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Chromosome X loci and spontaneous granulosa cell tumor development in SWR mice

Epigenetics and epistasis at work for an ovarian phenotype

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Abbreviations: *Actb*, *actin beta* gene; AIS, androgen insensitivity syndrome; *Ar*, *androgen receptor* gene; *CA*, Castaneus/Ei strain allele; Chr, chromosome; DHEA, dehydroepiandrosterone; dNTP, deoxyribonucleotide triphosphate mix; *FOXL2*, *forkhead box L2* gene; GC, granulosa cell; *Gct*, *Granulosa cell tumor susceptibility locus; GNAS*, *guanine nucleotide binding protein, alpha stimulating, complex locus; J*, SJL/Bm strain allele; Mb, million base pairs; MMLV, Moloney murine leukemia virus; ncRNA, non-coding RNA; qPCR, quantitative real-time PCR; *SW*, SWR/Bm strain allele; X_A, Chr X, transcriptionally active; X_I, Chr X, transcriptionally inactive; X_M, Chr X, maternally inherited; X_P, Chr X, paternally inherited; *XIC*, *X-inactivation center*

Females of the SWR/Bm (SWR) inbred mouse strain possess a unique susceptibility to juvenile-onset tumors originating from the granulosa cells (GC) of the ovarian follicles. Tumor susceptibility is an inherited, polygenic trait in SWR females, minimally involving an oncogenic *Granulosa cell tumor susceptibility* (*Gct*) locus on chromosome (Chr) 4 (*Gct1*), and two GC tumor susceptibility modifier genes mapped to distinct regions of Chr X (*Gct4* and *Gct6*). Shifts in the frequency of GC tumor initiation in the SWR female population from low penetrance to moderate penetrance, or phenotype switching between GC tumor-susceptible and GC tumor-resistant, is strongly influenced by the allelic contributions at *Gct4* and *Gct6*. In addition to the allele-specific effects, GC tumor susceptibility is controlled by the mode of X-linked transmission with a dominant, paternal parent-of-origin effect. We took advantage of the robust paternal effect with a recombinant male progeny testing strategy to resolve the *Gct4* locus interval to 1.345 million base (Mb) pairs. Based on the mapping resolution and the phenotype sensitivity to endogenous and exogenous androgen exposure, a promising candidate for *Gct4* identity is the *androgen receptor* (*Ar*) gene. We explored the mechanism of allelic variation for *Ar* between SWR (low penetrance allele) and SJL/Bm (SJL) (moderate penetrance allele) using an SWR.SJL-X congenic strain resource and a quantitative gene expression method. We report the low GC tumor penetrance allele of the SWR strain correlates with significantly reduced *Ar* transcript levels in the female ovary at the pubertal transition.

Introduction

In female patients, granulosa cell (GC) tumors of the ovary are classified into two general categories (adult-type or juvenile-type) based on both clinical and pathological criteria, such as tumor histology, nuclear morphology and the potential for disease recurrence.[1] As the name suggests, GC tumors of the juvenile-type are most commonly found in children and young women. A comprehensive review of 125 cases of juvenile-type GC tumors found that 78% of the cases occurred in individuals less than 20 y of age, with 44% of the cases presenting in girls 10 y of age or younger, suggesting a strong genetic contribution to susceptibility.[1] The relative rarity of cases of juvenile-type GC tumors has precluded human genetic linkage studies to identify candidate susceptibility genes or modes of transmission that confer tumor risk. A somatic mutation in the forkhead box L2 (FOXL2) transcription factor (p.C134W) has been identified in adult-type GC tumors with a high degree of consistency between independent research cohorts, suggesting a strong mechanistic link.[2,3] The etiology for juvenile-type GC tumors is not explained by the same *FOXL2* mutation, either germline or somatically acquired, suggesting a novel pathway of tumorigenic initiation. Lost or reduced expression of *FOXL2* has been associated with 50% of juvenile-type GC tumors of high grade, suggesting a role for FOXL2 as a suppressor of GC tumor progression, but not a requirement for tumor initiation.[4] To this end, we are pursuing tumor susceptibility genes in the SWR/Bm (SWR) mouse model of spontaneous, juvenile-onset GC tumor development that parallels the histological, endocrinological and developmental timing characteristics of human juvenile-type GC tumors.[5,6]

Approximately 1% of SWR inbred female mice develop unilateral or bilateral ovarian GC tumors that are easily identified by 8 weeks of age as large solid or cystic masses (**Fig. 1**).[5] GC tumor

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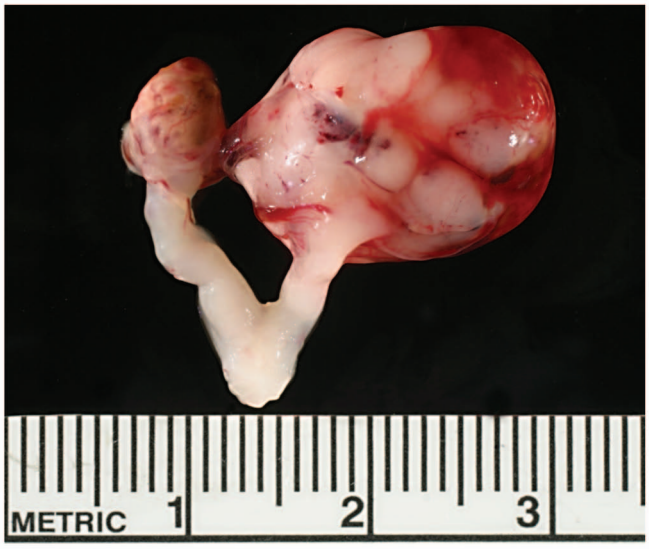

Figure 1. Bilateral GC tumor specimen. Spontaneous GC tumors in SWR-derived mice are easily recognized as large, 5–15 mm cystic or solid masses with blood filled spaces when compared with a normal ovary of 2–3 mm. This image shows a representative bilateral GC tumor specimen with the uterus still attached. The specimen was isolated from an 8 wk old SWR.SJL-X_3 congenic female that carries the *Gct4*^{J} modifier allele. The GC tumor on the left is less vascularized and more necrotic than the larger, highly vascularized tumor on the right.

initiation in SWR females is restricted to the time of ovarian maturation at puberty, within the window of 3–5 weeks of age.[7] Beyond this time frame, females that have not developed GC tumors are no longer at risk, retain their fertility and develop no other visible pathological changes in the ovary through 12 mo of age.[8] The penetrance of GC tumor initiation is significantly increased (≈20% incidence) in SWR females with the introduction of androgenic steroids at puberty, while introduction of estrogen suppresses tumor initiation, indicating a strong endocrine contribution to susceptibility.[9,10]

Genetic mapping studies for the GC tumor susceptibility phenotype in SWR female mice have been pursued as a means to elucidate novel pathways and genetic mechanisms for juvenile-onset GC tumorigenesis. These studies demonstrated that GC tumor susceptibility is a polygenic, heritable trait involving several *Granulosa cell tumor (Gct)* susceptibility loci on the autosomes and Chr X.[11,12] Both F_2 intercross and recombinant inbred strain mapping studies confirmed the SWR allele at the *Gct1* locus on Chr 4 is both necessary for GC tumor development and sensitive to dehydroepiandrosterone (DHEA). The *Gct1* locus has been fine-mapped to 1.31 Mb on mouse Chr 4 using a congenic strain mapping strategy.[6,12,13] When the SWR autosomal contribution to GC tumor susceptibility was genetically fixed, the activities of two independent, tumor incidence modifier alleles were revealed on Chr X: (1) *Gct4* was originally identified in the SJL inbred strain that served as a GC tumor resistant mapping partner for the SWR strain and (2) *Gct6* was revealed on distal Chr X following transfer of a 72.7 Mb Chr X donor segment from the Castaneus/Ei (CAST) inbred strain as part of an SWR.CAST-X congenic development strategy originally created to fine map *Gct4*.[11,14] The susceptibility loci identified in the mouse model do not overlap with candidate loci identified for human GC tumors based on somatically acquired mutations or altered expression in adult-type (*FOXL2*) or juvenile-type GC (*FOXL2* and *GNAS*) tumors; rather, they may reflect unique, heritable alleles that support juvenile-onset GC tumor initiation events.[2,3,15]

In the presence of an autosomal SWR strain background, allelic variation at *Gct4* modifies GC tumor incidence in the susceptible female population, increasing the phenotypic penetrance from ≤ 1% to ≈20% spontaneous tumor incidence in the absence of hormonal intervention. *Gct4*^{SW} is a low incidence allele, while *Gct4*^{CA} and *Gct4*^{J} support increased penetrance in F_1 generation daughters. Based on the allelic difference between *Gct4*^{SW} and *Gct4*^{J}, the locus was coarsely mapped with an SWR.SJL-X congenic strain series. The most informative subline—SWR.SJL-X_5—localized *Gct4* between the markers *DXMit45* and *DXMit170*, an ≈25 Mb interval encompassing the *Ar* gene and many other candidates.[14]

The CAST genome was introduced on Chr X in a new SWR.CAST-X congenic strain to increase the number of polymorphisms for high-resolution genetic mapping of the *Gct4* locus, but this divergent strain subsequently introduced a unique allele on Chr X that has a dominant influence on the GC tumor susceptibility phenotype. Allelic variation at the *Gct6* locus is either permissive or suppressive for spontaneous GC tumor initiation, with tumor suppressor activity contributed by the CAST strain allele (*Gct6*^{CA}).[13] In contrast, both *Gct6*^{SW} and *Gct6*^{J} are permissive alleles; thus, *Gct6* was not revealed as a functional GC tumor susceptibility locus in the earliest mapping crosses between SWR and SJL strains. We are pursuing the identity of the X-linked *Gct* candidates given their strong influence over spontaneous and androgen-induced GC tumorigenesis.

In addition to genetic variation, an epigenetic phenomenon in the form of a paternal parent-of-origin effect dominates the activity of the X-linked GC tumor susceptibility alleles in SWR female mice. This unexpected phenomenon was revealed through the differential activity of the *Gct4* and *Gct6* alleles derived from the SWR, SJL and CAST strains when the X-linked susceptibility alleles were maternally (X_M) or paternally (X_P) inherited by daughter offspring. In this research report, we have made use of a ten-generation SWR.CAST-X congenic strain resource to confirm the paternal, parent-of-origin effect for the tumor suppressor activity of the *Gct6* locus and to fine map the *Gct4* locus to a minimal genetic interval and short list of candidate genes with a recombinant male Chr X mapping strategy. Using SWR inbred and SWR.SJL-X_5 congenic subline females that retain *Gct4*^{J}, we report on the mechanism for allelic variation at *Gct4* related to the quantitative expression of the *Ar* gene in the ovary, as the primary candidate for *Gct4* identity.

Results

Paternal transmission of *Gct4*^{CA} and *Gct6*^{CA} allele effects for GC tumor susceptibility. Female mice derived from the homozygous SWR.CAST-X strain were examined for GC tumor susceptibility

Table 1. Ovarian GC tumor incidence in female offspring derived from SWR inbred, SWR.CAST-X congenic lines or reciprocal breeding crosses

Maternal strain	Paternal strain	Chr X_p alleles transmitted to daughter offspring	GC tumor incidence (%) of daughter offspring (n)	Androgen treatment at puberty
SWR.CAST-X	SWR.CAST-X	$Gct4^{CA}$; $Gct6^{CA}$	0% (50)	sham surgery
SWR.CAST-X	SWR.CAST-X	$Gct4^{CA}$; $Gct6^{CA}$	0% (87)	testosterone
SWR.CAST-X	SWR.CAST-X	$Gct4^{CA}$; $Gct6^{CA}$	0% (52)[a]	DHEA
SWR	SWR	$Gct4^{SW}$; $Gct6^{SW}$	17.6% (51)	DHEA
SWR.CAST-X	SWR	$Gct4^{SW}$; $Gct6^{SW}$	7.7% (65)[b]	DHEA
SWR	SWR.CAST-X	$Gct4^{CA}$; $Gct6^{CA}$	0% (58)[a]	DHEA
SWR	SWR.CAST-X	$Gct4^{CA}$; $Gct6^{CA}$	0% (50)	testosterone
SWR.CAST-X_1	SWR.CAST-X_1	$Gct4^{CA}$; $Gct6^{SW}$	20.4% (127)	-
SWR	SWR.CAST-X_1	$Gct4^{CA}$; $Gct6^{SW}$	27.0% (74)	-
SWR.CAST-X_1	SWR	$Gct4^{SW}$; $Gct6^{SW}$	4.9% (61)[c]	-

[a]Significantly reduced from the SWR inbred strain incidence following DHEA treatment (17.6%) ($p = 0.0012$). [b]Significantly greater than 0% incidence ($p = 0.038$). [c]Significantly reduced from SWR.CAST-X_1 homozygous congenic ($p = 0.0029$) or (SWR x SWR.CAST-X_1) incidence ($p = 0.0003$).

under the challenge of androgenic steroid supplementation. **Table 1** shows that SWR.CAST-X females homozygous for the CAST donor segment, inclusive of *Gct4* and *Gct6* loci, are resistant to GC tumor initiation despite the pubertal administration of DHEA or testosterone. Furthermore, tumor resistance is dominantly conferred by Chr X_p transmission of the CAST segment, as shown by the resistance of (SWR × SWR.CAST-X) F_1 generation female offspring to the development of GC tumors in the presence of DHEA or testosterone. When the CAST-X segment is maternally transmitted, complete tumor suppression is lifted and moderate GC tumor incidence is observed in (SWR.CAST-X × SWR) F_1 generation female offspring in the presence of DHEA (7.7%) (**Table 1**).

The SWR.CAST-X_1 congenic subline was derived from the SWR.CAST-X founder congenic strain to eliminate GC tumor suppression introduced by the $Gct6^{CA}$ locus while maintaining the $Gct4^{CA}$ locus. As an established homozygous colony, we observed that SWR.CAST-X_1 females have enhanced, spontaneous GC tumor incidence in the absence of exogenous androgenic stimulation (20.4%) (**Table 1**). In order to expedite *Gct4* mapping with the SWR.CAST-X_1 resource, we first confirmed that the paternal parent-of-origin effect was supported by SWR.CAST-X_1 males. SWR.CAST-X_1 breeders were put through a reciprocal breeding scheme with SWR mates and spontaneous GC tumor incidence was measured in the F_1 daughter offspring. We observed that paternal inheritance of the CAST-X_1 segment supported GC tumorigenesis incidence at a similar magnitude to the homozygous condition (27.0%), and that maternal inheritance of the same donor segment showed significantly reduced support for GC tumor initiation (4.9%) (**Table 1**). The allelic variants and robust paternal effect for the $Gct4^{CA}$ GC tumor susceptibility locus was subsequently applied to a male progeny testing scheme to refine the *Gct4* candidate gene region.

***Gct4* interval mapping.** Screening for informative progeny test males useful to map the *Gct4* locus progressed until we achieved a minimal genetic resolution and a short list of candidate genes. **Figure 2** shows the haplotype map for the resolved *Gct4* locus based on the six most informative Chr X_p haplotypes identified from hundreds of males genotyped and selected males chosen for progeny testing with SWR mates. The Chr X haplotype for each male in the vicinity of the *Gct4* locus is represented as a vertical column, while the overall GC tumor incidence observed in the F_1 daughter offspring is indicated below the haplotype for each tested male. The phenotype distinction for mapping *Gct4* was the stronger tumorigenic support of the $Gct4^{CA}$ allele vs. the weaker support of the $Gct4^{SW}$ allele. The allelic effects are confirmed by the GC tumor incidence of the F_1 daughter population for this subset of males tested, with $Gct4^{CA}$ supporting an average GC tumor incidence of 16% (48/301), vs 0% (0/75) recorded for the single $Gct4^{SW}$ carrier male shown (**Fig. 2**). Of note, 0% incidence in this case does not imply GC tumor resistance, since the *Gct6* locus in all males in this screen was genetically fixed to maintain a $Gct6^{SW}$ tumor susceptibility allele that is permissive for GC tumorigenesis. Informative males 1 through 6 refined the *Gct4* locus interval to 1.345 Mb between the markers *DXamd3* and *DXamd27*. **Table 2** lists the annotated genes within this interval that are candidates for *Gct4* identity, inclusive of the *Ar* gene.

Quantitative expression analysis of the *Ar* gene. The *Ar* gene was previously considered a candidate for *Gct4* identity based on the activity of androgens to stimulate GC tumor initiation in SWR inbred females, although no coding sequence polymorphisms were identified in the *Ar* gene between SWR and SJL strains that could explain the allelic differences between $Gct4^{SW}$ and $Gct4^{J}$.[11,14] Given the refined mapping resolution of 1.345 Mb for *Gct4* and retention of *Ar* as a candidate gene, we examined the potential for differential *Ar* gene expression between SWR and SWR.SJL-X_5 pubertal ovaries as an alternate explanation for the allelic difference between strains. **Figure 3** shows the gene expression ratios for *Ar* normalized to the *Actin* β (*Actb*) gene in the ovaries of SWR females collected at 4 weeks of age relative to age-matched homozygous SWR.SJL-X_5 females that have a higher (≈20 fold) spontaneous incidence of GC tumors.[14] Using a rigorous standard curve approach for qPCR analyses, we measured a statistically significant increase in *Ar* gene expression in the SWR.SJL-X_5 ovaries (1.6 ± 0.13-fold) relative to age-matched SWR ovaries.

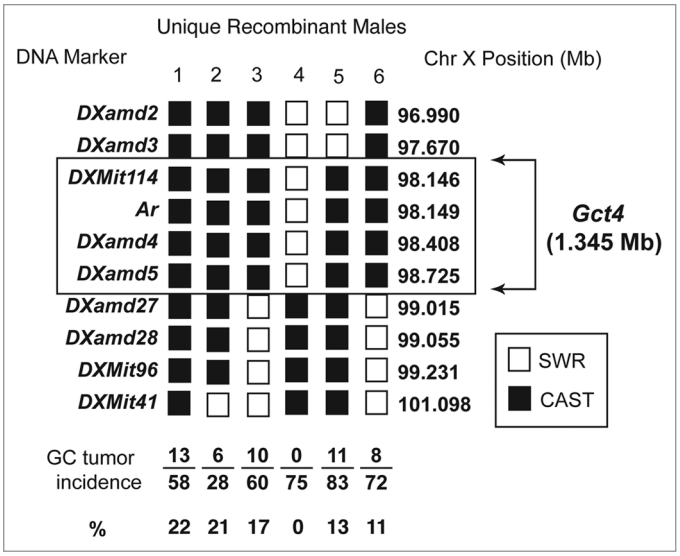

Figure 2. Male mice carrying unique combinations of CAST and SWR genome in the region of the *Gct4* locus on Chr X were mated to SWR dams and the GC tumor incidence measured in the F_1 generation female offspring. White boxes represent SWR alleles present at the DNA marker or gene and black boxes represent CAST alleles. Of the haplotypes shown for six unique animals, males 3, 4 and 6 place the distal interval boundary for the moderate GC tumor susceptibility allele $Gct4^{CA}$ at 99.015 Mb (marker *DXamd27*), while male 5 places the proximal boundary of the interval at 97.670 Mb (*DXamd3*). Only male 4 carries the GC tumor susceptibility allele $Gct4^{SW}$.

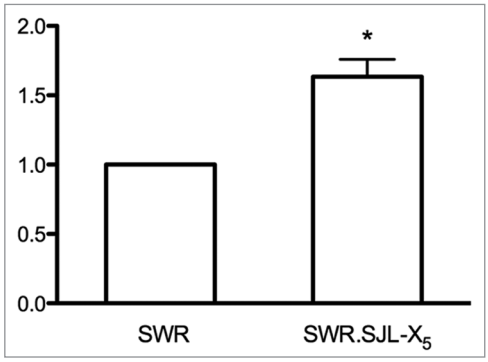

Figure 3. Quantitative expression of the *Ar* gene. Ovaries from a minimum of five individual females of the SWR and SWR.SJL-X_5 strains were compared for quantitative expression of the *Ar* gene relative to the *Actb* reference gene by qPCR. A significantly increased expression was measured in the SWR.SJL-X_5 ovaries, equal to 1.6 ± 0.13 fold that of the SWR strain (p = 0.0038).

Discussion

Ovarian tumors diagnosed in girls and young women are most commonly derived from germ cells (teratomas) or mesenchymal tissues (sex cord-stromal tumors), in contrast to the epithelial-derived ovarian carcinomas that usually appear in women of post-menopausal age. GC tumors of the sex cord-stromal class are found in women at both ends of the reproductive spectrum and are placed into two categories—juvenile-type and adult-type—based on differences in the age of onset and other cytological, histological and most recently, molecular genetic features.[2] Like adult-type GC tumors, juvenile-type cases have the potential for recurrence and life-threatening metastases, with the additional consequence of fertility loss if the ovaries are removed at an early age.[16-18] The SWR mouse model for juvenile-onset, spontaneous GC tumor development shares histological, endocrinological and malignant features with the human tumors, so we have pursued the identity of GC tumor susceptibility genes in the SWR mouse for translation to the human condition as a means to improve prevention, detection and therapeutic strategies. Through this genetic mapping process, we have uncovered a robust epigenetic contribution of the paternal Chr X in mice that has a major influence on GC tumor susceptibility.

All the biological evidence gathered from the SWR mouse model indicates that the site of action for *Gct* susceptibility genes lies within the GC cells. Alterations of the hormonal signaling profiles within the hypothalamic-pituitary-ovarian axis have been excluded by the fact that grafted, genetically susceptible ovaries can form tumors in fertile female mouse recipients that are not genetically susceptible.[7] GC tumors do not form, however, if the transplant recipients are gonadotropin-deficient (hypogonadal, *hpg/hpg*) mutants that do not undergo sexual maturation. The ovarian transfer experiments indicate that the normal endocrine stimulation during ovarian maturation is necessary and sufficient for GC tumor initiation, and that the maturing SWR hypothalamic-pituitary-ovarian axis is not unique in this regard.[7]

GC tumor initiation in SWR female mice is restricted to the time of ovarian maturation at puberty, suggesting that the *Gct* susceptibility genes have a short window of influence over GC fate.[7] During puberty in mice, the first wave of ovarian follicles mature, but these growing follicles subsequently undergo atresia rather than maturation and ovulation.[19] Follicular atresia also occurs in subsets of follicles in any subsequent reproductive cycle, but this does not correlate with GC tumor initiation potential in this model, suggesting that other GC attributes are unique at puberty. It has been reported that the GC progenitors for the earliest cohorts of ovarian follicles have a different cellular origin than subsequent cohorts of maturing follicles.[20] Molecular identification of the *Gct* candidates will open up complementary and functional approaches to address the hypothesis that a unique ovarian follicle cohort is the GC population of origin for juvenile-type GC tumors.

The SWR.CAST-X congenic strain and SWR.CAST-X_1 subline have supported our goal to fine-map the X-linked *Gct4* locus that functions as a modifier for GC tumor initiation events. Within the 1.345 Mb interval for *Gct4*, the *Ar* gene remains the primary candidate for shared identity with *Gct4* based on the testosterone sensitivity of the phenotype in SWR female mice. We have previously examined the *Ar* gene for coding polymorphisms between SWR, SJL and CAST strains, but this did not provide a clear association with their phenotypic effects, since polymorphisms were identified only in the CAST strain.[14] Further support for the *Ar* gene sharing identity with *Gct4* comes from our quantitative gene expression analysis between SWR and SWR.

Table 2. Candidate genes within the *Gct4* locus interval

	Gene symbol	Description	Ensembl ID (ENSMUSG000000)	Start location (Mb)	Gene type
1	*Ar*	Androgen receptor	46532	98.149	Protein coding
2	*Ophn1*	Oligophrenin 1	31214	98.554	Protein coding
3	*U6*	Novel snRNA	88616	98.814	ncRNA
4	*Yipf6*	Yip1 domain family, member 6	47694	98.936	Protein coding
5	*Stard8*	Start domain containing 8	31216	99.003	Protein coding

SJL-X_5 female ovaries. There is a modest but statistically significant increase in *Ar* expression in the SWR.SJL-X_5 ovary compared with the SWR ovary. We hypothesize a unique regulatory variant of the *Ar* gene exists in the SWR strain that reduces Ar protein production, such that exogenous androgen administration overcomes this difference and equalizes trait penetrance with SJL or CAST modifier alleles. The Ar protein is a known regulator of ovarian follicle maturation over the reproductive lifespan of the mouse.[21] Our model suggests Ar plays a similar role in the unique follicle cohort that has the potential for GC tumor initiation in SWR female mice. We analyzed the *Ar* expression pattern in a minimum of five females per strain and noted very consistent expression profiles, suggesting that intra-strain variation in *Ar* expression is not the primary contributor to the stochastic nature of GC tumor initation in this model system.

The *Ar* gene itself is not essential for life, and there are many variations in the coding sequence evident across human populations; some of these polymorphisms debilitate Ar's biological functions leading to clinical signs of androgen insensitivity syndrome (AIS) in males.[22] We have not observed any signs of AIS, aberrant male development or loss of fertility in SWR inbred strain males, suggesting that genetic polymorphisms leading to a modest reduction in the expression level of *Ar* does not measurably influence male development or fertility. Conversely, the *Ar* expression difference may be sex-specific and epigenetically regulated in SWR female ovaries harboring two copies of the *Ar* gene on Chr X; however, there is no evidence to date that the *Ar* gene escapes X-inactivation in mice. We are pursuing the genetic explanation as the primary hypothesis for strain-specific allelic differences at the level of transcriptional regulation, with complete sequence analysis of the *Ar* locus in the SWR strain.

The *Gct6* locus was an unexpected discovery that arose from our congenic mapping process and poses a good example of a functional epistatic interaction in vivo. The CAST allele at *Gct6* plays a tumor suppressor role in this model of reproductive cancer that is dependent upon endrocrine signaling. The SWR and SJL alleles for *Gct6* were essentially invisible in prior mapping strategies, as they had no differential modifying effect on the GC tumor incidence phenotype, nor did they behave as tumor suppressor loci. The $Gct6^{CA}$ allele suppresses the action of both DHEA and testosterone in female mice that carry the autosomal susceptibility alleles for tumor initiation; this suggests the $Gct6^{CA}$ allele interferes with the oncogenic mechanism triggered by the DHEA-responsive $Gct1^{SW}$ locus on Chr 4, or suppresses androgen signaling through Ar. The potential to identify a novel suppressor of androgenic hormone signaling based on $Gct6^{CA}$ identity may have direct benefit for GC tumor prevention or therapeutic targeting of other androgen-sensitive cancers.

The paternal effect for the X-linked GC tumor susceptibility alleles has not yet been mechanistically explained. When considering phenotypes controlled by Chr X in females, the process of Chr X inactivation must be considered. The X-inactivation center (*XIC*) at ≈100 Mb on mouse Chr X can exhibit strain-specific bias for which Chr maintains transcriptional activity (X_A) vs. the Chr that is functionally silenced (X_I). The CAST strain has a strong allele compared with other inbred strain alleles in the context of X_A bias.[23] The SWR.CAST-X and SWR.CAST-X_I lines include the *XIC* locus in the CAST congenic segment; however, bias in the X-inactivation ratios does not explain the paternal effect observed for the SWR.SJL-X_5 congenic line, which carries a relatively small piece of the SJL-derived Chr X that excludes the *XIC*.[14] Progress toward understanding this epigenetic phenomenon will proceed in parallel with the identification of candidate gene sequence polymorphisms that support allele-specific gene expression assays to address whether or not the ovary, or a specific subset of ovarian follicles, exhibit imprinted or biased expression of candidate GC tumor susceptibility genes. The paternal mode of transmission may or may not be relevant to the human condition, since there is significant variability at the level of epigenetic regulation for Chr X between mice and humans; for example, human female cells are estimated to have 15% of the X-linked genes escape X inactivation compared with 3% of the genes in female mice.[24] As the GC tumor susceptibility gene identities are translated to human cases, the significance of the mode of transmission will be further investigated for similar parent-of-origin effects.

In summary, we have identified two modifier genes on Chr X that impact juvenile-type GC tumor development in female mice. In search of a modifier locus for GC tumor susceptibility, the mapping resolution achieved for the *Gct4* locus has reduced the genetic interval with particular emphasis on the *Ar* gene as the primary candidate based on endocrine responsiveness and differential expression of strain-specific *Ar* alleles. The identification and validation of both *Gct4* and *Gct6* susceptibility genes in the mouse will provide a foundation for the pursuit of genetic and epigenetic features that contribute to pediatric cases of juvenile-type GC tumors.

Methods

Mouse housing and nutrition. This investigation proceeded across two institutions: The Jackson Laboratory (TJL) and Memorial University of Newfoundland (MUN). For *Gct* locus

mapping and progeny tests, SWR inbred, SWR.SJL-X_3, SWR.SJL-X_5, SWR.CAST-X and SWR.CAST-X_1 congenic mice were maintained in a research colony at TJL, housed under 14:10 h light/dark cycles with pasteurized NIH-31 diet (Purina Mills Intl.) plus HCL-acidified water (pH 2.8–3.2) ad libitum. Following an institutional move (AMD), the strains were transferred to a specific pathogen free barrier facility in the Faculty of Medicine at MUN. Mice were subsequently housed under 12:12 h light/dark cycles and provided Laboratory Autoclavable Rodent Diet 5010 food (Purina Mills Intl.) and autoclaved water ad libitum. Spontaneous and androgen-induced GC tumor susceptibility was measured and found comparable at the new facility before molecular analyses were performed.

Females were weaned at 20 to 23 d of age and housed in groups of 2 to 5 animals per cage in 27.9 cm (L) × 17.8 cm (W) × 12.7 cm (H) rodent cages with high-profile filtered lids, containing sterilized White pine shavings (TJL), or sterilized Bed-O-Cobs® corn-cob bedding material (MUN) (The Andersons). All animal procedures performed at each institution were approved by their respective Animal Care and Use Committees.

Hormonal interventions in SWR.CAST-X female mice. We have previously reported the development of a ten-generation (N_{10}) backcross congenic strain, SWR.CAST-X, that maintains an SWR strain autosomal background but carries a large, homozygous segment of the CAST genome on Chr X.[14] The CAST donor segment is 72.7 Mb from *DXMit109* to *DXMit35*, inclusive of the *Gct4* locus positioned near 95 Mb and the *Gct6* locus that lies ≈38 Mb distal to *Gct4*. Based on the observation of over 300 homozygous, young adult or aged breeding female mice, SWR.CAST-X females are resistant to spontaneous GC tumor development, despite the presence of fundamental GC tumor susceptibility alleles derived from SWR at Chr 4 (*Gct1*) and other autosomal loci linked to the phenotype. To test the extent of GC tumor resistance in the presence of androgenic challenge, SWR.CAST-X females homozygous for the CAST-X segment, or $N_{11}F_1$ generation females derived from reciprocal matings between SWR and SWR.CAST-X, were administered DHEA or testosterone (Steraloids Inc.). Androgens were administered to pre-pubertal females (age 20 to 24 d) in the form of a 1.0 cm steroid-powder filled capsule made from Silastic tubing (1.98 mm inner diameter × 3.18 mm outer diameter) (Dow Corning) capped with glass beads. Capsules were implanted subcutaneously on the back at the time of weaning under isoflurane anesthesia (Baxter Corporation), closed with stainless steel wound clips and treated post-operatively with carprofen (5 mg/kg BW) analgesic (Pfizer). For phenotypic examination, female mice were necropsied at 8 weeks of age and ovaries were examined for GC tumors. GC tumors present as unilateral or bilateral, cystic or solid hemorrhagic masses that are macroscopically identifiable (**Fig. 1**). Females with either unilateral or bilateral GC tumors were counted as one affected animal for statistical purposes.

Breeding strategies for the development of Chr X recombinant males. A GC tumor suppressor allele was first identified during a recombinant male progeny test strategy that was designed to map the robust tumor support conferred by the *Gct4*^{J} allele.[14] The first round of progeny testing revealed that *Gct4*^{J} and *Gct4*^{CA} had equivalent tumor modifier activity, and that a unique allele more distal on Chr X had tumor suppressor activity (*Gct6*^{CA}) when compared with the tumor permissive action of *Gct6*^{SW} or *Gct6*^{J}. To improve upon the 25 Mb mapping resolution for *Gct4* with the polymorphic potential of the SWR.CAST-X (*Gct4*^{CA}; *Gct6*^{CA}) congenic line, we recognized the recombinant male mapping strategy would fail if the *Gct6*^{CA} resistance locus inhibited GC tumor development, thereby masking the effect of the *Gct4* modifier action. We first set out to reduce the CAST-X congenic segment, creating one homozygous subcongenic line that re-instated tumor-permissive SWR alleles on distal Chr X at the *Gct6* locus. Following a similar N_2F_1 cross between SWR.CAST-X females and SWR males, a unique recombinant male was chosen to establish a new subline, SWR.CAST-X_1, that maintains CAST genome from *DXMit109* to *DXMit149* (124.5 Mb) and re-instates SWR genome on the more distal portion of Chr X. Of note, the founder male for SWR.CAST-X_1 was part of the first male progeny testing strategy, and the *Gct4*^{CA}*; Gct6*^{SW} combination induced 31% spontaneous GC tumor incidence in F_1 daughter generation offspring with SWR dams (n = 77) (reported as Male #10 in ref. 14). The robustness of the paternal, parent-of-origin effect for the *Gct4*^{CA} allele was tested with a measure of the spontaneous GC tumor incidence in a minimum of 50 females in the SWR.CAST-X_1 homozygous condition, and following reciprocal matings between SWR and SWR.CAST-X_1 breeders. Once the paternal parent-of-origin effect was confirmed with reciprocal crosses, the SWR.CAST-X_1 homozygous congenic subline was used to generate males with unique recombinations between SWR and the CAST-X_1 segment between *DXMit45* to *DXMit170*, the interval boundaries for *Gct4* determined prior with the SWR.SJL-X(n) congenic series.[14] Selected males with unique genetic recombinations across the *Gct4* interval were mated with SWR dams to determine the GC tumor incidence in F_1 generation daughters.

Genotyping protocol for recombinant male selection. All males with potential Chr X recombinations were genotyped prior to selection for progeny testing. Genomic DNA was isolated from 1–2 mm tail tip biopsies using a sodium hydroxide (NaOH) extraction procedure: tail tips were submerged in 500 μl of NaOH (50 mM) and heated at 95°C for 10 min, neutralized with 50 μl of TRIS-HCl (1M, pH 8.0), centrifuged at 13,000 rpm for 5 min and the supernatant transferred to a clean microcentrifuge tube. The DNA was PCR amplified for simple sequence length polymorphic (SSLP) markers around *Gct4* using a MasterTaq Kit (5 PRIME Inc.). Each 10 μL PCR reaction contained 5.55 μL of distilled water, 2 μL of 5× TaqMaster PCR enhancer heated to 65°C, 1 μL of 10X reaction buffer, 0.2 μL of 10 mM dNTPs (Life Technologies), 0.2 μL each of 10 μM forward and reverse primers (Integrated DNA Technologies), 0.05 μL of Taq DNA polymerase, and 1 μL of DNA template. PCR conditions were as follows: 97°C for 30 sec; 39 cycles of 94°C for 15 sec, 55°C for 30 sec, and 72°C for 30 sec; 72°C for 10 min; 4°C hold. Informative DNA markers were downloaded from the Mouse Genome Database (*DXMit114, DXMit96* and *DXMit41)*.[14,25] Custom DNA markers were also designed in-house around novel

SSLPs between CAST and SWR alleles in the region of *Gct4* (**Table S1**). SSLP PCR products were separated by horizontal electrophoresis in 4% MetaPhor agarose slab gels (Lonza), 1× Tris-Boric acid-EDTA buffer at 100 V for 3 h. The bands were visualized by ethidium bromide staining and imaged for allele scoring purposes with a U-Genius imaging system (Syngene). A restriction fragment polymorphism (RFLP) assay for the *Ar* gene informative for CAST and SWR allele distinction was performed as previously described.[14]

Male progeny testing and spontaneous GC tumor phenotyping. Males with unique recombinations around the *Gct4* locus were selected for progeny testing with multiple SWR dams to collect a minimum cohort of 50 F_1 generation daughter offspring for GC tumor phenotyping. At 8 weeks of age, F_1 daughter generation females were euthanized with carbon dioxide, necropsied and scored for spontaneous GC tumor development following macroscopic examination of the ovaries.

Quantitative PCR of the *Ar* gene. Mouse ovaries were collected from six SWR and five SWR.SJL-X_5 females at the age of 4 weeks for quantification of *Ar* transcript relative to the *Actb* gene. Two ovaries were pooled to create one RNA sample per female. Ovaries were collected and homogenized in RNase free tubes containing Trizol reagent (Life Technologies) and ceramic beads (Precellys, 1.4 mm) with two 15 sec pulses at 5,000 rpm on a Precellys 24 Dual Tissue Homogenizer (Bertin Technologies). Total RNA was isolated using the Trizol reagent protocol. Extracted RNA was purified using RNeasy MinElute Cleanup kit after DNase I (Qiagen) digestion according to the manufacturer's instructions, followed by storage at -80°C. All RNA samples used for downstream qPCR had 260/280 ratios between 2.02 and 2.07, and RNA integrity was visualized on 1% horizontal agarose gels. cDNA was synthesized from total RNA using the RETROscript kit (MMLV reverse transcriptase) with random decamers (Ambion). qPCR was performed using Power SYBR Green Mastermix (Applied Biosystems) on an Applied Biosystems 7900HT Real-Time PCR System. The *Ar* oligonucleotide primers (5'-TGT CAC TAC GGA GCT CTC ACT TGT; 5'-AAT CGT TTC TGC TGG CAC ATA GA) and *Actb* primers (5'-GGC TGT ATT CCC CTC CAT CG; 5'-CCA GTT GGT AAC AAT GCC ATG T) amplified 97 bp and 154 bp PCR products, respectively, that spanned the Exon 2-Exon 3 juncture of their corresponding cDNA targets. A minimum of six standard curves with correlation coefficients ≥ 0.993 were used to determine the average efficiency of the *Ar* (94.25%) and *Actb* (94.88%) PCR reactions. *Ar* amplification was normalized to *Actb* using the Pfaffl method and is represented as fold change of the SWR.SJL-X_5 ovaries (numerator) relative to the SWR ovaries (denominator).[26]

Statistical analysis. Spontaneous GC tumor incidence in female mice that possess an SWR autosomal complement and are homozygous for the *Gct4* allele from the SJL strain (*Gct4*^{J}) exhibit ≈20% spontaneous GC tumor incidence. Comparing this incidence to the low penetrance of SWR inbred females (≤ 1%) or a tumor resistant phenotype (0%), we determined a minimum sample size of n = 50 female progeny per recombinant male was required to provide sufficient statistical power. Individual comparisons of GC tumor incidence per paternal haplotype were made to the expected incidence of the *Gct4*^{CA}/*Gct6*^{SW} paternal contribution for *Gct4* mapping, or the *Gct4*^{CA}/*Gct6*^{J} contribution for *Gct6* mapping, using a Chi-Square analysis for proportions with a significance level of $p < 0.05$. A one-way t-test relative to the ratio of 1.0 was used to analyze the qPCR gene expression ratios. All statistical analyses were performed with Prism version 5.00b software (GraphPad).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Materials

Supplemental materials may be found here:
www.landesbioscience.com/journals/epigenetics/article/23399

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