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Depletion of the type 1 IGF receptor delays repair of radiation-induced DNA double strand breaks

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ABSTRACT

Background and purpose: IGF-1R depletion sensitizes prostate cancer cells to ionizing radiation and DNA-damaging cytotoxic drugs. This study investigated the hypothesis that IGF-1R regulates DNA double strand break (DSB) repair.

Methods: We tested effects of IGF-1R siRNA transfection on the repair of radiation-induced DSBs by immunoblotting and immunofluorescence for γ H2AX, and pulsed-field gel electrophoresis. Homologous recombination (HR) was quantified by reporter assays, and cell cycle distribution by flow cytometry.

Results: We confirmed that IGF-1R depletion sensitized DU145 and PC3 prostate cancer cells to ionizing radiation. DU145 control transfectants resolved radiation-induced DSBs within 24 h, while IGF-1R depleted cells contained 30–40% unrepaired breaks at 24 h. IGF-1R depletion induced significant reduction in DSB repair by HR, although the magnitude of the repair defect suggests additional contributory factors. Radiation-induced G2-M arrest was attenuated by IGF-1R depletion, potentially suppressing cell cycle-dependent processes required for HR. In contrast, IGF-1R depletion induced only minor radiosensitization in LNCaP cells, and did not influence repair. Cell cycle profiles were similar to DU145, so were unlikely to account for differences in repair responses.

Conclusions: These data indicate a role for IGF-1R in DSB repair, at least in part via HR, and support use of IGF-1R inhibitors with DNA damaging cancer treatments.

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The type 1 insulin-like growth factor receptor (IGF-1R) has a well-recognized role in promoting proliferation and cell cycle progression, and in protecting from apoptosis [1,2]. We and others established that the IGF-1R is up-regulated in primary prostate cancer, and is detectable in prostate cancer bone metastases [3–7]. Our recent demonstration of high and/or increasing IGF-1R expression with clinical prostate cancer progression is consistent with experimental evidence that IGF-1R expression is androgen-regulated in prostate cancer [8–10].

Targeting of the type 1 IGF receptor (IGF-1R) has been shown to enhance sensitivity to cytotoxic drugs and ionizing radiation, generally attributed to apoptosis induction [11–14]. In support of these data, IGF-1R inhibitory drugs enhance clinical effects of certain forms of chemotherapy, but negative Phase III results suggest

that we do not understand enough about the biology of the IGF-1R to use these new agents effectively [15].

The first data linking the IGF-1R to DNA repair came from a study of breast cancer: patients whose tumours over-expressed the IGF-1R were shown to be more likely to experience early recurrence within the irradiated site, indicating clinical radio-resistance [16]. Potential mechanisms connecting the IGF-1R to DNA repair include a link with the function of Ataxia Telangiectasia Mutated (ATM) that coordinates the response to DNA double strand breaks (DSBs; 17), and the reported interaction between the IGF-1R docking protein insulin-receptor substrate-1 (IRS-1) and RAD51, required for the strand invasion step of homologous recombination (HR) repair [18]. Additionally, an IGF-1R antibody was shown to inhibit the expression and activity of topoisomerase II, required for induction and repair of replication-associated DSBs [19].

We wished to investigate effects of IGF-1R targeting on the cellular response to DNA damage. Both IGF-1R antibodies and kinase inhibitors can affect the function of the closely-related insulin receptor, either directly or by down-regulating insulin receptors in IGF-1R:insulin receptor hybrids [20–22]. In order to study the

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specific contribution of IGF-1R to the DNA damage response, we used gene silencing to deplete IGF-1R in human prostate cancer cells. Our previous investigation using this approach demonstrated that IGF-1R knockdown sensitized prostate cancer cells to agents that induce DNA DSBs, but not to chemotherapeutic agents that kill cells without damaging DNA [23]. These results suggested that the ability to regulate apoptosis may not be the only means by which IGFs influence radio- and chemo-sensitivity. The aim of the current study was to investigate whether IGF-1R depletion modifies the response to DNA DSBs induced by ionizing radiation. Our data suggest that IGF-1R depleted prostate cancer cells show a delay in DNA repair, and support a role for the IGF axis in DSB repair via HR.

Materials and methods

Cell lines and siRNA transfection

Human prostate cancer cell lines DU145, PC3 and LNCaP were obtained from Cancer Research UK Laboratories, Clare Hall Hertfordshire, UK. DU145 cells are androgen independent, PTEN wild-type, PC3 are androgen independent, PTEN null, and LNCaP are androgen sensitive, mutant PTEN, IRS-1 null cells [24–27]. DU145 and LNCaP cells were cultured in RPMI 1640 medium, and PC3 cells in Hams F12 medium, each supplemented with 10% foetal calf serum (FCS). Cell line identity was verified by short tandem repeat multiplex assay (AmpF/STR® Identifier PCR Amplification Kit, Applied Biosystems), and all cell lines were negative for Mycoplasma when tested using the MycoAlert Mycoplasma Detection kit (Lonza Rockland Inc., Rockland, US). Cultures were transfected at 30–40% confluence using Oligofectamine (Invitrogen) with non-silencing Allstars (Qiagen) or scrambled sequence control siRNA, or IGF-1R siRNA (Hs_IGF1R_1, Qiagen; or IGF-1R R4 siRNA) as described [23,28]. Cells were irradiated in a Caesium-137 source (Gamma-Cell, Atomic Energy of Canada) at 3 Gy per minute.

Western blotting and immunoprecipitation

Forty-eight hours after siRNA transfection, cells were lysed in IGF-1R lysis buffer (50 mM HEPES pH 7.5, 100 mM NaCl, 10 mM EDTA, 1% Triton X-100, 4 mM sodium pyrophosphate, 2 mM sodium orthovanadate, 10 mM sodium fluoride, 1 mM PMSF or 150 mM Pefabloc (Roche), EDTA-free protease inhibitor cocktail, Roche) as described [17]. Lysates were analysed by SDS polyacrylamide gel electrophoresis and Western blotting, using primary antibodies to IGF-1R β (#3027, Cell Signaling Technology, CST), insulin receptor α (sc710, Santa Cruz), IRS-1 (#2382, CST) and β -tubulin (Sigma-Aldrich). Some cultures underwent initial lysis in IGF-1R lysis buffer to generate a Triton-soluble fraction, and the Triton-insoluble pellet was lysed in IGF-1R lysis buffer in which the Triton X-100 was replaced by 1% SDS. The SDS soluble fraction was sonicated for 10 s on ice at 70% maximum output on a Sonoplus GM70 sonicator (Bandelin Electronic, Berlin) prior to analysis by Western blotting for γ H2AX (#2577, CST) and heterochromatin protein-1 (HP1 α , loading control for SDS-soluble fraction; #2616, CST). Scanned images were quantified using ImageJ software and protein levels were corrected for loading. For immunoprecipitation, DU145 cells were serum-starved overnight and treated with 50 nM IGF-I (long-R3 IGF-I, Sigma) for 30 min prior to lysis in IGF-1R lysis buffer. Lysates were precleared using Protein A and G-Sepharose for 30 min at 4 °C on a rotating wheel. After centrifugation at 8000g for 1 min at 4 °C, precleared lysates were immunoprecipitated with IRS-1 antibody (as above) or rabbit IgGs (Sigma-Aldrich), overnight at 4 °C. Bound antibody was collected with Protein A and G-Sepharose for 90 min at 4 °C, washed 3 times with IGF-1R lysis buffer, and analysed by SDS-PAGE and immunoblotting for IRS-1 and RAD51 (14B4, Abcam).

Immunofluorescence

DU145 cells were seeded onto coverslips and after 24 h were siRNA-transfected as above. After 48 h, cells were irradiated (5 Gy), and at intervals were fixed in fresh 4% paraformaldehyde, 250 mM HEPES pH 7.4, 0.1% Triton X-100 for 20 min at 4 °C. After washing 3 times in ice-cold PBSA, cells were permeabilised in 0.5% TritonX-100 for 20 min on ice, washed and blocked with 5% FCS in PBS at room temperature for 30 min. Coverslips were incubated with γ H2AX antibody (#2577, CST) overnight at 4 °C, washed, and bound antibody was detected with Alexa Fluor 488-conjugated anti-rabbit secondary antibody (Invitrogen) in 5% bovine serum albumin (BSA) in PBS for 30 min at room temperature. After washing, DNA was stained with 1 μ M TO-PRO-3 (Invitrogen) in PBS, and coverslips were mounted in Fluoromount. Slides were examined by confocal microscopy (Bio-Rad) and nuclear foci were counted in 20–30 cells per condition.

Pulsed field gel electrophoresis (PFGE)

Methods for PFGE were based on [29,30]. The CHEF (contour-clamped homogeneous electric field) mammalian genomic DNA kit (Bio-Rad) was used to prepare agarose plugs containing 1×10^6 prostate cancer cells in 1% Clean-Cut agarose (Bio-Rad). Plugs were irradiated at 20 Gy, incubated at 37 °C, and at intervals of 2–36 h were lysed in 100 mM EDTA (pH 8.0), 0.2% sodium deoxycholate, 1% sodium lauryl sarcosine, 1 mg/ml proteinase K for 18 h at 50 °C. After washing four times in 20 mM TrisCl pH 8.0, 50 mM EDTA at room temperature, the plugs were loaded onto 1% agarose gels (Ultrapure DNA grade agarose, BioRad) in parallel with yeast chromosome size markers. PFGE was performed in TBE as described [29] using a CHEF-DRII apparatus (Bio-Rad, Richmond, CA) with an electric field reorientation angle of 120°, field strength 6 V/cm, pulses ramped from 50 to 60 s. After electrophoresis for 72 h at 4 °C, gels were stained for 30 min in 0.5 mg/ml ethidium bromide, destained in distilled water and imaged using a Gel Doc 2000 fluorimager (Bio-Rad).

Assay for homologous recombination (HR)

DU145 cells were transfected with HR reporter pDR-GFP, obtained from Professor Ian Hickson, Weatherall Institute of Molecular Medicine, Oxford, originally from Professor M. Jasin, Sloan-Kettering Cancer Center, New York [31]. Transfected clones were selected and expanded in 2 μ g/ml puromycin. Genomic DNA was extracted using the Wizard Genomic DNA extraction kit (Promega) to screen for pDR-GFP integration. GFP-specific primers (forward: 5'-CCTTCGGGCATGGCGGACTTG-3'; reverse: 5'-AGGGCGTTCGGCTTCTGG-3') were used in a PCR with an initial cycle of 95 °C for 12 min, 35 cycles of 94 °C for 30 s, 60 °C for 30 s, 72 °C for 40 s, and a final cycle of 72 °C for 10 min. Stable pDR-GFP transfectants were transiently transfected with control or IGF-1R siRNAs, and the following day with pCMV-Sce (provided by Professor Ian Hickson, WIMM Oxford) and pDsRed2-Mito (BD Clontech), using Genejuice transfection agent (Novagen). Two days later (3 days after siRNA transfection) cells were analysed by flow cytometry on a FACSCaliber flow cytometer (Becton Dickinson Biosciences), analysing 50,000 cells for each condition using CellQuest software (Becton Dickinson Biosciences). GFP-positive cells were calculated as a percentage of the transfected (pDsRed2-Mito-positive) population. Parallel cultures seeded on coverslips were harvested for fluorescent microscopy by fixing on ice using 4% paraformaldehyde in 250 mM HEPES pH 7.5, 0.1% Triton for 20 min. Coverslips were washed 4 times with PBS, mounted with Antifade Gold mounting medium (Molecular Probes), visualised

Fig. 1. IGF-1R depletion enhances radiosensitivity of human prostate cancer cells. (a) Cells were transfected with control (C, non-silencing) or IGF-1R (I) siRNA and after 48 h were lysed for Western blot analysis for IGF-1R β (arrow, IGF-1R proreceptor), insulin receptor- α , IRS-1 and β -tubulin. Right: levels of IGF-1R, insulin receptor and IRS-1 were quantified in three independent sets of cell lysates, corrected for β -tubulin loading and expressed as % levels in control-transfected DU145 cells. Transfection with IGF-1R siRNA induced significant IGF-1R depletion in DU145 ($p < 0.01$), PC3 ($p < 0.05$) and LNCaP ($p < 0.01$ by paired t -test) but did not significantly influence expression of insulin receptor or IRS-1. (b) Cells were siRNA-transfected as (a), and after 48 h were re-seeded into 10 cm dishes for clonogenic assay. After 4–6 h to allow adherence, cultures were irradiated and incubated at 37 °C to allow colony formation. Cell survival was expressed as % survival in unirradiated cultures. Points represent the mean of three independent assays, each with triplicate data points; bars, SEM. IGF-1R depletion (dashed line) enhanced radiosensitivity compared with controls (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ by t -test).

Table 1
Effect of IGF-1R depletion on cell cycle distribution after irradiation.

Cell line	SF1 (%)		SF10 (%)	
	Control siRNA	IGF-1R siRNA	Control siRNA	IGF-1R siRNA
DU145	90.0 $\pm$ 2.4	82 $\pm$ 1.0*	5.1 $\pm$ 0.4	1.5 $\pm$ 0.2***
PC3	82.1 $\pm$ 4.0	54.6 $\pm$ 3.0***	0.9 $\pm$ 0.2	0.1 $\pm$ 0*
LNCaP	45.9 $\pm$ 1.2	26.7 $\pm$ 2.5***	0.7 $\pm$ 0.3	0.4 $\pm$ 0.2

Data from assays shown in Fig. 1b are tabulated to show radiation survival, expressed as % survival in unirradiated cells (mean $\pm$ SEM of three independent assays) after 1 Gy (SF1) and 10 Gy (SF10) ionizing radiation. IGF-1R depleted DU145 and PC3 cells were significantly more radiosensitive than controls; LNCaP cells showed significant sensitization only at 1 Gy.

* $p < 0.05$; *** $p < 0.001$ by t -test.

on an Axioskop 2 plus microscope (Zeiss), and images were captured using Axiovision 3.1.

Cell cycle analysis

Cells were transfected with siRNAs and after 48 h were irradiated (5 Gy). At intervals, cells were fixed with ice cold 70% ethanol in PBS and stored at -20 °C for up to 2 weeks. After washing twice with PBS, cells were treated with 100 μ g/ml RNase A and 50 μ g/ml propidium iodide in PBS for 30 min at 37 °C. DNA content was quantified on a FACScaliber flow cytometer with CellQuest software.

Statistical analysis

Data were analysed using GraphPad Prism version 5 (GraphPad software, San Diego, California, USA), performing t -tests for comparison of two groups of data, and one way analysis of variance (ANOVA) for multiple (≥ 3) groups.

Results and discussion

We previously reported that IGF-1R depletion induces apoptosis and enhances the sensitivity of human prostate cancer cells to ionizing radiation and DNA damaging cytotoxic drugs [23]. The aim of the current study was to ask whether this effect is due, at least in part, to altered DSB repair. Initially we assessed IGF-1R expression in DU145, PC3 and LNCaP prostate cancer cells. Consistent with previous reports [23,27], PC3 and LNCaP cells expressed lower IGF-1R levels than DU145, and LNCaP cells lacked IRS-1 (Fig. 1a). We confirmed that transfection with IGF-1R siRNA induced depletion of IGF-1R, without influencing expression of insulin receptor or IRS-1 (Fig. 1a). In each subsequent experiment to analyse effects of this manipulation on radiosensitivity and DNA repair, IGF-1R depletion was confirmed by western blotting. Parallel cultures were used in clonogenic assays to assess cell survival after irradiation. Comparison of control-transfected cultures indicated that DU145 cells were relatively radioresistant, PC3 intermediate and LNCaP more radiosensitive. The same pattern of relative

radiosensitivity of DU145 and LNCaP cells had been noted previously [32]. Following IGF-1R depletion, there was evidence of enhanced sensitivity to ionizing radiation in DU145 and PC3 cells. Similar IGF-1R down-regulation was achieved in LNCaP cells, and was associated with sensitization to 1 Gy ionizing radiation; although statistically significant, effects at 3 and 5 Gy were modest and unlikely to be biologically important (Fig. 1b and Table 1). The results in all three cell lines were consistent with our previous report [23]. We did not check knockdown by fluorescence microscopy, although when we did this previously [33], we observed relatively uniform IGF-1R depletion in ~80–90% of cells, with ~10–20% retaining control IGF-1R levels. Therefore, it is possible that the results reported here could underestimate the true phenotype induced by IGF-1R depletion.

Ionizing radiation induces a variety of DNA lesions including single-strand breaks, abasic sites and cross-links, but DSBs are the most toxic [34]. DSB formation leads to rapid induction of foci containing γ H2AX, the phosphorylated form of the histone variant H2AX, required for recruitment of many factors that sense and repair DSBs. Thus, γ H2AX is a useful marker of DSB induction and resolution [35–37]. Effects of IGF-1R depletion on γ H2AX were measured initially by Western blotting. Unirradiated DU145 cells showed detectable γ H2AX signal, perhaps due to replication stress [38]. Levels of γ H2AX were enhanced by 5 Gy ionizing radiation, and control transfectants showed resolution of signal over 24–36 h (Fig. 2a and b). Following IGF-1R depletion, there was a delay in resolution of γ H2AX, which remained detectable for at least 36 h after irradiation (Fig. 2a and b). It is known that apoptosis can induce γ H2AX, typically in an ‘apoptotic ring’ around the periphery of the nucleus [39]. It is possible that this phenomenon could account, at least in part, for the apparent delay in γ H2AX resolution shown by Western blotting. Therefore, we also used immunofluorescence to monitor irradiation-induced γ H2AX focus formation. Occasional cells were seen with an ‘apoptotic ring’ or pan- γ H2AX staining (not shown), and these were omitted from quantification of γ H2AX foci. In undamaged cells there were few detectable γ H2AX foci, with a clear increase after irradiation, consistent with results of Western blotting (Fig. 2c). By 24 h, damage-induced γ H2AX foci had resolved in control-transfected cells. Following irradiation of IGF-1R depleted cells, γ H2AX foci were induced in a pattern paralleling that of the controls. However, there was a clear difference at later time points, with a significant excess of γ H2AX foci persisting at 24–36 h (Fig. 2c and d). This was not due to a large number of foci in a few cells, but rather persistence of foci in the majority of IGF-1R depleted cells: at 24 and 36 h control transfected DU145 cultures included 14% and 23% of cells with foci above background (undamaged) levels, compared with 85% and 64% of IGF-1R depleted cells. Therefore, characterization of the γ H2AX response indicated that IGF-1R depletion induced a delay in resolution of irradiation-induced γ H2AX.

Although γ H2AX foci form at DSBs, they are not specific for DSBs, and can be induced by other forms of DNA damage [38]. Therefore, we also performed pulsed field gel electrophoresis (PFGE) to quantify DSBs, to investigate whether IGF-1R depletion influenced the time-course of DSB repair. The DNA of DU145 cells was resolved by PFGE into three distinct bands, the upper representing intact chromosomal DNA that was retained in the wells. DSBs generate shorter DNA molecules that are able to migrate through the gel, creating an intermediate broad band/smear of DNA fragments (labelled * in Fig. 3a and c). A lower band, comprising diffuse fragments of relatively low molecular weight (labelled ** in Fig. 3a and c), was seen to increase after 2–6 h, and is likely to represent apoptotic DNA fragmentation. The intensity of the intermediate band, representing DNA that was fragmented by irradiation, was maximal immediately after irradiation, diminished with time, and in control-transfectants had resolved to basal

Fig. 2. IGF-1R depletion induces a delay in γ H2AX resolution. (a) DU145 cells were transfected with control or IGF-1R siRNA, and after 48 h were subjected to 5 Gy ionizing radiation. After 1–36 h, the cells were fractionated into Triton-soluble and Triton-insoluble, SDS-soluble extracts (see Methods), which were analysed by Western blotting for IGF-1R, γ H2AX and HP1 α as a loading control for the SDS-soluble fraction. (b) Three sets of independently-prepared lysates were analysed as in (a), and γ H2AX was quantified, corrected for HP1 α loading, and expressed as % maximal γ H2AX signal in control transfectants 1 h post-irradiation. Control transfectants showed peak γ H2AX induction 1 h post-irradiation, falling to basal levels at 24–36 h. In IGF-1R depleted cells there was an apparent reduction in γ H2AX signal 1–12 h post-irradiation, but at 24–36 h there was significant persistence of γ H2AX compared with controls (* p < 0.05, ** p < 0.01 by t -test). (c) DU145 cells were siRNA-transfected and irradiated (5 Gy) as in (a). At intervals, cells were fixed and stained for γ H2AX and DNA. The cells were examined by confocal microscopy, and representative cells are shown (original magnification 400 $\times$). (d) Foci were counted for 10–20 nuclei in each condition, and represented graphically as mean focus count per nucleus. After 1–4 h, both cell populations had similar numbers of foci. At 24–36 h, γ H2AX foci had resolved to basal levels in the control cells but persisted following IGF-1R depletion (* p < 0.05 by t -test).

Fig. 3. IGF-1R depletion induces a delay in DSB repair by PFGE in DU145 cells, but not in LNCaP cells. (a) DU145 cells were transfected with control or IGF-1R siRNA, and after 48 h were disaggregated and embedded in agarose plugs that were unirradiated (U) or irradiated at 20 Gy, and incubated at 37 °C. Repair was stopped at time-points up to 36 h, and the DNAs were analysed by PFGE. Image shows representative ethidium bromide-stained gel. Intact chromosomal DNA remained in the wells (arrow), while DNA that had been fragmented by DSB induction (*) was able to migrate into the gel. Intact and fragmented DNAs were quantified by densitometry, DNA fragmentation was calculated as a ratio of the intensity of the fragmented DNA to the sum of the intensity of the fragmented and intact DNA, and expressed as % maximum damage. Smaller fragments (**) may be products of apoptosis, and were not included in the calculation of DNA fragmentation. (b) The results of three independent experiments were plotted to show the time-course of DSB repair. Significant differences were seen at the 24 and 36 h time-points (* $p < 0.05$). (c) LNCaP cells were siRNA transfected, irradiated and used for PFGE as in (a), and a representative gel image is shown. (d) Graph shows the results of quantification of four independent assays in LNCaP cells (legend as b). Compared with DU145 cells, control-transfected LNCaP cells showed a slower rate of repair, and there was no difference in repair kinetics upon IGF-1R depletion.

(un-irradiated) levels by 36 h (Fig. 3a and b). There was a significant difference in the time course of appearance and resolution of fragmented DNA following IGF-1R depletion, with an increase in apoptotic DNA fragmentation, a predictable effect of IGF-1R depletion. Compared with control transfectants, there was also persistence of the intermediate products representing irradiation-induced damage, amounting to 30–40% excess of unrepaired DSBs at 24–36 h (Fig. 3a and b). The concordance between the PFGE results and γ H2AX quantification by Western blotting and immunofluorescence suggests that the rate of repair of radiation-induced DSB repair was impaired following IGF-1R depletion in DU145 cells.

Compared with DU145 cells, DSB repair in LNCaP was relatively slow and was incomplete at 24 h (Fig. 3c and d). The number of unrepaired DSBs generally correlates with the degree of cell killing after irradiation [34,40], and the detection of persisting damage at 24 h is consistent with the relative radiosensitivity of LNCaP cells (Fig. 1b and c). Similar differences were found in a previous study quantifying DSBs by neutral comet assay: DU145, PC3 and LNCaP cells showed delayed DSB repair compared with normal prostate epithelial cells, with evidence of more damage persisting at 24 h in LNCaP cells than DU145 [41]. In contrast, a second study using a modified electrophoresis protocol indicated that LNCaP cells repaired a greater fraction of DSBs during the initial fast phase of repair, and DU145 had more unrepaired DSBs at 24 h, although as here, LNCaP cells were found to be more radiosensitive than DU145 cells [32].

In contrast to findings in DU145 cells, LNCaP cells showed no change in the time course of DSB repair following IGF-1R depletion (Fig. 3c and d). Repair of DSBs is accomplished in mammalian cells by HR or non-homologous end joining (NHEJ). HR occurs via single strand annealing, when a DSB arises between two repeated sequences in the same orientation, or gene conversion, which uses an identical template, usually a sister chromatid, and results in high fidelity repair [42,43]. In considering why human prostate cancer cell lines respond differently to IGF-1R depletion, we note that LNCaP and PC3 cells lack functional PTEN, while DU145 expresses wild-type PTEN. A recent report suggested that IGF-1R inhibition radiosensitizes only PTEN wild-type cells, without effect in PTEN-null PC3 cells [44]. However, we have observed consistent radiosensitization of PC3 cells by IGF-1R depletion, both in the current study and previously [23]. In contrast, in IGF-1R depleted LNCaP cells we saw only modest radiosensitization (Fig. 1b and c) and no change in the time-course of DSB resolution (Fig. 3c and d). LNCaP cells are deficient in the adaptor protein IRS-1, which links the IGF-1R to the PI3K-AKT pathway [27]; see Fig. 1a). IRS-1 is reported to form IGF-regulated complexes with RAD51, the recombinase required for the strand invasion step of HR, thereby influencing RAD51 subcellular localization and function [18]. We speculated that the lack of IRS-1 could contribute to the relatively slow repair rate in LNCaP, noted also by [41], and to the inability to influence repair by IGF-1R depletion.

Therefore, to investigate directly whether IGF-1R depletion can influence DSB repair by HR, we used a recombination reporter that encodes inactive green fluorescent protein (GFP). When cleaved by the endonuclease I-Sce I, functional GFP can be restored by gene conversion using a donor fragment of GFP cDNA present on the same plasmid [31]. DU145 pDR-GFP stable transfectants were transiently transfected with control or IGF-1R siRNAs, and with pCMV-I-Sce and the transfection marker pDSRed2-Mito. Fluorescent cells were imaged by fluorescence microscopy (Fig. 4a) and quantified by flow cytometry (Fig. 4b). In control siRNA transfectants, ~7–8% of cells were GFP positive, indicating that they were competent to perform HR (Fig. 4b and c). These figures are consistent with 3–10% GFP positivity using this assay in other cell types [31,45]. Compared with controls, IGF-1R depletion resulted in ~30% decrease in the percent of GFP-positive cells, representing a significant reduction in the ability to accomplish HR ($p < 0.005$; Fig. 4b and c). This effect is consistent with the ability of IGF-I to promote HR in murine fibroblasts and murine and human mesangial cells [18,45], and is likely to contribute to the radiosensitization (Fig. 1b and c) and delay in DNA repair (Figs. 2 and 3) induced by IGF-1R depletion. The observed reduction in HR upon IGF-1R depletion could be compatible with the reported requirement for a functional IGF-1R to tyrosine phosphorylate IRS-1, disrupting interaction with RAD51 and allowing proper RAD51 function [18]. However, IRS-1 is predominantly cytoplasmic in DU145 cells (not shown), and we could not detect an interaction between IRS-1 and RAD51 (Fig. 4d). Consequently, we sought an alternative explanation for the HR defect induced by IGF-1R depletion in DU145 cells.

IGFs have well-recognized effects on cell cycle distribution, which is known to influence radiosensitivity and choice of repair pathway [34,40,46,47]. Therefore, we assessed effects of IGF-1R depletion on cell cycle distribution following 5 Gy ionizing radiation. Quantification of the sub-G1 population indicated that fewer than 10% of DU145 and LNCaP cells underwent apoptosis during the first 12 h post-irradiation, with a (non-significant) increase at 24 h following IGF-1R-depletion of DU145 but not LNCaP cells. These results paralleled the yield of relatively small DNA fragments, presumed due to apoptosis, on PFGE (marked ** on Figs. 3a and c). Apoptosis induction is highly likely to contribute to the ability of IGF-1R depletion to enhance radiosensitivity (Fig. 1). Viable DU145 cells showed a progressive reduction in the G1 population, with increasing G2-M arrest (from 26% to 40% of cells) in control transfectants 2–12 h after irradiation, and reverting to a profile similar to undamaged cells by 24 h (Table 2, upper). Following IGF-1R-depletion, there was more modest reduction in G1 and accumulation in G2-M after irradiation (G2-M population increased by ~7% at 4–6 h). A very similar pattern was observed in the LNCaP cell line, where post-irradiation G2-M arrest was attenuated by IGF-1R depletion (Table 2, lower). In considering the potential consequences of these effects, we note that mammalian cells are relatively radioresistant in S phase, with greatest radiosensitivity in early G1 [34]. An increase in the G1 population could, therefore, contribute to the ability of IGF-1R depletion to enhance radiosensitivity. However, given that IGF-1R depletion induced similar effects on cell cycle distribution in DU145 and LNCaP cells, it is unlikely that cell cycle effects *per se* could account for the differences in radiosensitivity and DSB repair induced by IGF-1R depletion in these two cell lines. In addition to influencing intrinsic radioresistance, cell cycle phase plays an important role in choice of DSB repair pathway, with access to a homologous template for repair by HR only during late S and G2 phases. Although the GFP reporter we used to quantify HR provides a repair template that is accessible independent of cell cycle phase, the loading of RAD51 filaments onto DNA is cell cycle dependent, requiring CDK-mediated phosphorylation of BRCA2 [48]. Therefore, it is

Fig. 4. IGF-1R depletion impairs the ability of DU145 prostate cancer cells to repair DSBs by homologous recombination. DU145 cells were stably transfected with pDR-GFP, and plasmid integration was confirmed by PCR in five puromycin-resistant clones (not shown). Stable transfectants were transiently transfected with control or IGF-1R siRNAs and 24 h later were transfected with pCMV-I-Sce I and pDSRed2-Mito as a transfection marker. After a further 48 h (i.e. 72 h post-siRNA transfection) cells were analysed by (a) light microscopy, showing a cluster of eight transfected (pDS-Red2-Mito positive) cells, of which two were GFP positive; (b) flow cytometry, showing representative results from one pDR-GFP clone. Homologous recombination efficiency was expressed as % red and green cells (i.e. upper right quadrant) as a percentage of the total transfected cells (i.e. upper right plus lower right quadrants). Cells in the lower left quadrant were untransfected, and there were occasional cells in the upper left quadrant (GFP positive, pDS-RedMito negative) that were omitted from the analysis. (c) Bar graph shows mean % GFP positivity from two independent assays of five DU145 pDR-GFP clones; bars, SEM. There was a significant reduction in repair by HR following IGF-1R depletion (** $p < 0.005$ by paired t test). (d) DU145 cells were serum-starved overnight and treated with solvent or 50 nM IGF-I for 3 h, shown previously to attenuate the IRS-1:RAD51 interaction detected in serum starved IGF-1R-transfected murine fibroblasts [18]. Whole cell extracts were immunoprecipitated with control IgG (C) or IRS-1 antibody and blotted for IRS-1 and RAD51. RAD51 was detected in whole cell extract but not in IRS-1 immunoprecipitates.

possible that the attenuated G2-M accumulation of IGF-1R depleted DU145 cells (Table 2) may contribute to the reduction in HR.

In conclusion, these results demonstrate a role for the IGF1R in the cellular response to DNA damage in DU145 prostate cancer cells, at least in part through effects on HR. However, the apparent 30–40% defect in DSB repair in DU145 cells (Fig. 3a and b) is in excess of the modest defect reported using PFGE to quantify repair of ionizing radiation-induced DSBs in cells defective in HR [46], and points to the possibility that NHEJ may also be defective. Finally, our findings suggest that there may be merit in combining IGF-1R inhibitory drugs with radiation therapy for primary prostate cancer, and potentially also with DNA damaging cytotoxic drugs as treatment for metastatic disease. Effective clinical application of this approach will require careful consideration of the scheduling of the IGF-1R therapeutic with damage [49], and of consequences for toxicity to IGF-1R-expressing normal tissues.

Table 2

Effect of IGF-1R depletion on cell cycle distribution after irradiation.

Hr	Sub-G1	G1	S	G2-M	Sub-G1	G1	S	G2-M
<i>DU145 control</i>					<i>DU145 IGF-1R depleted</i>			
0	2.1 ± 0.5	58.2 ± 5.3	13.6 ± 1.2	26.0 ± 3.6	4.0 ± 0.1	62.1 ± 5.9	13.5 ± 1.5	18.8 ± 6.0
1	1.8 ± 0.3	54.6 ± 3.0	17.0 ± 2.2	26.5 ± 0.5	5.1 ± 2.6	58.4 ± 3.1	14.2 ± 3.1	20.8 ± 4.0
2	2.1 ± 0.5	51.5 ± 7.6	17.2 ± 3.1	29.2 ± 3.9	4.1 ± 1.7	56.7 ± 0.9	14.4 ± 2.6	23.5 ± 1.2
4	2.9 ± 0.1	42.4 ± 2.4	17.2 ± 1.6	37.4 ± 0.7	4.0 ± 0.8	53.8 ± 4.5	14.8 ± 2.1	25.8 ± 3.0
6	2.3 ± 0.4	39.9 ± 5.0	18.3 ± 3.5	39.4 ± 1.1	4.0 ± 0.6	52.6 ± 6.6	15.2 ± 0.9	25.2 ± 6.9
12	3.2 ± 0.5	34.0 ± 6.0	22.9 ± 6.1	39.9 ± 0.4	3.4 ± 0.6	53.3 ± 9.9	16.1 ± 0.7	21.3 ± 7.9
24	6.0 ± 1.1	53.9 ± 5.6	16.3 ± 2.2	23.8 ± 1.6	11.3 ± 3.0	57.3 ± 0.7	12.7 ± 1.9	17.3 ± 3.0
<i>LNCAp control</i>					<i>LNCAp IGF-1R depleted</i>			
0	4.0 ± 3.4	47.8 ± 3.0	15.0 ± 0.7	27.1 ± 0.5	4.7 ± 4.0	61.1 ± 2.0	12.1 ± 0.6	20.3 ± 1.4
1	4.0 ± 3.5	45.0 ± 3.3	22.1 ± 1.2	23.6 ± 0.1	7.4 ± 5.7	69.2 ± 4.4	10.4 ± 0.8	12.9 ± 0.6
2	3.5 ± 2.9	41.2 ± 1.9	12.5 ± 0.5	30.1 ± 0.5	7.6 ± 6.6	61.1 ± 4.1	8.8 ± 0.7	21.0 ± 1.9
4	3.1 ± 2.6	36.7 ± 2.3	12.9 ± 0.5	37.1 ± 0.6	2.8 ± 1.7	58.4 ± 0.6	15.0 ± 0.3	23.5 ± 0.7
6	4.6 ± 3.2	33.2 ± 2.0	13.2 ± 0.6	37.8 ± 1.0	1.7 ± 1.2	58.1 ± 0.4	13.3 ± 0.2	26.3 ± 0.5
12	3.7 ± 2.3	36.2 ± 2.7	11.8 ± 0.35	40.7 ± 0.3	5.6 ± 3.8	60.1 ± 2.2	12.0 ± 0.5	19.8 ± 0.9
24	8.0 ± 6.0	52.1 ± 5.2	14.2 ± 0.4	22.4 ± 0.3	8.1 ± 5.7	63.4 ± 4.0	8.3 ± 0.5	17.5 ± 1.1

DU145 (upper) and LNCAp cells (lower) were transfected with control (non-silencing) or IGF-1R siRNA and after 48 h were irradiated (5 Gy). After incubation at 37 °C for 1–24 h, cell cycle distribution was analysed by flow cytometry, collecting 30,000 cells for each condition. Tables show results (mean ± range) of two independent analyses for each cell line.

Conflict of interest statement

The authors have no conflict of interest to declare.

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