Squamous Cell Tumors in Mice Heterozygous for a Null Allele of \textit{Atp2a2}, Encoding the Sarco(endo)plasmic Reticulum \textit{Ca}^{2+}-ATPase Isoform 2 \textit{Ca}^{2+} Pump* 

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Lynne H. Liu‡, Gregory P. Boivin§, Vikram Prasad‡, Muthu Periasamy¶, and Gary E. Shull†

From the Departments of ‡Molecular Genetics, Biochemistry and Microbiology and ¶Pathology and Laboratory Medicine, University of Cincinnati College of Medicine, Cincinnati, Ohio 45267 and the §Department of Physiology and Cell Biology, Ohio State University College of Medicine and Public Health, Columbus, Ohio 43210

Mutations in the human \textit{Atp2a2} gene, encoding sarco(endo)plasmic reticulum \textit{Ca}^{2+}-ATPase isoform 2 (SERCA2), cause Darier disease, an autosomal dominant skin disease characterized by multiple keratotic papules in the seborrheic regions of the body. Mice with a single functional \textit{Atp2a2} allele (the mouse homolog of \textit{Atp2a2}) were shown previously to have reduced levels of SERCA2 in heart and mildly impaired cardiac contractility and relaxation. Here we show that aged heterozygous mutant (\textit{Atp2a2}+/−) mice develop squamous cell tumors of the forestomach, esophagus, oral mucosa, tongue, and skin. Squamous cell tumors occurred in 13/14 \textit{Atp2a2}+/− mice but were not observed in age- and sex-matched wild-type controls. Hyperkeratinized squamous cell papillomas and carcinomas of the upper digestive tract were the most frequent finding among \textit{Atp2a2}+/− mice, and many animals had multiple tumors. Western blot analyses showed that SERCA2 protein levels were reduced in skin and other affected tissues of heterozygous mice. The development of squamous cell tumors in aged \textit{Atp2a2}+/− mice indicates that SERCA2 haploinsufficiency predisposes murine keratinocytes to neoplasia. These findings provide the first direct demonstration that a perturbation of \textit{Ca}^{2+} homeostasis or signaling can serve as a primary initiating event in cancer.

Sequestration of \textit{Ca}^{2+} in intracellular storage organelles, from which it can be released during \textit{Ca}^{2+} signaling events, is mediated by the SERCA1 family of \textit{Ca}^{2+}-ATPases. SERCA2 (gene locus symbol, \textit{Atp2a2} for mouse and \textit{ATP2A2} for human), the most widely expressed SERCA isoform, has two C-terminal variants (1, 2). SERCA2a is expressed primarily in heart, where it mediates cardiac muscle relaxation and maintains sarcoplasmic reticulum \textit{Ca}^{2+} stores on a beat to beat basis; SERCA2b is expressed in all tissues and is thought to be the most endoplasmic reticulum \textit{Ca}^{2+} pump (3). Gene-targeting studies in mice have shown that at least one functional copy of the \textit{Atp2a2} gene is essential for survival and that the loss of a single allele causes reductions in SERCA2 mRNA and protein in heart (4). Despite some compensation via alterations in the levels and phosphorylation status of phospholamban, which regulates the pump, cardiac muscle contractility and relaxation were impaired, and intracellular \textit{Ca}^{2+} transients in cardiac myocytes were reduced (4, 5).

Darier disease, an autosomal dominant skin disorder in humans (6), has been shown to be because of null mutations in one copy of the \textit{ATP2A2} gene (7), demonstrating that SERCA2 haploinsufficiency in keratinocytes can cause disease. To determine whether SERCA2 haploinsufficiency would lead to disease in mice as they age, we conducted an aging study using wild-type and \textit{Atp2a2}−/− mice. Subsequent analyses revealed that heterozygous mutants have a very high incidence of squamous cell carcinomas and papillomas in keratinized epithelial cells, the same cell type affected in human Darier disease. Although previous studies have provided suggestive evidence that perturbations of intracellular \textit{Ca}^{2+} homeostasis or signaling may contribute to cancer (8, 9), the data presented in the current study establish the first direct link between altered \textit{Ca}^{2+} handling and neoplasia.

**EXPERIMENTAL PROCEDURES**

**Mice**—The \textit{Atp2a2} gene was targeted previously by removing the promoter and first two exons, which eliminated expression of the mutant gene (4). \textit{Atp2a2}−/− mice of a mixed background (50% 129/SvJ, 50% Black Swiss) were crossed to obtain wild-type and \textit{Atp2a2}+/− mice, and genotypes were determined by polymerase chain reaction analysis of tail DNA as described previously (4).

**Aging Study and Histology**—Sex-matched heterozygous and wild-type sibling pairs were aged, and animals exhibiting evidence of morbidity, such as wasting, open sores, or apparent tumors, were euthanized. At necropsy, the skin, nails, and major organs were examined for gross lesions or abnormalities. The mouth and tongue were not examined in the first four \textit{Atp2a2}−/− mice exhibiting disease symptoms but were included in the protocol after we became aware of oral lesions in Darier disease patients (6). The heart, esophagus, stomach, tongue, and any diseased tissues noted during necropsy were removed and examined histologically. Tissues were fixed in 10% neutral buffered formalin, dehydrated through a gradient of alcohols, embedded in paraffin, sectioned, and stained with hematoxylin and eosin.

**Western Blot Analyses**—Proteins in tissue homogenates were separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose, and relative SERCA2 levels were determined as described previously (4) using a polyclonal SERCA2 antibody (10) raised against a fusion protein encompassing amino acids 362 to 705 of rat SERCA2, which is common to both SERCA2a and SERCA2b (antibody N1; provided by Jonathan Lytton, University of Calgary). Skin samples were from newborn mice; tongue and forestromach were from 6-, 9-, and 14-month-old mice (one animal of each age).

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† To whom correspondence should be addressed: Dept. of Molecular Genetics, Biochemistry and Microbiology, University of Cincinnati College of Medicine, 231 Bethesda Ave., Cincinnati, Ohio 45267-0524. Tel.: 513-558-0056; Fax: 513-558-1885; E-mail: shullge@ucmail.uc.edu.

**2** The abbreviation used is: SERCA, sarco(endo)plasmic reticulum \textit{Ca}^{2+}-ATPase.
RESULTS

The original objective of this study was to determine whether the loss of one Atp2a2 allele, which impairs cardiac function in young adult mice, would cause heart disease in older animals. We changed our objective when we observed two deaths of unknown causes (autolysis precluded detailed analysis of tissues, although one mouse had a prolapsed penis) among mutant mice of an aging cohort of 16 experimental pairs. The remaining mice were monitored over time, and 12 of the 14 Atp2a2+/− mice were examined when disease symptoms were clearly apparent, at ages ranging from 53–81 weeks. The two mutants that did not exhibit overt disease symptoms during the course of the study were euthanized and examined at 89 weeks of age. The wild-type controls, most of which showed no evidence of disease symptoms, were euthanized 4–5 weeks after the last Atp2a2+/− mice; their ages ranged from 81 to 94 weeks.

As shown in Fig. 1, the viability of Atp2a2+/− mice was markedly decreased compared with that of wild-type mice. Indications of disease among the Atp2a2+/− mice included growths on the face, abdominal masses, prolapsed penis, lethargy, and loss of weight. The benign skin lesions characteristic of Darier disease in humans were not apparent in Atp2a2+/− mice but, as described below, the affected cell type, keratinocytes, was the same. Although mild fibrosis was observed in some of the hearts from diseased Atp2a2+/− mice, there were no major cardiac lesions or hypertrophy when compared with wild-type controls. As summarized in Table I, the major histopathological correlates of morbidity in Atp2a2+/− mice were hyperkeratinized squamous cell tumors of the stomach, esophagus, tongue, oral mucosa, and skin (13/14 mice; 93%), with an average of more than two tumors per mouse. Two Atp2a2+/− mice exhibited extreme hyperkeratosis of a toenail, and another heterozygote had a large cystic mass containing keratin and bordered by squamous epithelium.

Among the 16 wild-type controls, two males died of unknown causes (at 58 and 86 weeks), a 94-week-old male had a hepatoceular adenoma, a 91-week-old male had an adenocarcinoma of the lung and hyperplasia of the forestomach, and an 82-week-old female had a mammary carcinoma, a leiomyosarcoma of one uterine horn, a lymphoma, and an alveolar adenoma. Squamous cell tumors or evidence of hyperkeratosis or dyskeratosis of the tissues that were affected in heterozygous mutants were not observed in the wild-type mice.

Squamous cell tumors of the forestomach (non-glandular mucosa) and esophagus occurred in 10/14 (71%) Atp2a2+/− mice. Six animals had hyperkeratinized squamous cell carcinomas of the forestomach (Fig. 2, A–C). Although less common than carcinomas, squamous cell papillomas were also observed in forestomachs of Atp2a2+/− mice (Fig. 2D). Papillomas of the esophagus occurred in 7/14 mutant mice (Fig. 2E). Even in the absence of overt tumors of the stomach and esophagus, hyperplastic changes in the epithelial lining of the forestomach and esophagus were frequently observed (Table I). Squamous cell tumors were observed in the tongue or oral mucosa of 9/10 of the Atp2a2+/− mice in which the mouth was examined. Four mice had carcinomas of the tongue (Fig. 2, F and G), three had papillomas of the tongue (Fig. 2H), and two had carcinomas of the oral mucosa (cheek) (Fig. 2I). Squamous cell tumors of the skin were seen in 5/14 mice and included two with carcinomas of the face (Fig. 2, J and K), two with carcinomas of the penis (Fig. 2L), and one with a papilloma of the penis. We have observed additional squamous cell tumors in retired Atp2a2+/− breeders but not in their wild-type mates; tumors in locations that were not seen in the 14 mutants described in Table I included carcinomas of the esophagus, lip, palate, and the skin adjacent to the vagina, penis, and anus.

Western blot analysis of tissue homogenates from unaffected wild-type and Atp2a2+/− mice (Fig. 3) showed that SERCA2 protein levels were reduced in skin, tongue, and forestomach of the mutants (relative to wild-type levels: skin, 66 ± 5%; tongue, 80 ± 2%; forestomach, 82 ± 3%). These data demonstrate that:

![Table I](http://www.jbc.org/)

### Table I: Distribution of squamous cell tumors and hyperplasia in aged Atp2a2+/− mice

| Sex | Age (weeks) | Esophagus | Stomach | Tongue/oral mucosa | Skin/nails |
|-----|-------------|-----------|---------|-------------------|-----------|
| M   | 89          | Hyperplasia| Carcinoma| Tongue carcinoma   | Ingual area-keratinized cyst |
| M   | 81          | Papilloma  | Carcinoma| Tongue papilloma   | Penis hyperplasia |
| M   | 74          | Papilloma  | Papilloma| NE1               | Facial growth carcinoma |
| M   | 68          | Papilloma  | Carcinoma| NE                | NE         |
| F   | 65          | Hyperplasia| Carcinoma| Tongue carcinoma   | Penis papilloma |
| M   | 76          | Hyperplasia| Carcinoma/papilloma/ hyperplasia| Oral mucosa carcinoma | Penic carcinoma |
| M   | 66          | Papilloma  | Hyperplasia| Tongue papilloma   | Penic carcinoma |
| F   | 64          | Papilloma  | Hyperplasia| NE               | Tongue papilloma |
| M   | 58          | Papilloma  | Carcinoma| Tongue carcinoma   | Toenail hyperkeratosis, dyskeratosis, parakeratosis |
| M   | 53          | Papilloma  | Carcinoma| Oral mucosa carcinoma | Toenail carcinoma |
| M   | 77          | Papilloma  | Hyperplasia| Tongue carcinoma   | Toenail carcinoma |

NE, not examined.
the loss of one Atp2a2 allele causes a reduction in SERCA2 protein levels in these tissues, as observed previously in heart (4).

DISCUSSION

Our results demonstrate that the loss of one Atp2a2 allele in mice, which causes reduced expression of the SERCA2 Ca\(^{2+}\) pump, leads to squamous cell tumors of the skin, oral mucosa, esophagus, and forestomach. Most of these tumors are rare in normal mice; among 4900 mice analyzed in the National Toxicology Program, there were only 11 cases of squamous cell tumors of the skin, 4 cases of squamous cell tumors of the oral cavity (any site), and only one squamous cell tumor of the esophagus (11). In the current study, we observed 30 squamous cell tumors among 14 Atp2a2\(^{1/2}\) mice and none in the wild-type controls. The high predisposition of Atp2a2\(^{1/2}\) mice to the development of squamous cell tumors provides compelling evidence that a perturbation of intracellular Ca\(^{2+}\) homeostasis and/or signaling can lead to cancer.

Squamous cell carcinomas and papillomas are not a common feature of Darier disease, although a number of case histories have been reported (12–14), consistent with the possibility that SERCA2 haploinsufficiency may cause a low incidence of skin cancer in humans. Similarly, although we did not observe skin lesions in the mouse model that resembled the keratotic papules seen in Darier disease, we did observe hyperkeratotic nail abnormalities and a keratinized cyst (Table I), both of which are reminiscent of findings in Darier patients (6, 15). Further studies will be needed to gain a clearer understanding of the similarities and dissimilarities in disease phenotypes between the two species. In both mouse and human, lesions resulting from SERCA2 haploinsufficiency arise in keratinized squamous epithelial cells, suggesting that there are similarities in
the initial stages of disease in the two species. Although there are species differences in the location of the affected tissues, this is likely to be because of differences in the distribution of keratinized squamous epithelial cells. In mice, this includes the skin, oral mucosa, tongue, esophagus, and forestomach (16), whereas in humans, it includes the skin and oral palate, where Darier lesions have been observed (6). In both humans and mice, keratinocytes have been shown to be highly sensitive to perturbations of calcium homeostasis (22). Depletion of Ca\textsuperscript{2+} stores (23) or the use of a Ca\textsuperscript{2+} influx inhibitor, carboxyamido-triazole, which would appear to be effective in the treatment of some cancers (9, 25, 26). It is conceivable that it could be used in a topical treatment for Darier lesions has been shown (24) to cause increased Ca\textsuperscript{2+} homeostasis and signaling in keratinocytes that occur as an immediate consequence of the mutation and the subsequent changes, including additional genetic mutations, that lead to cancer. Important issues to be addressed in future studies are the genetic and cellular mechanisms of tumorigenesis. It is conceivable that the tumors arise from cells that have lost the remaining wild-type allele; however, given the critical function of SERCA2 in maintaining endoplasmic reticulum Ca\textsuperscript{2+} stores and the severe effect of SERCA2 haploinsufficiency in keratinocytes of Darier disease patients, it seems more likely that the perturbation of Ca\textsuperscript{2+} homeostasis causes an increased susceptibility to the accumulation of genetic changes at other loci. Some of the immediate consequences of a reduction in SERCA2 activity with respect to intracellular Ca\textsuperscript{2+} homeostasis and signaling are reasonably well understood (20). Partial inhibition of SERCA2 by exposure to thapsigargin, a tumor promoter in murine two-stage skin carcinogenesis (21), increases the frequency of intracellular Ca\textsuperscript{2+} spikes in some cells (22). This suggested that an impaired ability to buffer elevations in cytoplasmic Ca\textsuperscript{2+} by removing it from the cytosol may decrease the interspike interval by stimulating Ca\textsuperscript{2+} release from the endoplasmic reticulum (22). Depletion of Ca\textsuperscript{2+} stores by Ca\textsuperscript{2+} release during signaling events or by treatment with thapsigargin has been shown to cause increased Ca\textsuperscript{2+} influx across the plasma membrane (23, 24). Interestingly, a Ca\textsuperscript{2+} influx inhibitor, carboxyamido-triazole, which would appear to have the potential to correct some of the imbalances in Ca\textsuperscript{2+} homeostasis resulting from mutations in the Atp2a2 gene (or its human homolog in Darier disease), has been shown to be effective in the treatment of some cancers (9, 25, 26). It is conceivable that it could be used in a topical treatment for Darier disease. Ultimately, the increased cytosolic Ca\textsuperscript{2+} levels and excitability of Ca\textsuperscript{2+} signaling mechanisms occurring as a direct result of SERCA2 haploinsufficiency may cause perturbations in gene expression (24, 27), nuclear Ca\textsuperscript{2+} homeostasis (28), DNA repair (28, 29), and cell cycle regulation (30, 31), with the resulting genetic instability contributing to cancer.

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