Autocrine human GH promotes radioresistance in mammary and endometrial carcinoma cells

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Abstract

Although recent advances in breast cancer treatment regimes have improved patient prognosis, resistance to breast cancer therapies, such as radiotherapy, is still a major clinical challenge. In the current study, we have investigated the role of autocrine human GH (hGH) in resistance to ionising radiation (IR)-based therapy. Cell viability and total cell number assays demonstrated that autocrine hGH promoted cell regrowth in the mammary carcinoma cell lines, MDA-MB-435S and T47D, and the endometrial carcinoma cell line, RL95-2, following treatment with IR. In addition, autocrine hGH enhanced MDA-MB-435S and T47D cell clonogenic survival following radiation exposure. The enhanced clonogenic survival afforded by autocrine hGH was mediated by JAK2 and Src kinases. Investigation into the DNA repair capacity demonstrated that autocrine hGH reduced IR-induced DNA damage in MDA-MB-435S and T47D cells. Functional antagonism of hGH increased RL95-2 sensitivity to IR in cell viability and total cell number assays, reduced clonogenic survival and enhanced the induction of DNA damage. Thus, autocrine hGH reduced sensitivity to treatment with IR in mammary and endometrial carcinoma cell lines *in vitro*, while functional antagonism of hGH sensitised endometrial carcinoma cells to IR. Functional antagonism of hGH, used in conjunction with radiotherapy, may therefore enhance treatment efficacy and improve the prognosis of patients with breast and endometrial cancer.

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Introduction

Radiotherapy is a frontline therapy used in the treatment of breast cancer and is used to treat the majority of patients with invasive breast cancer or ductal carcinoma *in situ*. Exposure of tumour cells to ionising radiation (IR) promotes cell death through the induction of DNA damage and results in a range of lesions including single-strand breaks and highly lethal double-strand breaks (DSBs; Jeggo & Lobrich 2006). Generation of DNA lesions activates DNA damage checkpoint pathways and subsequent DNA repair networks that are central to the cellular response to IR (Branzei & Foiani 2008). Resistance to radiation therapy is a major clinical obstacle; tumour response to

radiation can vary considerably, even in tumours displaying similar pathology.

Numerous extrinsic and intrinsic factors contribute to reduced cellular sensitivity to IR. The extent of tumour hypoxia is one well-established extrinsic factor affecting radioresponse; viable cells in hypoxic areas are more resistant to IR-induced cell death than normoxic cells (Gray *et al.* 1953, Nordsmark *et al.* 2005), a consequence of reduced DNA damage in hypoxic regions. Intrinsic factors include an enhanced capacity of cells to repair DNA damage, which can occur through alteration in the expression or activity of DNA repair enzymes (Dumont *et al.* 2009, Dutreix *et al.* 2010), cell cycle checkpoint control; the

activation of signal transduction cascades involved in tumour cell proliferation and survival such as the phosphatidylinositol 3-kinase (PI3K) pathway and mitogen-activated protein kinase (MAP kinase) pathway (Jameel *et al.* 2004, Begg *et al.* 2011), and through variation in the cellular concentration of thiols (e.g. glutathione and cysteine; Estrela *et al.* 2006). Increased expression in tumour cells of autocrine growth factors and growth factor receptors such as vascular endothelial growth factor (VEGFA), insulin-like growth factor 1 receptor (IGF1R) and members of the human epidermal growth factor receptor (HER) family have been demonstrated to promote radio-resistance through multiple mechanisms (Jameel *et al.* 2004, Dumont *et al.* 2009, Zaidi *et al.* 2009).

Accumulating evidence indicates a role for human GH (hGH) in human cancer (Perry *et al.* 2006, 2008, Kleinberg *et al.* 2009, Brooks & Waters 2010, Harvey 2010, Gallagher & Leroith 2011). Recent studies have demonstrated that humans with GH receptor (GHR) deficiency are protected from developing cancer (Shevah & Laron 2007, Gallagher & Leroith 2011, Guevara-Aguirre *et al.* 2011, Steurman *et al.* 2011); two recent studies observed that individuals with Laron syndrome, which results from a defect in the GHR, have a greatly reduced, almost absent, risk of cancer compared with individuals from the same family without the receptor deficiency (Guevara-Aguirre *et al.* 2011, Steurman *et al.* 2011). While studies investigating hGH expression in human breast and endometrial cancer have demonstrated that hGH expression is associated with specific histopathological features and survival outcomes for patients (Slater *et al.* 2006, Wu *et al.* 2011). In mammary carcinoma, hGH expression is positively correlated with lymph node metastasis, tumour stage, HER-2 status and proliferative index (Wu *et al.* 2011). Increased hGH expression has also been implicated in the pathogenesis of endometriosis and endometrial adenocarcinoma (Ayalon *et al.* 1982, Slater *et al.* 2006, Yurkovetsky *et al.* 2007, Pandey *et al.* 2008, Wu *et al.* 2011) and in endometrial carcinoma is associated with increased FIGO grade, myometrial invasion and ovarian metastases (Wu *et al.* 2011).

In recent studies, we have demonstrated that autocrine hGH promotes cell proliferation, cell survival and oncogenicity of human mammary epithelial and endometrial carcinoma cells, enhancing anchorage-independent cell growth and supporting tumour formation in immunodeficient mice (Zhu *et al.* 2005a, Pandey *et al.* 2008). Autocrine hGH also promotes cell migration/invasion and epithelial to mesenchymal transition in both mammary and

endometrial carcinoma cell lines (Mukhina *et al.* 2004, Pandey *et al.* 2008).

A radioprotective role for hGH has been suggested by cell culture and *in vivo* studies. Treatment of Chinese hamster ovarian (CHO-4) cells stably expressing the GHR with exogenous hGH protects them from cell death induced by γ -irradiation or the radiomimetic drug bleomycin in a dose-dependent manner (Madrid *et al.* 2002). Treatment of irradiated rat pituitary cells or human peripheral blood lymphocytes with recombinant GH increases cell survival (Chiarenza *et al.* 2000, Lempereur *et al.* 2003). While protection from radiation-induced apoptosis has also been observed in cell cultures derived from the rat embryonic brain cortex treated with GH-enriched media (Isla *et al.* 2002).

Administration of GH has also been demonstrated to be radioprotective in animal studies (Gomez-de-Segura *et al.* 1998, Vazquez *et al.* 1999, Mylonas *et al.* 2000, Raguso *et al.* 2002, Morante *et al.* 2003, Tekin *et al.* 2006, Isla *et al.* 2007, Chen *et al.* 2010). Treatment of rats with GH prevented body weight loss and decreased mortality following abdominal γ -irradiation (Gomez-de-Segura *et al.* 1998) and enhanced intestinal mucosal integrity (Alexandrides *et al.* 1998, Gomez-de-Segura *et al.* 1998). This protective effect following radiation was associated with decreased intestinal mucosal cell apoptosis (Mylonas *et al.* 2000, Morante *et al.* 2003) and an increased proliferative index (Gomez-de-Segura *et al.* 1998, Morante *et al.* 2003). hGH administration was also demonstrated to protect normal rat intestinal tissue from radiotherapy-induced damage but not implanted adenocarcinoma cells (Morante *et al.* 2003). Furthermore, rats pre-treated with GH before radiation exposure had decreased severity of radiodermatitis, a frequent complication of radiotherapy (Tekin *et al.* 2006). Such studies have led to the suggested clinical value of hGH in reducing radiation-induced toxicity.

There is less direct evidence in humans that hGH protects against radiotherapy-induced cell death, although one study found that elevated levels of expression *hGHR* mRNA and protein are associated with a poor response to radiotherapy in rectal cancer (Wu *et al.* 2006). Increased hGHR expression was also observed following treatment with radiotherapy when compared with pre-radiation samples (Wu *et al.* 2006).

As described, the majority of studies have investigated a radioprotective effect for exogenous GH on normal cells and tissues with the aim of reducing radiation-induced toxicity. As endogenous hGH expression is observed in a proportion of breast and endometrial tumours (Slater *et al.* 2006, Wu *et al.* 2011), we hypothesised that autocrine expression of

hGH would reduce cancer cell sensitivity to radiation-induced cell death, thus potentially reducing the effectiveness of radiotherapy. In this study, we investigated the effect of autocrine hGH on mammary carcinoma and endometrial carcinoma cell radiosensitivity and demonstrate that autocrine hGH reduces mammary carcinoma cell sensitivity to treatment with IR, while functional antagonism of hGH in endometrial carcinoma cells sensitises cells to IR and enhances IR-induced cell death, thus identifying compounds that antagonise the hGHR as potential radiosensitising agents.

Materials and methods

Cell lines and cell transfection

Two breast cancer cells lines (MDA-MB-435S and T47D) and an endometrial cancer cell line (RL95-2) were used in this study. MDA-MB-435S and T47D cells were cultured at 37 °C in 5% CO₂ in RPMI (Gibco) supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine. RL95-2 wild-type and stable cell lines (vec and hGH) were cultured at 37 °C in 5% CO₂ in Advanced DMEM/F-12 (Invitrogen) supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine (Gibco).

MDA-MB-435S, T47D and RL95-2 cells stably transfected with an expression plasmid containing the wild-type *hGH* gene, *GHI* (pcDNA3-hGH), under the control of a CMV promoter (cell lines designated MDA-hGH, T47D-hGH and RL95-hGH) have been described previously (Pandey *et al.* 2008, Bougen *et al.* 2011). It should be noted that the stably transfected MDA-MB-435S cell lines were originally reported to be derived from MDA-MB-231 cells (Bougen *et al.* 2011); however, recent short tandem repeat testing has identified that this cell line is MDA-MB-435S. The control cell lines are stably transfected with the empty pcDNA3 vector (cell lines designated MDA-vec, T47D-vec and RL95-vec). Pooled stable transfectants were used to minimise any effect of potential clonal selection.

For functional inhibition of hGH, the hGHR antagonist, B2036 (Pfizer, Chesterfield, MD, USA), was added to the medium (1000 nM) and media replaced every 2 days. Exogenous hGH was added to the medium at 0–100 nM and media replaced every 2 days. BSA (Sigma–Aldrich) was used as a protein control at equivalent concentrations where appropriate.

The following small molecule inhibitors were used: JAK2 inhibitor, AG490 (50 µM); c-SRC inhibitors, PP1 (20 µM) and PP2 (20 µM) and the inactive structural analogue control, PP3 (20 µM); all from Merck.

Extraction of RNA and semiquantitative RT-PCR

RNA extraction and semiquantitative reverse transcriptase (RT)-PCR was carried out as described previously (Amiry *et al.* 2009). Amplified RT-PCR products were visualised on a 1.5% agarose gel. The sequences of the nucleotide primers used in real-time PCR (qPCR) are provided in Table 1.

Real-time PCR analysis

For quantitative real-time PCR, total RNA was converted to cDNA using SuperScript III First-Strand Synthesis SuperMix for qRT-PCR (Invitrogen) as per manufacturer's instructions. Real-time PCR analysis was performed using an ABI 7900 real-time PCR system (Applied Biosystems, Foster City, CA, USA). Multiple gene markers distributed around the genome and three housekeeping genes were used for real-time PCR analysis using the SYBR GreenER qPCR SuperMix for ABI PRISM (Invitrogen). 5 ng total cDNA isolated from each stable cell line was added to a 20 µl reaction containing SYBR GreenER qPCR SuperMix for ABI PRISM and 200 nM of each primer. Triplicate reactions were performed for each marker in a 384-well plate using a two-step amplification program of initial denaturation at 95 °C for 10 min, followed by 40 cycles of 95 °C for 20 s and 60 °C for 30 s. A melting curve analysis step was carried out at the end of the amplification, consisting of denaturation at 95 °C for 1 min and re-annealing at 55 °C for 1 min. The geometric mean of Ct-value for each reaction was calculated. Amplification efficiencies were calculated according to the equation $E = 10^{(-1/\text{slope})}$ and ranged from 90 to 104% for all gene markers; no unspecific amplification or primer dimer was observed in any of the reactions as confirmed by the melt curve analysis.

Each change in gene expression is expressed as 'fold change' and is the average of three replicates. Positive and negative fold change indicates a respective increase or decrease in mRNA levels. To compensate for potential differences between markers, the relative expression was computed, based on the efficiency (*E*). Gene of interest (GOI) expression was normalised by reference genes (REF): β-actin and GAPDH.

Relative expression and fold change were calculated using the formulae:

$$\text{Relative expression (RE)}_{\text{Test Control}} = \frac{(E_{\text{GOI}})^{(C_{\text{tGOI Control}} - C_{\text{tGOI Test}})}}{(E_{\text{REF}})^{(C_{\text{tREF Control}} - C_{\text{tREF Test}})}}$$

$$\text{Fold change}_{\text{RE} > 1} = \text{RE}$$

$$\text{Fold change}_{\text{RE} < 1} = \frac{1}{\text{RE}}$$

Changes in relative expression > 1.5-fold were taken as significant.

Western blot analysis

Western blot analysis was carried out as described previously (Mertani *et al.* 2001) using the following antibodies: Rad-51 (Abcam, Inc., Cambridge, MA, USA; 1:1000) and anti-mouse secondary antibody conjugated to HRP (Sigma–Aldrich, 1:2000). Blots were stripped and re-probed using a β-actin antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1:20 000) as a loading control.

Table 1 The sequences of the oligonucleotide primers and methods used for quantitative real-time PCR

Gene	Symbol	Primer sequence (5'–3')	Amplicon size (bp)
Ataxia telangiectasia mutated	<i>ATM</i>	For: TGGATCCAGCTATTTGGTTTGA Rev: CCAAGTATGTAACCAACAATAGAAGAAGTAG	82
ATM related	<i>ATR</i>	For: TTCACCACAGGCACAATCAC Rev: TAGCCCGGATTACTTCATGG	125
ATP-dependent DNA helicase II	<i>Xrcc5/Ku80</i>	For: CGTGGATGTATGGGGAATCT Rev: CCTGCTGGGAATTTCTTGAG	117
ATP-dependent DNA helicase II, 70 kDa subunit	<i>Xrcc6/Ku70</i>	For: TGAAGACCCACATCAGCAAG Rev: TGCTTCTTCAGCCCACTCTT	100
Artemis	<i>DCLRE1C</i>	For: CTTGAGCCCAGGAGATTGAG Rev: TCCTGCGTAGCTGGGATTAC	140
Beta-actin	β-Actin	For: TTCCTGGGCATGGAGTC Rev: CAGGTCTTTGCGGATGTC	84
Breast cancer susceptibility 1	<i>BRCA1</i>	For: AGAGCGTCCCCTCACAAATA Rev: CCGTTTGGTTAGTTCCTGA	137
Breast cancer susceptibility 2	<i>BRCA2</i>	For: AAGCCCTTTGAGAGTGGAAG Rev: TCCATCTGGGCTCCATTAG	107
Checkpoint kinase 1	<i>Chk1</i>	For: AGTGGAGGAGAGCTTTTTGAC Rev: TGCAGATAAACCACCCCTGCC	104
Checkpoint kinase 2	<i>Chk2</i>	For: AGGGAAAGGAAAACGCCGTCC Rev: GCTTTACCTCTCCACAGGCAC	182
Histone family H2A, member X	<i>H2AFX</i>	For: CTTAGCCCAGGACTTTCAGA Rev: AAGATGGAGGGAGAGCTGAT	132
Mediator of DNA damage checkpoint 1	<i>MDC1</i>	For: ACCCCTGAATTCCAATCTCC Rev: TTTAGGTTCCAAGGGTGCAG	159
Meiotic recombination 11	<i>MRE11</i>	For: TGCTTCTGCCTTTAGTGCTG Rev: GCCTCTCCTTTGTTGGTTG	106
Nibrin	<i>NBN</i>	For: CTTCCAAGTTCTGGCTGCTT Rev: GCTCACCAATTTCTGTTCC	113
Rad50 homolog	<i>Rad50</i>	For: ACTGGCTTCACCATCAATCC Rev: TGGCATCTGAAGAGCAAATG	150
Rad51 homolog	<i>Rad51</i>	For: CATACGCTAGCTGTACCTGCC Rev: ACCATACCTCTCAGCCACTGCC	126
Rad52 homolog	<i>Rad52</i>	For: AGGCTCAGGCCACATAATTG Rev: TGACGTTTATTCTCCAGCAG	97
Rad54 homolog	<i>Rad54</i>	For: AAGAGGGCCAAACACTGATG Rev: AAACATTTCTGGCAACCTG	148
Replication protein A	<i>RPA</i>	For: GTGGCGATCTAGCTTTCAGC Rev: TCAGGCCTCTTCACTGTGTG	102
Telomerase reverse transcriptase	<i>TERT</i>	For: GGAGCAAGTTGCAAAGCATTG Rev: TCCCACGACGTAGTCCATGTT	182
X-ray repair cross complementing protein 2	<i>Xrcc2</i>	For: AAGCCTCGAGCTCATCAGAA Rev: ATCCTGTGCTTCACCACTTG	115
X-ray repair cross complementing protein 3	<i>Xrcc3</i>	For: CTCCCACAGGCTTTGAATTG Rev: ATTCTGGGATTCAAGTCCAG	137
X-ray repair cross complementing protein 4	<i>Xrcc4</i>	For: GAAGGGGAAACTGCAATCTG Rev: AAGCCAACCCAGAGAGATCA	138

ELISA

Cells were grown to confluence in six-well plates. The medium was then changed to serum-free medium for 24 h, and the cells were washed with PBS, pH 7.4, before preparation of cell lysate in 25 mM Tris–HCl, pH 7.4. The amount of hGH produced and secreted over a 24 h period into 2 ml serum-free medium was also estimated. ELISA for the quantification of hGH in cell lysates or secreted into the medium was performed according to the manufacturer's instructions. The protein concentration of cell lysates was determined using a Bradford protein assay.

IR treatment

IR treatment was conducted using a cobalt-60 source from an Eldorado G unit (FMHS, University of Auckland) at a dose rate of 0.5 Gy/min.

Total cell number assay

Cells (5×10^4 to 10×10^4) were seeded into six-well plates in full serum media. Following treatment with IR (0 or 4 Gy), cells were trypsinised with 0.5% trypsin and the cell number determined using a haemocytometer every 2 days for 8–10 days.

MTT assay

Cells were plated at different concentrations (MDA-vec/MDA-hGH: 1000 or 3000 cells/well; T47D-vec/T47D-hGH: 1500 or 4000 cells/well; RL95-vec/RL95-hGH and RL95-2 wild-type cells 3000 or 5000 cells/well) and irradiated 24 h later. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was conducted on days 5 and 10 as described previously (van de Loosdrecht *et al.* 1994).

Apoptosis assay

Apoptotic cell death was measured by fluorescent microscopic analysis of cell DNA staining patterns with Hoechst 33258 as described (Kaulsay *et al.* 2001). Cells were plated at 2×10^5 cells/well, changed into SF media and exposed to 4 Gy IR. Twenty-four and 48 h after radiation treatment, the cells were fixed, permeabilised and stained as described previously (Kaulsay *et al.* 2001). For B2036 studies, RL95-2 cells were treated with BSA or B2036 in serum-free media for 24 h before radiation treatment.

Cell survival clonogenic assay

Exponentially growing T47D-vec, T47D-hGH, MDA-vec and MDA-hGH cells were irradiated with 4 Gy IR. Twenty-four hour post-irradiation cells were trypsinised, plated in six-well plates in triplicates at 400 cells/well and cultured in 10% serum media for 14 days. For functional hGH antagonism, cells were pretreated with 1000 nM B2036 for 24 h before irradiation. Media containing B2036 or BSA (control) were replaced every 2 days. For antagonism of c-Src and JAK2, cells were treated with vehicle, PP1 (20 μ M), PP2 (20 μ M), PP3 (20 μ M) or AG490 (50 μ M) for 24 h before radiation exposure. After 24 h, cells were trypsinised, re-plated at 400 cells/well and cultured for 14 days in full serum media. Colonies were stained with crystal violet and counted. A colony was defined as a cluster of at least 50 cells.

3D Matrigel assay

Cell growth in the basement membrane extract, Matrigel, was assayed as described previously (Amiry *et al.* 2009). Briefly, RL95-2, T47D and MDA-MB-435S cells were suspended in 4% growth factor reduced Matrigel (BD Biosciences, San Jose, CA, USA) in 5% serum media in 96-well plates at 1000 cells/well. On day 4, cells were pretreated with 1000 nM B2036 or BSA, where indicated, for 24 h followed by 4 Gy IR. Media containing B2036 and BSA were replaced every 2 days. Cell viability was determined using Wst-1 (Roche) on day 9.

Neutral comet assay

A neutral comet assay was performed as described previously (Olive & Banath 2006). Briefly, irradiated cells were embedded in low melting temperature Seaplaque Agarose (Cambrex Bio Science, Rockland, ME, USA) on GelBond film (Lonza Rockland, Inc., Rockland, ME, USA). The cells were lysed overnight at 37 °C in neutral lysis solution (2% sarkosyl, 0.5 M Na₂EDTA and 0.5 mg/ml proteinase K, pH 8.0) and then washed in rinse buffer (90 mM Tris buffer, 90 mM boric acid and 2 mM Na₂EDTA, pH 8.5) three times. Slides were subjected to electrophoresis in 1 × TBE for 25 min at 20 V. Comets were stained with 10 μ g/ml propidium iodide for 20 min and rinsed in 400 ml distilled water to remove excess stain. At least 100 comet images from each slide were examined. Comet tail length and tail moment were analysed using Tritex CometScore software (Version 1.5; TriTek Corp., Sumerduck, VA, USA).

γH2AX flow cytometry assay

Flow cytometry was performed as described previously (MacPhail *et al.* 2003, Tanaka *et al.* 2006). Briefly, cells were seeded at 80% confluence in 10 cm plates in 10% serum media. After 24 h, cells were treated with IR (4 Gy). Following treatment, cells were harvested by trypsinisation at designated time points and fixed with 80% ethanol in PBS. Sample preparation entailed rehydration of cells in PST (4% FBS and 0.1% Triton-X-100 in PBS) for 10 min. Samples were then centrifuged (180 g, 5 min), resuspended in PST containing rabbit anti-phospho-Ser139-H2AX primary antibody (1:500; Sigma–Aldrich) and incubated at 4 °C overnight with gentle agitation. Samples were washed in cold PBS containing 2% FBS and resuspended in PST containing goat anti-rabbit FITC-conjugated secondary antibody (1:160; Sigma–Aldrich) and incubated for 2 h at room temperature on a rocker. Samples were washed again and resuspended in 400 µl PBS containing 10 µg/ml propidium iodide and were analysed on an FACSARIAII flow cytometer (BD Biosciences, San Jose, CA, USA).

Glutathione assay

Total cellular glutathione levels were determined in MDA-vec, MDA-hGH, T47D-vec and T47D-hGH using a glutathione colorimetric assay kit (Sigma–Aldrich) as per manufacturer's instructions. Approximately 1×10^8 cells from each cell line were used for sample preparation. 5-Thio-2-nitrobenzoic acid was measured spectrophotometrically at 412 nm.

Xenograft analyses

All experiments were carried out under a protocol approved by the University of Auckland Animal Ethics Committee. MDA-Vec or MDA-hGH (5×10^6) cells were suspended in 100 µl Matrigel and injected subcutaneously into the right hind quarter of female *Rag1* immunodeficient mice. Tumour cells were inoculated subcutaneously on the dorsal midline, 1.5 cm from the base of the tail. Tumour volume ($(\text{length} \times \text{width}^2) \times 3.14/6$) was determined with a digital calliper twice weekly. Tumour growth delay experiments were carried out with 165–180 mm³ tumours treated with IR (cobalt-60, 3 Gy/day at 0.5 Gy/min carried out over three consecutive days starting on day 12). Unanaesthetised mice ($n=6$ per treatment group) were restrained in jigs behind lead shielding to expose tumours to a lateral radiation beam. Tumour regrowth/regression was monitored up to day 19.

Statistical analysis

All numerical data are expressed as mean $\pm$ S.E.M. of triplicate determinants. Statistical significance between treatment groups was determined using an unpaired two-tailed *t*-test or a two-way ANOVA (Holm-Sidak) using SigmaPlot 11.0. All *in vitro* experiments were repeated at least three times. A single representative figure is shown.

Results

Autocrine hGH increases mammary and endometrial carcinoma cell viability and total cell number after exposure to IR

To determine the effect of autocrine hGH on IR-induced cell death, we used mammary carcinoma cell lines, MDA-MB-435S and T47D, stably transfected with an expression vector containing the *hGH* gene *GH1* (pcDNA3-hGH). These cell lines are designated MDA-hGH and T47D-hGH and have been described previously (Bougen *et al.* 2011). A second cell line transfected with an empty pcDNA3 vector was generated in parallel for control purposes (designated MDA-vec and T47D-vec respectively). Generation of stable forced expression of hGH in endometrial carcinoma cell line RL95-2, which was also used in this study, has been described previously (Pandey *et al.* 2008).

MDA-vec and T47D-vec cells do not produce detectable amounts of *hGH* mRNA or protein when cultured under serum-free conditions whereas MDA-hGH and T47D-hGH stable cell lines express *hGH* mRNA as demonstrated by semiquantitative RT-PCR (Bougen *et al.* 2011) and secrete hGH protein into the media, as demonstrated by western blot analysis (Bougen *et al.* 2011) and ELISA (184 ng/ml hGH (8.35 nM) secreted by MDA-hGH cells and 390 ng/ml (17.68 nM) hGH secreted by T47D-hGH cells over 24 h) (data not shown). Our previous study demonstrated that wild-type RL95-2 cells express endogenous *hGH* mRNA and protein with 0.5 ng/ml (22.73 pM) secreted into the media over 24 h as demonstrated by ELISA (Pandey *et al.* 2008). MDA-vec/MDA-hGH, T47D-vec/T47D-hGH and RL95-vec/RL95-hGH cells also express *hGHR* mRNA (Pandey *et al.* 2008, Bougen *et al.* 2011).

Radioreistance is characterised by enhanced tumour cell survival following exposure to IR. Due to evidence suggesting a radioprotective role for GH (Gomez-de-Segura *et al.* 1998, Vazquez *et al.* 1999, Mylonas *et al.* 2000, Isla *et al.* 2002, Madrid *et al.* 2002, Raguso *et al.* 2002, Lempereur *et al.* 2003,

Morante *et al.* 2003, Tekin *et al.* 2006), we investigated whether autocrine hGH enhanced mammary and endometrial cancer cell viability following treatment with IR. Three pairs of stably transfected cell lines (MDA-vec and MDA-hGH, T47D-vec and T47D-hGH and RL95-vec and RL95-hGH cells) were exposed to an initial treatment range of 0–8 Gy IR. Autocrine hGH significantly enhanced cell viability of MDA-MB-435S, T47D and RL95-2 cells at all doses of radiation tested (Fig. 1A, D and G). Following treatment with 2 Gy IR, autocrine hGH increased MDA-MB-435S cell viability by 1.5-fold, T47D cell viability by 1.5-fold and RL95-2 cell viability by 1.4-fold over 10 days ($P < 0.001$). At 4 Gy IR, autocrine hGH increased MDA-MB-435S cell viability by 1.6-fold (Fig. 1A),

T47D cell viability by 1.9-fold (Fig. 1D) and RL95-2 cell viability by 2.9-fold (Fig. 1G) over 10 days ($P < 0.001$).

Total cell number was determined over 7 days in serum-replete (10% FBS) conditions following treatment with 4 Gy IR. Exposure to IR significantly reduced increases in total cell number in all cell lines over 7 days (Fig. 1B, C, E, F, H and I). In addition, MDA-hGH cells exhibited significantly higher cell number at day 7 following radiation treatment compared with the MDA-vec cell line when expressed as a percentage of untreated controls (MDA-hGH $38.96 \pm 3.10\%$ vs MDA-vec $26.12 \pm 2.75\%$; $P < 0.05$). A similar trend was observed in T47D-hGH (T47D-hGH $46.91 \pm 3.18\%$ vs T47D-vec $26.30 \pm 0.84\%$;

Figure 1 Autocrine hGH increases cell viability and total cell number following treatment with ionising radiation. (A, D, F) MTT viability assay. Cells were exposed to IR doses of 0–8 Gy, cultured in serum-replete medium (10% FBS) and analysed on day 10. (B, E, H) Total cell number assay conducted in serum-replete medium after exposure to 4 Gy IR. (C, F, I) Total cell number assay results presented as percent of untreated control on the indicated days. (A, B, C) MDA-vec and MDA-hGH cell lines. (D, E, F) T47D-vec and T47D-hGH cell lines. (G, H, I) RL95-vec and RL95-hGH cell lines. Cells were plated at 5000 cells/well ($*P < 0.05$, $**P < 0.001$, $***P < 0.0001$). Experiments were repeated at least three times. A single representative figure is shown.

$P < 0.001$) and RL95-hGH (RL95-hGH $28.04 \pm 2.6\%$ vs RL95-vec $18.55 \pm 0.850\%$; $P < 0.05$) stable cell lines compared with the respective control cell lines following treatment with IR (Fig. 1B, C, E, F, H and I).

We also determined the effect of treatment with exogenous hGH (100 nM) on MDA-vec cell viability and MDA-vec/MDA-hGH total cell number following treatment with IR. No significant effect on MDA-vec cell viability was observed below 100 nM hGH following treatment with 4 Gy IR (Fig. 2A). hGH (100 nM) increased cell viability by 1.4-fold following exposure to 4 Gy IR and this concentration was used in subsequent assays (Fig. 2A).

A total cell number assay was conducted in which MDA-vec and MDA-hGH cells were exposed to 4 Gy IR and then treated with 100 nM hGH or BSA as a control. Treatment with exogenous hGH increased MDA-vec total cell number by 1.5-fold on day 9 following IR but had no significant effect on MDA-hGH total cell number (Fig. 2B and C). While exogenous hGH significantly increased MDA-vec total cell number following 4 Gy radiation compared with control BSA-treated cells (1.5-fold), it was not as effective as the protection afforded by autocrine hGH (2.2-fold; MDA-vec vs MDA-hGH BSA-treated cells; Fig. 2B and C). MDA-hGH cells secrete 8.35 nM hGH into the media over a 24 h period. Thus, the radioprotective effect of autocrine hGH was significantly more potent than 100 nM hGH added exogenously.

Autocrine hGH reduces mammary carcinoma cell apoptotic cell death and increases clonogenic survival after exposure to IR

Apoptotic cell death was determined in MDA-MB-435S and T47D cells after IR treatment. Untreated MDA-hGH and T47D-hGH cells exhibited significantly lower apoptotic cell death after 24 and 48 h in serum-free media when compared with respective control cell lines, MDA-vec and T47D-vec (Fig. 3A and B). Following treatment with 4 Gy radiation, MDA-vec cells exhibited significantly higher apoptotic cell death than MDA-hGH cells at both time points (Fig. 3A). When expressed as fold difference of untreated control, autocrine hGH reduced the effect of IR on MDA-MB-435S apoptotic cell death (MDA-hGH 1.29-fold vs MDA-vec 1.57-fold at 24 h (two-way ANOVA; $P < 0.05$) and 1.7- vs 1.75-fold at 48 h (two-way ANOVA; $P < 0.05$)). A more striking effect was observed with T47D cells; as with MDA-MB-435S, T47D-vec cells exhibited significantly higher apoptotic cell death than T47D-hGH cells at both time points (Fig. 3B). When expressed as

fold difference of untreated control, autocrine hGH reduced the effect of IR on T47D apoptotic cell death (T47D-hGH 1.38-fold vs T47D-vec 1.57-fold at 24 h (two-way ANOVA; $P < 0.01$) and 1.88- vs 2.33-fold at

Figure 2 The effect of exogenous hGH on mammary carcinoma cell radiation sensitivity (A) MTT viability assay. MDA-vec cells were exposed to 4 Gy IR and treated with 100 nM hGH or BSA (control) to determine the effect of exogenously added hGH on radiosensitivity. No significant effect on MDA-vec cell viability was observed below 100 nM hGH. (B) Total cell number assay conducted in serum-replete medium after exposure to 4 Gy IR. Cells were plated at 10 000 cells/well. Following exposure, cells were treated with 100 nM hGH or BSA (control) for 9 days. (C) Total cell number assay results presented as per cent of untreated control (* $P < 0.05$, ** $P < 0.001$).

48 h (two-way ANOVA; $P < 0.01$). Thus, autocrine hGH protects MDA-MB-435S and T47D cells from IR-induced apoptotic cell death.

A clonogenic survival assay was used to measure the ability of MDA-vec and MDA-hGH cells to form colonies after treatment with IR. Untreated MDA-hGH cells exhibited a 1.7-fold higher plating efficiency (PE) compared with MDA-vec cells ($P < 0.001$). This effect was enhanced following 4 Gy radiation treatment where MDA-hGH cells exhibited a 2.8-fold higher PE compared with MDA-vec cells ($P < 0.001$; Fig. 4A). Over repeat experiments, autocrine hGH increased overall clonogenic survival by 1.4-fold in MDA-hGH compared with MDA-vec cells following IR treatment ($P < 0.05$; $n = 6$). IR exposure reduced MDA-vec cell clonogenic survival by 1.7-fold, whereas radiation exposure was less effective in reducing MDA-hGH clonogenic survival, reducing it by 1.3-fold ($P < 0.05$; $n = 6$). Thus, MDA-hGH cells were significantly more resistant to IR-induced cell death than the control cell line in the clonogenic survival assay.

Clonogenic assays were also conducted with T47D-vec and T47D-hGH cells. Untreated T47D-hGH cells exhibited a 1.7-fold higher PE than T47D-vec cells ($P < 0.001$), and following 4 Gy treatment, T47D-hGH cells exhibited a fourfold higher PE than T47D-vec cells ($P < 0.001$; Fig. 4B). Over repeat experiments, autocrine hGH increased relative clonogenic survival following IR treatment by 1.5-fold in T47D-hGH compared with T47D-vec cells following IR treatment ($P < 0.05$; $n = 3$). IR exposure reduced T47D-vec clonogenic survival by 2.1-fold, whereas radiation exposure was less effective in reducing T47D-hGH clonogenic survival, reducing it by 1.3-fold ($P < 0.05$; $n = 3$). Thus, T47D-hGH cells were significantly more resistant to IR-induced cell death than the control cell line in the clonogenic survival assay.

Autocrine hGH significantly enhanced MDA-MB-435S and T47D cell growth in the extracellular matrix extract Matrigel after exposure to IR (Figs 4C and 3D). Untreated MDA-hGH cells exhibited a 1.3-fold higher cell viability in Matrigel than MDA-vec cells ($P < 0.05$), and following 4 Gy treatment, MDA-hGH cells exhibited a 1.6-fold higher cell viability than MDA-vec cells ($P < 0.05$; Fig. 4C). IR exposure reduced MDA-vec cell growth in Matrigel by 1.4-fold, whereas radiation exposure reduced MDA-hGH cell growth by 1.2-fold; however, this reduction was not significant by two-way ANOVA. Untreated T47D-hGH cells exhibited a 1.4-fold higher cell viability in Matrigel than T47D-vec cells ($P < 0.01$) and following 4 Gy treatment T47D-hGH cells exhibited a 1.8-fold higher cell viability than T47D-vec

cells ($P < 0.001$; Fig. 4D). IR exposure reduced T47D-vec cell growth in Matrigel by 1.7-fold, whereas radiation exposure reduced T47D-hGH cell growth by 1.3-fold ($P < 0.5$; two-way ANOVA).

The effect of autocrine hGH on sensitivity to IR *in vivo*

Xenograft studies were conducted to determine whether autocrine hGH protected mammary carcinoma cells against IR-induced damage *in vivo*. MDA-vec and MDA-hGH cells were inoculated subcutaneously into female *Rag1* immunodeficient mice. Tumours were treated with IR over three consecutive days starting on day 12 and tumour regrowth/regression was monitored on days 15 and 19. Both MDA-vec and MDA-hGH tumour volumes reduced following treatment with radiation; however, MDA-vec tumour volume

Figure 3 Autocrine hGH reduces apoptotic cell death following treatment with ionising radiation. (A) MDA-vec and MDA-hGH and (B) T47D-vec and T47D-hGH cells were cultured in serum-free RPMI and left untreated or exposed to 4 Gy IR. Apoptotic nuclei were counted at 24 and 48 h (* $P < 0.05$, ** $P < 0.001$, *** $P < 0.0001$). Experiments were repeated at least three times. A single representative figure is shown.

Figure 4 Autocrine hGH enhances clonogenic survival and growth in Matrigel following treatment with ionising radiation. (A and B) Clonogenic assay. Untreated or 4 Gy radiation-treated MDA-MB-435S (A) or T47D (B) cell lines were plated at 400 cells/well and cultured for 14 days in serum-replete media. Colonies with > 50 cells were counted and the plating efficiencies (PE) calculated. PE = (number of colonies formed/number of cells seeded) × 100%. (* $P < 0.05$, ** $P < 0.001$, *** $P < 0.0001$). (C and D) 3D Matrigel assay. MDA-MB-435S (C) or T47D (D) cells were treated with 4 Gy IR and cultured in growth factor reduced Matrigel for 9 days. Cell viability was determined using Wst-1. All *in vitro* experiments were repeated at least three times. A single representative figure is shown. (E) Xenograft analysis. *Rag1* immunodeficient mice were implanted with MDA-vec or MDA-hGH cells. Tumours were left untreated or subjected to fractionated IR treatment starting on day 11 post implantation and tumour volume measured on days 15 and 17.

decreased more significantly than MDA-hGH tumours (Fig. 4E). IR treatment resulted in a 37.79% decrease in the treatment volume for MDA-vec tumours on day 15, whereas MDA-hGH tumour volume decreased by 18.37% ($P < 0.05$; two-way ANOVA; Fig. 4E).

Enhanced clonogenic survival afforded by autocrine hGH is mediated by JAK2 and Src kinases

Both JAK2 and c-Src are concomitantly activated following binding of hGH to its receptor (Ling et al. 2003). To determine the relative contribution of JAK2 and c-Src to the autocrine hGH-stimulated increase in survival following IR, we conducted clonogenic survival assays in mammary carcinoma cells treated with pharmacological inhibitors of JAK2 (AG490) or Src kinases (PP1 and PP2). We also used the inactive structural analogue PP3 as a control in the experiments. Figure 5 demonstrates that the increased clonogenic survival afforded by autocrine hGH following IR is partially abrogated in both T47D-hGH and MDA-hGH cell lines following treatment with AG490, PP1 and PP2, but unaffected in the presence of the inactive

analogue PP3. PP1, PP2 and AG490 decreased both the basal (MDA-vec) and the autocrine hGH-stimulated increase in clonogenic survival in the MDA-MB-435S cell line (Fig. 5A), while only AG490 affected basal clonogenic survival in T47D cells (Fig. 5B). Thus, the protective effect of autocrine hGH against IR-induced cell death is mediated by both JAK2 and the c-Src family of proteins in these cell lines (Fig. 5A and B).

Autocrine hGH protects mammary carcinoma cells against IR-induced DNA damage

In order to assess whether autocrine hGH affected the induction of DNA damage following IR exposure in MDA-MB-435S and T47D cells, a neutral comet assay was performed. The neutral comet assay detects DNA DSBs, which are the principle cytotoxic lesion caused by IR. Neutral comet analysis indicated that endogenous levels of DSBs in untreated MDA-vec and MDA-hGH cells were similar (Fig. 6A). However, at 4 Gy IR treatment, MDA-hGH exhibited significantly reduced radiation-induced DNA damage, as measured by the tail moment (defined as the product of the tail

Figure 5 The protective effect of autocrine hGH against IR-induced cell death is mediated by both JAK2 and the c-Src family of proteins in MDA-MB-435S and T47D cells. (A) T47D and (B) MDA-MB-435S. Cells were treated with vehicle, PP1 (20 μ M), PP2 (20 μ M), PP3 (20 μ M) or AG490 (50 μ M) for 24 h before radiation exposure. After 24 h, irradiated cells were trypsinised and plated in six-well plates at 400 cells/well for 14 days. The data are represented as plating efficiency (PE) following IR treatment expressed as a percentage of the untreated control PE. Asterisks (**) indicates the changes observed in hGH-transfected cells are statistically significant compared with the control cell line (vec) ($P < 0.01$), whereas ¥ indicates changes after treatments observed between control (vec) cells are significant ($P < 0.05$) and § indicates changes after treatments observed between hGH transfected cells are significant ($P < 0.001$) when compared to vehicle treated cells. Experiments were repeated at least three times. A single representative figure is shown.

length and the fraction of total DNA in the tail; Fig. 6A), and by the percentage of DNA measured in the tail of comets (data not shown). Autocrine hGH prevented induction of DNA DSBs in MDA-hGH cells after treatment with 4 Gy IR, whereas IR induced a 1.7-fold increase in DNA DSB damage in MDA-vec cells at 4 Gy (Fig. 6A). When the IR dose was increased to 8 Gy, DNA damage was observed in both cell lines (Fig. 6A); however, at 8 Gy, the DNA damage induced in MDA-hGH cells was significantly reduced compared with MDA-vec cells. We also investigated DNA damage in T47D-vec and T47D-hGH cells following IR exposure. IR (4 Gy) induced DNA damage in both T47D-vec and T47D-hGH cell lines (Fig. 6C). However, DNA damage induced in

T47D-hGH cells was significantly reduced compared with T47D-vec cells following 4 Gy radiation as demonstrated by the tail moment.

DNA damage was also assessed by flow cytometry with cells immunofluorescently labelled with an antibody against phosphorylated histone H2AX (phospho-H2AX). H2AX is rapidly phosphorylated on serine 139 in response to DNA DSB damage (Rogakou *et al.* 1998). Phospho-H2AX plays a key role in the recruitment of DNA repair sensing and repair proteins after exposure to genotoxic agents such as IR (Paull *et al.* 2000, Celeste *et al.* 2002, Stiff *et al.* 2004). The level of phospho-H2AX in the control cell line (MDA-vec), as determined by flow cytometry, increased substantially after treatment with 4 Gy radiation with maximum phosphorylation observed at 3 h post treatment (Fig. 6D and F). In contrast, phospho-H2AX levels in MDA-hGH cells increased only slightly immediately after radiation treatment and returned to untreated levels within 3 h of treatment (Fig. 6E and F). Thus, autocrine hGH reduced levels of IR-induced DNA damage in the MDA-MB-435S cell line.

The reducing ability of intracellular sulphhydryl compounds such as glutathione has been implicated in determining cellular radiosensitivity (Estrela *et al.* 2006). As we have previously demonstrated that autocrine hGH increases mRNA expression of glutathione peroxidase (GPX), which catalyses the oxidation of glutathione, and glutamylcysteine synthetase (GCS), an enzyme involved in glutathione biosynthesis, in MCF-7 cells (Zhu *et al.* 2005b), we investigated whether autocrine hGH altered the intracellular levels of glutathione in MDA-MB-435S and T47D cells using a glutathione assay. However, no significant difference in total glutathione concentration was observed in MDA-vec vs MDA-hGH or T47D-vec vs T47D-hGH cell lines (data not shown).

Real-time PCR was conducted to determine mRNA levels of selected genes implicated in DNA repair, the cellular response to DNA damage and radioresistance. Expression levels of the majority of genes examined by real-time PCR were not affected by autocrine hGH. However, autocrine hGH increased mRNA levels of the breast cancer susceptibility gene, *BRCA2*, and the DNA repair protein involved in homologous recombination, X-ray repair cross complementing group-2 (XRCC2), in MDA-hGH cells compared with MDA-vec cells (Table 2). Consistent with our previous observations in MCF-7 cells (Emerald *et al.* 2007), autocrine hGH also increased mRNA levels of *TERT*, the catalytic subunit of telomerase in MDA-MB-435S cells (Table 2).

Figure 6 Autocrine hGH enhances DNA repair in mammary carcinoma cells. (A and B) Neutral comet assay. MDA-vec and MDA-hGH cells were treated with (A) 4 Gy or (B) 8 Gy IR and DNA double-stranded breaks were measured using the neutral comet assay. The tail moment correlates with the level of DSBs present in untreated (UT) cells, cells at 15 m repair time (15 m RP), cells at 1 h repair (1 h RP), 3 h repair (3 h RP) or 6 h repair (6 h RP). (C) Neutral comet assay conducted with T47D-vec and T47D-hGH cells treated with 4 Gy IR. (D and E) Phospho-H2AX flow cytometry assay. Phosphorylation of H2AX on serine 139 directly correlates with the presence of DSBs. Fluorescent histograms of cells subjected to IR followed by 0, 1, 3 and 6 h incubation. Increasing H2AX phosphorylation is indicated by a rightward shift along x-axis (D) MDA-vec and (E) MDA-hGH cells (values on the graphs indicate peak geo means). (F) Bar graph representation of the average phospho-H2AX fluorescent histogram geo means from three experiments (* $P < 0.05$, ** $P < 0.001$, *** $P < 0.0001$). Experiments were repeated at least three times. A single representative figure is shown.

Functional antagonism of autocrine hGH reduces RL95-2 cell viability, total cell number, clonogenic survival and DNA damage after treatment with IR

RL95-2 wild-type cells endogenously express and secrete hGH (Pandey *et al.* 2008) and hence provide a suitable system for investigating the effect of functional antagonism of hGH on post-radiation cell survival. Functional antagonism of hGH was achieved using the hGHR antagonist, B2036. B2036 is the peptide component of pegvisomant (Pfizer), which is FDA approved for the treatment of acromegaly (Kopchick *et al.* 2002, van der Lely & Kopchick 2006). It consists of a mutated hGH molecule that competitively binds the receptor but does not activate signal transduction. Treatment of RL95-2 cells with B2036 over 10 days in full serum media significantly reduced RL95-2 cell viability after 4 Gy IR compared with control cells treated with BSA (Fig. 7A). A marked effect was observed at day 5: B2036-treated cells exhibited 37.8% cell survival compared with 61.7% in BSA-treated cells ($P < 0.001$). A total cell number assay performed over 7 days demonstrated that

B2036 significantly reduced RL95-2 total cell number compared with control, BSA-treated cells (Fig. 7B and C). Combined treatment with B2036 and 4 Gy IR reduced RL95-2 total cell number additively when compared with either treatment with B2036 or IR alone (Fig. 7B and C). Combined treatment with B2036 and 4 Gy IR increased apoptotic cell death additively when compared with either treatment with B2036 or IR alone (Fig. 7D). In addition, combined treatment with IR and B2036 synergistically reduced RL95-2 cell clonogenic survival ($P < 0.001$; two-way ANOVA; Fig. 7E). In a 3D Matrigel assay, B2036 reduced RL95-2 cell growth in Matrigel after treatment with IR compared with BSA-treated control cells (Fig. 7F). Finally, in a neutral comet assay, B2036 significantly enhanced induction of DNA DSBs after exposure to radiation compared with BSA-treated controls (Fig. 7G). Thus, treatment with B2036 increases the sensitivity of RL95-2 cells to treatment with IR.

Discussion

Previous studies demonstrating a radioprotective effect for GH have investigated whether GH

Table 2 Quantitative real-time PCR analysis of the effect of forced expression of hGH in MDA-MB-435S breast cancer cells on mRNA levels of several key genes functionally involved in cell cycle, DNA repair and radioresistance

Gene symbol	Fold change	P
NBN	1.02	3.90×10^{-1}
MRE11 (MRE11A)	1.04	3.93×10^{-1}
RAD50	1.19	1.78×10^{-1}
RAD51	1.07	3.47×10^{-1}
RAD52	1.08	1.22×10^{-1}
RAD54 (ATRX)	1.01	4.73×10^{-1}
BRCA1	1.52	1.34×10^{-1}
BRCA2	1.61	4.82×10^{-2}
RPA	1.13	2.77×10^{-1}
H2AFX	1.34	3.92×10^{-2}
ATM	−1.00	4.90×10^{-1}
XRCC4	1.12	3.49×10^{-1}
XRCC2	1.52	2.91×10^{-2}
XRCC3	1.12	8.61×10^{-2}
ATR	1.37	1.01×10^{-1}
PRKDC	1.07	3.05×10^{-1}
LIGASE 4F	−1.19	1.74×10^{-1}
XRCC6/KU70	1.06	3.22×10^{-1}
XRCC5/KU80	1.01	4.75×10^{-1}
CHK1 (CHEK1)	−1.03	2.15×10^{-1}
CHK2 (CHEK2)	−1.20	2.22×10^{-1}
DCLRE1C	1.04	4.66×10^{-1}
ABL	1.31	5.75×10^{-2}
MDC1	1.29	4.91×10^{-2}
TERT	2.67	2.09×10^{-3}

administration reduces toxicity associated with radiation treatment in normal cells and tissues. As endogenous hGH expression is observed in a proportion of breast and endometrial tumours (Slater *et al.* 2006, Wu *et al.* 2011), we hypothesised that autocrine hGH would reduce cancer cell sensitivity to radiation-induced cell death, thus potentially reducing the effectiveness of radiotherapy. In the current study, we describe a novel role for autocrine hGH in radioresistance and protection from IR-induced DNA damage in mammary and endometrial carcinoma cell lines. Cell viability and total cell number assays demonstrated that autocrine hGH promoted MDA-MB-435S, T47D and RL95-2 cell regrowth following treatment with IR. We also observed that autocrine hGH had a protective effect against IR-induced DNA damage *in vitro* and tumour regression *in vivo*. Conversely, functional antagonism of endogenous hGH in RL95-2 cells increased the sensitivity of these cells to treatment with IR. To our knowledge, this is the first study demonstrating that hGHR antagonism enhances IR-induced cell death and indicates that hGHR antagonists may have potential as a novel radiosensitising agent *in vivo*.

Mechanisms contributing to these observations may be through regulation of cell proliferation and survival. Autocrine hGH promotes cell proliferation and reduces apoptotic cell death in numerous cell models (Perry *et al.* 2006, Harvey 2010). Binding of hGH to the hGHR activates signal transduction cascades including the PI3K/protein kinase B (AKT) and the MAP kinase cascades, both of which have been implicated in cellular resistance to IR (Liang *et al.* 2003, LoPiccolo *et al.* 2007, Begg *et al.* 2011). Autocrine hGH also protects numerous cell types from apoptosis (Perry *et al.* 2006, Harvey 2010). We observed here that autocrine hGH reduced the apoptotic response of T47D and MDA-MB-435S cells following radiation exposure. However, apoptotic cell death only plays a modest role in the response of most solid tumours to treatment and whether inhibition of apoptosis contributes to radioresistance in solid tumours remains contentious (Brown & Attardi 2005, Eriksson & Stigbrand 2010).

Although exogenous hGH also reduced radiosensitivity, we found that autocrine hGH reduced radiosensitivity more effectively. Differential effects of exogenous (thus mimicking endocrine) vs autocrine hGH in mammary carcinoma have been described by our laboratory in several studies (Xu *et al.* 2005, Perry *et al.* 2006, 2008, Brunet-Dunand *et al.* 2009). The mode of presentation of hGH to the cell may explain why exogenous hGH has disparate effects. Endocrine or pituitary hGH is secreted at high concentrations in a pulsatile manner, which contrasts with the low-level continuous secretion of autocrine hGH at extra-pituitary sites. This may lead to either transient or sustained activation of signal transduction pathways and is discussed in previous publications (Perry *et al.* 2006, 2008, Brunet-Dunand *et al.* 2009).

DNA damage induced by IR is primarily due to ionisation of water molecules within cells (Sharda *et al.* 2002). The resulting free radicals, especially the highly reactive hydroxyl radical, can interact with DNA, protein and lipids within a cell causing chemical damage. This form of damage to cells contributes to ~80% of the lethal effect of IR treatment (Sharda *et al.* 2002). We demonstrate here that autocrine hGH protected mammary and endometrial carcinoma cells from IR-induced DNA damage. Although we did not observe any effect on the DNA repair capacity using the neutral comet assay, an effect on DNA repair cannot be ruled out. A study by Madrid *et al.* (2002) demonstrated that the radioprotective effect mediated by exogenous GH in CHO-4 cells stably expressing the GHR was associated with an enhanced ability of the cells to repair damaged DNA, indicating that GH may

Figure 7 Functional inhibition of hGH in RL95-2 cells increases sensitivity to ionising radiation. RL95-2 cells express hGH endogenously. Cells were treated with either the hGHR antagonist B2036 (1000 nM) or BSA (1000 nM) for 24 h before treatment with IR. (A) MTT cell viability assay conducted in 10% serum media at 5 and 10 days post-radiation treatment (4 Gy) compared with BSA-treated control cells. Results are presented as a percentage of the control cells not exposed to IR. (B) Total cell number assay conducted in 10% serum media over 7 days. (C) Total cell number assay results from (B) presented as percent of untreated control on the indicated days. (D) Apoptotic cell death assay determined by Hoechst 33258 staining. Cells were treated with either B2036 or BSA for 24 h before treatment with IR and then serum starved for 24 h before assessing apoptotic nuclei. (E) Clonogenic survival assay. Cells were plated at 400 cells/well, treated with either B2036 or BSA for 24 h before treatment with 4 Gy IR and cultured for 14 days in 10% serum media. (F) Matrigel assay. Cells treated with either B2036 or BSA for 24 h before treatment with 4 Gy radiation in 5% FBS media. Cells were cultured for 9 days in media containing 10% FBS. (G) Neutral comet assay conducted after 4 Gy IR treatment in media containing 10% FBS (* $P < 0.05$, ** $P < 0.001$, *** $P < 0.0001$). Experiments were repeated at least three times. A single representative figure is shown.

activate pathways involved in DNA repair processes. Further investigation using assays interrogating different DNA repair pathways would determine whether autocrine hGH impacted on the DNA repair capacity of breast and endometrial cancer cell lines.

Protection against IR-induced DNA damage can also occur through increased cellular levels of thiols such as glutathione (Estrela et al. 2006). GCS is the rate-limiting enzyme for the synthesis of glutathione and increased GCS activity has been shown to be associated with the increased intracellular glutathione levels seen in many drug-resistant cancer cell lines

(Yao et al. 1995). As autocrine hGH increases mRNA levels of GCS in other breast cancer cell lines (Zhu et al. 2005b), we investigated whether autocrine hGH altered the intracellular levels of glutathione in the MDA-MB-435S and T47D cell lines used in this study. However, intracellular glutathione levels were not affected by autocrine hGH in these cell lines indicating that glutathione biosynthesis is unlikely to play a role in the protection against DNA damage afforded by autocrine hGH. Other cellular thiols not investigated here are also known to protect against IR-induced DNA damage and warrant further investigation.

Another means by which autocrine hGH may protect against DNA damage is by regulating superoxide scavenging enzymes. Autocrine hGH upregulates superoxide dismutase (SOD1) and catalase activity in mammary carcinoma cells in a p44/42 MAP kinase-dependant manner (Zhu *et al.* 2005b). Both enzymes play important roles in the cellular response to the oxidative damage caused by γ -radiation and certain chemotherapeutic drugs. SOD1 catalyses dismutation of highly damaging superoxide anions to H_2O_2 and O_2 , while catalase decomposes H_2O_2 to water and oxygen. The effective elimination of superoxide anions by SOD enzymes has been implicated in protecting cells against DNA damage (Elchuri *et al.* 2005, Southgate *et al.* 2006, Tennant & Kligerman 2011). Thus, regulation of other enzymes involved in oxidant protection may explain the protection afforded by autocrine hGH against IR-induced DNA damage and cell death and this is the subject of further investigation.

Expression of hTERT in human fibroblasts with short telomeres has been demonstrated to confer resistance to IR, etoposide, hydrogen peroxide and bleomycin (Blasco 2003, Rubio *et al.* 2004). While telomere length is closely correlated with cancer cell radiosensitivity (Ayouaz *et al.* 2008). Another mechanism contributing to autocrine hGH-mediated radioresistance may be through the upregulation of telomerase (Emerald *et al.* 2007). Autocrine hGH has been found to increase *hTERT* gene and protein expression, leading to an increase in telomerase activity in MCF-7 cells (Emerald *et al.* 2007). Consistent with these studies we observed that autocrine hGH increased *hTERT* mRNA expression in MDA-MB-435S cells (Table 2). Autocrine hGH also induced a modest increase in the level of *BRCA2* mRNA in MDA-MB-435S cells. *BRCA2* is involved in the repair of radiation-induced DSBs by homologous recombination. It would also be of interest to expand these studies to test other genes involved in DNA repair and the cellular response to radiation.

Another potential mechanism whereby autocrine hGH may mediate radioresistance is through increasing RAD51 protein expression. We observed increased RAD51 protein levels in MDA-hGH vs MDA-vector cell lines and T47D-hGH vs T47D-vec cell lines (Supplementary Figure 1, see section on supplementary data given at the end of this article). RAD51 over-expression has been demonstrated in numerous human tumour cell lines and primary tumour samples (Xia *et al.* 1997, Ohnishi *et al.* 1998, Maacke *et al.* 2000a,b, Collis *et al.* 2001, Raderschall *et al.* 2002, Qiao *et al.* 2005, Richardson 2005) and has been linked to reduced sensitivity to IR in numerous cell types, including breast

cancer (Maacke *et al.* 2000b, Russo *et al.* 2008, Le Scodan *et al.* 2010), prostate cancer (Collis *et al.* 2001) and glioma (Ohnishi *et al.* 1998). Elevated levels of RAD51 in breast cancer samples correlate with increased invasiveness and poor outcome in patients (Maacke *et al.* 2000b, Russo *et al.* 2008, Le Scodan *et al.* 2010). An increase in *RAD51* mRNA expression in MDA-hGH cells was not observed; however, RAD51 protein stability can be regulated post-transcriptionally by caspase-3-mediated proteolytic cleavage. Previous studies have suggested that maintenance of intact RAD51 contributes to abrogation of apoptosis following IR (Huang *et al.* 1999, Daboussi *et al.* 2002). In this respect, it is interesting to note that autocrine hGH increased mRNA levels of *BRCA2* in MDA-MB-435S cells (Table 2) as *BRCA2* has previously been demonstrated to protect RAD51 from proteolytic cleavage by caspase-3 (Brown *et al.* 2008).

A study by Morante *et al.* (2003) demonstrated that GH administered subcutaneously to rats prevented radiation injury to healthy intestinal cells, but did not protect colonic-implanted adenocarcinoma cell xenografts, or adenocarcinoma cells grown in culture against IR-induced cell death. In contrast, we observed a clear radioprotective effect mediated by autocrine hGH in three different cancer cell lines. The differential radioprotective effect observed in these studies is unlikely to be due to differences in GH administration. Although we have previously observed differential effects of exogenous vs autocrine hGH on breast cancer and endothelial cell growth, migratory capacity and gene expression (Xu *et al.* 2005, Perry *et al.* 2008, Brunet-Dunand *et al.* 2009), in the current study, we observed that, similar to autocrine hGH, exogenously added hGH promoted cell regrowth following IR exposure. However, in this regard, autocrine hGH was more effective than hGH added exogenously. The extent to which GH exerts a protective effect on various cancer cell lines following radiation exposure may therefore be cell line specific.

Our studies indicate that hGHR inhibition may sensitise a subset of hGH-responsive tumours to radiation therapy. However, multiple factors determine whether an agent modulates radiosensitivity *in vivo*. One point pertinent to this is the pro-angiogenic nature of autocrine hGH. We have previously demonstrated that autocrine hGH increases VEGFA expression in breast cancer cells and enhances tumour vascularisation in mouse xenografts (Brunet-Dunand *et al.* 2009). Increased tumour VEGFA expression leads to hyperproliferation of blood vessels, which might be expected to improve oxygenation within tumours. However, VEGFA also increases vascular permeability. Thus,

tumours with high VEGFA typically contain irregular, chaotic networks of leaky microvessels with heterogeneous blood flow and large intervessel distances. Consequently, tumours with high levels of VEGFA, which should act as a compensatory mechanism, continue to have regions of hypoxia (Karar & Maity 2009, Shinohara & Maity 2009). Paradoxically, inhibition of VEGFA expression in these tumours can cause the vessels to transiently ‘normalise’, resulting in decreased interstitial fluid pressure, improved blood flow and increased tumour oxygenation, thus improving radiosensitivity (Karar & Maity 2009, Shinohara & Maity 2009). Thus, functional antagonism of hGH has the potential to radiosensitise tumour cells *in vivo*; however, this needs to be determined experimentally.

As with any therapy, consideration of potential side effects is important. Some indication of potential side effects associated with hGHR antagonism may be obtained from patients with adult-onset GH deficiency, a clinical syndrome characterised by varying degrees of alterations in body composition, decreased bone mass, altered lipid and carbohydrate metabolism, decreased mood and general well-being and in some cases an increase in cardiac risk factors (Mathioudakis & Salvatori 2008). However, acute treatment regimens involving hGHR antagonism may not induce severe toxicities. Pfizer has conducted a clinical study that evaluated the safety of the hGHR antagonist, pegvisomant, given daily at high doses over several weeks and found that it was generally well tolerated in healthy subjects (Yin *et al.* 2007).

In conclusion, we have demonstrated that autocrine hGH enhances mammary carcinoma cell survival, clonogenic potential and reduces DNA damage following exposure to IR, while functional inhibition of hGH sensitises cells to IR-induced cell death. Agents capable of functionally antagonising hGH may therefore have clinical relevance as radiosensitising agents in hGH-expressing mammary tumours. Further studies investigating the clinical efficacy of such agents are warranted.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/ERC-12-0042>.

Declaration of interest

N M Bougen, M Steiner, M Pertziger, A Banerjee, S E Brunet-Dunand, T Zhu and J K Perry have nothing to declare; P E Lobie consults for Perseis Therapeutic and is an inventor on U.S. provisional patent application

no. 60/940939; and T Zhu and P E Lobie receive financial compensation from and have equity interest in Wuhan Long Ke Ltd.

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