by histological analysis as depicted in Figure 3c and by PCR with primers that were used to amplify the human MHC class II DQB2 region (data not shown). These observations illustrate the impaired immune system function of X-SCID rats and suggest that the animals may be important models for cancer and transplantation research.

# Discussion

In this study, we proved that targeted gene disruption using ZFN technology works well and provides for several advantages and possibilities when used in rats. First and foremost, knockout rats can be created in a four- to six-month time frame and with high efficiency at more than 20%. This is more favorable than the ES cell-based method for mice that usually takes 12–18 months. Given the high rate of germ line transmission, preliminary phenotypic analysis can be performed on G1 animals after intercrossing the initial G0 founders, thereby saving time and effort. Second, gene-targeting with ZFNs does not seem to be strain-dependent (unpublished data) and accordingly can be performed with any inbred strain. This is of great advantage

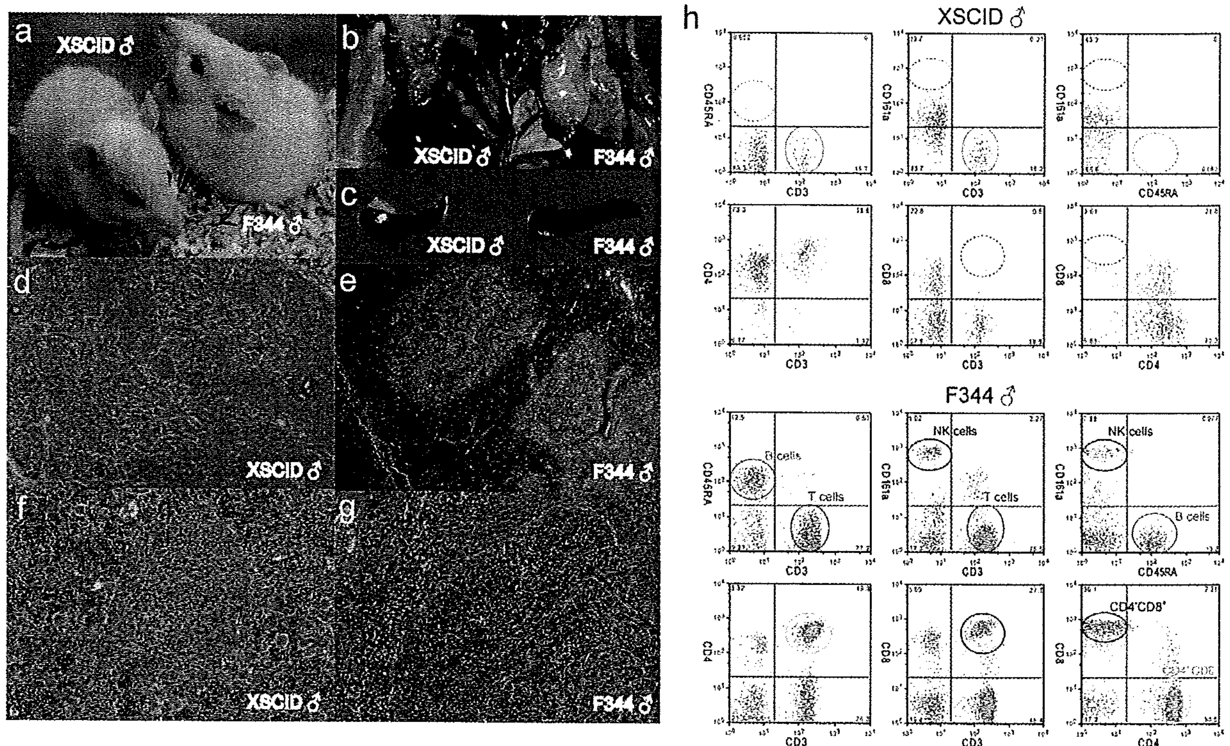


**Figure 2 Abnormal lymphoid development in X-SCID rats.** (a) Photograph of five week-old male X-SCID (*Il2rg*$^{-}$/Y) and F344 (+/Y) rats. (b) Thymus of X-SCID and F344 rats. (c) Spleen of X-SCID and F344 rats. (d, e) Histological analysis of the thymus of X-SCID (X40) and F344 (X40) rats. The thymus of the X-SCID rat was severely hypoplastic and consisted of an epithelial cell sheet. (f, g) Histological analysis of the spleen of X-SCID (X100) and F344 (X100) rats. In the X-SCID spleen, the white pulp was virtually devoid of lymphocytes and the red pulp was occupied by a variety of myeloid elements. (h) Dot plots representing CD3, CD45RA and CD161a for differentiation of T-, B- and NK cell sub-populations, and CD3, CD4 and CD8 for demarcation of T-cell sub-populations in peripheral blood lymphocyte cells. The numbers shown in the quadrants are mean percentages. The circled areas indicate cell populations that are referred to in the text.
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since other techniques like ENU mutagenesis differ in their efficiency when used with different strains. This provides a straight forward strategy for directly employing targeted gene disruption in the existing strain, thereby bypassing tedious and time-consuming backcrossing steps that generally take two to three years to complete. Third, ZFNs can be used to induce a wide variety of allelic changes covering small or wide deletions or insertions. They may be used to produce frameshifts or small in-frame deletions such as the 3-bp deletion that we observed. Given the reports on successful ZFN-targeted gene modification or correction by homologous recombination in mammalian cell cultures [15,24,25], it should be feasible to archive targeted knock-in technologies that have thus been far inaccessible without rat ES cells. Finally, since ZFN technology does not rely on using species-specific embryonic stem cell lines, it should be possible to adapt it to other mammalian species such as pigs, cattle, and monkeys, where it is possible to harvest and manipulate fertilized embryos.

The X-SCID rats established in this study provide not only a valuable *in vivo* model for evaluating drug treatment or gene therapy approaches, but also a system for assaying novel anticarcinogenic effects on transplanted malignancies. There is a growing need for animal models with which to carry out *in vivo* studies using human cells, tissues or organs as chimeras such as humanized models [26,27,28]. X-SCID and SCID mice homozygous for *Il2rg*- and *Prkdc*- alleles with a non-obese diabetic background are a powerful tool for the xenotransplantation of human tissues or potentially human ES/iPS cells. This could lead to advances in our understanding of human hematopoiesis, immunology, cancer biology, infectious diseases, and regenerative medicine [29,30,31]. Humanized rats, if generated by ZFN technology, could be powerful tools for pre-clinical testing during drug development and be better models in various fields of translational research.

## Materials and Methods

### Animals

All animal care and experiments conformed to the Guidelines for Animal Experiments of Kyoto University, and were approved by the Animal Research Committee of Kyoto University. F344-*Il2rg*$^{tm1Kyo}$ X-SCID rats are deposited at the National Bio Resource Project for the Rat in Japan (www.anim.med.kyoto-u.ac.jp/nbr).

### ZFN constructs

Custom-designed ZFNs plasmids for the rat *Il2rg* gene were obtained from Sigma-Aldrich. The design, cloning, and validation of the ZFNs was performed by Sigma-Aldrich [32]. ZFN design involved using an archive of pre-validated two-finger and one-finger modules [32,33]. The target region was scanned for positions where modules exist in the archive. This allowed the fusion of two or three such molecules to generate a five-finger

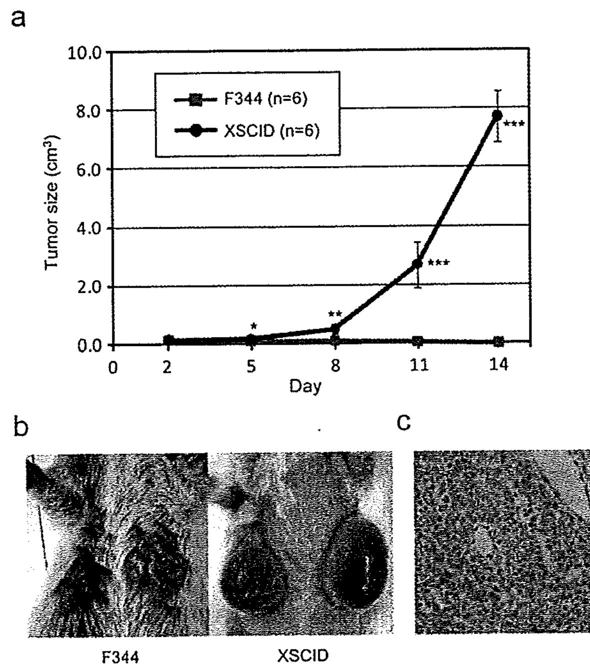


**Figure 3 Tumor development from the xenotransplantation of human ovarian cancer cells.** (a) Growth curve of tumor development after subcutaneous injection of A2780 human ovarian cancer cells in F344 and X-SCID rats. *P<0.01, **P<0.001, and ***P<0.0001, indicated in comparison with control F344. (b) The tumors became large and grew quickly about 11 days after injection in X-SCID rats but not in F344 rats. (c) Histology of the xenotransplanted tumors that formed in X-SCID rats (X400). No lymphocytic infiltration was detected in the tumors.
doi:10.1371/journal.pone.0008870.g003

protein that recognizes a 15 bp site on the top strand and the fusion of two to three different modules that recognize a 15 bp site on the bottom strand that lies 5–6 bp away. Measurements of ZFNs for gene disruption activity were performed using the Surveyor endonuclease (CEL-1) assay as described elsewhere [34]. Final candidate ZFNs were designed to recognize a site within the boundary between exon 2 and intron 2 of the *Il2rg* gene as shown in Figure S4.

## Microinjection of ZFN mRNA

To prepare ZFN mRNA, ZFN-encoding expression plasmids were linearized with *XhoI* and extracted with phenol-chloroform by the standard method. Messenger RNA was transcribed *in vitro* using a MessageMax$^{TM}$ T7 mRNA transcription kit (Epicentre) and polyadenylated using a A-Plus$^{TM}$ Poly(A) polymerase tailing kit (Epicentre). The resulting mRNA was purified using a MEGAClear$^{TM}$ kit (Epicentre) and finally resuspended in RNase-free water at 10 ng/µl for each ZFN. Approximately 2–3 pL of capped mRNA were injected into the male pronuclei of zygotes by the same method that was used to microinject DNA. Pronuclear stage embryos were collected from F344/Stm and TM/Kyo females six weeks of age that had been super-ovulated by injecting them with eCG (Serotropin, Asuka Pharmaceutical Co.) and hCG (Gonatropin, Asuka Pharmaceutical Co.). They were mated with males of the same respective strain. The mRNA solution was injected and embryos were cultured in KRB at 37.5°C with 5% $CO_2$ and 95% humidified air to promote their recovery. The embryos that survived were transferred to the oviduct of pseudopregnant females (Crlj:WI, 8–12wks).

## Analysis of genome editing at ZFN target sites

Genomic DNA was extracted from the tail, brain, heart, and liver using a GENEXTRACTOR TA-100 automatic DNA purification system (Takara). PCR for each was carried out in a total volume of 15 µl under the following conditions for 35 cycles: 94°C for 3 min for 1 cycle, 94°C for 30 sec, 60°C for 30 sec, and 72°C for 1 min. The final reaction mixture for each contained 100 ng of genomic DNA, 200 µM of each dNTP, 1.0 mM $MgCl_2$, 0.66 µM of each primer, and 0.4 U of Taq DNA polymerase (GibcoBRL).

For editing the ZFN cleavage site in the genome at the *Il2rg* locus, three primer sets were designed to amplify small 292-bp, middle 1509-bp, and large 3158-bp fragments as shown in Figure S4. The PCR products were directly sequenced using the BigDye terminator v3.1 cycle sequencing mix and the standard protocol for an Applied Biosystems 3130 DNA Sequencer. The products were also subcloned into the pCR4-TOPO vector (Invitrogen), and plasmid DNA was prepared and sequenced on a 3130 DNA Sequencer. All new sequence data is deposited in GenBank (GU294902-GU294925).

## Off-target site analysis

Off-target sites with the highest degree of similarity were identified by searching the rat genome (RGSCv3.4) for matches with the consensus sequence of each ZFP with appropriate spacing of 5–6 bp. A list of these target sites is provided in Supplementary Table 1. PCR primers were designed to flank the off-target sites as detailed in Table S6. Reactions were performed for the founder animals and the PCR products were directly sequenced on the 3130 DNA Sequencer.

## RT-PCR and Western blotting

Total RNA was extracted using Isogen reagent (Nippon Gene) from the spleen of five week-old rats. First strand cDNA was synthesized from 5 µg of total RNA that had been treated using DNase by using the oligo(dT)12–18 primer and SuperscriptII reverse transcriptase (Invitrogen). PCR was performed with the primers for *Il2rg* described in Figure S4 and with the *Gapdh* 5′-GGCACAGTCAAGGCTGAGAATG-3′ and 5′-ATGGTGGT-GAAGACGCCAGTA-3′. Western blotting was carried out using the cell lysates from the spleens of five week-old rats by the standard method. Signals were detected with antibodies against rat IL2RG (M-20, Santa Cruz Biotechnology) and β-actin (AC-40, Sigma Aldrich).

## Immunofluorescence and histological analyses

Complete necroscopy examinations were performed on five week-old *Il2rg*-deficient and wild-type male and female rats. Peripheral blood specimens were collected from the caudal vena cava. Serum immunoglobulin (Ig) levels were measured by enzyme-linked immunosorbent assay (ELISA) using Rat IgG, IgA and IgM ELISA Quantitation kits (Bethyl Laboratories). Blood parameters for a complete blood cell count, a WBC differential, and a reticulocyte count were measured using ADVIA 2120 flow cytometry (Block Scientific). For histopathology, tissues were fixed in Bouin's fluid and embedded in paraffin. The embedded tissues were then sectioned at 5–7 µm thickness at room temperature and stained with hematoxylin and eosin to permit evaluation by light microscopy.

Flow cytometric analyses of cell populations isolated from bone marrow, the spleen and peripheral blood were carried out using IOTest Anti-Rat CD3-FITC/CD45RA-PC7/CD161a-APC (Beckman Coulter) to differentiate T-, B- and NKcell sub-populations and IOTest Anti-Rat CD3-FITC/CD4-PC7/CD8-APC (Beckman Coulter) to enumerate T-cell sub-populations. Anti-CD45 monoclonal antibodies (Beckman Coulter) were used for the intracellular staining of lymphocytes. Mouse IgM, IgG1 and IgG2a antibodies (Beckman Coulter) were used as isotype-matched controls. The cell samples were treated with FcR-blocking reagent (Miltenyi Biotec) for 10 minutes, stained with the fluorochrome-conjugated antibodies for 30 minutes, and washed three times with PBS/10% FCS. Stained cell samples were analyzed with a four-color FACS flow cytometer (FACSCalibur, Becton Dickinson) using CellQuest software (Becton Dickinson).

### Tumor cell xenotransplantation

. The human ovarian cancer cell line A2780 was purchased from the European Collection of Cell Cultures (ECACC). Cells were cultured in RPMI1640 medium (GIBCO) with 10% heat-inactivated FBS (Hyclone). Subcutaneous injections of $2\times10^5$ A2780 cells with Matrigel (Becton Dickinson) were performed on five week-old female rats. Tumors were measured by length ($a$) and width ($b$) in millimeters using calipers, and tumor volumes ($V$) were calculated according to the relationship $V = ab^2/2$, where $a$ was the longer of the two measurements. Human-specific PCR primers were designed to amplify major histocompatibility complex class II DQ beta 2 (HLA-DQB2) at exon 4 as follows: 5′-CCTAGG-GTGGTCAGACTGGA-3′ and 5′-AAAATCCCCCAAAACA-AAGG-3′.

## Supporting Information

**Figure S1** PCR analysis of 13 mutant founders for the zinc-finger nuclease (ZFN) target site. For the analysis of the ZFN target site at the *Il2rg* locus, three primer sets were used to amplify small (a, 292-bp), middle (b, 1509-bp), and large (c, 3158-bp) fragments for PCR. See Figure S4 for further details. PCR fragments were electrophoresed through a 1-4% agarose gel. M: DNA molecular weight marker ϕX174-*Hae*III digest.
Found at: doi:10.1371/journal.pone.0008870.s001 (9.19 MB TIF)

**Figure S2** PCR analysis of genomic DNA isolated from several tissues. Three primer sets were used to amplify small (a, 292-bp), middle (b, 1509-bp), and large (c, 3158-bp) fragments for PCR. See Figure S4 for further details. Genomic DNA (T: tail, B: brain, H: heart, L: liver) was used as a template for PCR in zinc-finger nuclease-modified founders (numbers 28, 35, 40, and 53) and G1 rats. PCR fragments were electrophoresed through a 1–4% agarose gel. M: DNA molecular weight marker ϕX174-*Hae*III digest or Lambda DNA-*Hind*III digest.
Found at: doi:10.1371/journal.pone.0008870.s002 (6.28 MB TIF)

**Figure S3** Flow cytometric analysis of bone marrow lymphocyte cells (a) and spleen lymphocyte cells (b) from five-week-old F344 and X-SCID rats. Dot plots represent CD3, CD45RA, and CD161a for discrimination of T-, B-, and NK cell subpopulations; and CD3, CD4, and CD8 for demarcation of T cell subpopulations. The numbers shown in quadrants are mean percentages. Circled areas indicate cell populations referred to in the text.
Found at: doi:10.1371/journal.pone.0008870.s003 (6.66 MB TIF)

**Figure S4** Zinc-finger nuclease pairs designed against the *Il2rg* locus and primer sequences used for PCR analysis for the *Il2rg* gene. Each exon is underlined. The start codon is indicated by a red box. The three primer sets (small, middle, and large) used for the PCR analysis of *Il2rg* are shown by boxes. Primers used for the RT-PCR are shown as cDNA.
Found at: doi:10.1371/journal.pone.0008870.s004 (3.32 MB TIF)

**Table S1** Potential zinc-finger nuclease off-target sites.
Found at: doi:10.1371/journal.pone.0008870.s005 (0.14 MB DOC)

**Table S2** Backcrossing of zinc-finger nuclease-modified founders to F344/Stm rats.
Found at: doi:10.1371/journal.pone.0008870.s006 (0.16 MB DOC)

**Table S3** Intercrossing of zinc-finger nuclease-modified founders between males and females.
Found at: doi:10.1371/journal.pone.0008870.s007 (0.08 MB DOC)

**Table S4** Peripheral blood profiles of *Il2rg*-deficient (X-SCID) rats.
Found at: doi:10.1371/journal.pone.0008870.s008 (0.09 MB DOC)

**Table S5** Differential counts of the white blood cells of *Il2rg*-deficient (X-SCID) rats.
Found at: doi:10.1371/journal.pone.0008870.s009 (0.07 MB DOC)

**Table S6** Primer sequences for zinc-finger nuclease off-target analysis.
Found at: doi:10.1371/journal.pone.0008870.s010 (0.14 MB DOC)

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## Author Contributions

Conceived and designed the experiments: TM BV TS. Performed the experiments: TM AT KY HH TK. Analyzed the data: TM. Wrote the paper: TM.

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**Review**

# Inhibition of Colon Carcinogenesis by Dietary Non-Nutritive Compounds

Takuji Tanaka[1] and Shigeyuki Sugie[1]

[1]Oncologic Pathology, Kanazawa Medical University, 1–1 Daigaku, Uchinada, Ishikawa 920–0293, Japan

**Abstract:** Dietary habit is instrumental in about 50% of human colorectal cancers. Consumption of fruits and vegetables is associated with decreased risk of several types of cancer, including colorectal malignancy. These foods contain many non-nutritive as well as nutritive compounds, such as carotenoids, dithiolthiones, flavonoids, glucosinolates, indoles, isothiocyanates, monoterpenes, phenols, sterols, sulfhydryls and vitamins (including C, E and folate). There may be other unknown non-nutritive constituents in foods that can reduce cancer development. Studies using experimental chemical carcinogenesis models have indicated that several non-nutritive components, belonging to different chemical groups, in foods protect against certain types of cancer, including colorectal neoplasm. Many of these chemicals are known as potential "cancer chemopreventive agents" and are antioxidants that suppress carcinogenesis by (i) inhibiting phase I enzymes or blocking carcinogen formation, (ii) induction of phase II (detoxification) enzymes, (iii) scavenging DNA reactive agents, (iv) modulation of hormone homeostasis, (v) suppression of hyper-cell proliferation induced by carcinogen, (vi) induction of apoptosis, (vii) depression of tumor angiogenesis, and/or (viii) inhibition of certain phenotypic expressions of preneoplastic and neoplastic cells. Given the definite increase in the increase of colorectal cancer, we should determine the most effective mean of prevention and understand the mechanism(s) underlying successful prevention. There are critical interrelationships between diet, environmental factors and genetics that can affect cancer risk. However, non-nutritive compounds in fruits, vegetables and other dietary constituents (teas, spices and herbs) consumed as part of the diet have the ability to reduce cancer occurrence in pre-clinical animal carcinogenesis models. Although epidemiologic studies show similar associations, there have been very few intervention studies to date. This article describes our recent studies to determine whether several naturally occurring non-nutritive products from edible plants have any effective chemopreventive effects on colorectal carcinogenesis in rodents.
(J Toxicol Pathol 2007; **20**: 215–235)

**Key words:** colon carcinogenesis, chemoprevention, non-nutritive compounds, diet, rodents

## Introduction

Prevention of disease is an old and important concept. An important consideration in cancer research today is that exposure to pharmacologically active chemicals may play an important role in reducing the relative risks resulting from exposure to carcinogenic chemicals. Chemoprevention of cancer might be defined as the deliberate introduction of these selected non-toxic substances into the diet for the purpose of reducing cancer development. Numerous epidemiological studies on the relationship between diet and carcinogenesis have demonstrated the protective effect of consumption of fruits and vegetables against various forms of cancers[1,2]. A number of dietary compounds (Table 1) in diet are known to modulate the development of tumors in experimental animal models[3]. Epidemiological studies also suggest that specific, pharmacologically active agents present in the diet might reduce or increase the relative risk of cancer development. In regard to colorectal cancer (CRC), marked variations in dietary habits among the populations of different cultures and lifestyles have been associated with risk of this malignancy[4]. Also, there is an inverse correlation between the intake of vegetables/fruits and occurrence of CRC[1,5–7]. Thus, the relationship between risk of development of CRC and dietary habits is important when considering prevention of CRC[8], although the etiology of CRC is multifactorial and complex. Among dietary components, fiber has been found to reduce the risk of CRC development[9]. Also, green tea inhibits colorectal tumorigenesis[10]. However, some epidemiological data suggests that dietary fiber has no effect on recurrence of colorectal adenomas[11,12] and that green tea has no influence on the risk of stomach cancer[13].

Potential chemopreventive agents can be found both among buy nutrients and non-nutrients in diet.

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Mailing address: Takuji Tanaka, Department of Oncologic Pathology, Kanazawa Medical University, 1–1 Daigaku, Uchinada, Ishikawa 920–0293, Japan
TEL: 81-76-218-8116 FAX: 81-76-286-6926
E-mail: takutt@kanazawa-med.ac.jp

**Table 1.** Potential Non-Nutritive Compounds in Fruits, Vegetables and Spices that Inhibit Chemical Carcinogenesis

| | Class | Major food sources | Compounds |
|---|---|---|---|
| Flavonoids | Flavones | Celery, parsley, sweet red pepper, thyme | Tangeretin, Nobiletin, Apigenin, Chrysin, Diosmetin, Luteolin |
| | Flavonols | Apples, berries, broccoli cherries, fennel, kale, red wine, sorrel, grains, onions, tea | Quercetin, Rutin, Myricetin, Kaempferol |
| | Catechins | Tea, apples, cocoa, red wine | Epigallocatechin-3-gallate (EGCG) Epigallocatechin Epicatechin-3-gallate |
| | Flavanones | Citrus fruit, prunes, citrus peel | Naringenin, Fisetin, Hesperidin, Hesperitin, Taxifolin |
| | Isoflavones | Soybeans, legumes | Genistein, Daidzein |
| | Anthocyanidins | Grapes, cherries, raspberries | Cyanidin, Malvidin, Pelargonidin |
| Indoles | | Cruciferous vegetables | Indole-3-carbinol (I3C) 3,3' -diindolylmethane (DIM) |
| Isothiocyanates | | Cruciferous vegetables | |
| Lignans | | Grains, flaxseed, berries, chives, beverages | Matairesinol, Secoisolariciresinol, Enterodiol, Enterolactone |
| Organosulfur | | Allium vegetables: garlic and onions | Diallyl disulfide (DADS) |
| Terpenes | | Citrus, spices | D-Limonene |

Epidemiological and experimental studies have revealed that several micronutrients may have cancer preventive properties in several organs, including the large bowel[14]. Examples include vitamin A, vitamin C, $\beta$-carotene, selenium and calcium. The cancer chemopreventive abilities of two xanthophylls without provitamin A activity has been demonstrated in the rat colon[15], oral cavity[16] and urinary bladder[17]. Most of these compounds are antioxidants, which could serve as an explanation for their mode of action. Dietary fibers, a variety of ingestible carbohydrates[18] are well-known non-nutritive chemopreventives of colon tumorigenesis. Since the modifying effects of the major dietary factors on rodents colon carcinogenesis are heterogeneous[19], we focused on other non-nutritive inhibitors derived from vegetables and fruits in experimental colon carcinogenesis. Wattenberg[20] also suggested that some minor non-nutrients in the diet have protective effects on colon tumorigenesis. In 1985, he roughly classified chemopreventive agents into blocking and suppressing agents based on the time periods during which the agents appeared to have activity in animal models of carcinogenesis[21]. Since then, several naturally occurring compounds and synthetic chemicals have been intensively investigated for their chemopreventive ability on chemically-induced malignant epithelial neoplasms, including colon carcinoma. These have included such thins as the inorganic and organic selenium salts, phenolic antioxidants, non-steroidal anti-inflammatory drugs (NSAIDs) and ornithine decarboxylase (ODC) inhibitors. Our group has also found several natural chemopreventive agents against colon carcinogenesis. Indeed, food chemists and natural product scientists have identified hundreds of "phytochemicals" that are now being evaluated for the prevention of cancer[22]. Among the non-nutrient dietary components believed to exert a chemopreventive effect are flavonoids, polyphenolic derivatives of benzo-gamma-pyrone that are widely distributed in edible plants[23]. There are several major classes of flavonoids, which may occur as glycosides or aglycones. Total dietary intake of flavonoids has been estimated to be as high as 1 g/day, which is equivalent to 50,000 ppm in the diet[24], although more recent studies have indicated that intake varies widely[25].

This review is limited to mostly a few non-essential dietary components and to those which substantial documentation exists about an effect on the carcinogenesis process and for those in which a plausible mechanism of action can be postulated. We therefore list several non-nutritive chemopreventive agents against colon carcinogenesis[15,26–61] that we found in edible pants within our laboratory (Table 2–4). Also, the present report will introduce our recent data demonstrating the potential cancer chemopreventive abilities of capsaicin[48], rotenone[48], obacunone[47], limonin[47], nobiletin[49,60] and silymarin[54]. These are present in certain edible plants. It must be noted that response to individual components is assumed to be consistent with that occurring in a complex food matrix. Whether this is true or not needs to be adequately verified.

## Gene-Environment Interaction and Cancer Chemoprevention

Malignant epithelial neoplasm (cancer) is now considered to be primarily caused by the interaction of environmental factors (including dietary habit) with genetic, epigenetic and posttranslational events involved in the multistep process of cancer development[62]. Because

**Table 2.** Non-Nutritive Compounds that Inhibited Aberrant Crypt Foci (ACF) Formation in an ACF Bioassay in Our Laboratory

| Compounds | Dose | Carcinogens | Animals | % inhibition of ACFs | Reference no. |
|---|---|---|---|---|---|
| Rebaudioside A | 200 ppm | AOM | Rat | 19 | 28 |
| Liquiritin | | | | 6 | |
| Phyllodulcin | | | | 8 | |
| Hydrangenol | | | | 24 | |
| Oleanoic acid | | | | 36 | |
| Costunolide | | | | 22 | |
| Soyasaponin $A_2$ | | | | 16 | |
| Safflower oil | 12% | AOM | Rat | 47 | 31 |
| Perilla oil | 12% | | | 74 | |
| Perilla + Olive oil | 6% + 6% | | | 49 | |
| | 3% + 9% | | | 41 | |
| Olive oil + $\beta$-carotene* | 12% + 50 or 200 mg/kg/day* | AOM | Rat | 27 or 38 | 32 |
| Perilla + Olive oil + $\beta$-carotene* | 3% + 9% + 50 mg/kg/day* | | | 87 | |
| Perilla oil + $\beta$-carotene* | 12% + 50 mg/kg/day* | | | 91 | |
| *d*-Limonen | 5000 ppm | AOM | Rat | 32 | 29 |
| $\beta$-cryptoxanthin and hesperidin-rich powder | 500 ppm | AOM | Rat | 20 | 40 |
| Caffeine | 500 ppm | AOM | Rat | 30 | 43 |
| Quercetin | | | | 48 | |
| Garcinol | 100 ppm | AOM | Rat | 26 | 46 |
| | 500 ppm | | | 40 | |
| Zerumbone | 100 ppm | AOM | Rat | 14 | 50 |
| | 500 ppm | | | 46 | |
| Chalcone | 500 ppm | AOM | Rat | 51 | 52 |
| 2-hydroxychalcone | | | | 56 | |
| Extract of ginkgo leaves (*Ginkgo biloba*) | 50 ppm | AOM | Rat | 31 | 61 |
| | 500 ppm | | | 47 | |
| Bilobalide | 15 ppm | | | 26 | |
| | 150 ppm | | | 33 | |
| Powderd broccoli sprout | 20 ppm | AOM | Rat | 47 | 57 |
| | 100 ppm | | | 40 | |

AOM=azoxymethane.
* Oral administration (gavage).

dominantly inherited or familial cancers probably contribute to only a small percent of the total number of cases, it is quite important to identify the environmental modulators that influence non-familial risks[63]. Dietary habits are possibly a variable that markedly influences non-familial cancer risk. Some researchers have estimated that dietary habits are instrumental in about 60% of cancers in women and about 40% of cancers in men[62]. Although these are significant contributions, the true effect depends on the individual's genetic profile, the particular neoplasms, and the composition of the entire diet.

Although variability exists, fruit and vegetable consumption has often been inversely linked with the incidence of cancer[64,65]. The reason for the variability remains obscure, but it may be related to oxidative balance[66] or other physiological changes, as indicated in this review. Variations in pro- and antioxidant conditions that might arise from the absence or presence of food components is recognized as an influence on several essential cellular functions, including gene expression[67]. This homeostasis is unquestionably complex, as evident by the sensitivity of several kinases and transcription factors to rather subtle shifts in redox status[68].

The linkages between fruit and vegetable consumption and reduced cancer risk serve as sufficient evidence for the continued examination of individual foods or dietary components as modulators of the initiation, promotion and progression stages of carcinogenesis. A large number of agents with antioxidant properties are found in fruits and vegetables (Fig. 1). They include carotenoids, dithiolthiones, flavonoids, glucosinolates, indoles, isothiocyanates, monoterpenes, phenols, sterols, sulfhydryls

**Table 3.** Non-Nutritive Compounds that Inhibited Colonic Adenocarcinoma (ADC) in a Long-Term Bioassay in Our Laboratory

| Compounds | Dose | Carcinogens | Animals | % inhibition of ADCs | | Reference no. |
|---|---|---|---|---|---|---|
| | | | | Initiation | Post-initiation | |
| Chlorogenic acid | 250 ppm | MAM acetate | Hamster | ND* | 100 | 26 |
| $Mg(OH)_2$ | 500 ppm | MAM | Rat | ND | 77 | 27 |
| | 1000 ppm | acetate | | | 47 | |
| Astaxanthin | 100 ppm | AOM | Rat | ND | 39 | 15 |
| | 500 ppm | | | | 54 | |
| Canthaxanthin | 100 ppm | | | | 31 | |
| | 500 ppm | | | | 69 | |
| Juglone | 200 ppm | AOM | Rat | 7 | ND | 38 |
| Plumbagin | | | | 26 | | |
| Hydrangenol | | | | 17 | | |
| Satsuma mandarin juice | MJ** | AOM | Rat | ND | 49 | 45 |
| | MJ2*** | | | | 64 | |
| | MJ5**** | | | | 78 | |
| Columbin | 4 ppm | AOM | Rat | 36 | ND | 53 |
| | 20 ppm | | | 55 | | |
| | 100 ppm | | | 82 | | |
| Seed oil from bitter melon (*Momordica charantia*) | 100 ppm | AOM | Rat | 47 | | 59 |
| | 1000 ppm | | | 40 | | |
| | 1% | | | 17 | | |
| Pomegranate (*Punica granatum L.*) seed oil | 100 ppm | AOM | Rat | 46 | | 58 |
| | 1000 ppm | | | 53 | | |
| | 1% | | | 31 | | |

MAM=methylazoxymethanol and AOM=azoxymethane.
*Not determined; ** MJ contains 0.8 mg $\beta$-cryptoxantin and 79 mg hesperidin in 100 g juice; *** MJ2 contains 1.7 mg $\beta$-cryptoxantin and 84 mg hesperidin in 100 g juice, and **** MJ5 contains 3.9 mg $\beta$-cryptoxantin and 100 mg hesperidin in 100 g juice.

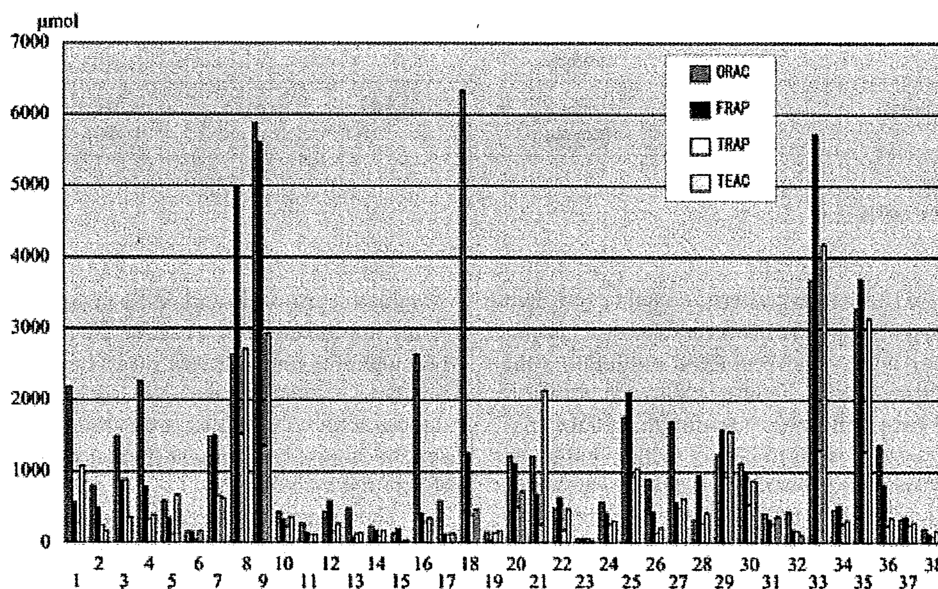



**Fig. 1.** Antioxidative activities of fruits and vegetables. 1, Apple (raw with skin), 2. Apricot (dried), 3. Asparagus (raw), 4. Avocado (raw), 5. Banana (raw), 6. Beans (green, boiled), 7. Beet (red), 8. Blackberries (raw), 9. Blueberries (raw), 10. Broccoli (raw, chopped), 11. Cabbage (green, raw), 12. Cantaloupe (raw), 13. Carrots (raw), 14. Cauliflower (raw), 15. Celery (raw), 16. Cherries (raw sweet), 17. Eggplant (Aubergine, raw), 18. Figs (dried), 19. Garlic (raw), 20. Grapefruit (pink/red, raw), 21. Grapes (white/green), 22. Kiwifruit, raw), 23. Lettuce (iceberg, raw), 24. Onion (raw), 25. Orange (raw), 26. Peach (raw), 27. Pear (raw), 28. Pepper (red/green, raw), 29. Pineapple (raw), 30. Plum (raw), 31. Potato (boiled, without skin), 32. Radish (raw), 33. Raspberries (raw), 34. Spinach (raw), 35. Strawberries (raw), 36. Tangerine, 37. Tomato (raw, red), 38. Watermelon (raw). ORAC, oxygen radical absorbance capacity; FRAP, Ferric reducing ability of plasma; TARP, Total radical trapping parameter assay; and TEAC, Trolox equivalent antioxidant capacity.

**Table 4.** Non-Nutritive Compounds that Suppressed Aberrant Crypt Foci (ACF) Formation in an ACF Bioassay and Colonic Adenocarcinoma (ADC) Development in a Long-Term Bioassay in Our Laboratory

| Compounds | Dose | Carcinogens | Animals | % inhibition of ACFs | % inhibition of ADCs | | Reference no. |
|---|---|---|---|---|---|---|---|
| | | | | Initiation | Initiation | Post initiation | |
| 1'-Acetoxychavicol acetate | 100 ppm | AOM | Rat | 41 | 54 | 45 | 34, 37 |
| | 200 ppm | | | 37 | ND* | | |
| | 500 ppm | | | ND | 77 | 93 | |
| Diosmin | 1000 ppm | AOM | Rat | 56 | 70 | 93 | 33 |
| Hesperidin | 1000 ppm | | | 63 | 93 | 79 | |
| Diosmin + Hesperidin | 900 + 100 ppm | | | 61 | 73 | 93 | |
| Auraptene | 100 ppm | AOM | Rat | 41 | 49 | 58 | 35, 39 |
| | 500 ppm | | | 56 | 65 | 65 | |
| Morin | 500 ppm | AOM | Rat | 63 | 43 | 61 | 4 |
| Defatted rice-germ | 2.5% | AOM | Rat | 20 | ND | | 42 |
| γ-aminobutyric (GABA)-enriched defatted rice-germ | | | | 32 | 43 | 73 | |
| Rice-germ | | | | 57 | 61 | 64 | |
| Ferulic acid | 250 ppm | AOM | Rat | 26 | 61 | 46 | 44 |
| | 500 ppm | | | 31 | 54 | 39 | |
| Capsaicon | 500 ppm | AOM | Rat | 40 | 60 | 28 | 48 |
| Rotenone | | | | 60 | 47 | 68 | |
| Obacunone | 200 ppm | AOM | Rat | 65 | ND | | 47 |
| | 500 ppm | | | 65 | 78 | 89 | |
| Limonin | 200 ppm | | | 55 | ND | | |
| | 500 ppm | | | 56 | 94 | 89 | |
| Nobiletin | 100 ppm | AOM | Rat | 50 | ND | 18 | 49, 60 |
| | 500 ppm | | | 55 | | 48 | |
| Silymarin | 100 ppm | AOM | Rat | 47 | 24 | 29 | 54 |
| | 500 ppm | | | 54 | 80 | 73 | |
| | 1000 ppm | | | 64 | ND | | |
| Conjugated linolenic and linoleic acids | 100 ppm | AOM | Rat | 19 | ND | | 51, 58, 59 |
| | 1000 ppm | | | 36 | | | |
| | 1% | | | 63 | 38 | | |
| Ethyl acetate extract of 'Kurosu' | 500 ppm | AOM | Rat | 21 | ND | 38 | 55, 56 |
| | 1000 ppm | | | 37 | | 56 | |
| | 2000 ppm | | | 67 | ND | | |

AOM=azoxymethane.
* Not determined

and vitamins, including folate, C and E. These dietary components likely have both complementary and overlapping mechanisms of action, including induction of detoxification enzymes, blockage of carcinogen formation, shifts in hormone homeostasis, slowing of cell division, induction of apoptosis and depression of tumor angiogenesis. Although several macronutrients are likely involved in the cancer process, they do not appear to totally explain the worldwide variance in cancer risk. Furthermore, it is possible that their impact is markedly influenced by several physiologically important dietary constituents. Thus, so-called functional foods (a name based on the ability of selected foods to have health benefits over and beyond the basic nutrition provided) continue to captivate the interest of scientists and legislators and, most importantly throughout the world, the consumer[69].

## Non-Nutritive Chemopreventive Compounds in Foods

To date, more than 500 compounds have been suggested to be potential modifiers of experimental carcinogenesis, including colon tumorigenesis[8,70–79]. Some of the major antioxidant constituents of fruit, vegetables, and

beverages are derived from phenolic phytochemicals synthesized through the shikimate pathway from tyrosine and phenylalanine[80]. Many of these exist as *O*-glycosides and *O*-methyl conjugates. Cinnamic acid, widely found in fruits and vegetables, is a transformation product of phenylalanine produced by the action of phenylalanine ammonia-lyase. Isoflavonoids[81], flavonoids[82], and lignans[83] are additional plant constituents that make up the three principal classes of phytoestrogens consumed by humans. Soy, a staple for Asians, is a major source of the isoflavonoids daidzein and genistein[84]. Flavonoids are also abundantly present in fruits. Quercetin and kaempferol are two commonly found flavonoids that are particularly profuse in apples, onions, and tea leaves. Plant lignans are present in many cereal grains, fruits, and vegetables and give rise to the mammalian lignans enterodiol and enterolactone. The richest sources of lignan precursors, such as secoisolariciresinol and matairesinol, are linseed (flaxseed) and other oil seeds. *Allium* foods, including garlic, onions, and leeks, provide a host of organosulfur compounds that may influence health. Terpenes are a group of hydrocarbons made up of building blocks of isoprene ($C_5H_8$) units that are widespread in nature. Most occur in plants as constituents of essential oils. Monoterpenes are composed of two units, such as limonene, citral, and camphor, whereas sesquiterpenes are made up of three units and include compounds such as humulene, which is a hops aromatic. Carotene is an example of an 8-isoprene or tetraterpene unit.

Fruit and vegetable consumption is not the only dietary factor that can influence cancer risk. Ingestion of green and black tea, herbs and spices has been reported to be inversely associated with cancer risk[10,85-87]. As reviewed by other investigators[70,71,79], numerous non-nutritives in foods (vegetables and fruits) can inhibit colon carcinogenesis in rodents.

## Preneoplastic Lesions for Colonic Neoplasms

It has been proposed that aberrant crypt foci (ACF, Fig. 2A and B) being present in the carcinogen-treated colons of rodents and in the colons of humans with a high risk for CRC could be employed to study modulators of colon carcinogenesis (Fig. 2C)[88], because ACFs are putative precursor lesions for CRC in rodents[89] and humans[90]. ACFs possess several biological aberrations, including gene mutations and amplification[89]. Also, alteration (decreased) of hexosaminidase activity is found in ACFs. Tsukamoto *et al.*[91] found downregulation of both hexosaminidase-$\alpha$ and hexosaminidase-$\beta$ in ACFs. ACFs also have increased cell proliferation activity compared with surrounding normal crypts[92,93]. We have also previously determined the hyper-cell proliferation activity of ACFs, especially dysplastic ACFs[94] (Fig. 3). Certain chemopreventive compounds have been reported to reduce such hyper-cell proliferation in ACFs[95,96] and to inhibit c-*myc* expression induced by methylazoxymethanol (MAM) acetate[97]. We have previously used the following two experimental bioassays to demonstrate the inhibitory action of compounds in colon carcinogenesis: (i) a 5-week short-term bioassay of ACFs for screening of natural compound in vegetables and fruits that have possible chemopreventive ability (Fig. 2C) and (ii) a long-term rat colon carcinogenesis model (Fig. 4A) for evaluating their inhibitory effects against colonic tumor development (Figs. 4B, C and D). Several biochemical and morphologic biomarkers are used in these bioassays (Table 5). Cell proliferation plays an important role in multistage carcinogenesis[72,98,99]. ODC and polyamines are intimately involved in normal cellular proliferation and are likely to play a role in carcinogenesis, including colon tumorigenesis[100,101]. The 5'-bromodeoxyuridine (BrdU)-labeling index, proliferating cell nuclear antigen (PCNA)-labeling index, and silver-stained nucleolar regions (AgNORs) number are also known to be proliferation biomarkers[76].

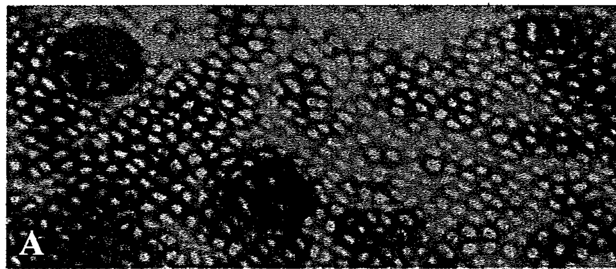
Current data suggests that the balance between the phase I carcinogen-activating enzymes and the phase II detoxifying enzymes is critical to determining an individual's risk for cancer[102]. Human deficiencies in phase II enzyme activity, specifically glutathione *S*-transferase (GST), have been identified and associated with increased risk for CRC[103]. Therefore, phase II detoxifying enzymes, such as GST and quinone reductase (QR), might be useful as biomarkers for chemopreventive studies.

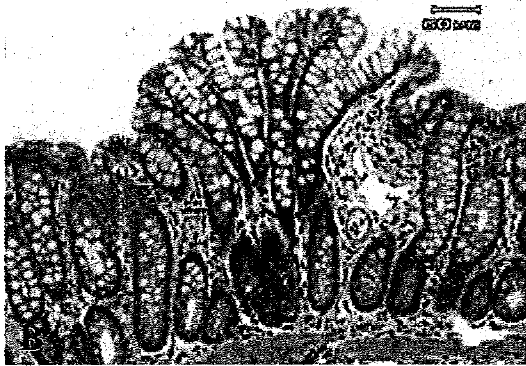
## Screening of Possible Chemopreventive Agents Against Colon Tumorigenesis Ability Using a 5-Week Short-Term ACF Bioassay

As the first bioassay for pilot studies, we investigated the modifying effects of test compounds on the development of ACFs. ACFs could be induced by weekly subcutaneous injections of azoxymethane (AOM, 15 mg/kg body weight, 3 times, or 20 mg/kg body weight, 2 times); test chemicals were added to the basal diet at various dose levels and administered to male F344 rats for 5 weeks, starting 1 week before AOM dosing (Fig. 2C). At the end of the study, the number of ACFs were counted, and expression of several biomarkers was examined. The biomarkers assayed included ODC activity and polyamine level in the colonic mucosa, number of AgNORs protein/nucleus in the colonic crypts and/or the activities of GST and QR in the colonic mucosa.

## Evaluation of the Chemopreventive Abilities of Selected Compounds Using a Long-Term Rat Colon Carcinogenesis Model

Based on the results of the pilot studies, a second bioassay was conducted to evaluate the chemopreventive effects of compounds, which were previously screened in the short-term pilot study, on colon carcinogenesis. Male F344 rats were given subcutaneously injections of AOM (15 mg/kg body weight, weekly, 3 times, or 20 mg/kg body weight, 2 times) to induce colonic adenoma and adenocarcinoma (Fig. 4B, C and D). For "initiation" feeding, oral



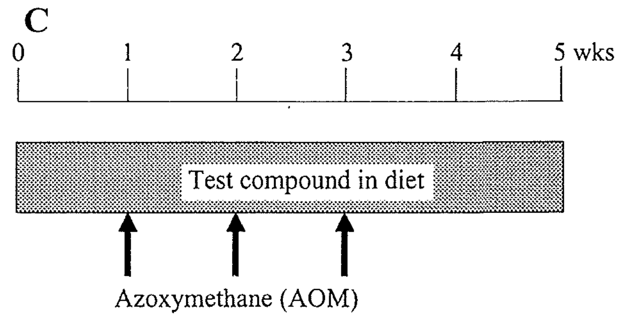



Fig. 2. Colonic lesisons 'induced by a colonic caracinoge zoxymethane (AOM). (A), Three large ACF in the colonic mucosa stained with methylene blue. (B) Histopathology of ACF stained with hematoxylin and eosin, bar=60 $\mu$m. (C) An ACF bioassay protocol for screening compounds that exert inhibitory activity of ACF formation in colon of rodents. Rats are given 2 (20 mg/kg bw) or 3 (15 mg/kg bw) weekly subcutaneous injections of AOM to induce colonic ACF. Test compounds are given during the study. At sacrifice (wk 5), number of ACF and expression of several biomarkers are assayed.

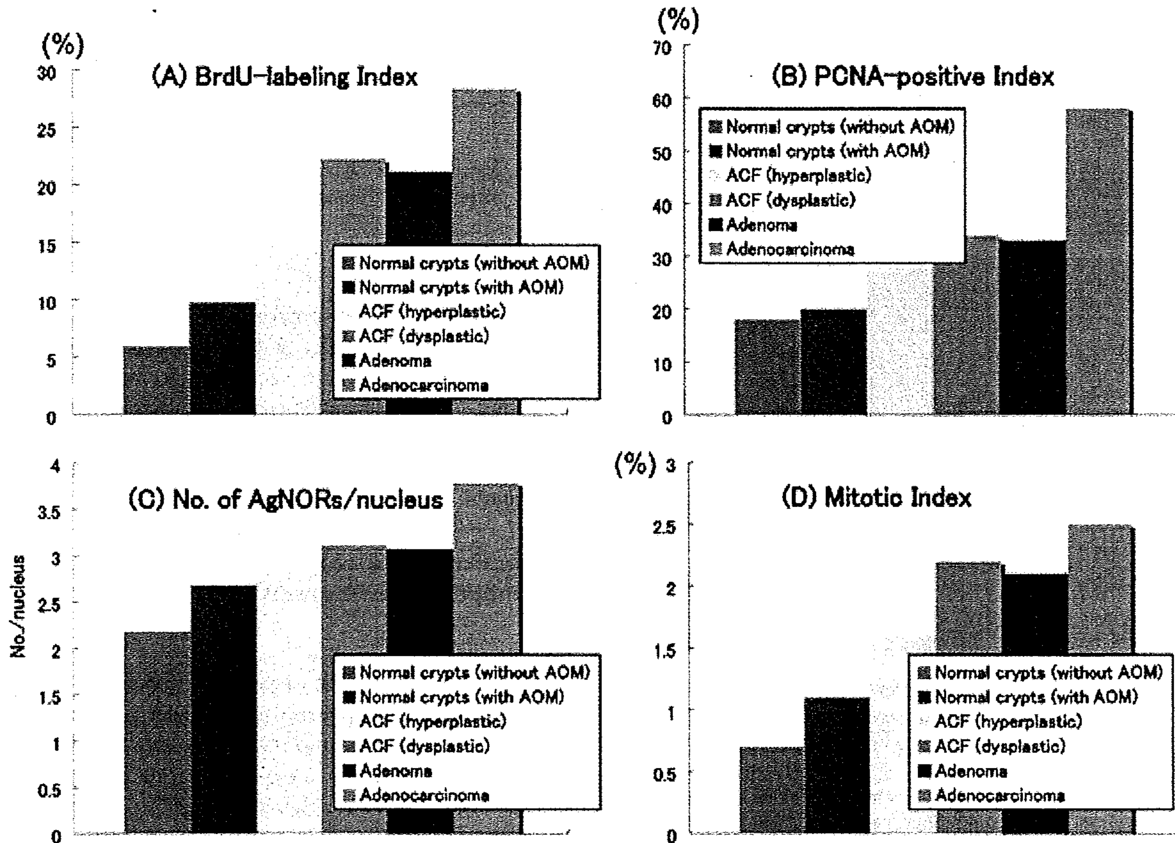



Fig. 3. Proliferative activities of the proliferative lesions induced by AOM in rats. (A) BrdU-labeling index, (B) PCNA-labeling index, (C) Number of AgNORs per nucleus, and (D) Mitotic index.

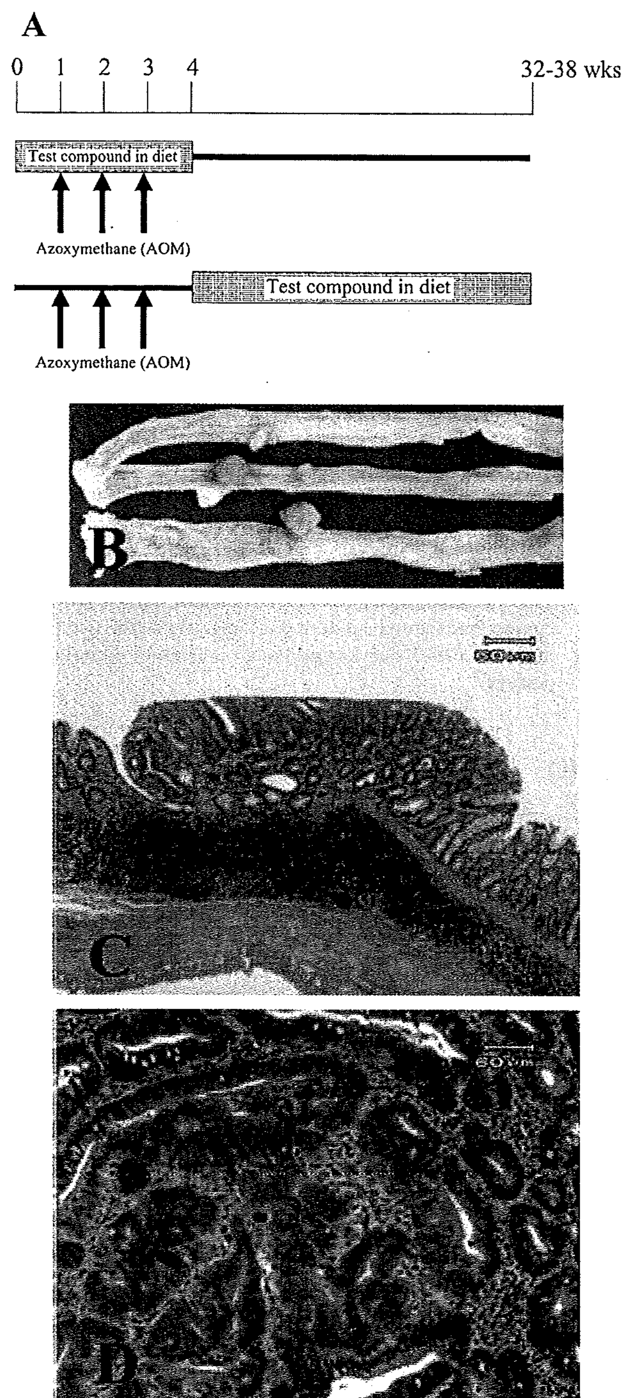



**Fig. 4.** (A) A long-term bioassay protocol for detecting compounds that can suppress development of colonic tumros in rats. Animals receive 2 (20 mg/kg bw) or 3 (15 mg/kg bw) weekly subcutaneous injections of AOM 2 to induce colonic neoplasms. Test compounds are given during the initiation stage and the post-initiation stage. At sacrifice (wks 32–38), incidence and multplicity of colonic neoplasma and expression of several biomarkers are assayed. (B) Macrosopic view of polypoid colonic tumors induced by AOM. (C) Tubular adenoma induced by AOM. bar=60 $\mu$m. (D) Tubular adenocarcinoma induced by AOM. bar=60 $\mu$m.

**Table 5.** Several Biomarkers Used for Detection of Chemopreventive Compounds Against Colon Carcinogenesis

| | Biomarkers |
|---|---|
| Proliferation activit | BrdU-labeling index, PCNA-labeling index, AgNORs number, Apoptotic index, Tumor-angiogenesis, etc. |
| Biochemistry | Carcinogen-DNA adduct or oxidative DNA adducts measures, ODC activity, Polyamine levels, CYP activity, GST activity, QR activity, MDA, 4-HNE |
| Histopathology | ACF, BCAC, MDF, Adenoma, Adenocarcinoma |

BrdU, 5'-bromodeoxyuridine; PCNA, proliferative nuclear antigen; AgNORs, silver-stained nucleolar regions; ODC, ornithine decarboxylase; GST, glutathione *S*-transferase; QR, quinone reductase; MDA, malondialdehyde; 4-HNE, 4-hydroxy-2(E)-nonenal; ACF, aberrant crypt foci; BCAC, $\beta$-catenin accumulated crypts; and MDF, mucin-depleted foci.

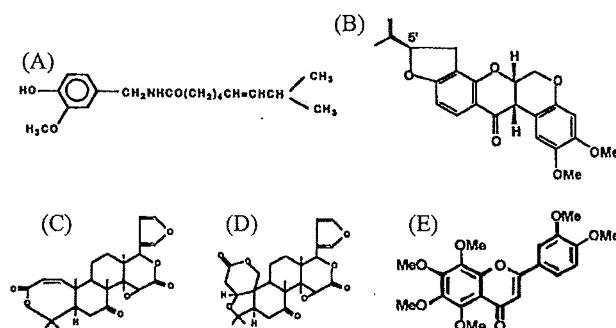



**Fig. 5.** Chemical structures of (A) capsaicin, (B) rotenone, (C) obacunone, (D) limonin, and (E) nobiletin.

administration of these compounds in the diet began 1 week before the AOM exposure and continued for 3 or 4 weeks, and for "post-initiation" feeding, experimental diets containing test compounds were given for 28 or 32 weeks, beginning 1 week after the last dosing of AOM. Several biomarkers (Table 5) were assayed at the end of the experiment.

## Examples for Potential Non-Nutritive Chemopreventive Agents

### *Capsaicin and rotenone*[48]

Capsaicin (Fig. 5A) is widely consumed as a food additive throughout the world, particularly in Southeast Asia and Latin American countries. Koreans are large consumers of capsicum fruit: the average daily per capita consumption of capsaicin may be as high as 50 mg[104]. Fresh capsaicin fruit contains about 0.02% capsaicin and dried ripe fruit contains about 0.5–1.0% capsaicin[105]. It is currently used as a versatile tool for the study of pain mechanisms and also for pharmacotherapy to treat several pain disorders because of its selective effects on the functions of a defined subpopulation of sensory neurons[106]. Excessive intake of hot

peppers containing capsaicin has been considered to be an irritant for the gastric mucosa and may be a risk factor for several gastrointestinal lesions, including gastric ulcers and cancer. However, some studies have suggested that capsaicin may have a beneficial effect on human peptic ulcers and certain types of cancer[105]. In animal carcinogenesis, capsaicin can inhibit cancer development in multiple organs such as the stomach, lung, and liver[105].

Rotenone (Fig. 5B) is a naturally occurring pesticide derived from root and back of the Derris and Lonchocarpus species, and is relatively harmless for mammals, especially after oral administration. Rotenone, deguelin and related compounds (rotenoids) are the active ingredients of botanical insecticides that have been used for at least 150 years to control crop pests. They have been used even longer as fish poisons by native tribes in order to obtain food in South America and East Africa and more recently in fish management to achieve the desired balance of species. The acute toxicity of rotenone to insects, fish, and mammals is attributable to inhibition of mitochondrial NADH:ubiquinone oxidoreductase activity as the primary target. Rotenoids are known not only as toxicants but also as candidate chemopreventive agents against liver tumors in mice[107], mammary tumors in rats[108], and skin tumors in mice[109]. Also, rotenoids can inhibit cell proliferation induced by a peroxisome proliferator in the mouse liver[107], and deguelin and three of its derivatives can inhibit phorbol ester-induced ODC activity as a biomarker of cancer chemopreventive potency[110,111].

In our study to determine the modifying effects of dietary feeding with administration of capsaicin and rotenone on AOM-induced colon tumorigenesis in male F344 rats, gavage with capsaicin and rotenone significantly elevated phase II enzymes, GST and QR, in the liver and colon. In an ACF bioassay, feeding with capsaicin and rotenone at a dose of 500 ppm for 4 weeks significantly inhibited ACF formation induced by AOM (20 mg/kg body weight, once a week for 2 weeks). Furthermore, at week 12, the treatments with capsaicin and rotenone significantly suppressed the number of large ACFs containing 4 or more crypts, which strongly correlates with tumor formation[112]. In a subsequent long-term study designed to confirm the protective effects of both compounds on ACFs development, dietary exposure of capsaicin (500 ppm) during the initiation phase (for 4 weeks) was found to significantly reduce the incidence of colonic adenocarcinoma (60% vs. 24%, 60% reduction, $P<0.05$). Feeding with rotenone (500 ppm) during the post-initiation phase (for 34 weeks) also reduced the frequency of colonic adenocarcinoma (60% vs. 19%, 68% reduction, $P<0.05$). Our results suggested that the two chemicals could inhibit the growth of colonic ACFs and suppress the progression of preneoplasia to malignancy. Subsequent long-term experiments have confirmed the results of the pilot study. The data concerning the incidence of colonic adenocarcinoma indicated that capsaicin can inhibit AOM-induced colon carcinogenesis when fed during the initiation phase, while rotenone exerted its chemopreventive action when fed during the post-initiation phase. This is the first report describing the preventive effects of capsaicin and rotenone in an animal model of colon carcinogenesis. Our data suggests that capsaicin and rotenone are potentially new dietary preventive agents against CRC development. In the study, rotenone had suppressing effects on AOM-induced colon tumorigenesis rather than blocking effects. The mechanism(s) by which rotenone exerts its inhibitory action when fed during the post-initiation is unknown, but feeding with rotenone reduced the PCNA-labeling index in colonic adenocarcinoma and the polyamine level in the colonic epithelium. These results may indicate that modulation of cell proliferation by feeding with rotenone accounts for part of its chemopreventive action. Our results suggest that rotenone has a possible cancer chemopreventive ability. However, Betarbet *et al.* recently reported that chronic exposure of rotenone reproduces several features of human Parkinson's disease in rats[113]. Therefore, chronic toxicity tests for rotenone should be done prior to its use as a chemopreventive drug.

*Obacunone and limonin*[47]

Limonoids are a group of triterpene derivatives present in the Rutaceae and Meliaceae families. Limonoids, including obacunone (Fig. 5C) and limonin (Fig. 5D), are also found in citrus seeds, commercial citrus juice and *Philodendron amurense* (Kihada). For example, commercial orange juice contains an average of 320 ppm limonoid glucosides[114]. These glucosides are responsible for the delayed bitterness of citrus juices and processed products[115]. Obacunone and limonin have been reported to enhance GST activity of various organs in mice[115]. Limonin and nomilin have been reported to inhibit forestomach, buccal pouch, lung and skin carcinogenesis in rodents[115]. However, the modifying effects of the citrus limonoids obacunone and limonin on large bowel carcinogenesis have not been reported. In our pilot study, we examined the modifying effects of obacunone and linonin on AOM-induced (20 mg/kg body wt, once a week for 2 weeks) formation of ACFs. Dietary feeding with both compounds at dose levels of 200 and 500 ppm during AOM exposure for 4 weeks ("initiation" feeding) or after AOM treatment for 4 weeks ("post-initiation" feeding) significantly inhibited ACF formation (55–65% reduction by "initiation" feeding, $P<0.001$; 28–42% reduction by "post-initiation" feeding, $P<0.05$). A subsequent long-term study was designed to confirm the protective effects of obacunone and limonin on ACF development. Dietary exposure to obacunone or limonin at a dose of 500 ppm during the initiation phase (for 4 weeks) was found to have significantly reduced the incidence of colonic adenocarcinoma (72% vs. 25% or 6%, $P<0.005$). Feeding with 500 ppm obacunone or limonin during the post-initiation phase (for 29 weeks) also reduced the frequency of colonic adenocarcinoma (72 vs 13%, $P<0.001$). The results of the pilot study suggest that the two chemicals tested can inhibit the growth of colonic ACFs and

suppress the progression of preneoplasia to malignancy. Subsequent long-term experiments confirmed the results of the pilot study. It should be noted, however, that since commercial orange juice contains 320 ppm of lirnonoid glucosides[114], the concentrations necessary to achieve the effects observed in our study would be approximately 12- to 30-fold higher than those obtained from normal dietary ingestion of these limonoids. Our data suggests that obacunone and limonin are potentially new dietary preventive agents against CRC development.

One possible mechanism for the suppression of colonic tumor development might be control of cell proliferation in the ACFs and/or "normal appearing" crypts of rats exposed to AOM. Increased cell proliferation has been suggested to play an important role in multistage carcinogenesis[116], including colon tumorigenesis[117]. There is a greater correlation between ACFs and reduction of the proliferating cell nuclear antigen labeling index of ACFs than between ACFs and reduction in the size of the proliferative components of ACFs in rats[95]. Overexpression of cyclin D1 has been reported in ACFs and adenocarcinomas in the mouse colon[118]. Overexpression of cyclin D1 plays an important role and is an early event in colon tumorigenesis. Therefore, we suspect that dietary administration of obacunone and limonin after AOM injections might lower cell proliferation activity in ACFs and/or colonic tumors. The results of our study clearly demonstrate the inhibitory effects of dietary obacunone and limonin on AOM-induced colon tumorigenesis. Further experiments, including preclinical efficacy and mechanistic studies, are warranted to fully evaluate these natural compounds for their cancer preventive properties and to understand their mode of action. Additional toxicity studies, such as genotoxicity, reproduction toxicity, acute oral toxicity and 2-year carcinogenicity trials should also be conducted prior to their use as chemopreventive drugs. One advantage of these compounds as chemopreventive agents in human trials is that, unlike synthetic chemopreventive agents, they are naturally occurring compounds that are produced endogenously in edible plants and are present in human foods.

*Nobiletin*[49,60]

Citrus fruit is a rich source of cancer inhibiting agents[77]. Nobiletin (5,6,7,8,3',4'-hexamethoxyfiavone) is a polymethoxy flavonoid extracted from citrus fruits (Fig. 5E)[119]. This compound has been reported to inhibit proliferation of human cancer cells[120] and exert anti-mutagenic activity[121]. These findings suggest a possible inhibitory effect of nobiletin on colon carcinogenesis. In the current study, the possible modifying effect of nobiletin on AOM-induced rat colon tumorigenesis was investigated. Also, several biomarkers for cancer chemoprevention studies were assayed for mechanistic investigation.

We first conducted an ACF bioassay to determine the modifying effects of dietary feeding with the polymethoxy flavonoid nobiletin isolated from Citrus unshiu on the development of AOM-induced colonic ACF in male F344 rats. We also assessed the effects of nobiletin on the cell proliferation activity of ACFs using a monoclonal antibody, MIB-5. The rats were given subcutaneous injections of AOM (15 mg/kg body weight) once a week for 3 weeks to induce ACFs, They also received an experimental diet containing 100 ppm or 500 ppm nobiletin for 5 weeks, starting one week before the first dosing of AOM. AOM exposure produced 139 ± 35 ACFs/rat at the end of the study (week 5). Dietary administration of nobiletin caused a significant reduction in the frequency of ACF: 70 ± 15 (50% reduction, $P<0.001$) at a dose of 100 ppm and 63 ± 10 (55% reduction, $P<0.001$) at a dose of 500 ppm. Feeding with nobiletin significantly lowered the MIB-5-index of ACFs. Also, dietary administration of nobiletin significantly reduced the prostaglandin (PG) $E_2$ content of the colonic mucosa. These findings might suggest a possible chemopreventive ability for nobiletin through suppression of the cell proliferating activity of ACFs during their development. A subsequent long-term experiment was conducted to investigate the inhibitory effects of dietary feeding with citrus nobiletin on AOM-induced colon tumor development in rats. Five-week old male F344 rats were initiated with two weekly subcutaneous injections of AOM (20 mg/kg bw) to induce colonic tumors. They were also given the diets containing 100 ppm or 500 ppm nobiletin for 34 weeks, starting one week after the last dosing of AOM. At the end of the study, the incidence of colonic adenocarcinoma were 67% in the AOM alone group, 55% in the AOM→100 ppm nobiletin group and 35% ($P<0.05$) in the AOM→500 ppm nobiletin group. Also, feeding with nobiletin reduced the cell-proliferation activity, increased the apoptotic index, and decreased the $PGE_2$ content in the colonic adenocarcinoma and/or colonic mucosa. These findings might suggest that citrus nobiletin has chemopreventive ability against AOM-induced rat colon carcinogenesis. In the study, administration of nobiletin reduced biosynthesis of $PGE_2$ in colonic adenocarcinomas and in their surrounding mucosa. Eicosanoids, including $PGE_2$, the metabolites of arachidonic acid (AA) through the lipoxygenase (LOX) and cyclooxygenase (COX) pathways, have a variety of biological activities. AA products synthesized via these pathways can modulate colon carcinogenesis[122], and some inhibitors of the AA cascade possess chemopreventive activity in colon carcinogenesis[123,124]. Although, we did not investigate expression of COX and LOX in the colonic mucosa in this study, nobiletin has been reported to suppress the COX-2 expression in RAW 264.7 cells treated with lipopolysaccharide[125] and interferon (IFN)-$\gamma$, suggesting that nobiletin may affect both pathways of AA. The results of this study suggest that dietary nobiletin has a beneficial effect on chemically-induced rat colon carcinogenesis. Our findings and those of other studies concerning the possible anti-metastatic ability of nobiletin[126,127] are of interest and suggest the need for further investigations of biological function and the mechanisms of nobiletin in fighting cancer

development. Recent studies[125,128] have demonstrated the inhibitory effects of nobiletin on carcinogenesis in the tissues other than the colon, as is the case of another citrus compound, auraptene[35,39,129].

*Silymarin*[54]

Silymarin, the collective name for an extract from milk thistle [*Silybum marianum* (L.) Gaertneri] is a naturally occurring polyphenolic flavonoid antioxidant[130]. It is composed mainly (approximately 80%, w/w) of silybin (also called silybinin, silibin or silibinin), with smaller amounts of other stereoisomers, such as isosilybin, dihydrosilybin, silydianin and silychristin. Silymarin protects experimental animals against the hepatotoxin $\alpha$-amanitin and has a strong antioxidant property. Other biologic properties have been reported for silymarin and its components, including inhibition of LOX[131] and PG synthetase[132]. For over 20 years, silymarin has been used clinically in Europe for treatment of alcoholic liver disease and as an anti-hepatotoxic agent. As a therapeutic agent, it is well tolerated and largely free of adverse effects[133]. It might be a potent anti-carcinogen against *in vitro* and *in vivo* carcinogenesis. However, the animal chemopreventive studies concluded to date have mainly been limited to skin[134,135], and only few studies have involved the digestive organs, including the colon[136]. The silymarin group of flavonoids (silybin, silychristin and silydianin) inhibits xanthine oxidase[137]. Silymarin induces G1 arrest in the human prostate carcinoma DU 145 cell and causes growth inhibition by inactivation of the erbB1-SHC signaling pathway leading to upregulation of Kip1/p27 followed by its increased binding with CDK causing a decrease in CDK- and cyclin-associated kinase activity[138]. These findings led us to evaluate the possible suppressing effects of dietary silymarin on the development of ACFs.

In the short-term ACF bioassay, the effects of silymarin on the development of AON-induced colonic ACFs, being putative precursor lesions for colonic adenocarcinoma, were studied to predict the modifying effects of dietary silymarin on colon tumorigenesis. Also, the activity of detoxifying enzymes, GST and QR, in the liver and colonic mucosa was determined in rats gavaged with silymarin. Subsequently, the possible inhibitory effects of dietary feeding with silymarin on AOM-induced colon carcinogenesis were evaluated using a long-term animal experiment In the short-term study, dietary administration of silymarin (100, 500 and 1,000 ppm in diet), either during or after carcinogen exposure, for 4 weeks caused significant reduction in the frequency of colonic ACFs in a dose-dependent manner. Silymarin given by gavage elevated the activity of detoxifying enzymes in both organs. In the long-term experiment, dietary feeding with silymarin (100 and 500 ppm) during the initiation or post-initiation phase of AOM-induced colon carcinogenesis reduced the incidence and multiplicity of colonic adenocarcinomas. The inhibition by feeding with 500 ppm silymarin was significant ($P<0.05$ by "initiation" feeding and $P<0.01$ by "post-initiation" feeding). Also, administration of silymarin in the diet lowered the PCNA labeling index and increased the number of apoptotic cells in adenocarcinomas. The $\beta$-glucuronidase activity, $PGE_2$ level and polyamine content were decreased in the colonic mucosa. These results clearly indicate the chemopreventive ability of dietary silymarin against chemically induced colon tumorigenesis and provide a scientific basis for progression to clinical trials of chemoprevention of human CRC. Our results are basically in agreement with those of Gershbein[139], who found that dietary feeding with silymarin (1,000 ppm) during the entire period of 1,2-dimethylhydrazine (DMH)-induced rat intestinal carcinogenesis significantly inhibited the development of large and small intestinal adenocarcinomas. We did not observe an inhibitory effect for silymarin on the incidence of small intestinal neoplasms. This may due to their low incidence and the use of a different carcinogen. Silymarin inhibited the growth of human breast[140] and prostate[138] cancer cell lines. Chemopreventive effects have been found for silymarin on mouse bladder carcinogenesis[141], rat tongue carcinogenesis, and rat prostate tumorigenesis[142]. Thus, silymarin may possess cancer chemopreventive ability in multiple organs. There are several mechanisms by which chemopreventive agents exert their inhibitory effects on tumorigenesis. AOM is an intermediate of the colonic carcinogen DMH and is metabolized by cytochrome P450 2E1, possibly by cytochrome 450 1A, and by the phase II carcinogen-detoxifying enzyme GST[143]. However, silymarin has no influence on liver P4502E1[144]. We[77] and others[145] have reported that certain chemopreventive agents inhibit the development of ACFs and carcinomas induced by AOM through induction of GST and QR. Also, epidemiologic observations suggest that consumption of certain cruciferous vegetables reduces the risk of CRC in individuals with null-type GSTM1[146]. Our results concerning GST and QR activities in the liver and colon could explain the decrease in ACF formation and the CRC development in rats given silymarin during the initiation phase. Further studies to assess the chemopreventive ability of silymarin are needed in different carcinogenesis models[147].

*Other compounds*

Our recent studies demonstrated that juices rich in hesperidin and $\beta$-cryptoxanthin could inhibit AOM-induced rat colon tumorigenesis[40,45]. Juices rich in hesperidin and $\beta$-cryptoxanthin also inhibit lung tumorigenesis in mice[148]. Thus, citrus fruit is a rich source of cancer inhibiting agents[77]. The rhizomes of *Zingiber zerumbet* Smith are used as an anti-inflammatory folk medicine in Indonesia[149]. A sesquiterpene zerumbone isolated from the rhizome is a potent inhibitor of 12-*O*-tetradecanoyl-13-acetate-induced Epstein-Barr virus activation[150], expression of inducible nitric oxide synthase (iNOS) and COX-2 expression in RAW 264.7 macrophages treated with lipopolysaccharide and IFN-$\gamma$ and $NO/O_2^-$ generation in leukocytes[151]. We demonstrated that dietary feeding with zerumbone is capable

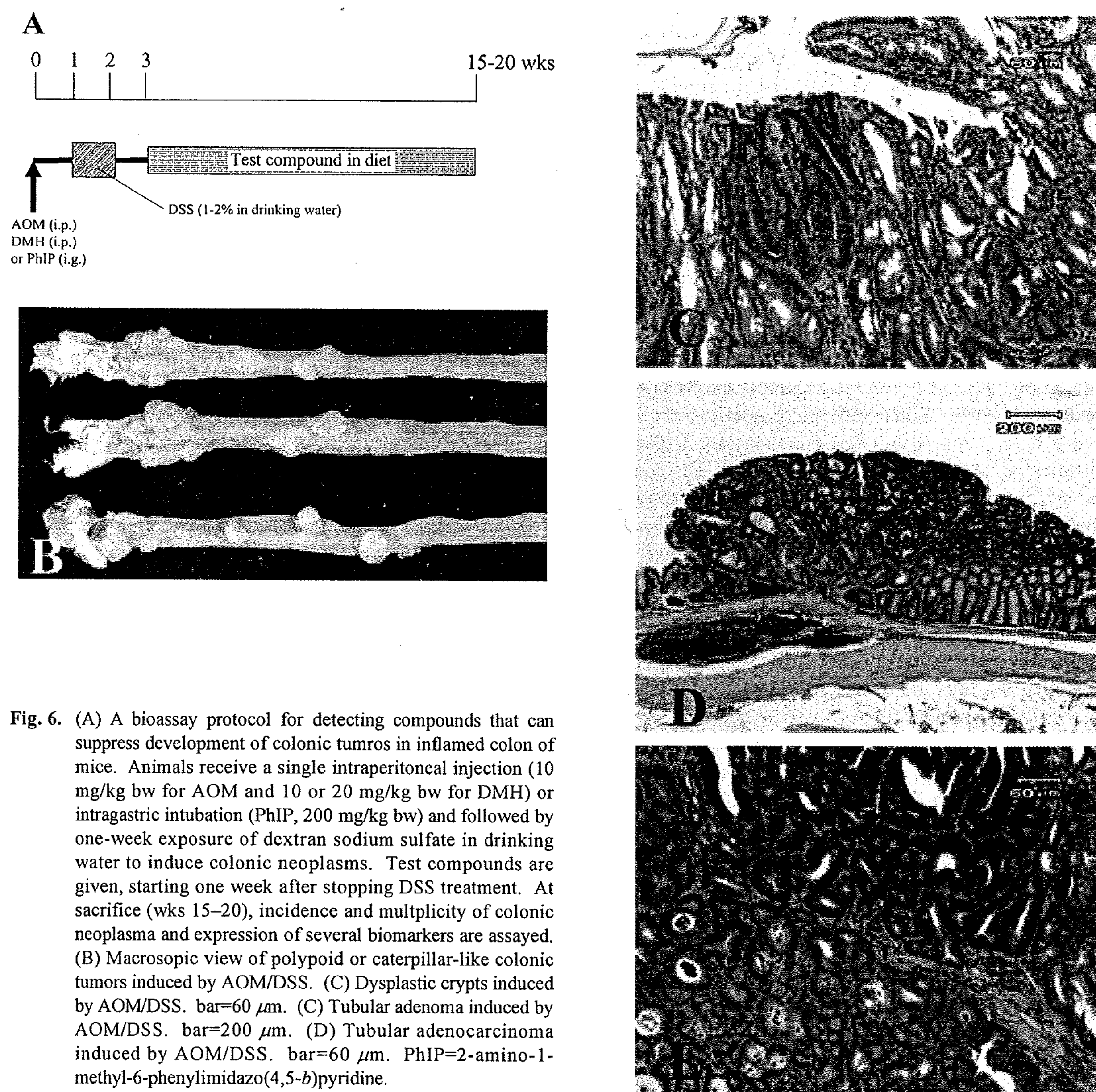



**Fig. 6.** (A) A bioassay protocol for detecting compounds that can suppress development of colonic tumros in inflamed colon of mice. Animals receive a single intraperitoneal injection (10 mg/kg bw for AOM and 10 or 20 mg/kg bw for DMH) or intragastric intubation (PhIP, 200 mg/kg bw) and followed by one-week exposure of dextran sodium sulfate in drinking water to induce colonic neoplasms. Test compounds are given, starting one week after stopping DSS treatment. At sacrifice (wks 15–20), incidence and multplicity of colonic neoplasma and expression of several biomarkers are assayed. (B) Macrosopic view of polypoid or caterpillar-like colonic tumors induced by AOM/DSS. (C) Dysplastic crypts induced by AOM/DSS. bar=60 $\mu$m. (C) Tubular adenoma induced by AOM/DSS. bar=200 $\mu$m. (D) Tubular adenocarcinoma induced by AOM/DSS. bar=60 $\mu$m. PhIP=2-amino-1-methyl-6-phenylimidazo(4,5-*b*)pyridine.

of suppressing AOM-induced ACF formation in rats[50]. Recent unpublished work has confirmed the findings of the ACF bioassay. A polyisoprenylated benzophenone, garcinol (also named camboginol) is present in Guttiferae. The dried rind of G. indica ('Kokum') containing garcinol (2–3%, w/w) is used as a garnish for curry and in traditional medicine in India. We have recently found the inhibitory effects for garcinol on AOM-induced ACFs[46], and a long-term experiment demonstrated that dietary garcinol at doses of 50 and 250 ppm suppresses AOM-induced rat colon carcinogenesis[152]. Ferulic acid (FA), which is widely found in brans of rice, wheat and barley, vegetables and other edible plants, can inhibit chemically-induced colon carcinogenesis[44]. Recently, Tsuda's group synthesized a new chemical, 3-(4'-geranyloxy-3-methoxyphenyl)-2-propenoate (EGMP), from the parent compound FA by adding a geranyl chain. They tested the chemopreventive efficacy of EGMP and FA on AOM-induced ACFs, since this compound is a more potent antioxidant than FA. They concluded that both compounds are effective in reducing ACF formation and that the effect of EGMP is more potent than that of FA[153]. More recently, our collaborating work with Dr. Epifano revealed that a novel prodrug of EGMP/FA is effective in inhibiting colitis-related colon carcinogenesis in mice[154] using our own mouse model[155] (Fig. 6).

## Discussion

Our recent data on the chemopreventive effects of naturally occurring compounds, capsaicin, rotenone,