

The Role of Estrogen Receptor β in Transplacental Cancer Prevention by Indole-3-Carbinol

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Abstract

In the present study, the efficacy of indole-3-carbinol (I3C), a key bioactive component of cruciferous vegetables, for prevention of cancer in offspring exposed *in utero* to the environmental carcinogen dibenzo[*def,p*]chrysene (DBC) was evaluated using an estrogen receptor β (ER β) knockout mouse model. I3C was provided either through the maternal diet coincident with carcinogen exposure during pregnancy or directly to offspring postinitiation with DBC. I3C was effective at reducing T-cell acute lymphoblastic lymphoma/leukemia (T-ALL)-related mortality in offspring only if provided via the maternal diet, although a gender difference in the role of ER β in mediating this response was evident. In female offspring, chemoprevention of T-ALL by maternal dietary I3C required expression of ER β ; survival in *Esr2* wild-type and heterozygous female offspring was more than 90% compared with 66% in *Esr2* null females. Alternatively, ER β status did not significantly impact the transplacental chemoprevention by I3C in males. The possible role of ER β in mediating lung carcinogenesis or chemoprevention by I3C was similarly complicated. Lung tumor incidence was unaltered by either dietary intervention, whereas lung tumor multiplicity was substantially reduced in *Esr2* null females on the control diet and marginally lower in *Esr2* null males exposed to I3C via the maternal diet compared with their wild-type and heterozygous counterparts. These findings suggest that I3C may act via ER β to prevent or suppress DBC-initiated transplacental carcinogenesis but that the involvement of this receptor seems to differ depending on the cancer type and gender of the offspring. *Cancer Prev Res*; 6(4); 339–48. ©2013 AACR.

Introduction

Indoles are degradation products of glucosinolates, which occur naturally in cruciferous vegetables, and have been widely studied for their chemopreventive properties (reviewed in ref. 1). One such bioactive food chemical, indole-3-carbinol (I3C), has been shown to prevent or reduce the risk of cancer in a number of animal models of carcinogenesis including, in part, cancer of the colon, mammary gland, skin, lung, and endometrium (see reviews in refs. 2, 3). Alternatively, dietary I3C can enhance tumorigenesis, particularly hepatocellular carcinogenesis in rat and trout (4, 5). The chemopreventive activity of I3C may be mediated by one or more of its acid concentration products, such as 3,3'-diindolylmethane (DIM). The anti-

cancer effects of I3C (or its derivatives) are facilitated by interaction with several nuclear transcription factors, including the aryl hydrocarbon receptor (AhR), the estrogen receptor (ER), Sp1, or NF- κ B to alter processes associated with carcinogen detoxification, cell-cycle progression, apoptosis, and DNA repair (reviewed in ref. 6).

The fetus is particularly sensitive to the toxic, teratogenic, and carcinogenic effects of environmental chemicals due to underdeveloped detoxification and elimination pathways and unique sensitivities to chemicals. Therefore, there is great concern that perinatal exposure to environmental chemicals could be linked to childhood and adult cancers. Polycyclic aromatic hydrocarbons (PAH) are ubiquitous in the environment and are derived from incomplete combustion of biomass and fossil fuels. The most potent PAH tested to date is dibenzo[*def,p*]chrysene (DBC), also called dibenzo[*a,l*]pyrene, a component of PAH mixtures in the environment (7). DBC is a multi-organ carcinogen that initiates cancers of the skin, endometrium, ovary, lung, and liver as well as lymphoma (8–11). A recent study by members of our research group showed that *in utero* exposure to DBC (15 mg/kg orally on gestation day 17) in mice results in a high incidence of mortality in young adults due to aggressive T-cell acute lymphoblastic lymphoma/leukemia (T-ALL; ref. 12), a disease that is also observed in human adolescents (13). All surviving offspring exposed to DBC *in utero* had lung tumors, and more than half of the males had liver tumors. Moreover, Yu and colleagues showed that

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dietary I3C consumed by the mother during gestation and lactation significantly reduced offspring mortality due to DBC-dependent T-ALL and reduced the number of lung tumors in offspring surviving to middle age (14). This study examined the influence of a responsive versus nonresponsive AhR phenotype in mediating the protective effect of I3C against these cancers, although neither maternal nor fetal AhR phenotype influenced the efficacy of I3C as an anticancer agent. While I3C is an effective chemoprotective compound transplacentally, the mechanism by which it blocks or suppresses tumorigenesis in this cancer model is currently not known. Many of the molecular actions of I3C or its derivatives involve direct interaction with the ER, modulation of ER-dependent gene expression, and/or alteration of estrogen metabolism pathways (15–19). Thus, it is possible that dietary I3C may act through the ER to exert its anticancer effects *in vivo*.

The main objective of this study was to evaluate the role of ER β , which is highly expressed in the fetal thymus and lung (20, 21), in modulating the chemopreventive properties of dietary I3C in an animal model of transplacental carcinogenesis using the ER β knockout mouse model. Our working hypothesis was that protection by dietary I3C against DBC-initiated transplacental cancer required expression of ER β in the offspring. Second, we also examined the timing of dietary I3C exposure, either administered via the maternal diet or fed directly to offspring after weaning. Herein, we show for the first time that transplacental cancer prevention by I3C administered to the dam during gestation/lactation requires at least one gene copy of ER β .

Materials and Methods

Chemicals

DBC (CAS# 191-30-0, formerly called dibenzo[*a,l*]pyrene) was obtained from the National Cancer Institute Chemical Reference Standard Repository and determined by high-performance liquid chromatography (HPLC) analysis to be 98% pure DBC (12). I3C was obtained from Sigma-Aldrich. All other chemicals used were of reagent grade and purchased from general laboratory suppliers.

Animals and design of transplacental cancer study

The Oregon State University Institutional Animal Care and Use Committee approved all procedures for the handling and treatment of mice in this study (protocol #3468). Mice were maintained in a pathogen-free vivarium at 20°C \pm 1°C and 50% \pm 10% humidity on a 12:12 hour dark:light cycle and housed in microisolator cages (Life Products, Inc.) with Care FRESH bedding. Breeding pairs of male B6.129P2-*Esr2^{tm1Unc}/J* (β ERKO mouse; *Esr2^{-/-}*) and female 129S1/SvImJ mice were obtained from Jackson Laboratories and acclimated to the vivarium for 1 week before breeding. The B6129SF1/J mouse strain was previously shown to be sensitive to DBC as a transplacental carcinogen (12). Because the β ERKO mouse had been backcrossed to C57BL6 mice for eight generations at Jackson Laboratories,

we bred *Esr2^{-/-}* males with 129S1/SvImJ females (*Esr2^{+/+}*) to generate heterozygous hybrids, B6129SF1/J-*Esr2^{+/-}* for carcinogen exposure (Supplementary Fig. S1). *Esr2* hybrid male and female mice were randomized to six experimental groups and paired for breeding. Females were monitored daily for detection of a vaginal plug, which was designated gestation day 0 (GD 0). On GD 17, dams in groups 1, 3, and 5 were dosed with an oral gavage of corn oil (5 mL/kg), whereas dams in groups 2, 4, and 6 were dosed with 15 mg/kg DBC in corn oil. The numbers of dams and offspring and the average litter sizes are presented in Supplementary Table S1.

AIN93G diet (Research Diets, Inc.) was provided to dams and then offspring until 3 months of age, at which point AIN93M diet was used for the remainder of the study. Pregnant mice and offspring in groups 1 and 2 were fed AIN93G/M diet without modification (control diet) until 10 months of age. Dams in experimental groups 3 and 4 were fed AIN93G diet supplemented with 2,000 ppm I3C (I3C:Maternal, or I3C:M diet) from GD 9 until weaning at 3 weeks of age; offspring were then fed unmodified AIN93G/M diet until 10 months of age. Dams in groups 5 and 6 were fed unmodified AIN93G throughout pregnancy and lactation; after weaning at 3 weeks, offspring were fed AIN93G/M diet supplemented with 2,000 ppm I3C (I3C: Offspring, or I3C:O diet) until 24 weeks of age followed by unmodified AIN93M diet until 10 months. Preparation, storage, and quality control analysis of I3C-supplemented AIN diet has been described previously (22). All diets were provided *ad lib.* and I3C-supplemented diets were replaced daily.

All mice were observed daily for any signs of distress or discomfort. Any mice exhibiting signs of morbidity, pain, or distress were humanely euthanized with an overdose of CO₂ followed by cervical dislocation, and then necropsied. At necropsy, presence of any tumor was noted (thymic lymphoma, lung, liver, ovarian, skin, etc.) and the number and size (diameter measured by digital caliper) of lung and liver tumors were recorded.

Genotyping protocol

All offspring were genotyped by PCR for presence of the *Esr2^{tm1Unc}* allele following the standard protocol provided by Jackson Laboratories. Briefly, genomic DNA was obtained from ear tissue samples (Qiagen DNeasy Blood and Tissue Kit) and then subject to standard PCR (Amplitaq Gold Master Mix; Life Technologies) using primers for the wild-type (oIMR3145, 5'-GTTGTGCCAGCCCTGTTACT), heterozygous (oIMR3146, 5'-TCACAGGACCAGACACCGTA), and mutant (oIMR3147, 5'-GCAGCCTCTGTTCCACATACAC) alleles. PCR products were separated by gel electrophoresis on a 2% agarose gel. Mice were classified as wild-type when a single PCR product of 106 bp was detected, as heterozygous when 2 products of 106 and 160 bp were detected, and mutant when only the 160 bp product was observed. The ratios of *Esr2* genotypes for offspring in each treatment group are presented in Supplementary Table S1.

Histopathology

In the present study, all mice surviving to 10 months were euthanized and necropsied as described earlier. Multiple tissues (thymus, lung, liver, kidney, spleen, testes, ovaries, colon, skin, and lymph nodes) were first examined by gross necropsy for abnormalities and then preserved in 10% formalin. Fixed tissues were processed to paraffin blocks and then subjected to hematoxylin and eosin staining for examination by a board-certified pathologist at the Utah Veterinary Diagnostics Laboratory (Logan, UT). As has been observed repeatedly in prior studies (12, 14, 23, 24), between 3 and 6 months of age, approximately half of the offspring initiated with DBC *in utero* succumbed to lymphoma that was characterized as T cell in origin (CD3⁺, B220⁻) with various antigen phenotypes (CD4⁻CD8⁺; CD4⁺CD8⁺), and thus, classified as T-ALL. In addition, lung carcinogenesis (hyperplasia, adenoma, adenoma with progression, and/or carcinoma) was evident in nearly all offspring surviving to 10 months of age, and some male offspring developed liver neoplasms. Histopathologic analysis of samples from the present study conducted at the Utah Veterinary Diagnostics Laboratory (in consultation with the Oregon State University Veterinary Diagnostics Laboratory, Corvallis, OR) confirmed the same disease profile (lymphoma, lung adenomatous hyperplasia and adenoma, and liver neoplasia) as was observed in prior studies using DBC as a transplacental carcinogen (12, 14, 23, 24). Representative histopathology images are shown in Supplementary Fig. S2.

Statistical analyses

Following the example previously published (24), the general strategy for analyses of individual offspring responses was (i) to account for cluster (litter) effects in offspring data, (ii) to assess main effects of the categorical predictors carcinogen, diet, *Esr2* genotype, and gender, and (iii) to assess *Esr2* genotype and gender effects within each diet group in DBC-initiated offspring. First, simple univariate analyses were conducted to determine the association between the categorical predictors carcinogen, diet, genotype, and gender and offspring survival (SAS 9.3, Lifetest procedure); all factors were considered potential significant contributors to the final model (log-rank test of equality over strata $P < 0.25$; Supplementary Fig. S3). All potential interactions between these experimental factors were similarly examined, although none were considered to be significant ($P > 0.25$). As the primary outcome of interest in this study was survival in carcinogen-exposed offspring, analyses of survival curves were limited to data for offspring initiated *in utero* with DBC using the SAS Lifetest procedure (Kaplan–Meier method with Šidák correction for multiple comparisons). Initial tests examined main effects of the categorical variables diet, *Esr2* genotype and gender, followed by pairwise tests to examine the effects of either *Esr2* genotype within each DBC/diet group. These pairwise analyses were conducted for all offspring, and then separately for male and female offspring. Similar analyses were conducted using proportional hazards (Cox) regression analysis (SAS

phreg procedure) with the robust sandwich score test to account for grouping of offspring by litter, which yielded comparable results (not shown). Graphical analysis of pairs of survival curves reasonably satisfied the proportional hazards assumption.

Analyses of lung tumor incidences were conducted using a quasi-likelihood logistic regression (SAS genmod procedure) where the apparent variation between litters was used to account for overdispersion in the grouped binomial data. Main effects of diet, gender, and *Esr2* genotype for DBC-initiated offspring were calculated, followed by pairwise analyses to determine effects of either *Esr2* genotype or gender within each DBC/diet treatment group. Similar results were obtained using logistic regression ignoring litters (SAS logistic procedure; results not shown). Mixed models analyses (SAS mixed procedure) of tumor multiplicities (log transformed) or tumor sizes were conducted with data for DBC-exposed offspring that survived to 10 months of age. Analyses were conducted in a stepwise fashion as described earlier with the parameter litters included as a random factor to account for data overdispersion. Again, these analyses were conducted first for all offspring and then separately for male and female offspring. Because of the relatively few liver tumors observed in this study, there was insufficient statistical power to reliably conduct a robust analyses of the data on liver tumor outcome.

Results

T-cell acute lymphoblastic/lymphoma–dependent survival

Although a few offspring died before weaning (anemia related to DBC exposure), there was not a significant effect of carcinogen or diet group on litter size or body weight at weaning (Supplementary Table S1). Offspring born to dams initiated *in utero* on gestation day 17 with 15 mg/kg DBC exhibited a high rate of mortality between 3 to 6 months of age due to aggressive T-ALL (Fig. 1A and Table 1), whereas deaths after 6 months of age were primarily attributed to lung cancer morbidity. Survival analyses were conducted using mortality data for death due to T-ALL (Fig. 1) or death due to any cause (results in Supplementary Table S2, figure not shown). When considering offspring survival data stratified according to diet group, survival in male offspring exposed to I3C via the maternal diet (91%) was significantly greater ($P = 0.003$) compared with offspring exposed to the control diet (61%; Fig. 1B). However, survival in offspring fed I3C directly (66%) was not significantly different from the control group ($P = 0.9896$). On the other hand, an overall significant effect of diet was not evident in females, where survival was 78% in control animals, 89% in I3C:M-treated offspring, and 75% in I3C:O-treated offspring (Fig. 1C).

Although only male offspring seemed to be responsive to the protective effects of maternal dietary I3C, beneficial effects of this intervention were observed in females when diet group and *Esr2* genotype were considered as nested

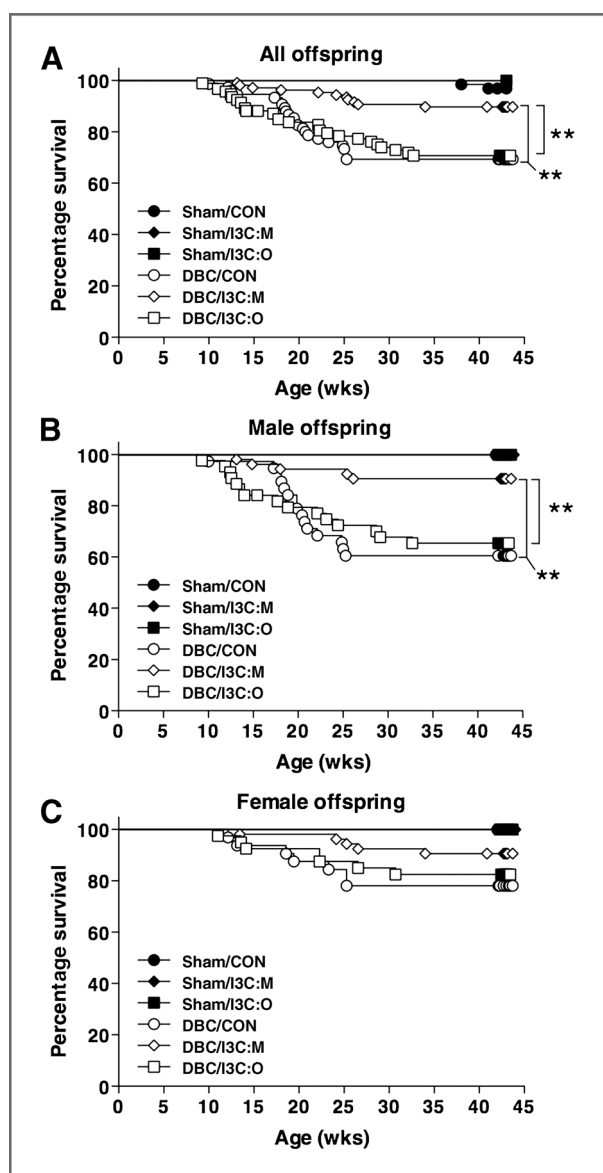


Figure 1. Impact of experimental diets and *Esr2* genotype on T-ALL-dependent mortality in offspring initiated *in utero* with DBC on gestation day 17. A–C, survival curves for all offspring (A), male offspring (B), and female offspring (C) according to carcinogen/diet groups: sham/control (●), sham/I3C:M (◆), sham/I3C:O (■), DBC/control (○), DBC:I3C/M (◇), and DBC/I3C:O (□). **, $P < 0.01$, significant difference in survival for the indicated pairwise comparisons as determined by Life tables (Kaplan–Meier method) analysis. Complete statistical results for these analyses (as well as results of similar analyses for mortality due to any cause, such as anemia or other cancers) are provided in Supplementary Table S1.

factors in the statistical analysis (Supplementary Table S2). Figure 2 shows the impact of *Esr2* genotype on survival of all offspring (Fig. 2A–C), males only (Fig. 2D–F), or females only (Fig. 2G–I) stratified by experimental diet. In the absence of any diet modification for dams or offspring, *Esr2* genotype did not significantly impact male or female offspring survival in the control diet group (Fig. 2D and G). However, in offspring exposed to I3C via the maternal diet,

lymphoma-dependent survival in females expressing one (100%) or both (97%) wild-type *Esr2* alleles was significantly greater ($P = 0.0051$ and 0.0058 , respectively) than survival in *Esr2* null females (60%; Fig. 2H). Alternatively, *Esr2* genotype did not significantly alter male offspring survival in the I3C:M diet group (Fig. 2E). Moreover, *Esr2* status in offspring fed I3C directly postcarcinogen exposure did not influence survival for either sex (Fig. 2F and I). Similar results were observed when deaths due to any cause were included in the statistical model (Supplementary Table S2).

Lung carcinogenesis in 10-month-old survivors

Morbidity due to lung tumorigenesis (adenoma and adenocarcinoma) was generally more than 80% in 10-month-old surviving male and female offspring initiated *in utero* with DBC compared with a spontaneous incidence of less than 12% and 3% in sham-initiated male and female offspring, respectively (Table 2). Dietary I3C did not significantly reduce lung tumor incidence whether administered via the maternal diet or fed directly to the offspring in either males or females (Supplementary Table S3). A modest, but significant, decrease in lung tumor incidence was observed in *Esr2* wild-type males in the control diet group compared with their heterozygous counterparts (Table 2).

Significant main effects of *Esr2* genotype on lung tumor multiplicity in 10-month survivors were detected in this study when considering DBC-exposed offspring irrespective of diet group (7.5 ± 1.2 tumors/animal for *Esr2* null offspring compared with 11.6 ± 1.1 and 12.4 ± 0.9 for wild-type and heterozygous offspring, respectively, $P \leq 0.01$), although this effect was predominant in females (Supplementary Table S2). On the other hand, significant main effects of experimental diet on lung tumor multiplicity were also observed, irrespective of *Esr2* genotype, primarily when both males and females were considered collectively: 9.2 ± 0.8 tumors/animal for offspring in the I3C:M diet group compared with 13.1 ± 1.4 and 13.7 ± 1.2 for offspring in the control and I3C:O diet groups, respectively ($P = 0.042$ and 0.006 , respectively).

A further examination of the interaction of diet and *Esr2* genotype suggested a complex response. *Esr2* null female offspring in the control diet group had significantly fewer lung tumors than their wild-type or heterozygous counterparts ($P = 0.0241$ and 0.0206 , respectively). However, one should accept this statistical observation with caution, given that few *Esr2* null females are present in the control diet group. On the other hand, *Esr2* null male offspring in the I3C:M diet group had (marginally) fewer lung tumors than *Esr2* wild-type (trend for significance, $P = 0.0822$) or heterozygous offspring ($P = 0.0257$). Even though these observations point to an interaction between the I3C:M diet and *Esr2* genotype in male and female offspring, a test for this interaction was, in fact, not significant ($P = 0.8795$), suggesting that the influence of *Esr2* status on lung tumor multiplicity was not strictly dependent on the experimental diet group. Finally, although a significant effect of *Esr2* genotype on lung tumor size was observed in control female

Table 1. Effects of experimental diet, gender, and *Esr2* genotype on offspring survival to 10 months of age

	All offspring		Male offspring		Female offspring	
	Any cause	T-ALL	Any cause	T-ALL	Any cause	T-ALL
Offspring stratified by diet group						
Sham-initiated						
Control	3.1% (2/65)	0.0% (0/65)	0.0% (0/26)	0.0% (0/26)	0.0% (0/39)	0.0% (0/39)
I3C:M	0.0% (0/65)	0.0% (0/65)	0.0% (0/34)	0.0% (0/34)	0.0% (0/31)	0.0% (0/31)
I3C:O	1.6% (1/64)	0.0% (0/64)	0.0% (0/29)	0.0% (0/29)	2.9% (1/35)	0.0% (0/34)
DBC-initiated						
Control	56.7% (68/120) ^a	30.7% (23/75) ^a	60.3% (35/58) ^a	39.5% (15/38) ^a	53.2% (33/62) ^a	21.6% (8/37) ^a
I3C:M	20.0% (24/120) ^b	10.3% (11/107) ^b	19.0% (11/58) ^b	9.4% (5/53) ^b	21.3% (13/61) ^b	11.1% (6/54) ^a
I3C:O	45.5% (55/121) ^a	29.0% (27/93) ^a	53.2% (33/62) ^a	34.1% (15/44) ^a	37.3% (22/59) ^{a,b}	24.5% (12/49) ^a
DBC-exposed offspring stratified by gender						
Male	44.1% (79/179) ^a	25.9% (35/135) ^a				
Female	37.4% (68/182) ^a	18.3% (26/142) ^a				
DBC-exposed offspring stratified by <i>Esr2</i> genotype						
Wild-type	28.2% (24/85) ^a	17.6% (13/74) ^a	30.2% (13/43) ^a	21.1% (8/38) ^a	26.2% (11/42) ^a	13.9% (5/36) ^a
Heterozygous	32.9% (56/170) ^a	18.0% (25/139) ^a	38.6% (34/88) ^a	23.9% (17/71) ^a	26.8% (22/82) ^a	11.8% (8/68) ^a
Null	50.8% (30/59) ^{a,b}	29.3% (12/41) ^a	54.8% (17/31) ^a	30.0% (6/20) ^a	46.4% (13/28) ^a	28.6% (6/21) ^a
DBC-exposed offspring stratified by diet, then genotype						
CON diet group						
Wild-type	38.7% (12/31) ^a	29.6% (8/27) ^a	37.5% (6/16) ^a	33.3% (5/15) ^a	40.0% (6/15) ^a	25.0% (3/12) ^a
Heterozygous	57.1% (32/56) ^a	31.4% (11/35) ^a	62.1% (18/29) ^a	42.1% (8/19) ^a	51.9% (14/27) ^a	18.8% (3/16) ^a
Null	66.7% (10/15) ^a	28.6% (2/7) ^a	66.7% (4/6) ^a	33.3% (1/3) ^a	66.7% (6/9) ^a	4.0% (1/4) ^a
I3C:M diet group						
Wild-type	4.0% (1/25) ^a	4.0% (1/25) ^a	7.7% (1/13) ^a	7.7% (1/13) ^a	0.0% (0/12) ^a	0.0% (0/12) ^a
Heterozygous	10.8% (7/65) ^a	4.9% (3/61) ^a	23.1% (3/31) ^a	6.7% (2/30) ^a	11.8% (4/34) ^a	3.2% (1/31) ^a
Null	41.7% (10/24) ^b	30.0% (6/20) ^b	38.5% (5/13) ^a	20.0% (2/10) ^a	45.5% (5/11) ^b	40.0% (4/10) ^b
DBC:I3C:O diet group						
Wild-type	37.9% (11/29) ^a	18.2% (4/22) ^a	42.9% (6/14) ^a	20.0% (2/10) ^a	33.3% (5/15) ^a	16.7% (2/12) ^a
Heterozygous	34.7% (17/49) ^a	25.6% (11/43) ^a	46.4% (13/28) ^a	31.8% (7/22) ^a	19.0% (4/21) ^a	19.0% (4/21) ^a
Null	50.0% (10/20) ^a	28.6% (4/14) ^a	66.7% (8/12) ^a	42.9% (3/7) ^a	25.0% (2/8) ^a	14.3% (1/7) ^a

NOTE: Mortality data are presented for offspring that died or were euthanized for any cause (anemia, lymphoma, other tumors [lung, liver, skin, or ovarian] or cause unknown) or for only offspring that succumbed to T-ALL. Different superscripts (a and b) indicate a significant difference within each comparison group (as indicated by row headings). Life tables analyses (Kaplan–Meier method; SAS lifetest procedure) of survival in DBC-initiated offspring were conducted in a stepwise manner, wherein death due to any cause or to T-ALL were considered separately. Effects of diet, gender, or genotype in sham-initiated animals were not considered as so few deaths occurred in these groups. Initial analyses included all data within the DBC exposure group stratified by gender, diet group, or genotype. Then, pairwise analyses for *Esr2* genotype within each DBC/diet group were conducted. Analyses were run irrespective of different genders, as well as separately for male and female offspring. Log-rank, Šidák-adjusted *P* values for pairwise comparisons between indicated data groups are available in Supplementary Table S2. Incidences of mortality or morbidity are reported as percentages (deaths/total).

offspring (Supplementary Table S3), this observation cannot be considered robust due to the small sample size for *Esr2* null females.

Liver carcinogenesis in 10-month-old survivors

Incidence, multiplicity, and size of liver tumors are presented in Supplementary Table S4. Because this study was not designed to examine liver cancer as a primary outcome, an insufficient number of offspring survived to 10 months of age to allow for a thorough statistical analysis of liver tumor data.

Discussion

Although dietary I3C has great potential in the field of cancer chemoprevention, the current lack of understanding of the mechanism(s) by which it exerts its chemopreventive properties has caused some concern about its possible use as a preventive and/or therapeutic agent for human carcinogenesis and its widespread, uncontrolled use as a dietary supplement by the public. Previously, members of our research team showed that I3C administered via the maternal diet during gestation and lactation conferred substantial protection to offspring against DBC-initiated transplacental

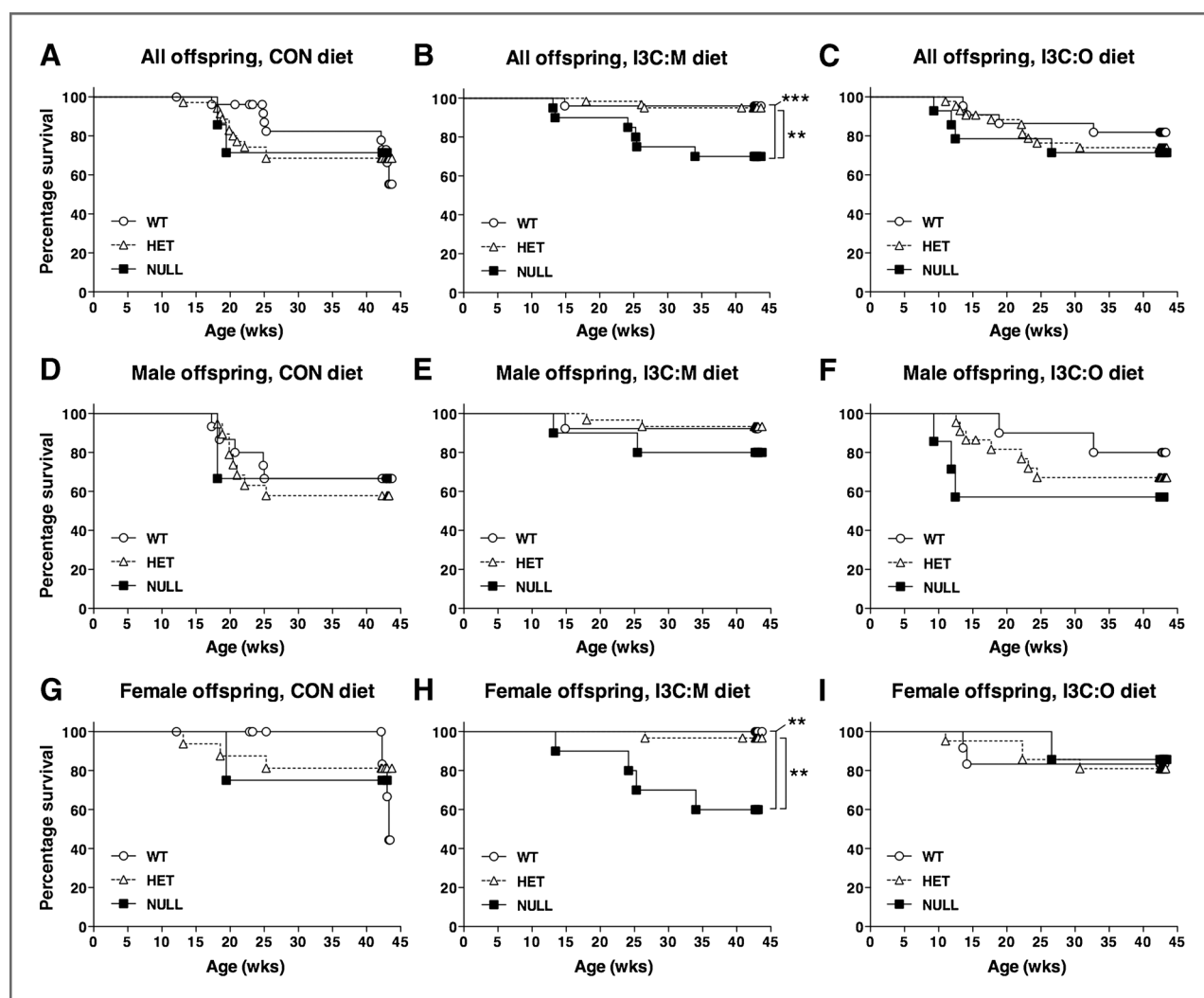


Figure 2. Impact of *Esr2* genotype on T-ALL-dependent mortality in offspring initiated with DBC *in utero* within each experimental diet group. A–I, survival curves for all offspring (A–C), male offspring (D–F), or female offspring (G–I) within each diet group: control (A, D, and G), I3C:M (B, E, and H) or I3C:O (C, F, and I). Data are stratified according to *Esr2* genotype: wild-type (○), heterozygous (△), and null (■). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, significant difference in survival for the indicated pairwise comparisons as determined by life tables (Kaplan–Meier method) analysis. Complete statistical results for these analyses (as well as results of similar analyses for mortality due to any cause, such as anemia or other cancers) are provided in Supplementary Table S1.

cancer (14). A primary goal of the present study was to examine the role of ER β in mediating transplacental chemoprevention by I3C. Our experimental design allowed us to test multiple key hypotheses related to I3C prevention of DBC-initiated transplacental cancer, specifically about the timing of intervention with dietary I3C and the role of ER β in mediating the anticancer effects of I3C *in vivo*.

We report herein for the first time that dietary I3C is effective at blocking DBC-initiated T-ALL only when administered via the maternal diet. Importantly, this transplacental chemoprevention requires expression of ER β in female offspring. Moreover, the protection conferred to these female offspring was substantial, as nearly all mice that were exposed to I3C during pregnancy and lactation and that carried at least one copy of the wild-type *Esr2* allele survived to adulthood. To our knowledge, this is the first

demonstration that ER β mediates the anticancer activity of I3C *in vivo*. On the other hand, protection by maternal dietary I3C was independent of *Esr2* genotype in males, as survival in all male offspring was substantially greater in this group compared with control animals or those fed I3C directly. This apparent difference in the role of ER β in mediating the chemopreventive activity of maternal dietary I3C against DBC-initiated T-ALL points to possible distinct modes of action for this bioactive food chemical, or its derivative(s), in males and females, although the physiologic basis for such a difference is not yet clear.

Before this study, no information was available about the beneficial or deleterious effects of I3C consumption by offspring that have been exposed *in utero* to a chemical carcinogen. Our results show that dietary I3C is most effective when administered coincidentally with the

Table 2. Effects of experimental diet, gender, and *Esr2* genotype on lung tumorigenesis in 10-month-old surviving offspring

	All offspring			Male offspring			Female offspring		
	Incidence	Multiplicity	Size	Incidence	Multiplicity	Size	Incidence	Multiplicity	Size
Offspring stratified by diet group									
Sham-initiated									
CON	1.6% (1/63)	1.0 ± 0.0	3.5 ± 0.0	0.0% (0/26)			2.6% (1/39)	1.0 ± 0.0	3.5 ± 0.0
I3C:M	6.2% (4/65)	1.5 ± 0.3	1.6 ± 0.3	11.8% (4/34)	1.5 ± 0.3	1.6 ± 0.3	0.0% (0/31)		
I3C:O	4.8% (3/63)	1.0 ± 0.0	1.3 ± 0.3	6.9% (2/29)	1.0 ± 0.0	1.5 ± 0.5	2.9% (1/34)	1.0 ± 0.0	1.1 ± 0.0
DBC-initiated									
CON	90.6% (48/53) ^a	13.1 ± 1.4 ^a	1.7 ± 0.1 ^{a,b}	87.5% (21/24) ^a	11.9 ± 1.6 ^{a,b}	1.9 ± 0.2 ^a	93.1% (27/29) ^a	14.0 ± 2.1 ^a	1.6 ± 0.2 ^a
I3C:M	91.7% (88/96) ^a	9.2 ± 0.8 ^b	1.9 ± 0.1 ^a	91.7% (44/48) ^a	9.8 ± 1.2 ^a	2.0 ± 0.1 ^a	91.7% (44/48) ^a	8.8 ± 1.1 ^a	1.7 ± 0.2 ^a
I3C:O	87.7% (57/65) ^a	13.7 ± 1.2 ^a	1.5 ± 0.1 ^b	92.9% (26/28) ^a	16.8 ± 1.7 ^b	1.5 ± 0.1 ^b	83.8% (31/37) ^a	11.0 ± 1.4 ^a	1.4 ± 0.1 ^a
DBC-exposed offspring stratified by gender									
Male	91.0% (91/100) ^a	12.2 ± 0.9 ^a	1.8 ± 0.1 ^a						
Female	89.5% (102/114) ^a	10.9 ± 0.9 ^a	1.6 ± 0.1 ^a						
DBC-exposed offspring stratified by <i>Esr2</i> genotype									
Wild-type	86.9% (53/61) ^a	11.6 ± 1.1 ^a	1.7 ± 0.1 ^a	80.0% (24/30) ^a	13.3 ± 1.8 ^a	1.9 ± 0.2 ^a	93.5% (29/31) ^a	10.2 ± 1.4 ^a	1.5 ± 0.1 ^a
Heterozygous	90.4% (103/114) ^a	12.4 ± 0.9 ^a	1.7 ± 0.9 ^a	96.3% (52/54) ^b	12.4 ± 1.1 ^a	1.7 ± 0.1 ^a	85.0% (51/60) ^a	12.4 ± 1.4 ^a	1.7 ± 0.2 ^b
Null	93.1% (27/29) ^a	7.5 ± 1.2 ^b	1.9 ± 0.3 ^a	92.9% (13/14) ^{a,b}	8.8 ± 2.1 ^a	2.1 ± 0.3 ^a	93.3% (14/15) ^a	5.8 ± 1.4 ^b	1.7 ± 0.4 ^b
DBC-exposed offspring stratified by diet, then genotype									
CON diet group									
Wild-type	84.2% (16/19) ^a	11.2 ± 2.1 ^a	1.7 ± 0.1 ^a	70.0% (7/10) ^a	10.6 ± 3.3 ^a	2.0 ± 0.3 ^a	100.0% (9/9) ^a	12.9 ± 2.9 ^a	1.5 ± 0.2 ^a
Heterozygous	96.0% (24/25) ^a	14.4 ± 2.0 ^a	1.7 ± 0.2 ^a	100.0% (12/12) ^a	11.9 ± 2.0 ^a	1.8 ± 0.3 ^a	92.3% (12/13) ^a	16.8 ± 3.4 ^a	1.5 ± 0.1 ^a
Null	80.0% (4/5) ^a	9.0 ± 4.3 ^a	2.6 ± 1.3 ^a	100.0% (2/2) ^a	16.5 ± 0.5 ^a	1.5 ± 0.3 ^a	66.7% (2/3) ^a	1.5 ± 0.5 ^b	3.7 ± 2.9 ^b
I3C:M diet group									
Wild-type	91.7% (22/24) ^a	10.0 ± 1.4 ^a	1.8 ± 0.2 ^a	83.3% (10/12) ^a	10.0 ± 2.3 ^{a,b}	2.2 ± 0.4 ^a	100.0% (12/12) ^a	8.6 ± 1.6 ^a	1.5 ± 0.2 ^a
Heterozygous	91.4% (53/58) ^a	10.5 ± 1.1 ^a	1.9 ± 0.2 ^a	96.4% (27/28) ^a	11.3 ± 1.6 ^a	1.8 ± 0.1 ^a	86.7% (26/30) ^a	9.6 ± 1.7 ^a	1.9 ± 0.4 ^a
Null	92.9% (13/14) ^a	4.6 ± 1.2 ^b	2.0 ± 0.3 ^a	87.5% (7/8) ^a	3.6 ± 1.1 ^b	2.5 ± 0.5 ^a	100.0% (6/6) ^a	5.8 ± 2.3 ^a	1.4 ± 0.2 ^a
I3C:O diet group									
Wild-type	83.3% (15/18) ^a	14.8 ± 2.5 ^a	1.4 ± 0.1 ^a	87.5% (7/8) ^a	20.6 ± 3.1 ^a	1.4 ± 0.1 ^a	80.0% (8/10) ^a	9.8 ± 2.9 ^a	1.5 ± 0.1 ^a
Heterozygous	83.9% (26/31) ^a	14.3 ± 1.7 ^a	1.5 ± 0.1 ^a	92.9% (13/14) ^a	14.8 ± 2.4 ^a	1.5 ± 0.1 ^a	76.5% (13/17) ^a	13.8 ± 2.4 ^a	1.5 ± 0.1 ^a
Null	100.0% (10/10) ^a	9.9 ± 2.4 ^a	1.4 ± 0.2 ^a	100.0% (4/4) ^a	14.0 ± 4.5 ^a	1.7 ± 0.3 ^a	100.0% (6/6) ^a	7.2 ± 2.2 ^a	1.3 ± 0.1 ^a

NOTE: Tallies for lung tumor incidence include only animals that survived to 10 months of age. Tumor multiplicity was calculated as the average number of tumors per tumor-bearing animal ± SEM. Average tumor size was estimated by the diameter (mm) ± SEM. For all statistical analyses, different superscripts indicate a significant difference within each comparison group (as indicated by row headings). P values of the differences of least squares means for the indicated comparisons are available in Supplementary Table S3. Effects of diet, gender, or genotype in sham-initiated animals were not considered as so few animals with lung tumors were observed in these groups. Analyses of lung tumor incidences in 10-month-old survivors initiated with DBC were conducted using a quasi-likelihood logistic regression (SAS genmod procedure) where the apparent variation between litters was used to account for overdispersion in the grouped binomial data. Mixed models analyses (SAS mixed procedure) of tumor multiplicities (log-transformed) and tumor sizes were conducted with data for DBC-exposed offspring that survived to 10 months of age. The parameter litters was included as a random factor to account for data overdispersion. Analyses were conducted for all DBC-initiated offspring, and then separately for male and female offspring.

carcinogen and that I3C provided either via the maternal diet or directly to the offspring does not increase risk of T-ALL, lung, or liver tumorigenesis in this transplacental cancer model. Others have shown that I3C fed long-term subsequent to carcinogen exposure in rat and rainbow trout promotes liver tumorigenesis (4, 5), although this phenomenon was not observed in our study, pointing to a likely species difference in the impact of I3C on liver tumor development. Similarly, Ogenesian and colleagues showed that consumption of I3C reduced hepatocarcinogenesis in mice that were initiated with diethylnitrosamine as infants (25).

Several mechanisms exist by which I3C or its acid condensation products can modulate the influence of estrogens on tumorigenesis including alteration of estrogen metabolism, direct interaction with the ER and modulation of estrogen signaling pathways (reviewed in ref. 6). Importantly, I3C and some of its derivatives are known ligands for the ER exhibiting both estrogenic and antiestrogenic effects in human breast cancer cells (26–28). The antiestrogenic properties of I3C and DIM in human breast and prostate cancer cells may be mediated by the AhR, showing that cross-talk exists between these 2 transcription factor signaling pathways (29–31). Alternatively, in the trout liver, I3C and DIM are potent xenoestrogens that show moderate affinity for the hepatic ER and induce a transcriptional profile similar to that of estradiol (32, 33). Finally, I3C and other acid condensation products modulate transcription of many genes that have the potential to counteract the proliferative effects of estrogen, including genes associated with growth arrest and apoptosis (15, 34–36).

Since the discovery in 1996 of two ER subtypes with unique expression patterns (37), the relative roles of ER α and β in development of cancer in both estrogen target and nontarget tissues has been a subject of great interest. ER β is the predominant form expressed in the human fetal thymus, and a large portion of lymphocytes in lymph nodes and multiple human lymphoma cell lines preferentially express ER β (20, 38). Estrogen is critical for thymus and lymphocyte development via ER α , whereas ER β mediates estrogen-induced thymic atrophy during early adulthood and pregnancy (39). Evidence suggests that the negative effects of estrogen on the immune system are mediated by ER β because lack of ER β expression in the aged ER β knockout mouse can lead to myeloproliferative disease, which resembles human chronic myeloid leukemia (40). The importance of estrogen in the immune system is also shown by increased risk of non-Hodgkin lymphoma in individuals expressing a polymorphism that enhances CYP17A1 activity, a key enzyme in the estrogen biosynthetic pathway (41, 42). However, in the present study, ER β status in offspring did not alter lymphoma-dependent survival in the absence of dietary intervention, suggesting that actions of endogenous estrogens via ER β do not have a strong influence on the development of T-ALL in this mouse model of transplacental carcinogenesis by DBC.

Estrogen plays an important role in lung development, particularly in females (21), and is likely responsible for the greater susceptibility of women to chronic pulmonary disease, lung cancer, and the deleterious effects of tobacco (43, 44). ER β is the sole subtype expressed in human fetal lung (20) and the predominant form expressed in mouse lung (21). Moreover, immunohistologic analysis of archived or surgically resected human lung tumors detected ER β in more than half of the tumors evaluated, whereas ER α was not expressed (45, 46). In the present study, we report that lack of ER β expression substantially reduced lung tumor multiplicity in surviving adult female offspring fed a control diet and in male offspring that were exposed to I3C via the maternal diet, although ER β status did not impact lung tumor incidence. The apparent contradictory observation that ER β facilitates chemoprevention of T-ALL by I3C, whereas the loss of ER β confers modest suppression of lung carcinogenesis (depending on the dietary regimen), suggests that the role of this nuclear receptor in mediating the anticancer activity of I3C may differ depending on the cancer type. However, the observation that lung tumor multiplicity in ER β null offspring was consistently lower compared with wild-type or heterozygous counterparts irrespective of dietary treatment (see DBC-exposed offspring stratified by genotype in Table 2 and Supplementary Table S3), points to a potential role of this steroid receptor in lung chemical carcinogenesis, while rendering any specific conclusions about the relative role of ER β in mediating I3C-dependent suppression of lung carcinogenesis speculative at this time. In addition, the loss of some offspring to lymphoma in this model of transplacental carcinogenesis can be considered a confounding factor when interpreting lung tumor outcome, especially considering the substantial difference in survival among some treatment groups. To decisively determine the role of ER β in mediating lung tumorigenesis in this cancer model, a different dosing strategy (lower DBC dose or intermittent dosing) or alternative mouse strain (e.g., A/J mouse) could be used to evaluate lung tumors without the confounding effects of T-ALL.

In summary, administration of I3C via the maternal diet provides substantial protection to offspring initiated *in utero* with the environmental carcinogen DBC, and ER β likely mediates this chemoprevention by I3C in female offspring. Collectively, results of this and prior studies by our research team lend weight to the argument that dietary I3C is an effective anticancer agent *in vivo*. Furthermore, our observations point to the critical gestation/lactation window of exposure for transplacental chemoprevention by I3C. However, the fact that this compound (or its bioactive derivative) may act via ER β , a critical component of the endocrine system, to modulate carcinogenesis may raise some concern about its suitability as a dietary supplement during pregnancy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: A.D. Benninghoff, D.E. Williams
Development of methodology: A.D. Benninghoff, D.E. Williams
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): A.D. Benninghoff
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): A.D. Benninghoff
Writing, review, and/or revision of the manuscript: A.D. Benninghoff, D.E. Williams

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