Stable Knockdown of *hCGβ* mRNA Expression in Bladder Cancer Cells Results in Significant Growth Inhibition

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Abstract. *Background: Expression of human chorionic gonadotropin beta subunit (hCGβ) by epithelial carcinomas is associated with a poor prognosis and has a proposed autocrine growth effect on cancer cells by inhibition of apoptosis. Material and Methods: We transduced the hCGβ-expressing bladder cancer cell line SCaBER with short hairpin (sh) RNA lentiviral gene-specific (CGB) constructs and determined its impact on the synthesis of hCGβ and the resultant effect on cancer cell growth. Results: Stable CGB gene-silenced clones exhibited a 60%-80% reduction in the level of hCGβ expressed and a reduced growth rate of more than 40% compared to wild-type SCaBER cells. Conclusions: shRNA Lentiviral particles achieve stable knockdown of hCGβ translation in the bladder cancer cell line SCaBER. This transforms the phenotype by reducing hCGβ expression and cell growth rate. This is consistent with the proposed autocrine/paracrine function of ectopic hCGβ expression during oncogenesis.*

The expression of human chorionic gonadotropin beta subunit (hCGβ) by epithelial carcinomas is associated with a poor prognosis for patients with bladder cancer (1). It is estimated that approximately 32% of all epithelial carcinomas secrete hCGβ (2) and that the autocrine effect of hCGβ on cancer cells is associated with the inhibition of apoptosis and tumour cell growth (3). The molecular evidence supporting a role for hCGβ in cancer was reinforced following the publication of phase I and phase II clinical studies which were carried out to determine the effect of vaccinating patients with common carcinomas against hCGβ (4, 5). Increased survival has been reported, along with a specific tumour suppressive function, within the antiserum of vaccinated patients (5). The function of ectopic hCGβ in bladder cancer has been examined *in vitro* by adding exogenous recombinant hCGβ protein to cell lines and also by reducing the availability of hCGβ by immunodepletion with anti-hCGβ vaccine or vaccine-derived antiserum (5-7). These studies indicate that the availability of hCGβ to the cells of the bladder squamous cell carcinoma (SCaBER) was essential for tumour survival. To assess the impact on inhibition of endogenous hCGβ, we transduced bladder cancer cells with short hairpin (sh) RNA lentiviral gene-specific (CGB) constructs and determined the impact on the synthesis of hCGβ and the resultant effect on cancer cell population growth.

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Materials and Methods

Lentiviral-based shRNAs that generate small-interfering RNAs (siRNAs) were used to transduce hCGβ-secreting cancer cells. Two different shRNA gene-specific constructs targeting different exonic regions of *CGB* gene were used in the study. Stable gene silencing was established by puromycin resistance clone selection. Reduction in the level of *CGB* mRNA was measured by real-time polymerase chain reaction (PCR) and protein detection by enzyme-linked immunosorbent assay (ELISA). The effect on cell population growth was then estimated by the MTS (tetrazolium salt reduction) assay.

shRNA lentiviral transduction. MISSION® shRNA Lentiviral Transduction Particles (Sigma Aldrich, Pool, UK) were used to knockdown the *CGB* in the hCGβ-expressing bladder cancer cell line SCaBER, derived from a squamous cell carcinoma of the human urinary bladder (ATCC, Rockville, Maryland, USA) which has been studied previously (3, 5-7). TRC1-pLKO.1-puro vector containing a hairpin insert with gene-specific sequence was used for cancer cell transduction according to the manufacturers'

Table I. *A short hairpin RNA (shRNA) which targets all human chorionic gonadotropin (CGB) genes designed against a CGB5 was constructed within the lentivirus vector pLKO.1-Puro. The inserted hairpin sequence contains gene-specific target sequence and loop region which correspond to sequence CTCGAG.*

shRNA lentiviral particles and clone number	Sequence targeting all *CGB* genes inserted into pLKO.1-puro vector	Target gene and exon position of sense and antisense sequences
TRCN0000082824 clone 1	GTGGTGTGCAACTACCGCGATCTCGAGAT CGCGGTAGTTGCACACCAC	*CGB5* mRNA NM_033043 exon 3 Sense strand: nt 588-608 Antisense strand: nt 588-608
TRCN0000082826 clone 2	CCGTGTGCATCACCGTCAACACTCGAGTG TTGACGGTGATGCACACGG	*CGB5* mRNA NM_033043 exon 2 Sense strand: nt 496-515 Antisense strand: nt 517-503

protocol in addition to hexadimethrine bromide (8 μg/ml), to enhance transduction efficiency. Sequences of inserts in shRNA constructs targeting the *CGB* gene (exon 2 or 3) (Acc. No. NM033043) are shown in Table I. Stable gene knockdown was established by cellular resistance to puromycin (500 ng/ml). Clones were isolated and several sub-clone cell lines were established. The Non-Target shRNA Control Vector (Sigma Aldrich, Pool, UK) containing an insert sequence that does not target any human gene but can activate RNA-induced silencing complex (RISC) and the RNAi pathway served as a negative control. In addition, MISSION® Control Vector pLKO.1-puro (no shRNA insert) (Sigma Aldrich, Pool, UK) along with untreated wild-type cells were used.

RNA extraction and cDNA synthesis. Quantitative real-time PCR. Total RNA was extracted from the selected cell clones using SV Total RNA Isolation System (Promega, Southhampton, UK). One microgramme of total mRNA was used for first-strand synthesis cDNA by Verso cDNA Kit (Thermo Scientific, Leicestershire, UK). CGB transcript quantity was determined by real-time PCR (Quantica, Techne, Stone, UK); using specific primers designed for amplification of any *CGB* genes coding hCGβ protein (NM_033043): forward primer 5'-CATGGGTGTGAACC ATGAGAAG-3' and reverse primer 5'-GTGCTAAGCAGTTGGT GGTGC-3' and the level of the housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*; NM_002046) forward primer 5'-CATGGGTGTGAACCATGAGAAG-3' and reverse primer 5'-GTGCTAAGCAGTTGGTGGTGC-3'. Real-time PCR was carried out according to the protocol for ABsolute™ Blue QPCR SYBR® Green Mix kit (Thermo Scientific). NTC (Non Template Control) contained complete qPCR master mix but no cDNA template. As a negative control we used non human cDNA from shrimp. All experiments were performed in triplicates and mean crossing point (Cp) values were calculated.

Relative quantification of the level of CGB mRNA transcripts. Relative quantification of *CGB* gene expression was calculated using the $\Delta\Delta C_p$ method described by Livak and Schmittgen (8). The level of transcription of *CGB* genes was normalised against the level for the housekeeping gene *GAPDH* and calculated relatively to the level of the gene expression in the cells transduced with Non-Target shRNA Control Vector (calibrator) using average crossing point values. Final results are expressed as percentage differences in CGB expression relative to calibrator gene expression set at 100%.

hCGβ-specific ELISA. Synthesis of hCGβ and release into culture media was determined by specific free hCGβ ELISA utilising monoclonal antibody FBT11 directed against epitope β6/7 of hCGβ and capture antibody – rabbit anti-hCGβ conjugated with horseradish peroxidise (4001-POD), which is a core antibody recognising β1 epitope. The assay has been validated and described previously (9-13). Standards used here were recombinant hCGβ (Sigma) these were calibrated against 1st International Reference Preparation (IRP) for hCGβ – (NIBSC, Potters Bar, UK) in a concentration range from 0.5 ng/ml to 50 ng/ml. The media from confluent cell cultures grown on 75 cm^2 flasks were collected and assayed to estimate the amount of secreted protein normalised to 1×10^6 cells.

Cell proliferation (MTS) assay. For viability assays 100 μl of complete growth medium in the cell culture wells was replaced with 20 μl of CellTiter 96® AQueous® One Solution Cell Proliferation Assay reagent (Promega). The plate was incubated at 37˚C in humidified atmosphere with 95% air, 5% CO_2 for 1-4 hours until colour was well developed and the absorbance was then measured at 490 nm on a Fluostar OPTIMA (BMG Labtech, Aylesbury, UK). Data were normalised against the optical density achieved for the control (set at 100%) and expressed as a percentage change in cell number.

Results

Effect of stable CGB gene silencing on protein expression and cell growth. Real-time PCR analysis of clones showed about 60%-80% (clone 1 and clone 2 respectively) reduction in the level of *hCGβ* mRNA transcripts when compared to SCaBER cells transduced with the Non-Target shRNA Control Vector (Figures 1-2). Similarly, the hCGβ protein concentration in culture media for clone 1 and clone 2 was reduced to 20% and 9% respectively. hCGβ concentration in exhausted culture media was reduced to 0.4 and 0.9 ng/ml compared to 4.4 ng/ml in medium from wild-type SCaBER cells over the same period and under the same conditions. After 72 hours in culture, populations of sub-clone cells, with stable knockdown of *CGB* expression, were reduced when compared with wild-type SCaBER cells as measured by the MTS assay (Figure 2). There was less than 5%

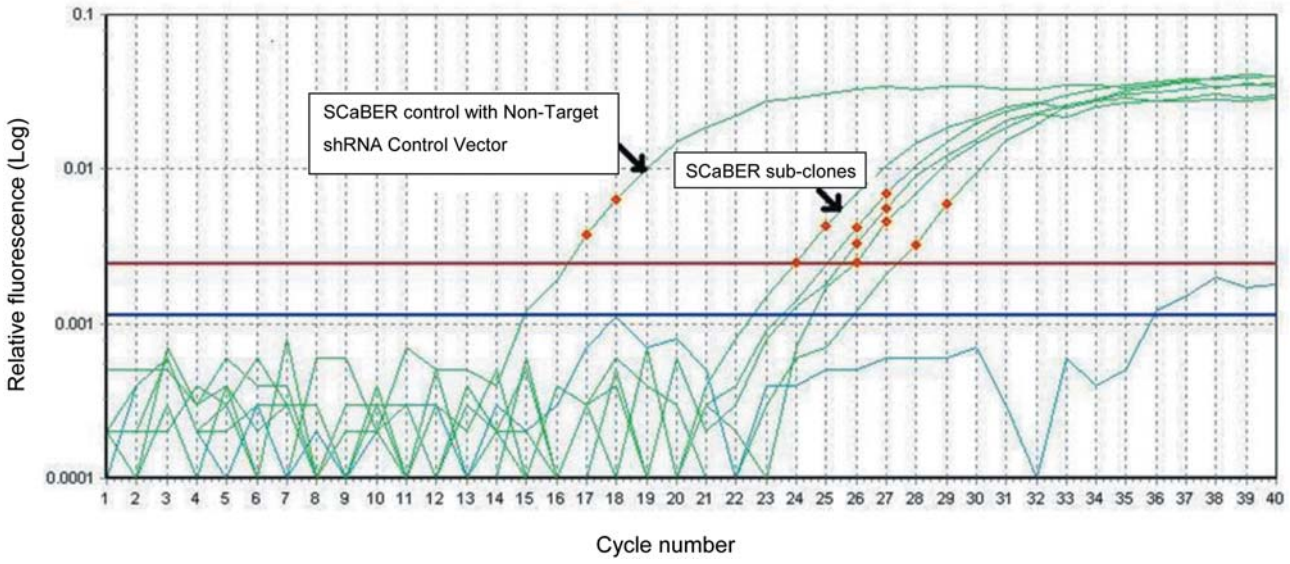

Figure 1. *Real-time PCR amplification curves to establish crossing points (Cp). Amplification of control clone SCaBER (CGB) gene expression following transfection with Non-Target shRNA Control Vector in comparison to the SCaBER clones stably transfected with shRNA targeting CGB gene. The control clone corresponds to Cp of 16.44, The lowest Cp (23.95) of the CGB-targeted clones corresponds to SCaBER clone 1 and the highest Cp (27.71) corresponds to SCaBER clone 2.*

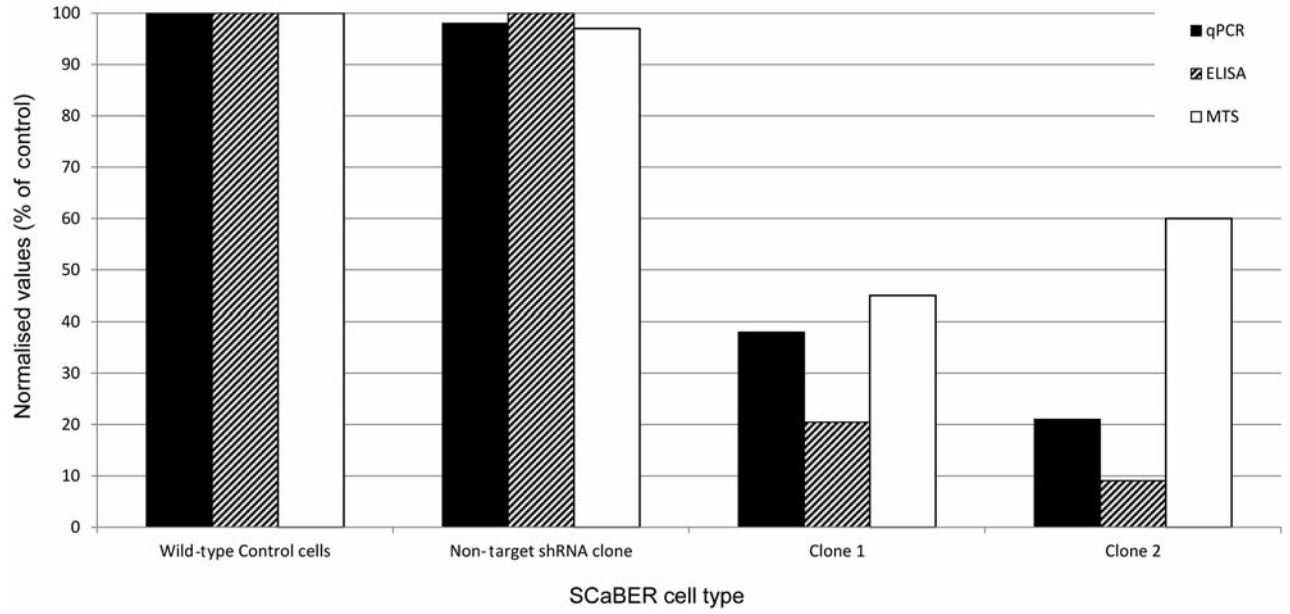

Figure 2. *Effect of human chorionic gonadotropin (CGB) knockdown by gene-specific short hairpin RNA (shRNA) lentiviral particles. Relative level of (CGB) mRNA measured by real-time PCR (qPCR) after silencing with (shRNA) target construct, amount of hCGβ in the culture medium estimated by ELISA and number of viable cells measured by MTS. Data were normalised to the level set at 100% measured for non-target shRNA transduced cells. A decreased mRNA level of (CGB) transcript to 38% in clone 1 and 21% in clone 2 was detected by real-time PCR. Level of hCGβ was reduced for clone 1 (to 20.45% of control) and clone 2 (to 9.09% of control). Cancer cell number was reduced in clone 1 by 55% and in clone 2 by 40%.*

difference in the levels of *w* transcripts, protein secretion and growth of cells between control (non-infected cells) and cells exposed to control vector non-target shRNA (see Figure 2).

Discussion

We have shown that epithelial carcinomas producing hCGβ are resistant to radiotherapy decline rapidly and metastasize

to a greater extent than those where no hCGβ is detected (1). Our studies and others have suggested that hCGβ has a direct autocrine function on the cells from which it is expressed (3, 14) and by reducing hCGβ in cell culture media (7) or the circulation (5), cell populations are significantly negatively impacted. In this study, we targeted the molecular mechanism controlling the autocrine loop at the gene expression level and used shRNA to stably knockdown and effectively silence hCGβ expression in the SCaBER cell line, thereby potentially inducing oncostasis. While the silencing efficiency was an improvement to earlier methods (14), complete silencing was not achievable and some hCGβ could still be detected in the media. In this study we achieved up to 80% reduction in the *hCGβ* mRNA transcripts expression and a similar level of reduction in hCGβ protein secreted into the medium compared with wild-type SCaBER cells and cells transduced with non-target shRNA. Furthermore, the populations of sub-clone with stable knockdown of *CGB* expression were significantly reduced (by 40% to 55%) in cell number when compared to control SCaBER cells. The results indicate that stable knockdown of hCGβ translation in the bladder cancer cell line SCaBER is possible when using shRNA lentiviral particles. The lentivirus-based transduction method is well-described and these *CGB*-knockdown cells can now be used for extensive experimentation into the role of hCGβ in bladder cancer malignancy. Once a reduction of hCGβ was demonstrated by real-time PCR and ELISA, the cell population studies showed a significant reduction (at least 40%) of cancer cell numbers following hCGβ knockdown. These data confirm our earlier studies which used immunodepletion of hCGβ and support the hypothesis that ectopic hCGβ expression plays a role in the growth of some epithelial carcinomas.

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