Elevated Circulating IGF-I Promotes Mammary Gland Development and Proliferation

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Animal studies have shown that IGF-I is essential for mammary gland development. Previous studies have suggested that local IGF-I rather than circulating IGF-I is the major mediator of mammary gland development. In the present study we used the hepatic IGF-I transgenic (HIT) and IGF-I knockout/HIT (KO-HIT) mouse models to examine the effects of enhanced circulating IGF-I on mammary development in the presence and absence of local IGF-I. HIT mice express the rat IGF-I transgene under the transthyretin promoter in the liver and have elevated circulating IGF-I and normal tissue IGF-I levels. The KO-HIT mice have no tissue IGF-I and increased circulating IGF-I. Analysis of mammary gland development reveals a greater degree of complexity in HIT mice as compared to control and KO-HIT mice, which demonstrate similar degrees of mammary gland complexity. Immunohistochemical evaluation of glands of HIT mice also suggests an enhanced degree of proliferation of the mammary gland, whereas KO-HIT mice exhibit mammary gland proliferation similar to control mice. In addition, HIT mice have a higher percentage of proliferating myoepithelial and luminal cells than control mice, whereas KO-HIT mice have an equivalent percentage of proliferating myoepithelial and luminal cells as control mice. Thus, our findings show that elevated circulating IGF-I levels are sufficient to promote normal pubertal mammary epithelial development. However, HIT mice demonstrate more pronounced mammary gland development when compared to control and KO-HIT mice. This suggests that both local and endocrine IGF-I play roles in mammary gland development and that elevated circulating IGF-I accelerates mammary epithelial proliferation.

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Mammary gland development is characterized by ductal proliferation and elongation. The initiation of ductal development begins when the terminal ends of the rudimentary ductal structures occupying the mammary epithelium enlarge and form terminal end buds (TEBs), sites at which cell proliferation is very active (1, 2). The TEBs repeatedly grow and bifurcate, extending into the mammary fat pad forming a system of primary branched ducts.

Development of the mammary gland is under strict hormonal control by both ovarian (estrogen and progesterone) and pituitary [prolactin and growth hormone (GH)] hormones (3, 4). IGF-I is an important growth factor regulating both ovarian and pituitary hormonal control, particularly for pubertal ductal growth. The postnatal effects of GH in mammary gland development are mediated predominantly by IGF-I (5–7). The action of estrogen and progesterone also are dependent on the presence of IGF-I (8). Consistent with these findings, IGF-I null female mice demonstrate significantly impaired mammary growth, highlighting the fundamental importance of this growth factor in mammary gland development (7).

IGF-I possesses characteristics of both an autocrine/paracrine (tissue) growth factor and an endocrine (circu-

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Abbreviations: BrdU, 5-Bromo-2-deoxyuridine; GH, growth hormone; HIT, hepatic IGF-I transgenic; IGF-IR, IGF-I receptor; IR, insulin receptor; KO-HIT, IGF-I knockout/HIT; LID, liver IGF-I deficient; LL, control; TEB, terminal end bud.
Circulating IGF-I is produced in the liver via the hypothalamic-pituitary axis and secretion of growth hormone. Extensive work has focused on the relative contributions of circulating and locally-derived IGF-I and is an ongoing area of debate. Both IGF-I and IGF-II, as well as the IGF-IR mRNAs, are expressed in the developing mammary gland. The ligands are expressed in TEBs but have distinct patterns of expression in mammary epithelium at different developmental stages and are found also in mammary stroma (9). Mammary expression of IGF-I, particularly in stroma, is induced by GH (10). IGF-IR mRNA is expressed throughout the mammary epithelium at all postnatal stages of growth (9). Local administration of des (1–3) IGF-I (a truncated form of IGF-I with reduced affinity for IGF binding proteins) to IGF-I null mice leads to increased TEB formation and branching in vivo (7). Experiments using conditionally expressed transgenes specific to mammary epithelium, have demonstrated that IGF-I expression via the MMTV-LTR promoter results in enhanced alveolar bud development in late adolescent virgin glands, and expression via the WAP milk protein promoter results in delayed involution of lactating glands (5, 11–14). These results have highlighted the importance of local sources of IGF-I in mammary gland development. Recent studies support distinct functions for locally produced epithelial and stromal IGF-I. A targeted deletion of IGF-I in the epithelium leads to decreased ductal branching whereas stromal sources of IGF-I mediate alveolar budding and proliferation during early pregnancy (15). These data support a unique role for local IGF-I, in mammary gland development, and imply that IGF-I derived from different cellular components in the mammary gland may have different biological functions.

Circulating IGF-I also can have an important influence on mammary gland development. Mice with moderate multiorgan deficiency of IGF-I (IGF-I m/m or midi mice), with a 70% reduction in serum IGF-I levels, have ducts that extend through the fat pad but fewer branching/bifurcation structures than do wild-type mice (16). In contrast, mice with a liver specific deletion of IGF-I [liver IGF-I-deficient (LID) mice], with a 75% reduction in circulating IGF-I but normal IGF-I in the mammary glands, demonstrate no differences in the extent of branching points when compared with control mice (16). Despite a similar reduction in serum IGF-I levels in both transgenic lines, only the midi mouse with reduced tissue IGF-I demonstrates impairment in mammary gland development.

Given that previous studies have suggested that local IGF-I is essential for mammary gland development, we wanted to examine the effects of elevated circulating IGF-I on mammary gland development in the presence and absence of local IGF-I. Our hypothesis was that tissue IGF-I combined with elevated circulating IGF-I would lead to enhanced mammary gland development and that in the absence of local IGF-I, even with high serum IGF-I, mammary gland development would be significantly reduced. To test this theory, we used the following three mouse models: 1) control mice, with normal levels of circulating and local IGF-I; 2) Hepatic IGF-I transgenic (HIT) mice, with normal tissue IGF-I but high circulating IGF-I due to a liver specific expression of a rat igf-1 transgene; and 3) mice with global ablation of the igf-1 gene but overexpression of the rat igf-1 transgene in liver [IGF-I knockout/ HIT (KO-HIT)], thus having increased serum IGF-I and absent local IGF-I (17). In this study we report that high circulating IGF-I can substantially accelerate mammary gland development when local tissue IGF-I levels are normal. However, contrary to what we had expected, elevated circulating IGF-I levels are sufficient for normal mammary gland development in the absence of local IGF-I.

Materials and Methods

Animals
All animal experimentation procedures were approved by the Animal Care and Use Committee of Mount Sinai School of Medicine. Animal care and maintenance were provided through the Mount Sinai Association Assessment and Accreditation of Laboratory Animal Care-accredited animal facility. Female HIT and KO-HIT mice (on FVB/N genetic background) were generated as previously described (17). Mice were housed in a clean mouse facility, fed standard mouse chow (Picolab rodent diet 5053; LabDiet, St. Louis, MO) and water ad libitum and were kept on a 12-h light, 12-h dark cycle.

Analysis of mammary gland development
The number four inguinal mammary gland was excised and spread onto a glass slide and fixed for 2–4 h in Carnoy’s fixative (60% ethanol, 30% chloroform and 10% glacial acetic acid). The fixed glands were hydrated, stained overnight in carmine alun, dehydrated, cleared in xylene overnight (Fisher Scientific, Pittsburgh, PA), and mounted with Mount-Quick mounting medium (Daido Sangyo Co., Tokyo, Japan) as previously described (18). Photographic documentation was performed using a stereomicroscope and MicroSuite FIVE imaging software (Olympus, Center Valley, PA). The extent of ductal branching was quantified by determining the number of intersecting branches along a line drawn between the leading edge of the ducts and the lymph node. Complexity was measured as the number of branches per unit length. Ductal extension into the fat pad was measured as the ratio of duct length to fat pad length. The number of TEBs per gland were counted, quantified, and are depicted per total gland area.

Histology
For histological procedures, the mammary glands were removed, fixed in 4% paraformaldehyde, and paraffin embedded.
Five-micrometer sections were deparaffinized and stained with hematoxylin and eosin according to standard protocols.

**Immunofluorescence**

To quantify 5-bromo-2-deoxyuridine (BrdU) incorporation (as detected by immunofluorescence), 6- to 8-week-old female mice were injected intraperitoneally with BrdU (Sigma Aldrich, St. Louis, MO) at 10 µl/gram of body weight, 2 h before sacrifice. Glands were fixed in 4% paraformaldehyde and embedded in paraffin. Sections were deparaffinized, rehydrated, subjected to 10 mM sodium citrate buffer antigen retrieval (microwave for 20 min), acid digestion for 45 min, blocking in TGB superblock [10% BSA, 0.05% NaN₃], 10% goat serum (Dako North America, Carpinteria, CA) and 0.5M Tris (pH 7.6) for 1 h and incubated overnight at 4°C with a mouse monoclonal antibody against BrdU (IIB5; 1:250; Santa Cruz Biotechnology, Santa Cruz, CA) and a rabbit polyclonal antibody against cytokeratin 14 (ck14; 1:200; Panomics, Redwood City, CA). Immunofluorescence staining was detected by incubation with secondary AlexaFluor 568-conjugated (red) donkey antirabbit IgG (1:500, Molecular Probes, Eugene, OR) and AlexaFluor 488-conjugated (green) goat antimouse IgG (1:100; Molecular Probes) for 2 h. Nuclei were counterstained with DAPI (0.5 µg/ml; Sigma Aldrich). Sections were then mounted using Fluorogel mounting medium (Electron Microscopy Sciences, Hatfield, PA). An Olympus AX70 fluorescence microscope (Olympus) was used to capture images from immunofluorescence staining with DP Manager version 3.1.1, 2008 (Olympus) software. Low power images were taken at a ×40 magnification to count the total number of BrdU-positive ductal elements. At least seven individual ×400 fields per group were captured for counting BrdU-positive cells. The ductal structure contained in each of these fields was identified, classified, and the number of total cells and BrdU⁺ cells were counted. Cells were classified as myoepithelial or luminal given the presence or absence of myoepithelial-specific ck14 staining, respectively. Immunolabeling using the luminal cell specific markers, cytokeratin 19 was used to confirm (1:50, Developmental Studies Hybridoma Bank, Iowa City, IA).

**Protein isolation and Western immunoblotting**

Tissue samples were lysed in buffer (pH 7.4) containing 50 mM Tris, 150 mM NaCl, 1 mM EDTA, 1.25% CHAPS (Roche, Indianapolis, IN), 1 mM sodium orthovanadate, 2 mM sodium fluoride, 10 mM sodium pyrophosphate (Sigma-Aldrich), 8 mM β-glycerophosphate (VWR, West Chester, PA) and Complete® Protease Inhibitor Cocktail (Roche). Concentrations of tissue proteins were determined by BCA protein assay using bovine serum albumin as a protein standard. Ten to 50 µg of cell lysates were resuspended in 3× loading buffer supplemented with DTT, de-natured by boiling at 96°C, subjected to SDS-PAGE (8% Tris-glycine gel) (Invitrogen, Carlsbad, CA), and transferred to nitrocellulose membrane (Bio-Rad, Hercules, CA). The membrane was sequentially blocked and probed with primary and secondary antibodies according to the manufacturers' instructions. The membrane was then analyzed by direct infrared fluorescence detection using the Odyssey Infrared Imaging System (Li-cor Biosciences, Lincoln, NE). Densitometric analysis was performed using the MacBas V2.52 software. The following primary antibodies were used: anti-phospho-IRb Y1150/51/IGF1Rb Y1135/36, anti-phospho-AKT (Ser 473), anti-AKT (pan) (40D4), anti-phospho-p44/42 MAP Kinase (Thr202/Tyr204), and anti-p44/42 MAP kinase (Cell Signaling Technology, Danvers, MA) and anti-IRb (Santa Cruz Biotechnology).

**Statistical analysis**

All data are presented as mean ± SEM. One-way ANOVA was used to test for differences among groups at each age. If one-way ANOVA revealed significance, Bonferroni post hoc comparisons followed (Statistica Software 6.0, StatSoft Inc, Tulsa, OK). P < 0.05 was considered statistically significant.

**Results**

HIT and KO-HIT mice were generated as previously described (17). Liver-specific IGF-I transgene expression in HIT and KO-HIT mice led to threefold increase in serum IGF-I above control mice at 4, 8, and 16 wk of age, as previously reported (17, 19). In agreement with previous reports, HIT mice with elevated serum IGF-I had increased body weight compared with control mice at 8 and 16 wk of age (Fig. 1A) (17, 19). Mammary gland weight of HIT mice, however, was less than that of wild-type control (LL) mice at 4 wk of age, but did not differ from LL mice at 8 and 16 wk of age (Fig. 1B). KO-HIT mice, with elevated serum IGF-I and total ablation of autocrine/paracrine IGF-I, did not significantly differ from LL mice in terms of body weight (Fig. 1A) or mammary gland weight (Fig. 1B).

HIT mice show enhanced mammary gland growth, whereas KO-HIT mice exhibit mammary gland development similar to that of control mice

To evaluate mammary gland development, whole mount preparation of the no. 4 inguinal gland was used (Fig. 2A). Overall, growth of the gland was more pronounced in the HIT mice compared with LL control mice; the HIT glands contained more and larger TEBs at 4 wk of age and increased ductal branching at 8 and 16 wk compared with LL control or KO-HIT glands (Fig. 2A). Morphometric analysis of whole mount preparations of 4-wk-old HIT virgin female mice confirmed more advanced mammary gland development than age-matched LL mice, both with regards to the degree of ductal extension (0.373 ± 0.03 vs. 0.286 ± 0.02 U, P < 0.05) and the number of the TEBs (6.3 ± 0.62 vs. 4.3 ± 0.21 U, P < 0.005) (Fig. 2, B and C). Eight-week-old HIT virgin female mice also demonstrated a greater degree of ductal extension than control mice (0.91 ± 0.06 vs. 0.85 ± 0.07), although this difference was not statistically significant. Additionally, mammary gland complexity, which represents the extent of ductal branching, was significantly increased in the HIT mice at 4 (10.9 ± 1.13% vs. 8.5 ± 0.34%, P < 0.05) and 16 (18.8 ± 0.75% vs. 10.7 ±
In our study, we observed that HIT mice showed enhanced mammary gland complexity when compared with control mice, whereas mammary glands of KO-HIT mice demonstrated a degree of mammary gland development equivalent to that of control mice.

**HIT mice show an enhanced degree of proliferation of the mammary gland, whereas KO-HIT mice exhibit mammary gland proliferation similar to that of LL mice**

To test whether the enhanced mammary gland development in HIT mice is the result of increased cellular proliferation rates (compared with KO-HIT and control mice), we monitored replicating cells by injecting 6- to 8-wk-old virgin HIT, KO-HIT, and LL mice with BrdU. Using immunofluorescence labeling performed on tissue sections collected 2 h postinjection, we were able to detect BrdU incorporation as a measure of S phase growth. Analysis of BrdU incorporation into mammary epithelium demonstrated that HIT female virgin mice had increased cell proliferation, measured by percentage of BrdU cells per total cells, in various epithelial structures. Epithelium was classified and scored separately as TEBs.
and ducts. Cells within TEBs were further defined as cap or body cells. A total of 43 TEBs were analyzed in LL mice, 50 TEBs were analyzed in HIT mice, and 25 TEBs were analyzed in KO-HIT mice. Thus, a large number of TEBs were analyzed for each genotype and there was variability across TEBs even within genotype. Ductal structures were analyzed separately by type as well as combined in total for an overall assessment of cellular proliferation in the mammary gland epithelium. The HIT mice showed increased BrdU incorporation in all types of epithelial elements evaluated, with an increase in the percent of BrdU+ cells within both TEBs (15.6 ± 3.4% vs. 4.4 ± 2.8%, P < 0.05) and ducts (10.8 ± 1.2% vs. 3.5 ± 2.0%, P = 0.01) (Fig 4, A–C). When TEBs were divided into cap cells and body cells, percent of BrdU+ cells were increased in HIT mice in body cells (14.6 ± 2.4% vs. 4.4 ± 2.1%, P < 0.05) but not cap cells. The cap cells showed the same trend as the body cells, however there was no statistically significant difference between HIT and LL mice. While the possibility of a type II error cannot be excluded, it is plausible that the decreased number of total BrdU+ cap cells prevents large numbers of cells from being counted and included in the analysis. With all structures combined, BrdU incorporation was increased threefold in the epithelium of HIT mice compared with LL mice (14.1 ± 0.4% vs. 4.7 ± 2.1%, P < 0.01). Thus, the HIT mice showed an increased cellular proliferation compared with control mice at 6–8 wk that correlates well with their enhanced mammary gland development and areas of increased epithelial density.

As expected, given the similar level of mammary gland development in KO-HIT and female control mice, we discovered that the degree of cellular proliferation, as assessed by BrdU incorporation, between these lines was comparable (Fig. 4A). KO-HIT mice showed a similar percent of BrdU+ cells (per total cells) to control mice in all TEBs in total count as well as when these structures were


scored separately as cap and body cells (Fig. 4A). KO-HIT mice also show equivalent rates of BrdU incorporation to control mice when ducts were evaluated (Fig. 4, A and B). Lastly, KO-HIT mice and control mice had equivalent percentages of BrdU/H11001 cells (per total cells) when all ductal structures were combined for analysis (Fig. 4A). Taken together, KO-HIT mice and control mice had similar rates of cellular proliferation in 6- to 8-wk-old virgin mammary epithelium.

HIT mice have a higher percentage of proliferating myoepithelial and luminal cells than do control mice, whereas KO-HIT mice have an equivalent percentage of proliferating myoepithelial and luminal cells to control mice

In vitro and in vivo studies have demonstrated that IGF-I stimulates breast cancer cell growth (20, 21). Higher circulating IGF-I levels have been found in breast cancer patients compared with normal controls (22–25). The ductal structures of the mammary gland are composed of an inner layer of luminal epithelial cells surrounded by an outer layer of myoepithelial cells (1). Traditionally luminal cells have been thought to be a more direct target for tumorigenesis than are myoepithelial cells (26). Myoepithelial cells dictate normal luminal polarization, have been postulated to inhibit dysregulated luminal cell growth in breast cancer (27), and may act as an overall tumor suppressor with inhibitory effects on tumor growth, invasion, and angiogenesis (28–31). However, recent work has demonstrated that tumorigenic myoepithelial cells, like stromal fibroblasts, can contribute to the invasive phenotype (32). In luminal type breast cancers, IGF signaling has been shown to be dysregulated; however, in normal development, the proliferation of cap cells (which have been demonstrated to be myoepithelial precursors) were found to be more sensitive to loss of IGF-IR (33). Thus, we wanted to determine whether HIT mice, with elevated serum IGF-I, and KO-HIT mice, with elevated serum IGF-I and absent local IGF-I, had different rates of luminal and/or myoepithelial cell proliferation relative to LL control mice.

BrdU+ cells were classified as myoepithelial cells or luminal cells based on the presence or absence of anti-cytokeratin-14 staining, respectively (Fig. 5A). Luminal cell identity was confirmed by using the luminal specific marker, cytokeratin 19 as well (please see Supplemental Figure 1 published on The Endocrine Society’s Journals Online web site at http://endo.endojournals.org). This analysis was used to calculate the percent of total myoepithelial and luminal cells that were BrdU+. The percent of myoepithelial cells that were BrdU+ was significantly higher in HIT mice than control mice (17.6 ± 0.59% vs. 6.1 ± 3.11%, P < 0.05) (Fig. 5B). In contrast, KO-HIT mice were similar to LL mice in terms of the percent of myoepithelial cells that were BrdU+ (Fig. 5B). The incidence of BrdU+ cells in the lumen was much smaller than in the myoepithelium for all three groups of mice studied. This is due to the greater total number of luminal cells than myoepithelial cells. However, within the groups of mice evaluated, HIT mice had an increased percentage of luminal cells that were BrdU+ relative to LL mice (0.12 ± 0.01% vs. 0.04 ± 0.01%, P < 0.01), while the KO-HIT mice were comparable to the LL mice (Fig. 5C). Thus, proliferation of both myoepithelial cells and luminal cells is greater in HIT mice than control mice and KO-HIT mice.

HIT, KO-HIT, and control mice do not exhibit different levels of receptor expression, phosphorylation, and expression of the downstream signaling cascades

The enhanced mammary gland development and cellular proliferation observed in the glands of HIT mice compared with control and KO-HIT mice could result from increased activation of the IGF-I receptor (IGF-IR) or insulin receptor (IR), as well as different expression levels of downstream signaling pathways. To explore this possibility, we performed protein extraction and Western blot analysis of mammary glands from HIT, KO-HIT, and con-
trol mice for IGF-IR, IR, and two major downstream targets of IGF-IR/IR signaling, MAPK/ERK and AKT. Densitometric analysis of Western blots failed to demonstrate a difference in the expression levels of the IGF-IR or the IR. Basal phosphorylation of total IGF-IR/IR combined was also similar among the three groups (Fig. 6). To evaluate the downstream signaling cascade, total and phosphorylated AKT and ERK levels were assessed; similar to receptor levels, no difference was observed for activation of these downstream targets between the three groups (Fig. 6). It is important to note that analysis of the signaling cascade was done for the whole gland and not solely the epithelium. In addition, it is possible that there is an alteration in the balance of IGF-IR vs. IR signaling in HIT mice, which cannot be discerned by the phosphorylated antibody. Lastly, it is possible that changes in signaling were not apparent at the time point studied but that changes might be measurable at an earlier time in development.

**Discussion**

The results of this study suggest that both local and circulating IGF-I play important roles in mammary gland development. When local IGF-I is normal, elevated serum IGF-I enhances mammary gland development. When local IGF-I is absent, elevated serum IGF-I is sufficient for normal mammary gland development but does not further enhance epithelial growth. HIT and KO-HIT mice have equal levels of serum IGF-I, however there is an increase in mammary gland complexity seen in HIT mice but not KO-HIT mice. Mammary gland complexity of KO-HIT mice only develops to a similar extent to that of LL control mice. Thus, the combination of increased circulating IGF-I with normal levels of local IGF-I in HIT mice, absent in KO-HIT mice, likely accounts for the increase in epithelial branching and ductal hyperproliferation that is seen in HIT mice compared with KO-HIT mice.

Our studies with the HIT and KO-HIT models support the idea that elevated circulating IGF-I can substitute for local IGF-I and normalize mammary gland development in IGF-I null female mice. As previous studies demonstrate, there is a unique and crucial role for local IGF-I in mammary gland development; therefore, we expected to see impaired mammary gland development in KO-HIT mice that are completely deficient of local IGF-I. The Midi mice, which have lower circulating as well as local IGF-I, have impaired mammary gland development. However, LID mice, which have only similarly reduced circulating IGF-I but normal local IGF-I, demonstrate normal mammary gland development (16). This suggests that local IGF-I is imperative for mammary gland development. Thus, it is surprising that the KO-HIT mice, with absent local IGF-I, have normal mammary gland development. However, LID mice have circulating total IGF-I levels that are approximately 25% the levels of control mice. It is possible that this reduced level is still sufficient for normal mammary gland development in the presence of local IGF-I, but in the absence of tissue IGF-I as in the Midi mice, lower circulating levels of IGF-I are...
insufficient. In the KO-HIT model, however, despite the complete absence of local IGF-I, mammary gland development is preserved, suggesting that elevated IGF-I can indeed overcome the absence of local IGF-I. At the same time, the HIT mice with intact local IGF-I and elevated circulating IGF-I have enhanced mammary gland development, suggesting that it is the balance between local and serum IGF-I that is important in normal mammary gland development. It is also possible that the absence of local IGF-I leads to enhanced mobilization of circulating IGF-I into the mammary gland through IGF binding protein transport and localization as a compensatory mechanism.

While mammary gland development of KO-HIT mice is normalized to the level of control mice, it is still less than that of HIT mice. This implies that IGF-I-dependent changes in the local environment could be responsible for the differential phenotypes. One possible explanation for this difference centers on the IGF-IR and IR signaling cascade, which would likely be affected by the presence or absence of local IGF-I. However, we did not observe a difference in the expression and basal phosphorylation of the IGF-IR and IR. In addition, no difference was seen in the major downstream signaling pathways suggesting that the classic IGF-I signaling cascade is not affected by absence of IGF-I in the KO-HIT mice or elevation in circulating IGF-I in the HIT mice, or that our acute experimentation did not detect these changes. It is possible that there is a lack of balance between IGF-I and IR signaling that may be contributing to the phenotypic difference observed in the HIT mice by altering further downstream pathways or cross talk pathways. In addition, studies have shown that overexpression of IGF-I in transgenic models does not enhance phosphorylation of the IGF-I signaling cascade, whereas intravenous (tail vein) injection of IGF-I results in a rapid and dose-dependent activation of the IGF-IR and downstream signaling cascade (34). Thus, it is possible that there are differences in signaling that are too subtle to be discerned by studying basal phosphorylation but would be observed after intravenous IGF-I injection.

The KO-HIT mice have absent ovarian IGF-I potentially leading to estrogen insufficiency that may affect mammary gland development (17). In fact, KO-HIT mice,
but not HIT mice, demonstrate reduced fertility, impaired estrous cycle, hypoplastic ovaries and uterus. Midi mice that were ovariectomized prepubertally and then treated with estradiol had the same impairment in mammary gland development that was observed in intact midi mice (16). In addition, when ovariectomized IGF-I null mutant mice were treated with estradiol, an extension of the ducts into the fat pad, but no lateral branching, was observed, further suggesting that ovarian hormones were not solely responsible for the deficit in mammary gland development (16). Thus, while the influence on ovarian hormones cannot be excluded, it is unlikely that this explains the difference in mammary gland development between the HIT and KO-HIT mice. Furthermore, KO-HIT mice, with increased circulating IGF-I, demonstrate normalized mammary gland development even in the face of estrogen deficiency, suggesting that increased IGF-I can overcome the effects of low estrogen on mammary gland development.
One would expect impaired mammary gland development in KO-HIT mice given their estrogen insufficiency. If this were observed, it would be unclear whether this defect was due to differences in IGF-I or ovarian hormones. However, as KO-HIT mice demonstrate normal mammary gland development, it is unlikely that ovarian hormonal differences are responsible for the phenotypic differences observed between HIT and KO-HIT mice. However, further experimentation would be required to completely elucidate the role of ovarian hormones in our model.

Our finding of increased epithelial growth and hyperproliferation in the HIT glands has potential implications for the ongoing debate on IGF-I and breast cancer risk. High levels of IGF-I have been associated with an increased risk of malignancy (22–25). The relative contribution of local and serum IGF-I to tumorigenesis has not yet been determined. Both myoepithelial and luminal cells are thought to play a role in the development of breast cancer. Myoepithelial cells have been traditionally thought of as “tumor repressor cells” as they delimit the duct and hence the extent of proliferation. In fact, the presence of an intact myoepithelial layer distinguishes ductal carcinoma in situ from invasive carcinoma (26). However, invasiveness may be promoted by altered or tumorigenic myoepithelial cells as well (32). Luminal cells, on the other hand, are thought to play a more direct role in tumor growth as they are likely the primary target for transformation in certain tumors (35). HIT female mice have elevated levels of both proliferating luminal and myoepithelial cells compared with control mice. This supports the idea that the myoepithelial cells may act as tumor suppressors in some circumstances, whereas in others, they may be tumorigenic. The significance of the increased proliferation rate of myoepithelial cells in this model is, however, not yet clear. Alternatively, the increased proliferation of luminal cells, together with the increased mammary gland complexity that we observed in HIT mice, may suggest that the HIT mice are more susceptible to tumorigenic cues and may have a greater ability to transform into tumorigenic cells. A study comparing tumor-type signatures does support that the luminal progenitor is a likely target for several different tumor types. If true, this would imply that merely targeting circulating IGF-I levels in breast cancer treatment may not be sufficient, as the local environment would need to be considered as well. Further study to evaluate breast cancer susceptibility in these mice is necessary to further establish whether elevated circulating IGF-I levels affects breast cancer risk differently in HIT and KO-HIT mice and what the contribution of myoepithelial and luminal cells are to this risk.

In conclusion, we found that elevated serum IGF-I can rescue mammary gland development in a setting of local IGF-I deficiency. However, when local IGF-I is normal, elevated serum IGF-I enhances mammary gland development, suggesting interplay between the actions of local and circulating IGF-I in the mammary gland. Further study is necessary to clarify what factors in the local environment are important for mammary gland development. Our study also raises interesting implications for the contribution of local and circulating IGF-I to cancer development, which remain to be further explored.

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