

# Genistein induces maturation of cultured human breast cancer cells and prevents tumor growth in nude mice<sup>1-3</sup>

Andreas I Constantinou, Aron E Krygier, and Rajeshwari R Mehta

**ABSTRACT** Results of recent studies in animal models of mammary carcinogenesis showed that the soybean isoflavone genistein is a chemopreventive agent. The objective of the present study was to determine whether soybean isoflavones can be used for the prevention of human breast carcinogenesis. Human adenocarcinoma cells that are either estrogen-receptor positive (such as MCF-7) or estrogen-receptor negative (such as MDA-MB-468) were used as our model system. Treatment of these cells with genistein concentrations of 15, 30, and 45  $\mu\text{mol/L}$  resulted in cell growth inhibition, which was accompanied by the expression of maturation markers. Maturation was monitored by the induction of intracytoplasmic casein and lipids and the membrane protein intercellular adhesion molecule-1. These maturation markers were optimally expressed after 9 d of treatment with 30  $\text{mmol}$  genistein/L. Both estrogen receptor-positive and -negative cells became differentiated in response to genistein treatments, suggesting that the antiestrogenic function of genistein is unrelated to the mechanism of cell differentiation. Daidzein, the other major isoflavone component of soybeans, did not induce differentiation in either MCF-7 or MDA-MB-468 cells. To explore the potential applications of this result, we used the nude mouse xenograft model of carcinogenesis. Treatment of either cell line with genistein before implantation into nude mice diminished the cells' tumorigenic potential. These data suggest that initiation of the differentiation program provides a protective effect against tumor growth in mouse xenografts. *Am J Clin Nutr* 1998;68(suppl):1426S-30S.

**KEY WORDS** Genistein, breast cancer cells, estrogen receptor, differentiation program, xenograft, daidzein, chemoprevention, MCF-7 cells, MDA-MB-468 cells

## INTRODUCTION

Treatment of preneoplastic or neoplastic cells with natural or synthetic agents that induce terminal differentiation at nontoxic concentrations may have applications in cancer prevention or treatment. Under the influence of such agents, proliferating tumor cells undergo terminal differentiation, cease dividing, and consequently lose their tumorigenic potential (1). This approach, known as differentiation therapy, has produced promising antitumor agents with no or low toxicity, and its popularity is now on the rise (2).

The natural products all-*trans*-retinoic acid and 13-*cis*-retinoic acid have preventive and therapeutic effects on carcinogen-induced premalignant and malignant lesions (3). All-*trans*-retinoic acid, which was initially recognized as an inducer of differentiation in promyelocytes, is now used to treat acute promyelocytic leukemia, with high rates of complete remission (4). A synthetic derivative of retinoids, *N*-(4-hydroxyphenyl)retinamide, was reported to up-modulate the expression of several differentiation markers in breast cancer cell lines independent of the hormone receptor status (5), and is currently being evaluated in clinical trials as a chemopreventive agent against breast carcinomas (6). Retinoids are useful in treating not only leukemias but also oropharyngeal cancers (7). Several other agents (such as vitamin D<sub>3</sub>, interferon- $\beta$ , and hexamethylene bisacetamide) are effective in the prevention or treatment, or both, of cancer through a mechanism that involves induction of differentiation (8-10).

There is evidence that soybean-based diets are associated with low incidences of breast and prostate cancers in humans, as is found in Asian populations (11). Initial animal studies suggested that genistein or daidzein or both could be the isoflavones responsible for this effect (12). More recently, genistein has been shown to be an effective chemopreventive agent during dimethylbenz[*a*]anthracene-induced mammary carcinogenesis in neonatal rats (13, 14). Genistein, however, was less effective as a chemopreventive agent in adult rats in the *N*-methyl-*N*-nitrosourea-induced mammary carcinogenesis model (15). These studies suggest that normal mammary gland development is influenced by genistein treatment, and the availability of undifferentiated progenitor cells in the mammary glands of neonatal is was a required component on genistein's cancer-protective action. These observations expanded previous reports that genistein induces differentiation in a variety of uncommitted tumor cells (16-19).

The ability to induce differentiation in a variety of cultured tumor cells, the natural origin, the lack of toxicity at physiologic concentrations, and the selective inhibition of tumor cell growth

<sup>1</sup>From the Department of Surgical Oncology, College of Medicine, University of Illinois at Chicago.

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<sup>3</sup>Address reprint requests to AI Constantinou, University of Illinois at Chicago, College of Medicine, Department of Surgical Oncology (M/C 820), 840 South Wood Street, Chicago, IL 60612. E-mail: andreasc@uic.edu.

of genistein make it an excellent candidate for differentiation therapy. Although genistein effectively induces differentiation in a variety of cell types, its effects on human breast adenocarcinomas (which are the most relevant to breast cancer prevention) have not yet been examined.

The objectives of the present studies were to 1) determine whether genistein or daidzein can induce mammary tumor cell differentiation and 2) determine whether induction of differentiation can be applied to the prevention of tumor growth. In these studies, we used 2 breast cancer cell lines, MCF-7 and MDA-MB-468, that, after treatment with the appropriate chemical inducer, can acquire a mature phenotype characterized by arrest of cell growth, presence of casein and lipids in the cytoplasm, expression of intercellular adhesion molecule-1 (ICAM-1), and loss of tumorigenic capacity when inoculated in the nude mice.

## MATERIALS AND METHODS

### Chemicals and reagents

Genistein (4',5,7-trihydroxyisoflavone) was purchased from Indofine Chemical Company (Somerville, NJ); anti-human casein was purchased from Accurate Chemical and Scientific Corp (Westbury, NY); mouse ICAM-1 was purchased from Biogenex Laboratories (San Ramon, CA); and Red Lipid was purchased from Rowley Biochemical Institute (Rowley, MA).

### Cells and culture conditions

MDA-MB-468 and MCF-7 cells were obtained from the American Type Culture Collection (Rockville, MD). MDA-MB-468 cells were cultured in minimum essential medium supplemented with 10% fetal bovine serum, penicillin (100 U/mL), and L-glutamine. MCF-7 cells were cultured in RPMI-1640 medium supplemented with 15% fetal bovine serum, penicillin (100 U/mL), and 2 mmol L-glutamine. All cells were plated at  $5 \times 10^4$  in 1 mL medium and cultured at 3°C in a humidified atmosphere of 8% CO<sub>2</sub> in air. Treatment with genistein was 24 h later at the specified doses. Cell numbers were measured by hemacytometer chamber counting and viability was monitored by trypan blue exclusion. At least 200 cells were counted for each data point. The means and SEMs were calculated from 6 independent determinations. For immunohistochemical purposes, cells were cultured in dishes containing cover slips and processed after 6 or 9 d.

### Immunohistochemical staining and visualization

The presence of casein was determined by immunohistochemical staining with mouse monoclonal antibody as described by Bacus et al (20). Cells cultured on cover slips were rinsed with phosphate-buffered saline (PBS) at room temperature and fixed in a quick dip of -20°C methanol-acetone solution. After blocking with milk (5%) for 10 min, the cells were incubated with either anticasein antibody (1:200) or ICAM-1 antibody overnight at 4°C. Slides were then rinsed with PBS and incubated for 15 min with secondary antibodies (biotinylated anti-mouse or anti-rabbit antibodies from Dako Corporation, Carpinteria, CA). The slides were rinsed in PBS and incubated for 15 min with streptavidin peroxidase conjugate (Dako Corporation). The cells were again rinsed with PBS and incubated for 10 min with 3-amino-9-ethylcarbazole chromogen (Biogenex Laboratories) and were then counterstained with hematoxylin.

A modified "Oil Red O in propylene glycol" technique was used for the lipid visualization (20). After the cover slips were placed in a special dish (Nunc Inc, Naperville, IL), they were rinsed with PBS (pH 7.6). Cells were then fixed by a quick dip in -20°C methanol. After fixation, cells were placed in absolute propylene glycol for 2 min at room temperature and then were placed for 30 min in an Oil Red O (1-8-[4-(dimethylphenylazo)dimethylphenylazo]-2-naphthalenol) staining solution. The slides were then dipped in 85% isopropanol, rinsed with deionized water, and counterstained with hematoxylin.

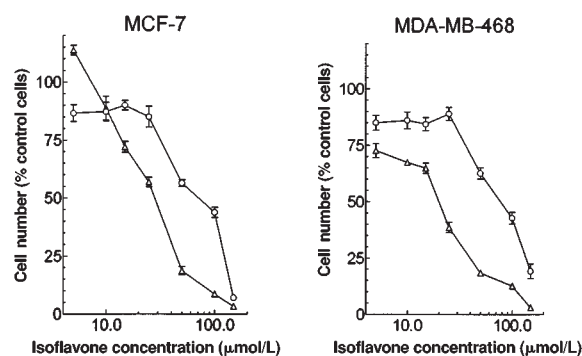
### In vivo tumor growth

Breast carcinoma cells were either untreated (controls) or treated for 2 or 6 d with genistein at 30 µmol/L. All cells were digested by trypsin and resuspended in Hanks balanced salt solution (GIBCO BRL, Gaithersburg, MD) to a final concentration of 10<sup>6</sup> cells/0.0001L and injected subcutaneously into the dorsal region of BALB/c athymic mice (Frederick Cancer Research Facility, Bethesda, MD). A total of 0.0003L ( $3 \times 10^6$  cells) were inoculated in each mouse. One week after inoculation, mice injected with MCF-7 cells also received estrogen in the form of 17β-estradiol (0.5 mg/pellet, 60-d release; Innovative Research of America, Sarasota, FL). Animals were examined twice weekly for development palpable tumors at the site of injection or other subcutaneous sites. Tumor volume was measured by using vernier calipers. Tumor doubling time was calculated as the number of days for the tumor to grow from "X" to "2X" volume. At termination of the experiments, the animals were killed, tumors were excised, and small pieces were fixed in 10% buffered formaldehyde solution and processed for hematoxylin and eosin staining for histopathologic examination. The animals were autopsied and examined for any evident tumor metastasis at distant visceral sites. The tumor latency period was determined as the time in days required for the tumor to show apparent, sustained, increasing volume from the initial volume of the injected suspension. Experiments were performed in groups of 5–6 animals.

## RESULTS

### Growth inhibition

The effect of the 2 major soybean isoflavones, genistein and daidzein, on MCF-7 and MDA-MB-468 cell growth is shown in **Figure 1**. Isoflavone concentrations tested ranged from 5 to 150 µmol/L. Genistein at 5 µmol/L had a small stimulatory effect on MCF-7 cell growth, but at higher concentrations there was a dose-dependent growth inhibitory action. The stimulatory effect at low concentrations was observed only in MCF-7 cells, and may have been due to genistein's estrogenic action at low concentrations. Genistein was more efficient in inhibiting MDA-MB-468 cells because the concentration required to inhibit 50% of cell growth (IC<sub>50</sub>) of MDA-MB-468 cells was 21 µmol/L compared with 31 µmol/L required for MCF-7 cells. Cell viability, determined by the trypan blue dye exclusion, remained >90% at concentrations of ≥45 µmol genistein/L; it began declining with increasing genistein concentrations (data not shown). Daidzein was not as effective as genistein at inhibiting the 2 cell lines. At concentrations of ≥25 µmol daidzein/L there was a small decrease in cell number compared with that of untreated controls.



**FIGURE 1.** Effect of increasing concentrations of genistein (○) or daidzein (△) on the growth of MCF-7 and MDA-MB-468 cells. Cells were plated at  $5 \times 10^7$ /L and treated 1 d later with the appropriate isoflavone at increasing doses. Cell numbers were determined after 4 d of continuous treatment and expressed as percentage of control cells, which were treated with the solvent dimethyl sulfoxide. Each data point represents the mean, and each bar the SEM, of 6 independent determinations.

### Induction of differentiation markers

The ability of genistein and daidzein to induce the expression of the mature phenotype in MCF-7 and MDA-MB-468 cells was evaluated by using immunohistochemical techniques. In these studies, we monitored the presence of neutral lipid droplets and casein, milk components characteristically secreted by differentiated mammary epithelial cells (21). ICAM-1 is an integral membrane glycoprotein (molecular mass 90000), the expression of which increases in response to heregulin, which then induces phenotypic differentiation in mammary cancer cells (22).

Less than 5% of the untreated MCF-7 or MDA-MB-468 cells stained positive for casein (panel g in **Figures 2** and **3**). The percentage of casein-secreting cells increased after treatment with genistein for 6 or 9 d in both cell lines. Although 95% of both cell types stained positive for casein when treated with genistein at doses of 15 or 30  $\mu\text{mol/L}$ , the intensity of the staining was higher when cells were treated with 30  $\mu\text{mol/L}$  genistein for 9 d (panels b and c in **Figures 2** and **3**).

A similar pattern was observed when the presence of the major milk protein, casein (types  $\beta$  and  $\kappa$ ), was assessed. In the untreated MCF-7 and MDA-MB-468 cells, no obvious lipid droplets could be observed (panel d in **Figures 2** and **3**). After cells were treated with 15 and 30  $\mu\text{mol}$  genistein/L, lipid droplets (orange stain) began to appear at 4 d posttreatment, but optimum production was seen after 9 d of treatment in both cell lines (panels e and f in **Figures 2** and **3**). The number and size of lipid droplets increased with increasing doses of genistein  $\leq 45$   $\mu\text{mol/L}$ . The difference in lipid content between cells treated with lower and higher concentrations of genistein was clearer in the MDA-MB-468 cells (compare panels e and f in **Figure 3**). A higher genistein dose (45  $\mu\text{mol/L}$ ) was also effective in inducing lipid production. Treatment of either cell line with genistein doses  $> 45$   $\mu\text{mol/L}$  or for periods longer than 9 d was cytotoxic, resulting in decreased production of neutral lipids.

The expression of ICAM-1 in MCF-7 cells in response to genistein treatment is shown in **Figure 2**, panels g, h, and i). Control MCF-7 cells were stained weekly with antibodies to ICAM-1 (panel g). After treatment with 15  $\mu\text{mol}$  genistein/L, the expression of this cell adhesion molecule increased clearly

(panel h), and even stronger staining was displayed in cells treated with 30  $\mu\text{mol}$  genistein/L (panel i).

In the MDA-MB-468 cells (**Figure 3**), some staining could be seen in the control cells and the cells treated with 15  $\mu\text{mol}$  genistein/L (panels g and h), but treatment with 30  $\mu\text{mol/L}$  (panel i) increased the intensity and percentage of positively stained cells. Immunohistochemical changes were accompanied by morphologic changes. Genistein-treated cells become flattened, and the cytoplasmic and nuclear areas became larger than in those in untreated cells (see **Figures 2** and **3**).

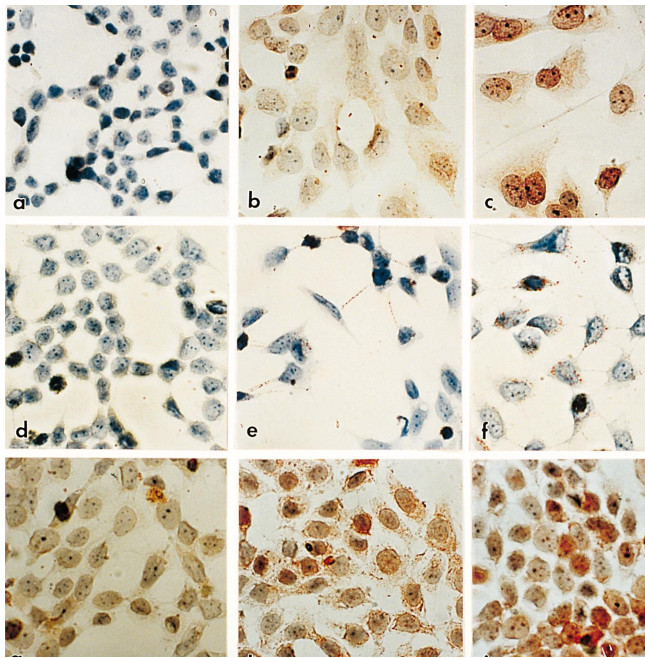
The effect of daidzein on maturation markers was evaluated at concentrations of 30, 45, and 90  $\mu\text{mol/L}$ . We included a higher concentration to compensate for the fact that daidzein's  $\text{IC}_{50}$  is higher than that of genistein. Although these daidzein treatments were performed for  $\leq 12$  d in both cell lines, we found no significant changes in the expression of either the milk components or ICAM-1 (data not shown).

### INHIBITION OF TUMORIGENICITY IN NUDE MICE

The experiments in nude mice were designed to evaluate the effect of prior treatment of MCF or MDA-MB-468 cell cultures on the incidence of cell-induced tumors in nude mice. Cultures of the 2 human cell lines were untreated or treated with genistein at 30  $\mu\text{mol/L}$  for 2 or 6 d. Cells were injected in mice as described in the Materials and Methods section, and tumor appearance and size were monitored. In MCF-7 cells treated for 6 d with genistein at 30  $\mu\text{mol/L}$ , the growth of palpable tumors was delayed by 4 wk compared with the group of mice injected with untreated MCF-7 cells. Two days of treatment had no effect on tumor latency. The rates of tumor growth, however, were unaffected by these treatments (data not shown). Six-day treatment of MDA-MB-468 cells with genistein entirely blocked tumor appearance; no animal developed tumors even after 12 wk of monitoring. The incidence was reduced after treatment for 2 d with genistein, but latency in the animals that developed tumors was identical to that of mice injected with the untreated MDA-MB-468 cells (see **Table 1**). Cell viability after treatment for 7 d 30  $\mu\text{mol}$  genistein/L was 70% for MCF-7 cells and 82% for MDA-MB-468 cells (as determined by trypan blue assay), indicating that the inhibition of tumor growth in nude mice was not due to cell killing. Slightly better survival was obtained in cells cultured in the same concentrations of daidzein.

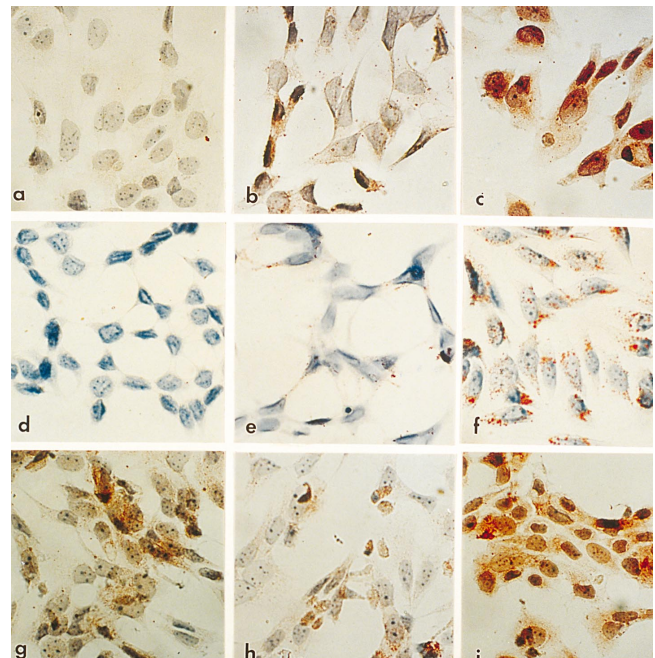
### DISCUSSION

The use of nontoxic concentrations of certain agents that promote terminal differentiation of human tumor cells is an appealing strategy in the fight against cancer (1–10). Previous studies have suggested that genistein and daidzein may be the chemopreventive components of soybeans (13, 14, 15). There are numerous reports on the effectiveness of genistein as an inducer of cell differentiation in a variety of tumor cells (reviewed in reference 16), including leukemia and melanoma cells (17, 18). Daidzein, the other major isoflavone of soybeans, has been generally found to be ineffective as a differentiating agent. Recently, however, daidzein has been reported to induce the mature phenotype in HL-60 and MEL cells (23, 24). We report here that genistein induces a mature phenotype in 2 breast cancer cell lines as determined by the detection of milk components (casein and lipid droplets) in the cytoplasm, expression of the adhesion protein ICAM-1 in the cell membrane, and morphologic alterations.



**FIGURE 2.** Induction of mature phenotype in MCF-7 cells as determined by reactivity with casein antibody (panels a, b, and c), neutral lipid production (panels d, e, and f), or reactivity with mouse intercellular adhesion molecule-1 antibody (panels g, h, and i). Cells were plated at  $5 \times 10^7/L$  and the next day were treated with genistein or dimethyl sulfoxide (control). Cells were treated as follows: with the solvent dimethyl sulfoxide, as shown in panels a, d, and g; with genistein at a final concentration of  $15 \mu\text{mol/L}$ , shown in panels b, e, and h; and with genistein at a final concentration of  $30 \mu\text{mol/L}$ , shown in panels c, f, and i. The red stain in the photomicrographs represents the stained protein visualized with biotinylated goat antimouse or antirabbit antibodies. Lipid visualization was as described previously (20). Magnification:  $\times 400$ .

Note, however, that many of the genistein actions described in cell-free systems either have not been reproduced in whole cells and animals or have required higher concentrations than those reported to be effective in cell-free systems. Plasma or serum concentrations of genistein and its metabolites, even in soy-product consumers, are lower than is required to inhibit tumor cell growth. The highest plasma concentrations of isoflavones are reportedly found in infants fed soy-based formulas. The plasma concentrations of soy isoflavones in these infants were in the range of 552–1775 ng/mL with mean genistein plasma concentration of 684 ng/mL or  $2.5 \mu\text{mol/L}$  (25). The concentrations in



**FIGURE 3.** Induction of mature phenotype in MDA-MB-468 cells as determined by either reactivity with casein antibody (panels a, b, and c), neutral lipid production (panels d, e, and f), or reactivity with mouse intercellular adhesion molecule-1 antibody (panels g, h, and i). Cells were plated at  $5 \times 10^7/L$  and the next day were treated with genistein or dimethyl sulfoxide (control). Cells were treated as follows: with the solvent dimethyl sulfoxide, as shown in panels a, d, and g; with genistein at a final concentration of  $15 \mu\text{mol/L}$ , shown in panels b, e, and h; and with genistein at a final concentration of  $30 \mu\text{mol/L}$ , shown in panels c, f, and i. The red stain in the photomicrographs represents the stained protein visualized with biotinylated goat antimouse or antirabbit antibodies. Lipid visualization was as described previously (20). Magnification:  $\times 400$ .

infants are almost an order of magnitude higher than typical plasma concentrations of Japanese adults ( $0.27 \mu\text{mol/L}$ ) consuming soy-rich diets (26). Thus, the genistein concentrations required to reduce tumor cell growth by 50% and induce tumor cell differentiation cannot be reached in either adult soy-product consumers or in infants consuming soy formulas. The average concentration of genistein in the plasma of infants is 6 to 12 times lower than what is required to elicit the above responses.

Regardless of its plasma concentrations, 2 main hypotheses on genistein's biochemical mode of action have been proposed. One is that the inhibition of protein tyrosine kinases initiates the dif-

**TABLE 1**


Treatment of cultured MCF-7 and MDA-MB-468 cells with genistein and its effects on their ability to give rise to tumors in nude mice

Treated cells	Length of treatment	Tumor incidence	Latency period	Tumor volume <sup>1</sup>
	<i>d</i>	<i>n</i> (%)	<i>d</i>	<i>mm</i> <sup>3</sup>
MCF-7 ( <i>n</i> = 5)	0	5 (100)	28	61
MCF-7 ( <i>n</i> = 5)	2	3 (60)	28	59
MCF-7 ( <i>n</i> = 5)	6	2 (40) <sup>2</sup>	56	70
MDA-MB-468 ( <i>n</i> = 6)	0	4 (66)	28	169
MDA-MB-468 ( <i>n</i> = 5)	2	1 (20)	28	150
MDA-MB-468 ( <i>n</i> = 5)	6	0 (0) <sup>2</sup>	—	—

<sup>1</sup>Data are based on measurements taken 3 wk after tumor appearance.

<sup>2</sup> $P < 0.05$ , chi-square test.

differentiation pathway through alterations in protein phosphorylation (27–31). We and others have suggested that genistein, by stabilizing DNA topoisomerase II to DNA, produces dynamic changes in the chromatin structure. These changes lead to alterations in gene expression that favor the differentiated phenotype (reviewed in references 16 and 19). The experiments presented here were designed to explore not the biochemical mechanism but rather the practical applications of genistein in breast cancer prevention and treatment.

The inability of differentiated breast cancer cells to give rise to tumors in nude mice suggests that genistein may find applications, at least in principle, in differentiation therapy of breast cancer. Because the estrogen-receptor-negative (ER<sup>-</sup>) cells (MDA-MB-468) were as susceptible to differentiation induction as the ER<sup>+</sup> cells (MCF-7), we concluded that genistein induces this process independently of its estrogenic or antiestrogenic action. The fact that MDA-MB-468 (ER<sup>-</sup>) cells treated for 6 d with genistein were unable to give rise to tumors in nude mice provides further support for the conclusion that the estrogenic properties of genistein can be dissociated from its differentiation-inducing properties in breast cancer cells. 

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