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# Growth hormone receptor antagonism suppresses tumour regrowth after radiotherapy in an endometrial cancer xenograft model.

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# ABSTRACT

Human GH expression is associated with poor survival outcomes for endometrial cancer patients, enhanced oncogenicity of endometrial cancer cells and reduced sensitivity to ionising radiation *in vitro,* suggesting that GH is a potential target for anticancer therapy. However, whether GH receptor inhibition sensitises to radiotherapy *in vivo* has not been tested. In the current study, we evaluated whether the GH receptor antagonist, pegvisomant (Pfizer), sensitises to radiotherapy *in vivo* in an endometrial tumour xenograft model. Subcutaneous administration of pegvisomant (20 or 100 mg/kg/day, *s.c.*) reduced serum IGF1 levels by 23% and 68%, respectively compared to vehicle treated controls. RL95-2 xenografts grown in immunodeficient NIH-III mice were treated with vehicle or pegvisomant (100 mg/kg/day), with or without fractionated gamma radiation (10×2.5 Gy over 5 days). When combined with radiation, pegvisomant significantly increased the median time tumours took to reach 3× the pre-radiation treatment volume (49 days *versus* 72 days; $p$=0.001). Immunohistochemistry studies demonstrated that 100 mg/kg pegvisomant every second day was sufficient to abrogate MAP Kinase signalling throughout the tumour. In addition, treatment with pegvisomant increased hypoxic regions in irradiated tumours, as determined by immunohistochemical detection of pimonidazole adducts, and decreased the area of CD31 labelling in unirradiated tumours, suggesting an anti-vascular effect. Pegvisomant did not affect intratumoral staining for HIF1α, VEGF-A, CD11b, or phospho-EGFR. Our results suggest that blockade of the human GH receptor may improve the response of GH and/or IGF1-responsive endometrial tumours to radiation.

**Abbreviations**

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|---|---|
| ATCC | American Type Culture Collection |
| EGFR | Epidermal growth factor receptor |
| FBS | Fetal bovine serum |
| GH | Growth hormone |
| HIF1α | Hypoxia inducible factor-1α |
| IGF1 | Insulin-like growth factor 1 |
| VEGF-A | Vascular endothelial factor-A |

# 1. INTRODUCTION

Radiotherapy is used to treat approximately 50% of all cancer patients, with varying success. Although recent advances in cancer treatment regimens have improved patient prognosis, failure of local control is still a major clinical challenge [1]. Increased expression in tumour cells of autocrine growth factors and receptors, as well as signal transduction cascades involved in tumour cell proliferation/survival, has been demonstrated to promote radioresistance through multiple means [2, 3]. For many common cancers, adding novel molecularly targeted agents to radiotherapy may increase cure rates [4, 5]. However, currently the EGFR antagonist cetuximab is the only molecularly targeted agent approved as a radiosensitiser. Thus, identification of novel molecularly targeted radiosensitisers addresses an important unmet clinical need [6, 7].

Growth hormone (GH) has a wide range of endocrine, autocrine and paracrine effects on growth and metabolism, following its secretion from the anterior pituitary and extra-pituitary sites. These can be through direct effects or through secondary stimulation of hepatic insulin-like growth factor 1 (IGF1) secretion. Substantial evidence implicates systemic circulating and extra-pituitary expression of GH and IGF1 in the pathogenesis and progression of cancer [8-14]. In animals and humans with disrupted GH receptor-mediated signal transduction, the incidence of cancer is significantly reduced [11, 12, 15, 16]. In endometrial cancer, human GH (hGH) expression is associated with specific histopathological features including higher International Federation of Gynecology and Obstetrics (FIGO) tumour grade, myometrial invasion, and ovarian metastases, in addition to a worse prognosis for patients [17, 18]. In addition, autocrine hGH enhances the oncogenic characteristics of endometrial cancer cells *in vitro* and increases the growth of RL95-2 tumours following stable forced expression [19].

Despite extensive *in vitro* studies demonstrating the potential utility of GH receptor antagonism for the purposes of treating cancer, studies investigating antitumour efficacy *in vivo* are limited. Pegvisomant, a clinically available GH receptor antagonist (Pfizer Inc.), is Food and Drug Administration (FDA) approved for the treatment of acromegaly, a debilitating disease characterised by excessive levels of GH, most frequently due to a GH-secreting pituitary adenoma [20]. Pegvisomant is an hGH analogue in which a single mutation in binding site 2 (G120K) prevents complete functional binding to the cell surface GH receptor dimer [21, 22], while 6 of 8 amino acid changes introduced into binding site 1 increase receptor affinity [23]. In addition, pegylation of the resulting receptor antagonist increases its pharmacokinetic half-life [21, 22]. A small number of xenograft studies have demonstrated antitumour efficacy for pegvisomant as a single agent [24-27]; however, it remains unclear whether pegvisomant can enhance tumour sensitivity to radiation.

Reports indicating that GH may be a radioprotective agent (reviewed in References [10, 28]) led us to investigate whether autocrine GH conferred resistance to ionising radiation in breast and endometrial cancer cell lines [29]. We demonstrated that autocrine GH enhances breast and endometrial cancer cell viability, clonogenic survival and DNA repair following treatment with ionising radiation [29]. Conversely, functional inhibition of GH signalling in endometrial cancer cells, using a specific GH receptor antagonist, sensitised cells to ionising radiation-induced cell death and enhanced the induction of DNA damage [29]. Similarly, Wu *et al.* recently demonstrated that combining recombinant GH with radiation increased clonogenic survival and reduced DNA damage in a colorectal cancer cell line [30], while expression of *GHR* mRNA or protein in rectal cancer predicted response of tumours to pre-operative radiotherapy [31].

The aim of the current study was to determine whether inhibition of the GH receptor with pegvisomant sensitises endometrial cancer cells to radiation treatment *in vivo,* using a xenograft model of human endometrial cancer. We used RL95-2 cells as they have previously been demonstrated to express low levels of GH and that antagonism of the GH receptor enhances radiation sensitivity of this cell line [19, 32].

# 2. MATERIALS AND METHODS

## 2.1 Cell lines and reagents

The human endometrial cancer cell line, RL95-2, was obtained from the American Type Culture Collection (ATCC). Cells were cultured at 37°C, 5% $CO_2$ in DMEM/F12 (GIBCO) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin, 100 µg/ml streptomycin and Glutamax. Pegvisomant was kindly supplied by Pfizer.

## 2.2 Determination of effective pegvisomant dose

All experiments were carried out under a protocol approved by the University of Auckland Animal Ethics Committee. Female specific pathogen-free NIH-III mice (approximately 22 g) were administered with 20 or 100 mg/kg/day pegvisomant or vehicle (1.36 mg of glycine, 36.0 mg mannitol, 1.04 mg $Na_2HPO_4$, and 0.36 mg $NaH_2PO_4 \cdot H_2O$ per 0.5 ml), subcutaneously (*s.c.*) every day for 5 days. Blood was collected 6 h after the final dose of pegvisomant by terminal cardiac puncture under $CO_2$ anaesthesia.

## 2.3 Xenograft studies

RL95-2 xenografts were established by the *s.c.* injection of $5\times10^6$ RL95-2 cells suspended in 50 µl serum free DMEM/F12 medium and Matrigel (1:1; BD Biosciences) 1 cm from the tail base on the midline. Mice were allocated to 4 treatment groups when tumours exceeded 100 $mm^3$ mean volume; the average volume at start of treatment was 173.6 $mm^3$, with a standard deviation of 55.1 $mm^3$. There was no significant difference in pretreatment volumes between groups. Mice were treated with vehicle or pegvisomant (*s.c.* 100 mg/kg every day for 7 days, then every second day until study end), with or without fractionated local tumour radiation (10×2.5 Gy over 5 days). The number of animals in each group was as follows: vehicle n=11; pegvisomant n=12; radiation n=11; radiation + pegvisomant n=11. Pegvisomant administration commenced 2 days prior to the initiation of radiation. Tumours were locally irradiated with an external beam cobalt-60 unit (dose rate 2.55 Gy/min) using a lateral beam with custom-designed lead collimators. Animals were held in restraining boxes without anaesthesia during irradiation. Tumours were measured three times weekly using calipers until they reached three times the pre-radiation treatment volume. Tumour volume was calculated as $\pi\ (L \times w^2) / 6$, where $L$ is the major axis and $w$ is the minor axis.

Once the volume endpoint was reached, blood was collected by terminal cardiac puncture under anaesthesia, 24 h after the final pegvisomant administration. The extent of hypoxia was evaluated by the hypoxia tracer pimonidazole. Mice were dosed *i.p.* with 60 mg/kg of pimonidazole (Hypoxyprobe-1 kit, Hypoxyprobe Inc) 90 min prior to euthanasia and tumours were 4% paraformaldehyde-fixed for immunohistochemistry.

## 2.4 IGF1 analysis

Serum IGF1 (ng/mL) was quantified by enzyme-linked immunosorbent assay (ELISA) (Mediagnost, Germany) as per the manufacturer's guidelines.

## 2.5 Immunostaining and determination of hypoxia in xenografts

Paraformaldehyde-fixed tumours were paraffin embedded, sectioned (5 µm), mounted on slides, deparaffinised, and rehydrated. Following antigen retrieval in either 0.01 M citrate buffer pH 6 (for Hypoxia inducible factor-1α (HIF1α), vascular endothelial factor-A (VEGF-A), CD11b, phospho-ERK1/2, phospho-epidermal growth factor receptor (EGFR) and pimonidazole antibodies) or 0.5 M Tris buffer pH 10 (for the CD31 antibody) for 1 h, sections were immunostained with antibodies against CD31 (Abcam, ab28364, 1/100),

HIF1α (Abcam, ab2185, 1/400), VEGF-A (Abcam, ab183100, 1/500), CD11b (Abcam, ab133357, 1/300), phospho-EGFR (Y1092) (Abcam, ab40815, 1/600); phospho-ERK1/2 (Thr202/Tyr204, Cell Signalling #4370, 1/150) or pimonidazole adducts (Hypoxyprobe, 1/100) and visualised with a Novolink polymer DS 250 Kit (Leica). Anti-HIF1α, CD11b, phospho-ERK1/2, phospho-EGFR and CD31 antibodies all recognise the mouse and human orthologues of the protein. The anti-VEGF-A antibody was human specific.

**2.6 Immunohistochemistry quantification**

Tumours (n=6 per treatment group) were sectioned and stained by immunocytochemistry as described above. Slides were examined and images were taken using an automated VSlide scanner (Metasystems). For pimonidazole analysis, the percentage of labelled area was quantitated for the entire tumour section under ×10 magnification. For HIF1α, VEGF-A, CD11b and CD31, at least six different fields (×20 magnification) were chosen randomly from each section. Images (TIFF files) were analysed using ImageJ/Fiji software [33]. For HIF1α, VEGF-A and CD31, thresholds were determined using at least three different images. The determined threshold was then used to analyse all images from sections that were stained in the same staining session (taken at ×20). The background level was calculated from control sections and subtracted for image analysis. CD11b was quantitated using point scoring under ×20 magnification. All immunohistochemistry quantification was performed blinded to treatment group and outcome.

**2.7 Statistical analysis**

Statistical significance was determined by one-way analysis of variance (ANOVA), ANOVA on ranks with Tukey's all-pairwise comparison, or paired *t* tests (within-group comparisons; pre- versus post-treatment). Differences in tumour growth delay studies were assessed using a log-rank test with Holm-Sidak multiple comparison analysis. Statistical analyses were performed using SigmaPlot version 12.5 (Systat Software Inc.). $p<0.05$ was considered significant.

## 3. RESULTS

To determine whether pegvisomant can prevent IGF1 production in NIH-III mice, animals (n=6) were treated with 5 daily *s.c.* doses of pegvisomant. Treatment with 20 mg/kg and 100 mg/kg pegvisomant reduced serum IGF1 concentrations by 23.0% (58 ± 29 (standard error of the mean, SEM) versus 453 ± 17 ng/ml, $p<0.05$, $p<0.001$) and 67.7% (versus 190 ± 10 ng/ml, $p<0.001$), respectively, when compared with vehicle-treated controls (Figure 1a). No significant bodyweight loss was observed following pegvisomant treatment at either dose level (Figure 1b).

Next, to determine if pegvisomant can prevent tumour growth alone or in combination with ionising radiation, NIH-III mice were inoculated with $5\times10^6$ RL95-2 cells in Matrigel and treated with pegvisomant *s.c.* 100 mg/kg/day for 7 days followed by every second day for up to 16 weeks, either as a single agent or combined with fractionated radiation (10 × 2.5 Gy fractions over the first five days). Radiation delayed tumour regrowth (measured as median time to 3×pre-radiation treatment volume) from 28 to 49 days ($p<0.001$, log-rank), while pegvisomant administered as a single-agent did not significantly affect RL95-2 tumour growth (Figure 2A & B). However, when combined with radiation, pegvisomant significantly increased the radiation-induced delay in tumour regrowth from 49 to 72 days ($p<0.001$, log-rank; pegvisomant + radiation versus radiation alone). There were no clinical signs of toxicity and no change in body weight relative to vehicle controls, following pegvisomant treatment (Figure 2C).

Serum IGF1 was measured in blood collected after tumours reached endpoint volume and 24 h after final pegvisomant administration. Pegvisomant every second day reduced serum IGF1 concentrations by 76.2% (522 ± 23 ng/ml versus 124 ± 3 ng/ml, $p<0.001$) in non-irradiated treatment groups and 45.3% (421 ± 26 versus 191 ± 10 ng/ml, $p<0.001$) in irradiated groups (Figure 3A). Serum IGF1 was also reduced slightly in the irradiated group when compared to non-irradiated controls (522 ± 23 versus 421 ± 26 ng/ml, $p<0.001$) (Figure 3A).

To investigate the mechanism by which pegvisomant might promote radiosensitisation, immunohistochemical analysis of markers of MAP Kinase signalling, hypoxia, and tumour vasculature was carried out when tumours reached endpoint volume. We observed reduced immunohistochemical staining of pERK1/2 in both the central and peripheral regions of pegvisomant-treated non-irradiated tumours when compared to vehicle-treated control tumours, indicative of inhibition of MAP Kinase signal transduction (Figure 3B). Staining with the hypoxia marker pimonidazole revealed that treatment with the combination of pegvisomant and radiation treatment increased tumoural hypoxic regions from 6.5 ± 0.7% to 11.6 ± 2.1% ($p<0.05$) when compared with radiation alone, suggesting pegvisomant had an anti-vascular effect in radiation-treated tumours (Figure 4A). We next evaluated tumoural expression levels of the hypoxia-induced transcription factor, HIF1α by quantitative immunohistochemistry. Radiation treatment resulted in a small increase in the intensity of HIF1α staining in the nucleus (Figure 4B). However, no change in HIF1α staining intensity was observed following treatment with pegvisomant (Figure 4B).

The extent of tumour vasculature was determined by analysing the area of CD31-labelled cells. CD31 staining was reduced 2.1-fold with pegvisomant treatment in non-irradiated tumours ($p<0.05$). However, pegvisomant did not affect the proportion of CD31-positive vessels in radiation-treated tumours (Figures 5A and B). No significant effect of either radiation or pegvisomant was observed on VEGF-A (Figures 5A and C) or the myeloid cell marker, CD11b (Figure 5A and D). Radiation increased staining for phospho-EGFR; however, pegvisomant did not affect the area of phospho-EGFR staining in irradiated or control tumours (Figures 5A and E).

# 4. DISCUSSION

Substantial evidence supports a role for the GH/IGF1 axis in cancer. However, limited studies investigating GH receptor antagonism in cancer models are available, and pegvisomant is yet to be tested in oncology clinical trials. We demonstrate here that pegvisomant delays the regrowth of RL95-2 tumours following fractionated radiation treatment. This is the first study to combine GH receptor antagonism with ionising radiation in an *in vivo* setting. As radiotherapy is used to treat approximately 50% of all cancer patients, agents that improve the efficacy of radiotherapy have the potential to improve treatment outcome in a significant proportion of patients.

Pulsatile secretion of GH from the anterior pituitary stimulates IGF1 production and secretion in the liver which is the primary source of circulating IGF1. Pegvisomant inhibits the actions of GH, but not secretion from the pituitary. Instead normalisation of plasma IGF-I concentration is the biochemical criterion by which efficacy is assessed in patients with acromegaly [34, 35]. We found that 100 mg/kg pegvisomant was effective at reducing circulating IGF1 in NIH-III mice and abrogated intratumoral activation of ERK1/2. 20 mg/kg was less effective at reducing circulating IGF1; however, as pegvisomant inhibits the human GH receptor much more effectively than the GH receptor in mice [21], it is likely that at this dose effective antagonism of the human GH receptor in RL95-2 cells would have been achieved. In human studies, *s.c.* injections of 80 mg (approximately 1.1 mg/kg) administered

daily to healthy subjects over 14 days reduced the mean circulating IGF1 concentration by 62% [36], consistent with much higher doses being required in mice than humans to antagonise the GH receptor and suppress circulating IGF1. We considered it important in the study design to suppress systemic IGF1, which exhibits cross-species activity, to parallel what would be expected in a human clinical setting, and so 100 mg/kg pegvisomant was used for the radiosensitisation study. As IGF1 has been linked to cancer and is a radioprotective agent [3, 37, 38], the reduction in circulating IGF1 concentrations may further impact on tumour growth and radiosensitivity [3, 38]. The reduction in serum IGF1 we observed following pegvisomant administration is consistent with other studies in mice. Divisova *et al.* observed approximately a 70% reduction in IGF1 with 100 mg/kg pegvisomant administered by daily *i.p.* injection for 14 days [25]. Dagnaes-Hansen *et al.* observed a 64-57% reduction in IGF1 with 60 mg/kg injected *s.c.* every second day over 30 days [24], while McCutcheon *et al.* observed a 20% reduction in circulating IGF1 with daily *s.c.* of 45 mg/kg over 8 weeks [26]. In a dose response study carried out by van Neck *et al.,* 20 mg/kg pegvisomant administered every 2 days, reduced serum IGF1 by 51% [39] which is greater than the reduction we observed. Differences in the mouse strain, injection route and composition of the vehicle control used may contribute to the observed variation in serum IGF1 concentrations across different studies.

When combined with radiation, pegvisomant significantly increased the median time tumours took to reach 3× the pre-radiation treatment volume. Although this is the first study to combine pegvisomant with radiation, there is precedent from other studies supporting anti-cancer activity for pegvisomant in several tumour models. Reported doses in these studies ranged from 60 mg/kg every second day to 250 mg/kg/day [24-27]. Studies from Adrian Lee's lab reported effective reduction in estrogen receptor-positive MCF-7 xenograft volume with 100 and 250 mg/kg/day pegvisomant *i.p.* [25]. But no effect was seen in the estrogen receptor-negative breast cancer cell lines MDA-MB-231 and MDA-MB-435. Dagnaes-Hansen *et al.*, found that 60 mg/kg *s.c.* every second day reduced the growth of colon cancer xenografts derived from Colo205, but not HT29 cells [24], while 45 mg/kg/day *s.c.* reduced the growth of xenografts generated with primary cell lines derived from fourteen human meningioma specimens [26]. Response to pegvisomant in most instances was linked to indirect effects via reduction of circulating IGF1, as MDA-MB-231 and MDA-MB-435 are unresponsive to IGF1. The lack of response observed in HT29 tumours may be due to the fact that these cell lines independently express relatively high levels of autocrine IGF1 and IGF2 which would not have been affected by pegvisomant treatment [24, 25]. However, response in the meningioma and colon cancer cell lines may also be linked to expression of autocrine hGH as this was not investigated.

The mechanisms contributing to delayed growth in irradiated tumours treated with pegvisomant are unclear. We have previously demonstrated that GH enhances vascularisation of MCF-7 xenografts and increases tumoural staining of vascular endothelial growth factor A (VEGF-A) [40]. Consistent with this, we observed reduced CD31 staining in unirradiated tumours treated with pegvisomant; however, this was not the case in irradiated tumours, and pegvisomant had no effect on VEGF-A staining either before or after radiation. RL95-2 cells expressed relatively high levels of VEGF-A though, and as the antibody did not recognise mouse VEGF-A, it was not possible to determine the effects of pegvisomant on expression of VEGF-A from endothelial or stromal cells of murine origin in the tumour microenvironment. We also assessed CD11b levels as previous studies had demonstrated that in glioblastoma tumour irradiation blocks local angiogenesis within the tumour microenvironment, and that revascularisation occurs through hypoxia-induced vasculogenesis and recruitment of

circulating progenitor cells [41, 42]. Initial recruitment of proangiogenic CD11b-positive monocytes to the microenvironment is a key step in this process [41, 42]. Consistent with this model, we observed a trend to higher numbers of $CD11b^+$ cells in irradiated tumours and suppression of this by pegvisomant, although these changes were not statistically significant. One other potential mechanism we considered was activation of the EGFR signal transduction pathway, as this can contribute to radiation resistance [43]. GH signalling has been demonstrated to promote EGFR kinase-independent EGFR phosphorylation and EGFR crosstalks with GH receptor signalling [44, 45]. However, pegvisomant did not affect the activation of EGFR in irradiated or control tumours, as determined by IHC staining of phospho-EGFR.

Pegvisomant increased tumour hypoxia in irradiated RL95-2 tumours, as determined by pimonidazole staining. Increased hypoxia in tumours is usually considered to be detrimental in the curability of tumors and is associated with treatment resistance, cell proliferation, and metastatic potential [46]. It is therefore interesting that a delay in tumour regrowth was observed in tumours treated with radiation and pegvisomant, despite an increase in the hypoxic fraction. However, our study did not assess whether pegvisomant treated tumours were more hypoxic prior to radiation treatment or during the regrowth period, as analysis was only carried out on pegvisomant-treated tumours at endpoint volume. Further studies will help us to understand the time-course of hypoxia induction. Hypoxia is also a characteristic of tumours that can be exploited therapeutically with drugs designed specifically to attack the hypoxic cell subpopulation [3, 47]; thus combining pegvisomant and ionising radiation with a hypoxia-activated prodrug may be of therapeutic relevance although it would be important to establish the time-course of induction of hypoxia during treatment with pegvisomant.

The RL95-2 cell line was selected based on previously reported endogenous expression of autocrine hGH, IGF1 responsiveness, and response to GH receptor antagonism in cell culture. Despite this, pegvisomant alone had no effect on tumour growth at dose levels comparable to those reported to delay tumour growth in other cell line xenograft models [24, 25]. Given outcomes from this study, and those described above [24-26, 48], there is clearly a need for careful characterisation of the expression of relevant receptors and autocrine growth factors, and the signal transduction pathways utilised, in order to predict the *in vivo* response of xenografted cell lines to pegvisomant. In this regard it should be noted that RL95-2 cells express the prolactin receptor (data not shown), and GH has been demonstrated to induce signal transduction through a GH receptor-prolactin receptor heterodimer in some cell lines which may impact on the effectiveness of GH receptor antagonism [49, 50]. Although we did not investigate whether GH-prolactin receptor heterodimers were present in RL95-2 cells, GH receptor antagonism has previously been shown to be effective in this cell line, reducing cell proliferation and survival, and enhancing radiation sensitivity *in vitro* [19, 29].

It is possible that GH receptor antagonism with pegvisomant may be more beneficial in a human clinical setting due to both the increased affinity of the antagonist for the receptor and efficient functional antagonism of systemic GH/IGF1 as well as autocrine GH. Human GH can bind and activate the mouse GH receptor effectively, whereas mouse GH does not activate the human GH receptor [51]. Therefore, the growth of GH-responsive human xenografts in mice is not promoted by pituitary/systemic GH, as would occur in the human setting. Thus, xenograft studies investigating the effect of pegvisomant on the growth of GH-responsive tumours would not benefit from blockade of systemic GH. Consequently, GH receptor antagonism with pegvisomant may prove to be more effective in humans. We cannot determine the relative contribution of autocrine GH blockade *versus* suppression of systemic GH/IGF1 to the delayed regrowth of RL95-2 tumours in our study, but it may be possible to

dissect out relative effects of autocrine and endocrine suppression by using a GH receptor inhibitor that is specific to the human GH receptor in xenograft studies.

In conclusion, we have demonstrated that pegvisomant combined with radiation delays the regrowth of RL95-2 tumours. As radiotherapy combined with surgery is used to manage Stage 2 endometrial cancer, blockade of the human GH receptor may improve the response of endometrial tumours which are GH and/or IGF1 responsive, to radiation. The radiosensitising activity of pegvisomant is unlikely to be limited to just endometrial cancer, so pegvisomant and radiation may also have application in other tumours that are GH- or IGF1-responsive, including breast and colon.

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# FIGURE LEGENDS

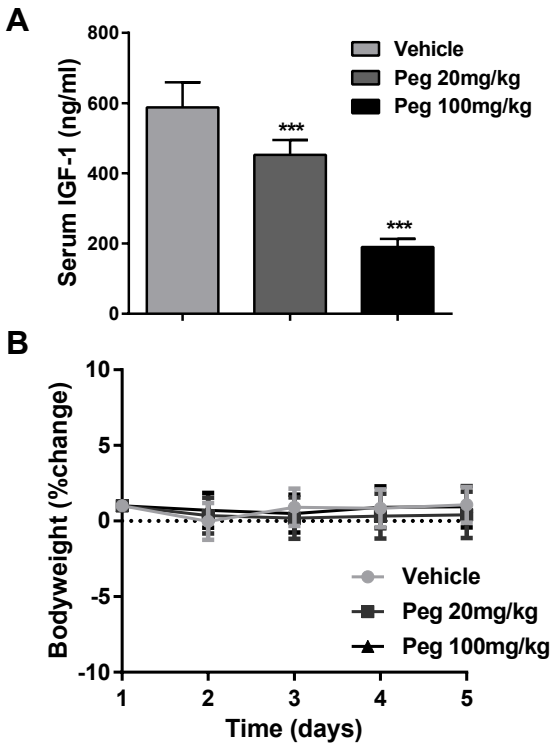
**Figure 1. Serum IGF1 concentration and bodyweight following treatment with pegvisomant.**
**A.** Mice were treated with *s.c.* administration of 20 or 100 mg/kg/day pegvisomant (Peg) for 5 days (*** $p<0.001$, one-way ANOVA, Tukey's post-hoc test). **B.** Bodyweight changes over the 5-day dosing period. Error bars and symbols represent mean ± SEM (n=6).

**Figure 2. Pegvisomant delayed the regrowth of RL95-2 tumours following exposure to ionising radiation.**
**A.** Tumour growth curves showing fold change in tumour volume (n=11-12 per group). Data points represent means (with errors not shown for clarity). Arrows indicate initiation of pegvisomant administration (P, Peg) and radiation (R, Rad). **B.** Kaplan-Meier survival plots depicting the activity of pegvisomant in combination with radiation. Pegvisomant increased the radiation-induced delay in RL95-2 tumour regrowth (time to 3×treatment volume; $p=0.001$ versus radiation alone; log-rank test, Holm-Sidak). **C.** Bodyweight changes over the dosing period. Error bars and symbols represent mean ± SEM (n=11-12).

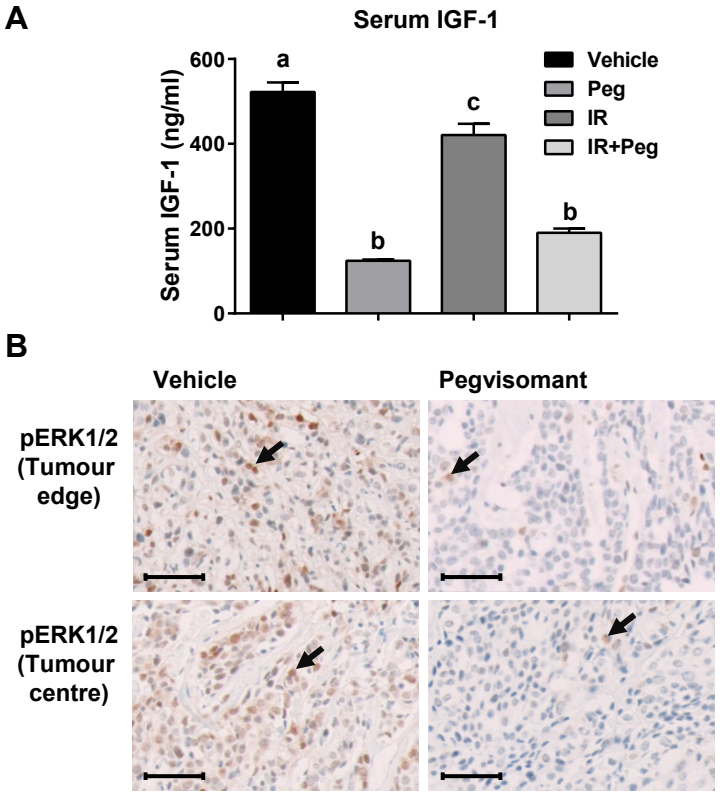
**Figure 3. Pegvisomant reduces serum IGF1 levels and intratumoural pERK1/2 expression.**
A. IGF1 concentrations in serum collected after tumours reached endpoint volume and 24 h after final pegvisomant (PEG) administration. Groups that do not share the same letter are significantly different from each other (*** $p<0.01$, one-way ANOVA, Tukey's post-hoc test). Error bars and symbols represent mean ± SEM (n=11-12 per group). (IR, ionising radiation) B. Immunohistochemical staining for phospho-ERK1/2 (indicated by arrows). Images were taken from the central and peripheral regions of unirradiated RL95-2 tumours treated with vehicle or pegvisomant (n=3 per group). Bar represents 50 μm.

**Figure 4. Pegvisomant increases tumour hypoxia in irradiated RL95-2 tumours.**
**A.** Immunohistochemical staining for pimonidazole adducts as indicated by arrows. Bar graph depicts quantification of the area of pimonidazole staining in irradiated tumours treated with vehicle or pegvisomant. Error bars and symbols represent mean ± SEM (n=6 per group). **B.** Immunohistochemical staining for HIF1α (indicated by arrows). IR, ionising radiation; Peg, pegvisomant.

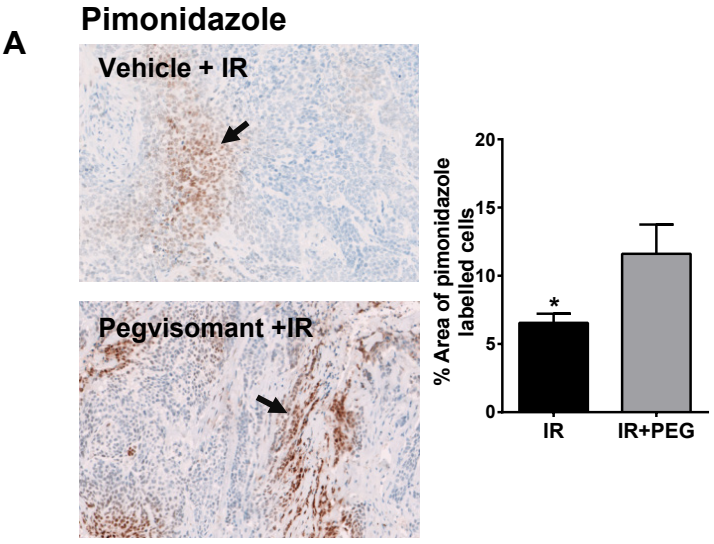
**Figure 5. Pegvisomant decreases vascularisation of unirradiated RL95-2 tumours**
**A.** Immunohistochemical staining of ***i.*** CD31 staining of endothelial cells (indicated by arrows), ***ii.*** VEGF-A staining (arrows), ***iii.*** CD11b positive cells as indicated with arrows and ***iv*** phospho-EGFR (pEGFR) in RL95-2 tumours (arrows). Bar graphs depict quantification of the area of **B.** CD31, **C**. VEGF-A, **D.** CD11b and **E.** phospho-EGFR staining. V, vehicle; P, pegvisomant; IR, ionising radiation; IR+P, combined radiation and pegvisomant. * $p<0.05$ (ANOVA on ranks, Tukey's all-pairwise comparison). Error bars and symbols represent mean ± SEM (n=6 per group).

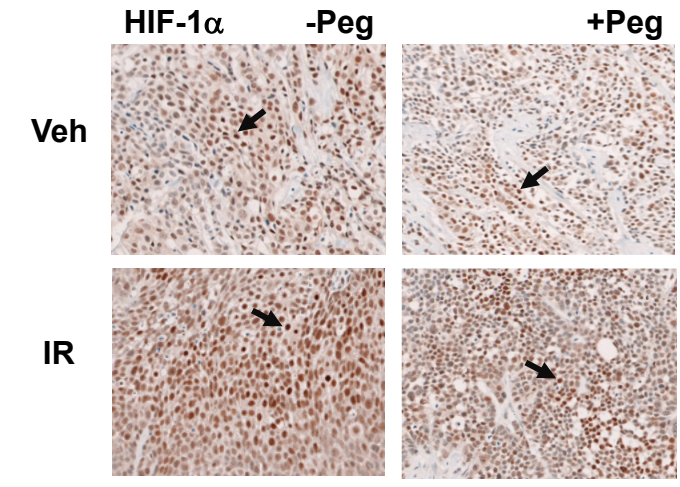



**Evans *et al.* Figure 1**

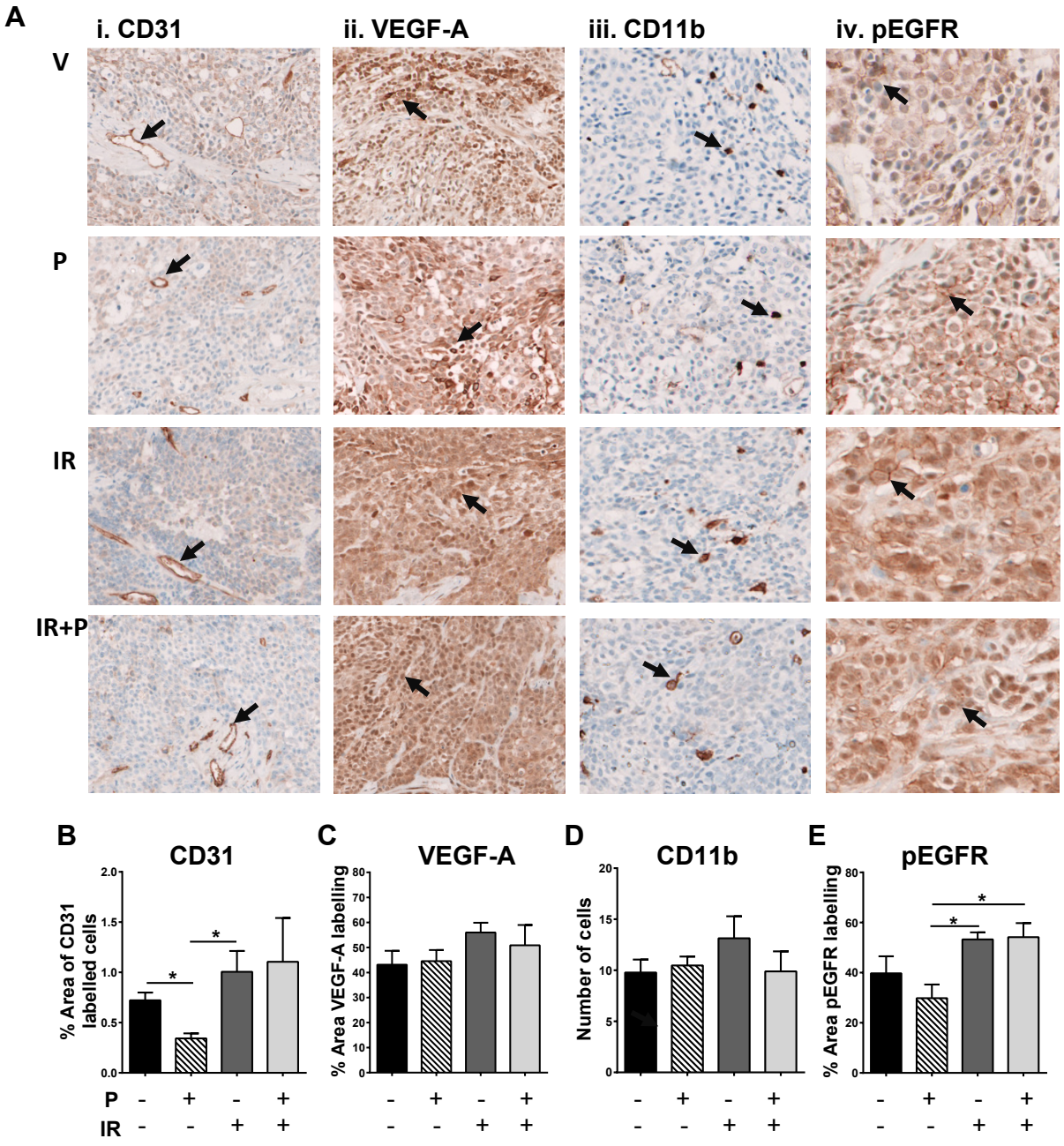



**Evans *et al.* Figure 3**




**Evans *et al.* Figure 4**




**Evans *et al.* Figure 5**