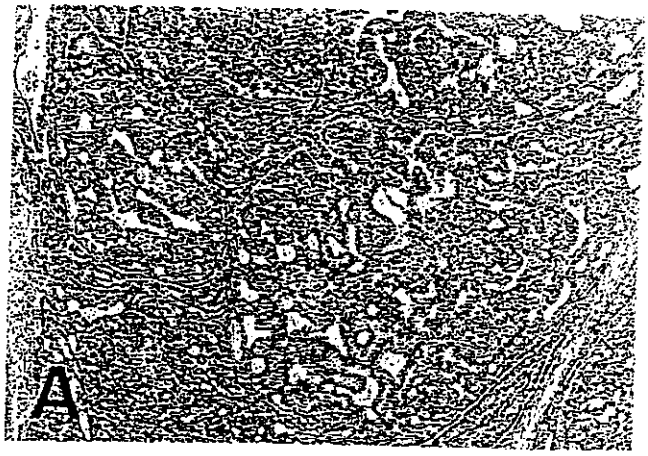
was 18% in the 10% miso group ($P<0.05$, data not shown) and 20% in the 10% miso+TAM group.

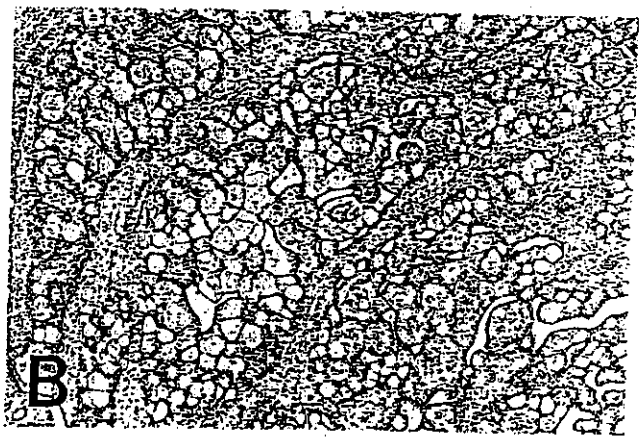
Chemopreventive effect of miso and TAM, alone and in combination The effects of 10% miso and TAM, alone and in combination, on the incidence and multiplicity of mammary tumors are shown in Fig. 2 and Table II. In the combination group, the tumor latency was greatly reduced, and there was a significant reduction in the incidence of palpable mammary tumors during the experiment compared to the control group ($P<0.0001$ or less). The multiplicity of palpable mammary tumors in all treatment groups was significantly reduced during the experiment compared to the control group ($P<0.01$). The incidence (%) and multiplicity (mean tumors/rat) of mammary tumors at termination were 91% and 4.5 in the control group, 77% and 2.4 ($P<0.05$) in the 10% miso group, and 68% ($P<0.01$) and 1.4 ($P<0.01$) in the TAM group. Tumor incidence and multiplicity in the combination group were 10% ($P<0.0001$ or less) and 0.2 ($P<0.0001$), and were also significantly decreased compared to the values in the TAM group ($P<0.01$ and $P<0.05$, respectively). Mean tumor size in the combination group was significantly reduced compared to both the control group and the TAM group ($P<0.001$ and $P<0.05$, respectively).

The BrdU index of the mammary tumors in each group is summarized in Table II. The BrdU index of mammary tumors in the groups given TAM was significantly decreased compared to the control group ($P<0.05$). The E_2 levels in serum and ERc levels in mammary tumors are summarized in Table III. The groups given miso and TAM, both alone and in combination, tended to have decreased serum E_2 levels. The serum E_2 levels in the groups given the miso diet were significantly decreased compared to the control group ($P<0.05$). In the 10% miso group, the maximum number of binding sites was significantly increased in the mammary tumors when compared with the control group ($P<0.05$).

Therapeutic effect of miso and TAM in combination The therapeutic effects of miso in combination with TAM on the regression of palpable mammary tumors after a 6-week treatment period are summarized in Table IV. At the conclusion of the diet period, mean percent tumor size in the control and TAM group was 160% and 141% of the pretreatment value, respectively. On the other hand, the value in the combination group decreased to 85% of the pretreatment value and was significantly different from the control and the TAM group ($P<0.01$ and $P<0.05$, respectively). At the conclusion of the experiment, there were no significant differences in body weight among the groups.

Histopathology of mammary tumors Although fibroadenoma has quite frequently been observed in 7,12-dimethylbenz[*a*]anthracene- or radiation-induced rat mammary tumors,[26,27] no fibroadenomas were observed in MNU-





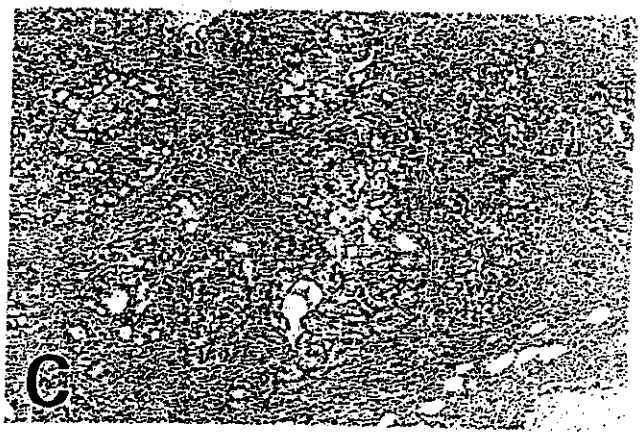


Fig. 3. Histopathology of mammary tumors in each group. All of the tumors were non-invasive papillotubular carcinoma, and photograph (A) shows a rat mammary tumor from the regular diet group. HE, ×20. Most of the neoplastic foci in the regular diet+TAM group (B) were accompanied by degenerative and vacuolated changes. HE, ×20. In the 10% miso diet+TAM group (C), heavy infiltrations of lymphoid cells were found in the stroma surrounding the tumor foci. HE, ×20.

induced rat mammary tumors in the present study. The histological appearance of the mammary tumor is shown in Fig. 3. All of the mammary tumors were non-invasive papillotubular carcinoma. The histopathology of mammary tumors in the control and the 10% miso groups was ordinary non-invasive papillotubular carcinoma (Fig. 3A), and no morphological difference between the two groups was apparent. On the other hand, most of the tumor foci in the TAM group exhibited vacuolated changes (Fig. 3B), and in the 10% miso+TAM group, heavy lymphoid cell infiltration was noted in the stroma surrounding the tumor foci (Fig. 3C).

DISCUSSION

It has been reported that soybean products in the diet reduce the risk of cancer.[28–31] In the present study, the soybean product miso significantly reduced the multiplicity of mammary tumors, indicating that a miso diet is useful in the prevention of mammary cancer. One of the candidate cancer-preventive agents in soybeans is genistein, the most abundant isoflavone in soybeans. Genistein is a potent inhibitor of tyrosine-specific protein kinases and modulates cell proliferation and transformation.[32] It also inhibits DNA topoisomerase I and II,[33] angiogenesis,[34] and the growth of cultured human gastric cancer cell lines[35] via apoptosis,[36] and arrests the cell cycle at G_2-M.[37] In addition, genistein has weak phytoestrogenic activity, with a uterotropic potency of about 1×10^{-5} that of diethylstilbestrol,[38] and it possesses antiestrogenic activity as well. It has been shown to compete with E_2 in receptor-binding assays[39,40] and to inhibit the estrogenic effects of estrone, estradiol, and diethylstilbestrol.[41] More recently, genistein has been shown to be present at higher levels in miso than in other soybean products such as soy powder, soy milk, tofu, natto, and soy sauce.[16] In our previous study, we clearly identified the presence of genistein by high-performance liquid chromatographic analysis in the serum of rats given a miso diet, but not in the serum of rats given a regular diet.[10] Thus, it is assumed that the consumption of miso-containing foods with significant levels of genistein is one possible mechanism of the protective effect against mammary cancer.

Consumption of soybean products has been shown to reduce circulating ovarian steroids in premenopausal women.[42] Several *in vitro* studies have found that genistein inhibits the biosynthesis of progesterone in bovine granulosa cells,[43] antagonizes transforming growth factor α-induced synthesis of estrogen in granulosa and theca cells,[44] and inhibits the enzyme activity of 17β-hydroxysteroid oxidoreductase type I,[45] an enzyme that converts estrone to E_2. Unlike some other flavonoids, isoflavones, including genistein are generally weak inhibitors of aromatase.[46] In the present study, the serum E_2 levels of the rats given miso were significantly reduced compared to those of the rats not given miso. E_2 stimulates breast cell proliferation and may promote breast tumor growth.[47] This suggests that miso reduces the amount of E_2 in serum, and thereby may reduce the risk of mammary cancer.

The etiology of cataract is uncertain but it is probably the result of age-related degenerative changes or metabolic factors in the lens epithelium or bow area. However, its prevalence can be modulated by alterations in sex hormone status.[48] Cataract is also one of the toxic effects of long-term administration of high doses of TAM in rats and humans.[49,50] In the present study, there was no difference regarding the appearance of cataract between the control and TAM groups, but the groups given miso tended to show decreased appearance of cataract. This suggests that miso has a protective effect against the appearance of cataract.

Atrophic change of the uterus is another toxic effect of TAM in rats.[49,51] In the present study, the uterine weight in the groups given TAM was significantly decreased compared to the groups not given TAM. This result should be attributable to the antiestrogenic effect of TAM on uterine tissue.[51]

It is documented that dietary restriction inhibits tumorigenesis in rodents.[52–54] In the present study, TAM-administered groups showed about 10–20% body weight reduction compared to the control, as evidenced by a suppression of the weight gain by 20–25 g. A two-year carcinogenicity study of TAM in rats showed that the growth rate was reduced in all groups treated with various doses of TAM.[49] This reduction in growth is believed to be a consequence of the pharmacological activity of TAM and related to changes in hormonal status.[49] On the other hand, our present results on the cumulative incidence of tumor-bearing rats and growth pattern in the TAM group are consistent with those found in animals given a 0.5 mg/kg diet of TAM by Anzano *et al.*,[20] using the same MNU-induced rat mammary carcinogenesis model. Thus, this systemic effect did not overtly affect mammary carcinogenesis in the present study.

TAM inhibits [^{3}H]thymidine uptake in the cells of preneoplastic lesions in the MNU-induced mammary carcinogenesis model.[55] *In vitro*, TAM inhibits the proliferation of human mammary cancer cells by preventing the transition of cells from the early G_1 phase to the mid-G_1 phase of the cell cycle, and as a result, cells accumulate in early G_1 phase, while the number of cells in S and G_2 plus M phases decreases.[56–58] Thus, TAM has a cytostatic effect. In the present case, the BrdU index of mammary tumors was significantly decreased in the groups given TAM and the values of BrdU index were comparable in all the groups given TAM. These results suggest that the antipro-

liferative activity may be mainly due to the effect of TAM on the mammary tumors.

We have successfully used the combination of miso and TAM for chemoprevention and for adjuvant therapy of established rat mammary cancer. To our knowledge, this is the first investigation of the chemopreventive potential of miso and TAM in combination. The increase in ERc levels of mammary tumors on the miso diet alone in the present study may point to another endocrine pathway mediating this potent antitumor effect, in addition to the decrease in the amount of E_2 in serum. The miso diet may increase the hormone dependency of mammary tumors and consequently increase the sensitivity of mammary tumors to TAM, producing a synergistic antitumor effect. Furthermore, the finding that heavy lymphoid cell infiltration was induced in the stroma surrounding the neoplastic foci may suggest another antitumor effect, involving immunomodulation rather than hormonal changes.

In summary, the soybean product miso is a useful agent for chemoprevention of MNU-induced rat mammary cancer, and is expected to have an excellent antitumor effect, especially when used in combination with TAM. Further investigation will be required to assess the usefulness and the precise mechanism of the miso and TAM in combination as a chemopreventive or therapeutic agent against mammary cancer, and to establish whether this strategy will be applicable to humans.

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REFERENCES

1) Tominaga, T. and Kuroishi, T. Epidemiology of breast cancer in Japan. *Breast Cancer*, 2, 1–7 (1995).
2) Kuroishi, T., Hirose, K., Tominaga, S., Ogawa, H. and Tajima, K. Prediction of future cancer mortality in Japan. *Jpn. J. Clin. Oncol.*, 22, 365–369 (1992).
3) Nomura, A., Henderson, B. and Lee, J. Breast cancer and diet among the Japanese in Hawaii. *Am. J. Clin. Nutr.*, 31, 2020–2025 (1978).
4) Hirayama, T. A large scale cohort study on cancer risks by diet—with special reference to the risk reducing effects of green-yellow vegetable consumption. *In* "Diet, Nutrition and Cancer," ed. Y. Hayashi, M. Nagao, T. Sugimura, S. Takayama, L. Tomatis, L. W. Wattenberg and G. N. Wogan, pp. 41–53 (1986). Japan Scientific Societies Press, Tokyo.
5) Troll, W., Wiesner, R., Shellabarger, C. J., Holtzman, S. and Stone, J. P. Soybean diet lowers breast tumor incidence in irradiated rats. *Carcinogenesis*, 1, 469–472 (1980).
6) Barnes, S., Grubbs, C., Setchell, K. D. R. and Carlson, J. Soybeans inhibit mammary tumors in models of breast cancer. *In* "Mutagen and Carcinogens in the Diet," ed. M. W. Pariza, H.-U. Aeschbacher, J. S. Felton and S. Sato, pp. 239–253 (1990). Wiley-Liss, New York.
7) Ito, A. Is miso diet effective for radiation injuries ? *Miso Sci. Technol.*, 39, 71–84 (1991) (in Japanese).
8) Watanabe, H., Takahashi, T. and Ishimoto, T. The effect of miso diet on small intestinal damage in mice irradiated by X-ray. *Miso Sci. Technol.*, 39, 29–32 (1991) (in Japanese).
9) Watanabe, H., Masaoka, Y., Gotoh, T., Fujimoto, N. and Ito, A. Effects of miso in reducing risk of liver and gastric tumors in experimental animals. *In* "Food Factors for Cancer Prevention," ed. H. Ohigashi, T. Osawa, J. Terao, S. Watanabe and T. Yoshikawa, pp. 351–354 (1997). Springer-Verlag, Tokyo.
10) Gotoh, T., Yamada, K., Yin, H., Ito, A., Kataoka, T. and Dohi, K. Chemoprevention of *N*-nitroso-*N*-methylurea-induced rat mammary carcinogenesis by soy foods or biochanin A. *Jpn. J. Cancer Res.*, 89, 137–142 (1998).
11) Masaoka, Y., Watanabe, H., Tanizaki, M., Ando, Y., Yamada, K., Gotoh, T., Fujimoto, N. and Ito, A. Effect of a miso diet on colonic aberrant crypt foci in F344 rats exposed to azoxymethane. *In* "Recent Advances in Gastroenterological Carcinogenesis 1," ed. E. Tahara, K. Sugimachi and T. Oohara, pp. 1181–1185 (1996). Monduzzi-Editore, Bologna.
12) Ito, A. and Watanabe, H. Recent topics on miso in the aspect of biological role and primary prevention of cancer. *Hiroshima J. Med. Sci.*, 47, 5–9 (1994) (in Japanese).
13) Messina, M. and Barnes, S. The role of soy products in reducing risk of cancer. *J. Natl. Cancer Inst.*, 83, 541–546 (1991).
14) Wang, H. J. and Murphy, P. A. Isoflavone content in commercial soybean foods. *J. Agric. Food Chem.*, 42, 1666–1673 (1994).
15) Coward, L., Barnes, N. C., Setchell, K. D. R. and Barnes, S. The antitumor isoflavone, genistein and daidzein, in soybean foods of American and Asian diets. *J. Agric. Food Chem.*, 41, 1961–1967 (1994).
16) Fukutake, M., Takahashi, M., Ishida, K., Kawamura, H., Sugimura, T. and Wakabayashi, K. Quantification of genistein and genistin in soybeans and soybean products. *Food Chem. Toxicol.*, 34, 457–461 (1996).
17) Hortobagyi, G. N. Overview of new treatments for breast cancer. *Breast Cancer Res. Treat.*, 21, 3–13 (1992).
18) Costa, A. Breast cancer chemoprevention. *Eur. J. Cancer*, 29A, 589–592 (1993).

19) Tominaga, T., Yoshida, K., Shimozuma, K., Hayashi, K. and Kosai, G. Effect of CGS 16949A plus tamoxifen on induced mammary tumors in rats. *Eur. J. Cancer*, 26, 600–603 (1990).
20) Anzano, M. A., Byers, S. W., Smith, J. M., Peer, C. W., Mullen, L. T., Brown, C. C., Roberts, A. B. and Sporn, M. B. Prevention of breast cancer in the rat with 9-*cis*-retinoic acid as a single agent and in combination with tamoxifen. *Cancer Res.*, 54, 4614–4617 (1994).
21) Weckbecker, G., Tolcsvai, L., Stolz, B., Pollak, M. and Bruns, C. Somatostatin analogue octreotide enhances the antineoplastic effects of tamoxifen and ovariectomy on 7,12-dimethylbenz(*a*)-anthracene-induced rat mammary carcinomas. *Cancer Res.*, 54, 6334–6337 (1994).
22) McCormick, D. L., Rao, K. V. N., Johnson, W. D., Bowman-Gram, T. A., Steele, V. E., Lubet, R. A. and Kelloff, G. J. Exceptional chemopreventive activity of low-dose dehydroepiandrosterone in the rat mammary gland. *Cancer Res.*, 56, 1724–1726 (1996).
23) Hochberg, R. B. and Rosner, W. Interaction of 16α-[^{125}I]iodo-estradiol with estrogen receptor and other steroid-binding proteins. *Proc. Natl. Acad. Sci. USA*, 77, 328–332 (1980).
24) Tominaga, T., Nomura, Y., Kobayashi, S., Yayoi, E., Wada, T., Enomoto, K., Iino, Y. and Mori, I. Estrogen receptor assay in human breast cancer tissue using 16α-^{125}I-estradiol-17β. *Prog. Med.*, 3, 399–404 (1983).
25) Ratcliffe, W. A., Carter, G. D., Dowsett, M., Hillier, S. G., Middle, J. D. and Reed, M. J. Oestradiol assays: applications and guidelines for the provision of clinical biochemistry service. *Ann. Clin. Biochem.*, 25, 466–483 (1988).
26) Shinha, D. K., Pazik, J. E. and Dao, T. L. Progression of rat mammary development with age and its relationship to carcinogenesis by a chemical carcinogen. *Int. J. Cancer*, 31, 321–327 (1983).
27) Cronkite, E. P., Shellabarger, C. J., Bond, V. P. and Lippincott, S. W. Studies on radiation-induced mammary gland neoplasia in the rat. *Radiat. Res.*, 12, 81–93 (1960).
28) Hirayama, T. Epidemiology of stomach cancer in Japan with special reference to the strategy for the primary prevention. *Jpn. J. Clin. Oncol.*, 14, 159–168 (1984).
29) Messina, M. J., Persky, V., Setchell, K. D. R. and Barnes, S. Soy intake and cancer risk: a review of *in vitro* and *in vivo* data. *Nutr. Cancer*, 21, 113–131 (1994).
30) Adlercreutz, H., Honjo, H., Higashi, A., Fotsis, T., Hämäläinen, E., Hasegawa, T. and Okada, H. Urinary excretion of lignans and isoflavonoid phytoestrogens in Japanese men and women consuming a traditional Japanese diet. *Am. J. Clin. Nutr.*, 54, 1093–1100 (1991).
31) Wu, A. H., Ziegler, R. G., Horn-Ross, P. L., Nomura, A. M. Y., West, D. W., Kolonel, L. N., Rosenthal, J. F., Hoover, R. N. and Pike, M. C. Tofu and risk of breast cancer in Asian-Americans. *Cancer Epidemiol. Biomarkers Prev.*, 5, 901–906 (1996).
32) Akiyama, T., Ishida, J., Nakagawa, S., Ogawara, H., Watanabe, S., Itoh, N., Shibuya, M. and Fukami, Y. Genistein, a specific inhibitor of tyrosine-specific protein kinases. *J. Biol. Chem.*, 262, 5592–5595 (1987).
33) Okura, A., Arakawa, H., Oka, H., Yoshinari, T. and Monden, Y. Effect of genistein on topoisomerase activity and the growth of [VAL 12]H-*ras*-transformed NIH3T3 cells. *Biochem. Biophys. Res. Commun.*, 157, 183–189 (1988).
34) Fotsis, T., Pepper, M., Adlercreutz, H., Hase, T. A., Montesano, R. and Schweigerer, L. Genistein, a dietary ingested isoflavone, inhibits cell proliferation and *in vitro* angiogenesis. *J. Nutr.*, 125, 790–797 (1995).
35) Yanagihara, K., Ito, A., Toge, T. and Numoto, M. Antiproliferative effects of isoflavones on human cancer cell lines established from the gastrointestinal tract. *Cancer Res.*, 53, 5815–5821 (1993).
36) Yanagihara, K., Numoto, M., Tauchi, H., Akama, Y., Yokozaki, H., Tahara, E., Kamiya, K. and Seito, T. Genetic status of p53 and induction of apoptosis by radiation or isoflavones in human gastric carcinoma cell lines. *Int. J. Oncol.*, 9, 95–102 (1996).
37) Matsukawa, Y., Marui, N., Sakai, T., Satomi, Y., Yoshida, Y., Matsumoto, K., Nishino, H. and Aoike, A. Genistein arrests cell cycle progression at G_2-M. *Cancer Res.*, 53, 1328–1331 (1993).
38) Martin, P. M., Horwitz, K. B., Ryan, D. S. and McGuire, W. L. Phytoestrogen interaction with estrogen receptors in human breast cancer. *Endocrinology*, 103, 1860–1867 (1978).
39) Shutt, D. A. and Cox, R. I. Steroid and phyto-estrogen binding to sheep uterine receptors *in vitro*. *J. Endocrinol.*, 52, 299–310 (1972).
40) Mathieson, R. A. and Kitts, W. D. Binding of phyto-estrogens and estradiol-17β by cytoplasmic receptors in the pituitary gland and hypothalamus of the ewe. *J. Endocrinol.*, 85, 317–325 (1980).
41) Folman, Y. and Pope, G. S. The interaction in the immature mouse of potent oestrogens with coumestrol, genistein and other utero-vaginotrophic compounds of low potency. *J. Endocrinol.*, 34, 215–225 (1966).
42) Lu, L-J. W., Anderson, K. E., Grady, J. J. and Nagamani, M. Effect of soya consumption for one month on steroid hormones in premenopausal woman: implication for breast cancer risk reduction. *Cancer Epidemiol. Biomarkers Prev.*, 5, 63–70 (1996).
43) Kaplanski, O., Shemesh, M. and Berman, A. Effects of phyto-estrogens on progesterone synthesis by isolated bovine granulosa cells. *J. Endocrinol.*, 89, 343–348 (1981).
44) Gangrade, B. K., Davis, J. S. and May, J. V. A novel mechanism for the induction of aromatase in ovarian cells *in vitro*: role of transforming growth factor α-induced protein tyrosine kinase. *Endocrinology*, 129, 2790–2792 (1991).
45) Makela, S., Davis, V. L., Tally, W. C., Korkman, J., Salo, L., Vihko, R., Santti, R. and Korach, K. S. Dietary estrogens act through estrogen receptor-mediated processes and

show no antiestrogenicity in culture breast cancer cells. *Environ. Health Perspect.*, **102**, 572–578 (1994).
46) Campbell, D. R. and Kurzer, M. S. Flavonoid inhibition of aromatase enzyme activity in human preadipocytes. *J. Steroid Biochem. Mol. Biol.*, **46**, 381–388 (1993).
47) Pike, M. C., Spicer, D. V., Dahmoush, L. and Press, M. F. Estrogens, progestogens, normal breast cell proliferation, and breast cancer risk. *Epidemiol. Rev.*, **15**, 17–35 (1993).
48) Lazenby, C., Westwood, F. R. and Greaves, P. Crescentic cataracts in Alderley Park rats. *Vet. Pathol.*, **30**, 70–74 (1993).
49) Greaves, P., Goonetilleke, R., Nunn, G., Topham, J. and Orton, T. Two-year carcinogenicity study of tamoxifen in Alderley Park Wistar-derived rats. *Cancer Res.*, **53**, 3919–3924 (1993).
50) Jaiyesimi, J. A., Buzdar, A. U., Decker, D. A. and Hortobagyi, G. N. Use of tamoxifen for breast cancer: twenty-eight years later. *J. Clin. Oncol.*, **13**, 513–529 (1995).
51) Jordan, V.C. Effect of tamoxifen (ICI 46,474) on initiation and growth of DMBA-induced rat mammary carcinoma. *Eur. J. Cancer*, **12**, 419–424 (1976).
52) Tucker, M. J. The effect of long-term food restriction on tumors in rodents. *Int. J. Cancer*, **23**, 803–807 (1979).
53) Birt, D. F., Pelling, J. C., White, L. T., Dimitroff, K. and Barnett, T. Influence of diet and calorie restriction on the initiation and promotion of skin carcinogenesis in the Sencar mouse model. *Cancer Res.*, **51**, 1851–1854 (1991).
54) Gillette, C. A., Zhu, Z., Westerlind, K. C., Melby, C. L., Wolfe, P. and Thompson, H. J. Energy availability and mammary carcinogenesis: effects of calorie restriction and exercise. *Carcinogenesis*, **18**, 1183–1188 (1997).
55) Osborne, M. P., Rupeno, J. F., Crowe, J. P., Rosen, P. P. and Telang, N. T. Effect of tamoxifen on preneoplastic cell proliferation in *N*-nitroso-*N*-methylurea-induced mammary carcinogenesis. *Cancer Res.*, **52**, 1477–1480 (1992).
56) Taylor, I. W., Hodson, P. J., Green, M. D. and Sutherland, R. L. Effects of tamoxifen on cell cycle progression of synchronous MCF-7 human mammary carcinoma cells. *Cancer Res.*, **43**, 4007–4010 (1983).
57) Sutherland, R. L., Green, M. D., Hall, R. E., Reddel, R. R. and Taylor, I. W. Tamoxifen induces accumulation of MCF-7 human mammary carcinoma cells in the G_0/G_1 phase of the cell cycle. *Eur. J. Cancer Clin. Oncol.*, **19**, 615–621 (1983).
58) Osborne, C. K., Boldt, D. H., Clark, G. M. and Trent, J. M. Effect of tamoxifen on human breast cancer cell cycle kinetics: accumulation of cells in early G_1 phase. *Cancer Res.*, **43**, 3583–3586 (1983).