Development and characterization of a novel rat model of estrogen-induced mammary cancer

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Abstract

The ACI rat model of 17β-estradiol (E2)-induced mammary cancer is highly relevant for use in establishing the endocrine, genetic, and environmental bases of breast cancer etiology and identifying novel agents and strategies for preventing breast cancer. E2 treatment rapidly induces mammary cancer in female ACI rats and simultaneously induces pituitary lactotroph hyperplasia and adenoma. The pituitary tumors can result in undesired morbidity, which compromises long-term studies focused on mammary cancer etiology and prevention. We have defined the genetic bases of susceptibility to E2-induced mammary cancers and pituitary tumors and have utilized the knowledge gained in these studies to develop a novel inbred rat strain, designated ACWi, that retains the high degree of susceptibility to E2-induced mammary cancer exhibited by ACI rats, but lacks the treatment-related morbidity associated with pituitary lactotroph hyperplasia/adenoma. When treated with E2, female ACWi rats developed palpable mammary cancer at a median latency of 116 days, an incidence of 100% by 161 days and exhibited an average of 15.6 mammary tumors per rat following 196 days of treatment. These parameters did not differ from those observed for contemporaneously treated ACI rats. None of the E2-treated ACWi rats were killed before the intended experimental end point due to any treatment-related morbidity other than mammary cancer burden, whereas 20% of contemporaneously treated ACI rats exhibited treatment-related morbidity that necessitated premature killing. The ACWi rat strain is well suited for use by those in the research community, focusing on breast cancer etiology and prevention.

Key Words
- ACWi rat
- ACI rat
- Copenhagen rat
- estradiol
- breast cancer

Introduction

Both endogenous and exogenous estrogens have been strongly linked to the etiology of breast cancer (Yager & Davidson 2006). Although it is clear that estrogen receptor (ER)-dependent pathways are essential for the development of a large fraction of breast cancers, the molecular mechanisms through which estrogens contribute to breast cancer etiology remain poorly defined. The ACI rat model of 17β-estradiol (E2)-induced mammary cancer serves as a unique and physiologically relevant model for defining the mechanisms through which estrogens contribute to...
breast cancer development, identifying genetic variants that determine susceptibility to breast cancer, and identifying agents and strategies for use in preventing breast cancer. Female ACI rats rapidly develop mammary carcinoma when treated continuously with physiological levels of E2 normally encountered during pregnancy (Shull et al. 1997). Concurrent treatment with tamoxifen dramatically diminishes the ability of E2 to induce mammary cancer in ACI rats, indicating an important role of ER-mediated pathways in mammary tumorigenesis (Li et al. 2002, Singh et al. 2011). The mammary cancers that develop in E2-treated ACI rats express ERα and progesterone receptor, are dependent upon estrogens for survival and growth, and exhibit non-random patterns of chromosome copy number alterations that mirror somatic copy number alterations frequently observed in breast cancers (Harvell et al. 2000, Adamovic et al. 2007, Ruhlen et al. 2009). Together, these data illustrate multiple important similarities between the mammary cancers induced by E2 in ACI rats and luminal type breast cancers in humans.

The ACI rat model of E2-induced mammary cancer has been extensively characterized genetically. Although female ACI rats are uniquely susceptible to mammary cancer induction by E2, female Copenhagen (COP) and Brown Norway (BN) rats are resistant (Shull et al. 1997, 2001, Spady et al. 1998, Shull 2007). Multiple quantitative trait loci (QTLs), designated estrogen-induced mammary cancer (Emca1) through Emca9, that harbor genetic determinants of susceptibility to E2-induced mammary cancer have been mapped in intercrosses between susceptible ACI rats and resistant COP or BN rats (Gould et al. 2004, Schaffer et al. 2006, Shull 2007). The existence of these Emca loci has been confirmed by generation and characterization of congeneric rat strains that harbor alleles from the resistant COP or BN strain on the genetic background of the susceptible ACI strain (Schaffer et al. 2013, Colletti et al. 2014). Moreover, each of the Emca loci is orthologous to genetic determinants of breast cancer risk mapped in genome wide association studies (Schaffer et al. 2013, Colletti et al. 2014). These data strongly suggest that these rat models of E2-induced mammary cancer share multiple genetic determinants of breast cancer risk with humans.

Continuous treatment with naturally occurring or synthetic estrogens induces pituitary lactotroph hyperplasia and/or adenoma in multiple inbred rat strains (Stone et al. 1979, Wiklund et al. 1981a, Spady et al. 1999a). ACI rats are among the most highly sensitive of inbred strains in this regard, and morbidity resulting from these pituitary tumors can compromise long-term studies focused on mammary cancer etiology and prevention (Shull et al. 1997, Spady et al. 1999a). By contrast, COP rats are only moderately sensitive and BN rats are relatively insensitive to the actions of estrogens in the induction of lactotroph hyperplasia/adenoma (Wendell & Gorski 1997, Spady et al. 1998, 1999a,c, Wendell et al. 2000, Strecker et al. 2005, Shull et al. 2007, Kurz et al. 2008, 2014). Multiple QTLs, designated estrogen-induced pituitary tumor (Ept1) through Ept14 have been mapped that determine the sensitivity to estrogen-induced lactotroph hyperplasia/adenoma in intercrosses between ACI and COP or BN rats (Strecker et al. 2005, Shull et al. 2007, Kurz et al. 2008, 2014). The data from these studies indicate that most of the QTLs that determine responsiveness of the pituitary lactotroph to estrogens segregate independently from the QTLs that determine susceptibility to E2-induced mammary cancer. The objective of this study was to utilize the knowledge gained from genetic studies of estrogen action in the pituitary and mammary glands to develop a novel inbred rat strain that retains the high degree of susceptibility to E2-induced mammary cancer exhibited by ACI rats, but lacks the treatment-related morbidity associated with pituitary lactotroph hyperplasia/adenoma. The data presented herein illustrate the unique phenotypic characteristics of such an inbred rat strain, designated ACWi. The ACWi rat strain is well suited for use by those in the community focusing on breast cancer etiology and prevention.

Materials and methods

Care, treatment, and phenotypic characterization of animals

All procedures involving live animals were approved by the Institutional Animal Care and Use Committee of the University of Wisconsin–Madison. ACI/SegHsd rats were obtained from Harlan Sprague–Dawley, Inc. (Indianapolis, IN, USA). COP/CrCrl rats were obtained from Charles River Laboratories (Wilmington, MA, USA). The ACI.COP-Ept1 and ACI.COP-Ept2 congenic rat strains were generated in our laboratory as described previously (Kurz et al. 2008). The ACWi rat strain (official strain designation: ACI.COP-(D3Rat130-D3Rat114) (D6Rat80-D6Rat146)/ Shul, RGD ID 9589088) was generated by intercrossing the ACI.COP-Ept1 and ACI.COP-Ept2 strains and selectively breeding the progeny to generate animals that were homozygous for COP alleles at both the Ept1 locus on rat chromosome 6 (Rno6) and the Ept2 locus on Rno3.

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The ACWi strain will be submitted to the Rat Resource and Research Center at the University of Missouri for cryopreservation and distribution. All rats were housed under controlled temperature, humidity, and 12 h light:12 h darkness conditions in facilities that were accredited by the American Association for Accreditation of Laboratory Animal Care and operated in accordance with The Guide for the Care and Use of Laboratory Animals. All procedures related to the care, propagation, genotyping, treatment with E2, evaluation for presence of mammary cancer, and assessment of pituitary hyperplasia/adenoa have been described (Shull et al. 1997, 2001, Spady et al. 1998, Harvell et al. 2000, Gould et al. 2004, Schaffer et al. 2006, 2013). The number of grossly discernible tumors, i.e., ~1 mm in diameter, was quantified at necropsy. Pituitary mass was measured as a quantitative phenotypic indicator of pituitary lactotroph hyperplasia/adenoa as discussed below. The animals were killed following 84 days of treatment to evaluate pituitary mass or following 196 (−4 to +1) days of treatment to evaluate mammary tumor burden together with pituitary mass. In the latter experiment, animals were killed before the desired experimental end point if necessary due to mammary tumor burden or another treatment-related morbidity.

Sources of genomic data

All genome coordinates presented in this manuscript are relative to rat genome assembly version 5.0. Whole-genome sequence data for ACI, COP, and BN rat strains were accessed through the Rat Genome Database and Ensembl Database (Laulederkind et al. 2013, Nigam et al. 2013, Flicek et al. 2014).

Statistical analyses

Latency was defined as the number of days separating initiation of E2 treatment and the first detection of a palpable mammary tumor. Phenotypes exhibited by ACWi rats were compared with those exhibited by contemporaneously treated ACI rats as well as to recent historical data from identically treated ACI and COP rats. Median latency was derived from Kaplan–Meier analyses. The log rank test was used to compare latencies between strains and to calculate the relative risk of each set of congenic rats in comparison with ACI rats. These analyses were carried out in MSTAT version 5.5 (Drinkwater 2013). Between-strain differences in mammary tumor multiplicity and pituitary mass at necropsy were evaluated using the Kruskal–Wallis and Wilcoxon rank sum tests with the Holm-Bonferroni method to correct for multiple pairwise comparisons. These analyses were carried out in R (R Core Team 2013). P values ≤ 0.05 were considered to be statistically significant for all tests.

Results

ACWi rats are highly susceptible to E2-induced mammary cancer

The susceptibility of ACWi rats to E2-induced mammary cancer was compared with that of contemporaneously treated ACI rats to determine whether or not ACWi rats retained the highly susceptible phenotype that is the characteristic of the parental ACI rat strain. The median latency to the appearance of palpable mammary cancer for E2-treated ACWi rats was 116 days and 100% of the treated rats developed mammary cancer by 161 days of treatment (Fig. 1). Median latency for contemporaneously treated ACI rats was 100 days and 100% of the treated rats exhibited palpable mammary cancers by 134 days of treatment. The time course to the appearance of palpable mammary cancer in ACWi rats did not differ significantly from that in ACI rats (P = 0.071). Because the mammary
cancer phenotypes exhibited by ACI rats and different ACI-derived congenic rats have been observed to be stable over time and between laboratories, the susceptibility of ACWi rats to E2-induced mammary cancer was also compared with historical data from identically treated ACI and COP rats evaluated in our laboratory. Median latency to the appearance of palpable mammary cancer in this larger group of E2-treated ACI rats was 123 days and 94% of the animals at risk developed mammary cancer by 196 days of treatment. As expected, COP rats were resistant to induction of mammary cancer by E2, relative to ACI and ACWi rats. The first palpable cancer in this group of E2-treated COP rats was observed following 154 days of treatment and only 33% of the treated COP rats developed mammary cancer during the course of treatment. The time course to the appearance of mammary cancer in E2-treated ACWi rats did not differ significantly from that exhibited by the group of ACI rats evaluated previously (P=0.184), but did differ significantly from that exhibited by the E2-treated COP rats (P<0.0001). Sham-treated control ACWi, ACI, and COP rats did not develop mammary cancer when evaluated over a 196-day time course.

Grossly discernable mammary tumors were enumerated at necropsy. The mean numbers of mammary tumors observed in contemporaneously treated ACWi and ACI rats were 15.6 and 20.5, respectively, and mammary tumor number did not differ between strains (P=0.2822; Fig. 2). Average mammary tumor number in the group of E2-treated ACI rats evaluated before the current study was 5.8 per rat, whereas average mammary tumor number in E2-treated COP rats was 0.3 per rat. Tumor number in ACWi rats differed significantly from that observed in these groups of ACI (P<0.0001) and COP rats (P<0.0001). Together, the data presented in Figs 1 and 2 clearly indicate that ACWi rats are as highly susceptible to E2-induced mammary cancer as ACI rats. The magnitude of the difference in average mammary tumor number observed between the contemporaneous and historical groups of ACI rats exceeded that normally observed between individual batches of ACI rats evaluated in our laboratory over the past several years. The bases for this observed difference between the contemporaneous and historical data are not known.

ACWi rats do not exhibit morbidity resulting from E2-induced pituitary lactotroph hyperplasia/adenoma

Continuous treatment of ACI rats with E2 or the synthetic estrogen diethylstilbestrol (DES) induces development of pituitary lactotroph hyperplasia/adenoma, and the resulting dramatic increase in pituitary gland mass and endocrine dysfunction (i.e., hyperprolactinemia) can result in morbidity that necessitates killing of an experimental animal before the intended experimental end point (Spady et al. 1999a). Therefore, we evaluated the impact on the survival of treatment-related morbidity that
resulted from any condition other than mammary cancer burden. No E2-treated ACWi or COP rats were killed due to any morbidity other than mammary cancer burden (Fig. 3). By contrast, 20% of the contemporaneously treated ACI rats and 49% of the E2-treated ACI rats evaluated in our laboratory over the past 4 years were killed due to morbidity other than mammary cancer burden before the intended experimental end point. The survival curves for the two groups of E2-treated ACI rats did not differ significantly (P = 0.3453), and these groups were combined for comparison with treated ACWi and COP rats (Fig. 3). Overall survival of E2-treated ACWi and COP rats was significantly higher relative to that observed in the combined population of E2-treated ACI rats (P = 0.0117 and P = 0.0019 respectively).

Pituitary gland mass was measured in all rats at necropsy. Pituitary mass in sham treated control ACWi, COP, and ACI rats averaged 10.2, 14.0, and 10.5 mg, respectively, and no significant differences were observed between strains (Fig. 4). E2 treatment increased pituitary mass 4.3-fold in ACWi rats, from an average of 10.2 mg in sham-treated control ACWi rats to an average of 43.5 mg in E2-treated ACWi rats (P = 0.0420). Pituitary mass was similarly increased 2.9-fold in E2-treated COP rats, relative to sham-treated control COP rats (P = 0.0013).

By contrast, E2 treatment increased pituitary mass 15.5-fold (P = 0.0006) in contemporaneously treated ACI rats and 18.5-fold (P < 0.0001) in the historical ACI population. Because pituitary mass did not differ significantly between these two groups of E2-treated ACI rats, they were combined to simplify between strain comparisons. Pituitary mass in E2-treated ACWi and COP rats differed significantly from that observed in E2-treated ACI rats (P < 0.0001 for both comparisons), whereas pituitary mass in treated ACWi and COP rats did not differ (P = 1). As noted above, a significant fraction of E2-treated ACI rats exhibited treatment-related morbidity and were killed before the desired experimental end point; i.e., 196 days of treatment. Average durations of E2 treatment in the ACWi, COP, and ACI populations were 192, 196, and 185 days respectively.

Ept1 and Ept2 were originally mapped in male F2 progeny that were generated in crosses between ACI and COP and treated with DES for 12 weeks, and the actions of these QTLs were subsequently confirmed in female congenic rats treated with E2 for 12 weeks (Strecker et al. 2005, Kurz et al. 2008). Therefore, we also evaluated pituitary mass in female ACWi and ACI rats that were treated with E2 for 12 weeks (Fig. 5). Treatment of ACWi rats with E2 for 12 weeks increased pituitary mass by 3.3-fold, from 10.3 to 34.2 mg (P = 0.0710). By contrast,
12 weeks of E₂ treatment increased pituitary mass by 5.5-fold in ACI rats ($P=0.0130$). Average pituitary mass in treated ACWi rats was significantly less than in ACI rats following 12 weeks of treatment ($P=0.0350$), further indicating that ACWi rats are less sensitive than ACI rats in regard to the induction of pituitary lactotroph hyperplasia/adenoma by E₂.

The gross appearance of the pituitary glands of E₂-treated rats also differed upon comparison by rat strain. The glands of E₂-treated ACWi and COP rats were enlarged, but were normal in shape and color (Spady et al. 1999a,b). The pituitary glands of E₂-treated ACI rats were enlarged and were usually abnormal in shape and red in color, which results from the presence of abundant dilated and congested vascular channels and focal regions of hemorrhage (Spady et al. 1999a,b).

**Discussion**

Published genetic data from reciprocal intercrosses between ACI and COP rats indicate that the QTLs that determine susceptibility to E₂-induced mammary cancer segregate independently of those QTLs that determine the sensitivity of the pituitary lactotroph population to...
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Current study with published data indicates that the pituitary mass in identically treated ACI.COP-Ept1 and ACI.COP-Ept2 congenic rats and selectively breeding to homozygosity for COP alleles at Ept1 on Rno6 and Ept2 on Rno3 (Kurz et al. 2008). This strategy was chosen because: i) for those QTLs where COP alleles reduced the sensitivity of the pituitary lactotroph to estrogen, Ept1 and Ept2 exerted the greatest effects on phenotypic variance and ii) pituitary lactotroph responsiveness in ACI.COP-Ept1 and ACI.COP-Ept2 congenic rats differed most from that of ACI rats (Strecker et al. 2005, Kurz et al. 2008). The responsiveness of the pituitary lactotroph population to E2 in ACWi rats, as indicated by quantification of pituitary mass, a surrogate indicator of absolute lactotroph number (discussed below), was dramatically reduced relative to ACI rats, and did not differ from that of COP rats. Comparison of the data from the current study with published data indicates that the combined actions of COP alleles at Ept1 and Ept2 on induction of pituitary lactotroph hyperplasia/adenoama in ACWi rats dramatically exceed the actions of COP alleles at either Ept1 or Ept2 alone (Kurz et al. 2008). Whereas pituitary mass in ACWi rats treated with E2 for 28 weeks averaged 43.4 mg, pituitary mass in identically treated ACI.COP-Ept1 and ACI.COP-Ept2 rats averaged 142.7 mg (P<0.0001 vs ACWi) and 75.9 mg (P=0.0033 vs ACWi) respectively.

The sensitivity of the pituitary lactotroph population to the induction of hyperplasia/adenoama by estrogen is rat strain-specific and highly heritable (Stone et al. 1979, Wiklund et al. 1981a,b, Wendell et al. 1996, Shull et al. 1997, 2007, Wendell & Gorski 1997, Spady et al. 1999a,c, Strecker et al. 2005). Pituitary mass is frequently used as a quantitative phenotype for estrogen-induced lactotroph hyperplasia/adenoama (Wiklund et al. 1981a,b, Wendell et al. 1996, Wendell & Gorski 1997, Spady et al. 1999a,c, Strecker et al. 2005, Shull et al. 2007). Because pituitary mass is directly correlated with pituitary DNA content and the level of prolactin in the systemic circulation, this phenotype serves as an accurate surrogate indicator of absolute lactotroph number (Wiklund et al. 1981a, Spady et al. 1999a,c, Tachibana et al. 2006, Kurz et al. 2008). Estrogen-induced lactotroph hyperplasia/adenoama results in sustained hyperprolactinemia (Spady et al. 1999a). Prolactin plays important roles in mammary gland development and lactation, and has been implicated in the etiology of breast cancer (Clevenger et al. 2003, Rose-Hellekant et al. 2003). ACWi rats remain as highly susceptible as parental ACI rats to E2-induced mammary cancer while the sensitivity of the pituitary lactotroph population in ACWi rats to induction of hyperplasia/adenoma is significantly attenuated relative to ACI rats. These data further confirm that the molecular events that give rise to these two different estrogen-induced

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**Figure 7**

Genetic relationships between the Ept2 pituitary tumor locus and the Emca5 mammary cancer locus. (A) Rat chromosome 3 (RNO3) is illustrated as an ideogram that denotes defined cytogenetic bands. (B) Ept2 was mapped to RNO3 in intercrosses between ACI and COP rats (Strecker et al. 2005). The location of the Ept2 LOD peak is illustrated by the upward arrow above a horizontal black bar that indicates the 95% CI for Ept2, which was defined by interval mapping analyses. Also illustrated is the Ept2 congenic interval; the horizontal black bar indicates the segment of RNO3 that is known to be derived from COP rats, the open termini of this horizontal bar indicate areas of recombination between the ACI and COP genomes, and the remainder of RNO3 is known to be derived from ACI rats (Kurz et al. 2008). (C) Emca5 was mapped to RNO3 in an intercross between BN and ACI rats (Schaffer et al. 2006). The locations of the Emca5 LOD peak, the Emca5 95% CI, and the Emca5 congenic interval (Colletti et al. 2014) are illustrated as described for Ept2. (D) The upward arrow indicates the location of the region of RNO3 that is orthologous to the breast cancer risk locus in humans linked to single-nucleotide polymorphism rs2284378 in a genome wide association study (Siddiq et al. 2012). The overlap between the rat and human loci suggest these species share genetic determinants of breast cancer susceptibility (Colletti et al. 2014).
neoplasms are influenced by distinct genetic determinants. Moreover, these data further illustrate the lack of a direct association between pituitary tumor mass, hyperprolactinemia, and susceptibility to E2-induced mammary cancer in the rat models.

The morbidity associated with pituitary lactotroph hyperplasia/adenoma in humans and estrogen-sensitive rat strains, such as ACI, results in large part from the effect of expanded pituitary mass on those tissues adjacent to the pituitary gland, primarily the hypothalamus and the optic nerves, as well as from endocrine imbalances, specifically hyperprolactinemia and/or deficiency of the other hormones normally produced by other pituitary cell types (Spady et al. 1999a, Molitch 2002, Sam & Molitch 2005, Colao & Savastano 2011). In this study, 46% of ACI rats treated with E2 to induce mammary cancer exhibited treatment-related morbidity unrelated to mammary cancer burden and were killed before the desired experiment end point. By contrast, 0% of E2-treated ACWi rats were killed before the completion of the study due to morbidity other than mammary cancer burden. In an effort to reduce treatment-related morbidity in E2-treated ACI rats, Ravoori et al. explored the use of a lower dose of E2 to induce mammary cancer, relative to that used in our studies. Although the lower E2 dose, achieved by reducing the length of the silastic tubing implant, was effective in reducing treatment-related morbidity, an extra 60 days of treatment was required to attain an equivalent mammary tumor multiplicity observed in ACI rats treated with the larger implants/higher dose of E2 (Ravoori et al. 2007). Thus, ACWi rats offer clear advantages over ACI rats to those investigators who perform long-term studies focused on the etiology and prevention of E2-induced mammary cancer using these physiologically relevant rat models.

The ACWi rat strain was developed by intercrossing ACI.COP-Ept1 and ACI.COP-Ept2 rats followed by iterative breeding with selection for COP alleles at Ept1 and Ept2. It is noteworthy that the Ept2 congenic interval on RNO3 extends from D3Mgh21 (48.4 Mb) to D3Rat142 (171.5 Mb) and overlaps almost entirely with Emca5, a mammary cancer susceptibility QTL that was mapped in an intercross between susceptible ACI and resistant BN rats (Fig. 7; Strecker et al. 2005, Schaffer et al. 2006, Kurz et al. 2008, Colletti et al. 2014). Data presented herein as well as data published previously indicate that COP alleles across the Ept2 congenic interval do not impact susceptibility to E2-induced mammary cancer, whereas BN alleles across the Emca5 congenic interval dramatically reduce mammary cancer susceptibility (Kurz et al. 2008, Colletti et al. 2014). Together, these data suggest that the genetically related ACI and COP rat strains share alleles at Emca5. The peak LOD region of Emca5 is orthologous to a breast cancer risk locus mapped to human chromosome 20q11 that spans three genes, ASIP, RALY, and EIF2S2 (Schaffer et al. 2006, Siddiq et al. 2012, Colletti et al. 2014). ASIP encodes agouti signaling protein, which regulates skin and hair pigmentation in humans and multiple other mammalian species, including rats (Kanetsky et al. 2002, Bonilla et al. 2005, Suzuki 2013). It is interesting to note that BN alleles at Emca5 confer upon ACI.BN-Emca5 congenic rats a black, non-agouti, coat color phenotype, indicating that ACI and BN rats harbor different alleles at Asip (J D Shull, unpublished observations). By contrast, both ACI and ACI.COP-Ept2 congenic rats exhibit the agouti coat color, indicating that ACI and COP strains share alleles at Asip. An evaluation of available whole-genome sequence data for the ACI, COP, and BN rat strains further supports the assertion that ACI and COP rats share alleles across the peak LOD region of Emca5, including the Asip locus, whereas BN rats differ across this region. Together, these comparative genomic data indicate that BN rats harbor a genetic variant that inhibits production or function of agouti signaling protein and suggest a possible functional association between this genetic variant and mammary cancer susceptibility.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement
K L Dennison and N B Samanas generated the ACWi rat strain. K L Dennison, N B Samanas, Q E Harenda, M P Hickman, N L Seiler, and L Ding generated data. K L Dennison and J D Shull evaluated data and prepared the manuscript. J D Shull conceived and directed the study and was the primary author of the manuscript.

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