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Preclinical study

The growth hormone receptor antagonist pegvisomant blocks both mammary gland development and MCF-7 breast cancer xenograft growth

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Summary

Mammary gland development is dependent upon the growth hormone (GH)/insulin-like growth factor-I (IGF-I) axis, this same axis has also been implicated in breast cancer progression. In this study we investigated the effect of a GH antagonist, pegvisomant (Somavert®, Pfizer), on normal mammary gland development and breast cancer xenograft growth. Intraperitoneal administration of pegvisomant resulted in a 60% suppression of hepatic IGF-I mRNA levels and upto a 70–80% reduction of serum IGF-I levels. Pegvisomant administration to virgin female mice caused a significant delay of mammary ductal outgrowth that was associated with a decrease in the number of terminal end buds and reduced branching and complexity of the gland. This effect of pegvisomant was mediated by a complete inhibition of both GH and IGF-IR-mediated signaling within the gland. In breast cancer xenograft studies, pegvisomant caused shrinkage of MCF-7 xenografts, with an initial 30% reduction in tumor volume, which was associated with a 2-fold reduction in proliferation and a 2-fold induction of apoptosis. Long-term growth inhibition of MCF-7 xenografts was noted. In contrast, pegvisomant had no effect on MDA-231 or MDA-435 xenografts, consistent with primary growth of these xenografts being unresponsive to IGF-I both *in vitro* and *in vivo*. In MCF-7 xenografts that regressed, pegvisomant had only minor effects upon GHR and IGF-IR signaling. This data supports previous studies indicating a role for GH/IGF in mammary gland development, and suggests that pegvisomant maybe useful for the prevention and/or treatment of estrogen receptor positive breast cancer.

Introduction

The growth hormone (GH)/insulin-like growth factor-I (IGF) axis can have both endocrine and autocrine/paracrine effects in multiple tissues and represents one of the major regulators of organ growth in the mammal [1]. GH receptor (GHR) is a single transmembrane class I cytokine receptor that has no tyrosine kinase activity [2]. Upon binding GH, GHR undergoes dimerization and activates downstream signaling intermediates such as janus kinase (JAK2) and signal transduction and activator of transcription 5 (STAT5). In contrast, IGF-IR is a receptor tyrosine kinase that exists as a heterotetramer [3]. IGF-IR activation leads to phosphorylation of multiple downstream signaling intermediates, and some of these are shared with GH, e.g. insulin receptor substrates (IRSs) [4]. IGF-IR signaling can lead to proliferation, survival and differentiation, and is also critical for cell transformation [5].

GH is an important regulator of mammary gland development [6]. Early studies from the Kleinberg laboratory using hypophysectomized and oophorectomized rodents showed that GH directs mammary gland development [6], and subsequently that GH acts directly upon stromal GHR to induce IGF-I, which then acts upon mammary epithelial cells to induce proliferation and development [7]. Genetic studies in rodents have subsequently confirmed that the GH/IGF axis is essential for pubertal mammary gland development [8,9]. The GH/IGF axis is also important in breast cancer development and progression [10]. GH deficient rodents are completely resistant to carcinogen-induced mammary tumorigenesis [11], and readministration of GH restores susceptibility to carcinogen [12]. Furthermore, transgenic mice expressing a GH antagonist showed reduced DMBA-induced mammary tumors [13]. There is also strong evidence that IGF-I (both endocrine and autocrine/paracrine) is critical for breast cancer progression

[14]. For instance, several studies have shown that serum IGF-I levels can predict breast cancer risk, suggesting a role for endocrine IGF-I in cancer development and progression [15–17]. Consistent with this, mammary tumors are decreased in mice with low serum IGF-I levels [18], and growth of MCF-7 xenografts is reduced in GH deficient $lit^{-/-}$ mice which show reduced circulating IGF-I [19]. We have recently shown that intravenous tail vein administration of IGF-I in mice leads to rapid IGF-IR signaling in both normal tissues and cancer xenografts [20], supporting the idea that tumors are sensitive to endocrine IGF action. The last year has seen the emergence of several strategies to block IGF-IR signaling which have been successful at inhibiting breast cancer xenograft growth and metastasis [21–24].

Pegvisomant (Somavert®, B2036-PEG, Pfizer) is a recently developed growth hormone receptor antagonist that can normalize serum IGF-I levels in approximately 90% of patients with acromegaly [25,26]. Pegvisomant is a recombinant protein that is structurally similar to human GH, but has mutations that block receptor activation [27,28]. Previous studies have shown that pegvisomant can block growth of MCF-7 breast cancer cells transfected to overexpress autocrine GH [29], and also block meningioma [30] and colon [31] xenograft growth. We have used pegvisomant to test whether pharmacological inhibition of GH action affects mammary gland development and human breast cancer xenograft growth. Our results show that pegvisomant is a potent GH antagonist, reducing circulating IGF-I levels, and completely eliminating both GH and IGF-IR signaling in the mammary gland. This resulted in a block of ductal elongation and branching in the mammary gland. In addition, pegvisomant was able to block the growth of human MCF-7 xenografts, supporting other evidence that these tumors are sensitive to circulating mouse IGF-I [19,20]. These studies suggest that pegvisomant may be useful in the treatment of estrogen receptor positive breast cancer.

Materials and methods

Materials

General materials and chemicals were purchased from Sigma (St. Louis, MO, USA) unless otherwise noted. Pegvisomant was provided in powder form by Pfizer, and reconstituted in distilled water. All tissue culture reagents were purchased from Invitrogen (Carlsbad, CA, USA) unless otherwise stated.

Animals

All procedures were conducted in accordance with the *NIH Guide for the Care and Use of Laboratory Animals*, and were approved by the IACUC of Baylor College of Medicine. Mice were maintained on a 12 h light, 12 h dark schedule with *ad libitum* access to laboratory chow (Picolab Rodent Diet 20, Lab Diet 5053, PMI Nutrition International Inc., Brentwood, MO) and water. For determining the effect of pegvisomant on development, 3.5 week old female virgin FVB/N mice (Charles River Laboratories, Inc., Wilmington, MA, USA) ($n=4$–6/group) were given daily intraperitoneal (IP) injections of either pegvisomant (250 mg/kg dissolved in water) or vehicle (PBS) and mammary glands harvested at 4, 4.5, 5.5, 7.5, 9.5 and 13.5 weeks of age. Mice were injected with BrdU 2 h prior to sacrifice. Mammary glands were then harvested, whole-mounted or snap-frozen in liquid nitrogen and kept in −80 °C until further analysis. To determine the effect of pegvisomant upon mammary gland branching, 5 week old female virgin CD1 mice (Charles River, Laboratories, Inc., Wilmington, MA, USA) ($n=4$/group) were treated with pegvisomant (100 or 250 mg/kg) or vehicle and mammary glands analyzed by whole mount after 3 weeks of treatment. Estrous stage was assessed in mice by vaginal pap smear as published previously [32].

In all xenograft experiments we used 4–6 week old athymic nude (Nu/Nu) mice (Charles River Laboratories, Inc., Wilmington, MA, USA). MCF-7, MDA-231, or MDA-435 human breast cancer cells were grown orthotopically as subcutaneous xenografts in the #3 thoracic mammary fat pad ($n=7$–11/group). For MCF-7, mice were injected with 0.5×10^7 cells. Mice were surgically implanted with pellets designed to deliver a continuous dose (0.72 mg/pellet) of estrogen over 90 days (Innovative Research of America, Sarasota, FL, USA). Implantation of the estrogen pellet was done 2 days prior to cell injection in order to stimulate development and growth of MCF-7 tumors. For MDA-231 and MDA-435, 0.5×10^7 cells were injected. Tumor size was measured by digital caliper twice a week and tumor volumes were estimated according to the formula for an ellipse (short dimension)$^2\times$(long dimension)/2. For MCF-7, in three individual experiments, xenografts were grown until they were ~70, ~150, or ~300 mm^3, and mice were then randomized to receive vehicle (PBS) or pegvisomant (daily i.p., 100 or 250 mg/kg) and harvested 4 days after the beginning of the treatment. Mice were injected with BrdU 2 h prior to sacrifice. BrdU only incorporates into the DNA of cells that are replicating DNA (S-phase) and is thus a very specific marker of cell proliferation [33]. Tumors were then harvested, a representative part cut for histological analysis and the rest snap-frozen in liquid nitrogen and kept in −80 °C until further analysis. In addition, mice were also followed up until the animals had to be euthanized (~1000 mm^3). Tumors were then harvested, representative part cut for histological analysis and the rest snap-frozen in liquid nitrogen and kept in −80 °C until further analysis.

Serum IGF-I analysis

Blood was collected by cardiac puncture under isoflurane anesthesia, allowed to clot for 1 h at room

temperature and serum collected after centrifugation. IGF-I was analyzed by enzyme-immuno-assay kit (Diagnostics Systems Laboratories, Inc., Webster, TX, USA) according to the manufacturer's instructions.

IGF-I mRNA analysis

Total RNA was extracted from tissues (liver or mammary gland – snap-frozen after sacrifice) using Trizol (Life Technologies, Inc., Carlsbad, CA, USA). A 1 μg of RNA was reverse transcribed using Moloney Murine Leukemia Virus reverse transcriptase using a SuperScript system (Life Technologies, Inc.) in a final volume of 20 μl. Negative controls for reverse transcription (RT) were generated in the absence of RT enzyme. Each PCR reaction contained 1 μg of cDNA. The sequences of the primers are as follows: IGF-I 5-sense primer: 5′-GGACCAGAGACCCTTTGCGGGG-3′, IGF-I 3-antisense primer: 5′-GGCTGCTTTTGTAGGCTTCA GTGG-3′; β-actin primers: 5′-AACAGAACTTAG-GACGAGGG-3′, 5′-GGAAACCAGGTTGTCAGTC-3′. All primers were synthesized by MWG Biotech (High Point, NC, USA). These primers generate cDNAs of 209 and 267 bp for IGF-I and β-actin, respectively. PCR conditions were as follows: 94 °C for 10 min, followed by 25, 30 or 35 cycles including denaturation at 94 °C for 1 min and extension at 60 °C for 1 min. Gene expression levels were normalized to the corresponding level of β-actin mRNA.

Whole mount quantitative analysis

Whole mount preparations were performed on #4 inguinal mammary glands as described previously [32]. Quantitation was performed by scanning the whole mount and measuring area and distance. Distance of ductal growth was measured by ruler using the edge of the lymph node distal to the nipple as a reference point. Fat pad area filled was counted by tracing the area of the fat pad, and then the area of the epithelium (using Adobe Photoshop), and then calculating area using NIH Image Pro. Fat pad filled represents the area of the epithelium divided by the area of the fat pad×100. Total branch points were calculated in a 2.5×field that was adjacent to the distal side of the lymph node. TEBs were identified as being at end of a duct at the leading edge of the gland (furthest from the nipple in the #4 gland) and had an area that was at least three-times larger than the end of a duct or a side-branch present in the middle of the gland.

Histological analysis

Histological analysis was performed on xenografts or #3 thoracic mammary glands that had been fixed in 4% paraformaldehyde overnight at 4 °C and then placed in paraffin. For analysis of proliferation, mammary tissue sections or xenografts were stained with for BrdU incorporation or Tunel for apoptosis as described previously [32]. For counting of BrdU and Tunel, mammary gland or xenografts sections were first blinded, and then three representative images (200×) captured by digital camera by two independent observers. For the mammary gland, pictures were taken directly of TEBs (for TEB data Figure 2), or in the adult 13 week old mice experiment (data not shown) pictures were taken randomly throughout the gland (no TEBs were present). Positive cells in the mammary gland were counted by hand and represented a specific count of TEB positive cells (Figure 2) or a random count of all epithelial cells (ductal and lobular) in the adult 13 week old virgin mice. Positive cell number was corrected for the total number of cells counted by the counterstain. Each photograph had a similar number of cells counted. Positive cells from the xenografts were counted using an Alpha Innotech 7000 since the xenograft is fairly homogenous in cell number and cell type in contrast to the mammary gland which contains multiple cell types. The results represent the average of the two counts by the independent observers.

Immunoblot analysis

Mammary glands were harvested, snap-frozen in liquid nitrogen, homogenized and lysed in TNESV buffer as described previously. Cells grown *in vitro* were lysed in 5% SDS solution. Equal amounts of total protein extracts were separated by electrophoresis on a 6% (GH signaling) or 8% (IGF signaling) polyacrylamide gel and transferred by electroblotting into a nitrocellulose membrane (Schleicher&Schuell BioScience, Inc., Keene, NH, USA). The membranes were blotted overnight against phospho-specific antibodies: p-JAK2 1:1000 (Cell Signaling Technology, Beverly, MA, USA), p-STAT5A/B 1:1000 (Upstate Group, Inc., Lake Placid, NY, USA), p-IGF-IR (Cell Signaling Technology, Beverly, MA, USA), p-IRS-1 1:1000 (Biosource International, Inc., Camarillo, CA, USA), p-AKT 1:1000 (Cell Signaling Technology, Beverly, MA, USA), p-ERK1/2 1:1000 (Cell Signaling Technology, Beverly, MA, USA). Total antibodies blotting was performed at room temperature for 2 h: JAK2 1:1000 (Upstate Group, Inc., Lake Placid, NY, USA), STAT5A/B 1:1000 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), IGF-IR (Cell Signaling Technology, Beverly, MA, USA), IRS-1 1:1000 (Upstate Group, Inc., Lake Placid, NY, USA), AKT 1:1000 (Cell Signaling Technology, Beverly, MA, USA), ERK1/2 1:50,000 (Upstate Group, Inc., Lake Placid, NY, USA). Anti-rabbit or anti-mouse antibodies (Amersham Biosciences, Piscataway, NJ, USA) were used as a secondary antibody. Blots were developed using the enhanced chemiluminesence (ECL) procedure (Pierce Biotechnology, Inc., Rockford, IL, USA) and acquired and densitometrically analyzed using an Alpha Innotech 7700.

Cell stimulation and proliferation assay

Cells were plated into 6 cm Petri dishes at 0.5×10^6 cells per plate in growth medium and were grown for 24 h.

Cells were subsequently starved in SFM for 24 h and afterwards stimulated with either vehicle or GH (SIGMA, 0–1000 ng/ml) or IGF-I (GroPep, Adelaide, Australia, 100 ng/ml) for 15 min. After lysis in 5% SDS solution the lysates were stored at −20 °C until immunoblot analysis. Cell proliferation was performed by plating cells at 2×10^4 cells per well in 24 well plates and counting triplicate well daily using a coulter counter.

Statistical analysis

Statistical comparisons between pegvisomant versus vehicle (saline) groups on serum IGF-1 levels, proliferation, apoptosis, mammary gland branching, and number of TEBs were performed using two sample *t*-tests. For xenograft experiments, tumor volume measurements over time were analyzed using longitudinal models; specifically, fixed effects model was implemented to compare tumor volume between control versus pegvisomant groups at each time point (Figure 5a) while random coefficients modeling was employed to compare slopes of tumor growth over time between control versus pegvisomant groups (Figure 6a–c).

Results

Pegvisomant lowers hepatic IGF-I mRNA levels and circulating IGF-I levels

To examine the role of pegvisomant in mammary gland development and breast cancer xenograft growth *in vivo*, we first examined the ability of pegvisomant to reduce serum IGF-I levels in different genetic strains of mice (outbred CD-1, inbred FVB/N, and athymic nu/nu). Treatment of 3.5 week old female virgin FVB/N mice for 10 weeks with 250 mg/kg pegvisomant caused a 60% ($p<0.01$) decrease in hepatic IGF-I mRNA levels (Figure 1a). Similar results were also obtained in CD-1 and athymic nude mice, and also with shorter times of administration (2, 4 or 6 weeks, data not shown). Consistent with this decline in IGF-I mRNA levels, treatment of FVB/N, or athymic mice for 8 days caused a dose-dependent reduction in circulating IGF-I levels (Figure 1b), with 250 mg/kg pegvisomant causing a ~75% reduction ($p<0.001$). This reduction remained constant when pegvisomant was given for longer periods (upto 10 weeks). To determine the duration of effectiveness of pegvisomant, we treated mice for 8 days and then stopped administration and measured serum IGF-I levels daily thereafter (24, 48 or 72 h) (Figure 1c). As expected, 24 h following pegvisomant administration, serum IGF-I levels were suppressed by 83% ($p<0.001$). Levels remained suppressed for at least 72 h following withdrawal. To determine the periodicity of dosing required to achieve constant suppression of serum IGF-I, mice were given pegvisomant daily, every other day, or every 3 days for 9 days and then serum IGF-I levels analyzed. Every day dosing suppressed serum IGF-I levels by 75%. Every other day dosing only suppressed levels by 62%, and dosing every 3 days suppressed levels to 54% (data not shown). Thus maximal suppression of serum IGF-I levels was achieved by daily dosing and thus for all further experiments we treated mice daily with either 100 or 250 mg/kg pegvisomant.

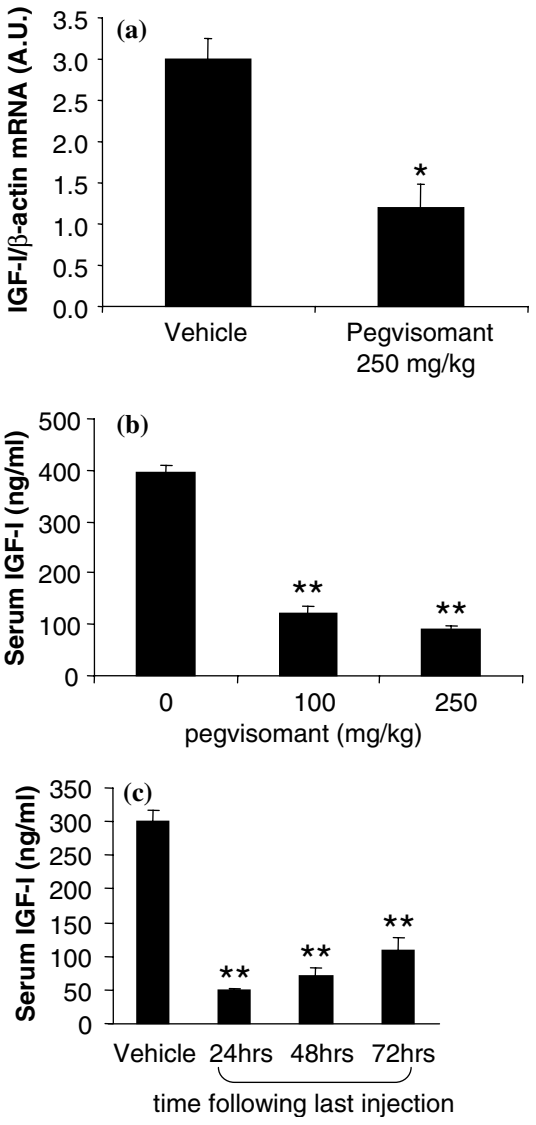

Figure 1. Pegvisomant lowers hepatic IGF-I mRNA and serum IGF-I levels in the mouse: (a) FVB/N female mice ($n=5$ per group) were treated daily with pegvisomant (250 mg/kg) or vehicle (saline) by intraperitoneal injection for 10 weeks. Mice were then sacrificed and mRNA extracted from liver. RT-PCR was performed for IGF-I and *β*-actin as a loading control. Band intensity was measured by densitometry. Bars represent the average absorbance units (A.U.) ± S.E.M. $*p<0.01$. Similar results were found when pegvisomant was administered for 1, 2, 4 or 6 weeks. (b) CD-1 female mice ($n=5$ per group) were treated with pegvisomant (100 or 250 mg/kg) or vehicle (saline) by intraperitoneal injection for 2 weeks and serum IGF-I levels measured by EIA. Bars represent the average serum IGF-I concentration ± S.E.M. ** $p<0.001$ (pegvisomant treatment compared to vehicle). (c) FVB/N female mice ($n=5$ per group) were treated with pegvisomant (250 mg/kg) or vehicle (saline) by intraperitoneal injection for 8 days, and at this point treatment was stopped, vehicle treated mice were sacrificed, and then pegvisomant treated mice were sacrificed after 24, 48 or 72 h. Bars represent the average serum IGF-I concentration ± S.E.M. ** $p<0.001$ (24, 48 or 72 h compared to vehicle).

Pegvisomant delays pubertal ductal development

The GH/IGF axis is critical for normal mammary ductal development [7]. However, studies to date have generally used either surgical (hypophysectomy and oophorectomy) or genetic (transgenic and gene targeted deletion) methodologies; both methods have the potential for confounding results caused by deletion or compensation by other hormones. Therefore, to test whether short-term postnatal pharmacologic blockade of GHR action in intact wild-type rodents would mimic previous data from surgical and genetic models, female FVB/N mice ($n = 5$ per group) were treated from 3.5 weeks of age with pegvisomant or with saline control and mammary ductal development analyzed by whole mount analysis. Whole mounts from mice after 2, 4 or 6 weeks of treatment are shown as examples (Figure 2a). Measurement of ductal outgrowth past the lymph node revealed a significant reduction in ductal outgrowth in pegvisomant treated mice (Figure 2b). An identical result was found when this data was expressed as a percentage of fat pad filled (data not shown). This decrease was apparent after just 1 week of treatment, and persisted throughout the experiment, such that the pegvisomant treated mammary glands never grew out as far as the controls. A potential reason for the delay in outgrowth was an apparent reduction in TEBs in the pegvisomant treated gland (Figure 2b). After 1 week of treatment, vehicle treated glands had 5.25 ± 0.75 TEBs per gland whereas the pegvisomant group of mice significantly decreased to 2 ± 0.4 per gland ($p < 0.01$). More importantly, a large increase in TEB number (upto 8.8 ± 1.0 per gland) that occurred in vehicle treated mice at 5.5 weeks of age was completely blocked in pegvisomant treated glands which had 1.6 ± 0.5 TEBs per gland ($p < 0.001$). To confirm that the decrease in ductal outgrowth was not due to an indirect effect of pegvisomant administration on ovarian cycling, we examined estrus cycle in mice by vaginal smear. Daily examination of mice revealed no difference in cycling in pegvisomant treated versus saline treated mice (data not shown).

Histological analysis of mammary glands from the pegvisomant treated mice showed no apparent abnormalities, apart from the reduction in TEB number. We hypothesized that the decrease in ductal outgrowth was probably due to a decrease in proliferation in the TEB, similar to that found in IGF-IR-null mammary glands [34]**.** Due to the rapid decline in TEB number following pegvisomant administration, and the small number of TEBs for analysis after long-term pegvisomant treatment, we measured proliferation in TEBs (by BrdU incorporation) in mice after 0.5 or 1 week of pegvisomant. We found no significant difference in the percentage of BrdU stained cells in TEBs after 0.5 weeks (saline $34.3 \pm 3.1\%$ vs. pegvisomant $30.5 \pm 5.4\%$) or 1 week (saline $27.0 \pm 4.2\%$ vs. pegvisomant $28.2 \pm 2.0\%$) of treatment. In addition we found no significant difference in apoptosis as assessed by cleaved caspase 3 immunohistochemistry (data not shown). However, we did find that pegvisomant glands showed reduced bifurcation of TEBs (Figure 2c), which may have accounted for the inability of pegvisomant treated glands to increase their TEB number. We conclude from these results that postnatal blockade of GHR action inhibits mammary gland development, and that this is not due to an affect on TEB proliferation, but perhaps via an initial decrease in TEB number (within 1 week), and the failure to increase TEB number at 5.5 weeks.

Pegvisomant reduces side-branching in the mammary gland

Several recent papers have shown that genetic loss of IGF-I or its receptor inhibits mammary gland side branching [34,35]. In our studies of ductal elongation in FVB mice (Figure 2), a reduction in branching was noted. However, the mammary gland in FVB/N strain of mice does not exhibit as a complex branching structure as other strains of mice. Therefore, to examine the effect of pegvisomant further, we assessed branching in CD-1 outbred mice, as the mammary glands in these mice exhibit extensive side branching. Five-week-old CD-1 female mice were treated with pegvisomant or saline for 3 weeks. The initial treatment was delayed to 5 weeks of age compared to 3 weeks of age in Figure 2 to allow the mammary gland to undergo extensive ductal elongation (at 5 weeks of age approximately 70% of the fat pad was filled), and then the pegvisomant was administered for 3 weeks at a time of extensive side-branching morphogenesis. In this experiment we also examined the effect of reducing the dose of pegvisomant to 100 mg/kg. Whole mount analysis revealed a dramatic reduction in side branching in pegvisomant treated mice, with an apparent dose-response relationship (Figure 3a). Branch points were quantified by counting (Figure 3b) and revealed a 47% reduction in branch points with 100 mg/kg pegvisomant compared to saline ($p < 0.01$). There was a trend ($p = 0.09$) to a greater reduction in branch points with 250 mg/kg pegvisomant (62% vs. 47%).

Pegvisomant does not affect mammary gland proliferation in adult virgin mice

Embryonic targeted gene deletion studies that have shown an effect of the GH/IGF axis on pubertal ductal outgrowth can not address the role of the GH/IGF axis in maintenance of the adult virgin gland. We therefore tested the effect of 2 weeks of pegvisomant administration in 10 week old female FVB/N mice. While serum IGF-I levels were decreased by ~70% as expected (saline 224.2 ± 10.6 ng/ml vs. pegvisomant 67.7 ± 10.3, $p < 0.001$), there was no obvious difference in mammary gland morphology by both whole mount and histological analysis (data not shown). We also measured proliferation (BrdU staining) within the gland and found no significant difference (saline $2.76 \pm 1.2\%$ vs. pegvisomant $3.3 \pm 1.1\%$, $p = 0.7$). This suggests that proliferation and

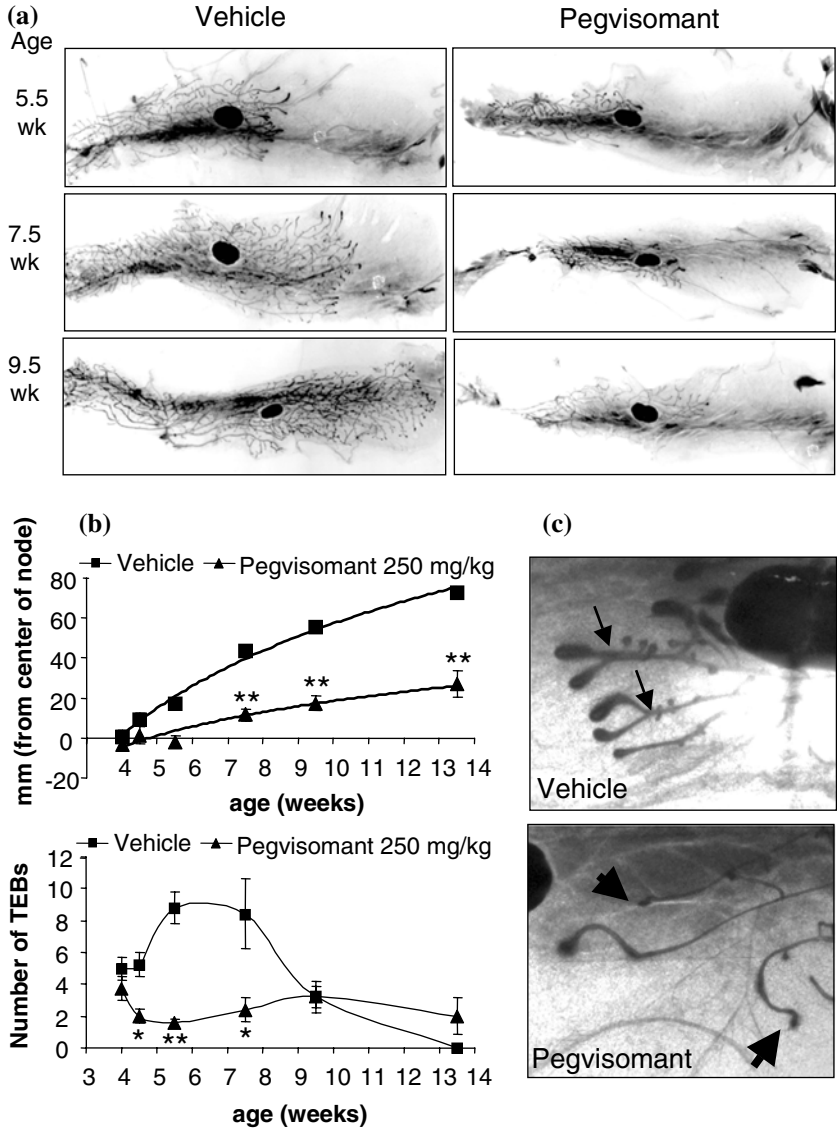

Figure 2. Pegvisomant delays ductal outgrowth: Female FVB/N mice ($n = 5$ per group) at 3.5 weeks of age were treated with pegvisomant (250 mg/kg) or vehicle (saline) for 0.5, 1, 2, 4, 6 or 10 weeks. (a) Mammary gland whole mounts from the inguinal (#4) mammary gland. The dark spot in the center is a lymph node and serves as a point of reference in the gland (0.8× magnification). Representative images after 2, 4 or 6 weeks of pegvisomant (mice were 5.5, 7.5 and 9.5 weeks of age) are shown. (b) Morphometric analysis of whole mounts was performed by imaging the glands using a flatbed scanner and then making measurements using a ruler (measurement of furthest TEB from the center of the lymph node). Each point represents the average distance (mm) of 4–6 mice ± S.E.M. TEBs were counted from the whole mounts and are expressed as average number per mammary gland ± S.E.M. (c) High magnification image of TEBs from mice treated for 2 weeks with pegvisomant. Note the bifurcation of the TEB in vehicle treated glands (small arrow) and the lack of bifurcation in pegvisomant treated glands. Additionally, note the reduced size of the TEB in pegvisomant treated glands (large arrows). * $p < 0.01$, ** $p < 0.001$.

homeostasis of the mature mammary gland is not dependent upon the GH/IGF axis.

Pegvisomant blocks both GH and IGF signaling within the mammary gland

To examine how pegvisomant inhibited ductal outgrowth and branching, we examined the proximal signaling intermediates of the GH and IGF signaling pathways in mammary glands from FVB mice treated for 2, 4 or 6 weeks from Figure 2. Results are shown for mice treated with pegvisomant for 6 weeks, but were essentially the same for all time points. For GH signaling we measured phosphorylation of JAK2 (which directly binds and is phosphorylated by GHR) and STAT5 (which is phosphorylated by JAK2). p-JAK2 and p-STAT5 were readily detectable in mammary glands from saline treated mice, but levels were virtually eliminated by pegvisomant, consistent with an effective blockade of GHR (Figure 4a).

For IGF-IR signaling we examined phosphorylation of the receptor (p-IGF-IR) and its downstream signaling intermediate insulin receptor substrate-1 (IRS-1) which is bound and directly phosphorylated by IGF-IR (but also potentially indirectly phosphorylated via GH activated JAK2). Consistent with evidence implicating GH-induction of IGF action within the mammary gland, we were also able to detect p-IGF-IR and p-IRS-1 in the

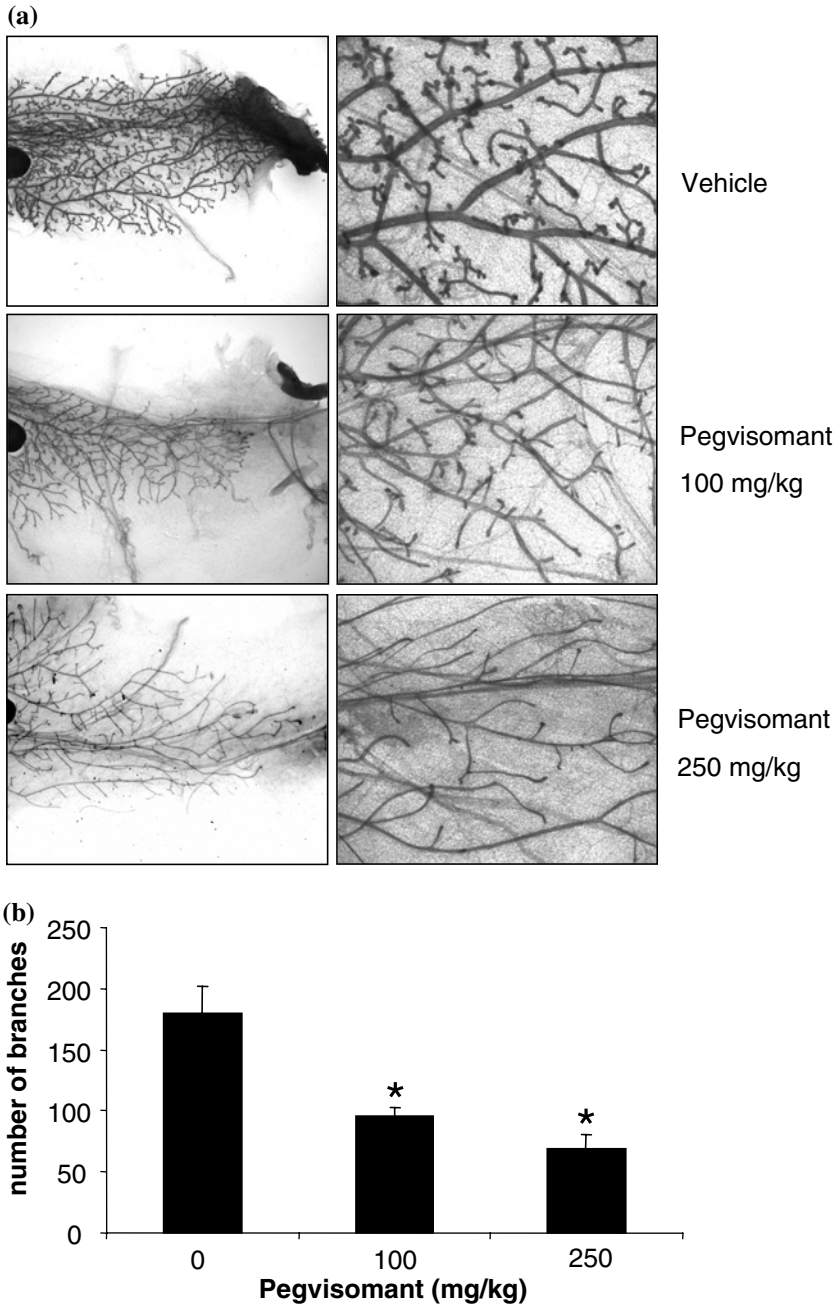

Figure 3. Pegvisomant reduces mammary gland branching: CD-1 female mice ($n = 4$ per group) at 5 weeks of age were treated for 3 weeks (until 8 weeks of age) with pegvisomant (100 or 250 mg/kg) or vehicle (saline). (a) Whole mounts of inguinal (#4) mammary glands. The right panels represent higher power images of the left panels. The lymph node is at the far left of the image. (b) Morphometric analysis of whole mounts. Each bar represents the average number of branch points for four mice ± S.E.M. * $p < 0.01$.

mammary gland which was eliminated by pegvisomant (Figure 4b). The loss of p-IGF-IR indicated a direct loss of IGF-I-mediated signaling, whereas the loss of p-IRS-1 may represent both inhibition of IGF-IR and also GH-regulated JAK2 activity (see above). Interestingly, pegvisomant did not alter IGF-IR levels, but caused a dramatic reduction in IRS-1 levels, suggesting that the GH or IGF signaling pathway are critical to maintain IRS-1 levels in the mammary gland. We confirmed this reduction of IRS-1 levels by pegvisomant in all of our studies in mice, and also found a similar effect in the rat mammary gland (data not shown). To examine pathways downstream of IGF-IR and IRS-1 we examined phosphorylation of AKT and ERK1/2, however, we did not detect any difference in either of them (data not shown).

Pegvisomant causes regression of MCF-7 human breast cancer xenografts via a reduction in proliferation and an increase in apoptosis

We next tested the effect of pegvisomant on the growth of human breast cancer xenografts. MCF-7 cells express estrogen receptor (ER) and progesterone receptor and

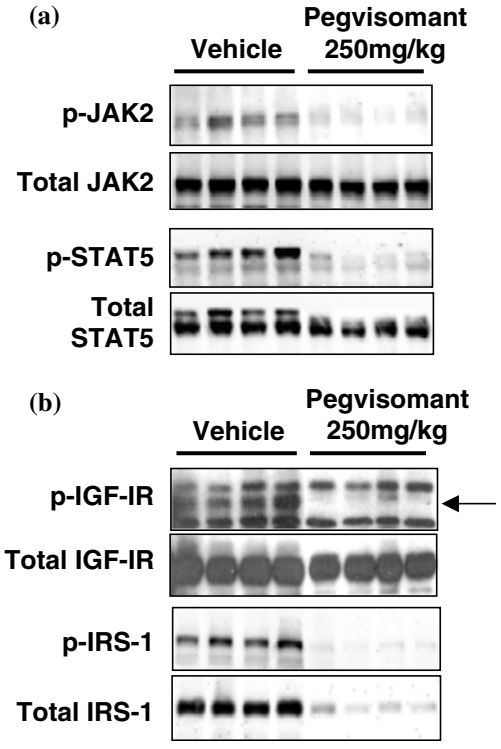

Figure 4. (a and b) Pegvisomant inhibits GH and IGF signaling in the mammary gland: Mammary glands from mice treated for pegvisomant or vehicle for 6 weeks (9.5 weeks of age). Tissue was crushed, lysed and 30 ug of protein separated by SDS-PAGE and immunoblotted for phosphorylated and total JAK2, STAT5, IGF-IR and IRS-1. The phospho-IGF-IR protein is highlighted by an arrow. The antibody recognizes two non-specific bands above and below pY-IGF-IR which serve as loading controls. Specificity of the pY-IGF-IR antibody was confirmed by immunoprecipitation of IGF-IR followed by immunoblotting with the pY-IGF-IR antibody which only detected the middle band (data not shown).

are sensitive to IGF-I [36]. Previous studies have shown that MCF-7 xenograft growth was reduced in mice that have low circulating IGF-I levels due to a mutation in the GHRH gene [19]. We therefore grew MCF-7 xenografts in athymic nude mice implanted with an estrogen pellet, and when tumors reached a size of ~70 mm they were randomized to saline or pegvisomant treatment (Figure 5a). An initial treatment for 3 days with pegvisomant caused a significant 30% reduction in tumor volume compared to vehicle ($p < 0.05$). This was repeated (including increasing tumor size to 150 or 300 mm^3 at randomization) and similar reductions were observed (data not shown). The reduction in tumor volume was similar with both 100 and 250 mg/kg pegvisomant. To examine the mechanism of pegvisomant-mediated tumor regression, we examined proliferation (BrdU IHC) and apoptosis (Tunel assay) in xenografts following 3 days of pegvisomant administration (Figure 5b). Pegvisomant caused a 2-fold reduction in the percentage of BrdU positive cells (saline 9.7 ± 1.0 vs. pegvisomant 4.8 ± 0.6, $p < 0.01$). In addition pegvisomant caused a 2-fold induction of Tunel-positive cells (saline 7.8 ± 2.5 vs. pegvisomant 19.8 ± 2.1, $p < 0.05$). This increase in apoptosis and decrease in proliferation presumably accounted for the tumor shrinkage.

Pegvisomant blocks the growth of MCF-7 xenografts, but not MDA-435 or MDA-MB-231

Following our short-term administration studies in Figure 5, we next asked what the long-term effect of pegvisomant was on ER-positive MCF-7 and other ER-negative breast cancer cell lines (MDA-MB-231 and MDA-435) (Figure 6). For MCF-7, we used two different strains, MCF-7L and MCF-7B, both of which express ER and respond to IGF-I [37]. Treatment of MCF-7L xenografts with pegvisomant caused a reduction in tumor volume over the first 2 weeks, similar to that seen previously in Figure 5. A comparison of the slopes of tumor growth over time between vehicle versus pegvisomant revealed a significant reduction in tumor growth ($p < 0.001$). Pegvisomant was also able to block xenograft growth at 100 mg/kg (data not shown, $p < 0.01$), and there was no significant difference between the effect of 100 or 250 mg/kg pegvisomant ($p = 0.77$). MCF-7B xenografts gave a similar result to MCF-7, with an initial decrease in tumor volume following pegvisomant administration (data not shown). In contrast to MCF-7 cells, MDA-435 and MDA-MB-231 xenograft growth was unaffected by pegvisomant (Figure 6b and C, $p = 0.6$ and 0.35, respectively), which is in keeping with data indicating that these ER-negative cells lines fail to proliferate in response to IGF-I *in vitro* and *in vivo* [38,39].

Effect of pegvisomant on signaling in MCF-7 xenografts

To determine if the effect of pegvisomant on MCF-7L xenografts was due to direct blockade of GHR action, or due to the reduction in synthesis of IGF-I, we first examined the sensitivity of the cell lines used (MCF-7L, MDA-231, and MDA-435) to GH and IGF-I *in vitro*. Similar to previous studies, MCF-7L cells were growth arrested by incubation in serum-free media (SFM), but growth was stimulated by IGF-I (100 ng/ml) (data not shown). In contrast to this, GH at concentrations of upto 1000 ng/ml had no effect Similar data indicating a lack of effect of GH in MCF-7 cells has been previously reported, however, these cells can respond to autocrine GH produced by stable transfection with a GH plasmid [29]. Consistent with the proliferation data, IGF-I was able to simulate IGF-IR and IRS-1 phosphorylation in MCF-7L, whereas GH did not induce phosphorylation of JAK2 or STAT5 (data not shown). MDA-231 and MDA-435 proliferation was unaffected by IGF-I or GH (data not shown).

MCF-7 cells do not produce autocrine IGF-I and do not respond to exogenous GH (although they can respond to autocrine GH [29,40]). This suggests therefore that effect of pegvisomant *in vivo* is to block mouse-derived IGF-I stimulation of MCF-7 xenograft *in vivo*. To test this directly we examined GH and IGF signaling in the MCF-7 xenografts that regressed following 3 days of pegvisomant treatment. MCF-7 xenografts showed highly variable levels of p-JAK2 and p-STAT5

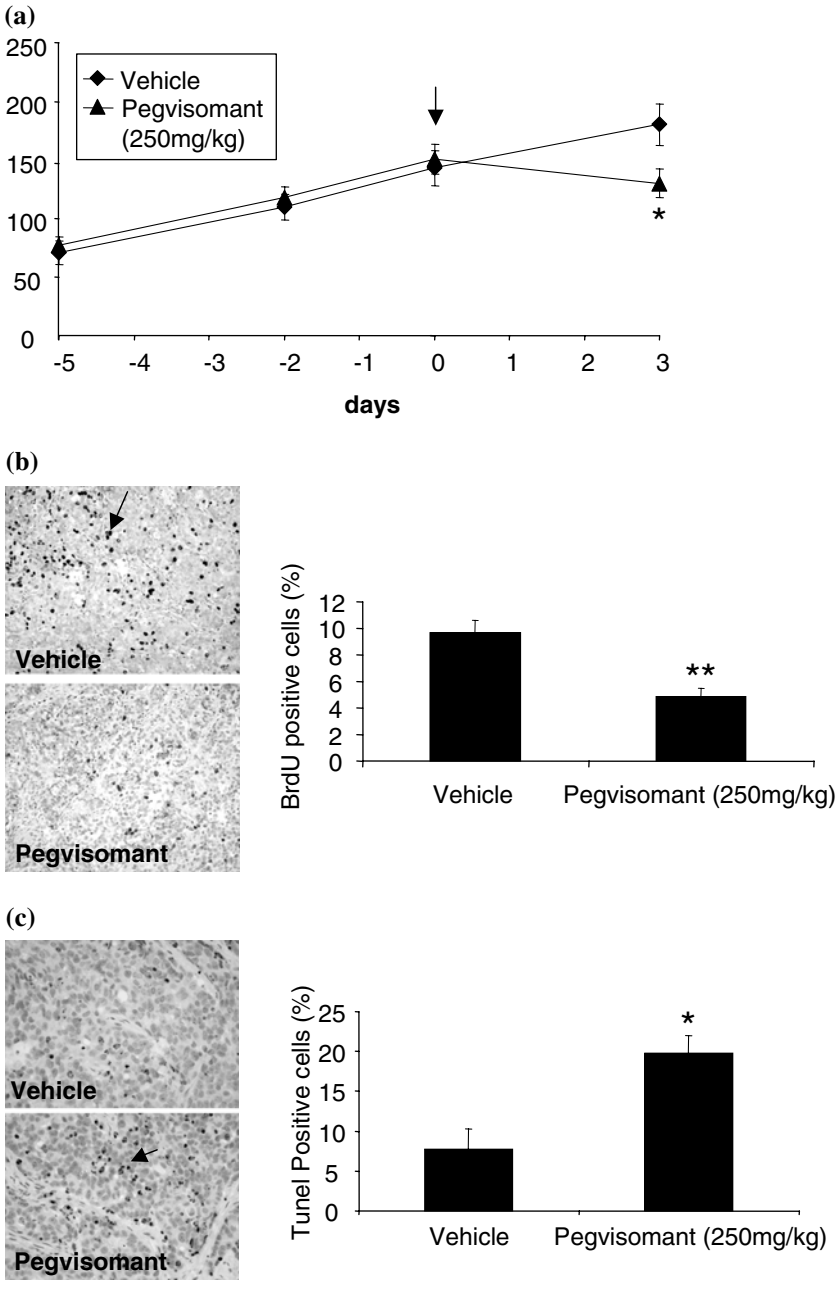

Figure 5. Pegvisomant causes regression of MCF-7 xenografts via a reduction in proliferation and an increase in apoptosis: (a) MCF-7 breast cancer xenografts were grown orthotopically in athymic mice until they reached a volume of ~70 mm^3 and then randomized (day 0 – arrow) to receive daily pegvisomant (250 mg/kg) or vehicle. Each point represent the average cubic volume ($n = 19$ xenografts vehicle, $n = 21$ xenografts pegvisomant) ± S.E.M. *$p < 0.05$ (vehicle vs. pegvisomant at day 3). (b) MCF-7 xenografts from Figure 5a, were harvested after 3 days of pegvisomant and then xenografts were immunostained for BrdU (left panels). Positive cells are highlighted by an arrow. Right panel represent counting of BrdU positive cells. Each bar represents the average of four xenografts ± S.E.M. **$p < 0.001$. (c) MCF-7 xenografts were processed by Tunel Assay (left panels). Positive cells are highlighted by an arrow. Right panel represent counting of Tunel-positive cells. Each bar represents the average of four xenografts ± S.E.M. *$p < 0.01$.

(Figure 7a). Densitometric analysis revealed a reduction with pegvisomant in both p-JAK2/total JAK2 and p-STAT5/total STAT5, although the reductions did not reach statistical significance ($p = 0.2$ and 0.18, respectively) due to the large variation between individual tumors. p-IGF-IR and p-IRS-1 levels were mildly reduced in pegvisomant treated tumors (Figure 7b). Quantitative densitometry (Figure 7c) showed that after correction for total levels of protein, the reduction in p-IGF-IR/IGF-IR was not significant, however, there was a significant ($p < 0.05$) reduction in p-IRS-1/IRS-1. Considering the dramatic reduction in total IRS-1 levels seen in both the MCF-7 xenografts and the mammary gland (Figure 4), it seems that part of pegvisomant's action maybe via neutralization of an IRS-1 mediated signal. Despite the changes in IRS-1 signaling, we were unable to detect a change in p-AKT or p-ERK1/2 within the tumors (data not shown).

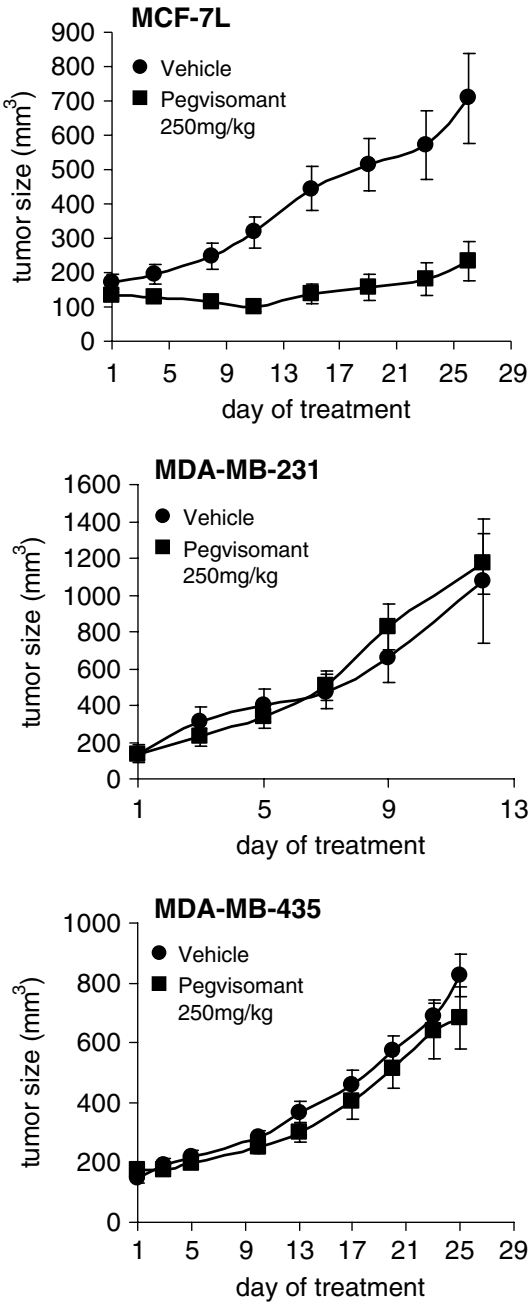

Figure 6. Pegvisomant delays the growth of MCF-7 xenografts, but has no effect on MDA-435 and MDA-MB-231 xenografts: MCF-7, MDA-435, and MDA-MD-231 xenografts were grown orthotopically in athymic nude mice (MCF-7 mice received an estrogen implant). When xenografts reached between 100 and 200 mm^3 in size, the mice were randomized to receive pegvisomant (250 mg/kg) or vehicle daily by IP injection. Each point represents the average of between 6 and 10 xenografts ± S.E.M.

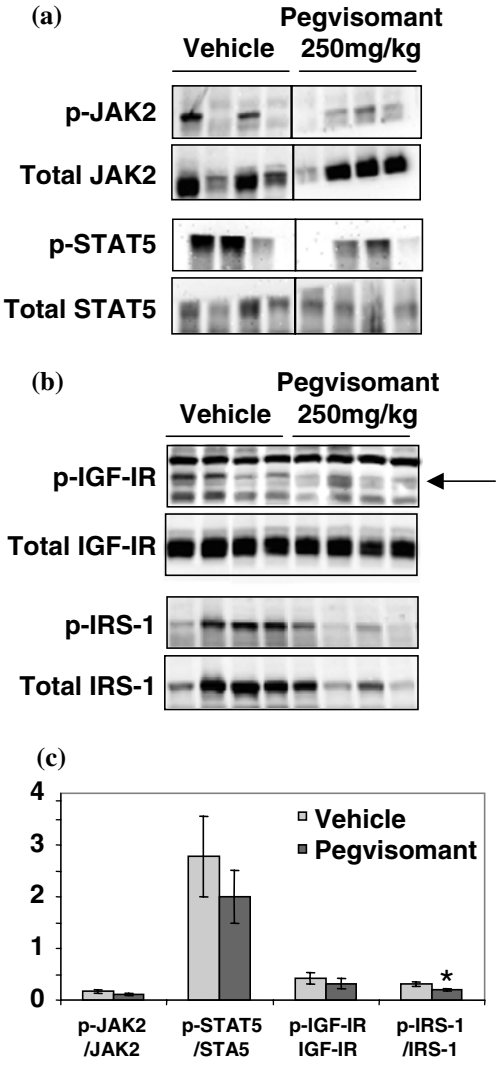

Figure 7. Pegvisomant inhibits IRS-1 phosphorylation in MCF-7 xenografts: MCF-7 xenografts were harvested following 4 days of treatment with pegvisomant (250 mg/kg) or vehicle and frozen in liquid N_2. Tissue was crushed, lysed and 30 ug of protein separated by SDS-PAGE. Immunoblotting was performed for (a) phosphorylated and total JAK2, STAT5, or (b) phosphorylated and total IGF-IR and IRS-1. As in normal mammary glands in Figure 4, the pY-IGF-IR antibody detected two non-specific bands above and below the specific pY-IGF-IR band and the specific IGF-IR band is highlighted by and arrow and was confirmed by immunoprecipitation. (c) Densitometry of A and B. Values represent the ratio of absorbance of phosphorylated protein divided by the total protein ± S.E.M. $*p < 0.05$.

Discussion

The GHR antagonist pegvisomant is a useful new treatment in the management patients with acromegaly [41]. The GH/IGF axis has been implicated in both normal mammary gland development, but also in breast cancer risk and progression [42]. In this manuscript we have used pegvisomant to assess the effect of pharmacological blockade of GHR on both normal mouse pubertal mammary gland development, and also on the growth of human breast cancer xenografts. We find that pegvisomant delays ductal outgrowth and reduces branching in the mammary gland, associated with a direct blockade of both GHR and IGF-IR signaling in the gland. Pegvisomant is also able to cause regression of MCF-7 xenografts by inhibiting proliferation and causing apoptosis that is associated with reduced IRS-1 levels and phosphorylation.

Studies from Kleinberg's laboratory have previously shown that GH is a critical regulator of ductal elongation in the mammary gland, and that GH acts via local production of IGF-I [7]. Our studies support this, showing that pharmacological blockade of GHR delays

ductal elongation. We also noted that GHR blockade resulted in reduced IGF-IR phosphorylation, supporting the notion that GH stimulates ductal growth via IGF-I.

Surprisingly, blockade of mammary gland growth was not associated with a reduction of proliferation within the TEB, even thought we were clearly able to show complete blockade of JAK2, STAT5, IGF-IR and IRS-1 phosphorylation. This result is in conflict with previous genetic studies suggesting that IGF-I and IGF-IR acts upon the terminal end bud to stimulate proliferation [8,34]. It is possible that differences in results may result from the different methodologies for receptor blockade. For instance, pharmacologic blockade of GHR, which indirectly blocks IGF-I signaling through IGF-IR may not completely eliminate signaling to the extent that is achieved with genetic deletion of the IGF-IR. Thus, IGF-IR may still be available under GHR blockade to signal to other critical downstream signaling intermediates. Additionally, the reduction in all of the signaling intermediates seen on a whole cell lysates (Figure 4) may not be representative of the signaling present just within the TEB. Studies are ongoing to try and determine if this is the case.

As there were no changes noted in TEB proliferation or apoptosis, we hypothesized that the delay of ductal outgrowth was caused by the decrease in TEB number and size. After 1 week of pegvisomant, TEB number decreased by ~60% compared to saline treated mice. This decrease in TEB number may be due the fact that TEBs appeared much smaller and some seemed to regress. However, the decrease may also be partly due to an apparent decrease in bifurcation of TEBs, and thus an inability to increase TEB number. An unexpected result was the inability of the pegvisomant treated glands to continue slowly growing, but not be able to 'catch-up' to the vehicle treated glands even after prolonged treatment. In fact, the slope of the curve for the pegvisomant treated glands suggested that ductal development was slowing down at 13 weeks, similar to vehicle treated mice. Indeed, mammary glands from two out of the four pegvisomant treated mice at 13 weeks of age had no TEBs and were morphologically similar to mammary glands from control mice (apart from the lack of fat pad filled). One hypothesis for the slowing down of ductal outgrowth in the presence of pegvisomant is reduced replicative capacity of the TEB due to a lower number of stem/progenitor or transit amplifying cells such that after 10 weeks of treatment, the TEB simply does not have enough renewal capacity left to complete ductal extension. Supporting this, transcription profiling of embryonic, neural, hematologic, and mammary stem cells, found that GHR was one of only 10 genes enriched in all four types of stem cells, with GHR being 10-fold elevated in mammary stem cells compared to differentiated mammary epithelial cells [43]. Thus, pegvisomant maybe blocking the self-renewal of stem/progenitors, or maybe inhibiting their progression to transit amplifying cells. This hypothesis would be consistent with the lack of change in proliferation levels in the pegvisomant TEBs as these tend to reflect the bulk of terminally differentiating cells. This progenitor hypothesis could be tested by treating mice with pegvisomant and then transplanting mammary glands and analyzing their capacity for ductal elongation and self-renewal.

Evidence from several laboratories suggests that IGF signaling may regulate branching within the mammary gland. Kleinberg initially noted in IGF-I-null mice that the limited ductal outgrowth showed dramatically reduced bifurcation and side branching [8]. IGF-IR-null mammary gland outgrowths were subsequently shown to have a similar phenotype [34]. A recent study has shown that the reduction in bifurcation and branching is due to an effect of local IGF-I synthesis, as a reduction of branching is seen in midi mice (which have a decrease in overall IGF-I production) but is not seen in mice exhibiting a specific reduction in just circulating IGF-I by having the liver IGF-I gene deleted [35]. We also noted that pegvisomant caused a dramatic reduction in side branches and bifurcation, clearly supporting the previous genetic data.

We have used serum IGF-I levels as a biomarker for pegvisomant blockage of GH action due to the relative ease of measuring the high circulating IGF-I levels. However, this is not meant to imply that pegvisomant delays ductal elongation and branching by reducing circulating IGF-I levels. Indeed, virtually all of the literature on GH/IGF action in the mammary gland argues against this scenario. For example, GH induces IGF-I mRNA in the mammary gland [44], and IGF-I-null mice have severely perturbed ductal elongation [8], but liver specific deletion of the IGF-I gene does not affect mammary branching [35]. These data all argue for a local paracrine role of GH-induced IGF-I. Consistent with this model, we found that pegvisomant was able to effectively block GHR action in the mammary gland, and that this was also associated with inhibition of IGF-IR action. We were not, however, able to measure IGF-I levels in the mammary gland to directly determine if there had been a reduction of local IGF-I synthesis, as levels were below the sensitivity of the assay. Further experiments are planned to directly address whether the inhibition of mammary gland by pegvisomant is via a disruption of local or systemic IGF-I action by directly administering pegvisomant to the mammary gland.

The same GH/IGF axis that is critical for normal mammary gland development seems to become deregulated and critical for breast cancer growth and progression [42]. The last several years has seen the successful inhibition of human cancer xenograft growth using strategies to target IGF-I [45,46] or IGF-IR [21–24,47]. Here we have used an alternative and potentially complimentary strategy, by inhibiting GHR and subsequent blockade of IGF-I synthesis, to inhibit breast cancer xenograft growth. The blockade of MCF-7 xenograft growth occurs with an inhibition of IRS-1 action. We were unable to definitively prove whether the loss of IRS-1 signaling is via an inhibition of upstream

GH or IGF-IR signaling, or if this affect is simply via direct targeting and lowering of IRS-1 levels. Interestingly we and others have reported significant synergism between the ER and IGF signaling pathways [48], and there are many reports of the lack of IGF-I stimulated growth of ER-negative breast cancer cells [39]. Consistent with this, we have previously shown that MDA-435 growth *in vitro* and as xenografts is not affected by a dominant-negative IGF-IR [24]. However, it should be noted that pegvisomant has the putative ability *in vivo* to block breast cancer growth by a two-pronged attack, inhibiting both GHR and IGF-IR. At present we do not know what downstream pathways are inhibited by pegvisomant. Immunoblot analysis on regressing tumors showed no significant changes in p-AKT or p-ERK1/2. It is possible that other GH/IGF signaling pathways maybe critical for this regression such as an IGF-IR interaction with integrins (e.g. $\alpha v\beta 3$) or adhesion pathways (e.g. FAK), or that pegvisomant maybe targeting as yet undefined signaling pathways.

The ability of pegvisomant to cause MCF-7 xenograft regression, which was associated with a 2-fold induction of apoptosis, points to an emerging literature suggesting that blockade of growth factor receptor signaling will not only be cytostatic, but also cytotoxic. For instance, a new antibody that blocks IGF-IR also inhibited MCF-7 xenograft growth, and this was mainly via an increase in apoptosis [21]. Notably, other anti-IGF-IR antibodies [23], or IGF-IR tyrosine kinase inhibitors [47] have successfully blocked MCF-7 xenograft growth.

Pegvisomant has already shown success in restoration of serum IGF-I levels in patients with acromegaly. However, its clinical use may go much further than this. The GH/IGF axis has been implicated in a number of cancers. Mice that have reduced GH [11] or IGF-I [49–51] are refractory to a number of cancers. Furthermore, restoration of GH [12] or IGF-I [49, 51] levels restores susceptibility to tumorigenesis. Consistent with this, pegvisomant has been reported to inhibit the growth of human meningioma [30] and colon cancer xenografts [31]. Based on this, we predict that pegvisomant maybe effective in the treatment of many cancers, including that of the breast.

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