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Hypoxia regulates FGFR3 expression via HIF-1α and miR-100 and contributes to cell survival in non-muscle invasive bladder cancer

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Background: Non-muscle invasive (NMI) bladder cancer is characterised by increased expression and activating mutations of FGFR3. We have previously investigated the role of microRNAs in bladder cancer and have shown that FGFR3 is a target of miR-100. In this study, we investigated the effects of hypoxia on miR-100 and FGFR3 expression, and the link between miR-100 and FGFR3 in hypoxia.

Methods: Bladder cancer cell lines were exposed to normoxic or hypoxic conditions and examined for the expression of FGFR3 by quantitative PCR (qPCR) and western blotting, and miR-100 by qPCR. The effect of FGFR3 and miR-100 on cell viability in two-dimensional (2-D) and three-dimensional (3-D) was examined by transfecting siRNA or mimic-100, respectively.

Results: In NMI bladder cancer cell lines, FGFR3 expression was induced by hypoxia in a transcriptional and HIF-1α-dependent manner. Increased FGFR3 was also in part dependent on miR-100 levels, which decreased in hypoxia. Knockdown of FGFR3 led to a decrease in phosphorylation of the downstream kinases mitogen-activated protein kinase (MAPK) and protein kinase B (PKB), which was more pronounced under hypoxic conditions. Furthermore, transfection of mimic-100 also decreased phosphorylation of MAPK and PKB. Finally, knocking down FGFR3 profoundly decreased 2-D and 3-D cell growth, whereas introduction of mimic-100 decreased 3-D growth of cells.

Conclusion: Hypoxia, in part via suppression of miR-100, induces FGFR3 expression in bladder cancer, both of which have an important role in maintaining cell viability under conditions of stress.

Bladder cancer is the 9th most prevalent cancer in the world (Ploeg *et al*, 2009). In the United Kingdom, there are ~10 000 new cases of bladder cancer diagnosed each year. The majority (90%) of cases of bladder cancer are derived from the urothelium and are termed urothelial cancers (UC; Luis *et al*, 2007; Office for National Statistics, 2009). There are two subtypes of UC (Wu, 2005; McConkey *et al*, 2010). Around 20% of patients present with muscle invasive (MI) cancer associated with higher mortality (McConkey *et al*, 2010). However, the majority of patients (70–80%) are diagnosed with low-grade non-muscle invasive (NMI) disease. The NMI cancer is associated with a low incidence of progression but a high recurrence rate (McConkey *et al*, 2010; Pollard *et al*, 2010). Thus, although not fatal, NMI cancer requires long-term monitoring of patients and regular surveillance, making this the most expensive cancer per patient to manage.

The two subtypes of UC have distinct underlying aetiologies. The MI cancer is associated with inactivating mutations in the *TP53*, *RB* and *PTEN* genes (Castillo-Martin *et al*, 2010; Goebell and

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Knowles, 2010). In contrast, NMI cancer is characterised by increased expression and activating mutations of *FGFR3*, *H-RAS* and phosphotidylinositol-3-kinase (*PI3K*; Castillo-Martin *et al,* 2010; Pollard *et al,* 2010). *FGFR3* in particular has an important role in the pathogenesis of bladder cancer, either by mutation in NMI cancer or by increased expression in invasive bladder cancer (Tomlinson *et al,* 2007). It is one of four members of the FGFR family of receptor tyrosine kinases that serve as cell surface receptors for the FGF ligands. Signalling by the FGFRs leads to the activation of multiple intracellular signalling pathways including the extracellular signalling-regulated kinase/mitogen-activated protein kinase (ERK/MAPK) cascade and PI3K signalling (L'Hote and Knowles, 2005). Germline gain-of-function mutations in *FGFR3* that either allow the receptor to signal in the absence of ligand or enhance signalling upon ligand stimulation are associated with achondroplasia and hypochondroplasia (L'Hote and Knowles, 2005). On the other hand, germline loss-of-function mutations have been associated with CATSHL syndrome and characterised by tall stature and loss of hearing (Toydemir *et al,* 2006). In addition to bladder cancer, *FGFR3* mutations have also been identified in myeloma (Foldynova-Trantirkova *et al,* 2012) and cervical cancer (Dai *et al,* 2001), with aberrant fusion proteins being described in glioblastoma multiforme (Singh *et al,* 2012). Small molecule inhibitors (Lamont *et al,* 2011) and blocking antibodies (Martinez-Torrecuadrada *et al,* 2005) against FGFR3 inhibit the growth of bladder cancer cell lines *in vitro* and xenografts *in vivo*.

Hypoxia is a key feature of many solid tumours including bladder cancer (Turner *et al,* 2002; Ioachim *et al,* 2006; Ord *et al,* 2007; Chai *et al,* 2008; Tickoo *et al,* 2011). The cellular effects of hypoxia are primarily mediated by hypoxia inducible factor (HIF), a heterodimer of one of two possible α-subunits (HIF-1α or HIF-2α) and a β-subunit. In normoxia, the α-subunits are hydroxylated by prolyl hydroxylases (PHDs), which targets them for ubiquitination and degradation. In hypoxia, the activity of the PHDs is inhibited, leading to the stabilisation of the α-subunit and its dimerisation with the β-subunit. The HIF transcription factor binds to HIF response elements (HREs) on DNA to drive the expression of target genes that regulate diverse biological processes including angiogenesis (VEGF), metabolism (LDHA) and invasion (LOX; Brahimi-Horn and Pouyssegur, 2009).

An emerging class of molecules whose expression is regulated by hypoxia are microRNAs (miRNAs). MicroRNAs are small, single-stranded RNA molecules that bind with imperfect complementarity to the 3′ untranslated region (UTR) of target genes and primarily suppress the translation of the cognate protein (Pillai *et al,* 2007). By expression profiling, we have previously identified a number of miRNAs differentially regulated in distinct UC subtypes (Catto *et al,* 2009). It has also been shown that miRNA expression may predict tumour grade and therefore recurrence or progression (Gottardo *et al,* 2007; Catto *et al,* 2009; Neely *et al*, 2010).

Although FGFR3 can be mutated or overexpressed in UC, the mechanisms underlying altered FGFR3 expression in NMI bladder cancer are not well understood. We have previously shown that miR-100 is downregulated in NMI bladder cancer and that this may be responsible for increased FGFR3 expression (Catto *et al,* 2009). In this study, we investigated the effect of hypoxia on regulating the expression of FGFR3 and uncovered a role for both HIF-1α and miR-100 in regulating FGFR3 levels and downstream signalling pathways.

MATERIALS AND METHODS

Cell culture. Cell lines RT4, RT112 and T24 were obtained from Cancer Research UK Cell Services (Clare Hall Laboratories, London, UK). The 97-7 mutant S249C FGFR3 cell line was obtained from Margaret Knowles, The University of Leeds, UK. Cell lines were grown in appropriate media, containing antibiotics, with supplemental material from Sigma-Aldrich (Dorset, UK). Exposure of cell cultures to hypoxia (1% or 0.1% oxygen (O_2)) was undertaken in a hypoxia incubator (MiniGalaxy A, RS Biotech, Scotland, UK) or in a hypoxic workstation (In Vivo$_2$, Ruskinn Technology, Bridgend, UK) in parallel with cells maintained in normoxic conditions (5% CO_2, 37 °C, 21% O_2).

RNA extraction. Cells were lysed with Tri Reagent (Sigma-Aldrich) and extracted using chloroform, followed by ethanol precipitation. RNA quality and quantity were confirmed using the NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Waltham, MA, USA).

Reverse transcription and qPCR for miRNAs. Hsa-miRNA expression was assessed by real-time PCR according to the TaqMan MicroRNA Assay protocol (Applied Biosystems, Warrington, UK). The cDNA was synthesised from 350 ng of total RNA using either TaqMan miRNA-specific primers and the TaqMan MicroRNA Reverse Transcription Kit or TaqMan Megaplex Primer Pool A, together with the TaqMan Reverse Transcription Kit. Quantitative real-time PCR was performed on either on the Rotor-Gene 3000 (Corbett Research, Qiagen, West Sussex, UK) or 7900HT Fast Real Time PCR System (Applied Biosystems). For each miRNA, each sample was assayed in triplicate. Fold changes in miR-100 expression between treatments and controls were determined by the 2-DDCt method (Livak and Schmittgen, 2001), normalising the results as appropriate, using some or all of the small nRNAs RNU44 and RNU48.

Reverse transcription and qPCR for mRNA. Total RNA was reverse transcribed to cDNA using random primers and the High Capacity cDNA Synthesis Kit (Applied Biosystems). The expression of FGFR3 and CAIX along with the internal control gene *ACTIN* was assayed by quantitative PCR (qPCR) using Sybr Green (Bioline Reagents Ltd, London, UK). Primer sequences are as follows: ACTIN_F: 5′-ATTGGCAATGAGCGGTTC-3′, ACTIN_R: 5′-GGA TGCCACAGGACTCCAT-3′, CAIX_F: 5′-CTTGGAAGAAATC GCTGAGG-3′, CAIX_R: 5′-TGGAAGTAGCGGCTGAAGTC-3′, FGFR3_F: 5′-GCCTCCTCGGAGTCCTTG-3′, FGFR3_R: 5′-CGA AGACCAACTGCTCGTG-3′.

SiRNA/miRNA mimic and anti-miR transfection protocol. Cells were reverse transfected with the Oligofectamine transfection reagent (Invitrogen, Paisley, UK) according the manufacturer's instructions. Briefly, complexes were made in OptiMEM (Invitrogen) with 6 μl of oligofectamine and 20 nM final concentration of siRNA, mimics or anti-miRs. Cells were plated in full media without antibiotics at a density of 3×10^5 cells per 6-cm dish to which the complexes were added. The following day, the media was changed and the cells treated as appropriate. The pooled siGENOME siRNA against FGFR3, pooled siRNA control, miRNA mimics and anti-miRs and appropriate controls were all purchased from Dharmacon (Lafayette, CO, USA). The siRNA sequence against HIF-1α and HIF-2α are as follows: siHIF-1α: 5′-UCAA GUUGCUGGUCAUCAG-3′, siHIF-2α: 5′-UAACGACCUGAAG AUUGAA-3′.

Western blot. Protein lysates were prepared in the urea lysis buffer (6.2 M urea, 5 mM DTT, 1% SDS, 10% glycerol) with added protease (Roche, Indianapolis, IN, USA) and phosphatase (Sigma-Aldrich) inhibitors. Lysates were quantified using the Bio-Rad Dc System (Bio-Rad, Hertfordshire, UK). Proteins were separated on standard SDS–polyacrylamide gels, transferred onto PVDF membranes and incubated with primary antibodies overnight at 4 °C. Antibodies against FGFR3, phospho-Thr^{202}/Tyr^{204}-MAPK, phospho-Ser^{473}-protein kinase B (PKB), total MAPK and total PKB antibodies were all purchased from Cell Signaling Technologies (Danvers, MA, USA). The antibody against ACTIN was purchased

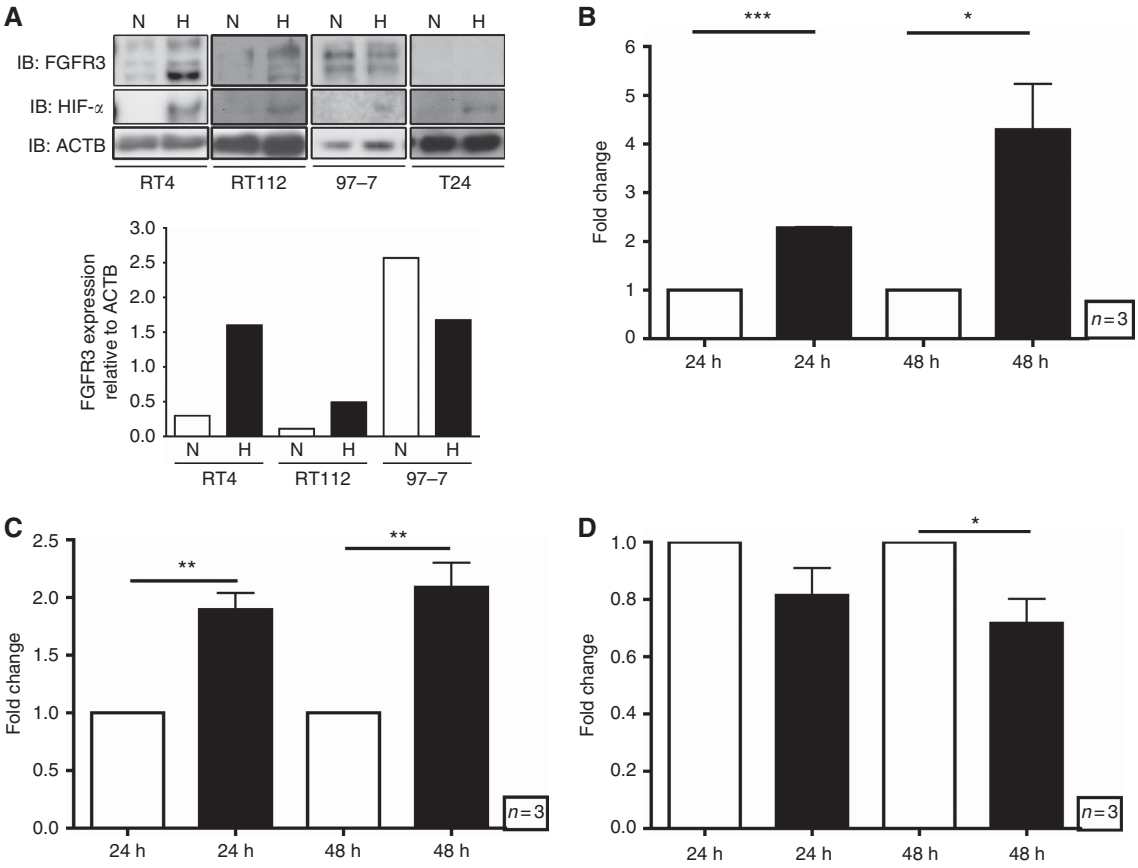

Figure 1. Regulation of FGFR3 expression by hypoxia. (**A**) Expression of FGFR3 at the protein level in the bladder cancer cell lines RT4, RT112, T24 and 97-7. Cells were cultured in normoxia (N) or 0.1% O_2 (H) for 24 h and whole cell lysates were probed for FGFR3, HIF-α (RT4 and 97-7: HIF-1α; RT112 and T24: HIF-2α) and ACTB. Quantification of representative western blots are shown. (**B–D**) Expression of FGFR3 mRNA in (**B**) RT4, (**C**) RT112 and (**D**) 97-7 cells after exposure to normoxia (white bars) or 0.1% O_2 (black bars) for the indicated time. (**A**) is representative of at least three independent experiments, (**B–D**) are mean and s.e.m. of three independent experiments. $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.

from Sigma-Aldrich. The HRP-conjugated secondary antibodies were purchased from Dako (Cambridgeshire, UK) and incubated for 1 h at room temperature. All antibodies were used at a dilution of 1 : 1000. ECL (GE Healthcare, Uppsala, Sweden) was used and the signal was visualised by exposing to film. Where required, membranes were stripped with the Pierce Stripping Buffer (Thermo Scientific, Rockford, IL, USA) before being reprobed with a new antibody.

Correlation of miR-100 with tumour hypoxia *in vivo*. We have previously generated an *in vivo* hypoxia gene expression metagene signature in breast cancer; the median expression of this signature significantly correlated with tumour hypoxia (Winter *et al*, 2007; Buffa *et al*, 2010). MicroRNAs expression profiling has also been performed on these tumour samples (Buffa *et al*, 2011). The correlation of miR-100 expression to the hypoxia score of the tumours was assessed by Spearmann correlation.

Luciferase assays. Genomic region (1.7 kb) immediately upstream of the transcription start site of the human *FGFR3* gene was cloned upstream of luciferase in the pGL3-basic backbone (Promega, Southhampton, UK). The Renilla luciferase vector pRL-TK (Promega) was used as a transfection control. Transfection of RT112 cells was performed using Fugene HD (Promega), according to the manufacturers' instructions. Cells were exposed to normoxia, 0.1% O_2 or normoxia and treated with 1 mM dimethyloxalylglycine (DMOG; Sigma-Aldrich) for 24 h. Cells were lysed and assayed for luminescence with the Dual Luciferase Reporter Assay System (Promega).

Cellular proliferation assays. For two-dimensional (2-D) growth assays, cells were reverse transfected with the indicated reagents. The following day, cells were harvested and plated at a density of 1500 cells per well of a 96-well tissue culture plate. The cells were allowed to adhere for 24 h and then placed in either normoxia or 0.1% O_2 for further 48 h. Cell viability was assessed with the sulphorhodamine assay (Houghton *et al*, 2007). For each transfection, data from five wells was taken.

For three-dimensional (3-D) growth assays, cells were reverse transfected with the indicated reagents. The following day, cells were harvested and plated at a density of 5000 cells per well of a 96-well ultra-low attachment plate (Corning, Amsterdam, The Netherlands), pelleted by centrifugation and allowed to grow as spheroids in normoxia for the indicated time. Spheroid volumes were calculated by extrapolation from their radii. For each transfection, data from 10 wells was taken.

Statistical analyses. Unless otherwise noted, all statistical analyses were performed with the unpaired Student's *t*-test.

RESULTS

Hypoxia induces FGFR3 expression in NMI bladder cancer cell lines. The expression of FGFR3 was first investigated in four bladder cell lines after exposure to hypoxia. Three cell lines, RT4, RT112 and 97-7, were derived from low-grade bladder cancer whereas the third cell line, T24, was derived from a grade-3 highly malignant bladder cancer. The specificity of the antibody was

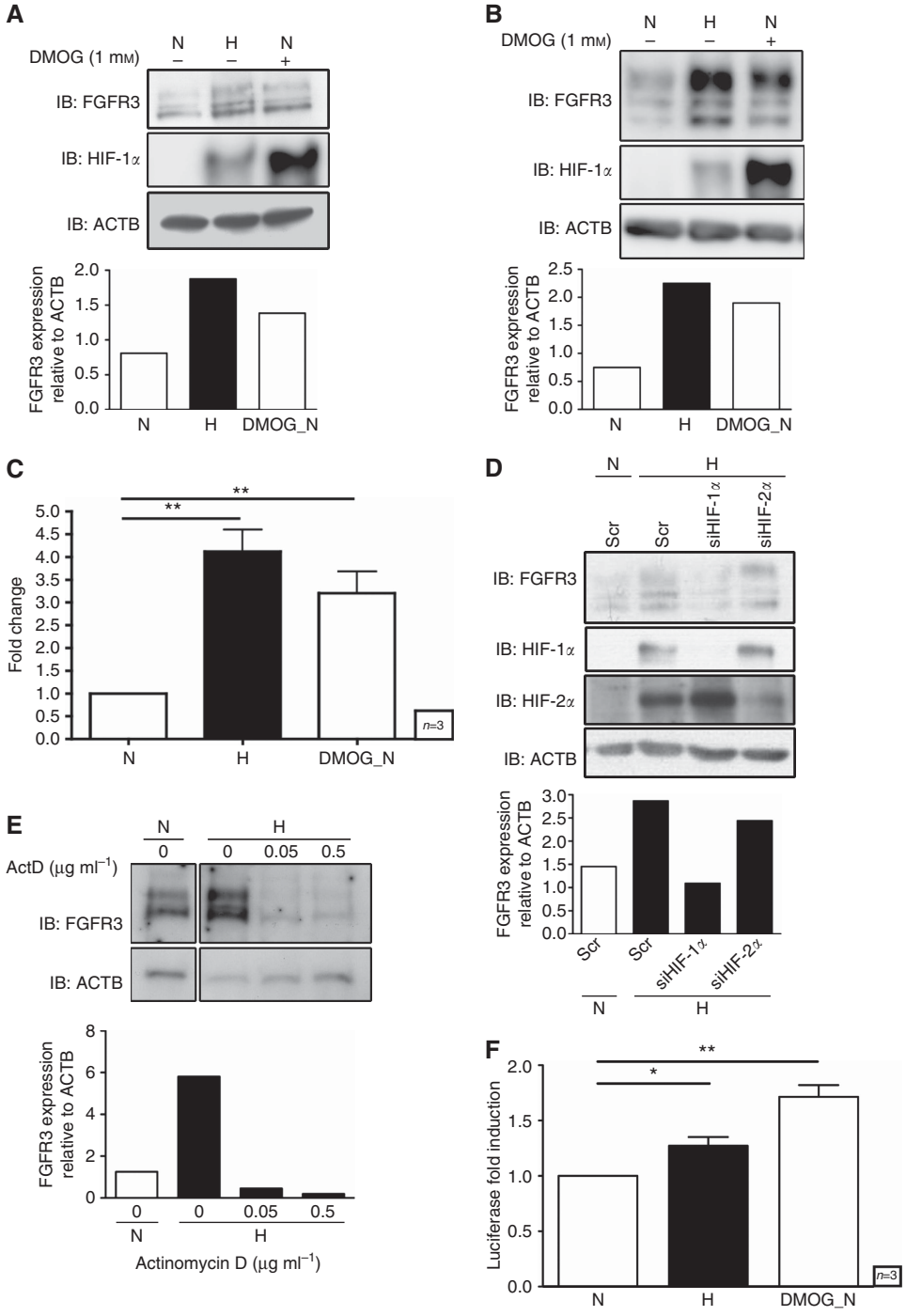

Figure 2. Mechanism of regulation of FGFR3 in hypoxia. (**A**) RT4 and (**B**) RT112 cells were cultured in normoxia (N), 0.1% O_2 (H) or in normoxia and treated with DMOG for 24 h, and whole cell lysates were probed for FGFR3, HIF-1α and ACTB. Quantification of representative western blots are shown. (**C**) Expression of FGFR3 mRNA in RT4 cells cultured in normoxia (N), 0.1% O_2 (H) or in normoxia and treated with DMOG for 24 h. (**D**) Expression of FGFR3 protein in RT4 cells cultured in normoxia (N) or 0.1% O_2 (H) for 24 h after transfection with scramble (Scr) siRNA or siRNA against HIF-1α or HIF-2α. Quantification of a representative western blot is shown. (**E**) Expression of FGFR3 protein in RT4 cells cultured in normoxia (N) or 0.1% O_2 (H) and treated with the transcription inhibitor actinomycin D (ActD). Quantification of a representative western blot is shown. (**F**) Luciferase induction from an FGFR3 promoter reporter construct transfected into RT112 cells cultured for 24 h in normoxia (N), 0.1% O_2 (H) or in normoxia and treated with 1 mM DMOG. (**A** and **D**) data are representative of two independent experiments, (**B** and **E**) data are representative of one experiment, (**C** and **F**). Data are mean and s.e.m. of the mean of three independent experiments. $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.

validated by performing western blot analyses after siRNA knockdown of FGFR3. The antibody was found to cross-react to a number of bands with molecular weights ranging from 120 to 150 kDa (Supplementary Figure 1); these are most likely to be alternative spliced products of the FGFR3 transcript (Martinez-Torrecuadrada *et al*, 2005), although post-translational modification is also possible. In both RT4 and RT112 cells, increased expression of FGFR3 protein was observed after exposure to 24 h in hypoxia (Figure 1A). In 97-7 cells, FGFR3 was expressed in both normoxic and hypoxic conditions but

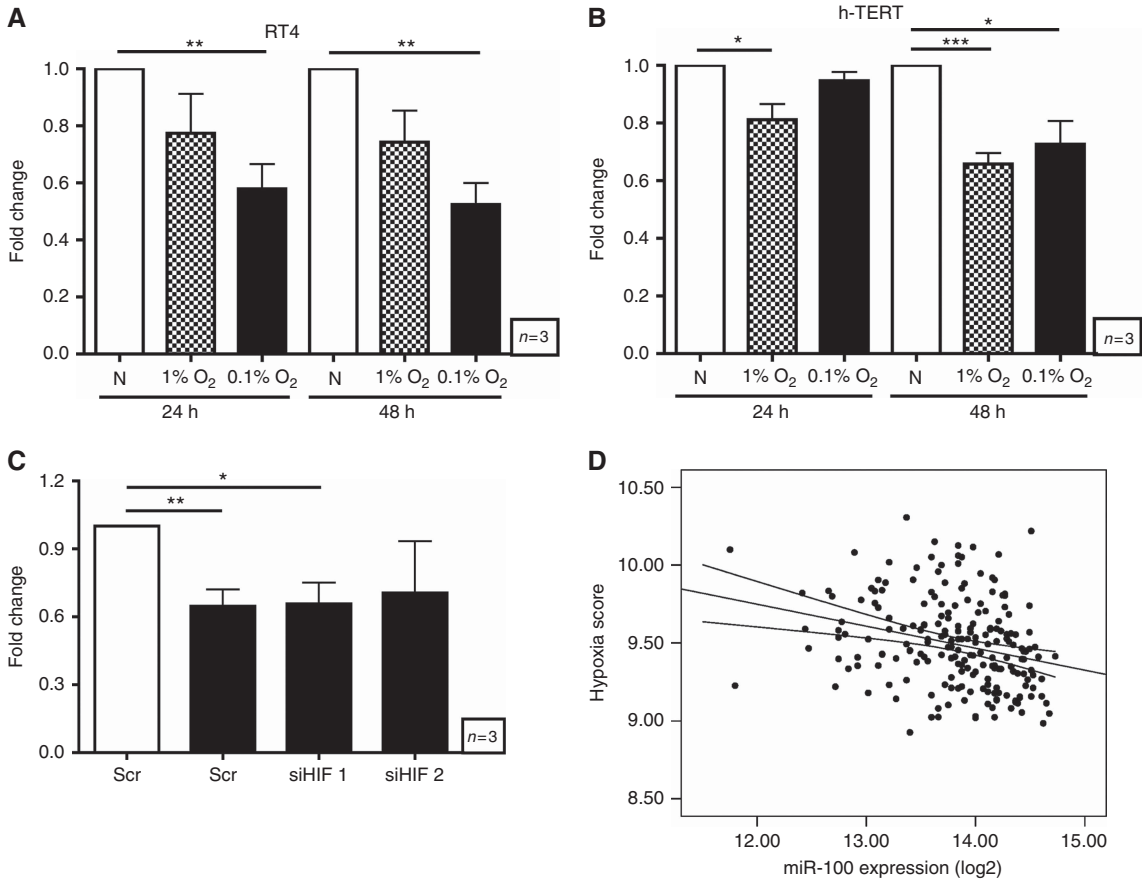

Figure 3. Relationship between miR-100 expression and hypoxia. (**A** and **B**) Expression of miR-100 in (**A**) RT4 and (**B**) h-TERT cells exposed to normoxia (white bars), 1% O_2 (hatched bars) or 0.1% O_2 (black bars) for the indicated time. (**C**) Expression of miR-100 in RT4 cells cultured in normoxia (white bar) or 0.1% O_2 (black bars) for 24 h after transfection with scramble (Scr) siRNA or siRNA against HIF-1α or HIF-2α. (**D**) Correlation of miR-100 expression with the hypoxia score of 219 primary breast cancer samples. (**A**–**C**) Data are mean and s.e.m. of three independent experiments. *$P<0.05$, **$P<0.01$, ***$P<0.001$.

further induction in hypoxia was not seen. In contrast, no expression of FGFR3 was detectable in T24 cells (Figure 1A). The induction of FGFR3 was also reflected at the mRNA level, where expression levels were induced in RT4 and RT112 but not in 97-7 cells at both 24 and 48 h (Figures 1B–D). The stabilisation of HIF by hypoxia was observed at the protein level (Figure 1A) and was also confirmed by the induction of the robust HIF-1α target gene *CAIX* (Supplementary Figures 2A–D).

To further confirm the hypoxic induction of FGFR3 expression, RT4 and RT112 cells were treated with DMOG, a PHD inhibitor, in normoxia. Inhibiting PHD activity led to an increase in HIF protein levels in normoxia in both RT4 and RT112 cells (Figures 2A and B). Concomitant with this stabilisation of HIF, increased levels of FGFR3 protein were observed in both cell lines (Figures 2A and B). In RT4 cells, this increase at the protein level was accompanied by a concomitant increase in FGFR3 mRNA levels (Figure 2C). We next sought to determine the HIF dependence of FGFR3 expression. Knocking down HIF-1α but not HIF-2α expression significantly decreased FGFR3 mRNA (Supplementary Figure 3) and protein (Figure 2D) after exposure of RT4 cells to hypoxia. Furthermore, treatment of these cells with the transcription inhibitor actinomycin D also prevented the induction of FGFR3 protein levels in hypoxia (Figure 2E).

Using MatInspector (Cartharius *et al,* 2005) to search for putative HREs within the promoter region of FGFR3, two canonical RCGTG motifs were identified. However, these were not enriched when chromatin immunoprecipitations were performed with HIF-1α (data not shown). Next, 1.7 kb of the *FGFR3* gene upstream region was cloned into a luciferase plasmid to look at promoter activity. We were unsuccessful in transfecting RT4 cells with plasmid DNA. In RT112 cells, both exposure to hypoxia and treatment with 1 mM DMOG led to significant luciferase induction (Figure 2F), suggesting that this genomic region conferred hypoxia-responsive transcriptional activity.

miR-100 expression is suppressed by hypoxia. We have previously shown that FGFR3 is regulated by miR-100 expression in bladder cancer (Catto *et al,* 2009). To determine whether miR-100 may have a role in the hypoxic regulation of FGFR3 levels, miR-100 expression in RT4 cells after exposure to hypoxia was examined. Exposure to hypoxia for 24 and 48 h led to a significant decrease in miR-100 levels in RT4 (Figure 3A); miR-100 levels were unaffected by hypoxia in RT112, 97-7 and T24 cells (Supplementary Figure 4). To better understand the effect of hypoxia on miR-100 expression, its expression was also examined in the immortalised, but otherwise relatively normal, bladder urothelial line h-TERT; miR-100 levels were also suppressed by hypoxia in these cells (Figure 3B). To confirm that suppression of miR-100 was a specific outcome of hypoxia and not a general consequence of repression of the miRNA machinery, the expression of the robust hypoxia-induced miRNA, miR-210, was investigated. miR-210 was induced by hypoxia in all cell lines investigated (Blick *et.al*, manuscript in preparation).

The hypoxic suppression of miR-100 could not be reversed by knocking down either HIF-1α or HIF-2α (Figure 3C). We have previously generated an *in vivo* hypoxia metagene signature that is

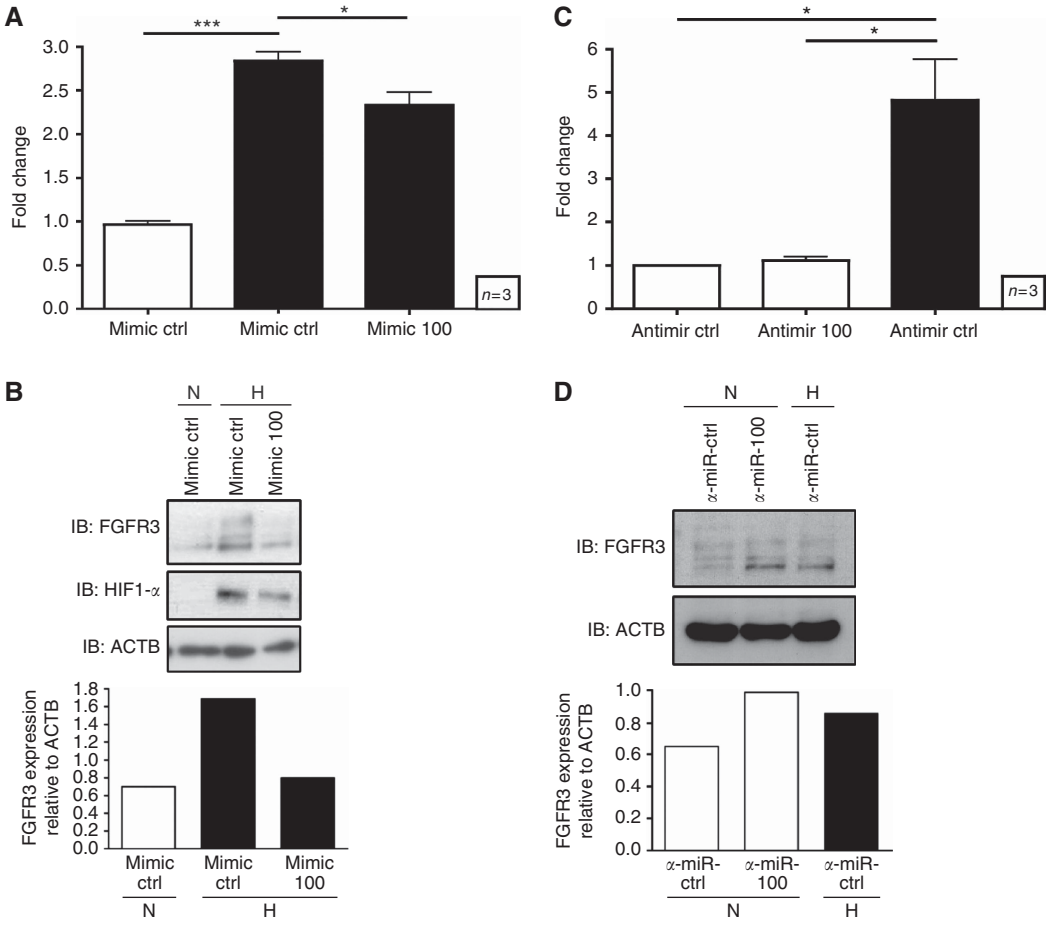

Figure 4. Regulation of FGFR3 by miR-100. (**A**) FGFR3 mRNA expression was examined in RT4 cells transfected with a miRNA control mimic or mimic-100 and exposed to normoxia (white bar) or 0.1% O_2 (black bars) for 24 h. (**B**) FGFR3 protein expression was examined in RT4 cells transfected with a miRNA control mimic or mimic-100, and exposed to normoxia (N) or 0.1% O_2 (H) for 24 h. Quantification of a representative western blot is shown. (**C**) FGFR3 mRNA expression was examined in RT4 cells transfected with a control miRNA inhibitor (anti-miR-ctrl) or miR-100 inhibitor (anti-miR-100), and exposed to normoxia (white bars) or 0.1% O_2 (black bar) for 24 h. (**D**) FGFR3 protein expression was examined in RT4 cells transfected with a control miRNA inhibitor (anti-miR-ctrl) or miR-100 inhibitor (anti-miR-100), and exposed to normoxia or 0.1% O_2 (H) for 24 h. Quantification of a representative western blot is shown. (**A** and **C**) Data are mean and s.e.m. of three independent experiments, (**B** and **D**) data are representative of at least two independent experiments. $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.

a robust indicator of tumour hypoxia (Winter *et al*, 2007; Buffa *et al*, 2010). To examine the relationship between miR-100 and hypoxia *in vivo*, we used our breast cancer tumour samples for which both miRNA and mRNA expression profile data is available (Buffa *et al*, 2011). In 219 breast cancer tumour samples, a significant inverse correlation between miR-100 expression and tumour hypoxia score was observed (Figure 3D).

It has previously been shown that the MYC oncogene suppresses transcription from the miR-100 locus (Cairo *et al*, 2010). As FGFR3 signalling can activate MYC (Zingone *et al*, 2010), we investigated whether FGFR3 signalling in hypoxia was responsible for reducing miR-100 levels. However, knocking down FGFR3 levels in RT4 did not affect miR-100 levels (data not shown).

miR-100 contributes to regulating FGFR3 levels in RT4 cells. The biological relevance of miR-100 on regulating FGFR3 expression in bladder cancer was investigated by perturbing miR-100 levels in RT4 cells by transfecting either mimic-100 in hypoxia (when miR-100 levels are low) or anti-miR-100 in normoxia (when miR-100 levels are high). The transfection of mimic-100 into RT4 cells modestly inhibited the hypoxic induction of FGFR3 mRNA (Figure 4A) and strongly inhibited FGFR3 expression at the protein level (Figure 4B). Importantly, although transfection of RT4 cells with anti-miR-100 in normoxia did not increase FGFR3 mRNA levels (Figure 4C), FGFR3 protein levels were increased after transfection of anti-miR-100 (Figure 4D). Thus, levels of miR-100 have a role in regulating FGFR3 protein levels in RT4 cells.

FGFR3 and miR-100 regulate cellular proliferation of RT4 cells. Activation of FGFR3 initiates signalling cascades in the cell primarily mediated by the MAPK and PI3K cascades. Therefore, the effects of FGFR3 and miR-100 on the activation of these downstream pathways was investigated. Activation of MAPK signalling ultimately leads to phosphorylation of the terminal kinase in the cascade, MAPK (p42/44 MAPK) whereas PI3K activation leads to the phosphorylation of PKB (Marshall *et al*, 2011). Transfection with siRNA against FGFR3 efficiently decreased FGFR3 protein levels (Supplementary Figure 1). FGFR3 knockdown reduced phosphorylation of Ser^{473} on PKB (Figure 5A) and the dual phosphorylation of Thr^{202}/Tyr^{204} on MAPK (Figure 5B). This effect was more prominent under hypoxic conditions, where knockdown of FGFR3 led to an almost complete loss of PKB and ERK phosphorylation. In hypoxia, transfection of mimic-100 led to a modest decrease in phosphorylation of both PKB and MAPK (Figures 5A and B). Transfection of anti-miR-100 in normoxia did not appreciably alter phosphorylation of MAPK

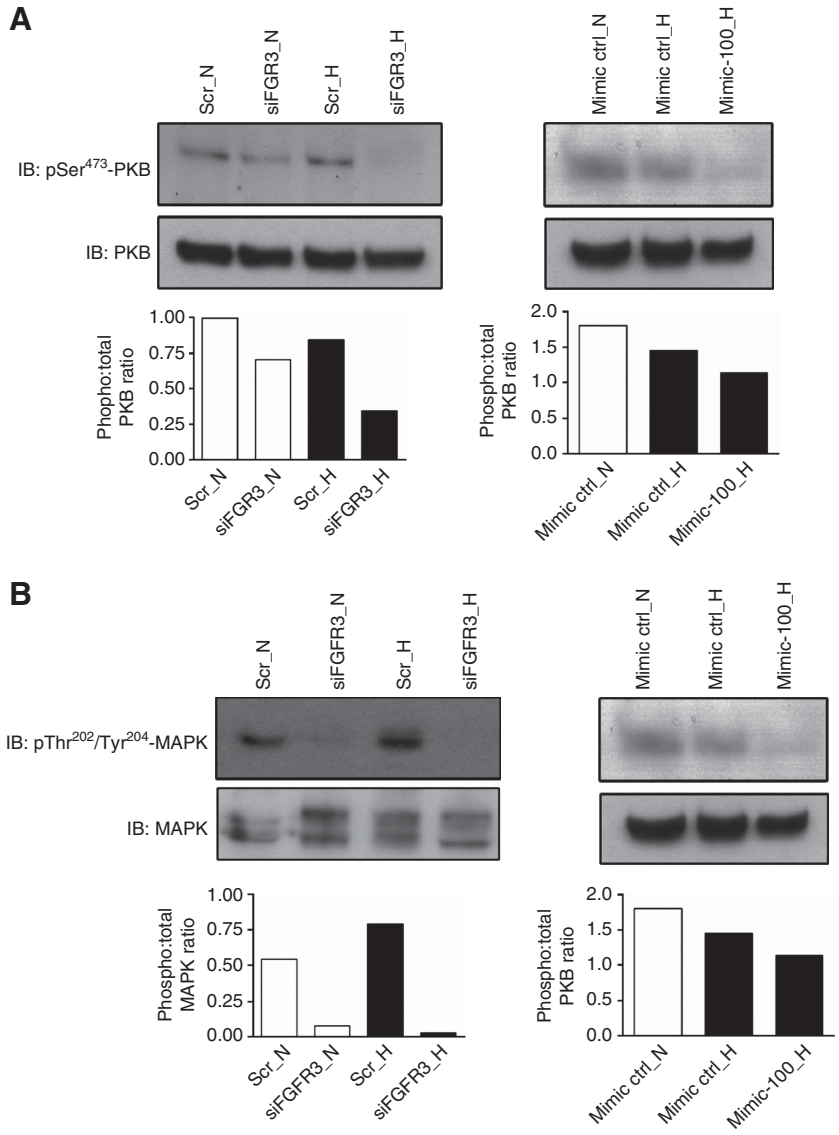

Figure 5. Regulation of PKB and MAPK activation by FGFR3 and miR-100. Whole cell lysates of RT4 cells transfected with scramble (Scr) or siFGFR3 siRNA (left panels) or miRNA control mimic or mimic-100 (right panels), and cultured in normoxia (N) or 0.1% O_2 (H) for 24 h were probed for (**A**) phospho-Ser^{473}-PKB and total PKB, and (**B**) phospho-Thr^{202}/Tyr^{204}-MAPK and total MAPK. Quantification of representative western blots are shown. (**A** and **B**) Data are representative of at least two independent experiments.

(Supplementary Figure 5). Thus, increased levels of FGFR3 have an important role in maintaining MAPK and PKB signalling in hypoxia.

It is well established that MAPK and PKB activation are potent mitogenic signals. Thus, the effects of miR-100 and FGFR3 on cell viability was investigated. In 2-D growth, knockdown of FGFR3 reduced proliferation of RT4 cells in normoxia and hypoxia (Figure 6A). In contrast, transfection of mimic-100 did not affect the 2-D growth (Figure 6B). To assess the effects of FGFR3 and miR-100 on 3-D growth, cells were grown as spheroids. FGFR3 knockdown significantly reduced the 3-D growth of RT4 cells (Figures 6C and E). In addition, transfection of mimic-100 also reduced spheroid volume of these cells (Figures 6D and F).

DISCUSSION

Hypoxia is an important aspect of tumour biology. It is known that in addition to its effects on metabolic reprogramming, hypoxia can lead to the activation of RTK signalling. HIF-1α directly targets the C-MET receptor for transcriptional induction (Pennacchietti *et al,* 2003). Furthermore, HIF-2α has been implicated in increased expression of the epidermal growth factor receptor by enhancing translation (Franovic *et al,* 2007; Wang and Schneider, 2010). In this study, we show for the first time that FGFR3 is induced by hypoxia in bladder cancer cell lines. Thus, tumour hypoxia may represent an additional mechanism for increased levels of FGFR3 in bladder cancer, and could be relevant in other tumours that have been shown to express FGFR3 including head, neck (Marshall *et al,* 2011) and liver cancer (Gauglhofer *et al,* 2011).

The upregulation of FGFR3 is dependent on transcription as mRNA levels were increased after exposure to both hypoxia and the hypoxia-mimetic DMOG, and treatment with the transcription inhibitor actinomycin D prevented the accumulation of FGFR3 protein after exposure to low oxygen. The increased expression of FGFR3 was primarily dependent on HIF-1α, as knockdown of this isoform of the α-subunit had a stronger effect on suppressing FGFR3 levels in hypoxia. It should be noted that in these experiments, the knockdown of HIF-2α was incomplete; thus,

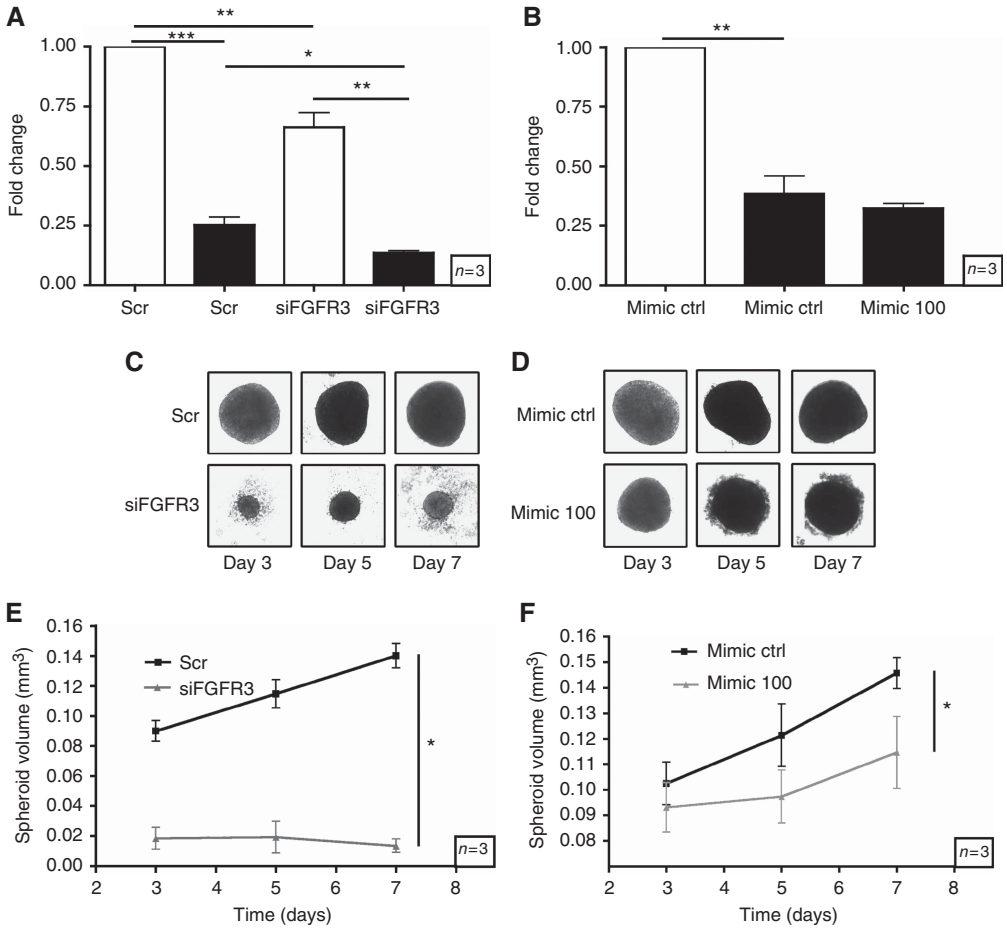

Figure 6. Regulation of RT4 proliferation by FGFR3 and miR-100. (**A** and **B**) Cell viability of RT4 cells in normoxia (white bars) or 0.1% O_2 (black bars) after transfection with (**A**) scramble (Scr) siRNA or siRNA against FGFR3 or (**B**) miRNA control mimic or mimic-100. (**C** and **D**) RT4 cells grown as spheroids over a time course after transfection with (**C**) scramble (Scr) siRNA or siRNA against FGFR3 or (**D**) miRNA control mimic or mimic-100. (**E** and **F**) Quantification of (**C**) and (**D**), respectively. (**A**–**F**) Data are mean and s.e.m. of three independent experiments. *$P<0.05$, **$P<0.01$, ***$P<0.001$.

although FGFR3 levels were unaffected after knockdown, HIF-2α may still have a role in regulating its expression in hypoxia.

Although the FGFR3 promoter region did confer hypoxic transcriptional activity in a luciferase reporter assay, we have been unable to immunoprecipitate any HREs in this genomic region. Thus, either HREs exist in the region that we have not investigated or the hypoxic induction of FGFR3 is dependent on other transcription factors that act in syngergy/downstream of HIF. The coordinated regulation of FGFR3 expression by additional pathways warrants further investigation, as p73 and p63, members of the p53 family of transcription factors, have been shown to induce FGFR3 transcription (Sayan *et al,* 2010).

To further understand the potential mechanisms by which FGFR3 levels may be increased under hypoxia, the role of miR-100 was explored. We have previously shown that miR-100 can target FGFR3 and that the expression of these two molecules at the mRNA levels were negatively correlated in the bladder cancer (Catto *et al,* 2009). In both RT4 and h-TERT cells, hypoxia led to a suppression of miR-100 levels. We do not have a data set of matched miRNA and gene expression profiles available for bladder cancer. Thus, we interrogated our breast cancer cohort for which we have both miRNA (Buffa *et al,* 2011) and mRNA expresssion data, and in which we have previously employed a hypoxia scoring system to stratify tumours (Winter *et al,* 2007; Buffa *et al,* 2010). Importantly, *in vivo*, the expression of miR-100 was anti-correlated to tumour hypoxia in breast cancer, suggesting that the ability of hypoxia to decrease miR-100 levels extends to other tumour types.

The mechanism of downregulation of hypoxia-regulated genes, including miRNAs, is unclear. It is thought that the majority of genes downregulated by hypoxia are not direct targets of HIF (Mole *et al,* 2009). Indeed, the hypoxic suppression of miR-100 could not be reserved by knockdown of either HIF-1α or HIF-2α. Although the majority of cellular responses to hypoxia are mediated by HIF, a number of HIF-independent events in hypoxia have been reported, including the induction of haeme oxygenase-1 in Chinese hamster ovary cells (Wiesener *et al,* 1998; Wood *et al,* 1998) and ICAM-1 in monocytes (Winning *et al*, 2010). It is likely that one of the other stress–response pathways is responsible for regulating the expression of a subset of genes under hypoxia (Koumenis and Wouters, 2006).

In RT4 cells, miR-100 expression contributes to the regulation of FGFR3 levels, as transfection of anti-miR-100 increased FGFR3 levels in normoxia and introduction of mimic-100 decreased FGFR3 levels in hypoxia. As the suppression of miR-100 was not dependent on the HIF-α subunits, yet hypoxic induction of FGFR3 could be reversed by HIF-1α knockdown, we conclude that two parallel mechanisms operate to maintain increased FGFR3 levels in hypoxia. Of the two, the HIF-1α-dependent pathway is likely to have the central role, with the suppression of miR-100 serving to augment increased FGFR3 levels. The ability to circumvent miRNA-mediated regulation of gene expression can be advantageous to cells. Indeed, it has recently been shown that in glioblastoma, a duplication of chromosome 4p16.3 generates a FGFR3-TAAC3 fusion protein in which the 3′ UTR of FGFR3 is

lost. The fusion protein is insensitive to regulation by the miR-99, a member of the miR-100 family, resulting in increased expression of the aberrant protein and enhanced tumour growth in xenografts models (Parker *et al,* 2013).

Functionally, FGFR3 and miR-100 are responsible for regulating cell proliferation of RT4 cells. Of note is the observation that knocking down FGFR3 expression in RT4 cells led to a dramatic decrease in cell viability in normoxia and hypoxia, concomitant with a decrease in the activation of PKB and MAPK signalling. Thus, even in cells expressing the FGFR3 without a point mutation, autocrine and/or paracrine signalling clearly have an indispensible role in cell viability. We found knockdown of FGFR3 had a more dramatic effect on reducing the phosphorylation of MAPK and PKB in cells exposed to hypoxia compared with their normoxic counterparts. This strongly suggests that increased levels of FGFR3 has a crucial role in maintaining the viability of RT4 cells exposed to hypoxia.

The role of miR-100 was found to be more subtle, with a decrease in cell viability observed in 3-D cultures but not 2-D growth. Thus, the role of miR-100 is more pronounced in stressful cellular conditions, when nutrient supply is perhaps more limiting and oxygen gradients are steeper. A single miRNA is likely to regulate the expression of multiple target genes, although the effect on each may be subtle (Selbach *et al,* 2008). Indeed, it has recently been reported that miR-100 can target mTOR (Nagaraja *et al,* 2010). Under more severe conditions of 3-D growth, miR-100 is likely to have a role in regulating cell proliferation by targeting additional pathways, such as mTOR, which has a key role during stress (Tickoo *et al,* 2011).

In summary, we have shown for the first time that hypoxic regulation of FGFR3 occurs at the transcriptional level but is also partly dependent on suppression of miR-100 expression. This represents a novel mechanism for regulating FGFR3 in bladder cancer that may provide an additional mechanism for increased expression of this growth-promoting pathway that could contribute to the pathogenesis of this disease. The link with hypoxia may explain the strong association with the papillary phenotype, where it has been shown that the luminal cells away from the vascular core are hypoxic. This may provide selection for upregulation of FGFR3 and maintenance of the papillary phenotype. FGFR3 overexpression may provide a positive effect by preventing progression to MI disease; the loss of this potentially protective mechanism requires additional studies. However, the clear effect of FGFR3 on signalling and growth under hypoxia also suggests a potential synthetic lethal approach, targeting the hypoxic cells away from the vascular core.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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