AIB1/SRC-3 Deficiency Affects Insulin-Like Growth Factor I Signaling Pathway and Suppresses v-Ha-ras-induced Breast Cancer Initiation and Progression in Mice

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ABSTRACT

Although the amplified in breast cancer 1 (AIB1) coactivator is amplified and overexpressed in breast cancers, its role in mammary carcinogenesis remains unknown. We demonstrate that during mammary development and tumorigenesis, the elevation of AIB1 level and its nuclear localization correlate with normal and transformed mammary epithelial proliferation, whereas its lower expression and cytoplasmic localization correlate with mammary epithelial quiescence and differentiation. In this study, the role of AIB1 in breast tumor initiation, progression, and metastasis was studied by generating AIB1+/−, AIB1−/−, and AIB1−/− mice harboring the mouse mammary tumor virus/Ha-ras (ras) transgene that induces breast tumors. Breast tumor incidence was reduced dramatically in the intact AIB1−/−-ras virgin mice and inhibited completely in the ovarioectomized AIB1−/−-ras mice. Breast tumor latency was delayed significantly in AIB1−/−-ras virgin mice with natural estrous cycles, multiparous mice with cyclically elevated reproductive hormones, and virgin mice bearing pituitary isografts with persistently elevated hormones. Although AIB1 deficiency significantly suppressed mammary tumorigenesis under all of the concentrations of ovarian hormones, it did not affect the promotional role of ovarian hormones on mammary tumorigenesis, suggesting that AIB1 and ovarian hormones contribute to mammary carcinogenesis through different pathways. AIB1 deficiency did not alter the expression of estrogen and progesterone-responsive genes in the mammary gland, but it caused partial resistance to the insulin-like growth factor I because of a significant reduction in the insulin receptor substrates. The impaired insulin-like growth factor I pathway in AIB1−/−-ras mammary epithelium and tumor cells was responsible in part for the suppression of mammary tumorigenesis and metastasis caused by inhibition of cell proliferation and migration. These results suggest that a more effective strategy to control breast cancer is to target AIB1-mediated and ovarian hormone-initiated pathways.

INTRODUCTION

Breast cancer is the most frequently occurring cancer among women in developed countries (1). Among multiple mammary oncogenic factors, ovarian steroids, including estrogen and progesterone, are not only essential hormones for normal development and function of the mammary gland but also critical risk factors for the initiation and progression of breast cancers. Clinical and animal studies have demonstrated that depletion of ovarian steroids or ablation of estrogen receptor (ERα) or progesterone receptor (PR) significantly reduces breast cancer risk and strongly suppresses mammary gland tumorigenesis (2–7). Accordingly, aromatase inhibitors and estrogen antagonists are used for breast cancer prevention and treatment (7).

ER and PR are members of the nuclear receptor (NR) superfamily, which contains a large group of hormone-inducible transcription factors and activates gene expression through recruiting multiple coactivators (1, 8, 9). The p160 steroid receptor coactivator (SRC) family contains three homologous NR coactivators, including SRC-1, the transcription intermediary factor 2 (TIF2 or GRIP1), and the amplified in breast cancer 1 (AIB1) coactivator (also known as p/CIP, RAC3, ACTR, TRAM1, and SRC-3; Refs. 8, 9). Although the in vivo functional relationships between these SRC family members and individual transcription factors have not been defined fully, biochemical and gene transfer analyses have shown that these p160 coactivators interact with ligand-bound NRs and amplify their transcriptional activities through recruiting downstream essential coactivator complexes (9). These coactivator complexes are responsible for the remodeling of chromatin, the change of chromosome topology, and the assembly of basal transcription machinery (10, 11). Therefore, the levels and functional states of SRC family proteins may play a central role in NR-regulated gene expression and modulate hormonal sensitivities and cancer risks in hormonal target tissues such as the mammary gland.

A potential link between AIB1 and breast cancer was strongly suggested by the initial identification of the AIB1 gene in the highly amplified 20q12 chromosomal region of human breast cancer cells (12, 13). Subsequent independent surveys demonstrated that the AIB1 gene is amplified in 4.8–9.5% of human breast tumors (12, 13), and its mRNA and protein are overproduced in 10–64% of breast tumors with or without ER and PR (12, 14, 15). More surprisingly, AIB1 overproduction is associated with high levels of HER-2/neu and with tamoxifen resistance in tamoxifen-treated invasive breast tumors (15, 16). Increased number of polyglutamine repeats in the AIB1 protein also is associated with higher breast cancer risk in women with BRCA1/2 mutations (17). Furthermore, AIB1 can be recruited to the estrogen-responsive cyclin D1 promoter to enhance cyclin D1 expression in breast cancer cells; therefore, reduction of AIB1 in these cells slows down cell proliferation in culture and grafted tumors in nude mice (18, 19). These findings suggest that elevated AIB1 expression or function correlates with higher breast cancer risk and enhances breast cancer cell growth under in vitro or ex vivo conditions. However, the role of AIB1 in the initiation and progression of breast cancer in vivo is completely unknown.

We demonstrate that AIB1 expression levels and subcellular localizations are associated with mammary epithelial proliferation, differentiation, and malignant states. In the mouse mammary tumor virus/v-Ha-ras (ras) transgenic mice with expression of the v-Ha-ras oncogene in their mammary epithelial cells, AIB1 deficiency significantly delays mammary tumor latency, reduces mammary tumor frequency, and suppresses primary tumor growth and metastasis to the lung. Surprisingly, inactivation of AIB1 suppresses mammary tumor initiation and progression in presence and absence of ovarian hormones. Our data also indicate that AIB1 deficiency results in partial impairment of the insulin-like growth factor I (IGF-I) signaling pathway and thereby inhibits cell proliferation and migration, which may

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be responsible for the suppression of mammary tumorigenesis and metastasis in AIB1 null mice.

MATERIALS AND METHODS

Mice. The AIB1+/− mouse line with 129SvEv background was generated by breeding 129SvEv female mice with the chimeric mice produced from microinjection of targeted 129SvEv embryonic stem cells as described previously (20). The transgenic mice with FVB/N background were described previously and obtained from Charles River Laboratories (Wilmington, MA; Ref. 21). AIB1+/− female mice were intercrossed with the transgenic male mice to generate AIB1+/−×AIB1+/− mice. Because female ras transgenic mice could have problems with lactation, female AIB1+−/H11001 mice were ovariectomized (OVEX) at age 3 weeks (22). Intact virgin mice were housed for monitoring breast tumor occurrence, tumor growth, and metastasis under different hormonal conditions: (a) intact virgin mice were used for PCR to analyze AIB1 and ras genotypes as described previously (20, 22). All of the surgical and experimental procedures performed on mice were in accordance with the NIH guidelines outlined in the Guide for Care and Use of Laboratory Animals.

Examination of Breast Tumor Development. Female AIB1+/−/−, AIB1+/−/+, and AIB1+/−/− mice were used in four experiments to examine breast tumor occurrence, tumor growth, and metastasis under different hormonal conditions: (a) intact virgin mice were housed for monitoring breast tumor occurrence, tumor growth, and metastasis in AIB1 null mice. Entire mammary glands and cryostat-prepared mammary gland sections of AIB1+/− mice were used for 5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside (X-Gal) staining. X-Gal staining was performed as described previously (20).

Semiquantitative Reverse Transcription-PCR and Real-Time Reverse Transcription-PCR. RNA samples were extracted from mouse inguinal mammary glands and mammary tumors using the TRIzol reagent (Invitrogen, Carlsbad, CA). For semiquantitative reverse transcription-PCR, single-strand cDNA was transcribed using 1 μg RNA, random hexamers, and reverse transcriptase. PCR conditions, including annealing temperature, number of cycles, magnesium concentrations, and amounts of cDNA templates and primers, were optimized for each assay to determine linear amplification. The primer pair for PR was 5′-ggcctgagactcactaatgcgc and 5′-ggcagagccagacctgtgtaactctcag and 5′-tggcctgagactcactaatgcgc and 5′-gctactgagactcactaatgcgc. The primer pair for Wnt-4 was designed previously (29). The primers for IGF-I were 5′-gctactgagactcactaatgcgc and 5′-gctactgagactcactaatgcgc. The primers for transforming growth factor β were 5′-gctactgagactcactaatgcgc and 5′-tggcctgagactcactaatgcgc. Analysis of β-actin expression was performed simultaneously as total cDNA input control. The primer pair for amplification of β-actin was 5′-cgctgagactcactaatgcgc and 5′-gctactgagactcactaatgcgc. PCR products were analyzed by electrophoresis with agarose gel containing ethidium bromide.

Real-time reverse transcription-PCR was performed as described previously (26). The primers and TaqMan probe were designed according to the mouse AIB1 cDNA sequence. The forward and reverse primers were 5′-acaagacgccacaagactg and 5′-gctactgagactcactaatgcgc. The TaqMan probe was 5′-cagctactgagactcactaatgcgc. Real-time reverse transcription-PCR was performed with total RNA samples and the One Step Master Mix reagent using the ABI 7700 Sequence Detection System (Applied Biosystems, Foster City, CA). Parallel measurements of the 18S RNA, which is the most consistently expressed RNA in all of the cell types, were performed as endogenous controls (Applied Biosystems). The expression levels of AIB1 mRNA were normalized to the 18S RNA concentrations.

RPA and in Situ Hybridization. RNA extracted from mouse inguinal mammary glands and breast tumors was used for RNase protection assay (RPA). The template DNA for the ras riboprobe was amplified from the purified DNA of the ras transgenic mice by PCR with a pair of primers, 5′-gcctgctgctgctgctgctg and 5′-gcctgctgctgctgctgctg. The TaqMan probe was 5′-cagctactgagactcactaatgcgc. Real-time reverse transcription-PCR was performed with total RNA samples and the One Step Master Mix reagent using the ABI 7700 Sequence Detection System (Applied Biosystems, Foster City, CA). Parallel measurements of the 18S RNA, which is the most consistently expressed RNA in all of the cell types, were performed as endogenous controls (Applied Biosystems). The expression levels of AIB1 mRNA were normalized to the 18S RNA concentrations.

Western Blot Analysis. Western blot analysis was performed as described previously (31). Antibodies against PR (SC-7208), AIB1 (SC-1306), and IGF-I receptor β (IGF-IR; SC-713) were purchased from Santa Cruz Biotechnology, Santa Cruz, CA. Antibodies against insulin receptor substrate 1 (IRS-1; Cat. #06248) and IRS-2 (Cat. #06506) were from Upstate Biotechnology (Lake Placid, NY). Antibody against β-tubulin (T5293) was from Sigma (St. Louis, MO). Antibodies for Akt (R422; Cat. #06506) and phosphorylated Akt (MPK; 06–182), and activated MAPK (VK8031) were from Zymed, Upstate Biotechnology, and Promega (Madison, WI, respectively).

Development of Mammary Tumor Cell Lines and Mouse Embryonic Fibroblasts. Solid breast tumors were isolated from AIB1+/−/− mice, washed in PBS, and minced in 0.25% trypsin-EDTA solution (Invitrogen) containing 3.5 mg/ml collagenase. After 1 h of digestion at 37°C, released cells and minced tissues were pelleted, washed in PBS, and cultured for 24 h in DMEM with 4.5 g/l glucose, 10 μg/ml insulin, 1 mg/ml collagenase, and 10% FCS. Cells were cultured in the same medium without collagenase and the medium was changed every 3 days. Individual...
epithelial colonies were trypsinized and transferred into 24-well plates. These purified epithelial tumor cells were expanded for cell growth and migration analyses.

To obtain mouse embryonic fibroblasts (MEFs), AIB1+/+ and AIB3−/− mouse embryos at the stage of E13.5 were minced into small pieces, soaked in 0.25% trypsin-EDTA cold solution at 4°C overnight, and digested for 30 min at 37°C. After digestion, DMEM containing 4.9 g/l glucose, 10% FCS, and 7 μM β-mercaptoethanol was added, and cell suspension was prepared by pipetting vigorously. Tissue clumps were removed by natural sedimentation, and cell suspension was transferred to 10-cm culture dishes at the plating density of three dishes per embryo. When cells grew to confluence, they were stored in a liquid nitrogen tank. Cell migration assays with MEFs were carried out with cells at passage 3.

Cell Migration Analysis. Cell migration assays were performed with the 48-well chemotaxis chamber mounted with gelatin-coated polycarbonate membrane with 8-μm pores (Neuro Probe, Inc., Gaithersburg, MD). The bottom wells were filled with DMEM containing 5% serum. Mammary tumor cells or MEFs were suspended in serum-free DMEM and loaded into the top wells (2 × 10⁴ cells/well). After cells were cultured for 12 h, cells attached to the top surface of the membrane were removed by moving the membrane against a wiper. Cells on the bottom surface of the membrane were fixed in methanol and stained with H&E for counting.

[3H]thymidine Incorporation Assay. AIB1+/+ and AIB1−/− mammary tumor cells were plated in 24-well plates (10⁵ cells/well) and cultured overnight in DMEM containing 10% serum. Cells were starved in serum-free DMEM containing 0.1% BSA for 24 h and then treated with or without IGF-I in serum-free medium containing 0.1% BSA for 20 h. [3H]thymidine was added (1 μc/ml/well), and cells were cultured in the presence of IGF-I for 4 h. Cells were washed with cold PBS and incubated with 10% trichloroacetic acid for 10 min. After being washed with 95% ethanol, cells were lysed with 0.3 ml of 0.2M NaOH and 0.2% SDS for scintillation counting.

RESULTS

Expression and Localization of AIB1 in the Mouse Mammary Gland and Breast Tumor. Different levels of AIB1 mRNA were detected in different phases of mammary gland development and in mammary tumors in mice (Fig. 1A). AIB1 mRNA was detectable by real-time reverse transcription-PCR in the mammary glands of 6-week-old young virgin mice, but AIB1 mRNA levels in the mammary glands of 12-week-old mature mice were reduced to 40% of that in the 6-week-old mice because of absence of terminal end buds (TEB). At early (day 5), middle (day 12), and late (day 18) stages of pregnancy, the levels of AIB1 mRNA in the mouse mammary glands were increased ~2-, 3-, and 4-fold, respectively, when compared with the AIB1 mRNA levels in the mature virgin female mice. At lactation (day 12) and involution (day 4) stages, the AIB1 mRNA decreased to levels seen in 6-week-old virgin female mice. Surprisingly, in mice with expression of the ras transgene in the mammary epithelium (21), AIB1 mRNA levels were elevated by 2-fold in the mammary glands with ductal hyperplasia and by 10-fold in the mammary tumors when compared with mature virgin mice. No AIB1 mRNA was detectable in AIB1−/− mammary tumors, which validated the aforementioned measurements for AIB1 mRNA (Fig. 1A).

Using the AIB1 heterozygous mice harboring a knock-in LacZ sequence at the AIB1 gene locus, we identified the mammary ductal branches as a major site of AIB1 promoter activity by X-Gal staining of the whole-mounted virgin mammary glands (20). Consistent with the AIB1 mRNA expression (Fig. 1A), the endogenous AIB1 promoter directed the expression of β-galactosidase to the mammary gland in all of the other developmental phases (Fig. 1B, a and b). X-Gal staining of the AIB1−/− mammary gland sections demon-

Fig. 1. AIB1 mRNA expression and protein localization in mammary gland and tumor. A, real-time reverse transcription-PCR measurements of relative AIB1 mRNA levels in the mammary glands at different developmental stages. RNA samples were prepared from inguinal mammary glands or breast tumors of two or more mice and assayed in triplicate. Y6w and v12w, 6- and 12-week-old female virgin mice; P5, P12, and P18, pregnant days 5, 12, and 18; brd, involution day 4; Hyp, mammary glands with hyperplasia in ras transgenic mice; and T=WT and T=ko, AIB1+/+ras and AIB1−/−ras mammary tumors. B, detection of AIB1 promoter activity by 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) staining. Inguinal mammary glands were isolated from AIB1+/− virgin (a and c) and pregnant (b and d) mice with a knock-in β-galactosidase reporter. X-Gal staining was performed with either the entire glands (a and b) or gland sections isolated by cryostat. Strong X-Gal staining (blue color) was observed in the mammary gland (a and b) and cross-sections of mammary ducts (c) and alveoli (d). SC, stroma cells; DEC, ductal epithelial cells; and ME, myoepithelial cells. C, immunohistochemical staining (dark brown) with AIB1 antibody, showing the distribution of AIB1 protein in terminal end buds (e) in 6-week-old mice, in mammary ducts in matured virgin mice (f) and in alveoli of pituitary isograft-stimulated glands (d) and lactating (g) glands. Note the cytoplasmic localization of AIB1 (f). Strong AIB1 immunoreactivity was located mainly in the nuclei of ras-induced mammary tumor cells (e). No AIB1 immunostaining could be detected in the AIB1−/− mammary glands (g). CC, cap cells; and BC, body cells. D, immunoblotting for AIB1 in mammary gland (Ma), hyperplastic mammary gland (Hyp), and breast tumor (Tu) in AIB1+/−ras (WT) and AIB1−/−ras (KO) mice. Tubulin was used as loading control.
strated that the β-galactosidase activity was located in the myoepithelial and luminal epithelial cells (Fig. 1B, c and d). There was no detectable β-galactosidase activity in the AIB1+/+ mammary gland, which confirmed the specificity of X-Gal staining (data not shown). These results suggest that the AIB1 promoter is activated in the mammary epithelial cells.

To examine the cellular and subcellular localizations of the AIB1 protein in the mammary gland, IHC was performed with AIB1-specific antibodies. AIB1 immunoreactivity was observed at high levels in the nuclei of cap cells but at lower levels in TEB body cells in the young virgin mice (Fig. 1C, a). In the mammary glands of matured virgin mice, relatively lower AIB1 immunoreactivity was found in the nuclei of myoepithelial and luminal epithelial cells (Fig. 1C, b). AIB1 immunoreactivity was increased in the mammary epithelial cells of mice at middle pregnant stage and of mice with a pituitary isograft and in the nuclei of breast tumors in the rats transgenic mice when compared with those in normal mammary glands of mature virgin mice (Fig. 1C, c–e). Interestingly, strong AIB1 immunoreactivity was observed in the cytoplasm of the ductal epithelial and alveolar epithelial cells at pregnant day 18 and during lactation (Fig. 1C, f; data not shown). Consistent with an increase in epithelial population that expresses higher AIB1, AIB1 protein concentrations were increased moderately in the hyperplastic mammary glands and dramatically in the breast tumors in the rats transgenic mice when compared with those in normal mammary glands of mature virgin mice (Fig. 1D). AIB1 protein was not detectable in the mammary glands and tumors of AIB1−/− mice by either IHC or immunoblot analysis (Fig. 1, C and D). These results demonstrate that cellular concentrations and subcellular localization of the AIB1 protein in the mammary gland are cell type, developmental stage, and malignant state specific, suggesting that the levels of AIB1 are regulated differentially at different stages of mammary gland development and tumorigenesis.

Inhibition of Breast Cancer Initiation and Progression in AIB1−/−-ras Virgin Mice. To assess the role of AIB1 in breast cancer, we generated AIB1+/+, AIB1+/−, and AIB1−/− mice harboring the ras transgene by breeding AIB1−/− mice with the ras transgenic mice and compared their mammary gland morphology and mammary gland tumorigenesis (20, 21). Whole mount staining revealed that the mammary gland morphology in AIB1+/−-ras, AIB1+/−-ras, and AIB1−/−-ras female mice was similar at prepubertal, peripubertal, and postpubertal stages by age 11 weeks (Fig. 2A, a–d). By age 17 weeks, multifocal nodules were detected in the whole mount mammary glands, and many mammary intraepithelial neoplasia (MIN) lesions were observed in the mammary gland sections in most of the AIB1+/−-ras and AIB1+/−-ras mice (Fig. 2, A, e and B, e). By age 40 weeks, in situ and malignant adenocarcinomas were observed in the mammary glands of most AIB1+/−-ras and AIB1−/−-ras virgin mice (Fig. 2, A, g and i, and B, g and i). In contrast, the MIN lesions and mammary tumors were observed only in a small proportion of AIB1−/−-ras virgin mice older than 35 weeks (Fig. 2, A, h and B, h). Approximately one half of the AIB1−/−-ras virgin mice exhibited normal mammary gland morphology even by age 80 weeks (Fig. 2, A, j and B, j). These results suggest that the ras-induced breast tumor initiation and progression are delayed or suppressed in AIB1−/−-ras virgin mice.

Significant Decrease in Mammary Tumor Incidence in the Intact AIB1+/−-ras Virgin Mice. In the AIB1+/−-ras and AIB1+/−-ras virgin mice, palpable breast tumors were first observed at ages 14 and 18 weeks, respectively. One half of the AIB1+/−-ras mice (n = 38) and AIB1+/−-ras mice (n = 46) developed breast tumors by age 32.5 and 42 weeks, respectively. By age 70 weeks, all of the AIB1+/−-ras and AIB1+/−-ras virgin mice developed palpable breast tumors (Fig. 3A). There was no statistical difference (P = 0.38) in the latency of tumor appearance between these two groups, sug-
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Fig. 3. Inactivation of AIB1 suppresses mammary tumorigenesis under different hormonal conditions. A, reduction of breast tumor incidence in AIB1−/−-ras virgin mice. Percentage of mice without palpable breast tumors is plotted against their ages. The tumor-free curve of AIB1−/−-ras mice is significantly different from those of other groups (P < 0.0001; log-rank test; n = 34 for AIB1−/−-ras, 41 for AIB1+/--ras, and 25 for AIB1+/--ras). B, mammary gland morphology in adult AIB1−/−-ras and AIB1+/--ras mice after ovariectomy at age 3 weeks. Arrows indicate the undeveloped mammary gland trees. C, mammary tumor-free curves for ovariectomized AIB1−/−-ras and AIB1+/--ras mice. Log-rank test, P < 0.01; n = 16 (AIB1+/--ras) and 10 (AIB1−/−-ras). D, representative morphologies of the pituitary isograft-stimulated mammary glands in AIB1+/--ras and AIB1−/−-ras mice. E, mammary tumor-free curves for AIB1−/−-ras and AIB1+/--ras mice with pituitary isografts. T50 = 29 (AIB1+/--ras) and 53 (AIB1−/−-ras) weeks. F, delay in breast tumor latency in multiparous AIB1−/−-ras mice. The T50 values are 26, 28, and 42 weeks for AIB1+/--ras (n = 34), AIB1−/−-ras (n = 28), and AIB1−/−-ras (n = 22) multiparous mice, respectively. P = 0.0002 (log-rank test).

suggesting that inactivation of one of the two AIB1 alleles does not significantly affect breast tumor onset induced by the ras oncogene.

The first palpable breast tumor in AIB1−/−-ras virgin mice was not observed until age 40 weeks, a delay of 26 weeks. By age 80 weeks, when all of the AIB1+/--ras mice developed tumors, only 25% (7 of 28) of AIB1−/−-ras virgin mice developed breast tumors (Fig. 3A). Therefore, the breast tumor latency was remarkably longer and the breast tumor frequency was significantly lower (P < 0.0001) in AIB1−/−-ras virgin mice than those observed in AIB1+/--ras and AIB1−/−-ras virgin mice. These results indicate that AIB1−/−-ras virgin mice are significantly resistant to ras-induced mammary tumorigenesis.

Complete Suppression of Breast Tumor Development in OVE
X AIB1−/−-ras Mice. To address whether the detrimental role of AIB1 in facilitating mammary tumor development was associated with ovarian steroids, we analyzed breast tumor development in AIB1+/--ras and AIB1−/−-ras mice in which ovarian hormones were depleted by prepubertal ovariectomy. Mouse mammary gland ductal growth during pubertal development depends on the estrogen secreted from ovari es (32). Prepubertal ovariectomy equally arrested mammary gland ductal elongation in AIB1+/--ras and AIB1−/−-ras mice and restricted their mammary glands to small regions close to the nipple (Fig. 3B). In the OVEX AIB1+/--ras mice, the first palpable breast tumor was observed at age 28 weeks. By age 38 weeks, palpable breast tumors were observed in 50% of the OVEX AIB1+/--ras mice. By age 64 weeks, palpable breast tumors developed in 64% of the OVEX AIB1+/--ras mice (Fig. 3C). In contrast, none of the OVEX AIB1−/−-ras mice developed palpable breast tumors by age 75 weeks (Fig. 3C). The mammary gland morphology revealed by whole mount staining also confirmed that the mammary glands of the OVEX AIB1−/−-ras mice were tumor free when the mice were killed (data not shown). These results demonstrate that depletion of ovarian hormones extends the mammary tumor latency from T50 = 32.5 weeks in the intact AIB1−/−-ras mice to T50 = 58 weeks in the OVEX AIB1−/−-ras mice and the inactivation of AIB1 together with depletion of ovarian hormones completely inhibits mammary tumorigenesis in AIB1−/−-ras mice. The mammary tumor frequency (64% by 75 weeks) observed in the OVEX AIB1+/--ras mice was much higher than that observed in the intact AIB1−/−-ras virgin mice: only 25% of these mice developed breast tumors by age 80 weeks (Fig. 3A).

Delay of Hormone-Stimulated Mammary Tumorigenesis in AIB1−/−-ras Mice. To assess the role of AIB1 in breast tumorigenesis under conditions with strong and persistent hormonal stimuli, we analyzed breast tumor development in AIB1+/--ras and AIB1−/−-ras mice carrying pituitary isografts. Implantation of pituitary isografts into the kidney capsule is known to elevate significantly circulating levels of progesterone, prolactin, and estrogen in rodents (33). To achieve persistent hormonal stimulation, we isolated pituitaries from their wild-type or AIB1−/−- ras female littermates and implanted one pituitary isograft into the kidney capsule of each AIB1−/−-ras or AIB1−/−-ras recipient mouse. The success of pituitary transplant was confirmed by observing the luteinized ovarian follicles and the increased mammary ductal density as described previously (34). When pituitary isografts were received at age 6 weeks, the mammary ductal density and the number of alveoli in AIB1−/−-ras or AIB1−/−-ras recipient mice were increased dramatically by 3 weeks post-transplantation (Fig. 3D). By age 28 and 38 weeks, palpable breast tumors were detected in 50% (T50 = 28 weeks) and 95% of AIB1−/−-ras mice with pituitary isografts, respectively (Fig. 3E). However, palpable breast tumors developed in 50% of the AIB1−/−-ras mice with pituitary isografts but not until age 51 weeks (T50); tumors did not develop in 88% of the AIB1−/−-ras mice until age 61 weeks (Fig. 3E). This delay was significant (P < 0.001) when compared with the tumor development in the AIB1+/--ras mice with pituitary isografts. Our results indicate that although the enhanced hormonal stimuli originating from the pituitary isografts significantly promoted mammary tumor development in AIB1+/--ras and AIB1−/−-ras mice as compared with those mice with the same genotypes without pituitary isografts, the mammary tumor latencies in AIB1−/−-ras mice remain significantly longer than in AIB1+/--ras mice.

To analyze the effects of AIB1 deficiency on breast tumor development during natural reproductive cycles that modulate levels of hormones in a cyclic fashion, we examined breast tumor development in multiparous AIB1+/--ras, AIB1−/−-ras, and AIB1−/−-ras mice by pairing them with wild-type male mice starting at age 6 weeks. Palpable breast tumors occurred in multiparous AIB1+/--ras and AIB1−/−-ras mice with 50% incidence by age of 26 and 28 weeks, respectively, and their tumor latencies were not statistically different (P = 0.6). However, mammary tumors did not develop in multiparous AIB1−/−-ras female mice to a 50% incidence until age 41 weeks; this was significantly slower (P < 0.001) than in multiparous AIB1+/--ras and AIB1−/−-ras mice (Fig. 3F). These results indicate that the loss of AIB1 function also significantly delays the breast tumor onset in mice cyclically exposed to endogenous reproductive hormones.

Slower Mammary Tumor Growth Rate in AIB1−/−-ras Mice.
To determine whether AIB1 deficiency affects breast tumor growth rate, the L and W of the first palpable breast tumor in each mouse were measured once a week for 8 weeks, and the tumor volumes were estimated by (L × W)2/2 as described previously (22). The average
Although the average number of tumors per mouse was less in AIB1+/−-ras mice than in AIB1+/−-ras mice. Breast tumor growth rate in AIB1+/−-ras mammary tumor cells to the lung in AIB1+/−-ras mice. Interestingly, the average growth rate of breast tumors in AIB1+/−-ras mice was significantly slower than that in AIB1+/−-ras and AIB1+/−-ras mice either with or without pregnancy history (Fig. 4, A and B). These results indicate that loss of AIB1 function also inhibits breast tumor growth after the palpable tumors appear.

To determine whether inactivation of AIB1 also reduces breast tumor multiplicity, the number of breast tumors was counted at the end point of observation for each mouse bearing at least one tumor. The average breast tumor numbers per mouse in the intact AIB1+/−-ras, AIB1+/−-ras, and AIB1+/−-ras wild-type mice were 2.3 ± 1.3 (n = 8), 2.7 ± 1.2 (n = 11), and 1.8 ± 0.7 (n = 5), respectively. The average breast tumor numbers per mouse in the AIB1+/−-ras, AIB1+/−-ras, and AIB1+/−-ras multiparous mice were 2.7 ± 1.1 (n = 11), 2.9 ± 1.6 (n = 13), and 2.1 ± 1 (n = 9), respectively. Although the average number of tumors per mouse was reduced in AIB1+/−-ras-bearing mice at least one breast tumor, the differences were not statistically significant (P > 0.05) when compared with that in AIB1+/−-ras and AIB1+/−-ras mice.

Decrease in Metastasis Frequency of Breast Tumor Cells to Lung in AIB1+/−-ras Mice. To address whether AIB1 plays a role in breast cancer metastasis, we examined the metastatic capacities of mammary tumor cells to the lung in AIB1+/−-ras, AIB1+/−-ras, and AIB1+/−-ras mice. Mice were killed when the diameter of the largest breast tumor reached 2 cm. Three sagittal lung sections spaced at 300-μm were prepared for histopathologic examination of each mouse (25). Focal lung tumors were observed in 42% (5 of 12) of AIB1+/−-ras mice and in 39% (7 of 18) of AIB1+/−-ras mice. Importantly, the frequency of metastasis to lung was reduced significantly to 17% (2 of 12) among AIB1+/−-ras mice, significantly lower than those in AIB1+/−-ras and AIB1+/−-ras mice (P = 0.05; Fig. 4, C and E). The mammary gland origin of these lung tumors was confirmed by reverse transcription-PCR analysis of β-casein mRNA expression. The β-casein gene encodes for a milk protein that is expressed specifically in cells with mammary epithelial origin (5, 35). The β-casein mRNA was detected in breast tumors and lungs with solid tumors isolated from AIB1+/−-ras and AIB1+/−-ras mice but not in normal lungs of wild-type mice (Fig. 4D). These results indicate that AIB1 deficiency reduces breast tumor metastasis.

To examine the changes in cell behavior responsible for the partial suppression of breast tumor metastasis in AIB1+/−-ras mice, AIB1+/−-ras and AIB1+/−-ras mammary tumor cell lines (two lines for each) were developed from primary breast tumors and used for transwell assays to measure their migration ability. Our analysis revealed that the migration ability of AIB1+/−-ras breast tumor cells was significantly lower than that of AIB1+/−-ras tumor cells (Fig. 4F). Furthermore, we also detected that AIB1+/−-MEFs migrated much slower than AIB1+/− MEFs in the same assay system (Fig. 4F). These results suggest that AIB1 plays an intrinsic role in the regulation of cell mobility and that the lower metastasis frequency of breast tumor cells observed in AIB1+/−-ras mice may be related in part to their impaired migration ability.

Comparable Expression of the ras Transgene and MAPK Activation. To determine whether AIB1 deficiency alters the expression of the ras transgene in mammary gland, RPAs were performed using RNA samples isolated from mammary glands and tumors and an antisense RNA probe complementary to the 5′ untranslated sequence of the ras mRNA (GenBank accession no. X00740; Ref. 30). Because the ras transgene is expressed in the mammary epithelium, the expression of K-18, an epithelial cell marker, was counteranalyzed to normalize the expression levels of the ras in the epithelial cells. As expected, the ras mRNA was detected only in AIB1+/−-ras, AIB1+/−-ras, and AIB1+/−-ras mammary glands but not in mammary...
AIB1 deficiency suppresses breast cancer

Expression of ERα, PR, and Their Regulated Genes. To address the question of whether the loss of AIB1 affects ER and PR functions in the mammary gland, we analyzed the expression levels of ERα, PR, and some of their regulated genes. The ERα mRNA was equally expressed in the mammary glands of AIB1+/−/−-ras and AIB1+/−-ras mice, but it was low in the ras-induced breast tumors (Fig. 7A). The ERα immunoreactivity and the number of ERα-positive mammary epithelial cells were similar between young and mature mammary glands of AIB1+/−/−-ras and AIB1+/−-ras virgin mice (Fig. 7B). The number of ERα-positive cells was decreased significantly in the mammary lesions of hyperplasia and MIN in AIB1+/−/−-ras and AIB1+/−-ras mice, and they were undetectable in the breast tumors of AIB1+/−-ras and AIB1+/−-ras mice (Fig. 7B). These results suggest that loss of AIB1 function reduces cell proliferation in the mammary TEBS, regions with hyperplasia and MIN, and in tumor lesions in which high levels of cell proliferation are present. These results also correlate with the reduction of mammary tumor incidence and the delay in breast tumor latency in AIB1+/−-ras mice.
Down-Regulation of the IGF-I Signaling Pathway in AIB1<sup>−/−</sup>-ras Mice. The IGF-I signaling pathway plays an important role in mammary gland tumorigenesis (42). Because AIB1<sup>−/−</sup> mouse embryonic fibroblasts are resistant to IGF-I, we analyzed the levels of several components of the IGF-I signaling pathway in AIB1<sup>−/−</sup> mammary gland and tumor cells (20, 43). In young and mature virgin mammary glands, the levels of IGF-I mRNA were barely detectable by semiquantitative reverse transcription-PCR. Interestingly, IGF-I mRNA was increased significantly in the breast tumors of AIB1<sup>−/−</sup>-ras mice. IGF-I mRNA also was detected in the breast tumors of AIB1<sup>−/−</sup>-ras mice, but its levels were much lower than those in AIB1<sup>−/−</sup>-ras tumors (Fig. 8A). From the same set of RNA samples, highly elevated transforming growth factor β1 mRNA levels also were found in breast tumors, but there were no differences between AIB1<sup>−/−</sup>-ras and AIB1<sup>−/−</sup>-ras tumors with respect to transforming growth factor β1 expression (Fig. 8A).

The protein levels of IGF-IRβ, IRS-1, and IRS-2 in the virgin mammary glands, the levels of IGF-I mRNA were barely detectable by semiquantitative reverse transcription-PCR. Interestingly, IGF-I mRNA was increased significantly in the breast tumors of AIB1<sup>−/−</sup>-ras mice. IGF-I mRNA also was detected in the breast tumors of AIB1<sup>−/−</sup>-ras mice, but its levels were much lower than those in AIB1<sup>−/−</sup>-ras tumors (Fig. 8A). From the same set of RNA samples, highly elevated transforming growth factor β1 mRNA levels also were found in breast tumors, but there were no differences between AIB1<sup>−/−</sup>-ras and AIB1<sup>−/−</sup>-ras tumors with respect to transforming growth factor β1 expression (Fig. 8A).

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mammary glands were undetectable by immunoblot analysis (data not shown). Variable levels of IGF-IRβ protein were detected among mammary glands of AIB1+/+ and AIB1−/− mice at pregnant (day 18) and lactating (day 15) stages without significant correlation with AIB1 genotypes (Fig. 8B; data not shown). In contrast, IRS-1 and IRS-2 proteins were increased dramatically in the lactating glands of AIB1+/+ and AIB1−/− mice (Fig. 8B). The IGF-IRβ protein levels were similar in AIB1+/+−ras and AIB1−/−ras mammary glands and also equally increased in AIB1+/+−ras and AIB1−/−ras breast tumors (Fig. 8C). The IRS-1 protein levels in AIB1−/−ras mammary glands and breast tumors were reduced dramatically as compared with AIB1+/+−ras mammary glands and breast tumors (Fig. 8C). The IRS-2 levels in AIB1−/−ras breast tumors also were significantly lower than those in AIB1+/+−ras breast tumors (Fig. 8C). These data indicate that AIB1 is required for up-regulation of IRS-1 and IRS-2 in mammary glands and breast tumors.

To evaluate the biological consequences caused by the decrease of IRS-1 and IRS-2 in AIB1−/−ras breast tumors, we measured IGF-I-induced DNA synthesis by [3H]thymidine incorporation in cultured AIB1+/+−ras and AIB1−/−ras breast tumor cells. The DNA synthesis in AIB1+/+−ras tumor cells was increased 4- and 4.7-fold after treated with 1 and 10 nM IGF-I, respectively. However, the DNA synthesis was increased only 2.5- and 3.6-fold after identical IGF-I treatments of AIB1−/−ras cells (Fig. 8D). These results indicate that the AIB1-deficient breast tumor cells are partially resistant to IGF-I.

DISCUSSION

Our data demonstrated that AIB1 is expressed mainly in the myoepithelial and luminal epithelial compartments of the mammary gland and in ras-induced mammary tumor cells. Higher concentrations of AIB1 mRNA and protein in mammary glands are associated with specific mammary gland developmental stages with more active epithelial proliferation, such as mammary glands in 6-week-old versus 12-week-old mice and mammary glands in pregnant versus virgin mice, or with more epithelial cells, such as mammary glands in pregnant versus virgin mice. The additional increase of AIB1 concentrations in the mammary tumor samples may represent a combined increase in AIB1 expression level and mammary tumor cell population because AIB1 immunoreactivity in individual tumor cells is comparable with that in the mammary epithelial cells of pregnant mice but stronger than that in the mammary epithelial cells of mature virgin mice. Interestingly, AIB1 protein is localized in the cell nuclei in the mammary glands of virgin and early pregnant mice and in ras-induced mammary tumor cells, which is consistent with its function as a transcriptional coactivator in the nucleus. However, when the mammary epithelium is differentiated fully at late pregnancy and lactation stages, AIB1 is localized mainly in the cytoplasm of luminal epithelial cells, which should impede the involvement of AIB1 in the nucleus for transcription. These results suggest that elevated AIB1 level and its nuclear localization may promote normal and transformed mammary epithelial proliferation and that its lower expression and cytoplasmic localization may facilitate mammary epithelial quiescence and differentiation.

In this study, the specific in vivo role of AIB1 in breast cancer initiation and progression was evaluated by comparing ras-induced mammary tumorogenesis in AIB1+/+−, AIB1−/+−, and AIB1−/− mice under different hormonal conditions. We found that inactivation of AIB1 reduced mammary epithelial proliferative lesions, suppressed breast tumor formation, retarded breast tumor growth, and decreased metastasis rate. Surprisingly, AIB1 deficiency significantly extended breast tumor latencies in mice with normal, elevated, or depleted ovarian hormones, indicating that the AIB1-mediated pathway for breast tumorogenesis is distinct from those mediated by ovarian hormones and their cognate receptors. This notion also is supported by our results showing that the expression of ERα, PR, and their target genes was unaffected in the mammary glands of AIB1−/−ras mice and by the comparison of breast tumor latencies between the OVEX AIB1+/−−ras mice and the intact AIB1−/−−ras mice. The tumor latency in the OVEX AIB1+/−−ras mice without ovarian hormones was T50 = 43 weeks or T50 = 58 weeks, which was significantly faster than that (T50 = 80 weeks) in the intact AIB1−/−−ras virgin mice with ovarian hormones (Fig. 3, A and C). Therefore, the oncogenic role of AIB1 in the mammary gland is independent of or not limited to its coactivator function for ovarian steroid-activated ER and PR, although AIB1 has been shown to mediate estrogen-induced cell proliferation in culture (15, 18, 44).

Conversely, the promotion of mammary tumorigenesis by elevated ovarian hormones and the inhibition of mammary tumorigenesis by ovariectomy were observed in AIB1+/+−ras and AIB1−/−−ras mice, suggesting that AIB1 is not essential for hormonal promotion of mammary tumorigenesis. Consequently, AIB1 and ovarian hormones may additively contribute to the ras-induced mammary tumorigenesis, causing rapid mammary tumor development in AIB1+/−−ras mice with pituitary isografts or pregnant experience. Therefore, a more effective strategy to control breast cancer will need to target AIB1-mediated and ovarian hormone-initiated pathways, which should create a condition similar to that in the OVEX AIB1+/−−ras mice, in which mammary tumor development was suppressed completely.

Expression of the v-Ha-ras oncprotein in the mammary epithelium constitutively activates the MAPK pathway and induces mammary tumorigenesis through enhancing cell proliferation, cell motility, and steroid sensitivity (Fig. 9; Ref. 45). Our data demonstrated that the
presence or absence of AIB1 affects neither the expression levels of the ras transgene nor the activation of MAPK in the mammary glands and tumors in virgin, multiparous, and pituitary-isografted mice, suggesting that AIB1 is not required for the function of the mouse mammary tumor virus transgene promoter and the activation of MAPK. However, it has been shown that protein kinases, including MAPK and IκB kinase, can phosphorlate AIB1, and its phosphorylation leads to nuclear translocation and enhanced AIB1 coactivator function (43, 46, 47). Therefore, AIB1 deficiency should affect specific gene expression regulated by those transcription factors that require AIB1 as coactivator (Fig. 9). Additional studies will be required to characterize the specific transcription factors associated with AIB1 and their direct target genes responsible for AIB1-enhanced mammary tumorigenesis in vivo.

The IGF-I signaling pathway plays a crucial role to enhance mitogenesis, cell survival, and cell invasive behavior (48). The binding of IGF-I to its tyrosine kinase receptor, IGF-IR, causes phosphorylation of insulin receptor substrates (IRS-1 and IRS-2) and activation of phosphatidylinositol 3’-kinase, which is followed by further activation of downstream signaling components, including Akt and Rac (Fig. 9; Refs. 45, 48). Our data demonstrated that the levels of IGF-I expression and IRS-1 and IRS-2 proteins were decreased significantly in AIB1+/− mammary glands and/or tumors, indicating that AIB1 is required for maintenance of these signaling components of the IGF-I pathway. Consistent with the decrease in IGF-I expression in the AIB1+/− mammary tumors, the IGF-I level also was lower in the blood of AIB1+/− mice (20). Although the lower IGF-I levels in the tumors or in the entire mice may have potential inhibitory effects on mammary carcinogenesis, the partial resistance of the cultured AIB1+/−-ras mammary tumor cells to IGF-I clearly indicates that AIB1 has an autonomous role in the mammary epithelial cells for IGF-I response. In agreement with this, a previous study also showed that AIB1-deficient MEFs were resistant to IGF-I-induced proliferation (43). Furthermore, a recent study also demonstrated that depletion of AIB1 in cultured cells resulted in a reduction in the Akt protein and thereby inhibited cell growth (49). These results collectively suggest that AIB1 deficiency causes down-regulation of multiple components of the IGF-I signaling pathway and thereby suppresses mammary epithelial proliferation, tumorigenesis, and metastasis. The important impact of AIB1 on growth factor signaling pathways, including the MAPK and phosphatidylinositol 3’-kinase/Akt pathways, also may explain in part why human breast cancers with high levels of AIB1 and HER-2 are more malignant and tamoxifen resistant and lead to a worse survival rate (Fig. 9; Ref. 16).

In summary, we have discovered that AIB1 is expressed in the mammary TEB, myoepithelial, and luminal epithelial cells. The expression levels and subcellular localizations of AIB1 are regulated in accordance with the stages of mammary gland development and differentiation. AIB1 expression is slightly elevated in the ras-induced breast tumor cells. Inactivation of AIB1 suppresses the ras-induced breast cancer initiation, progression, and metastasis in mice with natural, depleted, and elevated ovarian hormones, which are accredited partially to impaired IGF-I signaling pathway and decreased cell proliferation and migration. In addition, future studies should identify other growth regulation pathways involving AIB1 in carcinogenesis. These data suggest that AIB1 is an oncogene, whose signaling pathway may contain useful targets for prevention and treatment of breast cancers.

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