Mice Lacking the Amplified in Breast Cancer 1/Steroid Receptor Coactivator-3 Are Resistant to Chemical Carcinogen–Induced Mammary Tumorigenesis

Shao-Qing Kuang, Lan Liao, Shu Wang, Daniel Medina, Bert W. O’Malley, and Jianming Xu

Department of Molecular and Cellular Biology, Baylor College of Medicine, Houston, Texas

Abstract
Amplified in breast cancer 1 (AIB1; steroid receptor coactivator-3, p/CIP, RAC3, ACTR, TRAM-1, or NCoA-3) is a transcriptional coactivator for nuclear receptors and certain other transcription factors and is a newly defined oncogene overexpressed in human breast cancer. Although the role and molecular mechanism of AIB1 in normal physiology and in breast cancer are currently under intensive investigation, the role of AIB1 in determination of the susceptibility of mammary gland to chemical carcinogens remains uncharacterized. In this study, we used back-crossed FVB wild-type (WT) and AIB1 mutant mice to assess the role of AIB1 in mammary gland development and in carcinogen-induced tumorigenesis. We show that mammary ductal growth was delayed in AIB1−/− mice with FVB strain background, and mammary ductal outgrowths emanating from the AIB1−/− mammary epithelial transplants in WT mice also were attenuated, indicating that the role of AIB1 in mammary ductal growth is a mammary epithelial autonomous function. In mice treated with the chemical carcinogen 7,12-dimethylbenz[a]anthracene (DMBA), AIB1 deficiency protected the mammary gland, but not the skin, from tumorigenesis. AIB1 deficiency suppressed the up-regulation of the insulin receptor substrate (IRS)-1 and IRS-2 and thereby inhibited the activation of Akt, expression of cyclin D1, and cell proliferation. The suppression of these components for insulin-like growth factor-1 signaling might be partially responsible for the decreased DMBA-induced mammary tumor initiation and progression in AIB1−/− mice. Our results suggest that AIB1 may serve as a potential target for prevention of carcinogen-induced breast cancer initiation and for treatment of breast cancer progression. (Cancer Res 2005; 65(17): 7993-8002)

Introduction
Amplified in breast cancer 1 (AIB1) belongs to the p160 steroid receptor coactivator (SRC) family that also contains SRC-1 and the transcription intermediary factor 2 (TIF2; also known as GRIP1 or SRC-2; refs. 1, 2). Biochemical and cellular transfection assays have shown that SRC coactivators interact with nuclear receptors, such as estrogen receptor (ER) and progesterone receptor (PR), and certain other transcription factors, such as activator protein-1, serum response factor, and nuclear factor-κB. SRC coactivators amplify the transcriptional activities of these transcription factors through recruiting chromatin modification enzymes to the promoter/enhancer regions of their target genes (1–6). These chromatin modification enzymes include acetyltransferases, such as CBP, p300, and P/CAF, and methyltransferases, such as CARM1 and PRMT1 (1, 2). As the cellular levels of these SRC coactivators are usually limiting in normal cells, the changes of their concentrations and activities are thought to play remarkable roles in the regulation of gene expression (2). Multiple lines of evidence have shown that the transcriptional activities of SRC coactivators are regulated by multiple mechanisms involving protein phosphorylation, acetylation, translocalization, and degradation, suggesting that these coactivators can serve as targets for multiple signaling pathways activated by hormones, growth factors, cytokines, ubiquitins, and xenobiotic agents to regulate gene expression (7–10).

In the SRC family, elevated expression of SRC-1 and AIB1 has been observed in human breast cancers. In normal human mammary epithelial cells, SRC-1 levels are very low (11). However, SRC-1 protein levels are elevated in 46% of HER-2/neu–positive, but only 6% of HER-2/neu–negative, breast cancers, indicating that overexpression of SRC-1 in breast cancer is associated with HER-2/neu oncogene expression. Furthermore, SRC-1 levels in breast cancers are positively associated with nodal positivity and resistance to endocrine therapy and are inversely associated with disease-free survival (11–13). The AIB1 gene is amplified in 5% to 10% breast cancers and overexpressed in 30% to 60% breast cancers (3, 14, 15). Interestingly, AIB1 amplification and overexpression were detected in both ER positive and negative breast cancers (14, 15). In tamoxifen-treated breast cancer patients, high levels of AIB1 protein were found associated with HER-2/neu expression, tamoxifen resistance, and poor disease-free survival (16). These clinical data suggest that SRC-1 and AIB1 play important roles in breast cancer. Indeed, recent studies have shown that transgenic expression of an active AIB1 isoform in mouse mammary epithelial cells increased mammary epithelial proliferation (17). Overexpression of the full-length AIB1 in mouse mammary epithelial cells led to mammary hyperplasia and the development of malignant mammary tumors, indicating that AIB1 overexpression is sufficient to initiate mammary gland oncogenesis (18).

We showed previously that AIB1 is expressed in the epithelial compartment of the mammary gland (19, 20). Inactivation of AIB1 in mice partially attenuated mammary ductal growth during puberty and lobular alveolar development in response to estrogen and progesterone treatment (19, 21). Recently, we also showed that AIB1 deficiency impairs the insulin-like growth factor-I (IGF-I) signaling pathway, inhibits cell proliferation and migration, and significantly suppresses the mouse mammary tumor virus (MMTV)-v-Ha-ras oncogene-induced mammary tumor initiation and progression (20). These results indicate that AIB1 plays a
critical role in the mammary tumorigenesis mediated by pathways involving the membrane-associated tyrosine kinase receptors and their downstream protein kinases. However, the role of AIB1 in mammary carcinogenesis induced by DNA mutagens, such as chemical carcinogens, is unclear. In this study, we used an established 7,12-dimethylbenz[a]-anthracene (DMBA)–induced mouse mammary tumor model to assess the in vivo role of AIB1 in the mammary carcinogenesis triggered by chemical carcinogen exposure. We show that inactivation of AIB1 in mice significantly delayed the latency and reduced the frequency and growth rate of the mammary tumors induced by DMBA. AIB1 deficiency attenuated the up-regulation of several components of the IGF-I/Akt/cyclin D1 signaling pathway, which might be responsible for the suppression of DMBA-induced mammary tumor development in AIB1−/− mice. The fact that inactivation of AIB1 suppresses the carcinogen-induced mammary tumorigenesis in mice suggests that AIB1 is a potential target for prevention and treatment of human breast cancer.

Materials and Methods

Mice. The targeted AIB1 mutant mice with a mixed 129SvEv and C57BL/6J strain background were described previously (19). These mice were backcrossed with wild-type (WT) FVB mice for seven generations before their offspring were interbred to produce WT, AIB1−/−, and AIB1+/− mice. The AIB1 genotype was analyzed by PCR as described previously (19). All animal protocols were approved by the Baylor College of Medicine Animal Care and Use Committee.

Transplantation of mammary epithelium and examination of mammary glands. Mammary gland epithelial transplantation was done as described.1 Briefly, the entire rudiment mammary ducts in 3-week-old small piece (inguinal fat pads from the nipple to the proximal mammary lymph node. A pituitary gland was removed from a male littermate and implanted under the kidney capsule of the recipient mouse. The mammary ductal growth was retarded in AIB1−/− mice, suggesting that AIB1 plays an important role in mammary gland development (19, 21). The AIB1 mutant mice were initially generated by breeding the chimeric mice derived from the AIB1-targeted embryonic stem cells with the 129SvEv strain background with WT C57BL/6J mice (19). Previous studies showed that mice with mixed 129SvEv and C57BL/6J genetic background were less sensitive to chemical carcinogen–induced DMBA treatment and examination of tumorigenesis. DMBA was purchased from Sigma (St. Louis, MO) and dissolved in cottonseed oil at a concentration of 5 mg/mL. To induce tumorigenesis in mice without pituitary isografts, 8-week-old female WT, AIB1−/−, and AIB1+/− mice were fed weekly by gastric intubation with 1 mg DMBA in 0.2 mL for 6 consecutive weeks. To induce tumorigenesis in mice under pituitary isograft stimulation, pituitaries from WT siblings were implanted under the right kidney capsule of female WT, AIB1−/−, and AIB1+/− recipient mice at 5 weeks of age. These recipient mice were fed weekly with 1 mg DMBA for 3 consecutive weeks starting at 8 weeks of age. Pituitaries remained in place for the duration of the experiment. All of the pituitary isografts were checked for viability after necropsy as described (23).

After DMBA treatment, mammary glands were examined in a time course to check mammary tumor initiation and progression by whole-mount staining and by sectioning the paraffin-embedded tissues and H&E staining as described previously (20, 24). Palpation for mammary tumors was carried out weekly for each DMBA-treated mouse. Once the initial palpable mammary tumors (~0.5 mm in diameter) were detected, the tumor length (L) and width (W) were measured once weekly. The tumor volume was calculated by (L × W²) / 2 as described (20, 24, 25). Occurrence of skin tumors also was recorded. Tumor-free curves were generated by using the Prism 3.0 software (GraphPad Software, San Diego, CA). Statistical differences were compared by either log-rank test for tumor-free curves, χ² test for tumor incidence, or unpaired t test for tumor volume. A significant difference was determined at P ≤ 0.05.

Mice were euthanized when the primary tumor reached 1.5 cm in diameter or when mice became moribund. The number of mammary tumors developed in each mouse was counted and recorded. Mammary tumors were collected for further histologic and molecular analysis. Ovaries were carefully examined for ovarian tumors.

RIA of estradiol and progesterone. Blood samples were collected from DMBA-treated mice bearing pituitary isografts at the time of sacrifice. Serum samples were prepared and stored at −80°C until assayed for 17β-estradiol and progesterone by using the DSI-4400 and DSI-3900 RIA kits (Diagnostic Systems Laboratories, Webster, TX).

Immunohistochemistry. Immunohistochemistry was done on deparaffinized and rehydrated mammary tissue sections prepared after 4% paraformaldehyde–fixation and paraffin embedding (20, 26, 27). Primary antibodies against IGF-I receptor (IGF-IR), AIB1, and cyclin D1 were purchased from Santa Cruz Biotechnology. Antibodies against the insulin receptor substrate (IRS-1) and IRS-2 were from Upstate. Antibodies against Akt and phospho-Akt (Ser473) were from Cell Signaling Technology (Beverly, MA).

Rnase protection assays. Total RNA was extracted from mouse inguinal mammary glands and tumors using the Trizol reagent (Invitrogen, Carlsbad, CA). The mouse mCYC-1 multiprobe template set for detecting mRNAs of cyclins A2, B1, C, D1, D2, D3, A1, and B2 and control mRNAs of L32 and the glyceraldehyde-3-phosphate dehydrogenase was purchased from BD Pharmingen (San Jose, CA). Antisense RNA probes were labeled using T7 RNA polymerase and [32P]UTP according to the Maxiscript kit protocol (Ambion, Austin, TX). Rnase protection assay (RPA) was done according to the manufacturer's instruction using the RPAIII kit (Ambion).

Results

Mammary ductal morphogenesis is delayed in AIB1−/− mice with different genetic backgrounds. Our previous studies showed that the mammary ductal growth was retarded in AIB1−/− mice compared with their WT siblings, suggesting that AIB1 plays an important role in mammary gland development (19, 21). The AIB1 mutant mice were initially generated by breeding the chimeric mice derived from the AIB1-targeted embryonic stem cells with the 129SvEv strain background with WT C57BL/6J mice (19). Previous studies showed that mice with mixed 129SvEv and C57BL/6J genetic background were less sensitive to chemical carcinogen–induced tumors.


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mammary tumorigenesis, and carcinogen treatment of these mice frequently caused ovarian tumors (23). To achieve an appropriate strain background for defining the role of AIB1 in carcinogen-induced breast cancer, we back-crossed AIB1−/− mice with WT mice with the FVB strain background for seven generations. AIB1−/− mice from the seventh generation with ~99% FVB strain background were intercrossed to generate WT, AIB1+/−, and AIB1−/− mice that were analyzed in the present study. Staining of whole-mounted mammary glands isolated from 6- and 8-week-old females with FVB strain background revealed that the mammary ductal growth was significantly delayed in AIB1−/− mice compared with WT mice (Fig. 1A; data not shown). Because the reduced mammary ductal morphogenesis was observed in AIB1−/− mice with either FVB or 129SvEv/C57BL/6J strain backgrounds, we conclude that the role of AIB1 in mammary ductal growth under natural physiologic conditions is independent of genetic background.

The attenuated mammary ductal morphogenesis in AIB1−/− mice is caused by amplified in breast cancer 1 deficiency in the mammary epithelial cells. To determine whether the attenuated mammary ductal growth in AIB1−/− mice was due to the loss of AIB1 function in the mammary epithelial cells or an alteration of the systemic endocrine system, we did mammary epithelial transplantation between female siblings with different AIB1 genotypes at 3 weeks of age. The WT mammary epithelium and the AIB1−/− mammary epithelium were transplanted into the cleared right and left inguinal fat pads of the same WT host mice. When ductal outgrowths were examined in 10 weeks after the transplantation, the mammary epithelial ducts emanating from the AIB1+/− transplants were significantly shorter than those from the WT and AIB1−/− transplants (Fig. 1B, a-d). The number of AIB1−/− mammary ductal branches from the AIB1−/− transplants also was significantly less than those from the WT transplants in the same WT host mice (Fig. 1B, a-d). Because the targeted AIB1 gene locus carried a β-galactosidase indicator that was specifically expressed in AIB1+/− and AIB1−/− mammary epithelial cells (19), we assayed the in situ β-galactosidase activity in the mammary fat pads of WT recipients bearing the mammary ductal outgrowths from either AIB1+/− or AIB1−/− mammary epithelial transplants. These assays showed that the β-galactosidase activity was specifically detected in all mammary ducts in the cleared WT fat pads, indicating that these mammary ducts indeed originated from the transplanted mammary epithelium (Fig. 1B, c and d). These results show that the reduced mammary ductal morphogenesis in AIB1−/− mice was caused by the AIB1 deficiency in the mammary epithelial cells and not by systemic changes of endocrine factors. Thus, AIB1 plays an autonomous role in the regulation of mammary ductal growth in the mammary epithelium.

To examine the response of the mammary ducts from the transplanted WT and AIB1−/− mammary epithelium to the elevated hormonal stimulation in the same WT recipients, we isolated pituitaries from their male siblings and implanted one pituitary into the kidney capsule of each female WT recipient at 2 weeks after receiving the mammary epithelial transplants. It is known that the pituitary isograft in the kidney capsule significantly increases the circulating levels of progesterone and prolactin and moderately increases the circulating level of estrogen (28–30). Interestingly, when the reconstituted mammary glands in the recipient mice were examined at 10 weeks after transplantation or 6 weeks after receiving the pituitary isografts, extensive mammary ductal side branches and numerous lobuloalveolar were developed along the mammary ducts derived from the transplanted WT and AIB1−/− mammary epithelium, and no appreciable differences in the mammary gland morphology could be observed between the

![Figure 1](https://example.com/figure1.png)

Figure 1. AIB1 deficiency reduces mammary ductal morphogenesis. A, representative whole-mounted inguinal mammary glands of 8-week-old female WT and AIB1−/− mice. Note the decreased AIB1−/− mammary ductal growth. B, mammary gland transplantation assay. a and b, WT and AIB1−/− mammary epithelial tissues were transplanted into right and left cleared fat pads of WT recipient mice and examined by whole-mount staining in 10 weeks after the transplantation; c and d, X-gal staining of the constitutive mammary glands of the transplanted AIB1+/− (c) and AIB1−/− (d) mammary epithelium and the cleared WT fat pads at 10 weeks after the transplantation; e and f, WT recipient mice bearing transplanted WT (e) and AIB1−/− (f) mammary epithelium were stimulated by pituitary isografts at 2 weeks after the transplantation and the mammary glands were examined at 10 weeks after the transplantation. Arrows, locations of implanted mammary tissues.
transplanted WT and AIB1−/− mammary glands (Fig. 1B, e and f). These results suggest that the highly enhanced hormonal stimulation provided by the pituitary isograft can rescue the delayed mammary ductal morphogenesis caused by AIB1 deficiency in the mammary epithelium.

Amplified in breast cancer 1 deficiency selectively suppresses 7,12-dimethylbenz[a]anthracene–induced mammary carcinogenesis. DMBA is a known chemical carcinogen that causes DNA damage and induces tumorigenesis in the mammary gland, ovary, and epidermis and leukemia (23, 29, 30). To examine the role of AIB1 in chemical carcinogen–induced neoplasia, we treated 8-week-old female WT, AIB1+/−, and AIB1−/− mice with DMBA once weekly for 6 consecutive weeks and recorded the DMBA-induced tumorigenesis. Mammary tumors were developed in 45% (5 of 11), 44% (11 of 25), and 11% (2 of 19) of DMBA-treated WT, AIB1+/−, and AIB1−/− mice, respectively, indicating that the frequency of mammary tumor formation in AIB1−/− mice is significantly lower than in WT and AIB1+/− mice (P < 0.05, χ² test). The mammary tumor latency after the initial DMBA treatment also was significantly extended in AIB1−/− mice compared with WT and AIB1+/− mice (P < 0.05, log-rank test; Fig. 2A). In contrast, DMBA-induced skin tumors were observed in 45% (5 of 11), 48% (12 of 25), and 39% (7 of 18) of WT, AIB1+/−, and AIB1−/− mice, respectively. There were no significant differences (P > 0.05, χ² test) in the skin tumor frequencies and latencies among these three groups (Fig. 2B). The skin tumor occurrence also was not associated with the mammary tumor occurrence. Ovarian tumors were only observed in one of the DMBA-treated AIB1+/− mice. Taken together, these results indicate that inactivation of AIB1 selectively protects the mammary glands from DMBA-induced carcinogenesis under natural ovarian hormonal conditions.

AIB1−/− mice are more resistant to pituitary isograft-promoted and 7,12-dimethylbenz[a]anthracene–induced mammary tumorigenesis. The pituitary isograft under the kidney capsule continuously secretes prolactin, which in turn stimulates the ovaries to synthesize and secrete progesterone and estradiol, providing a hormonal environment similar to that observed in the pregnant mouse (23, 28). The elevation of these hormones actively induces mammary epithelial proliferation and alveolar development and strongly accelerates chemical carcinogen– and oncogene-induced mammary carcinogenesis (23, 29, 30). To define the role of AIB1 in hormone-regulated mammary gland development, we did whole-mount staining to evaluate the relative responses of the WT and AIB1−/− mammary glands to pituitary isograft stimulation. To be consistent with the established protocol of DMBA-induced mammary tumorigenesis, the pituitary was implanted under the kidney capsule of WT and AIB3−/− mice at 5 weeks of age and their inguinal mammary glands were examined when the mice received the pituitary isograft for 3 weeks. Our analysis showed that both WT and AIB1−/− mammary glands exhibited extensive ductal side branching and alveolar budding, a morphology similar to that observed in mid-pregnant mice (Fig. 3A, a and B, a). There were no obvious differences in relative responses of WT and AIB1−/− mammary glands to the pituitary isograft stimulation, which was consistent with the comparable responses of the transplanted WT and AIB1−/− mammary glands to the pituitary isograft stimulation (Fig. 1B, e and f). These results suggest that AIB1 is not essential for mammary gland morphogenesis under significantly enhanced hormonal (progesterone and prolactin) conditions.

To evaluate the contribution of AIB1 to hormone-promoted and chemical carcinogen–induced chemical carcinogenesis, we implanted a male donor pituitary under the kidney capsule of each female WT or AIB1−/− mouse at 5 weeks of age and treated these mice with DMBA once weekly for 3 weeks starting at 8 weeks of age. By 5 weeks of pituitary isograft stimulation and 2 weeks after DMBA treatment, the mammary glands of both WT and AIB1−/− mice exhibited a morphology similar to that observed in mid- to late-pregnant mice (data not shown). This mammary gland morphology persisted through the entire experimental period in both WT and AIB1−/− mice with the pituitary isograft. By 5 weeks after DMBA treatment, WT mammary glands showed large lobules (Fig. 3A, c and d). In contrast, the same large lobules were not observed in AIB1−/− mammary glands until 16 weeks after DMBA treatment (Fig. 3B, c and d). Development of small mammary tumors in WT mammary glands also was observed significantly earlier than in AIB1−/− mammary glands (compare e and g in Fig. 3A with e and g in Fig. 3B). Although mammary ductal and lobuloalveolar hyperplasia, mammary intraepithelial neoplasia (MIN), hyperplastic alveolar nodule, multilayered squamous metaplasia, unusual squamous cysts with central keratin debris, and invasive adenocarcinomas were observed in H&E-stained mammary gland sections of both WT and AIB1−/− mice, these pituitary isograft-promoted and DMBA-induced hyperproliferative lesions were significantly delayed in AIB1−/− mice compared with WT mice (compare f, e, and h in Fig. 3A with f, d, and h in Fig. 3B).

These results indicate that AIB1 deficiency delays the initiation and/or progression of DMBA-induced mammary tumorigenesis under these hormonal conditions.

Figure 2. AIB1 deficiency selectively suppresses DMBA-induced mammary tumorigenesis. A, DMBA-induced mammary tumor development in WT (n = 11), AIB1−/− (+/−; n = 25), and AIB1−/− (−/−; n = 19) mice. The frequency and latency of the palpable mammary tumors in AIB1−/− mice was statistically different from that of WT and AIB1−/− mice (P < 0.05, log-rank test). B, DMBA-induced skin tumor formation in WT (n = 11), AIB1−/− (n = 25), and AIB1−/− (n = 19) mice. There were no statistic differences in skin tumor frequencies and latencies among these three groups of mice (P > 0.05, log-rank test).
Importantly, palpable mammary tumor in AIB1 WT and AIB1+/- respectively. Palpable mammary tumors were observed in 50% of pituitary isografts, only 2 of 31 WT mice developed ovarian tumors (b, d, f, and h). Red arrows, in situ mammary tumors in whole-mounted mammary glands. B, stained whole-mount inguinal mammary glands isolated from AIB1+/- mice with pituitary isografts just before DMBA treatment (a) and after 16, 31, and 44 weeks of DMBA treatment (c, e, and g), and H&E-stained tissue sections were prepared from their contralateral inguinal mammary glands or tumors (b, d, f, and h). Of note, the DMBA-induced mammary tumor formation is delayed in AIB1+/- mammary glands compared with WT mice under pituitary isograft stimulation.

To quantify the incidence and latency of the pituitary isograft-promoted and DMBA-induced mammary tumor development, we recorded the time required for formation of palpable mammary tumors. Palpable mammary tumors were first detected in 9 and 7 weeks after DMBA treatment in WT and AIB1+/- mice, respectively. Palpable mammary tumors were observed in 50% of WT and AIB1+/- mice with pituitary isografts by 16 and 18 weeks after DMBA treatment (Fig. 4A). Sixty-two percent (23 of 31) WT and 86% (25 of 29) AIB1+/- mice developed palpable mammary tumors by 39 weeks after DMBA treatment. Other mice died of DMBA-induced leukemia or other types of cancers (see next paragraph). There were no statistical differences in mammary tumor incidences and latencies between WT and AIB1+/- mice.

Importantly, palpable mammary tumor in AIB1+/- mice with the pituitary isografts was first detected at 17 weeks after DMBA treatment, and 50% of AIB1+/- mice did not develop mammary tumors until 31 weeks after DMBA treatment (Fig. 4A). Palpable mammary tumors were observed in 16 of 22 (73%) AIB1+/- mice by 52 weeks after DMBA treatment. Although the incidence of the pituitary isograft-stimulated and DMBA-induced mammary tumors in AIB1+/- mice was similar to that observed in WT and AIB1+/- mice, these results indicate that the mammary tumor latency of AIB1+/- mice was significantly longer than that of WT and AIB1+/- mice ($P < 0.0001$, log-rank test).

In DMBA-treated WT, AIB1+/-, and AIB1+/- mice bearing pituitary isografts, only 2 of 31 WT mice developed ovarian tumors and 1 of 29 AIB1+/- mice developed skin tumor. In addition, mice that did not develop solid tumors might have died of DMBA-induced leukemia as described in previous studies (23).

AIB1+/- mammary tumors grow slower than wild-type mammary tumors. The growth rate of the primary mammary tumor in each mouse was measured by estimating its volume once weekly after it was detected by palpation. The average growth rate of mammary tumors in WT mice with pituitary isografts was similar to that in AIB1+/- mice with pituitary isografts. In contrast, the average volume of mammary tumors in AIB1+/- mice was significantly smaller than in WT mice by 4 weeks after palpable mammary tumors were detected ($P < 0.05$, unpaired and two-sided t test; Fig. 4B). These results showed that DMBA-induced mammary tumors grow much slower in the absence of AIB1.

The number of breast tumors per tumor-bearing mouse was comparable among WT, AIB1+/-, and AIB1+/- mice at the experimental end point ($P > 0.05$, two-sided F test), which were 2.7, 2.6, and 2.3 tumors per mouse, respectively. Histopathologic analysis of mammary tumors developed in WT, AIB1+/-, and AIB1+/- mice did not reveal obvious histologic differences. Most mammary tumors examined in the pituitary isografted mice were squamous adenocarcinomas and type B mammary adenocarcinomas, whereas those mammary tumors in mice without pituitary isografts were predominantly type B mammary adenocarcinomas.

Amplified in breast cancer 1 deficiency reduces cell proliferation in the mammary epithelium and neoplasia lesions induced by pituitary isograft and 7,12-dimethylbenz[a]-anthracene treatment. To search for cellular mechanisms responsible for the delayed mammary tumor initiation and growth, we analyzed normal mammary epithelial and DMBA-transformed mammary epithelial proliferation by immunostaining PH3, a specific marker of cell mitosis (24). The fractions of PH3-positive
mammary epithelial cells in 8-week-old WT and AIB1−/− mice were similar, ~6% of total mammary luminal epithelial cells. On pituitary isograft stimulation for 3 weeks, the PH3-positive cells were examined for each section. *, P < 0.05, unpaired t test. B, AIB1 deficiency inhibits the increase in cell proliferation induced by pituitary isograft and chemical carcinogen. Proliferating mammary epithelial cells or tumor cells on each WT and AIB1−/− mammary gland or tumor section were identified by the anti-PH3 antibody. The cell proliferation index (%) was determined by the ratio of PH3-positive cells to total counted mammary epithelial or tumor cells. For each group, mammary sections were prepared from four animals, and 500 to 1,000 cells were examined for each section. A, a, Pituitary isograft-stimulated AIB1−/− mammary glands (Fig. 5A, a-d). The estradiol level was statistically higher in WT mice than in AIB1+/− and AIB1−/− mice (P < 0.05 and P < 0.01, unpaired and two-sided t tests) and the estradiol level in AIB1+/− mice also was significantly higher than that in AIB1−/− mice (P < 0.01, t test). In contrast, the progesterone levels were similar in these mice [13.0 ± 10.8 (n = 19), 12.5 ± 6.9 (n = 12), and 12.3 ± 9.9 (n = 12) pg/mL in WT, AIB1+/−, and AIB1−/−, respectively]. These results indicate that AIB1 deficiency attenuates the estradiol increase induced by the pituitary isograft.

Figure 4. A, pituitary isograft-stimulated and DMBA-induced mammary carcinogenesis is delayed in AIB1−/− mice. The percentage of pituitary-isograft-stimulated mice free of palpable mammary tumors is plotted against the time in weeks after administration of DMBA. The number (n) of mice in WT, AIB1+/+, and AIB1−/− groups is indicated. B, AIB1−/− mammary tumors grow slower than WT mammary tumors. After palpable mammary tumors were detected, their volumes were estimated weekly as described in Materials and Methods. Points, average; bars, SD. *, P < 0.05, unpaired t test. C, AIB1 deficiency inhibits the increase in cell proliferation induced by pituitary isograft and chemical carcinogen. Proliferating mammary epithelial cells or tumor cells on each WT and AIB1−/− mammary gland or tumor section were identified by the anti-PH3 antibody. The cell proliferation index (%) was determined by the ratio of PH3-positive cells to total counted mammary epithelial or tumor cells. For each group, mammary sections were prepared from four animals, and 500 to 1,000 cells were examined for each section. A, a, Pituitary isograft-stimulated AIB1−/− mammary glands (Fig. 5A, a-d). The estradiol level was statistically higher in WT mice than in AIB1+/− and AIB1−/− mice (P < 0.05 and P < 0.01, unpaired and two-sided t tests) and the estradiol level in AIB1+/− mice also was significantly higher than that in AIB1−/− mice (P < 0.01, t test). In contrast, the progesterone levels were similar in these mice [13.0 ± 10.8 (n = 19), 12.5 ± 6.9 (n = 12), and 12.3 ± 9.9 (n = 12) pg/mL in WT, AIB1+/−, and AIB1−/−, respectively]. These results indicate that AIB1 deficiency attenuates the estradiol increase induced by the pituitary isograft.

Next, we compared the expression patterns and relative levels of ERα and PR in WT and AIB1−/− mammary glands and DMBA-induced mammary tumors by immunohistochemistry. ERα and PR immunoreactivities were detected only in a proportion of the mammary epithelial cells as reported previously (20, 31, 32). The relative intensity of ERα and PR immunoreactivities and the number of ERα- and PR-positive cells were similar in the WT and AIB1−/− mammary epithelium and in the pituitary isograft-induced WT and AIB1−/− mammary alveoli (Fig. 5, a-d in B and C). The number of ERα- and PR-positive cells was significantly decreased in the hyperplastic lesions induced by DMBA and was undetectable in the advanced breast tumors in both WT and AIB1−/− mammary glands (Fig. 5, e and f in B and C).

Finally, we measured SRC-1 and TIF2 (SRC-2) mRNA levels to check whether AIB1 (SRC-3) deficiency would result in a compensatory up-regulation of SRC-1 and TIF2 expression. Quantitative real-time reverse transcription-PCR assays revealed that SRC-1 and TIF2 expression levels were quite different among individual DMBA-induced mammary tumors and not correlated with the presence or absence of AIB1. The average ratios of SRC-1 and TIF2 mRNA concentrations to the 18S RNA concentration in DMBA-induced WT and AIB1−/− mammary tumors were not significantly different (P > 0.05, unpaired t test). These results suggest that the change of SRC-1 and TIF2 expression in the DMBA-induced mammary tumors is not influenced by AIB1 deficiency.
Amplified in breast cancer 1 deficiency down-regulates the insulin-like growth factor-I/phosphatidylinositol 3-kinase/Akt/cyclin D1 pathway in 7,12-dimethylbenz[a]anthracene–induced mammary tumors. The IGF-I/phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway plays a critical role in breast cancer initiation and progression (20, 33). To address the question of whether inactivation of AIB1 suppresses DMBA-induced mammary tumorigenesis through alteration of the IGF-I/PI3K/Akt pathway, we examined several essential components of this signaling pathway. Low levels of IGF-IR were detected in the mammary gland extracts and much higher levels of IGF-IR were found in the extracts of DMBA-induced mammary tumors from both WT and AIB1–/– mice, suggesting that IGF-IR is overexpressed in DMBA-induced mammary tumor cells regardless of AIB1 deficiency. IRS-1 and IRS-2 proteins were undetectable on immunoblots of the mammary gland extracts from WT and AIB1–/– virgin mice. Interestingly, robust levels of IRS-1 and IRS-2 proteins were identified in DMBA-induced WT mammary tumors but not in DMBA-induced AIB1–/– mammary tumors (Fig. 6A). Total Akt protein remained unchanged in WT and AIB1–/– mammary glands and tumors (Fig. 5A). The activated form of Akt, phosphorylated Akt S473, was observed in DMBA-induced mammary tumor cells regardless of whether inactivation of AIB1 suppresses DMBA-induced mammary tumors than in AIB1–/– mammary tumors (Fig. 6C). Accordingly, the cyclin D1 protein also was much higher in DMBA-induced WT mammary tumors than in AIB1–/– mammary tumors (Fig. 6D). These results suggest that AIB1 is required for IGF-I/PI3K/Akt signaling pathway–stimulated up-regulation of cyclin D1 during the DMBA-induced breast tumor initiation and progression.

Discussion

Because the profile of DMBA-induced mammary gland tumorigenesis in mice can be modified by different strain backgrounds (23), we back-crossed our original AIB1 mutant mice containing a mixed C57BL/6j and 129SvEv strain background with mice with a pure FVB strain background. The back-crossed AIB1–/– virgin mice with the FVB strain background exhibited delayed mammary ductal morphogenesis during puberty, which was similar to that observed in AIB1–/– mice with the mixed strain background (19). These results indicate that the effect of AIB1 deficiency on mammary ductal growth is independent of mouse genetic background. We also showed that the ductal outgrowths emanating from the AIB1–/– mammary epithelial transplants also

Figure 5. ERα, PR, and AIB1 expression in mammary gland and DMBA-induced mammary tumors. A, immunohistochemical staining of AIB1. AIB1 immunoreactivity was detected in the mammary epithelial cells of the 8-week-old virgin mice (a) and pituitary–isografted mice (b) and in the DMBA-induced mammary tumor cells (c) but not detected in the mammary gland of pituitary-isografted AIB1–/– mice (d). Note the AIB1 immunoreactivity in b and c was higher than that in a. B, immunohistochemical staining of ERα in 8-week-old WT and AIB1–/– virgin mammary glands (a and b), in pituitary-isografted WT and AIB1–/– mammary glands (c and d), and in DMBA-induced WT and AIB1–/– mammary tumors (e and f). C, immunohistochemical staining of PR in 8-week-old WT and AIB1–/– virgin mammary glands (a and b), in pituitary-isografted WT and AIB1–/– mammary glands (c and d), and in DMBA-induced WT and AIB1–/– mammary tumors (e and f). Bars, 100 μm.
were retarded in the fat pads of WT recipient mice compared with the outgrowths of WT mammary epithelial transplants in the WT recipient mice. These results confirmed that AIB1 plays an intrinsic epithelial autonomous role in the mammary gland ductal growth, which is consistent with the AIB1 expression noted in the mammary epithelial compartment (19, 20). Because of the partial lethality of AIB1−/− mice at prenatal and neonatal stages (19), we encountered difficulties to generate enough number of female AIB1−/− donor and recipient mice and were unable to examine the outgrowth of WT and AIB1−/− mammary epithelium in the fat pads of AIB1−/− recipient mice in this study. Therefore, we could not exclude a possible role of AIB1 in the mammary stromal tissue for mammary gland development.

Interestingly, both endogenous and transplanted WT and AIB1−/− mammary glands exhibited similar development of side branches and lobular alveoli when they were stimulated by the elevated hormones derived from the pituitary isografts, suggesting that enhanced hormonal stimulation can compensate for the developmental defect caused by AIB1 deficiency. Although the exact mechanisms responsible for this compensation is not clear, the combination of elevated progesterone and prolactin or the partially redundant function among SRC family members may contribute to this functional compensation on significantly enhanced hormonal stimulations (1, 34, 35). In agreement with the interpretation that the persistently enhanced hormonal stimulation is able to improve certain initial deficits in the mammary gland development, other studies also have shown that multiple cycles of pregnancy and lactation were able to restore mammary gland morphology and function in certain mutant mice with partially compromised mammary gland development (36, 37).

In WT and AIB1−/− mice that did not receive the pituitary isografts, DMBA treatment mainly induced mammary tumors, and AIB1+/- mice were unaltered compared with WT and AIB1−/− mice. However, the susceptibility of AIB1−/− mice to DMBA-induced mammary tumorigenesis was significantly reduced, manifesting much lower mammary tumor incidence and much longer mammary tumor latency compared with DMBA-treated WT and AIB1−/− mice. These results indicate that inactivation of AIB1 selectively protects the mammary gland from tumorigenesis induced by chemical carcinogens, such as DMBA. This tissue-selective role of AIB1 in carcinogenesis may be partially accredited to its high expression level in the mammary epithelium and low expression level in the skin (19), to its regulatory function in the tissue microenvironment.
expression of the IGF-1 signaling pathway components (discussed below; refs. 7, 18, 20, 38), and to its transcriptional coactivation function for ERα and PR in the mammary gland (1, 16, 23, 39). Although the relative contribution of AIB1 to specific pathway-mediated tumorigenesis is not clear at this stage, our results show that suppression of AIB1 function increases the resistance of the mammary gland to the chemical carcinogen–induced mammary tumorigenesis.

In WT and AIB1 mutant mice that received the pituitary isografts, DMBA treatment predominantly induced mammary gland tumors, and skin tumors were rare. It likely was because the elevated hormones that were directly and indirectly derived from the pituitary isografts selectively promoted the susceptibility of the mammary epithelial cells to the carcinogen by stimulating the mammary epithelial proliferation and increasing the mammary epithelial population (23, 28–30). Consequently, the latency of DMBA-induced mammary tumors in the mice with pituitary isografts was much shorter than the latency in the mice without pituitary isografts. Most mice developed mammary tumors before they developed the skin tumors. Interestingly, the enhanced hormonal conditions caused by the pituitary isografts accelerated the DMBA-induced mammary tumorigenesis in both WT and AIB1 mutant mice, suggesting that AIB1 is not absolutely essential for hormonal promotion of chemical carcinogen–induced breast cancer. This conclusion is consistent with our previous findings showing that AIB1 was not essential for ERα-mediated gene expression and ductal morphogenesis of the mammary gland (19–21).

Most importantly, our results suggested that AIB1 deficiency significantly delayed the DMBA-induced mammary carcinogenesis in mice with or without the pituitary isografts. Further analyses revealed that AIB1 expression was elevated during the DMBA-induced mammary tumorigenesis, recapitulating the situation in human breast cancer where AIB1 was found to be overexpressed (3, 16). AIB1 deficiency mainly suppressed the proliferation of mammary epithelial cells and DMBA-induced mammary tumor cells and the DMBA-induced mammary tumor growth. Consistent results also were observed in another mouse model of human breast cancer where mammary tumors were induced by the MMTV-\(v\)-Ha-ras oncogene (20). Because the DMBA and the ras use different molecular mechanisms to induce mammary tumorigenesis and AIB1 deficiency suppresses both the DMBA-induced and the ras-induced mammary tumorigenesis, the molecular mechanism by which AIB1 promotes breast cancer risk may involve a common pathway, such as the IGF-1 signaling pathway, which can modulate the susceptibility of the mammary epithelium to the tumorigenesis induced by both DMBA and the ras oncogene.

Indeed, we found that AIB1 deficiency inhibited the up-regulation of IRS-1 and IRS-2 in DMBA-induced mammary tumor cells, which in turn suppressed the overactivation of the downstream protein kinase Akt and the overexpression of cyclin D1 in the pituitary-stimulated mammary gland and in the DMBA-induced mammary tumor cells. In agreement with this finding, our previous work showed that inactivation of AIB1 suppressed the up-regulation of IRS-1 and IRS-2 expression in the ras-induced mammary tumor cells and resulted in partial IGF-1 resistance in these cells (20). Inversely, other studies showed that overexpression of AIB1 could enhance Akt activity and cyclin D1 expression in cultured cells and in the mammary tumors induced by the MMTV-AIB1 transgene (18, 38). Therefore, the overexpression of AIB1 may cause up-regulation of IRS-1 and IRS-2 and overactivation of the downstream IGF-1 signaling components to promote cell proliferation and mammary tumorigenesis.

A lesser increase in the estrogen level was observed in the pituitary isograft-stimulated AIB1\(^{+/−}\) mice compared with the pituitary isograft-stimulated WT mice. However, this difference in estrogen level was unlikely responsible for the suppression of DMBA-induced mammary tumorigenesis in AIB1\(^{+/−}\) mice based on the following observations. First, the estrogen level was still significantly increased in AIB1\(^{+/−}\) mice after stimulated by the pituitary isografts. Second, the AIB1\(^{+/−}\) mammary glands showed a similar response to the pituitary isograft stimulation as WT mammary glands in terms of ductal side branching and lobular alveolar development. Finally, the expression level of PR, an ERα target gene in the mammary epithelial cells, was comparable in AIB1\(^{+/−}\) and WT mammary glands on pituitary isograft stimulation.

In summary, our study showed that AIB1 plays a mammary epithelial autonomous role in the regulation of mammary ductal growth and tumorigenesis. Inactivation of AIB1 function selectively protects the mammary gland over the skin from DMBA-induced tumorigenesis and inhibits mammary tumor growth. The suppression of IRS-1 and IRS-2 elevation during the DMBA-induced mammary tumorigenesis by AIB1 deficiency may be responsible, at least in part, for preventing the PI3K/Akt/cyclin D1 pathway from overactivation and the target cells from overproliferation. These findings suggest that AIB1 may serve as a potential target for prevention of breast cancer initiation as well as for treatment of breast cancer progression.

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