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14. ABSTRACT

Prolonged exposure to estrogens is considered a major risk factor for development of breast cancer. When treated with the naturally occurring estrogen, 17β-estradiol, ACI rats develop mammary cancers in over 90% of the population at risk. Genetic crosses between the susceptible ACI rat and resistant Copenhagen (COP) or Brown Norway (BN) rats identified a region on chromosome 5 (Emca1) that modified the development of estrogen-induced mammary cancer. The purpose of this research is to determine the role of Emca1 in the development of mammary cancer. To define the role of Emca1 in the development of mammary cancer, a congenic line was developed (ACI.BN-Emca1) in which the resistant BN allele of Emca1 was introgressed onto an ACI background. To determine the number of modifiers that reside within Emca1, four additional congenic lines that encompass overlapping regions of RNO5 within the Emca1 region were developed. Characterization of these congenic lines indicated that at least three modifiers of estrogen-induced mammary cancer reside within Emca1. Analysis of microarray data identified ten genes that may modify estrogen-induced mammary cancer within Emca1.

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ACI rat, estrogen, estradiol, cell proliferation, mammary cancer

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Table of Contents

INTRODUCTION: Prolonged exposure to estrogens is considered a major risk factor for development of breast cancer. When treated with the naturally occurring estrogen, 17β-estradiol, ACI rats develop mammary cancers in over 90% of the population at risk. Genetic crosses between the susceptible ACI rat and resistant Copenhagen (COP) or Brown Norway (BN) rats identified a region on chromosome 5 (*Emca1*) that modified the development of estrogen-induced mammary cancer. The purpose of this research is to determine the role of *Emca1* in the development of mammary cancer. To define the role of *Emca1* in the development of mammary cancer, a congenic line was developed (ACI.BN-*Emca1*) in which the resistant BN allele of *Emca1* was introgressed onto an ACI background. To determine the number of modifiers that reside within *Emca1*, four additional congenic lines that encompass overlapping regions of RNO5 within the *Emca1* region were developed. Characterization of the congenic lines indicated that at least three modifiers of estrogen-induced mammary cancer reside within *Emca1*. Analysis of microarray data identified ten genes that may modify estrogen-induced mammary cancer within *Emca1*.

BODY: The following accomplishments are documented according to the approved statement of work:

Task 1: **Evaluate the impact of *Emca1* on the development of estrogen-induced mammary cancer.**

This task was completed in the second year of the grant.

•**Results**: ACI.BN-*Emca1* (*Emca1*) rats exhibited increased latency to the development of estrogen-induced mammary cancer and decreased incidence in the population at risk (Figure 1A). In addition, *Emca1* rats developed significantly fewer mammary tumors than ACI rats (Figure 1B). However, there was no difference in the gross appearance of the mammary tissue from ACI and *Emca1* rats following 12 weeks of estrogen treatment (Figure 2), suggesting that *Emca1* does not affect the proliferative response of the mammary gland to estrogen. Instead, *Emca1* appears to delay the transformation of mammary hyperplasia to mammary cancer.

•**Status**: Completed

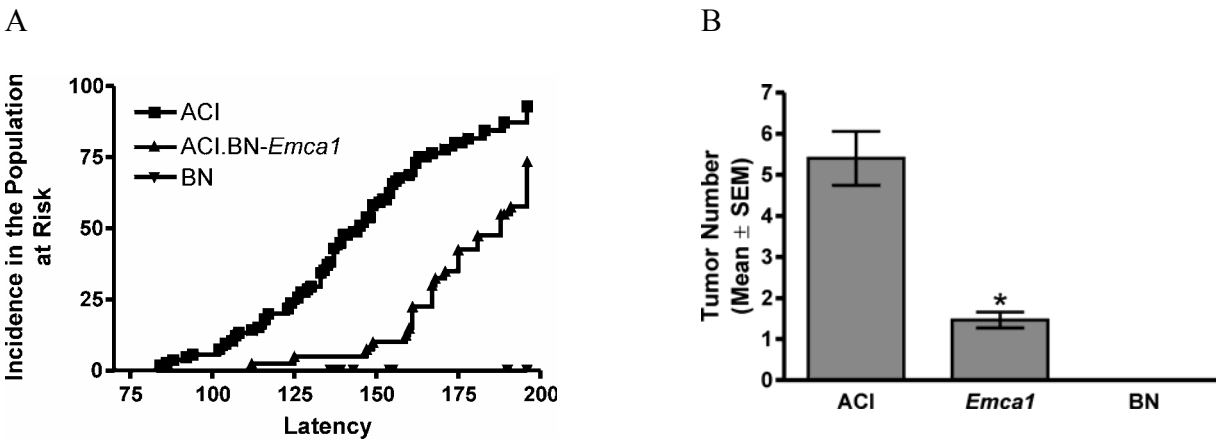

Figure 1. Impact of *Emca1* on estrogen-induced mammary cancer. ACI, BN and ACI.BN-*Emca1* rats were treated with estrogen beginning at nine weeks of age. Rats

were sacrificed following 28 weeks of estrogen treatment or when necessary due to treatment related morbidity. The number of tumors was determined at necropsy. A. Kaplan-Meier survival analysis of ACI rats (105) and BN (41) rats treated as part of this project and previous experiments and *Emca1* rats (39). B. Tumor number at necropsy for ACI (77), *Emca1* (39) and BN (50) rats that were sacrificed at or prior to 196 days of E2 treatment.

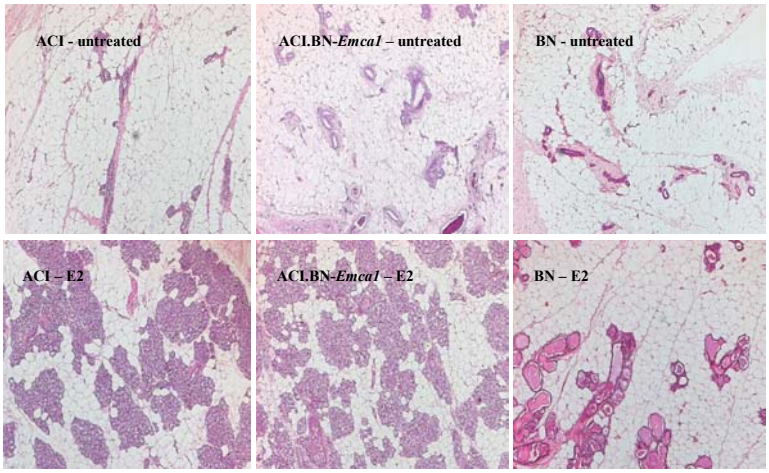

Figure 2. Rat strain-specific responsiveness of mammary tissue to 12 weeks of E2 treatment. The upper panel shows sham treated mammary tissue from ACI, *Emca1*, and BN female rats. The bottom panel shows 12 week estrogen-treated mammary tissue from the corresponding strain of female rats.

Task 2: Establish more precisely the location of the genes that confer and/or modify susceptibility to estrogen-induced mammary cancer.

Because additional genotyping and statistical analysis did not provide insight into regions on chromosome 5 that could modify estrogen-induced mammary cancer, these analyses were not utilized to more precisely define the genes within the *Emca1* locus that modify susceptibility to estrogen-induced mammary cancer. Instead, microarray analysis of 12 week estrogen-treated mammary tissue was utilized to determine changes in gene expression and to identify genes within the *Emca1* locus that are differentially regulated by estrogen. During this reporting period, microarray data were analyzed using Gene Chip Operating Software (GCOS) and dCHIP software.

•**Experimental Design**: Total RNA was isolated from approximately 40 mg of 12 week E2-treated mammary tissue from ACI (5) and *Emca1*(5) female rats using the Absolutely RNA miniprep kit from Stratagene. Two micrograms of RNA was labeled and hybridized to the Affymetrix Rat 230 2.0 microarray by personnel in the UNMC Microarray Core Facility.

•**Results**: GCOS was used to assess the data quality. Data from all five ACI and *Emca1* microarray data sets were within the recommended quality control parameters. dChip was used to normalize all arrays and to identify differentially expressed genes. Genes were identified as differentially expressed if 1) the fold change was ≥ 1.5; 2) the absolute difference of the group means was ≥ 100; 3) the T-test P value was ≤ 0.05; and 4) the gene was present in at least 20% of the samples. Data sets were also selected in which genes were present in one group of samples and absent in the other group of samples. Table 1 summarizes the changes in gene expression identified in these comparisons.

Table 1. Summary of changes in gene and EST expression in 12 week E2-treated mammary tissue.

Comparison	# Changes	# on Chromosome5

ACI vs Emca1		
Changed 1.5 fold	40	11
Present ACI, absent Emca1	10	4
Absent ACI, present Emca1	0	0

Of the forty genes differentially expressed, eleven of the genes resided on RNO5 within the *Emca1* region. Ten additional genes were considered present in the ACI and absent in the *Emca1*. Of these ten genes, four resided on RNO5 within the *Emca1* region. A list of these 15 genes is included Table 2. These analyses indicate that 1) genes within *Emca1* are differentially expressed after 12 weeks of estrogen treatment; and 2) the *Emca1* region on RNO5 modifies the expression of other genes outside the congenic interval. Thus, these analyses provide important tools to identify genes, both globally and within *Emca1*, that are differentially expressed as a result of estrogen treatment. Quantitative real-time PCR is underway to verify the differential expression of five selected genes.

Table2. Genes differentially expressed in *Emca1* compared to ACI following 12 weeks of E2 treatment.

Gene Symbol	Gene Description	RNO5 location (Mb)	Mean Expression ACI	Mean Expression Emca1	Fold Change	Within Congenic Subline
Changed 1.5 fold						
--	Transcribed sequence with moderate similarity to protein pdb:1LBG (E. coli) B Chain B, Lactose Opero	21948815-21949253	416.33	194.32	-2.14	---
--	Similar to carbonic anhydrase VIII; CA-related protein; carbonic anhydrase-related protein; carbonic	21951217-21951671	666.66	262.62	-2.54	---
--	Transcribed sequences	23458632-23459020	406.98	217.5	-1.87	---
--	Transcribed sequences	40707066-40707434	210.28	33.03	-6.37	---
--	Transcribed sequence with strong similarity to protein sp:O95477 (H.sapiens) ABC1_HUMAN ATP-binding	70495509-70496084	396.46	902.35	2.28	*Emca1a*
--	Transcribed sequences	106242158-106242826	89.34	230.5	2.58	*Emca1b*
Pde4b	phosphodiesterase 4B	123356922-123538838	351.95	177.9	-1.98	*Emca1b*
--	Similar to 2610027C15Rik protein (LOC362597), mRNA	145514197-145514715	1569.69	623.36	-2.52	*Emca1c*
--	Similar to CD2-associated protein (LOC362598), mRNA	145517809-145518662	305.6	54.97	-5.56	*Emca1c*
--	Zinc finger protein (pMLZ-4) mRNA, 3' untranslated region	155048406-155049967	370	190.51	-1.94	*Emca1c*
Tas1r2	taste receptor, type 1, member 2	158463161-158463867	296.49	152.43	-1.95	*Emca1c*

Gene Symbol	Gene Description	RNO5 location (Mb)	Within Congenic

			Subline
Present in ACI and absent in Emca1			
--	Transcribed sequences	61462857-61463366	*Emca1a*, *Emca1b*
--	Similar to nbla10363 (LOC298065), mRNA	65123670-65124140	*Emca1a*, *Emca1b*
--	Similar to hypothetical protein D4Ertd765e (LOC298381), mRNA	129286647-129289467	*Emca1b*
--	Transcribed sequences	146781125-146781543	*Emca1c*

• **Status**: This task is completed.

Task 3: Characterize additional congenic lines carrying specific intervals of *Emca1* to determine the effect of genotype on susceptibility to estrogen-induced mammary cancer.

- Analysis of the pattern of LRS peaks during the progression of mammary identified three potential regions of interest on RNO5. In addition, *cdkn2a* was identified as a potential candidate because the gene is located at the peak LRS value shown in Figure 3 and a congenic line was developed that contained approximately 8 million base pairs (Mb) of BN RNO5 introgressed onto an ACI background. During this reporting period, the impact of estrogen-induced mammary cancer was characterized in these four additional congenic lines.

• **Experimental Design**: Treat females from the four *Emca1* congenic sublines (*Emca1a*, *Emca1b*, *Emca1c*, and *Emca1-p16* (Figure 3) with estrogen for 28 weeks beginning at nine weeks of age.

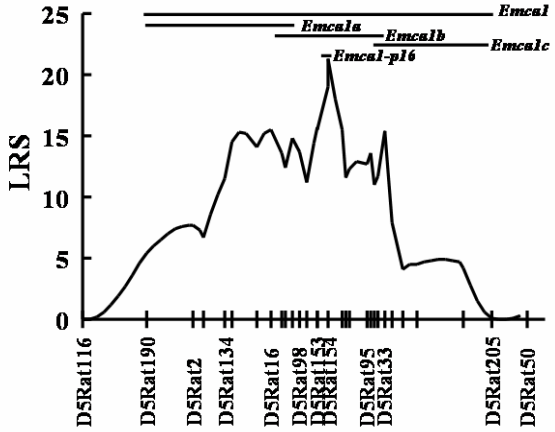

Figure 3. Boundaries of the four *Emca1* congenic sublines. The *Emca1* locus has been divided and multiple congenic lines developed to better define the region of interest on RNO5. Lines represent the region on RNO5 encompassed by the congenic sublines.

• **Results**: The four additional congenic lines exhibited a significant increase in latency to the development of mammary cancer (Figure 4) compared to ACI rats, but were not significantly different from the *Emca1* rats. In addition, *Emca1b* and *Emca1c* rats also exhibited a decreased incidence of mammary cancer in the population at risk that was similar to *Emca1* rats.

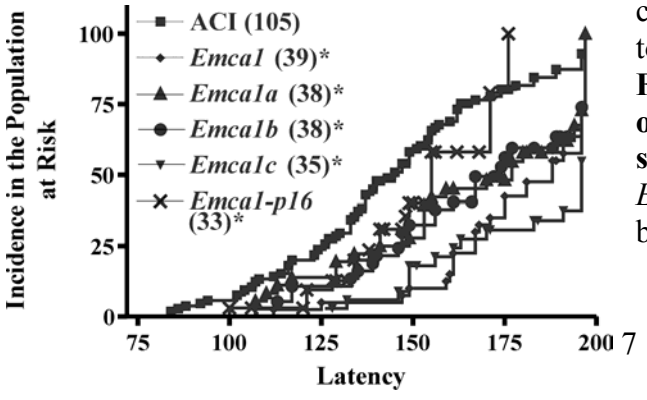

Figure 4. Impact of estrogen on development of mammary cancer in *Emca1* congenic sublines. ACI, *Emca1a*, *Emca1b*, *Emca1c* and *Emca1-p16* rats were treated with estrogen beginning at nine weeks of age. Rats were

sacrificed following 28 weeks of estrogen treatment or when necessary due to treatment related morbidity. Kaplan-Meier survival analysis of the *Emca1* congenic sublines. The combined ACI and *Emca1* survival curve are depicted for comparison purposes. * Indicates a significant difference in the congenic lines compared to the ACI.

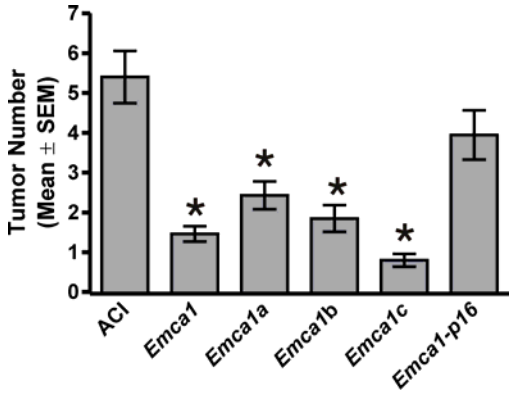

Figure 5. Impact of estrogen on the number of mammary cancers in *Emca1* congenic sublines. The number of tumors at necropsy was analyzed for the rats in Figure 4 above. Rats treated with estrogen for less than 196 days were not included in this analysis. Rats treated with estrogen for 196 days that were mammary cancer negative at necropsy were given a tumor number of zero. Mean mammary cancers ± the standard error of mean (SEM) at necropsy for ACI (77), *Emca1* (39), *Emca1a* (28), *Emca1b* (33), *Emca1c* (30) and *Emca1-p16* (18) rats is shown. * Indicates a significant difference in the congenic lines

The number of mammary cancers per rat in the *Emca1a*, *Emca1b*, and *Emca1c* congenic lines differed significantly from the number of mammary cancers in the ACI rat, but did not differ significantly from the number of mammary cancers in the *Emca1* rats. Although the *Emca1-p16* rats exhibited a significant decrease in the latency to the development of mammary cancers, the average number of mammary cancers per rat was significantly increased compared to the *Emca1*, *Emca1a*, *Ecma1b* and *Emca1c* rats, but did not differ significantly from the parental ACI rats, indicating that this region on RNO5 delays the development of mammary cancers, but does not impact mammary cancer multiplicity. When treated with estrogen, ACI rats also develop pituitary hyperplasia commonly referred to as pituitary tumors. We have mapped the regions of the rat genome associated with estrogen-induced pituitary hyperplasia. Although RNO5 has not been associated with a pituitary phenotype in the ACI rats, the *Emca1-p16* rats exhibited an extreme pituitary phenotype that was not exhibited by the *Emca1*, *Emca1a*, *Emca1b* or *Emca1c* congenic lines, suggesting that this region of RNO5 could harbor a regulator of estrogen-induced pituitary growth that is suppressed in the other four congenic lines. These data indicate that at least two strong and one weak modifier of esstrogen-induced mammary cancer reside within the *Emca1* locus on RNO5.

• **Status**: This task is completed.

KEY RESEARCH ACCOMPLISHMENTS:

• Analysis of the proliferative response to estrogen of the *Emca1*, *Emca1a*, *Emca1b*, *Emca1c*, and *Emca1-p16* congenic lines.
• Analysis of changes in gene expression in the ACI.BN-*Emca1* congenic line compared to the parental ACI.
• Identification of 15 genes/ESTs that are differentially expressed in 12 week estrogen-treated mammary tissue of *Emca1* rats compared to ACI rats.

REPORTABLE OUTCOMES:

Schaffer, B.S., Lachel, C.M., Pennington, K.L., Murrin, C.R., Strecker, T.E., Tochacek, M., Gould, K.A., Meza, J.L, McComb, R.D., and Shull, J.D. Genetic Bases of Estrogen-Induced Tumorigenesis in the Rat: Mapping of Loci Controlling Susceptibility to Mammary Cancer in a Brown Norway X ACI Intercross. *Cancer Research* (in press).

Shull, J.D., Lachel, C.M., Strecker, T.E., Spady, T.J., Tochacek, M., Pennington, K.L., Murrin, C.R., Meza, J.L., Schaffer, B.S., Flood, L.A., Gould, K.A. Genetic bases of renal agenesis in the ACI rat: mapping of *Renag1* to chromosome 14. *Mammalian Genome* (in press).

Schaffer, B.S., Kurz, S.K., Pennington, K.L., Hansen, K., Tochacek, M., Adamovic, T., Levan, G., and Shull, J.D. Development and characterization of congenic rat strains to identify genes residing on rat chromosome 5 (RNO5) that determine susceptibility to 17β-estradiol-induced mammary cancer in the ACI rat. 2006 AACR 97th Annual Meeting

Schaffer, B.S., Tochacek, M., Pennington, K.L., Meza, J.L., and Shull, J.D. Evidence that *Emca8*, a locus on RNO5, harbors multiple determinants of susceptibility to E2-induced mammary cancer in the ACI rat. 2005 (Poster) Cold Spring Harbor Rat Genomics and Models.

Gould, K.A., Murrin, C.R., Flood, L.A., Pennington, K.L., Schaffer, B.S., Tochacek, M., McComb, R., Meza, J.L., Wendell, D., and Shull, J.D. Genetic mapping of *Eutr1*, a locus controlling E2-induced pyometritis in the Brown Norway rat, to RNO5. *Mammalian Genome*. 2005 Nov; 16(11): 854-864.

Schaffer, B.S., Tochacek, M., Pennington, K.L., Meza, J.L., and Shull, J.D. Evidence that *Emca1* is a genetic determinant of E2-induced mammary tumor incidence and tumor multiplicity in the ACI rat. Era of Hope – Department of Defense Breast Cancer Research Program Meeting. 2005 (Oral and Poster)

Gould, K.A., Tochacek, M., Schaffer, B.S., Reindl, T.M., Murrin, C.R., Lachel, C.M., VanderWoude, E.A., Pennington, K.L., Flood, L.A., Bynote, K.K., Meza, J.L., Newton, M.A. and Shull, J.D. Genetic Determination of Susceptibility to Estrogen-Induced Mammary Cancer in the ACI Rat: Mapping of *Emca1* and *Emca2* to Chromosomes 5 and 18. *Genetics.* 2004 Dec; 168(4):2113-25.

Strecker, T.E., Spady, T.J., Kaufman, A.E., Shen, F., McLaughlin, M.T., Pennington, K.L., Meza, J.L., Schaffer, B.S., Gould, K.A., and Shull, J.D. Genetic Bases of Estrogen-Induced Pituitary Tumorigenesis: Identification of Genetic Loci Determining Estrogen-Induced Pituitary Growth in Reciprocal Crosses between the ACI and Copenhagen Rat Strains. *Genetics*. 2005 Apr; 169(4):2189-97. .

Schaffer, B.S., McLaughlin, M.T., Tochacek, M., Pennington, K.L., Meza, J.L. and Shull, J.D. Confirmation of *Emca1*, a locus that modifies development of estrogen-induced mammary tumors, in the ACI.BN-*Emca1* congenic rat strain. XVth International Workshop on Genetic Systems in the Rat. 2004 (Oral).

Schaffer, B.S., McLaughlin, M.T., Tochacek, M., Pennington, K.L., Meza, J.L., McComb, R.D. and Shull, J.D. Characterization of estrogen-induced mammary cancer in the ACI.BN-*Emca1* congenic rat: evidence that *Emca1* inhibits mammary carcinoma. San Antonio Breast Cancer Symposium. 2004 (Poster).

CONCLUSIONS:

The *Emca1*, *Emca1a*, *Emca1b*, *Emca1c* and *Emca1-p16* congenic lines exhibited delayed latency to the development of estrogen-induced mammary cancer. The *Emca1*, *Emca1a*, *Emca1b* and *Emca1c* congenic lines also exhibited decreased incidence within the population at risk and decreased tumors per rat when compared with the susceptible ACI parental rats indicating that at least three modifiers of estrogen-induced mammary cancer reside within the *Emca1* locus on RNO5. Of the 15 genes that were differentially expressed between the *Emca1* and ACI rats, four genes did not reside within any of the *Emca1* sublines and are not potential candidate genes for the *Emca1* region on RNO5 (Table 2). Multiple candidate genes were identified for the *Emca1a*, *Emca1b* and *Emca1c* congenic sublines (Table 2). Because the regions of RNO5 encompassed by *Emca1a* and *Emca1c* do not overlap, these congenic lines must contain different modifiers of estrogen-induced mammary cancer. Potential candidate genes for *Emca1a* include a transcribed sequence with similarity to the human ATP binding cassette gene, a transcribed sequence similar to hypothetical protein D4Ertd765e, a sequence similar to nbla10363, and a transcribed sequence on which little is known. Potential candidates for *Emca1c* include a protein similar to CD2-associated protein, the zinc finger protein pMLZ-4, a taste receptor, a protein similar to 2610027C15Rik, and a transcribed sequence. The region of RNO5 that encompasses the *Emca1b* congenic line overlaps with *Emca1a*, *Emca1c* and may harbor both the *Emca1a* and *Emca1c* modifiers of E2-induced mammary cancer. However, the region of *Emca1b* that does not overlap with either *Emca1a* or *Emca1c* encompasses the *Pde4b* gene that has been shown to be overexpressed in breast cancer and is a strong candidate gene for estrogen-induced mammary cancer. Thus, it is possible that *Emca1a*, *Emca1b* and *Emca1c* each contain different modifiers of estrogen-induced mammary cancer. No gene that was differentially expressed resided within the *Emca1-p16* congenic line suggesting that an additional modifier(s) of estrogen-induced mammary cancer is yet to be identified. A search of genes resident on RNO5 using NCBI Map Viewer indicated that 64 genes or predicted genes reside within the *Emca1-p16* region between 106.29 and 114.80 Mb. Both the *cdkn2a* and *cdkn2b* genes, which reside within this region, were below the level of sensitivity of the microarray analysis and are considered good candidates for the *Emca1-p16* congenic line. Expression of these genes is being analyzed by another member of the lab and was not presented here. Preparation of a manuscript that describes these results is currently underway. In summary, we have shown that *Emca1* contains at least three modifiers of estrogen-induced mammary cancer. We have also identified potential candidate genes for *Emca1*, but additional research is required to verify the role of these genes in estrogen-induced mammary cancer in the ACI rat.