

***Igf1r* is not required for AIB1-induced mammary hyperplasia and ductal branching**

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1. ABSTRACT

The oncogene AIB1 (amplified in breast cancer 1) is a transcriptional coactivator which is up-regulated in many types of tumors including breast cancer. Studies with cell lines and animal models reveal that AIB1 interacts with the IGF-I signaling pathway at different molecular levels. To determine whether AIB1-dependent cell growth requires IGF-I signaling, we deleted the *Igf1r* gene specifically in the mammary gland of transgenic mice which overexpress AIB1 and are characterized by the development of epithelial hyperplasia, a pre-neoplastic change in breast tissue. Loss of *Igf1r* alone reduced cell proliferation, ductal branching and fat pad occupancy in comparison with wild-type glands. However, in the transgenic mice that overexpress moderate levels of AIB1, the absence of *Igf1r* had a minimal effect on epithelial hyperplasia and ductal branching in the mammary gland. Thus, our results confirm the essential role of *Igf1r* in mammary gland morphogenesis and demonstrate that overexpression of AIB1 circumvents the requirement for the *Igf1r* pathway in promoting epithelial growth during mammary development.

2. INTRODUCTION

Overexpression of the transcriptional coactivator AIB1 (amplified in breast cancer 1) has been linked to cancer in humans (reviewed in (1)) and mice (2). Many studies have shown that AIB1 affects several transduction pathways, including IGF-I/Phosphatidylinositol 3-Kinase (PI3K)/AKT, HER2/neu, NF-KB, and E2F1 signaling pathways (reviewed in (3)). Among them, the IGF-I/PI3K/AKT has been the most extensively characterized. Different lines of evidence with animal models, human tumors, and cell lines have demonstrated that AIB1 is involved in the regulation of AKT activity and in the expression levels of components of the IGF-I/PI3K/AKT signaling pathway.

AIB1 depletion in the human breast cancer cell line MCF-7 reduced IGF-I-induced colony formation (4). Cyclin D1, Bcl-2, ERK2, IGF-IR, and IRS-1 expression levels were also reduced, suggesting that AIB1 activity was important to maintain AKT signaling in MCF-7 cells. This regulation also occurred in the presence of anti-estrogens, revealing new pathways independent of the estrogen

receptor. In the prostate cancer cell lines LNCaP and PC3, AIB1 overexpression promoted cell growth by augmenting AKT activity (5). Additional increase in AKT activity was not observed when LNCaP cells were treated with androgens, indicating that AIB1 functions through steroid-independent pathways to regulate AKT activity. Conversely, AIB1 downregulation resulted in reduced cellular size and decreased AKT activity in these cells. Accordingly, overexpression of AIB1 in LNCaP cells revealed augmented expression levels of several components of the PI3K/AKT pathway, including IGF-I, IGF-II, IRS-1, IRS-2, PIK3CA (PI3K catalytic subunit), and AKT1, whereas AIB1 depletion decreased the levels of these proteins (6). Noteworthy, AIB1 is recruited to the promoter regions of IGF-I, IGF-II, IRS-1, IRS-2, PIK3CA, AKT1, and Bcl-2, suggesting a direct function of AIB1 in the transcription of these genes. These observations suggest that AIB1 is required for IGF-I-dependent cell proliferation and survival in breast and prostate cancers.

Results in animal models agree with those from cell lines. Mammary glands of AIB1 transgenic mice show high levels of phosphorylated IGF-IR and AKT indicating that PI3K/AKT pathway is more active in these animals (2). The increased activity was due to an augment in IGF-I circulating levels. In contrast, deletion of AIB1 in mice reduced IGF-I levels and resulted in growth retardation (7-9). Low IGF-I levels in AIB1-null mice were the result of a rapid degradation of IGF-I. In the absence of AIB1, transcription of the vitamin D receptor (VDR)-target gene IGF-binding protein 3 (IGFBP3) was reduced, resulting in the instability of IGF-I (10). Interestingly, although both acid-labile subunit (ALS) and IGF-binding proteins form complexes with IGF-I to maintain its circulating concentration and endocrine function (reviewed in (11)), lower IGFBP3 levels in AIB1-knockout mice did not correlate with changes in ALS or growth hormone (GH). In addition, levels of IRS-1 and IRS-2 were significantly reduced when AIB1 was depleted in mice expressing the oncogene *Ras* driven by the mouse mammary tumor virus promoter (MMTV) (12). This result further supports a role for AIB1 in maintaining the expression levels of IGF-I signaling molecules relevant for tumor development.

In another study, it was reported that AIB1-knockout mice treated with the carcinogen 7,12-dimethylbenz[*a*]anthracene (DMBA) exhibit reduced mammary ductal morphogenesis and delayed onset of palpable tumors in comparison with wild-type (WT) controls (13). Hence, AIB1 deficiency protected against DMBA-induced tumorigenesis. Levels of IRS-1, IRS-2, p-AKT, and cyclin D1 were augmented in DMBA-induced WT tumors but not in DMBA-induced AIB1-null tumors. This study revealed that AIB1 is required for IGF/PI3K/AKT signaling pathway-stimulated upregulation of cyclin D1 during DMBA-induced breast tumor initiation and progression. All together, these results suggest that AIB1 is an important modulator for mammalian cell growth and that IGF-I signaling is an important effector of its physiological function.

The AIB1 splice variant delta3 in which exon 3 is deleted, is overexpressed in MCF-7 breast cancer cells, as well as in breast tumor tissue (14). A transgenic mouse harboring the AIB1-delta3 isoform was generated in which its expression was driven by the human cytomegalovirus immediate early gene 1 (*hCMVIE1*) promoter (15). Although no tumors were seen, mice showed increased mammary proliferation and gland mass together with increased expression levels of IGF-IR, cyclin D1, and C/EBPbeta liver-enriched inhibitory protein (LIP) isoform. These results suggested that AIB1 functions through different pathways. To analyze the role of AIB1 during the first steps of breast cancer, we generated a mouse model which overexpresses moderate levels of AIB1 in the mammary gland (AIB1-tg) (16). Similarly to the AIB1-delta3 tg mice, AIB1-tg mice did not generate tumors. However, they displayed mammary hyperplasia at the onset of puberty. Interestingly, primary mammary epithelial cultures derived from AIB1-tg mice presented enhanced proliferation and augmented levels of cyclin D1 and E-cadherin. Therefore, overexpression of AIB1 may represent one of the pre-neoplastic changes in breast tissue.

The role of the IGF system in the regulation of the mammary gland development and tumorigenesis has been documented thanks to the study of transgenic and knockout mice as well as mammary gland transplantation experiments (17, 18). IGF-I signaling mediates mammary ductal development through the stimulation of epithelial growth and terminal end bud development (19). Similarly, AIB1 enhances mammary gland growth and development after menarche (8). AIB1-knockout mice showed altered regulation of IGF-I expression in specific tissues and a cell-autonomous defective response to IGF-I (7). However, the role of IGF-I in AIB1-mediated mammary gland development and breast tumor initiation has not yet been established. In order to understand the role of IGF-I in the growth and pre-neoplastic changes induced by AIB1 overexpression, we have used the Cre-lox strategy to delete the *Igf1r* gene specifically in the mammary epithelium. Our results show that deletion of *Igf1r* results in retarded epithelial growth together with a poorer occupancy of the mammary fat pad. In contrast, AIB1 overexpression obviates the need for the IGF-IR for mammary gland morphogenesis and proliferation, since loss of this receptor had little effect on the hyperplastic phenotype displayed by AIB1-tg mice. These results suggest that the early development of AIB1-induced breast tumors may be independent of IGF-IR signaling.

3. MATERIAL AND METHODS

3.1. Mice

AIB1-tg mice that overexpress moderate levels of AIB1 in the mammary epithelium were generated in our laboratory in C57Bl/6J genetic background and have previously been described (16). MMTV-Cre transgenic mice (B6129-TgN(MMTV-Cre)4Mam, strain 003553, was purchased from The Jackson Laboratory. Mice harboring loxP sequences flanking exon 3 of the *Igf1r* gene were generated in the Department of Mouse Genetics and Metabolism at the University of Cologne, and have been

previously described (20). Breeding crosses were set up to generate four different genotypes: *Igf1r^{loxP/loxP}*, *Igf1r^{loxP/loxP}/AIB1-tg*, *Cre/Igf1r^{loxP/loxP}* and *Cre/Igf1r^{loxP/loxP}/AIB1-tg*. Mice were maintained in ventilated cages and housed at the pathogen-free barrier area of the Prince Felipe Research Centre. For the quantification of ductal mammary gland occupancy, branching and PCNA staining, females were subjected to vaginal smear analysis and selected in proestrus stage of the estrous cycle. Vaginal smears were evaluated for the cytological composition taking into account the relative proportions of the desquamated cellular types (squamous, intermediate, and parabasal cells), the infiltration of polymorphonuclear leukocytes, and the presence of mucus, and the appearance of the vaginal fluid.

3.2. PCR analysis

Genotype of mice was assessed by PCR analysis of genomic DNA from tail biopsies using the following primers: 5'GGCCCCGGCCCCAAGCTTG3' and 5'CGTGAATCACTGGCCAGTGGATCC3' for AIB1-tg; 5'TCCCTCAGGCTTCATCCGAA3' and 5'CTTCAGCTTTGCAGGTGCACG3' for loxP; deletion of exon 3 was verified with oligos 5'TTATGCCTCCTCTCTTCATC3' and 5'CTTCAGCTTTGCAGGTGCACG3'; oligos 5'AGTTGCTGAAGGGAGGGTAGCGGC3' and 5'TGACCAGGCCGTCTCTGTGTAATTGG3' for *Rasgrf1* gene were used as an internal positive control; Cre transgene was genotyped following the recommendations by the supplier (http://jaxmice.jax.org/protocolsdb/f?p=116:2:3733608331999007::NO:2:P2_MASTER_PROTOCOL_ID,P2_JRS_CODE:552,003551). For Reverse Transcriptase-PCR (RT-PCR), RNA was isolated (TRIZOL, GibcoBRL) from tissues and digested with DNase followed by purification through QIAGEN columns (RNeasy kit). Subsequently, the RT reaction (SuperScript, Invitrogen) was done with 0.5 µg of total RNA and oligo dT. RT-PCR was performed with 25ng RNA-equivalent cDNA using the following oligos for AIB1: 5'GCTTGAACATCCTTTGACTGG3' and 5'TAAGATTGGCAGATATCAGCTCAGC3'; for testing deletion of exon 3 in the mRNA: 5'AGGAGAAGCCCATGTGTGAG3' and 5'CAGGAAGGACAAGGAGACCA3'; and for expression of Cre transgene 5'GACCAGGTTCGTTCACTCATGG3' and 5'AAATCCATCGCTCGACCAGTTT3'. *Gapdh* was used as a house keeping gene with oligos 5'ACCACAGTCCATGCCATCAC3' and TCCACCACCCTGTTGCTGTA3'.

3.3. Western blot

Protein lysates were done with commercial lysis buffer (Cell Signaling) supplemented with protease cocktail inhibitors (Roche), NaCl to make a final concentration of 150mM, 1mM AEBSF (Roche), 1mM NaF. Fifty mg of each protein lysate were transferred to Immobilon-P membranes (Millipore, Bedford, Mass.) and blotted with the indicated antibodies: monoclonal anti-AIB1 (611105, BD), polyclonal IGF-IRbeta (3027, Cell Signaling), polyclonal Cre (69050, Novagen) and monoclonal beta-actin (A1978, SIGMA).

3.4. Mammary epithelial cell culture

Mammary glands from 12-13 week-old virgin females were collected from sacrificed animals, carefully minced in DMEM/F12 media without serum and digested with 1 mg/ml Collagenase A (Roche) for 45 minutes at 37°C. Digested tissue was washed once and resuspended in DMEM/F12 media supplemented with 5% FBS, 50 microg/ml gentamicin, 5 microg/ml insulin, 1 microg/ml hydrocortisone, 10 ng/ml murine EGF and 5 microg/ml linoleic acid. Mammary epithelial cells were grown in incubators at 37°C with 5% CO₂.

3.5. Immunohistochemistry

Tissues were fixed overnight in 10% neutralized buffered formalin at 4°C and embedded in paraffin by standard procedures. Sections of 4 µm thickness were processed for immunohistochemical analysis following antigen retrieval. Cross-sections were stained with anti-PCNA antibody (PC10, Santa Cruz Biotech.) and counterstained with hematoxylin.

4. RESULTS AND DISCUSSION

In order to evaluate the role of IGF-I signaling in AIB1-mediated mammary epithelial growth we used the Cre-lox technology to delete the *Igf1r* gene in the mammary gland of AIB1-tg mice that moderately overexpress AIB1 (15). Both Cre and AIB1 transgenes were driven under the MMTV promoter. Deletion of exon 3, which codes for the IGF-I ligand domain, has been shown to produce a frame shift, resulting in an intact mRNA with a stop codon in exon 4 (21, 22). Therefore, we used recombinant mice harboring loxP sites flanking exon 3 to target the *Igf1r* locus (20). Crosses were set up so that we could generate the four different genotypes of interest: wild-type (WT), deletion of *Igf1r* in the mammary epithelium (*Igf1r^{ΔExon3}*), transgenics for AIB1 (AIB1-tg), and both *Igf1r^{ΔExon3}/AIB1-tg*. Genotyping of mice also included a set of oligos for Cre transgene that when inherited, resulted in the deletion of flanked exon 3 (Figure 1). No growth variations among different litter groups were observed, suggesting that these mutant females did not display impaired lactation. The expression pattern correlating with these genotype groups was further confirmed by RT-PCR. We previously characterized the expression of AIB1 transgene with the onset of puberty and this expression increased during the next 4 weeks (16). Therefore, we analyzed females at 7 weeks and 11 weeks of age, providing enough time for the transgene to exert their effect during the development of mammary ducts. As expected, expression of AIB1 and Cre in the mammary glands was clearly detected at 7 weeks of age (Figure 2A), as well as in primary cultures derived from mammary glands (Figure 2B). In addition, Cre expression coincided with the deletion of floxed exon 3 (*Igf1r^{ΔExon3}*). The fatty stroma does not express MMTV transgenes and, therefore, contributed to the undeleted *Igf1r* allele that was also detected in the gel (Figure 2A). It is also possible that some of the undeleted *Igf1r* may result from epithelial expression, since MMTV-Cre functions incompletely in mammary epithelium due to a mosaic expression pattern (23). However, at 14 weeks this contribution is minor and over time, most cells lose

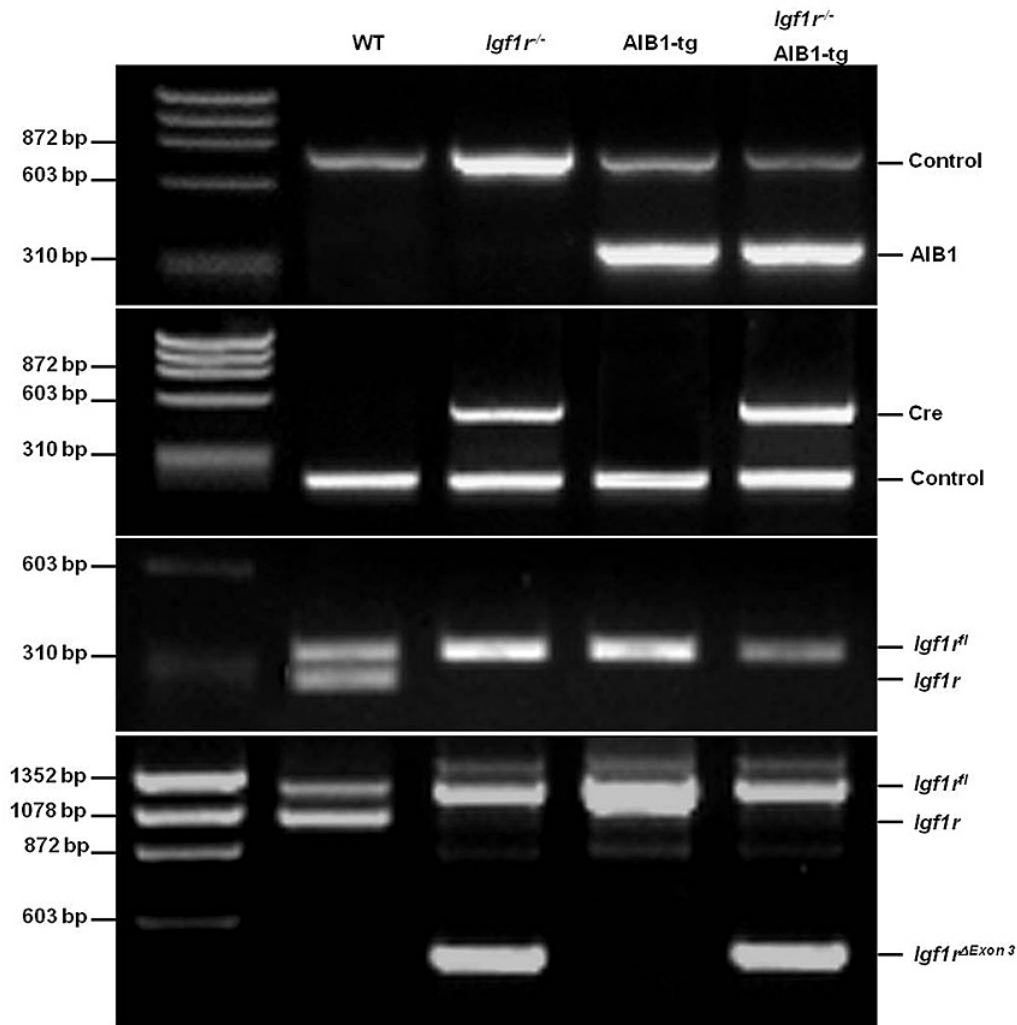


Figure 1. PCR strategy to genotype littermates for AIB1 and Cre transgenes, and the loxP-flanked exon 3 of the *Igf1r* gene. DNA isolated from tail biopsies was subjected to four sets of PCR reactions to define mouse genotype and to confirm the genomic deletion of exon 3 (*Igf1r*^{ΔExon3}). In parallel, one positive control for the PCR reaction was included for each transgene (Control). Reactions were resolved in a 2 % agarose gel. Based on the results of these PCRs, the four genotype groups were assigned as indicated on the top of each line (WT: wild- type; *Igf1r*^{-/-}: deletion of exon 3 in *Igf1r* occurring when both Cre and *Igf1r*^{fl} were concomitantly inherited; AIB1-tg: AIB1 transgenic mice; *Igf1r*^{-/-} AIB1-tg: mice with deletion of exon 3 in *Igf1r* plus transgenics for AIB1).

targeted gene function presumably due to additional rounds of Cre-mediated recombination (24). Moreover, primary culture of mammary epithelial cells barely expressed IGF-IR in *Igf1r*^{-/-} mice (Figure 2B), further supporting that exon 3 of *Igf1r* gene was also deleted within the mammary epithelium.

Mammary ductal outgrowth and branching was analyzed in 7- and 11-week-old virgin females. As previously reported (16), AIB1-tg epithelium from 7-week-old females exhibited extensive ductal branching with prominent terminal end buds (TEBs) (compare magnified panels, Figure 3). These characteristics of the AIB1-tg epithelium also persisted in fully mature mammary glands at 11 weeks of age. Mammary epithelium in AIB1-tg mice

also showed increased fat pad occupancy as well as secondary branching (Figure 3). Importantly, mammary gland branching depends on paracrine IGF-I signaling (25). In addition, IGF-IR expression localizes to the proliferating ductal epithelium of the mammary glands (26). Hence, based on AIB1-dependent regulation of the IGF-I signaling pathway, we hypothesized that IGF-I may mediate AIB1-induced proliferation. Specific deletion of *Igf1r* in the mammary epithelium of 7-week-old virgin females resulted in reduced ductal branching and morphogenesis (Figure 3). At 11 weeks, fat pad of *Igf1r*^{-/-} virgin females showed extended ductal outgrowth, probably due to the proliferative action of repetitive estrous cycles. However, the reduced branching and fulfill of the fat pad still persisted (compare 7 vs. 11 weeks of *Igf1r*^{-/-} in Figure 3).

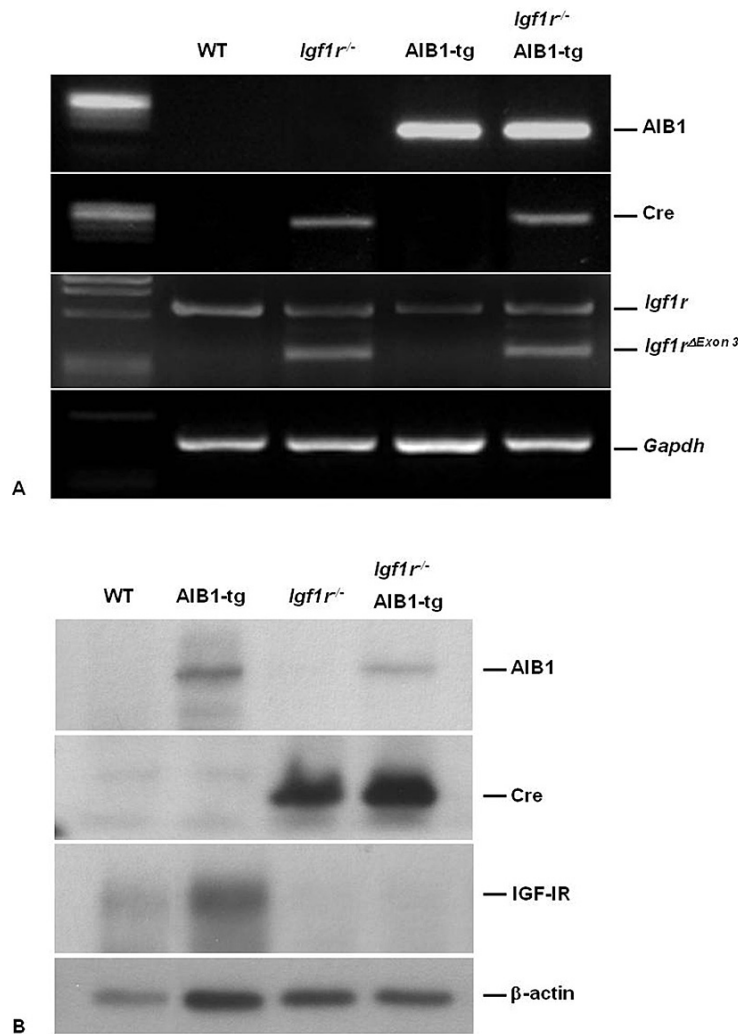


Figure 2. Expression profiles for AIB1 and Cre transgenes, together with *Igf1r* isoforms. (A) Total RNA isolated from the mammary gland of 7-week-old virgin females was used in four RT-PCR reactions to confirm the different genotype groups. Reactions were further resolved in a 2 % agarose gel. *Gapdh* was used as a positive control to validate each set of reactions. (B) Primary cultures of mammary epithelial cells derived from mammary glands of 12-13-week-old virgin females were analyzed by western blot using the indicated antibodies. Antibodies against β-actin were used as loading control.

Noteworthy, expression of the AIB1 transgene in the *Igf1r*^{-/-} genetic background greatly restored ductal branching. This rescue of the phenotype was obvious even at 7 weeks of age, suggesting that there are other signaling pathways, different from IGF-IR, that mediate AIB1-dependent ductal branching and outgrowth.

We quantified the percentage of filled fat pads in adult mice. Consistent with the observed morphology in whole mount mammary glands, the area occupied by the epithelium within the fat pad was significantly increased in AIB1-tg versus WT mice and was reduced in *Igf1r*^{-/-} mice (Figure 4A). Noteworthy, this response and the reduction in lateral side branching occurring in *Igf1r*^{-/-} mammary glands were significantly rescued by AIB1 overexpression (Figure 4A,B), both at 7 and 11 weeks. These observations suggest that the role for AIB1 in ductal morphogenesis as well as in

pre-tumoral lesions is independent of *Igf1r* and indicate that additional genetic/epigenetic modifications are subsequently required to further develop breast tumors. Although enhanced IGF-I signaling may represent one of the triggering modifications required for tumor development, our results show that this event is not required for AIB1-induced ductal branching and hyperplasia.

To evaluate cell proliferation under the four different genotype backgrounds we performed immunohistochemical analysis of PCNA in mammary gland tissue sections. As shown in the representative images of Figure 5A, and in agreement with the morphology observed in the whole mount studies, PCNA immunoreactivity was abundant in ductal epithelial cells of AIB1-tg versus WT mice; in contrast, PCNA-positive cells

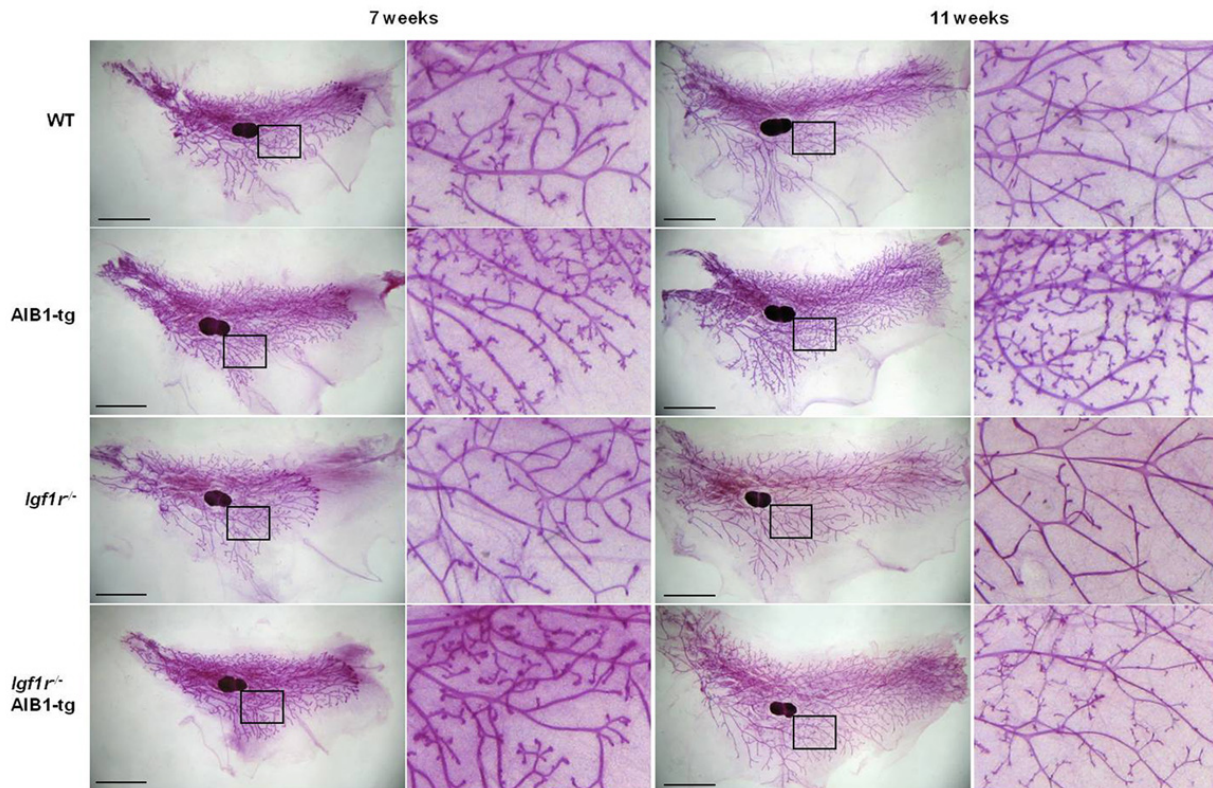


Figure 3. *Igf1r* is not required for AIB1-induced hyperplasia and ductal branching in the mammary epithelium. Representative whole mount staining of the four genotype groups. At least 10 animals were analyzed for each genotype. Mammary fat pads were isolated from 7-week-old and 11-week-old virgins. Right panels for each age column represent a magnification of the framed area in the image of the whole mammary gland. Mammary glands were dissected, mounted on glass slides, and stained as described in <http://mammary.nih.gov/tools/histological/Histology/index.html>. Scale bars: 5 mm.

were scant in *Igf1r*^{-/-} mice, but AIB1 transgene expression in *Igf1r*^{-/-} / AIB1-tg mice rescued the positivity for PCNA. Figure 5B shows the quantitative analysis of these results, which revealed that AIB1-tg significantly increased mammary epithelial cell proliferation independently of IGF-IR expression, although the proliferative activity in *Igf1r*^{-/-} / AIB1-tg was not as high as in AIB1-tg alone, suggesting that IGF-IR may also contribute to some extent to the proliferative and morphological changes observed in AIB1-tg with intact *Igf1r*. Taken together these results support that cellular proliferation and mammary hyperplasia induced by AIB1 overexpression does not necessarily require IGF-IR signaling and, therefore, other pathways may account for the increased proliferation observed in AIB1-tg mice.

As a transcriptional coactivator, AIB1 can promote the expression of estrogen receptor and E2F1-dependent transcription of cyclin D1 (27, 28), as well as the expression of genes important for initiation of DNA replication (29). These AIB1 molecular targets may therefore explain its role in cell proliferation independently of IGF-I. In addition, prevention of apoptosis could also be another mechanism that consequently favors the hyperplasia observed in AIB1-tg mice. In this regard, we have recently described that AIB1 downregulates the pro-

apoptotic factor DRO1 (30), a tumor suppressor also repressed by several other oncogenes (31). Thus, DRO1 and IGF-IR signaling could represent two AIB1-induced pathways important to inhibit apoptosis, but independent of AIB1-induced proliferation. Another regulatory mechanism is the subcellular redistribution of AIB1. We found that nuclear shuttling of AIB1 correlates with cell cycle progression in non-cancer cells (16), but this distribution is altered in cancer cell lines (32). This subcellular trafficking is dependent on IKK and importin alpha-3 (33-35), thus constituting another potential mechanism for IGF-1-independent regulation of AIB1. Further studies are therefore warranted to identify the pathways that are required for AIB1-induced hyperplasia and the ensuing progression to cancerous growth, which should help predict the clinical outcome of breast tumors and may constitute potential targets for improving both diagnostic tools and therapies for breast cancer.

5. ACKNOWLEDGEMENTS

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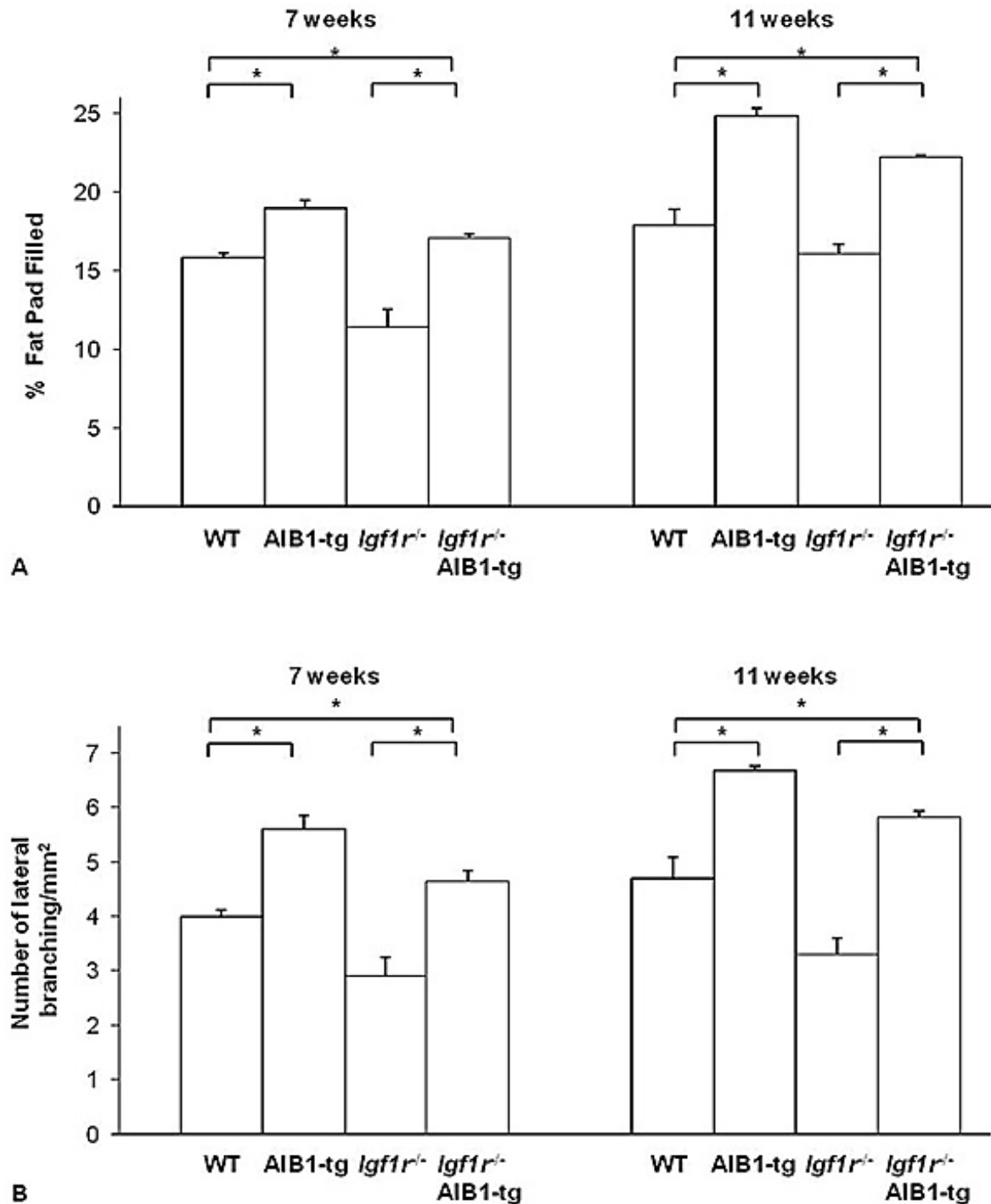


Figure 4. Increased fat pad occupancy in AIB1-tg does not require *Igf1r*. (A) Reduction in the percentage of fat pad filled (% FPF) in the mammary glands of *Igf1r*^{-/-} was significantly rescued by AIB1 overexpression. Images of whole mount mammary glands were taken with a Leica microscope and analyzed with ImageJ 1.42q software (Broken Symmetry Software). Percentage of FPF was measured by dividing ductal area vs. whole fat pad area in at least three animals for each genotype. *: p<0.05. (B) Number of lateral branching per square mm was determined. Images of whole-mount mammary glands were analyzed with SigmaScan Pro v.5.0.0. A 3x3 mm overlying grid layer was combined with the whole-mount images. The number of ductal branch points was counted from 2 fields (7-week-old) or 3 fields (11-week-old) per whole mount in at least three mice for each genotype. Values represent average number of ductal branch points per square millimeter. *p<0.05.

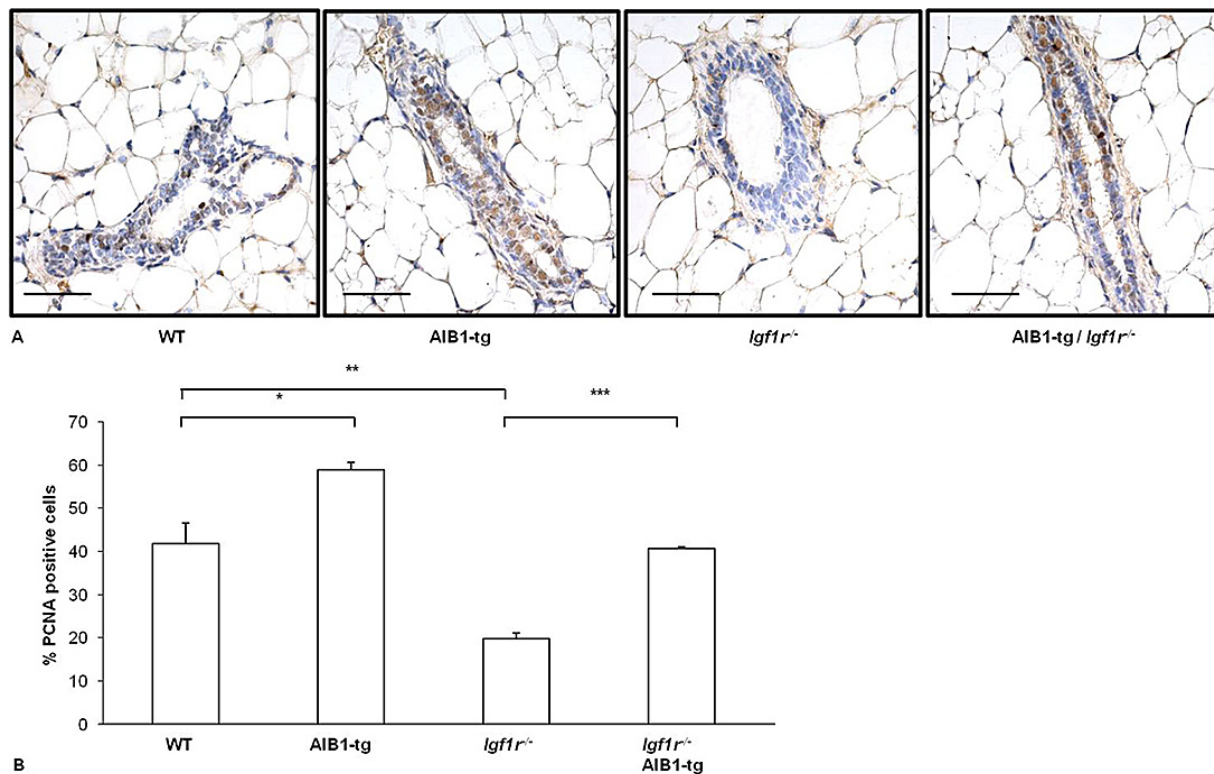


Figure 5. Increased proliferation in mammary epithelial cells of AIB1-tg does not require *Igf1r*. (A) Sections for each genotype were stained with anti-PCNA antibodies to visualize proliferative nuclei and counterstained with hematoxylin. Scale bars: 50 microm. (B) Proliferation was estimated by counting positive PCNA nuclei in mammary gland tissue sections. The number of PCNA-positive cells was counted in 10 fields per slide from three animals of each genotype. Hematoxylin-stained nuclei were scored as a reference for total cell number. *: $p=0.004$; **: $p=0.001$; ***: $p=10^{-5}$.

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