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Downregulation of IGF1R Expression Inhibits Growth and Enhances Cisplatin Sensitivity of Head and Neck Squamous Cell Carcinoma Cells In Vitro

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Abstract

A lentivirus-mediated doxycycline-inducible pTRIPZ shRNAmir plasmid targeting IGF1R transcript was transfected into two head and neck squamous cell carcinoma (HNSCC) cell lines to silence IGF1R expression and to assess the effect of its downregulation on cisplatin sensitivity in vitro. In Cal27-regIGF1R and SCC25-regIGF1R cell lines, IGF1R protein expression was reduced by more than 90% after 72 h of incubation with doxycycline. Both basal and IGF-stimulated pIGF1R, pAKT, and pERK were significantly reduced, without influence on total AKT and ERK expression. Downregulation of the IGF1R was associated with decreased proliferation and cell viability in both cell lines. Reduced IGF1R expression was also associated with increased sub-G0/G1-phase and G0/G1-phase populations and decreased S-phase and G2/M-phase populations. IGF1R down-regulation enhanced sensitivity to cisplatin with decrease of cisplatin IC_{50} from 15 to 7.1 in Cal27-regIGF1R cells and from 11 to 6.3 in SCC25-regIGF1R cells. Cisplatin exhibited increased pro-apoptotic activity by annexin V staining and PARP cleavage in both cells lines when cultured in doxycycline. Thus, in two HNSCC cell lines in vitro, reduced IGF1R expression results in reduced growth rate and increased sensitivity to cisplatin. Thus, IGF1R downregulation and/or inhibition may serve as a useful adjunct to platinum-based cytotoxic chemotherapy.

Keywords IGF1R · Oral cavity cancer · Squamous cell carcinoma

Introduction

Head and neck squamous cell carcinoma (HNSCC) include tumors that involve the mucosal surfaces of the oral cavity, pharynx, larynx, and nasal cavity [1]. Current therapeutic options for HNSCC include surgery, radiation therapy, chemotherapy, or a combination of treatments [2]. Chemotherapy plays an important role in treatment, especially of advanced or recurrent disease; cisplatin is commonly used, either alone or in combination with other chemotherapeutic drugs. The effectiveness of chemotherapy is often inadequate due to development of drug resistance. Thus, exploration of the molecular basis of HNSCC chemotherapeutic resistance is an important step toward improving survival of patients who suffer from this cancer [3, 4].

The insulin-like growth factor-1 receptor (IGF1R) is a hetero-tetrameric transmembrane glycoprotein. Once a ligand is bound, the IGF1R is autophosphorylated, activating its tyrosine kinase function. The IGF1R then engages adaptor molecules such as insulin receptor substrates (IRSs) and Shc, leading to activation of downstream protein kinases, including the phosphatidylinositol-3-kinase (PI3K)/AKT and mitogen activated protein kinase (MAPK)/ERK1/2 signaling pathways that regulate growth and survival of cancer cells [5].

Abnormal expression and/or activity of the IGF1R has been associated with tumor growth and poor prognosis in several human cancers, including the myeloma, lung, and

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prostate [6, 7]. Recent studies in HNSCC demonstrated that IGF1R expression was significantly higher in grade II and III tumors than in patients with grade I disease and that elevated IGF1R expression correlated with poorer survival [8]. In other cancer types, IGF1R activity may be more important and may be elevated without increased IGF1R expression level. Studies using siRNA, dominant negative mutants, and antisense molecules directed against the IGF1R mRNA have shown that decreased IGF1R expression is associated with decreased radio- and chemoresistance and with induction of apoptosis [9].

Hence, the IGF1R is regarded as a target for the development of novel anticancer therapy in selected tumor types, and multiple IGF1R antagonist agents are being tested clinically for their therapeutic potential [10, 11]. These include small molecule inhibitors of the IGF1R tyrosine kinase, anti-IGF1R antibodies, and anti-IGF antibodies [13]. These agents have entered phase II and III clinical trials for various human cancers [14, 15]. An important feature of the anti-IGF1R antibodies is their ability to downregulate IGF1R by promoting receptor internalization; their anti-tumor activity is existing in vitro and in vivo model systems, with favorable safety profile [16]. IGF1R tyrosine kinase inhibitors (that also inhibit the insulin receptor) and antibodies that neutralize both IGF-1 and IGF-2 (which can act through the insulin receptor) have the potential therapeutic advantage of targeting both IGF1R and insulin signaling pathways [17]. The available data from the clinical trials with agents targeting the IGF1R have been promising [18]; nevertheless, several clinical development programs have since been discontinued due to unsatisfactory results [12, 13].

We hypothesize that suppression of IGF1R expression in HNSCC will increase sensitivity to conventional chemotherapy. To test this hypothesis, we used an inducible shRNAmir expression system targeting the IGF1R in two oral cavity squamous cell carcinoma (HPV-negative) cell lines and assess whether downregulation of IGF1R expression increases sensitivity of these cell lines to cisplatin.

Materials and Methods

Tissue Culture and Reagents

Cal27, SCC25 and HEK293T cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and were grown in DMEM/F12 medium supplemented with 5% FBS and 1% penicillin/streptomycin. Cell cultures were maintained in a humidified atmosphere of 5% CO_2 and 95% air at 37C. Cisplatin, doxycycline, puromycin, crystal violet, and 5-bromo-2′-deoxyuridine (BrdU) were purchased from Sigma-Aldrich (St. Louis, MO, USA), alamarBlue and trypan blue were obtained from Invitrogen (Carlsbad, CA, USA).

Propidium Iodide (PI), FITC mouse anti-BrdU, and FITC Annexin V (BD Biosciences, San Jose, CA, USA), anti-AKT, anti-pAKT (Ser473), anti-IGF1R, anti-ERK1/2, and anti-pERK1/2, were purchased from Cell Signaling Technology (Beverly, MA, USA). Anti-pIGFR and anti-β-actin were obtained from Millipore (Bedford, MA, USA).

pTRIPZ-shRNAmir Lentivirus Production and Cell Line Transduction

Inducible shRNA constructs (TRIPZ shRNAmir) targeting the IGF1R (catalog numbers RHS4696, RHS4697, and RHS4743), negative control shRNA (non-silencing-TRIPZ lentiviral inducible shRNAmir control), and RNAintro TRIPZ-inducible lentiviral shRNAmir transfection kit were obtained from Open Biosystems (Thermo Fisher Scientific, Rockford, IL, USA). IGF1R-TRIPZ lentiviral shRNAmir starter kit was used to produce the inducible shRNA according to the manufacturer's instructions. One clone from *Escherichia coli* stocks containing TRIPZ shRNAmir plasmids was grown in 5 ml 2× LB low salt on a shaker at 37C for 18 h. pTRIPZ shRNA DNA was isolated using a Qiagen Maxprep Kit. DNA concentration was measured using a Nano Drop H spectrophotometer (Thermo Scientific). HEK293T cells (5.0×10^3) were seeded in a 10-cm plate for 24 h and then transfected with 37.5 μg trans-lentiviral TM packaging mix, 9 μg of pTRIPZ shRNA plasmids, and 187.5 ml of Arrest-In TM transfection reagent in serum and antibiotic-free medium. Medium was replaced with fresh serum and antibiotic-containing medium 4 h later. Medium containing viruses was harvested 48 and 72 h after transfection, filtered (0.45 mm falcon filters, Millex HV; Millipore), and concentrated by ultracentrifugation at 3×10^4 RPM at 4 C for 12 h. Cal27 and SCC25 cells were plated at 30% density in 24-well plate, and 24 h later, virus at multiplicity of infection (MOI) of 10 for IGF1R and 20 for negative control was added to the cells for 4 h and then replaced with fresh media and cultured for 48 h. Cells were trypsinized and reseeded in 6-well plate, and cells were selected for the puromycin resistance gene by adding 3 μg/ml puromycin for negative control and the IGF1R shRNA-transfected cells. Cells were maintained in puromycin for at least three successive passages to ensure full selection of the resistant cells. Cells were induced by adding 2 μg/ml doxycycline (Sigma) in the culture media for 3 days and examined by fluorescence microscopy to detect expression of the turboRFP shRNA reporter gene.

Determination of Functional Titer and MOI

To establish a functional titer of lentiviral particles, HEK293 cells in 24-well plates were plated at a density of 5×10^4 cells/well and cultured overnight in humidified 37C incubator with 5% CO_2. Transfection media (TM) containing 10 μg/ml

polybrene in serum-free and antibiotic-free DMEM was freshly prepared. Culture medium was removed, and 225 μl TM was added to each well of the 24-well plate. Lentiviral particles were diluted to 5-fold serial dilutions in TM (5, 25, 125, 625-fold) using a flat-bottom 96-well plate. Twenty-five microliters of diluted lentiviral particles was added to each well. Following incubation for 24 h, doxycycline 2μg/ml was added to the wells to induce the expression of Turbo-RFP. Turbo-RFP expression in the cells was observed under a fluorescence microscope 72 h after transduction with the TRIPZ lentivirus. Turbo-RFP positive colonies were counted at × 10 magnification in three arbitrary fields using ImageJ software (National Institutes of Health, Bethesda, MD, USA). The functional titer was calculated as transducing units per milliliter (TU/ml) using the following formula: TU/ml = no. of TurboRFP positive colonies counted × dilution factor / volume of diluted lentiviral particles used. This functional titer was used to calculate the volume of lentiviral particles required to transfect Cal27 and SCC25 cells at the desired MOI. MOI was calculated from the formula MOI = (TU/ml) / no. of cells [19, 20].

Western Blot Analysis

Western immunoblots were obtained from total protein extracts as described previously [21, 22]. Briefly, treated cells in 6-cm dishes were washed with ice-cold phosphate-buffered saline (PBS) containing 2 mmol/l sodium orthovanadate. Cells were collected by scrapping in 1 ml cold PBS, centrifuged at 13,000 rpm for 5 min at 4 C and the supernatants were discarded. Cells pellets were re-suspended in 50 μl lysis buffer, vortexed, and incubated at 4 C for 30 min followed by centrifugation at 13,000 rpm for 15 min at 4 C. The supernatants were collected, and the concentrations of the supernatant were measured with a BCA protein assay kit (Pierce, Rockford, lL, USA). Proteins were resolved on a 12% SDS-PAGE by loading 25 μg protein and then transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, (Bedford, MA, USA). The membranes were incubated with blocking buffer (1% casein in PBS) (Bio-Rad, Hercules, CA, USA) for 60 min at room temperature (RT). The membranes were incubated with primary antibody overnight at 4 C. Proteins were visualized using an infrared-emitting conjugated secondary antibodies anti-mouse 680 Alexa Fluor (Molecular Probes, Eugene, OR, USA) or anti-rabbit IRDYE 800 (Rockland Immunochemicals, Gilbertsville, PA, USA) diluted 1:15,000 for 60 min. Band intensities were quantified using Odyssey imaging system (LI-COR Biosciences, Lincoln, NE, USA).

alamarBlue Cell Proliferation Assay

Cell suspensions treated with either vehicle or 2 μg/ml doxycycline were plated at 5×10^3 cells per well in 96-well plates and were incubated for 72 h. alamarBlue was added to each well according to the manufacturer's protocol. Cells were incubated for 3 h at 37 C, and the fluorescence at 540 nm was recorded.

Trypan Blue Cell Viability Assay

Cells were plated at 5×10^4 cells per well in 12-well plates. Wells in triplicates were treated with either vehicle or 2 μg/ml doxycycline and incubated for 72 h. Medium was evacuated from each well were collected and wells were trypsinized; anchored and floating cells were combined and centrifuged at 1000 rpm for 3 min at 4 C. Cells were resuspended in 1 ml PBS. One hundred microliters of the suspension was stained with 100 μl trypan blue. Living and dead (blue) cells were counted using the TC20 Automated Cell Counter (Bio-Rad, Hercules, CA, USA).

Colony Formation Assay

Cells treated with vehicle or 2 μg/ml doxycycline were seeded at 250 cells per well in 6-well plates and incubated for 14 days; during this period, the medium was changed twice per week with fresh media ± 2 μg/ml doxycycline. Plates were washed with ice-cold PBS; the colonies were fixed with ice cold methanol for 15 min, stained with 2% crystal violet, and counted. Colonies consisting of ≥ 50 cells were scored.

Flow Cytometry Analysis of Cell Cycle

Cells were seeded in 6-cm dishes at 2.5×10^4/dish, treated with vehicle or 2 μg/ml doxycycline for 72 h to achieve a steady and maximal state of IGF1R knockdown. Cells were treated with cisplatin for 72 h, followed by incubation with BrdU at 1:1000 for 6 h. Cells were trypsinized, collected, washed twice in cold PBS solution, and then fixed in 95% ethanol. Cells pellets were re-suspended in 1 ml of 2 N HCl and 0.5% Triton X-100, incubated at (RT) for 30 min. Cells were pelleted and re-suspended in 1 ml of 0.1 M NaB_4O_7 (pH 8.5) and incubated for 30 min at RT. Cells were pelleted and re-suspended in 0.5 ml of anti-BrdU antibody dilute 1:50 in PBS containing 1% BSA and 0.2% Tween-20 and then incubated at RT for 60 min. Cell pellets were re-suspended in 10 ml of PBS/BSA/Tween containing FITC-conjugated rabbit anti-mouse secondary antibodies (1:25) and incubated at RT for 30 min in the dark. Cell suspensions were passed through a cell strainer to remove aggregates and collect single cells in proper flow cytometry tubes. The relative proportions of cells in the G0/G1, S, and G2/M phases were determined by FACS Calibur™ Flow Cytometry. Analysis was performed using Flow Jo v8.5 (Flowjo, LLC—Ashland, OR, USA).

Flow Cytometry Evaluation of Apoptosis

Cells were grown in 6 cm dishes to 50% confluence in 5% FBS ± 2 µg/ml doxycycline for 72 h. Cells were incubated with the desired concentration of cisplatin for another 72 h. The conditioned medium from each well was collected; cell monolayers were trypsinized, resuspended in the corresponding conditioned medium, centrifuged at 3000 rpm for 3 min at 4 C, washed twice with cold PBS, and resuspended in annexin V-FITC and PI according to the manufacturer's recommendations (Millipore). Flow cytometry was conducted in the University of Virginia (UVA) Flow Cytometry Core Facility with marker combinations as follows: annexin V and PI negative; cells in early apoptosis are annexin V positive and PI negative; cells in late apoptosis are annexin V and PI positive; necrotic/dead cells are annexin V negative and PI positive [23]. Acquisition was performed on a FACS Calibur™ Flow Cytometry. Analysis was performed using Flow Jo v8.5 (Flowjo, LLC—Ashland, OR, USA).

Statistical Analysis

All statistical analyses were performed using Prism version 3.0 (GraphPad Software, Inc., La Jolla, CA, USA). Data were analyzed using two-way analysis of variance (ANOVA), followed by post-hoc Bonferroni to test significances between treatment groups. Data are presented as means ± standard error or the mean, and $p < 0.05$ was considered significant.

Results

Establishment of Doxycycline-Inducible IGF1R-shRNAmir Expressing Cells Lines

Lentiviral doxycycline-inducible pTRIPZ-shRNAmir constructs directed against IGF1R were used to transfect Cal27 and SCC25 HNSCC cells. The construct also contains a doxycycline-inducible TurboRFP as a reporter gene co-expressed with the shRNA sequence. A negative control pTRIPZ plasmid containing a non-silencing shRNAmir construct was also used. The transfected cells were selected with puromycin to generate Cal27-regIGF1R and SCC25-regIGF1R cells with the IGF1R-shRNAmir construct and Cal27-nonreg and SCC25-nonreg with the non-silencing shRNAmir construct. Cells were seeded in 6-well plates with vehicle or 2 µg/ml doxycycline for 3 days to induce shRNAmir and TurboRFP expression. Cells were then examined by florescence microscope for the expression of the reporter Turbo-RFP protein as marker of successful transfection and doxycycline inducibility (Fig. 1a). Immunoblot with anti-IGF1R showed that doxycycline-induced IGF1R-shRNAmir reduced the IGF1R protein level by 90% relative to the
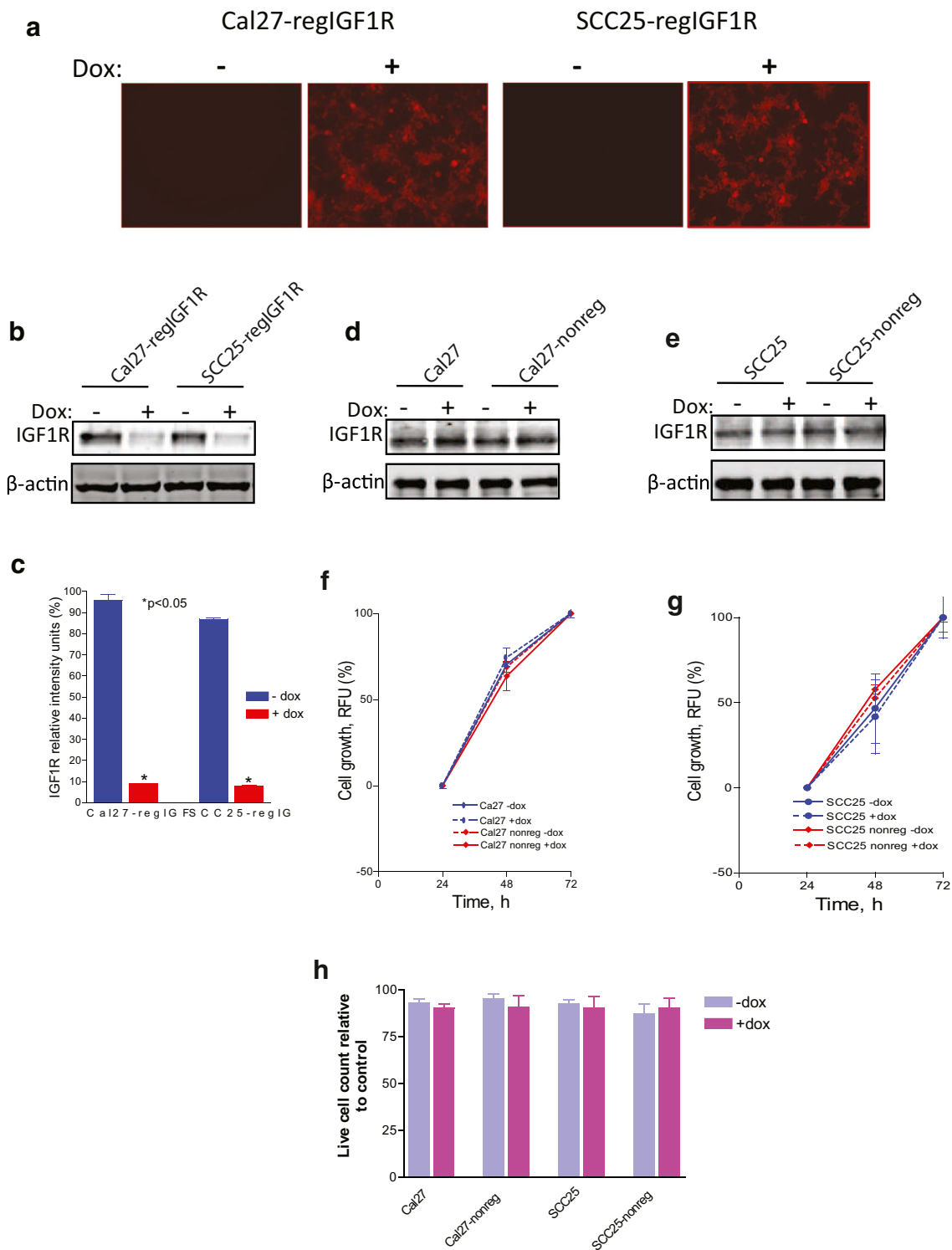
Fig. 1 Induction of shRNAmir-IGF1R with doxycycline reduces IGF1R ▶ expression. Indicated cell lines were grown in the absence or presence of 2 µg/ml doxycycline for 3 days. Cal27 and SCC25, parental cell lines. Cal27-regIGF1R and SCC25-regIGF1R, cell lines transfected with IGF1R-pTRIPZ-shRNAmir. Cal27-nonreg and SCC25-nonreg, cell lines transfected with non-silencing pTRIPZ-shRNAmir (negative control). **a** Fluorescence microscopy demonstrates expression of the reporter TurboRFP in the presence of doxycycline. **b** Representative immunoblot demonstrating regulation of IGF1R expression by doxycycline. **c** Quantification of densitometric analysis of three such immunoblots showing relative intensity of IGF1R band normalized to β-actin band. **d, e** Representative immunoblots demonstrating no impact of doxycycline or negative control plasmid on IGF1R expression. **f, g** Parental Cal27, Cal27-nonreg, SCC25, and SCC25-nonreg cells were grown in 96-well plates in the absence or presence of 2 µg/ml doxycycline as indicated. Proliferation was measured using the alamarBlue assay at 24, 48, and 72 h. Data are expressed as net fluorescence. Values represent means ± SEM of three independent experiments performed in triplicate and demonstrate no effect of non-silencing shRNAmir expression or dox treatment. **h** Cell viability by trypan blue assay at 72 h. Data are expressed as average live cell counts ± SEM for three independent experiments performed in triplicate. No effect of non-silencing shRNAmir expression or dox treatment. Asterisk indicates significant difference between −dox and +dox, $p < 0.05$

untreated cells (Fig. 1b, c). Untransfected parental cells and cells transfected with the non-silencing control did not show any changes in the level of the IGF1R in the absence or the presence of doxycycline (Fig.1d, e), indicating that doxycycline alone has no effect on IGF1R expression and that the downregulation of IGF1R occurs only when the IGF1R-shRNAmir is expressed.

Parental cells and cells transfected with the non-silencing shRNAmir (Cal27-nonreg and SCC25-nonreg) were treated with vehicle or 2 µg/ml doxycycline and cell proliferation was determined at 24, 48, and 72 h using alamarBlue (Fig. 1f, g). Cell viability was evaluated at 72 h by trypan blue assay (Fig. 1h). Parental Cal27 and SCC25 cells or cells expressing the non-silencing shRNA do not show any significant difference in the cell proliferation or cell viability, indicating that transfection and doxycycline treatment, separately or combined, do not affect the proliferative and survival behavior of the parental cell lines.

Downregulation of IGF1R Decreases Phosphorylation of AKT and ERK

Cal27-regIGF1R and SCC25-regIGF1R cells in 6 cm dishes were incubated with 2 µg/ml doxycycline for 3 days to induce IGF1R-shRNAmir expression. Ten nanomolars of des[1–3]IGF-1 (desIGF1) was used to activate the IGF1R. des[1–3]IGF-1 is an N-terminally truncated form of IGF-1 that binds to the IGF1R with high affinity but has substantially reduced affinity for the IGFBPs. (Residues 1–3 and 49–51 of IGF-1 are crucial for interaction with the IGFBPs, and

a Cal27-regIGF1R   SCC25-regIGF1R

Dox: − +   − +

b   Cal27-regIGF1R   SCC25-regIGF1R
Dox: − +   − +
IGF1R
β-actin

d   Cal27   Cal27-nonreg
Dox: − +   − +
IGF1R
β-actin

e   SCC25   SCC25-nonreg
Dox: − +   − +
IGF1R
β-actin

c *p<0.05

IGF1R relative intensity units (%)

− dox
+ dox

Cal27-regIGF1R SCC25-regIGF1R

f Cell growth, RFU (%)

Ca27 -dox
Cal27 +dox
Cal27 nonreg -dox
Cal27 nonreg +dox

Time, h

g Cell growth, RFU (%)

SCC25 -dox
SCC25 +dox
SCC25 nonreg -dox
SCC25 nonreg +dox

Time, h

h Live cell count relative to control

-dox
+dox

Cal27   Cal27-nonreg   SCC25   SCC25-nonreg

mutations in these domains reduce IGFBP affinity [24].) As some HNSCC cell lines are known to secrete high levels of IGFBPs, we routinely use desIGF1 to stimulate the IGF1R, thus eliminating IGFBP effects on IGF1R signaling. Cells were collected after 10 min of IGF1R activation and processed for immunoblot (Fig. 2). Stimulation with desIGF1 increased

IGF1R, AKT, and ERK1/2 phosphorylation in cells not treated with doxycycline. In cells treated with doxycycline and thus expressing shRNAmir, the level of IGF1R was reduced with concomitant decrease in IGF-stimulated pIGF1R, pAKT, and pERK1/2 levels; levels of total AKT and ERK1/2 were unaffected by doxycycline.
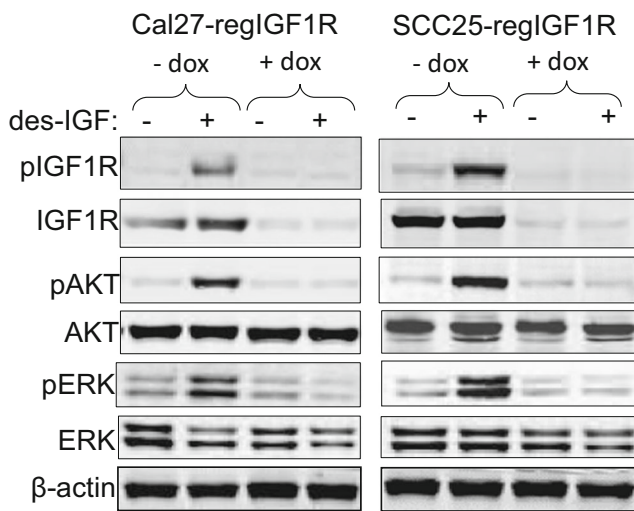
Fig. 2 Suppression of IGF1R expression reduces IGF-induced AKT and ERK phosphorylation. Cal27-regIGF1R and SCC25-regIGF1R cells were incubated in the absence or presence of 2 μg/ml doxycycline for 3 days and then treated with vehicle or 10 nM des [1–3] IGF for 10 min. Immunoblots were performed on whole cell lysates with the indicated antibodies. The blots shown are representative of three independent replicate experiments

IGF1R Downregulation Exhibits Growth Inhibitory Effect on HNSCC Cells

Cal27 and SCC25 cells transfected with IGF1R-shRNAmir (Cal27-regIGF1R and SCC25-regIGF1R) or with non-silencing shRNAmir (Cal27-nonreg and SCC25-nonreg) were treated with vehicle or 2 μg/ml doxycycline, and cell proliferation was determined 72 h by alamarBlue. Expression of IGF1R-shRNAmir and resulting downregulation of the

IGF1R results in a reduction in cell proliferation that is detected after 3 days of continuous exposure to doxycycline ($p < 0.05$) (Fig. 3a). This reduction in the cell proliferation was accompanied by a significant reduction in cell viability as evaluated by trypan blue assay (Fig. 3b). Cells expressing the non-silencing shRNAmir did not demonstrate any significant difference in the cell proliferation or cell viability, indicating that the growth effect is dependent on knockdown of the IGF1R.

IGF1R Downregulation Alters Cell Cycle Distribution in HNSCC Cells

The effect of IGF1R downregulation on the cell cycle distribution was determined 72 h after doxycycline treatment using flow cytometric analyses of Cal27-regIGF1R and SCC25-regIGF1R cells labeled with BrdU/FITC. Compared to cells without doxycycline treatment, doxycycline-treated Cal27-regIGF1R and SCC25-regIGF1R cells showed the following: (1) slight increase in the fraction of cells in Sub-G0/G1 phase (4.1% and 5.1%, respectively with dox vs. 3.2% and 2.6%, respectively without dox); (2) an increase in the fraction of cells in the G0/G1 phase (49% and 54%, respectively with dox vs. 38% and 39%, respectively without dox, $p < 0.05$); (3) a decrease in the proportion of cells in the S phase (26% and 30%, respectively with dox vs. 36% and 35%, respectively without dox, $p < 0.05$); and (4) a decrease in the G2/M phase (14.7% and 15%, respectively with dox vs. 22% and 20%, respectively without dox). Control cells without doxycycline treatment had a significantly lower G0/G1 cell population and higher S population (Table 1).
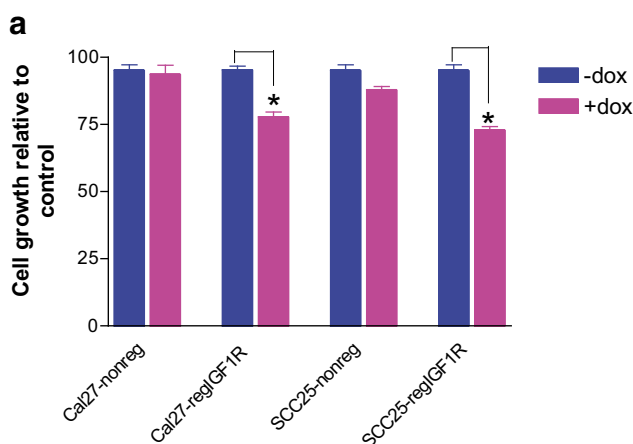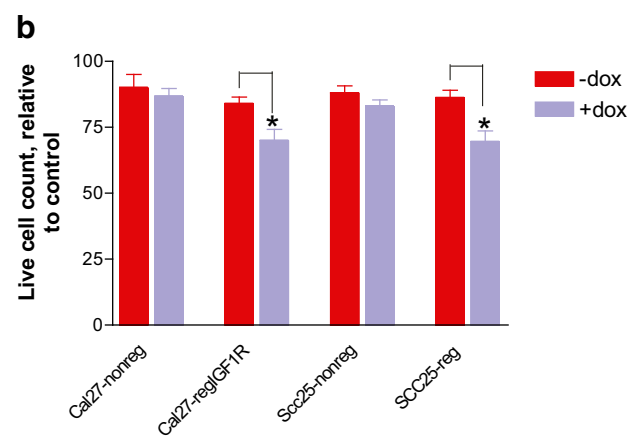

Fig. 3 IGF1R downregulation reduces HNSCC cell growth and viability. Cal27-regIGF1R, SCC25-regIGF1R, and their negative controls Cal27-nonreg and SCC25-non-reg respectively were grown in 96-well plates in the absence or presence of 2 μg/ml doxycycline as indicated. **a** Proliferation was measured using the alamarBlue assay at 72 h. Data are expressed as net fluorescence after subtracting background. Values

represent means ± SEM of three independent experiments performed in triplicate. **b** Cell viability by trypan blue assay at 72 h. Data are expressed as average live cell counts ± SEM for three independent experiments performed in triplicate. Asterisk indicates significant difference between −dox and +dox, $p < 0.05$

Table 1 Comparison of cell cycle distribution in OSCC cells (mean ± SD) (*P < 0.05)

Cell type	Cell cycle phases % M ± SEM			
	Sub-G1	G0/G1	S	G2/M
Cal27-regIGF1R, −dox	3.2 ± 0.1	38.7 ± 0.9	36.3 ± 2.3	21.7 ± 1.2
Cal27-regIGF1R, +dox	4.1 ± 0.6	49.0 ± 2.1*	26.3 ± 0.9*	14.7 ± 1.5
SCC25-regIGF1R, −dox	2.6 ± 0.9	39.7 ± 1.2	35.3 ± 1.9	19.7 ± 0.9
SCC25-regIGF1R, +dox	5.1 ± 0.6	54.3 ± 1.5*	30.0 ± 3.5*	15.0 ± 0.6

IGF1R Downregulation Enhances Growth Inhibitory Effect of Cisplatin on HNSCC Cells

Cal27-regIGF1R and SCC25-regIGF1R (or Cal27-nonreg and SCC25-nonreg) cells were treated with increasing concentrations (2.5, 5, 10, 15, and 25 μM) of cisplatin in the absence and presence of 2 μg/ml doxycycline. Cell proliferation was determined after 72 h using alamarBlue (Fig. 4a). Treatment with cisplatin alone caused a dose-dependent reduction in cell number at 72 h. The cisplatin IC_{50} was 15 and 11 μM for Cal27-regIGF1R and SCC25-regIGF1R, respectively. After reduction in IGF1R expression, the cisplatin IC_{50} decreased to 7.1 and 6.3 μM, respectively. There was a significant difference between the growth inhibition caused by cisplatin in IGF1R-depleted cells (+dox) compared to cisplatin in control cells (−dox, $p < 0.05$). Table 2 summarizes the growth inhibitory effect at different concentrations of cisplatin in the presence or absence of doxycycline. The enhanced sensitivity to cisplatin with IGF1R reduction was accompanied by a significant reduction in the cell viability as determined by trypan blue assay (Fig. 4b). This effect was absent in cells transfected with non-silencing shRNAmir. The effect of chronic exposure (14 days) of Cal27-regIGF1R cells to 2.5, 5, and 10 μM cisplatin in the presence or absence of 2 μg/ml doxycycline on clonogenic survival was evaluated using a colony formation assay. Doxycycline alone caused a nonsignificant reduction in the number of colonies formed. Cisplatin at 2.5, 5, and 10 μM resulted in 17%, 33%, and 92% reductions respectively in colony formation compared to untreated cells ($p < 0.05$). In the presence of doxycycline resulting in reduced IGF1R expression, reductions of 33%, 57%, and 97% were observed ($p < 0.05$). Control cell expressing non-silencing shRNAmir showed no difference in the number of colonies formed with or without dox (Fig. 4c).

IGF1R Knockdown Enhances the Apoptotic Effect of Cisplatin on HNSCC Cell Lines

The effect of IGF1R downregulation on the induction of apoptosis by cisplatin treatment of Cal27-regIGF1R and SCC25-regIGF1R cells was examined by flow cytometry. Cells were incubated with 5, 10, and 25 μM cisplatin either alone or in combination with 2 μg/ml doxycycline for 72 h. Cells were processed for flow cytometry using annexin V-FITC and PI as described in "Materials and Methods." Figure 5a demonstrates the cell populations in early (lower right quadrant) and late (upper right quadrant) apoptosis at 10 μM cisplatin dose. The apoptotic rates of Cal27-regIGF1R and SCC25-regIGF1R at various concentrations of cisplatin are summarized in Table 3 and Fig. 5b. The apoptotic rates (early apoptotic and late apoptotic) of Cal27-regIGF1R and SCC25-regIGF1R 72 h after doxycycline induced IGF1R downregulation increased from 3 and 5 to 3.5 and 7% ($P > 00.5$) respectively. The percentage of apoptotic cells treated with 5, 10, and 25 μM cisplatin alone increased to 8% and 19%, and 40%, respectively in Cal27-regIGF1R and to 12%, 25%, and 33% in SCC25-regIGF1R ($p < 0.05$). With IGF1R downregulation, this apoptotic rate respectively increased to 14%, 40%, and 52% in Cal27-regIGF1R and to 25%, 50%, and 56% in SCC25-regIGF1R ($p < 0.05$).

Apoptosis was also assessed using immunoblot for PARP cleavage as shown in Fig. 5c. Cal27-regIGF1R and SCC25-regIGF1R cells either untreated or pretreated with doxycycline for 3 days were incubated with 2.5, 5, 10, or 25 μM cisplatin for 72 h followed by immunoblot analysis of whole cell lysates. Cisplatin caused a dose-dependent induction in PARP cleavage. Combined treatment with cisplatin and doxycycline caused a cisplatin dose-dependent significant increase in PARP cleavage with 3–4-fold increases in the PARP cleavage (determined by densometric analysis of the band intensity) was noticed at 5, 10, and 25 μM. Cisplatin also induced pIGF1R, pAKT, and pERK in dose dependent. IGF1R downregulation suppressed the cisplatin induced IGF1R, AKT, and ERK activation which shows that induction of apoptosis via PARP cleavage was associated with decrease in the survival signals via decrease phosphorylation of IGF1R/AKT/ERK pathway.

Discussion

The effects of IGF1R downregulation on the sensitivity of HNSCC to the chemotherapeutic agent cisplatin have been tested in vitro. Two HNSCC cell lines that express high levels of IGF1R were used. A post-transcriptional gene silencing mechanism was used to target the IGF1R via a doxycycline-
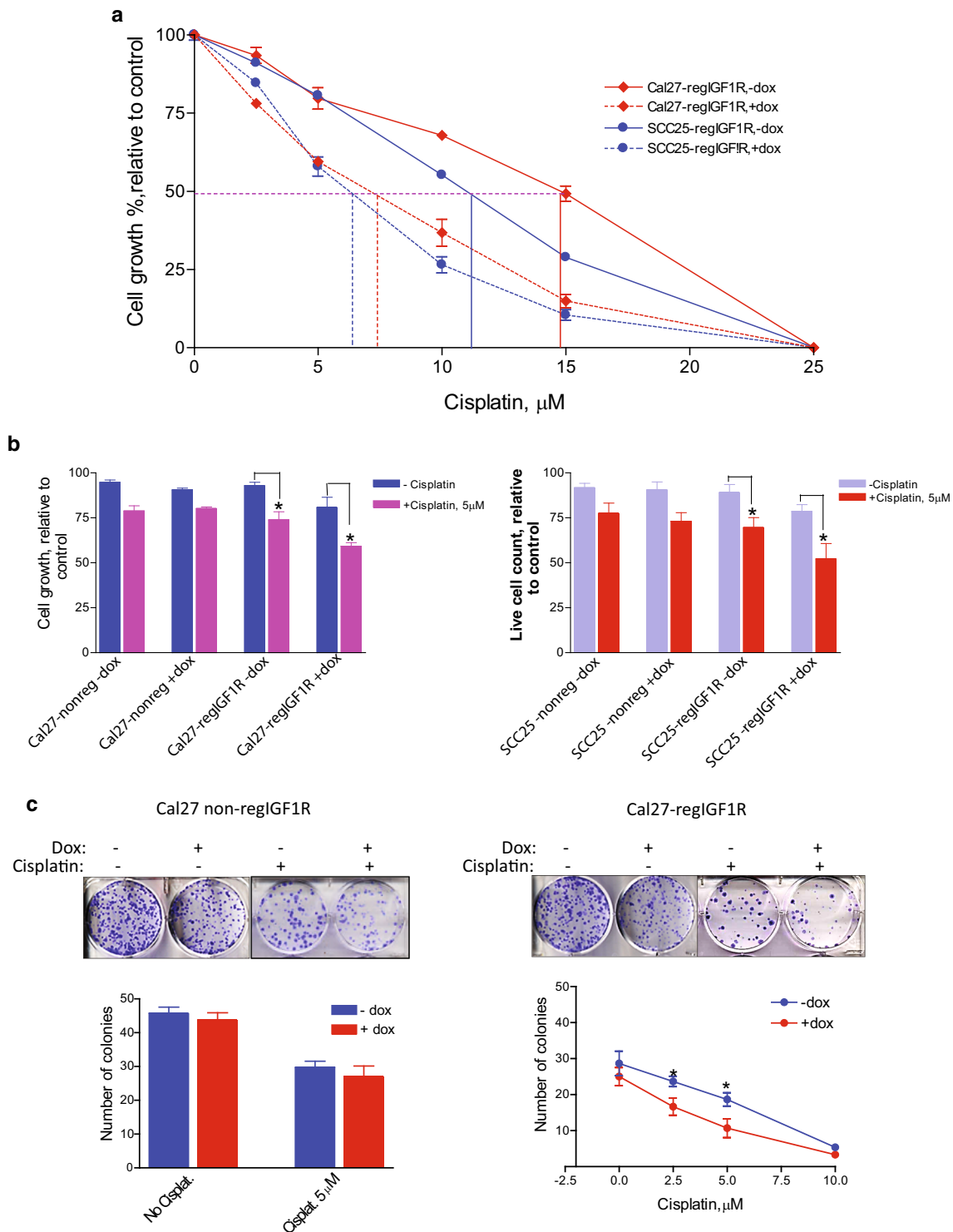
Fig. 4 Impact of IGF1R knockdown on sensitivity to cisplatin. Cal27-regIGF1R and SCC25-regIGF1R cells were grown for 3 days in the absence or presence of 2 μg/ml doxycycline to alter IGF1R expression. They were then treated with cisplatin for 72 h. **a** Proliferation was measured using the alamarBlue assay. Data are expressed as average net fluorescence as a percentage of cells not treated with cisplatin (each cell line and dox treatment set to 100%). **b** Cell viability was measured using the trypan blue assay. Data are expressed as average live cell counts ± SEM for three independent experiments performed in triplicate. Asterisk indicates $p < 0.05$ compared to control. **c** Colony formation assay was performed on Cal27-regIGF1R cells and Cal27-nonreg cells in the absence or presence of 2 μg/ml doxycycline. Cells were treated with cisplatin dose as indicated. Image shows representative stained colonies. Graph quantifies three independent experiments showing mean colony number ± SEM. Asterisk indicates significant difference between −dox and +dox at given cisplatin concentration, $p < 0.05$

Table 2 The growth inhibitory effect of cisplatin in IGF1R downregulated Cal27-regIGF1R and SCC25-regIGF1R cells (*P < 0.05)

Cisplatin/μM	Cal27-regIGF1R cell growth inhibition (%)			SCC25-regIGF1R, cell growth inhibition (%)		
	−dox	+dox	p	−dox	+dox	p
2.5	6.5	21.5	< 0.05	9	15	< 0.05
5	20	42	< 0.05	20	41	< 0.05
10	31.5	62.5	< 0.05	45	72.5	< 0.05
15	50	85	< 0.05	72.5	89.7	< 0.05
25	90	95	< 0.05	90	95	< 0.05

inducible short hairpin RNA construct (TRIPZ shRNAmir). The resultant cell lines, Cal27-regIGF1R and SCC25-regIGF1R, demonstrate reduction in IGF1R with exposure to doxycycline. IGF1R protein expression was reduced by more than 90% within 72 h. The decrease in IGF1R level is associated with abrogation of IGF-stimulated growth and survival signals, including the AKT and ERK pathways. These signaling changes were mirrored by the suppressed proliferation, decreased cell viability, and reduced clonogenicity after continuous IGF1R downregulation.

Exploration of cell cycle changes associated with IGF1R downregulation showed increases in the sub-G0/G1 and G0/G1 fractions. There was also a decrease in the proportion of cells in the S and G2/M phases. These changes in cell cycle distribution are consistent with the inability of the IGF1R downregulated cells to promote G1 to S transition resulting in G0/G1 cell cycle delay and a subsequent reduction in the S phase and G2/M population. This is consistent with the finding of inhibited cell proliferation.

Cisplatin is the mainstay of many chemotherapeutic regimens used in a wide variety of solid tumors, including HNSCC. The suppressing effect on tumor growth is limited due to severe toxicity particularly nephrotoxicity and hepatotoxicity [25]. Thus, enhanced of the chemotherapeutic effects of cisplatin at the tumor would be expected to have clinical relevance. The effect of IGF1R downregulation on chemosensitivity to cisplatin was tested in Cal27-regIGF1R and SCC25-regIGF1R cell lines. Cisplatin inhibited proliferation, viability, and colony formation in a dose-dependent fashion. Downregulation of the IGF1R enhanced the growth inhibitory effect of cisplatin with significant 50% reduction in IC$_{50}$ in both cell lines. At all dose tested, IGF1R downregulation increased chemosensitivity to cisplatin. Flowcytometric examination of different cell populations treated with cisplatin demonstrated that cisplatin significantly increases the rate of apoptosis. IGF1R downregulation in the presence of doxycycline significantly augmented the pro-apoptotic effect of cisplatin; this was assessed by flow cytometry for annexin V staining and immunoblot for PARP cleavage.

The results of this study are consistent with a large body of evidence which suggests that IGF1R signaling plays a vital role in chemotherapy resistance in a wide variety of tumors [26–28].

IGF1R inhibition sensitizes breast cancer cells to cisplatin [29]. Knockdown of IGF1R enhances chemosensitivity to cisplatin in human lung adenocarcinoma A549 cells [30]. Inhibition of IGF1R signaling using RNAi enhances sensitivity to adriamycin chemotherapy liver cancer cell lines [31, 32] and the effect of the 5-fluorouracil (5-FU) in SW480 colon cancer cells [33]. In pancreatic cancer xenografts, the chemosensitivity to gemcitabine was enhanced by concomitant treatment with an anti-IGF1R antibody [34]. Furthermore, inhibition of IGF1R with an antisense molecule increases sensitivity of bladder cancer cell [35] or prostate cancer cells to cisplatin, mitoxantrone, and paclitaxel [36]. This is the first demonstration of this phenomenon in HNSCC, a tumor type where cisplatin resistance portends very poor clinical outcomes.

In addition to apoptosis, additional mechanisms could be relevant to the enhancement of cisplatin sensitivity with IGF1R down regulation. Prior reports have indicated that cytotoxic drugs can induce IGF1R activation leading to drug resistance via activation of pro-survival/anti-apoptotic pathways [37]. In the present study, HNSCC cell lines treated with cisplatin demonstrated a dose-dependent increase in IGF1R, AKT, and ERK1/2 phosphorylation; this activation of pro-survival signaling was reduced by IGF1R downregulation. Thus, IGF1R downregulation or targeted IGF1R inhibition has the potential to block this compensatory pathway in HNSCC treated with cisplatin. Furthermore, there is evidence that a functional IGF1R is required for repair of DNA double strand breaks caused by chemotherapeutic drugs [38]. IGF1R downregulation or targeted IGF1R inhibition has the potential to interfere with DNA damage repair resulting in enhanced drug sensitivity and increased cell death.

Clinical studies have shown that upregulation of IGF1R expression during treatment is a poor prognostic factor, and such patients may benefit from targeted IGF1R inhibition [11, 39]. Though phase I/II studies in many cancer including breast, lung, prostate and pancreatic carcinoma with agents that target IGF1R have demonstrated a wide range of responses ranging from progressive disease to complete response [40–42], most of phase III clinical trials failed to show a clear benefit from targeting IGF1R in combination with conventional treatment [43]. However, IGF1R expression was not a selection factor in these clinical trials, and it is likely

Fig. 5 IGF1R downregulation enhances cisplatin-induced apoptosis. Cal27-regIGF1R and SCC25-regIGF1R cells were plated in the absence (−dox) or presence (+dox) of 2 μg/ml doxycycline for 3 days followed by treatment with cisplatin for 72 h. **a** Cells were collected, stained with annexin V-FITC and PI, and subjected to flow cytometry. Representative dot blots are shown for treatment with 10 μM cisplatin (Cisp10), with percentage population in early and late apoptosis indicated in the lower right and upper right corners, respectively. **b** Quantification of three independent experiments using 5 μM (Cisp5), 10 μM (Cisp10), and 25 μM (Cisp25) cisplatin. Data are shown as mean ± SEM. Asterisk indicates significant difference between −dox and +dox at given cisplatin concentration, $p < 0.05$. **c** Adherent and non-adherent cells were collected, and whole cell lysates were subjected to immunoblot with the indicated antibodies. Fold change in the PARP cleavage was determine by densometric analysis of the band intensity
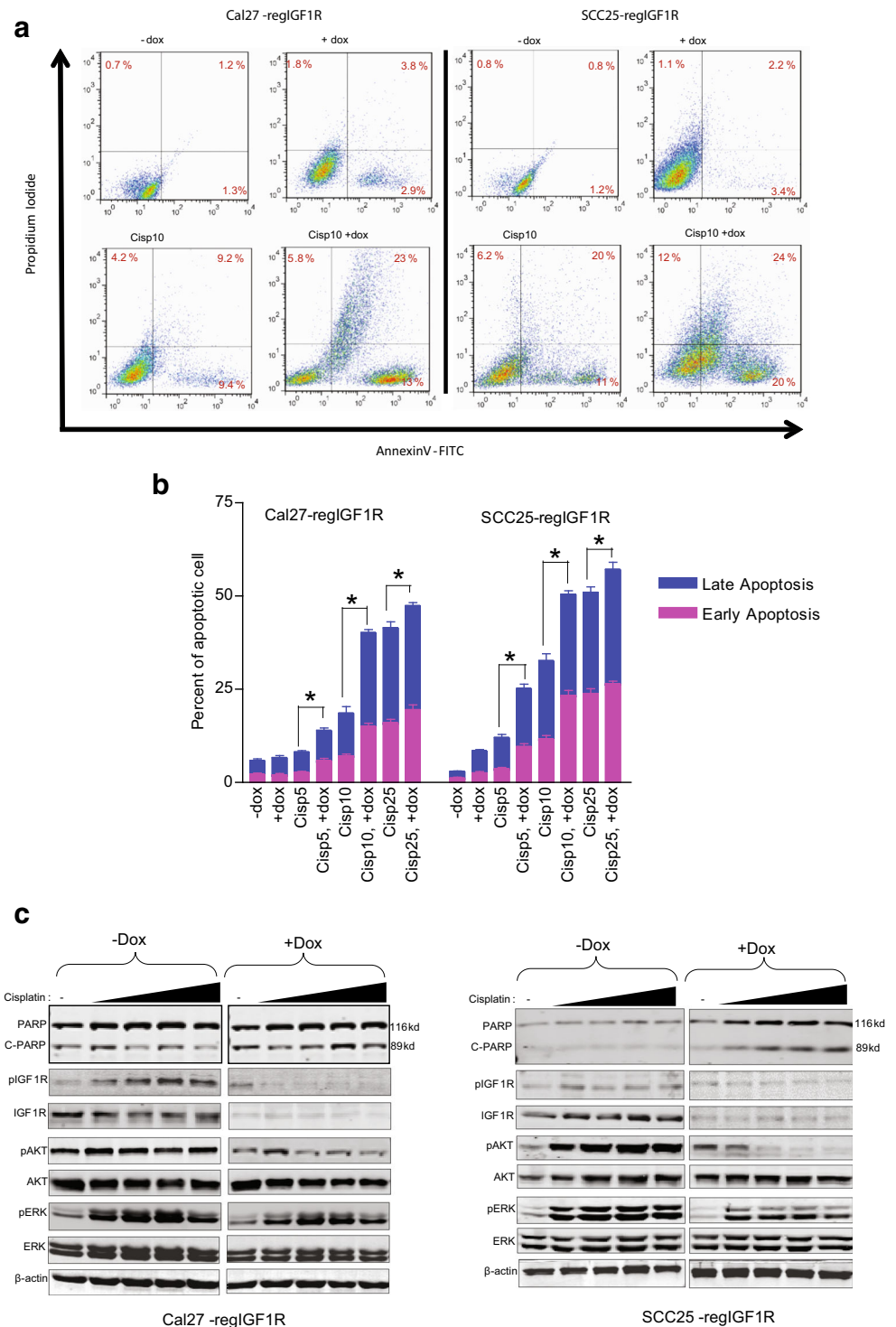
that IGF1R expression and/or activity are important predictors of response to IGF1R antagonists [44, 45]. Thus, understanding the cellular effects of IGF1R downregulation or inhibition and the related impact on sensitivity to chemotherapeutic and other targeted therapeutic agents is necessary for identification of reliable predictive biomarkers of response to combination therapy [5, 22, 46].

It should be noted that prior studies of HNSCC have reported that outcomes are particularly poor for patients with HPV-negative tumors. Dale et al. noted the association of high IGF1R expression with higher T-stage and HPV-negativity, with a tendency toward shorter OS and indicated the potential value of adding targeted anti-IGF1R therapy to selected HPV-negative tumors with high IGF1R expression [47]. Both

Table 3 Effect of cisplatin on the apoptotic rate in IGF1R downregulated Cal27-regIGF1R and SCC25-regIGF1R cells

Cell treatment	Cal27-regIGF1R			SCC25-regIGF1R		
	Apoptosis M ± SEM		p value	Apoptosis M ± SEM		p value
	Early	Late		Early	Late	
−dox	1.2 ± 0.3	3.7 ± 0.4	> 0.05	1.2 ± 0.2	1.8 ± 0.1	> 0.05
+dox	1.1 ± 0.3	3.6 ± 0.5		2.6 ± 0.3	2.9 ± 0.3	
Cisp5	2.7 ± 0.3	5.5 ± 0.4	< 0.05	3.7 ± 0.3	8.4 ± 0.8	< 0.05
Cisp5 + dox	5.8 ± 0.7	8.2 ± 0.8		9.7 ± 0.7	15.6 ± 1.1	
Cisp10	7.1 ± 0.5	11.5 ± 1.7	< 0.05	11.7 ± 0.9	21.0 ± 1.8	< 0.05
Cisp10 + dox	15.0 ± 0.9	25.1 ± 0.8		23.4 ± 1.4	27.1 ± 0.9	
Cisp 25	16.0 ± 0.9	25.5 ± 1.6	< 0.05	23.9 ± 1.3	27.2 ± 1.4	< 0.05
Cisp25 + dox	19.5 ± 1.3	27.9 ± 0.8		26.5 ± 0.6	30.6 ± 2.0	

Cal27 and SCC25 cell lines are HPV-negative, which may represent the HNSCC tumors most likely to respond to reduced IGF1R signaling. Further study should evaluate the comparative impact of IGF1R inhibition/antagonism in HPV-related vs. HPV-unrelated disease.

In conclusion, the results of this in vitro study provide the evidence that the IGF1R axis is an important mediator of cisplatin sensitivity in HNSCC through regulation of both cell cycle progression and apoptosis. It has been demonstrated that a doxycycline-inducible shRNAmir effectively suppresses IGF1R expression in Cal27 and SCC25 HNSCC cell lines, leading to enhanced cisplatin toxicity in vitro. These results encourage further exploration of IGF1R downregulation or inhibition as a potential method for chemosensitization in head and neck cancer treatment.

Compliance with Ethical Standards

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