LH analog and dietary isoflavones support ovarian granulosa cell tumor development in a spontaneous mouse model

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Abstract

The reproductive hormone environment is an important influence upon spontaneous ovarian granulosa cell (GC) tumor development in genetically susceptible (SWR×SWXJ-9) F1 female mice: androgenic support during puberty stimulates tumorigenesis, while exposure to 17 β -estradiol (E₂) suppresses tumor initiation. We sought to determine whether gonadotropic stimulation was sufficient to initiate GC tumors in a grafted model system, and to determine the potential for dietary isoflavones (genistein and daidzein) as alternatives to E₂ for tumor chemoprevention *in vivo*. Isolated ovaries from pre-pubertal (SWR×SWXJ-9) F1 females were transferred to the kidney capsule of host mice homozygous for the hypogonadal (*hpg/hpg*) and severe combined immunodeficiency (*scid/scid*) mutations. CB17; HPG-*Prkdc*^{scid} *Gnrh1*^{hpg}/Bm host mice received either follicle-stimulating hormone (FSH), or a functional analog for LH human chorionic gonadotropin for 2 consecutive weeks, at which time the ovary grafts were examined for evidence of tumor initiation. LH analog administration, but not FSH, initiated GC tumorigenesis in the graft system, suggesting that the LH surge at puberty initiates GC tumor development in genetically susceptible female mice. To assess the chemopreventive potential of phytoestrogens, GC tumor frequency was compared between (SWR×SWXJ-9) F1 females reared on an isoflavone-free diet versus a diet supplemented with 125 μ g/g each of the isoflavones daidzein and genistein. It was observed that (SWR×SWXJ-9) F1 females reared on isoflavone-supplemented diet maintained significantly higher GC tumor frequency (22%) than females reared on isoflavone-free diet (11%), and that non-tumor-bearing siblings reared on the isoflavones had significantly increased ovarian weight, indicative of an overall stimulation of the reproductive hormone axis. The stimulation of GC tumorigenesis by isoflavones, which contrasts with the chemopreventive action of E₂ (2.5 mg/kg) administration during pubertal maturation, may result from general stimulation of ovarian growth, and the inability of the genistein and daidzein supplements to suppress LH secretion.

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Introduction

Juvenile-type granulosa cell (GC) tumors of the ovary are classified as sex-cord stromal tumors, representing the second most frequent class of ovarian tumors appearing in girls younger than 20 years of age (Young *et al.* 1984). GC tumors present immediate endocrinological and reproductive complications in infants, girls, and young women, and a potential life-threatening situation if tumors have acquired malignant characteristics, yet little is known about the etiology of this pediatric cancer (Kalfa *et al.* 2005). The low frequency of juvenile-type GC tumor

appearance in the general population has motivated researchers to utilize animal models for investigations of the genetic and hormonal stimuli that support GC tumor development. Female mice of the inbred strain SWR/Bm (hereafter written SWR) are genetically susceptible to the onset of juvenile-type ovarian GC tumors at a low population frequency (1–2%), and pose a unique spontaneous system for these investigations (Beamer *et al.* 1998a).

The SWR mouse ovarian tumors are initiated during puberty, between 3 and 4 weeks of age, and are macroscopically evident by 6 weeks as solid or cystic

hemorrhagic masses homogeneously comprising proliferative GCs (Beamer *et al.* 1985). The tumors secrete estrogen and inhibin, a characteristic common to human juvenile-type GC tumors, and they progress to a malignant carcinoma with metastatic potential, similar to the progression observed in some human cases (Tennent *et al.* 1990, Gocze *et al.* 1997). The endocrinological and genetic investigations of SWR mice and related recombinant inbred strains derived from SWR revealed two interesting features: first, manipulation of the reproductive hormone environment with exogenously supplied androgenic substrates in pre-pubertal females can significantly increase GC tumor frequency, from ~2 to 30% of the susceptible population developing tumors (Beamer *et al.* 1988, Tennent *et al.* 1993). Secondly, spontaneous GC tumor frequency is increased to ~25% in first generation daughters of the (SWR×SWXJ-9) cross that introduced supportive tumor susceptibility alleles on mouse chromosome X (Beamer *et al.* 1998b, Dorward *et al.* 2003). Overall, the ability to increase tumor frequency by genetic or hormonal means increased the functionality of this mouse model, providing sufficient experimental power to further investigate the biological factors that are either tumor supportive or chemopreventive, and the opportunity to translate these findings into risk reduction strategies for human patients.

The stimulation of GC tumor frequency in pre-pubertal SWR mice when treated by a dietary supplement or s.c. capsule of testosterone or dehydroepiandrosterone (DHEA) has been replicated in an grafted model system (Beamer *et al.* 1993). This system requires grafting the pre-pubertal (<22 days) genetically susceptible ovaries under the kidney capsule of doubly homozygous hypogonadal (*hpg/hpg*) and severe combined-immunodeficient (*scid/scid*) hosts. The *scid/scid* mutation prevents graft rejection, since homozygous *scid/scid* mice lack functional B and T cell surveillance, and the *hpg/hpg* mutation abrogates gonadotropin release from the pituitary, such that the gonads are not stimulated to produce steroid hormones (Cattanach *et al.* 1977, Bosma *et al.* 1983, Mason *et al.* 1986). The *hpg/hpg*, *scid/scid* host animal thus represents a gonadotropin- and gonadal hormone-free system, useful for exogenous hormonal manipulation of the ovary graft environment. Important evidence was revealed by the grafting experiments with regards to both the endocrinological support mechanisms and genetic etiology of this tumor system: 1) without reproductive hormone stimulation of the *hpg/hpg*, *scid/scid* hosts, no GC tumors were evident in the susceptible ovary grafts, while tumors could be

initiated in fertile *scid/scid* hosts, indicating that normal endocrine stimulation provided by an intact hypothalamic–pituitary axis is both necessary and sufficient for GC tumor development; 2) the GC tumor susceptibility genes function autonomously within the ovary and GCs, as evidenced by tumor initiation in the grafted ovaries when *hpg/hpg*, *scid/scid* hosts were administered testosterone or DHEA; and 3) confirmed the developmental window for tumor susceptibility during puberty, as was previously suggested by the spontaneous GC tumor frequency patterns observed in the tumor-susceptible breeding colonies (Beamer *et al.* 1985). The ovary transplant experiments suggested that peak spontaneous GC tumor susceptibility was recapitulated when both the ovary and recipient were at the pubertal stage, between 21 and 24 days in the mouse (Beamer *et al.* 1993). This suggested that events connected to the transition of puberty are highly relevant to GC tumor initiation in this mouse model, within the context of follicular differentiation in the ovary and the onset of gonadotropin cycles that stimulate follicular development. To address the specific role of the pituitary gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH), for support of GC tumor initiation, we have utilized the *hpg/hpg*, *scid/scid* model to evaluate GC tumor development in ovary grafts following independent stimulation by an LH analog human chorionic gonadotropin (hCG) or FSH administration.

The window for hormonal sensitivity to GC tumor initiation in genetically susceptible female mice suggested that an equivalent window might exist for tumor prevention. It was previously observed that continuous administration of E₂ in the diet or by s.c. capsule beginning at 21–22 days of age could significantly reduce GC tumor frequency (Beamer *et al.* 1988). This tactic for tumor prevention is less than promising, since females are rendered infertile due to negative feedback regulation of continuous high serum E₂ on gonadotropin release. We have utilized our highest spontaneous frequency colony, represented by (SWR×SWXJ-9) F1 females with a population GC tumor frequency of ~25%, as a model to test two experimental strategies to mimic the chemopreventive activity of continuous E₂. First, we tested the effect of short-term E₂ administration (2.5 mg/kg) during the developmental window for GC tumor initiation (21–26 days), and secondly, we tested whether dietary isoflavones might provide alternatives for chemoprevention against spontaneous GC tumorigenesis in this mouse model. The isoflavone components of plants, commonly called ‘phytoestrogens’, are of

considerable interest to the scientific community for their potential impact on reproductive, cardiovascular, and bone health as a result of their pharmacologic activity to bind and transactivate estrogen receptors (Esrs; Kuiper *et al.* 1998). Our investigation suggests that LH is the gonadotropic signal required for GC tumor initiation at puberty in juvenile female mice, and that E₂ administered during the window of tumor susceptibility can prevent tumor initiation, likely via suppression of gonadotropin release. Furthermore, in contrast to the generalization that isoflavones have anti-tumor action, we observed that genetically susceptible female mice exposed to the isoflavones, genistein and daidzein, in the diet showed an overall increased frequency of GC tumor initiation, coincident with isoflavone stimulation of ovarian growth.

Materials and methods

Mice

All mice were produced and housed in our research colony at The Jackson Laboratory (Bar Harbor, ME, USA) under 14 h light:10 h darkness cycles. Mice were provided with autoclaved NIH-31 diet (6% fat PMI Nutrition International, Brentwood, MO, USA; unless a custom diet is specified) and HCl-acidified water (pH 2.8–3.2) *ad libitum*. Animals were weaned at 20–23 days of age and housed in groups of three to five in 329 cm² polycarbonate cages containing sterilized white pine shavings. Genetically susceptible female mice were derived from the first filial (F1) generation of SWR inbred females mated to recombinant inbred SWXJ-9 males, and are hereafter described as (SWR × SWXJ-9) F1 females. Double mutant hypogonadal (*hpg/hpg*) and severe combined immunodeficient (*scid/scid*) CB17; HPG-*Prkdc*^{*scid*} *Gnrh*^{*hpg*}/Bm mice (Jax Stock No. 002038) were derived from breeders homozygous for the *scid/scid* mutation, but heterozygous for the *hpg/+* mutation, since homozygous females and males are infertile, lacking both circulating gonadotropins and gonadal hormones as a result of a deletion in the gonadotropin-releasing hormone (*Gnrh*) gene (Cattanach *et al.* 1977, Mason *et al.* 1986). Offspring were genotyped for *hpg/hpg* homozygosity by PCR, to identify individuals homozygous for the *Gnrh* gene deletion (wild-type product = 712 bp, mutant product = 586 bp). Genomic DNA isolated from tail tip biopsy (2 µl) was added to a 24 µl reaction mix: 19.75 µl H₂O, 2.5 µl 10× concentrated Clontech Advantage buffer, 0.25 µl Clontech Advantage Taq polymerase (BD Bioscience-Clontech), 0.5 µl dNTPs (2.5 mM), and

0.5 µl each of primers A, B, and C (10 µM): primer A (5'-CACATCTGTAGCCACAGTCC-3'), primer B (5'-AGCTCCGAGGCTGTCACTGG-3'), and primer C (5'-GCTTGGAGAGCTGTAAGGTC-3'). PCR cycles were as follows: 94 °C 1 min, 35 cycles of 94 °C 30 s and 68 °C 3 min, followed by 68 °C 3-min final extension phase. In addition to PCR genotyping, male and female host gonads were visually inspected prior to ovary grafting to confirm sexual immaturity and hypogonadism. All animal procedures were approved by the Animal Care and Use Committee of The Jackson Laboratory.

Grafting studies

Ovary pairs were isolated from pre-pubertal (SWR × SWXJ-9) F1 (15–20 days) females killed by cervical dislocation. Ovaries were immediately transferred under the left kidney capsule of *hpg/hpg*, *scid/scid* male or female host recipients anesthetized with 2,2,2-tribromoethanol (Sigma-Aldrich Chemical Co.) and gonadotropic or hormonal stimulus simultaneously initiated (Cunliffe-Beamer 1983). The gonadotropins, rat FSH-B1 (15 µg/day), ovine FSH S-18 (3 µg/day; National Institutes of Health (NIH) Pituitary Hormone Program, Bethesda, MD, USA), or hCG (15 IU/day; Wyeth-Ayerst, Madison, NJ, USA), were administered in sterile PBS via s.c. Alzet osmotic pumps (Durect Corp., Cupertino, CA, USA). 1007D Alzet pumps were replaced after 1 week, to achieve 2 weeks of continuous gonadotropin exposure. Following this period, at an ovary graft age of ~5 weeks, the hosts were killed by cervical dislocation and the grafts fixed in Bouin's solution for histological assessment.

DHEA was administered via s.c. capsule made from a 1 cm length of silastic tubing (Cat No. 508-009, Fisher Scientific, Pittsburgh, PA, USA), solid packed with DHEA (Sigma-Aldrich Chemical Co.) and plugged with 3 mm diameter glass beads (Fisher Scientific), as previously reported (Beamer *et al.* 1988). The ovary grafts remained in the DHEA-treated hosts for 5 weeks before the assessment of GC tumor growth (ovary graft, 8 weeks of age) and fixation of the graft in Bouin's solution for histological examination. In cases where the host diet was modified, the custom diets (see below) were introduced to *hpg/hpg*, *scid/scid* hosts 5–7 days prior to ovary grafting.

Short-term exposure to E₂

To determine whether short-term E₂ exposure at puberty was equally effective as continuous E₂ to prevent GC tumor initiation, we administered an s.c. injection of E₂ dissolved in 100 µl peanut oil vehicle

(2.5 mg/kg BW) at 22 ± 1 days of age, and once again 3 days following the first injection. Each litter was divided into one of two treatment arms, and control females from each litter received two injections of the peanut oil vehicle on the same schedule.

Custom rodent diets

Two isocaloric experimental diets were introduced to female mice genetically susceptible to spontaneous ovarian GC tumors: 1) AIN-76A is a purified rodent diet devoid of isoflavone-containing ingredients, with casein as the major protein source (Harlan Teklad, Madison, WI, USA) and 2) a custom diet formulation based on AIN-76A that we have named AIN-D/G, was supplemented at Harlan Teklad with 125 µg/g each of the isoflavones daidzein and genistein (Indofine Chemical Co., Hillsborough, NJ, USA) for a total of 250 µg isoflavone per gram of diet. The SWR female and SWXJ-9 male breeders used to generate tumor-susceptible (SWR×SWXJ-9) F1 females are usually fed the open formula diet NIH-31 diet with 6% fat. Historically, this diet supports 25% spontaneous ovarian GC tumor frequency in the F1 daughter offspring (Beamer et al. 1998b). When experimental diets were introduced, the SWR and the SWXJ-9 breeders were placed on the experimental diet (AIN-76A or AIN-D/G) at an early age (4 weeks), such that their daughter offspring would be exposed to the dietary constituents *in utero*, throughout infancy and puberty, until the time of necropsy and tumor assessment at 8 weeks of age.

Ovarian histology and follicle staging

A minimum of 12 ovaries from females aged 24 days from both isoflavone-free and isoflavone-supplemented diet groups were fixed with Bouin’s fixative, serially sectioned (5 µm sections) and stained with H&E for follicle staging. Every fifth section was examined for growing follicles that were categorized as primary (one GC layer), secondary (two GC layers), pre-antral (three or more GC layers), or antral (three or more layers plus an antral space), and follicles were counted only if the sections transversed the germinal vesicle, in order to prevent duplicated counts.

Serum assays for FSH and LH

Mouse serum FSH RIAs and LH sandwich immunoassays were performed at the University of Virginia Center for Research in Reproduction Ligand Assay and Analysis Core (NICHD (SCCPRR) Grant U54-HD28934, <http://www.healthsystem.virginia.edu/>

internet/crr/methodspage.cfm). Serum was collected from coagulated whole-blood samples, and stored at -20°C until dry ice shipment to UVA. Assays were performed in duplicate unless volume was limiting.

Statistical analysis

χ^2 Analysis for proportions was used to evaluate GC tumor frequencies between treated and control groups at a significance level of $P < 0.05$. Since it was uncertain what effect the treatments would have on GC tumor frequency, power calculations to determine sufficient sample size were based on ongoing estimates of tumor frequency during the dietary manipulation experiments, with $\alpha = 0.05$ and $\beta = 0.20$. For measurement of the effect of E_2 on tumor frequency, the minimum sample size was estimated at 47 individual animals, and for the isoflavone study, the minimum sample size was estimated at 113 individual animals. Follicle stages were calculated as a proportion of the total counted, and means and standard errors of the proportions are presented in the results sections. Statistical analyses were performed on arcsine-transformed proportion data. ANOVA was used to compare the continuous variables of body and organ weights, serum FSH and LH, with a significance level of $P < 0.05$ (JMP Statistical Software, Version 6.0; SAS Cary, NC, USA).

Results

Gonadotropin stimulation of GC tumorigenesis

The gonadotropic stimulus for spontaneous GC tumor initiation in genetically susceptible (SWR×SWXJ-9) F1 females was examined in an grafting model system utilizing double mutant *hpg/hpg*, *scid/scid* recipients. Table 1 shows the summary of histological observations for grafted pre-pubertal (21–22 days) ovaries when treated for 2 consecutive weeks with hCG or FSH. Both ovine and rat FSH stimulated the growth of ovarian follicles, such that the number of GC cells per follicle was greatly increased in an organized pattern, with most follicles having developed an antrum. In a

Table 1 Response of pre-pubertal (3 weeks; SWR×SWXJ-9) F1 ovarian grafts to 14 days of continuous gonadotropin exposure

Gonadotropin	<i>n</i>	Normal histology	Blood-filled cyst	Pre-neoplastic follicles
Rat FSH	9	7	2	0
Ovine FSH	8	7	1	0
HCG	14	3	4	7

few sections, there was evidence for blood-filled cysts, but there was no evidence for ovarian GC tumors in 17 independently treated grafts (Fig. 1A and B). In contrast, hCG stimulated the development of pre-neoplastic follicles, with evidence for cystic blood-filled spaces and irregular proliferative masses of GC cells in 7 out of 14 independent grafts (Fig. 1C and D). This suggests that LH provides the upstream stimulus for GC tumor initiation in cases of spontaneous GC tumor development in (SWR×SWXJ-9) F1 female mice.

In the absence of gonadotropic support, ovary grafts failed to thrive, with no evidence of follicular growth or neoplasia (Fig. 1E and F).

Effect of short-term E₂ administration

Short-term administration of E₂ was accomplished by two injections of E₂ in oil vehicle during the pubertal window between 21 and 26 days of age. It was observed that short-term E₂ administration during this period significantly reduced GC tumor frequency from 27% (13/48) in the oil vehicle-treated control group to 4% (2/49) in the E₂-treated group. Four females treated with E₂ were fertility tested at 8 weeks of age to confirm that short-term E₂ exposure did not affect subsequent reproductive potential. E₂-treated females produced litters of equal size and with equivalent latency as females provided with the oil vehicle control (data not shown).

Figure 1 Follicular appearance of (SWR×SWXJ-9) F1 ovaries (Ov) grafted under the kidney (K) capsule of *hpg/hpg, scid/scid* hosts following 2 weeks of continuous gonadotropin administration. FSH stimulated GC proliferation and the development of antral follicles (A and B). In contrast, hCG-triggered GC tumor initiation, evident as pre-neoplastic foci (PNF) in the ovary grafts, with irregular masses of GCs and blood pooling (C and D). In the absence of gonadotropic support, ovarian grafts showed no evidence of growth at 10- or 14-day post-engraftment (E and F). All tissue sections were stained with hematoxylin/eosin (scale bar=1 mm).

Dietary isoflavones and spontaneous GC tumorigenesis

Reproductive performance of the SWR and SWXJ-9 breeder pairs on the two experimental diets was consistent with breeders maintained on NIH-31 diet, indicating no adverse effects of isoflavone depletion or supplementation to the overall fertility of these animals. Table 2 shows the GC tumor frequencies recorded in the (SWR×SWXJ-9) F1 females reared on the experimental diets. There was a significant increase in GC tumor frequency in the (SWR×SWXJ-9) F1 population reared on the AIN-D/G diet (~22%) supplemented with isoflavones, when compared with the AIN-76A isoflavone-free diet (~11%). Table 2 also summarizes body and organ weight data for female mice necropsied at the age of 8 weeks; these data are sub-categorized by tumor status, whether the females had a GC tumor (unilateral or bilateral), or macroscopically normal ovaries. There were no significant differences in the body weights of females on either diet regardless of tumor-bearing status; since these diets are calorie matched and equally digestible, this suggests no overt changes in food consumption with either diet. Of those females with GC tumors, it was evident that the tumors were larger in females fed the AIN-D/G diet, as shown by the normalized weight of GC tumors. In cases where the tumors were bilateral, the larger tumor was entered into this calculation. In females without GC tumors, the ovary pair weight was also significantly increased in females raised on AIN-D/G versus AIN-76A, suggesting an overall stimulation of ovary growth by the isoflavone supplement. Uterine weights were significantly increased in tumor-bearing animals from either diet group, as has been observed previously, but no differences were observed in females with normal ovaries fed either experimental diet.

The significant increase in ovarian weight measured at 8 weeks for females fed AIN-D/G suggested that the

isoflavone supplement of daidzein and genistein supported ovarian growth. We subsequently examined juvenile females at 24 and 30 days of age from the AIN-76 and AIN-D/G diet groups to measure body and organ weight parameters, and serum gonadotropin concentrations at the age of GC tumor initiation. Figure 2A–E shows the body weight, and ovary and uterine weights normalized to body weight, along with the serum FSH and LH concentrations measured in 24- and 30-day-old females. The pattern of increased ovarian weight in AIN-D/G- versus AIN-76A-fed females was evident at both 24 and 30 days, indicating that enhanced ovarian growth was established prior to the onset of puberty. No significant differences in serum FSH or LH were observed at 24 or 30 days of age, although the variability was still high within this genetically homogenous population of animals, and despite their being raised in a controlled environment on a uniform diet. The significant increase in ovarian and uterine weight observed at 24 days of age in AIN-D/G-reared mice suggested puberty acceleration, which could be an outcome of isoflavone supplementation. Follicle staging of 24-day-old ovaries obtained from both diet groups did reveal an increased proportion of large pre-antral follicles in animals receiving the isoflavone supplement, with a concomitant reduction in the proportion of follicles at the primary stage (Table 3). In addition, several instances of multi-oocyte follicles were observed in ovary sections of isoflavone-supplemented females, but not the isoflavone-free group (data not shown).

The overall reduction in spontaneous GC tumor frequency observed in (SWR×SWXJ-9) F1 females raised on AIN-76A suggested a diet lacking in isoflavones reduced GC tumor susceptibility. To address whether this ‘protection’ carried over as permanent defense against a tumor-promotive substance, and whether the dietary isoflavone content of AIN-D/G was independently capable of supporting GC tumor initiation, we utilized the *hpg/hpg*, *scid/scid*

Table 2 Body and organ weight measurements for 8-week-old (SWR×SWXJ-9) F1 females reared on AIN-76A or AIN-D/G diet (mean±S.E.M.)

Base diet	GC tumor frequency	Tumor status	Body weight, BW (g)	GC tumor weight/BW (mg/g)	Ovary pair weight/BW (mg/g)	Uterine weight/BW (mg/g)
AIN-76A	30/273 (10.9%)	Normal (n=130)	18.27±0.15	–	0.37±0.01	4.31±0.12
		Tumor bearing (n=18)	18.68±0.34	4.75±1.19	–	6.76±0.45*
AIN-D/G	23/105† (21.9%)	Normal (n=82)	18.53±0.15	–	0.43±0.01†	4.65±0.17
		Tumor bearing (n=23)	19.17±0.25	24.89±4.36‡	–	7.47±0.18‡

*Significant difference ($P<0.05$) between tumor- and non-tumor-bearing animals in the AIN-76A-fed group. †Significant difference ($P<0.05$) between the diet groups for similar measurement under similar tumor-bearing condition. ‡Significant difference ($P<0.05$) between tumor- and non-tumor-bearing animals in the AIN-D/G group.

Figure 2 Mean $\pm$ S.E.M. for body, organ weights, and serum gonadotropins of pubertal (SWR $\times$ SWXJ-9) F1 females raised on an isoflavone-free (AIN-76A) diet or a supplemented (AIN-D/G) diet containing the isoflavones genistein (125 μ g/g) and daidzein (125 μ g/g). Minimum group size $n=45$ for (A) body weight (BW), (B) ovary weight/BW, and (C) uterine weight/BW measurements. For serum FSH (D), minimum group size $n=18$, and for serum LH (E), $n=20$. Significant differences ($P<0.05$) determined by ANOVA are indicated by *.

graft model. Table 4 shows that GC tumor frequency could be stimulated to 27% when an s.c. DHEA capsule was simultaneously inserted at the time of ovary grafting, this suggests that AIN-76A feeding does not prevent GC tumor initiation in the presence of exogenous androgenic tumor support, and reduction of spontaneous GC tumor frequency observed in the group reared on AIN-76A may result from the immediate changes in endocrine environment. When *hpg/hpg*, *scid/scid* hosts were fed AIN-76A or AIN-D/G, and received ovary grafts from the colonies maintained on AIN-76A or AIN-D/G, no GC tumors were evident, indicating that the isoflavone supplement

present in the AIN-D/G diet did not independently support GC tumor initiation (Table 4).

Discussion

Juvenile SWR female mice are genetically susceptible to the development of spontaneous ovarian GC tumors between 3 and 4 weeks of age when the ovary and hypothalamic–pituitary axis undergo reproductive maturation. Genetic susceptibility is inherent within the GCs of the SWR ovary, and tumor initiation requires endocrine support provided by the intact hypothalamic–pituitary axis. We have grafted

Table 3 Follicle growth in (SWR $\times$ SWXJ-9) F1 female ovaries at 24 days: influence of isoflavone-supplemented diet

Diet	Primary	Secondary	Pre-antral	Antral
AIN-76A	0.389 $\pm$ 0.017	0.303 $\pm$ 0.014	0.134 $\pm$ 0.016	0.171 $\pm$ 0.011
AIN-D/G	0.317* $\pm$ 0.015	0.291 $\pm$ 0.012	0.224* $\pm$ 0.014	0.169 $\pm$ 0.009

*Significant difference ($P<0.05$) between AIN-76A and AIN-D/G groups.

Table 4 GC tumor frequency in 8-week-old (SWR×SWXJ-9) F1 ovary grafts transferred to *hpg/hpg*, *scid/scid* hosts at 3 weeks: a comparison of isoflavone-free or supplemented diet effects for tumor prevention or promotion

Ovary graft source diet	<i>hpg/hpg</i> , <i>scid/scid</i> host diet	Capsule implant	GC tumor frequency
AIN-76A	AIN-76A	Empty	0/35 (0%)
AIN-76A	AIN-76A	DHEA	9/33 (27%)
AIN-76A	AIN-D/G	Empty	0/21 (0%)
AIN-D/G	AIN-D/G	Empty	0/25 (0%)

genetically susceptible ovaries into immunodeficient, hypogonadal recipient mice in order to determine which gonadotropic stimulus – LH or FSH – supports GC tumor initiation. The observation that the LH analog hCG stimulates pre-neoplastic follicle development in ovary grafts, while FSH stimulates only follicle growth, suggests that LH is the tumor initiation stimulus during pubertal maturation of the juvenile animal. This observation is consistent with the fact that androgenic steroids, downstream products of LH-stimulated thecal cells, are also capable of stimulating GC tumor initiation in the graft system (Beamer *et al.* 1993). Continuous LH stimulation has been associated with various conditions of ovarian pathology in several transgenic and gene knockout models, including the development of cystic, hemorrhagic follicles, or sex-cord stromal tumors (Matzuk *et al.* 1992, Risma *et al.* 1995, Couse *et al.* 1999, Britt *et al.* 2000, Danilovich *et al.* 2001). However, these genetically engineered mouse models contrast strikingly with the SWR model system, since they pose adult-onset pathology resulting from chronic, elevated gonadotropic stimulation, which is not required for juvenile-onset GC tumorigenesis in SWR female mice. To date, no association has developed between mutations in the gonadotropins, gonadotropin receptor, or inhibin genes and juvenile GC tumor development in children, nor do these genes overlap with the known tumor susceptibility loci identified in SWR mice (Shen *et al.* 1996, Watson *et al.* 1997, Beamer *et al.* 1998b, Fuller *et al.* 1998, Dorward *et al.* 2005). Thus, the pursuit of the tumor susceptibility genes operative in the SWR spontaneous mutant strain will provide novel candidates for investigation of both the genetic determinants and the endocrinological interactions that support human cases of juvenile-onset GC tumorigenesis.

An inherent property of GC tumor development in SWR female mice is the restricted window for GC tumor susceptibility during puberty. This window of

susceptibility is emphasized under conditions of androgenic exposure, which stimulates tumor frequency in pre-pubertal females, but not in females aged beyond the susceptibility window. This investigation has now confirmed that successful chemoprevention by E₂ overlaps the period of GC tumor initiation. The tumor-preventive action of continuous exposure to elevated E₂ initiated prior to puberty is recapitulated by short-term E₂ exposure during the pubertal maturation window. Building on the evidence for LH-triggered GC tumor initiation, we now propose that the mechanism of action for high-dose E₂ chemoprevention is to suppress gonadotropins, and more specifically LH release from the anterior pituitary, thus delaying gonadotropic cyclicity until the window for tumor susceptibility is passed.

As an alternative to E₂, we also examined the chemopreventive potential of dietary isoflavones on GC tumor frequency in genetically susceptible mice. Generally, the term ‘phytoestrogen’ is applied to plant-derived isoflavones, lignans and coumestans, that have weak estrogenic activity *in vivo* and *in vitro*, via binding and transactivation of (Esr1 and Esr2) receptors, as well as non-hormonal activities, such as inhibition of tyrosine kinases (Akiyama *et al.* 1987, Kuiper *et al.* 1998). Our study took into account data that commercially available rodent chows, including the NIH-31 chow utilized by The Jackson Laboratory, contain significant amounts of isoflavones contributed by soy and alfalfa ingredients (Thigpen *et al.* 1999). We therefore compared GC tumor frequency and reproductive status between tumor-susceptible females reared on a commercially available isoflavone-free synthetic diet (AIN-76A), versus the same diet supplemented with a 1:1 combination of genistein and daidzein, to emulate the major constituents and peak concentration in the NIH-31 diet. In contrast to E₂ administration, a total dietary isoflavone supplement of 250 µg/g did not prevent GC tumor development; rather, our study provided consistent evidence that isoflavones supported GC tumor initiation in this spontaneous tumor model. First, GC tumor frequency dropped significantly in females reared on the isoflavone-free diet, where 11% were tumor-bearing relative to the expected frequency of ~25% on the isoflavone-containing NIH-31 diet. Secondly, GC tumor frequency rebounded significantly from 11 to 22% when isoflavones were reintroduced to the maternal diet, and genetically susceptible female offspring were exposed *in utero*, during lactation, puberty, and young adulthood. The inability of isoflavones to prevent GC tumor initiation could be explained by the fact that gonadotropin levels were not

reduced by the dietary isoflavone supplementation during the critical window for tumor initiation. This finding agrees with the genetic evidence that signaling via the *Esr1* receptor is most important for negative feedback regulation of gonadotropin release, while pharmacological data show that the isoflavones genistein and daidzein show preferential binding and transactivation of the *Esr2* receptor subtype (Kuiper *et al.* 1998, Couse *et al.* 2003).

The ability of isoflavones to support GC tumor development in this animal model raises the question as to their mechanism of action. The genistein- and daidzein-supplemented diet did not support GC tumor initiation in genetically susceptible ovaries grafted into *hpg/hpg*, *scid/scid* recipients, suggesting that the isoflavones cannot stimulate GC tumor initiation independently from gonadotropic stimulation. The supportive action of the isoflavones for GC tumor initiation may be a function of their *Esr2* receptor subtype specificity, acting via transactivation of *Esr2* to support GC differentiation and follicular development *in vivo* (Sar & Welsch 1999, Hegele-Hartung *et al.* 2004, Couse *et al.* 2005, Emmen *et al.* 2005). The isoflavone combination of genistein and daidzein did increase the proportion of large growing follicles in genetically susceptible female mice, which may effectively render a larger population of GCs responsive to the genetically programmed tumorigenic events. Multi-oocyte follicles were also observed in juvenile ovaries from isoflavone-supplemented females, as previously reported following neonatal exposure to genistein (Jefferson *et al.* 2006). Although the mechanism of action is not yet fully elucidated, the finding for a tumor-supportive interaction between juvenile GC tumor susceptibility genes and isoflavones in female mice is disturbing. Pediatric GC tumors may arise during infancy and isoflavones can be measured in the amniotic fluid of pregnant mothers consuming soy products (Engel *et al.* 2006). Furthermore, infants fed soy-based formula are exposed to high concentrations of genistein and daidzein, in excess of 13 000 times the normal concentration of plasma E_2 equivalents (Setchell *et al.* 1997).

Tumor susceptibility genes associated with human juvenile- and adult-onset GC tumors have not yet been identified, although multiple mouse models that develop late-onset GC tumors have been engineered through transgenic overexpression of LH, and knock-outs for the FSH receptor (*Fshr*) and inhibin α (*Inha*) genes (Matzuk *et al.* 1992, Risma *et al.* 1995, Danilovich *et al.* 2001). GC tumor susceptibility in mice that overexpress LH is influenced by the genetic background of the transgenic strain, with at least three

genes predicted to modify tumor susceptibility (Keri *et al.* 2000). The SWR mouse model for spontaneous juvenile-type GC tumor development shares common elements with the genetically engineered models, since tumor initiation can be triggered by LH and tumor susceptibility is a polygenic trait. The downstream effector of LH seems to be androgenic stimulation in SWR ovary, as both testosterone and DHEA can stimulate tumor development in a grafted, genetically susceptible ovary without gonadotropic influence (Beamer *et al.* 1993). One major difference between the spontaneous tumors that arise in inbred SWR mice and other genetically engineered models is the narrow developmental window for GC tumor susceptibility during puberty, and the heightened level of sensitivity to initiating factors such as LH or androgens at normal physiological levels. Identification of the genetic alleles that support GC tumor development in SWR mice will be very informative, to compare with the biological pathways that are sufficient to support GC tumorigenesis in engineered model systems, such as dysregulation of Wnt and β -catenin tumor suppressor genes (Boerboom *et al.* 2005, 2006). In addition, successful positional cloning efforts in SWR mice to identify the relevant ovarian tumor susceptibility genes will open up a more detailed investigation as to the mechanism by which specific isoflavones contribute to GC tumor initiation in the ovary.

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