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THE ROLE OF FREE BETA SUBUNIT OF HUMAN CHORIONIC GONADOTROPIN IN HIGH-GRADE SEROUS CANCER

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Declaration

The work described in this thesis was performed by the candidate, except where due acknowledgement has been made.

This work was undertaken in the Functional Genomics Group at the Kolling Institute of Medical Research, Royal North Shore Hospital. I declare that no part of this work has been submitted previously for the purpose of obtaining a degree or diploma in any other university.

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Dedication

This thesis is dedicated to my family and loving husband.

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Abstract

Introduction: Ovarian cancer is one of the most lethal gynaecological cancers in the developed world. Development of chemoresistance to platinum-based drugs is a key factor for the high morbidity seen in this malignancy. Epithelial ovarian cancer (EOC) is the most common form of ovarian cancer, with high-grade serous cancer (HGSC) accounting for up to 75% of EOC. The β-subunit of human chorionic gonadotropin (hCGβ) is elevated in a number of epithelial cancers, including ovarian cancer, and is often associated with aggressive and metastatic disease with poor clinical outcomes. Studies have shown evidence of the biological activity of hCGβ in epithelial cancer including proliferation, apoptosis, and migration. The level of hCGβ has also been shown to be associated in chemoresistance in small-cell lung cancer patients.

Aims and Methods: The aim of this thesis was to investigate the role of hCGβ in modulating oncogenic functions and drug resistance in HGSC cell line models. The expression levels of the genes encoding hCGβ, *CGB*, and secreted hCGβ were determined by qRT-PCR and ELISA, respectively, in a panel of eight HGSC cell lines: A2780, A2780cis, CaOV-3, HEY, OVCAR-3, OV202, PEO-1 and SKOV-3. hCGβ was downregulated with two siRNAs or overexpressed using an expression plasmid vector in order to determine its role in cell proliferation, migration, adhesion and response to platinum-based drugs. The quantitative proteomic technique, isobaric tags for relative and absolute quantitation (iTRAQ), was employed to determine the mechanism by which hCGβ might be involved in the response of HGSC cells to cisplatin. A selected number of proteins found to be dysregulated were validated by western blotting.

Results and Discussion: All eight HGSC cell lines expressed *CGB* transcript(s) and secreted hCGβ, with SKOV-3 and HEY cells being the highest expressers. HEY cells and the pair of cisplatin sensitive/resistant cell lines, A2780 and A2780cis, were chosen for functional studies. The effect of hCGβ on cell proliferation was cell type dependent, as downregulation of hCGβ significantly decreased proliferation of A2780cis and HEY cells, but its effect on A2780 cells seemed to be dependent on the siRNA used to downregulate hCGβ. This suggested that the two siRNAs targeting hCGβ may have some differences in their actions. Overexpression of hCGβ had no effect on proliferation in any cell line, suggesting that a threshold level may be reached beyond which hCGβ had no effect. Downregulation of hCGβ increased cell adhesion of HEY and A2780cis cells on the various extracellular matrix (ECM) proteins, which suggested that hCGβ may be inhibitory to cell adhesion. Cell migration was not influenced by hCGβ.

hCGβ may have a role in the response of HGSC cells to cisplatin, as downregulation of hCGβ in A2780cis and HEY cells increased sensitivity to cisplatin; however, this effect was not seen in the cisplatin sensitive A2780 cells. This suggested that hCGβ may be involved in the response of HGSC cells towards cisplatin but only when cells have acquired resistance to cisplatin. An increase in drug sensitivity was also observed when cells were treated with carboplatin and oxaliplatin, particularly in A2780cis cells. This was not surprising for carboplatin which is thought to have a similar mode of action to cisplatin, but unexpected for oxaliplatin as it is thought to have a different mode of action. These results suggested that hCGβ may be involved in a common mechanism of action for all three drugs.

iTRAQ and pathway analysis revealed a number of proteins and pathways that were differentially regulated when cells were treated with cisplatin following hCGβ

downregulation, compared to cisplatin treatment alone. Validation by western blotting revealed that the wings apart-like homolog (WAPAL) and sirtuin 1 (SIRT1) proteins were both downregulated when cells were treated with cisplatin following hCGβ downregulation compared to cisplatin treatment alone; however, this effect was dependent on the siRNA used to target hCGβ, indicating that the two siRNAs worked by different mechanisms to confer cisplatin sensitivity. A compensatory upregulation of the highly homologous *LHB* gene (encoding the β-subunit of luteinising hormone) by one siRNA but not the other provided a plausible explanation as to why the two siRNAs had some different effects. Interestingly, one of the siRNAs decreased the level of SIRT1 independent of cisplatin treatment, suggesting that SIRT1 could be a secondary target of the siRNA and therefore be a contributing factor to the increased sensitivity to drug treatment. Downregulation of SIRT1 increased cisplatin sensitivity in A2780cis cells but not HEY cells. From this result it can be inferred that the increased sensitivity toward cisplatin following downregulation of hCGβ was not caused by a decrease in SIRT1 alone.

Conclusion: This study has demonstrated that hCGβ is potentially involved in cell proliferation, adhesion and response to platinum-based drugs in HGSC cells. However, further work on the mechanism by which hCGβ regulates cellular responsiveness to platinum-based drugs would be needed with the view to establishing a targeted therapeutic approach that could have future implications on how chemoresistance is managed in ovarian cancer.

Abstracts associated with this thesis

Sinnappan S., Baxter RC. and Marsh DJ. A role for the free beta subunit of human chorionic gonadotropin in sensitivity of high grade serous cancer cells to platinum-based chemotherapeutics, 3rd Lowy Cancer Symposium, 4-6th May 2015, Sydney, Australia

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Sinnappan S., Xiaolang Y., Baxter RC. and Marsh DJ. Does the human chorionic gonadotropin β subunit play a role in cisplatin sensitivity in epithelial ovarian cancer? Sydney Cancer Conference, September 26-28th 2012, Sydney, Australia.

Sinnappan S., Xiaolang Y., Baxter RC. and Marsh DJ. Does the human chorionic gonadotropin β subunit play a role in cisplatin sensitivity in epithelial ovarian cancer? ASMR NSW Scientific Meeting, June 4th 2012, Sydney, Australia.

Sinnappan S., Xiaolang Y., Baxter RC. and Marsh DJ. A role for the free beta subunit of the human gonadotropin in sensitivity of epithelial ovarian cancer cells to platinum-based chemotherapeutics, Scientific Research meeting, November 20th-21st 2012, Sydney, Australia.

Sinnappan S., Molloy MP., Baxter RC. and Marsh DJ. A phosphoproteomic approach to investigate signalling pathways regulated by gonadotropins in ovarian cancer. 28th Scientific Research Meeting, November 1st-2nd 2011, Sydney, Australia.

Abbreviations

ANOVA	analysis of variance
BSA	bovine serum albumin
cDNA	complementary DNA
CO_2	carbon dioxide
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
FBS	fetal bovine serum
FIGO	International Federation of Gynaecology and Obstetrics
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
h	hour
hCG	human chorionic gonadotropin
hCGβ	β-subunit of human chorionic gonadotropin
HGSC	high-grade serous ovarian cancer
HMGB	High-Mobility Group Protein
iTRAQ	isobaric tags for relative and absolute quantitation
L	litre
LH	luteinising hormone
LHβ	β-subunit of luteinising hormone
LIMK1	LIM Domain Kinase 1
min	minute
mL	millilitre
M	molar
mM	millimolar
mRNA	messenger RNA
MMR	mismatch repair
ng	nanogram
OSE	ovarian surface epithelium
PARP	poly (ADP-ribose) polymerase

PBS	phosphate buffered saline
PCR	polymerase chain reaction
qRT-PCR	quantitative reverse transcription polymerase chain reaction
RNA	ribonucleic acid
RNase	ribonuclease
RT	room temperature
SDS	sodium dodecyl sulfate
S.E.M.	standard error of the Mean
shRNA	Short hairpin RNA
siRNA	small interfering RNA
SIRT1	sirtuin1
TCGA	The Cancer Genome Atlas
TE	trypsin/EDTA
TP53	tumour suppressor p53
µg	microgram
µM	micromolar
µL	microlitre

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Chapter 1 Literature Review

1.1 Ovarian cancer

Ovarian cancer is one of the most lethal gynaecological cancers in the developed world, and accounts for half of the gynaecological cancer-related deaths of women in Australia [1, 2]. The lack of specific symptoms, poor diagnostic methods, diagnosis at an advanced stage and development of drug resistance are key factors for the high morbidity seen in this malignancy. Ovarian cancer is predominantly diagnosed in postmenopausal women with 60% of new diagnoses being in patients 60 years and over. Of all ovarian cancer diagnosis, 3-17% are in women aged 40 years and under [3]. Within the first 5 years of diagnosis, the survival rate for older women is significantly lower than for women diagnosed at 30 years of age or younger [2]. The survival rates of patients with ovarian cancer is also linked to the stage of diagnosis with 80-90% cure rates for patients with stage I cancer (when the malignancy is confined to the ovaries) dropping to 20-30% when patients are diagnosed at an advanced stage (III and IV, when the tumour(s) have metastasised beyond the pelvis) [4-8]. Unfortunately, only 20-25% of ovarian cancers are detected at stage I [5, 8].

1.1.1 Familial risk factors

Between 5 to 15% of ovarian cancers are considered to be caused by hereditary germline mutations [8, 9]. Women with a family history of breast and ovarian cancer, in particular first degree relatives, have a higher risk of developing ovarian cancer [6, 10, 11]. Among the best studied hereditary risk factors contributing to

ovarian cancer are germline mutations in the breast cancer type 1 and 2 genes (*BRCA1* and *BRCA2*) [10-13]. The lifetime risk of ovarian cancer in the general population jumps from 1.8% to 30–65% and 15-30% in carriers of the *BRCA1* and *BRCA2* mutations, respectively [8, 14-16]. Interestingly, the two genes also seem to have differential levels of risk based on age, with women carrying *BRCA1* mutations presenting with disease predominantly before 50 years of age and *BRCA2* mutation carriers after 50 years [16, 17]. Bilateral prophylactic oophorectomy is a preventative procedure chosen by some women who are carriers of a *BRCA1* or *BRCA2* mutation [18, 19].

Mutations in mismatch repair genes *MLH1* and *MSH2* which are linked to hereditary nonpolyposis colorectal cancer (HNPCC or Lynch syndrome) have also been associated with an increased risk (up to 7%) of developing ovarian cancer [8, 20, 21].

1.1.2 Protective factors

The use of oral contraception has been shown to play a protective role in the development of ovarian cancer [22, 23]. Studies have also shown that the longer the duration of use of the oral contraceptive, the better the protective outcome. In addition, oral contraception has been shown to have a long term protective effect against ovarian cancer, even after the cessation of use, but only up to 20 years since the last use [24-27]. The use of oral contraceptives has also been found to have a protective effect in women who are carriers of *BRCA1/2* germline mutations who are at increased risk of developing ovarian cancer as discussed in section (1.1.1) [28].

Parity (child birth) has been shown to have an inverse effect on the risk of developing ovarian cancer [29-32].

The protective role of oral contraception and parity supports two theories about the development of ovarian cancer: the incessant ovulation theory and the gonadotropin theory [33-35]. These two theories will be further discussed in section 1.2.

1.1.3 Symptoms

Ovarian cancer has been referred to as the 'silent killer' as the disease is asymptomatic or symptoms may only occur at an advanced stage of disease [36]. However, symptoms can arise at earlier stages of the disease but are often ignored or misdiagnosed [37]. These symptoms include abdominal distention, pelvic and abdominal pain and fatigue but since these symptoms are non-specific to ovarian cancer, the disease can be dismissed or misdiagnosed [38].

1.1.4 Detection

Ovarian cancer can be detected and diagnosed by transvaginal ultrasound and determining the level of serum biomarker CA-125. CA-125 is elevated in 50% of patients with stage I ovarian cancer and up to 90% in patients with an advanced stage of disease [6]. Though CA-125 has been used for several decades as a biomarker for ovarian cancer it has a number of drawbacks: 1. high levels of CA-125 can be detected in early pregnancy, during the menstrual cycle and in benign conditions

such as endometriosis [39, 40]; 2. elevated levels of CA-125 have also been shown in other cancers such as breast [41], gastric [42] and non-Hodgkin lymphoma (NHL) [43]; and, 3. twenty percent of ovarian cancer tumours do not express CA-125 [44]. CA-125 however has been shown to be a valuable biomarker in monitoring the progress of ovarian cancer during treatment and follow-up of recurrent disease [45-47].

Alternate or additional biomarker(s) for the detection of ovarian malignancies have been studied and one such marker that has been extensively investigated is the human epididymis secretory protein 4 (HE4). HE4 has been shown to be superior to CA-125 as it is able to better distinguish between benign and malignant pelvic masses and can detect malignancies at stage I of the disease [48, 49] which is thought to be due to HE4 being released earlier than CA-125 [50]. The combination of HE4 and CA-125 has been shown to be more specific at distinguishing between malignant and benign tumours compared to either biomarker alone, leading to the development of the Risk of Ovarian Malignancy Algorithm (ROMA) [48, 49]. ROMA incorporates HE4 and CA-125 as well as menopausal status, to determine the likelihood of finding malignant abnormalities [51, 52]. Although HE4 or ROMA has had promising results, studies have also shown it does not outperform CA-125 as a predictor of ovarian cancer [53].

OVA1 is an assay approved by the FDA in 2009 for pre-surgical prediction of pelvic malignancies. OVA1 is a multivariate index assay encompassing five biomarkers: CA-125, 2-microglobulin, apolipoprotein A1, transthyretin, and transferrin [54]. It has been shown to be more sensitive and effective at detecting advanced and early stage malignancies in both pre- and postmenopausal women

compared to CA-125 alone [55]. Studies have also shown that OVA1 is capable of detecting malignancies (up to 76%) missed by standalone CA-125 assay [56].

1.1.5 Staging

The Federation of International Gynaecology and Obstetrics (FIGO) staging system which is based on surgical and pathological observations is the most commonly used staging system, established in 1988 (Rio de Janeiro). Since its establishment, FIGO has undergone revision, with the current version, outlined in Table 1-1, approved in 2012 [57, 58].

Table 1-1 FIGO nomenclature for ovarian carcinomas

Stage	Description
Stage I	Tumour confined to ovaries or fallopian tube(s) IA: Tumour limited to one ovary (capsule intact) or fallopian tube; no tumour on ovarian or fallopian tube surface; no malignant cells in the ascites or peritoneal washings. IB: Tumour limited to both ovaries (capsules intact) and fallopian tubes; no tumour on ovarian or fallopian tube surface; no malignant cells in the ascites or peritoneal washings. IC: Tumour limited to one or both ovaries or fallopian tubes, with any of the following: IC1: Surgical spill IC2: Capsule ruptured before surgery or tumour on ovarian or fallopian tube surface. IC3: Malignant cells in the ascites or peritoneal washings.
Stage II	Tumour involves one or both ovaries or fallopian tubes with pelvic extension (below pelvic brim) or primary peritoneal cancer IIA: Extension and/or implants on uterus and/or fallopian tubes and/or ovaries. IIB: Extension to other pelvic intraperitoneal tissues.
Stage III	Tumour involves one or both ovaries or fallopian tubes, or primary peritoneal cancer, with cytologically or histologically confirmed spread to the peritoneum outside the pelvis and/or metastasis to the retroperitoneal lymph nodes IIIA1: Positive retroperitoneal lymph nodes only (pathologically proven): IIIA1(i) Metastasis up to 10 mm in greatest dimension. IIIA1(ii) Metastasis >10 mm in greatest dimension. IIIA2: Microscopic extrapelvic (above the pelvic brim) peritoneal involvement with or without positive retroperitoneal lymph nodes. IIIB: Macroscopic peritoneal metastasis beyond the pelvis up to 2 cm in greatest dimension, with or without metastasis to the retroperitoneal lymph nodes. IIIC: Macroscopic peritoneal metastasis beyond the pelvis more than 2 cm in greatest dimension, with or without metastasis to the retroperitoneal lymph nodes (includes extension of tumor to capsule of liver and spleen without parenchymal involvement of either organ).
Stage IV	Distant metastasis excluding peritoneal metastases Stage IVA: Pleural effusion with positive cytology. Stage IVB: Parenchymal metastases and metastases to extra-abdominal organs (including inguinal lymph nodes and lymph nodes outside of the abdominal cavity).

Table extracted and adapted from [57, 58]

1.1.6 Histological subtypes

Ninety percent of ovarian cancers are malignant epithelial cancers (EOC) and there are five distinguished histological types: high-grade serous ovarian carcinoma (HGSC), endometrioid carcinoma (EC), clear-cell carcinoma (CCC), mucinous carcinoma (MC), and low-grade serous carcinoma (LGSC). Characteristics of the histological subtypes are outlined in Table 1-2.

Table 1-2 Characteristics of histological subtypes of ovarian cancer

Classification	Incidence	Characteristics
high-grade serous carcinoma (HGSC)	60-80% [59]	HGSC are the most common and aggressive of ovarian cancers accounting for more than two thirds of EOC cases and are thought to originate from precursor lesions from the fimbria of the fallopian tube [60, 61]. Up to 80% of HGSC present at an advanced stage and respond to conventional chemotherapy [58, 60]. Though these tumours are generally initially responsive to chemotherapy, they develop drug resistance resulting in poor patient survival outcomes [62].
low-grade serous carcinoma (LGSC)	<5% [60, 63]	LGSC are thought to originate from serous or adenofibroma cystadenoma [64] and borderline tumours as well as the fallopian tube [65, 66]. They are often resistant to platinum-taxane based chemotherapy [67, 68].
mucinous carcinoma (MC)	3% [60, 63, 65]	MC consists of a mix of borderline, non-invasive or invasive carcinomas as well as cystadenomas [69]. 80% of MC are cystadenomas with low proliferative potential [69]. The origin of MC is still under speculation and mostly unknown and is thought to arise from metastatic gastrointestinal tumours [60, 65, 70, 71].
endometrioid carcinoma (EC)	10% [60, 63]	EC are thought to arise from atypical endometriosis or endometriotic cysts [60, 72, 73].
clear-cell carcinoma (CCC)	10% [60, 63]	CCC are thought to rise from atypical endometriosis or endometriotic cysts [60, 72] and do not respond well to platinum-taxane based chemotherapy [74, 75].

1.1.7 Aetiology

Ovarian cancer is a complex disease and the underlying molecular events involved in tumorigenesis of the cancer are poorly understood. However, some of the molecular events or pathways that are known to be implicated in the development of the different histological subtypes of ovarian cancer are summarised in Table 1-3 and are discussed further in this section.

Table 1-3 Dysregulated molecular pathways in the different histological subtypes of ovarian cancer

Histological subtypes	Molecular pathway	Reference
High-grade serous carcinoma (HGSC)	Mutations in *TP53* and *BRCA1*/2 and hypermethylation of *BRCA1*	[14, 62, 76-78]
Low-grade serous carcinoma (LGSC)	Mutations in *BRAF* and *KRAS*	[8, 79, 80]
Mucinous carcinoma (MC)	Mutations in *BRAF* and *KRAS*	[8, 80, 81]
Endometrioid carcinoma (EC)	Mutations in *PTEN*, *PIK3CA, BRAF*, *KRAS* and *ARID1A*	[65, 77, 82, 83]
Clear-Cell carcinoma (CCC)	Mutations in *PTEN*, *PIK3CA* and *ARID1A*	[8, 84]

1.1.7.1 TP53

TP53 is the most common and well-studied tumour suppressor gene in ovarian cancer [85] and mutations in *TP53* occur in up to 96% of HGSC [62, 76] but are less frequent in the other four histological subtypes [77, 84]. *TP53* mutations are also more frequent at a later stage of tumour progression, with mutations more prevalent in stages III and IV (58%) compared to I and II (27%) which could suggest that *TP53* mutations occur at a later stage of cancer development [86]. Contrary to this notion, the fact that *TP53* mutations have been identified in low stage HGSC [86-88], and in precursor lesions [66, 89] suggests that *TP53* mutations may be an early event in the development of HGSC.

1.1.7.2 KRAS and BRAF

Activating mutations in *KRAS* and *BRAF* which code for the V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (KRAS) and proto-oncogene B-RAF (BRAF) proteins, respectively, are commonly found in mucinous, endometrioid and LGSC compared to HGSC [64, 81, 90, 91]. KRAS and BRAF are upstream regulators of mitogen-activated protein kinase (MAPK) and mutations in *KRAS* and *BRAF* result in constitutive activation of the MAPK pathway [77]. Interestingly, the two mutations are mutually exclusive with tumours carrying either a *KRAS* or a *BRAF* mutation [79].

1.1.7.3 Phosphotidylinositol 3-kinase (PI3-kinase)/AKT pathway

The phosphatidylinositol 3-kinase (PI3K)/AKT pathway which is involved in a number of cellular processes including survival, apoptosis, cell cycle arrest and DNA

repair, has been found to be dysregulated in ovarian cancer [92, 93]. Phosphatase and tensin homolog (PTEN) is an antagonist of the PI3K pathway and inactivating mutations in the *PTEN* gene occur in 14–21% of endometrioid cancer [77]. Although *PTEN* mutations were initially associated with only endometrioid cancer [90], they have since been shown in other histological subtypes including HGSC [62, 76, 94] and mucinous cancer [95]. In addition to *PTEN*, other genes such as *PIK3CA* and *AKT2* which are involved in the PI3K pathway have also been shown to be amplified in ovarian cancer [96, 97]. *PIK3CA* which codes for the human p110α subunit of PI3K is amplified in 40% of ovarian cancer [97].

1.1.7.4 ARID1A

Somatic mutations in AT-rich interactive domain1A gene (*ARID1A)* which codes for the BAF250a protein have been reported in almost 50% of clear cell carcinoma and 30% of endometrioid cancers [82]. BAF250a forms part of the SWI/SNF complex which is involved in chromatin remodelling and cellular processes such as proliferation, DNA repair and tumour suppression [98]. It is speculated that mutation in *ARID1A* and loss of BAF250a is an early event in the development of the cancer from endometriosis as they are detected in preneoplastic lesions [82]. In a study by Weigand *et al.* mutations in *ARID1A* were not present in HGSC tumours [82].

1.1.7.5 BRCA1/2

As discussed in section 1.1.1 carriers of *BRCA1*/2 mutations have an increased risk of ovarian cancer. In addition to inactivating germline mutations, somatic mutations in *BRCA1*/2 have been identified in serous ovarian cancer [62, 76, 99-

101]. Hypermethylation of the promoter region *BRCA1* leading to loss of *BRCA1* has also been documented in HGSC [62, 76].

1.2 Site of origin

The site of origin for ovarian cancer is a controversial and evolving topic of research. A widely accepted, but highly disputed, theory is that epithelial ovarian cancers arise from the cells of the ovarian surface epithelium (OSE) or ovarian epithelial inclusions (OEI) [9, 59, 102]. An explanation behind this train of thought is explained by the incessant ovulation theory put forward by Fathalla which suggests that the constant rupture and repair of the OSE during ovulation predisposes the cells to malignant transformations [33]. In support of this theory, Schildkraut *et al.* found that high levels of *TP53* mutations in ovarian cancer tumours were associated with higher number of ovulatory cycles [103]. Interestingly, the risk of ovarian cancer also seems to be associated with the number of ovulation cycles, particularly in women in their 20s [104, 105]. Furthermore, pregnancy and oral contraceptives which allows for a break in the ovulation cycle have been found to decrease the risk of ovarian cancer [30, 106]. Another theory which suggests that EOC cancer may develop from OSE is the gonadotropin hypothesis which suggests that high levels of pituitary gonadotropins during ovulation, in particular of follicle stimulating hormone (FSH) and luteinising hormone (LH), directly leads to malignant transformation of ovarian epithelium [107, 108]. In agreement with this theory factors such as multiple pregnancies, breast feeding and oral contraception which lead to a reduction of these hormones, seem to play a protective role in ovarian cancer [105, 106, 109]. Some studies have shown that fertility treatments

which increase the levels of FSH and LH also increase the risk of ovarian cancer [30, 110, 111]; however, subsequent studies have shown little or no link between fertility treatment and increased risk of ovarian cancer [1, 10, 106, 112].

One of the main criticisms of the OSE being the site of origin of EOC is that EOC cells are morphologically distinct to OSE cells often displaying a Müllerian phenotype. This discrepancy has been explained by the suggestion that cells of the OSE differentiate into a Müllerian-like phenotype and invaginate into the stroma forming small OEI mesothelial cysts before metaplasia into EOC cells [59, 102, 113]. A major criticism for this explanation is that intermediate precursor lesions have been rarely identified [59, 102].

A more recent and increasingly accepted theory about the site of origin of EOC specifically for HGSC is that they arise from fallopian tube [65, 71, 114, 115]. In support of this theory samples from prophylactic salpingo-oophorectomy specimens from *BRCA* mutation carriers displayed high levels of dysplasia and early serous malignancies often referred to as serous tubal intraepithelial carcinomas (STICs) or tubal intraepithelial carcinoma or (TIC) at the fimbriated end of the fallopian tube [61, 89]. These precursor lesions contained abnormal immunohistochemical expression of p53 and *TP53* mutations similar or identical to HGSC [59, 61, 116]. STICs also have upregulated levels of cyclin E1, Rsf-1 and fatty acid synthase which is also observed in HGSC [66]. Despite this evidence for the origin of serous ovarian cancer, a consensus of the origin of ovarian cancer has not been reached perhaps due to the diverse nature of the different histological subtypes.

1.2.1 Type I and II EOC

EOC can be classified into two broad tumour groups based on morphological, immunohistochemical and molecular characteristics, and subsequent clinical presentations: Low-grade-Type I and high-grade-Type II [59, 71, 90, 117]. Type I cancers are composed of low-grade serous, mucinous, endometrioid or clear cell carcinomas and account for 25% of EOC [71]. These cancers are often detected at an early stage, have a less aggressive growth and a low-malignant potential (LMP). They are however less likely to respond to, or are even resistant to, conventional platinum-taxane based chemotherapy [81]. Type I tumours are thought to develop from serous borderline tumours from OEIs or serous cystadenoma which develop into invasive carcinoma [59]. Type II cancers account for 75% of EOC and include high-grade serous and endometrioid cancers as well as undifferentiated carcinomas [66, 71]. These tumours are poorly differentiated, and are generally diagnosed at a late stage (III-IV) [118]. Type II tumours are more aggressive and genetically unstable, with greater gene copy number abnormalities compared to type I tumours; however, they do generally respond initially to conventional chemotherapy. Type I serous cancer have wild-type *TP53* and *BRCA1*/*BRCA2* and often present with mutations in *BRAF*, *KRAS*, *PTEN*, *PIK3CA* and *ARID1A* [66, 81, 119]. Type II in particular HGSC, almost always have a mutation in *TP53* (up to 96%), and can also have *BRCA1* or *BRCA2* mutations [76].

1.2.2 Clinical management

The current treatment of ovarian cancer is primary cytoreductive surgery (surgical resection or debulking) to remove as much of the macroscopic tumour as possible, followed by adjuvant combination chemotherapy consisting of platinum-based chemotherapeutic agents such as carboplatin, in combination with a taxane such as paclitaxel. Optimal cytoreduction has been linked to better patient survival [120]. Furthermore, in patients whose tumours cannot be completely removed, it is thought that cytoreduction improves the ability of chemotherapeutic agents to penetrate remaining tumour deposits. In some cases, neoadjuvant chemotherapy is employed before interval cytoreduction, when extensive metastasis has occurred and/or primary debulking is not possible due to poor health of the patient [118, 121]. However, studies have reported conflicting results of the benefit of neoadjuvant therapy over primary cytoreductive surgery; van der Burg *et al.* showed that neoadjuvant therapy was more beneficial [122] while others have shown patients are no better or even worse off with neoadjuvant therapy compared to primary cytoreduction [120, 123]. Despite the arguments for neoadjuvant or primary cytoreduction followed by adjuvant therapy, it is clear that optimal cytoreduction is a primary prognostic factor for overall patient survival [122, 123].

Genotyping patients either at germline or somatic levels has given rise to personalised treatment that is specific to a patient. One example is a new line of treatment that has been approved in patients who carry *BRCA1/2* mutations. Tumour cells that carry these mutations are unable to repair double stranded breaks (DSBs) in the DNA by homologous recombination. The enzyme poly(ADP-ribose) polymerase is involved in the repair of single stranded breaks (SSBs) in DNA by excision repair.

PARP inhibitors prevent the repair of SSBs, thus leading to DSBs. In this case DSBs cannot be repaired by the mutant BRCA proteins, thus leading to synthetic lethality and subsequent cell death [124]. Following a Phase II study in platinum-sensitive relapsed HGSC patients, the FDA approved (in December 2014) the first PARP inhibitor Lynparza (olaparib) in patients with advanced disease carrying *BRCA* mutations.

1.3 Human chorionic gonadotropin

Human chorionic gonadotropin (hCG) is a gonadotropic hormone important in the maintenance of early pregnancy, in particular rescue of the corpus luteum which produces progesterone required for the maintenance of pregnancy. hCG is predominantly produced by the syncytiotrophoblasts of the placenta. Levels of hormone can be detected in the maternal serum 8-10 days after ovulation near the time of implantation of a fertilised egg. After 7 weeks of gestation, progesterone production by the placenta takes over the function of the corpus luteum [125]. Though the main function of hCG in pregnancy seems to be the rescue of the corpus luteum, there is increasing evidence that hCG may be involved in cellular differentiation and angiogenesis [126, 127]. Shi *et al.*, proposed that hCG may be involved in the differentiation of cytotrophoblast into syncytiotrophoblasts by showing that the addition of exogenous hCG to cytotrophoblasts in culture resulted in the cells beginning to merge into multinucleated cells and increased cadherin production which indicate differentiation into syncytiotrophoblasts [126]. Berndt *et al.* showed that exogenous hCG could be involved in endometrial angiogenesis which could also be important in pregnancy [127]. They found that hCG increased

proliferation of endothelial cells (HUVEC) and endometrial epithelial cells (EEC) (isolated from the endometrium of fertile women) as well as increased the VEGF production in EEC. In addition, the e*x vivo* aortic ring assay (with rat aortic rings cultured in collagen) and *in vivo* mouse matrigel plug assay also showed increased angiogenesis by increased microvessel outgrowth and haemoglobin respectively in the presence of exogenous hCG [127].

1.3.1 Structure of hCG

hCG is a heterodimeric glycoprotein consisting of non-covalently linked α- and β-subunits. It has a total molecular mass of 36 kDa composed of 237 amino acids, 92 amino acids in the α-subunit and 145 amino acids in the β-subunit [128, 129]. hCG belongs to the gonadotropin-glycoprotein family which includes FSH, LH and thyroid-stimulating hormone (TSH). Interestingly all four hormones share the same α-subunit but differ in their β-subunit resulting in different and specific biological roles [130]. Both the α- and β-subunits are required for hCG to interact with its extracellular receptor [131]; however, it is the β-subunit which is responsible for specific hormone activity [132]. FSH and TSH have specific cellular receptors; however LH and hCG interact with the same receptor known as the luteinising hormone/chorionic gonadotropin receptor (LHCGR) (Figure 1.1). The β-subunit of hCG (hCGβ) is highly homologous to the β-subunit of LH (LHβ), sharing 82% sequence homology. LHβ is composed of 121 amino acids whereas hCGβ has 145 amino acids [133, 134]. The major difference between hCGβ and LHβ is the additional 24 amino acids at the carboxyl terminal of hCGβ [134]. The glycosylation sites within these extra amino acids are thought to contribute to the longer half-life

and higher biological potency of hCG compared to LH (hours compared to minutes) [133, 135-138].

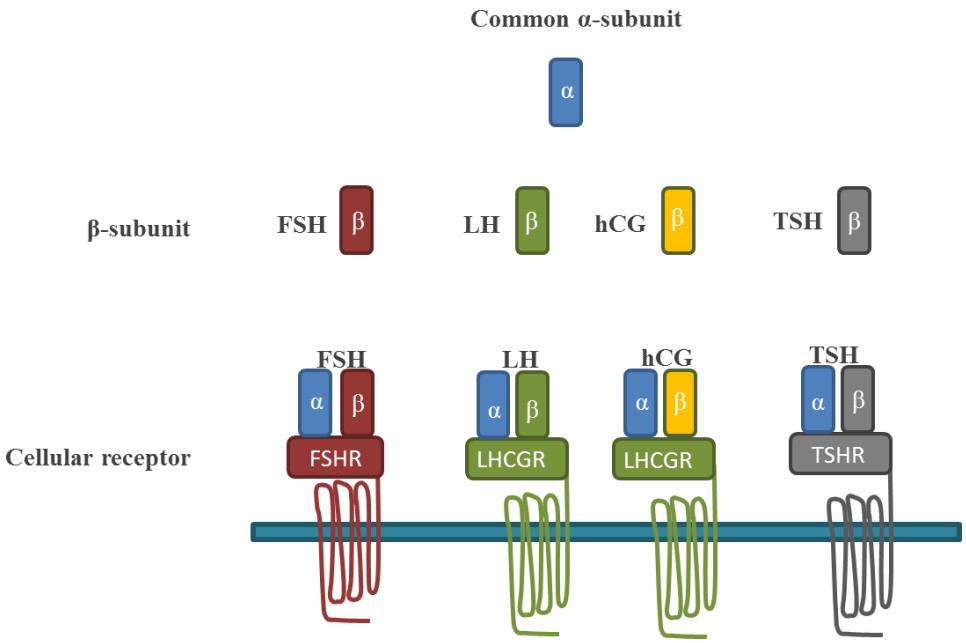


Figure 1.1 α- and β subunits of the gonadotropin-glycoprotein family

The follicle stimulating hormone (FSH), luteinising hormone (LH), thyroid stimulating hormone (TSH) and human chorionic gonadotropin (hCG) share a common α-subunit but each has a unique β-subunit. FSH and TSH interact with different cellular receptors, FSHR and TSHR receptively, but LH and hCG share a common LHCGR receptor.

Although LH and hCG bind to the same receptor, share a high sequence homology and promote progesterone production, they are important in different biological settings. LH, which is secreted from the anterior pituitary gland, is predominantly involved in the menstrual cycle regulating follicular maturation and induction of ovulation; whereas the main role of hCG is the maintenance of pregnancy [133].

hCG is heavily glycosylated with 25–30% of the protein's molecular weight composed of both N- and O-linked oligosaccharides [132, 139]. The α-subunit

contains two N-linked glycans whereas the β-subunit contains two N-linked glycans and four additional O-linked glycans. The position on the sugar residues is depicted in Figure 1.2. The degree of glycosylation has been found to not only affect protein stability but also the biological activity of the hormone [140, 141].

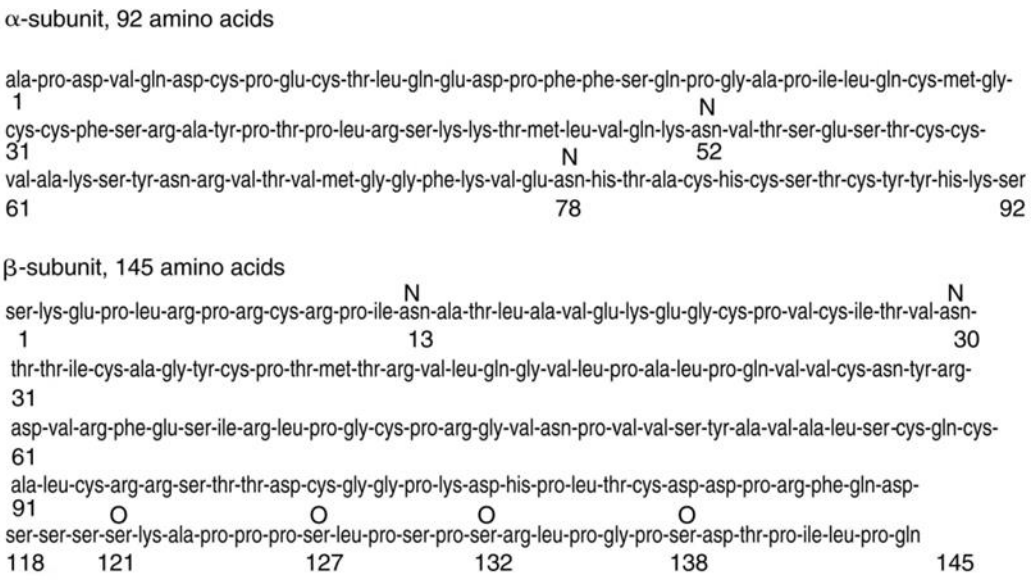


Figure 1.2 Amino acid sequence of hCG α-subunit and β-subunit

Numbers indicate amino acid residue positions and N and O indicate the positions of N- and O-linked oligosaccharides.

Image extracted from Cole[139]

hCG also bears resemblances to the proteins of the cysteine knot family which includes transforming growth factor β (TGF-β), neuronal growth factor (NGF) and platelet-derived growth factor beta (PDGFB) due to the presence of cysteine knot disulphide bonds [132, 142]. The structural similarity of hCG to the cysteine knot family is thought to contribute to its biological activity which may be independent of the presence of the α-subunit and binding to LHCG receptor (for further discussion, see section 1.5).

1.3.2 Isoforms of hCGβ

So far in this thesis, hCG has been discussed as a single molecule, however it occurs in at least four known physiological isoforms which include hCG, hyperglycosylated (h-hCG), free hCGβ and pituitary hCG [133, 143]. These different isoforms seem to have different functions which are summarised in Table 1-4. As this thesis will focus on hCGβ, its function will be discussed in detail in sections 1.4 and 1.5.

Table 1-4 Isoforms of human gonadotropin

Isoform	Produced by	Function
hCG	villous syncytiotrophoblast cells	-Rescue of the corpus luteum and progesterone production [144, 145] -Maintenance of pregnancy [133, 145, 146] -Foetal growth and development [146] -Angiogenesis of uterine vasculature [147-150]
hyperglycosylated hCG (h-HCG)	cytotrophoblast cells and choriocarcinoma cells	-Normal function: Implantation of pregnancy e.g. invasion of cytotrophoblast/ trophoblast [151, 152] -Choriocarcinoma: Antiapoptotic and growth of choriocarcinoma cells [153]
free β-subunit	non-trophoblastic malignancies	-Proliferation, anti-apoptotic and migration [154-156]
pituitary hCG	anterior pituitary	-Generally unknown role however like LH maybe be involved in the menstrual cycle [143]

Table adapted from Cole et al. [143]

In addition to these isoforms, hCG can also be detected in a shorter proteolytically degraded form consisting of nicked-hCGβ and the core fragment of hCGβ (hCGβcf) which can be detected in the placenta, blood and urine [157].

1.3.3 Expression of hCG subunits

The expression of the α- and β-subunits is thought to be controlled by different regulatory pathways due to imbalance of expression of the two subunits: specifically the α-subunit is found to be produced in excess compared to the β-subunit [158]. The α-subunit is encoded by a single gene located on chromosome 6q12-q21; however, the β-subunit of hCG (hCGβ) is encoded by multiple genes arranged in a gene cluster of six nonallelic genes located on chromosome 19q13.3 organised in tandem and inverted pairs along with the *LHB* gene which codes for the β-subunit of luteinising hormone (Figure 1.3) [130, 159]. These genes share 89-99% nucleotide sequence identity [160] and have been thought to have evolved from the ancestral *LHB* gene [161]. *CGB1* and *CGB2* are considered to be psueudogenes and though their gene transcripts have been detected in both the placenta [162] and pituitary [163] their function and protein product remains to be identified.

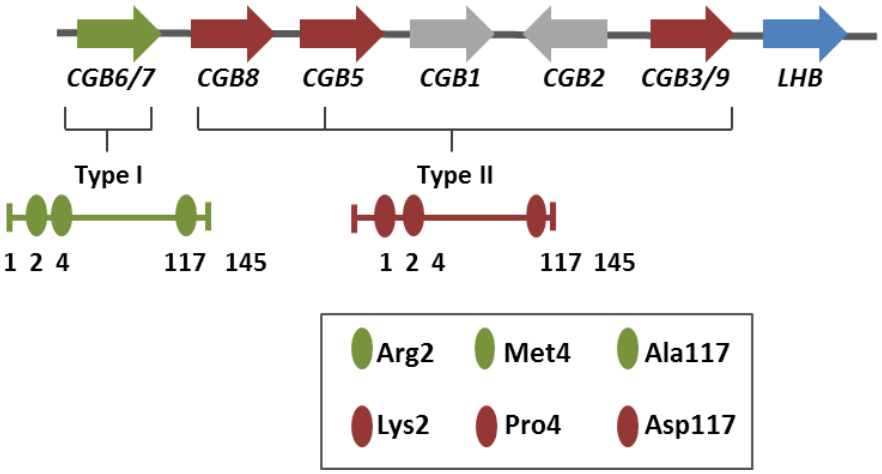


Figure 1.3 Gene cluster on chromosome 19q13.3 encoding the β-subunit of human chorionic gonadotropin

CGB6/7 are type I genes coding for hCGβ with an arginine, methionine and alanine at positions 2, 4, and 117 respectively and CGB3/9, CGB5, and CGB8 are type II genes coding for hCGβ with a lysine, proline and aspartic acid at positions 2, 4, and 117 respectively. CGB1 and CGB2 are pseudogenes.
Image extracted and adapted from Jameson et al. [164]and Aldaz-Carroll [165]

Two structurally different isoforms (types I and II) of hCGβ are expressed which differ in a single amino acid at position 117: type I hCGβ is encoded by *CGB6*/7 (alleles) and has an alanine at position 117, and type II hCGβ encoded by *CGB3*/9 (alleles), *CGB5*, and *CGB8* has an aspartic acid at this position. In addition to the difference in the amino acid at position 117, type I gene products have an arginine and methionine at positions 2 and 4, respectively whereas, type II gene products have a lysine and proline at positions 2 and 4 respectively (Figure 1.3) [165]. Expression of the two isoforms seems to be tissue-specific with type I genes expressed in normal nontrophoblastic tissues and type II genes expressed in the normal trophoblastic tissue and non-trophoblastic malignant tumours [166, 167]. Type I genes have also been shown to be expressed in renal cell carcinoma which is a non-trophoblastic tumour [168]. However it should be noted that a general tissue

specific pattern of gene expression cannot be made. For example Dirnhofer *et al*. also found that *CGB7*, *CGB5*, *CGB3*, *CGB8* and *CGB1/2* were expressed in the pituitary [163]; and Bo and Boime found that all six *CGB* genes were expressed in the placenta albeit at varying levels: *CGB5> CGB3 = CGB8>CGB7, CGB1/2 [162]*.

1.4 hCG and cancer

Elevated expression of hCGβ is common in trophoblastic cancers and germline tumours and monitoring the levels of hCGβ as a biomarker for prognosis, relapse and therapeutic response has been well established in these cancers [134, 169-171].

hCGβ is expressed by a number of non-trophoblastic epithelial cancers e.g. in bladder [172, 173], cervical [174] and pancreatic [175] cancers and is often associated with aggressive disease and poor survival outcomes [134]. Interestingly, it is the monomeric β-subunit (hCGβ) and not the intact dimer which is predominantly expressed by epithelial cancers [134, 176]. Often the presence of hCGβ is a hallmark of aggressive and metastatic disease and is associated with poor clinical outcome [177, 178]. High hCGβ levels have also been associated with tumours which are resistant to radiotherapy [179] and chemotherapy [180]. Expression of hCGβ has been well documented in ovarian cancer and is summarised in (Table 1-5).

Table 1-5 Detection of hCG/hCGβ in serum, ascites or tumour tissue from ovarian cancer patients

% Expression (cohort size)	Detected in	Comments
33% (N=173)	Serum	Strong association between high levels of hCGβ and poor survival. Patients with high levels of hCGβ had poorer survival rates - 19% compared to 65% in patients with normal levels of hCGβ [181].
29% (N=146)	Serum	The frequency of hCGβ elevation correlated with the stage of disease with 12% in stage I and 82% in stage IV [182].
100% (N=15)	Tissue	High levels of hCGβ transcript in ovarian cancer tissue compared to almost no expression in normal tissue [183].
41% (N=27)	Serum and ascites	The ratio of hCG/hCGβ levels was found to be elevated in serum and ascites fluids of patients with ovarian cancer [184].
36% (N=73)	Serum	High levels of hCGβ correlated with poor survival outcome of patients [185].
67% (N=123) 68% (N=156)	Serum Tissue	Higher levels of hCG were detected in malignant tumours compared to benign tumours. In mucinous carcinomas expression of hCG was significantly higher at stage III compared to stage I [186].

Survival studies have shown a negative correlation between high levels of hCGβ and survival in patients with ovarian cancer [181, 182]. In one study Vartiainen and colleagues showed that the frequency at which hCGβ was elevated in patients with ovarian cancer correlated with the stage of the disease and poor survival outcomes. They found that elevated hCGβ occurred at a frequency of 82% in patients with stage IV disease compared to 12% with stage I [182]. Another study also by

Vartiainen *et al.* found that the combination of hCGβ and p53 expression levels was a strong prognostic marker in patients with serous ovarian cancer (Figure 1.4). They found that the five-year survival for patients with either elevated serum hCGβ levels or aberrant p53 expression was 44% but only 14% in patients who had both elevated hCGβ levels as well as aberrant p53 expression. The five-year survival outcome for patients with normal hCGβ and p53 expression was 82% [181].

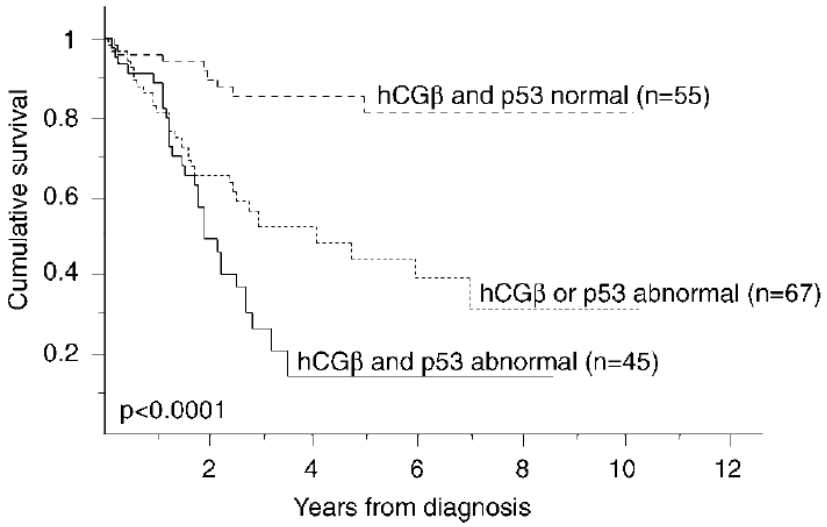


Figure 1.4 Overall disease-specific survival in 167 patients with serous ovarian carcinoma in relation to serum hCGβ and p53 tissue expression

Figure extracted from Vartiainen et al. [181]

The value of hCGβ as a tracker of disease progression in ovarian cancer is not well established but Grossman *et al.* showed that the ratio of hCG/hCGβ correlated with tumour burden in a 47 year old patient with ovarian cancer who had undergone surgical invention as well as chemotherapy (Figure 1.5) [184].

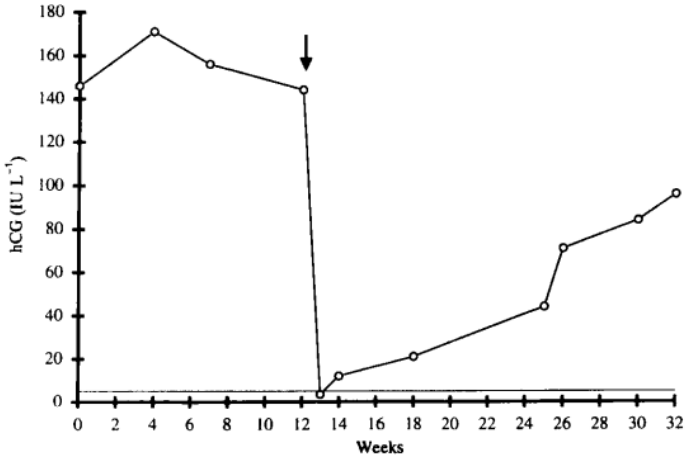


Figure 1.5 Serial expression of hCG/hCGβ serum concentration in a 47 year old woman with ovarian cancer

During chemotherapy without clinical response (0-12 weeks) hCG/hCGβ levels were elevated. At 12 weeks when the tumour was surgically removed (arrow) levels dropped, followed by an increase as the tumour began to relapse.

Figure extracted from Grossman et al. [184]

1.5 Evidence to support biological activity of hCGβ

Since the free β-subunit of hCG cannot interact with the LHCG receptor it was originally thought to have no functional biological role; however, a number of studies have shown evidence of its biological activity in epithelial cancer including proliferation, apoptosis and malignant transformation and this is further discussed in sections 1.5.1 to 1.5.4.

1.5.1 hCGβ and cell proliferation

Gillot *et al.* observed that exogenous hCGβ could promote proliferation of bladder cancer cell lines T24, SCaBER, RT112 and 5637 in a dose dependent manner shown by the tetrazolium salt reduction assay (MTT) [154]. T24 cells

produced the least amount of secreted hCGβ but showed the highest proliferative response to exogenous hCGβ. This group also showed that the proliferative effect of hCGβ could be reversed with the addition of anti-hCGβ anti-serum in a dose dependent manner and that the anti-serum could only inhibit cell growth in bladder cancer cell lines that produced endogenous hCGβ [154].

1.5.2 hCGβ and cell apoptosis

hCGβ has been shown to be involved in preventing apoptosis in some cancer cell lines [155, 187]. Janowaska *et al.* showed that downregulation of hCGβ in the cervical carcinoma cell line HeLa, caused an increase in the population of cells undergoing apoptosis (shown by cell cycle analysis) [187]. Butler *et al*. found more evidence in support of the anti-apoptotic role of hCGβ by showing that exogenous hCGβ reversed the apoptotic effects of TGF-β1 in a dose dependent manner in bladder cancer cell lines [155]. They proposed that due to the structural similarity between hCGβ and TGFβ (discussed in section 1.3.1), hCGβ may be competing with dimeric TGFβ for the TGFβ receptor. This is a plausible theory, given that it has been found that like some members of the cysteine knot family, hCGβ can form homo-dimers which are required for receptor interaction [188]. Therefore, even if hCGβ cannot interact with the LHCG receptor, it may be able to participate in cellular processes by binding an alternate receptor.

1.5.3 hCGβ and cell migration and invasion

Wu *et al*. showed that overexpression of hCGβ in prostate carcinoma cell lines caused a change in cellular morphology which increased their migratory characteristics [156]. The cells' morphology changed from rounded cells to more elongated shapes with increased cellular protrusions, decreased E-cadherin expression and increased migration and invasion through matrigel. A successive paper also from Wu and colleagues, showed that activation of ERK1/2 and subsequent upregulation of matrix metalloproteinase-2 (MMP-2) were the mechanism by which hCGβ induced invasion and migration in a prostate cell line model DU145 [189]. They also demonstrated that hCGβ could increase motility of the human glioblastoma cell line U87MG by the same mechanism [190].

1.5.4 hCGβ and malignant transformation

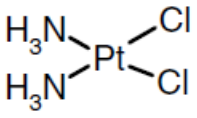
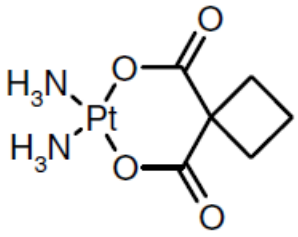
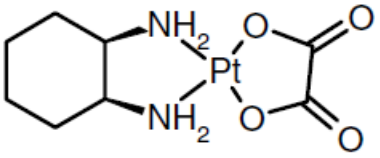
Whether hCGβ is a driver of cancer progression or can actually transform normal cells into malignant cells was studied by Guo *et al*. [191]. This study showed that overexpression of hCGβ in OSE cells caused an increase in proliferation, anchorage independent growth and a decrease in apoptosis by mechanisms that increased pro-survival proteins such as Bcl-X_L, as well as a decrease in the pro-apoptotic protein phospho-Bad. They also found that xenografts of these transformed cells were tumorigenic in nude mice.

1.6 Platinum-based chemotherapeutics in ovarian cancer

Cis-platinum(II) diammine dichloride (cisplatin) was the first platinum-based chemotherapeutic drug approved by the FDA in 1978. Though cisplatin has been a successful cytotoxic agent, it has a number of toxic side effects which include oto-, neuro- and nephrotoxicity. Cyclobutane-1,1-dicarboxylic acid platinum(II) (carboplatin) is a second generation platinum anti-cancer drug introduced in 1989 and is more stable and has fewer side effects compared cisplatin [192, 193]. However, resistance and cross-resistance of cisplatin and carboplatin is common and has led to the development of a third generation of platinum-therapeutics, out of which, oxaliplatin has proven to be the most successful [192]. The chemical structure of cisplatin, carboplatin and oxaliplatin is depicted in Table 1-6. Despite their molecular differences, the primary target of platinum drugs is thought to be DNA, resulting in cytotoxicity (discussed in detail in section 1.6.2).

The use of cisplatin in conjunction with cyclophosphamide and later paclitaxel showed that these combinations improved patient survival outcomes compared to cisplatin treatment alone [194, 195]. The cisplatin-paclitaxel combination was then replaced with carboplatin-paclitaxel combination and is now accepted as standard for the treatment of ovarian cancer, in particular advanced ovarian cancer [196]. Interestingly, the International Collaborative Ovarian Neoplasm (ICON) 3 trial showed that the addition of paclitaxel did not improve the benefit of using carboplatin as a single agent treatment [197].

Table 1-6 Chemical structure of cisplatin, carboplatin and oxaliplatin

Drug	Structure
Cisplatin (cis-dichlorodiammineplatinum(II))	
Carboplatin (cis-diammine(1,1-cyclobutanedicarboxylato)platinum(II))	
Oxaliplatin ([1*R*,2*R*]-1,2-cyclohexanediamine-*N*,*N*′)oxalate(2-)-*O*,*O*′platinum(II))	

Structures extracted from Turner and Mascorda [198]

1.6.1 Uptake of platinum-based drugs

Though cisplatin has been widely used for a number of decades, the actual mechanism by which it enters cells is yet to be fully understood. It was initially thought that the drug might enter cells through passive diffusion but the fact that the side effects (nephrotoxicity, ototoxicity) seem to be specific to certain cell types implies that there may be specific drug transporters, perhaps expressed by specific cell and/or tissue types responsible for cisplatin transport [199]. Furthermore, studies have found a link between lowered cisplatin accumulation and resistance which cannot be explained by mere diffusion of this drug [200]. Indeed, there is increasing literature that suggests that cisplatin is transported across cell membranes by active

means through membrane transporters. Some of the transporters include the copper transporters 1 and 2 (CTR1 and CTR2) [201, 202], the P-type copper-transporting ATPases (ATP7A and ATP7B) [203, 204], multidrug extrusion transporter 1 (MATE1) [199] and the multidrug resistance-associated protein 2 (MRP2) [205].

1.6.2 Mechanism of action of platinum-based drugs

Cisplatin, carboplatin and oxaliplatin are administered as prodrugs and are activated inside the cell by aquation (hydrolysis), which is initiated by the low chloride environment [206]. Two water molecules replace two chloride ions, bidendate cyclobutanedicarboxylate and bidentate oxalate ions, on cisplatin, carboplatin and oxaliplatin respectively [198].

Hydrolysis of the drugs is important for their biological activity, as once they are aquated, the drugs become positively charged and can interact with nucleophilic DNA, RNA and proteins; but preferentially bind to the N-7 position on the imidazole ring of purines, guanosine and adenosine of DNA, forming monoadducts and intra-, inter- and DNA-protein cross links (Figure 1.6) [192, 207]. The DNA-adducts cause distortion of the DNA helix which leads to interference with DNA replication and transcription and subsequently leads to apoptosis [208-210].

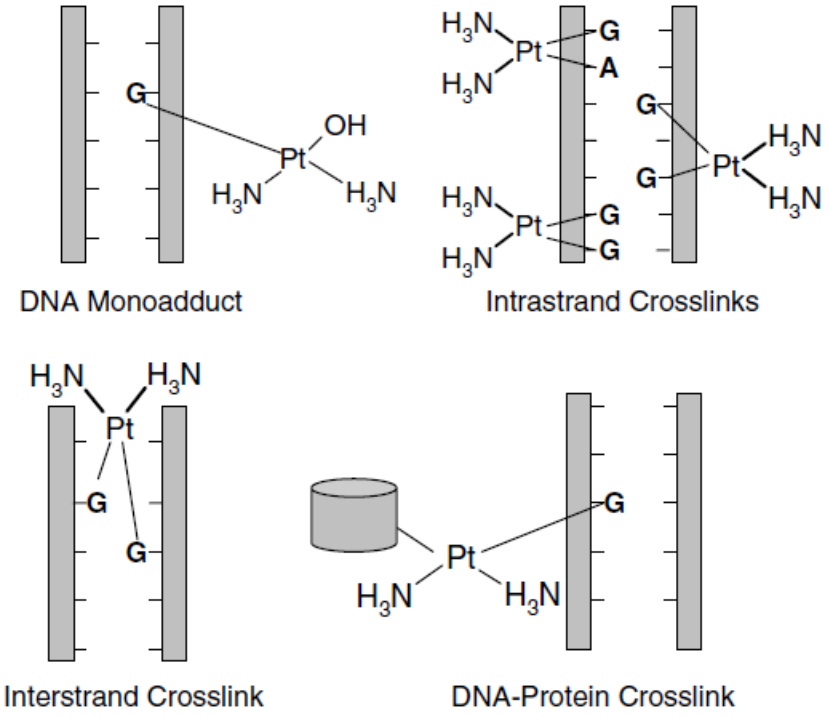


Figure 1.6 DNA-adducts formed by platinum-based chemotherapeutics

Image extracted from Rabik and Dolan [192]

About 90% of cisplatin-DNA adducts are 1,2- or 1,3-intrastrand cross links [206]. Carboplatin is thought to be therapeutically equivalent to cisplatin and forms similar DNA adducts to cisplatin which could explain their similar mechanism of action as well as cross-resistance [192, 211]. However, a higher concentration of carboplatin is required compared to cisplatin to produce equivalent anti-tumour effects due to the higher stability and lower DNA interaction of carboplatin [211]. Oxaliplatin is as potent (and sometimes more potent) as cisplatin. Despite having similar, but fewer DNA adducts, oxaliplatin causes the same number of DNA strand breaks as does cisplatin [212-214]. Oxaliplatin has a different mechanism of action and no reported cases of cross-resistance to cisplatin which is possibly due to the

difference in the way the DNA is distorted by its bulky 1,2-diaminocyclohexane (DACH) ring [214].

It is thought that the 1,2-intra strand crosslinks caused by cisplatin is the major driver of apoptosis. It is believed that the High-Mobility Group Protein (HMGB) family are able to bind to these DNA-lesions, preventing DNA replication and transcription as well as preventing the lesions from being repaired, leading to the activation of pro-apoptotic signals [192, 215]. Apoptosis is also thought to be induced by activation of the endoplasmic reticulum (ER)-stress pathway.

1.6.3 Platinum-Resistance

The initial response rate to platinum therapy in patients with ovarian cancer is 70-80%; however, development of resistance to the drug is common with patients relapsing within two years of initial treatment resulting in a 5-year patient survival rate of only 15–20% [216-219]. Resistance is multifactorial and includes drug inactivation, reduced drug accumulation, increased DNA repair and tolerance to DNA damage, as well as failure to induce apoptosis (Figure 1.7) [192, 219].

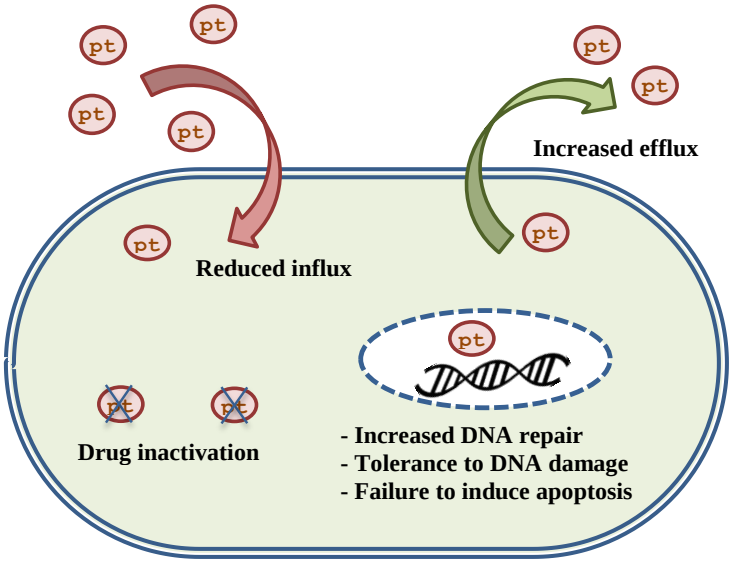


Figure 1.7 Mechanisms of platinum-based (cisplatin) drug resistance

Platinum resistance, in particular to cisplatin (pt), is multifactorial involving a number of mechanisms. Cisplatin can either be exported out of the cells through efflux pumps, have reduced accumulation due to mutations or low expression of entry pumps/ transporters, and once inside the cells the drug can be inactivated by thiol containing proteins and the damaged DNA can be repaired and/or tolerated leading to failure to activate cisplatin-adduct induced apoptosis.

1.6.3.1 Drug inactivation by thiol containing proteins

Cisplatin has a tendency to react with thiol containing proteins forming insoluble sulphides and limiting its reactivity with DNA. Increased levels of thiol containing proteins/peptides, e.g. glutathione (GSH), metallothionein and thioredoxin, have been correlated with increased cisplatin resistance [192, 220-222]. Enzymes involved in the regulation of GSH levels such as gamma-glutamylcysteine synthetase and gamma-glutamyl transpeptidase have been shown to be upregulated in cisplatin-resistant ovarian cancer cell lines [223]. Ishikawa and Ali-Osman showed that in L1210 leukemia cells, cisplatin formed a complex with GSH which was subsequently expelled from the cells by the ATP-dependent glutathione

transporter pumps (GS-X pump), providing another perspective on how GSH and glutathione transporters may be associated with cisplatin resistance [224]. Interestingly, GSH has been shown to also reduce the toxic effects of cisplatin. In fact, one study found that administering GSH in conjunction with cisplatin in patients with ovarian cancer reduced the toxic effects of cisplatin and improved their overall quality of life; however, the effect of administered GSH on cisplatin sensitivity was not considered [225].

1.6.3.2 Reduced drug accumulation

Studies have shown a correlation between reduced sensitivity to cisplatin and reduced intracellular levels of cisplatin which could be due to reduced influx or increased efflux of the drug [226]. As previously mentioned (section 1.6.1), cisplatin transport into and out of the cell can be regulated through heavy metal transporters, e.g. the CTR 1/2 copper transporters [199]. It is therefore plausible that irregularities in these transporters could result in reduced drug accumulation and resistance to platinum compounds. A study by Larson *et al*. using an isogenic pair of *CTR1*(+/+) and *CTR1*(-/-) mouse embryonic fibroblasts showed that deletion of this copper transporter reduced intracellular accumulation of cisplatin and increased cell survival [202]. The same group also showed that increased exposure of *CTR1* (+/+) fibroblast cells to cisplatin significantly reduced CTR1 expression. In addition, they showed that *CTR1* (-/-) cells not only had reduced intracellular levels of cisplatin, but also less carboplatin and oxaliplatin; however, the effect on oxaliplatin levels was less than that of cisplatin. This result demonstrated that oxaliplatin was less dependent on the CTR1 transporter, suggesting that its mode of intracellular transport is different from that of cisplatin. These results were in agreement with Holzer *et al.* who

reported that *CTR1*(-/-) embryonic fibroblasts cells accumulated lower levels of cisplatin and carboplatin and were also less sensitive to the drugs compared to the wild-type *CTR1* (+/+) cells. Interestingly, they showed that accumulation of oxaliplatin in *CTR1*(-/-) cells was also lower compared to *CTR1* (+/+) cells but this was only evident at low concentration of oxaliplatin and not at high concentrations of the drug; suggestive of additional means by which oxaliplatin enters the cell [227]. Lee *et al*. showed that high expression of CTR1 in tumour tissue from patients with ovarian cancer was associated with higher sensitivity to platinum-based treatment and improved survival [228].

CTR2 like CTR1 is a copper transporter; however, its cellular distribution is different from CTR1. CTR1 is predominantly a plasma membrane protein whereas CTR2 is expressed in late endosomes and lysosomes, as well as on the plasma membrane [229, 230]. Interestingly, the links between CTR2 and CTR1 expression and cisplatin sensitivity are different. Lee *et al*. showed that patients with ovarian cancer who had low expression levels of CTR1 along with high expression levels of CTR2 were resistant to platinum-based therapy and had poor survival outcomes [228]. Furthermore, *in vitro* studies have shown that downregulation of CTR2 in mouse embryonic fibroblasts with either *CTR1* (+/+) or *CTR1* (-/-) showed increased sensitivity to cisplatin and carboplatin and drug accumulation independent of CTR1 expression [201]. Blair *et al*. also showed in a panel of six ovarian carcinoma cell line models, a positive correlation between CTR2 expression and IC_{50} levels for cisplatin (indication of drug sensitivity) [201].

The copper-transporting P-type adenosine triphosphate proteins ATP7A and ATP7B which regulate copper efflux, are elevated in some ovarian cancers resulting in a negative correlation with cisplatin sensitivity [204, 220, 231]. Katano *et al*.

observed that the expression of either ATP7A or ATP7B was increased in cisplatin resistant (A2780/CP, 2008/C13*5.25, and IGROV-1/CP) cell lines compared to their cisplatin-sensitive parental line (A2780, 2008, and IGROV-1) and was associated with reduced intracellular cisplatin accumulation, formation of fewer DNA adducts and reduced sensitivity to cisplatin [203]. The ATP-binding cassette (ABC) transporters e.g. *ABCB1* which codes for the multi-drug efflux pump MDR1 P-glycoprotein (MDR1 or P-gp), has been associated with chemoresistance [232]. *In vitro* work by Yang *et al.* showed that the cisplatin-resistant ovarian cancer SKOV3/CIS cell line expressed low levels of MDR1 mRNA which they attributed to the increase in the levels of the micro RNA miR-130a [233]. Ren *et al*. on the other hand showed that MDR1 was not involved in cisplatin-resistance in cisplatin-resistant A2780 cells [234]. Patch *et al.* reported that promoter fusion and translocation in the 5' region of *ABCB1* was observed in 8% of HGSC patient with recurrence, resulting in upregulation of MDR1; however, authors of this study attributed MDR1 expression to resistance to paclitaxel which was part of the combination treatment for HGSC [62]. Expression of another member of the ABC transporters which has also been implicated with cisplatin resistance in cell lines is the multidrug resistance protein 2 (MRP2, ABCC2) or the canalicular multiple organic anion transporter (cMOAT) [235, 236]. However, studies in patients with ovarian cancer, found that MRP2 expression was not associated with response to platinum-based chemotherapy, progress-free survival or overall survival time [237-239]. Interestingly, MRP2 can be localised in the cytoplasmic or nuclear member and Surowiak *et al*. observed that cisplatin resistance in patients with ovarian carcinoma could be attributed its nuclear localisation [240].

1.6.3.3 Defects in DNA damage repair, increased DNA repair and tolerance to DNA damage

Platinum-resistant cancer cells have been shown to evade activation of pro-apoptotic pathways by employing a number of different mechanisms to overcome DNA damage induced by platinum-based compounds. These mechanisms which include defects in the DNA damage repair pathways, increased DNA repair and tolerance to DNA damage are discussed further in this section.

Formation of DNA adducts has been shown to lead to cell cycle arrest and in the case of cisplatin, this is thought to occur predominantly in the S and G_2 phases of the cell cycle. This break in the cell cycle is thought to give cells a chance to repair DNA damage and prevent activation of the DNA-damage induced apoptosis pathway [241, 242].

Mismatch repair (MMR) proteins are important mediators of DNA repair, cell cycle arrest and activation of apoptosis, failing repair of damaged DNA. MMR proteins are important in the recognition of cisplatin induced DNA adducts, and mutations or aberrations in expression levels of some of the genes/proteins associated with the MMR system have been linked to cisplatin resistance [208, 214, 220, 241, 243]. For example, loss of MutS protein homologue 2 (MSH2) and MutL protein homologue 1 (MLH1), which are inducers of cisplatin-induced cell cycle arrest and apoptosis, have been associated with cisplatin resistance in ovarian cancer [220, 244, 245]. Interestingly, the lack of cross-resistance between cisplatin and oxaliplatin is proposed to be due to the MMR system not being able to recognise oxaliplatin-DNA adducts [246].

Nucleotide excision repair (NER) is another mechanism through which some cancers can repair cisplatin-DNA adducts and is an important determinant of cisplatin sensitivity. NER has also been implicated in the activation of apoptosis induced by cisplatin [242, 247]. Patients with ovarian cancer who are resistant to cisplatin have been shown to have elevated levels of the DNA repair genes XP complementation group (*XPA*, *XPG*) and excision repair cross-complementation group I (*ERCCI*) which are involved in NER [192, 248-252]. Saldivar *et al*. observed allelic variations of the *XPA* and *XPG* genes in ovarian tumours which were linked to a poor response to cisplatin treatment [253]. Elevated levels of the *ERCC1* gene which encodes the excision repair cross-complementation group 1 (ECCR1) in tumour samples have been linked to clinical resistance or poor survival in a number of tumours including colorectal [254], non-small-cell lung [255], and ovarian cancer [252, 256, 257]. Steffensen *et al*. observed that expression levels of ECCR1 negatively correlated with patient response to platinum-based therapy but also noted that it was not an indicator of patient survival in ovarian cancer [257]. Studies have also shown that single nucleotide polymorphisms in *ECCR1* can be a predictor of how well patients with ovarian cancer respond to platinum-based chemotherapy, but is not a predictor of overall survival [258-260].

Though the NER system has been shown to have a preferential affinity for the less common 1,3 intrastrand cross links compared to the more common 1,2 intrastrand cross links formed by cisplatin, the 1,2 cross links seemed to be more rapidly repaired [261]. A plausible explanation for these conflicting observations is that trans-lesion DNA replication or repair of damaged DNA is facilitated by high mobility group proteins (e.g. HMGB1), which have an affinity for 1,2 intrastrand

cross links and induce MMR opposite the 1,2 intrastrand which in turn facilitates DNA repair by NER [261, 262].

BRCA1/2 genes are often deregulated through somatic or germline mutations in EOC and are involved in homologous recombination DNA repair. *BRCA1/2* mutations in ovarian tumours have been linked to high sensitivity towards cisplatin [263]. However, even in *BRCA1/2* mutant carriers, the occurrence of platinum resistance is common. Though PARP inhibitors have proven to be useful in this group of patients, reversion of *BRCA2* to wild-type has been shown, leading to disease relapse and poor patient outcomes [62, 264, 265].

Epigenetic changes which include DNA methylation, histone modification, and posttranslational gene regulation by micro-RNAs (miRNAs), which can regulate gene expression independent of DNA sequence, have been associated with platinum-resistance [266, 267]. For example, studies have shown hypermethylation of promoter regions of the DNA damage repair proteins *BRCA1* and *MLH1*, leading to the loss of expression of these genes, is associated with platinum-resistance ovarian cancer [62, 268-271].

Tolerance of cisplatin-DNA damage has been observed in cisplatin-resistant ovarian cancer cell lines compared to the parental cisplatin sensitive cell lines [221, 272]. The theory behind DNA damage tolerance is that adducts formed by cisplatin can be bypassed by DNA replicative enzymes during DNA replication.

1.6.3.1 Failure of apoptotic pathway

Apoptosis induced by DNA damage is central to the cytotoxic effects of platinum-based drugs. A network of pathways is involved in regulating apoptosis and interception of one, or more of these pathways can be a mechanism employed by cancer cells to evade cell death. Apoptosis can be triggered through the intrinsic or extrinsic pathway [273, 274]. The intrinsic pathway involves disruption of the mitochondrial membrane and an array of protein regulators such the B-cell lymphoma-2 (BCL-2) family proteins, BCL-2-associated X protein (BAX), caspases, and PI3K/AKT pathway. The extrinsic pathway is triggered by binding of ligand to the death receptors, e.g. the TRAIL receptor, leading to activation of molecules involved in apoptosis such as caspases [273, 274].

A key player in the intrinsic pathway is the tumour suppressor p53 which is mutated in almost 38-50% of cancers [275], which could confer inherent resistance to cisplatin induced apoptosis [276]. Loss of p53 function however, does not exclusively render cells resistant to cisplatin [219, 220]. Aurora kinase A is elevated in a number of cancers and *in vitro* data suggests that it is involved in the destabilisation and degradation of p53 leading to the loss of wild-type p53 and is a proposed mechanism of cisplatin resistance [277]. Dysregulation of PIK3/AKT pathway, as discussed in section 1.1.7.3, has also been linked to cisplatin resistance in a range of cancers including ovarian cancer [220, 274].

1.6.4 hCGβ and resistance to chemotherapy

The link between elevated levels of hCGβ and poor survival outcomes in patients with cancer is well studied [278]; however, its link to chemoresistance is still not

determined. Both patient studies and *in vivo* data exist to suggest that hCGβ may have a role in chemoresistance. Szturmowicz *et al*. aimed to determine whether the level of hCGβ in the serum of patients with small-cell lung cancer could be used a prognostic factor or used to reclassify the cancer into different subtypes [180]. They found that serum levels of hCGβ was elevated in 21 of 156 patients (14%) which correlated with poor survival outcomes (5% compared to 21% 2-year survival). What was interesting about their data is that 73% of patients with normal levels of hCGβ responded to chemotherapy, compared to 48% of patients with elevated hCGβ levels. This data suggests that hCGβ could be involved in resistance to chemotherapy.

A study by Berman *et al*. used xenografts of tumours established from patients with small cell lung cancer which had differing responsiveness to the chemotherapeutic drug cyclophosphamide. One chemosensitive xenograft (HX78) which was never exposed to cyclophosphamide was made resistant by repeated exposures to the drug [279]. They found that when the xenografts were maintained in culture the cyclophosphamide resistant (HX78Cy) line produced up to five times more hCGβ (detected in the media by radioimmune assay) compared to the parental chemosensitive line (HX78) [280]. This suggests that the levels of secreted hCGβ could be linked to chemoresistance.

1.7 Hypothesis and aims

Given that hCGβ is expressed by a number of epithelial cancers including ovarian cancer and evidence suggests that it has a biological role in some cancer cell line models, we sought to study the role of hCGβ in cellular processes in ovarian cancer cell lines.

Central hypothesis: hCGβ is expressed in HGSC cell lines and has a role in proliferation, migration, adhesion and sensitivity to platinum-based drugs.

Aim 1: Characterise the expression of *CGB* and secreted hCGβ levels in HGSC cell line models.

Aim 2: Determine the role of hCGβ on proliferation, migration and adhesion of HGSC cell lines.

Aim 3: Determine the role of hCGβ in sensitivity of HGSC cell lines to the platinum-based drugs cisplatin, carboplatin and oxaliplatin.

Aim 4: Determine the mechanism by which hCGβ may regulate sensitivity of HGSC cells to cisplatin treatment by detecting global protein changes using the quantitative proteomic technique isobaric tags for relative and absolute quantitation (iTRAQ).

Chapter 2 Material and Methods

2.1 Chemicals and Reagents

A list of chemicals and reagents used in this thesis are outlined in Table 2-1.

Table 2-1 List of chemicals and reagents

Item	Catalogue #	Manufacturer
Ampicillin Sodium Salt	A9518-5G	Sigma-Aldrich Pty. Ltd., Castle Hill, NSW, Australia
Agar	214010	Bacto Laboratories Pty. Ltd., Mt Pritchard, NSW, Australia
β-mercaptoethanol	M3148-25ML	Sigma-Aldrich Pty. Ltd., Castle Hill, NSW, Australia
3(N-Morpholino) propanesulfonic acid (MOPS)	M1254-1KG	Sigma-Aldrich Pty. Ltd., Castle Hill, NSW, Australia
Bromophenol blue	114391-25G	Sigma-Aldrich Pty. Ltd., Castle Hill, NSW, Australia
Carboplatin	C2538-100MG	Sigma-Aldrich Pty. Ltd., Castle Hill, NSW, Australia
Cell Gibco Cell Dissociation Buffer enzyme-free, PBS	13151-014	Life Technologies Corporation, Mulgrave, VIC, Australia
CellTiter 96® AQueous One	G3581	Promega, Alexandria NSW, Australia
Cisplatin	4319H	Hospira Australia Pty Ltd VIC , Australia
Cytosine beta-D-arabinofuranoside	C1768-100MG	Sigma-Aldrich Pty. Ltd., Castle Hill, NSW, Australia
Dimethyl Sulfoxide (DMSO)	67-68-5	Sigma-Aldrich Pty. Ltd., Castle Hill, NSW, Australia

RNase-Free DNase Set	79254	Qiagen Pty. Ltd., Chadstone, VIC, Australia
Dulbecco's Phosphate buffered saline (PBS)	21600-010	Life Technologies Corporation, Mulgrave, VIC, Australia
Ethanol, absolute	E7023-500ML	Sigma-Aldrich Pty. Ltd., Castle Hill, NSW, Australia
Ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid tetrasodium salt (EGTA)	E8145-10G	Sigma-Aldrich Pty. Ltd., Castle Hill, NSW, Australia
Ethylenediaminetetraacetic acid (EDTA)	0105-5009	Astral Scientific Pty. Ltd., Caringbah, NSW, Australia
Fetal Bovine Serum (FBS)	FBS500-S	AusgeneX Pty. Ltd., Oxenford, QLD, Australia
Glycerol	15524	Sigma-Aldrich Pty. Ltd., Castle Hill, NSW, Australia
Glycine	VWRC10119CU-5KG	Sigma-Aldrich Pty. Ltd., Castle Hill, NSW, Australia
Hybond-C-Extra Nitrocellulose Membrane	RPN303E	Crown Scientific Pty. Ltd., Minto, NSW, Australia
Methanol	5005-10L	Thermo Fisher Scientific Australia Pty. Ltd., Scoresby, VIC, Australia
NuPAGE Novex 4-12% Bis-Tris gels	Gel NP0321BOX	Life Technologies Corporation, Mulgrave, VIC, Australia
Opti-MEM® Reduced Serum Medium	31985-062	Life Technologies Corporation, Mulgrave, VIC, Australia
Oxaliplatin	O9512-5MG	Sigma-Aldrich Pty. Ltd., Castle Hill, NSW, Australia
Ponceau S	P7170-1L	Bio-Rad Laboratories Pty. Ltd., Gladesville, NSW, Australia
Propan-2-ol (isopropanol)	425-2.5L	Thermo Fisher Scientific Australia Pty. Ltd., Scoresby, VIC, Australia

Ribonuclease A (RNaseA)	R6513-10MG	Sigma-Aldrich Pty. Ltd., Castle Hill, NSW, Australia
RPMI 1640	R0278-50ML	Sigma-Aldrich Pty. Ltd., Castle Hill, NSW, Australia
SeeBlue® Plus2 Pre-stained Protein Standard	LC5925	Thermo Fisher Scientific Australia Pty. Ltd., Scoresby, VIC, Australia
Sodium dodecyl sulfate (SDS)	L3771-500G	Sigma-Aldrich Pty. Ltd., Castle Hill, NSW, Australia
Sodium chloride	BIOSB0476-5kg	Astral Scientific Pty. Ltd., Caringbah, NSW, Australia
Sucrose	S0389-1KG	Sigma-Aldrich Pty. Ltd., Castle Hill, NSW, Australia
Sodium fluoride	201154-5G	Sigma-Aldrich Pty. Ltd., Castle Hill, NSW, Australia
Sodium orthovanadate	S6508-10G	Sigma-Aldrich Pty. Ltd., Castle Hill, NSW, Australia
Triton® X-100	T9284-500ML	Sigma-Aldrich Pty. Ltd., Castle Hill, NSW, Australia
Tryptone	211705	Bacto Laboratories Pty. Ltd., Mt Pritchard, NSW, Australia
Trypsin-EDTA	T4049	Sigma-Aldrich Pty. Ltd., Castle Hill, NSW, Australia
Tween-20	P5927-500ML	Sigma-Aldrich Pty. Ltd., Castle Hill, NSW, Australia
Yeast extract	212750	Bacto Laboratories Pty. Ltd., Mt Pritchard, NSW, Australia

2.2 Commercial kits

A list of commercial kits used in this thesis is outlined in Table 2-2.

Table 2-2 List of commercial kits

Kit	Catalogue #	Manufacturer
10X Gene Expression Master Mix	4369016	Life Technologies Corporation, Mulgrave, VIC, Australia
MycoAlert™ Mycoplasma Detection Kit	LT07-318	Lonza, North Sydney, NSW, Australia
Cell Line Nucleofector® Kit L	VCA-1005	Lonza, North Sydney, NSW, Australia
Cell Line Nucleofector® Kit V	VCA-100V	Lonza, North Sydney, NSW, Australia
ECM Cell Adhesion Array Kit, colorimetric	ECM540	Merck Millipore, Bayswater, VIC, Australia
Chorionic Gonadotropin beta Human ELISA kit	ab108638	Abcam, Melbourne, VIC, Australia
PureYield™ Plasmid Midiprep System	A2495	Promega, Alexandria, NSW, Australia
Pierce® BCA Protein Assay	23227	Thermo Fisher Scientific Australia Pty. Ltd., Scoresby, VIC, Australia
RNeasy Kit	74034	Qiagen Pty. Ltd., Chadstone, VIC, Australia
Super Signal® West Dura Stable chemiluminescent substrate	PIE34075	Thermo Fisher Scientific Australia Pty. Ltd., Scoresby, VIC, Australia
Super Signal® West Pico Stable chemiluminescent substrate	PIE34080	Thermo Fisher Scientific Australia Pty. Ltd., Scoresby, VIC, Australia
SuperSignal™ West Femto Maximum Sensitivity Substrate	34095	Thermo Fisher Scientific Australia Pty. Ltd., Scoresby, VIC, Australia

Superscript III Reverse Transcription Kit	18080-400	Life Technologies Corporation, Mulgrave, VIC, Australia
X-tremeGENE 9 DNA Transfection Reagent	06365787001	Roche Products Pty. Ltd., Dee Why, NSW, Australia

2.3 Routine equipment

A list of equipment used routinely in this thesis is outlined in Table 2-3.

Table 2-3 List of Equipment

Equipment	Manufacturer
ABI 7900 HT Fast real-time PCR	Life Technologies/Applied Biosystems, USA
epMotion 5070	Eppendorf, Hamburg, Germany
Fijifilm LAS-4000 imaging system	Fijifilm Australia, Brookvale, NSW, Australia
Incucyte™ FLR Kinetic Imaging System	Essen Bioscience, MI , USA
Moxi Z	Gene Target Solutions Pty Ltd., Dural, NSW, Australia
NanoDrop ND-1000 spectrophotometer	NanoDrop Technologies, Wilmington DE, USA
Veritas™ Microplate	Promega Corporation, Alexandria, NSW, Australia
Victor Multilabel Plate Reader	Perkin Elmer, Australia

2.4 Cell lines

All cell lines used were classified as human serous epithelial ovarian cancer cells at the commencement of this thesis. OVCAR-3 and SKOV-3 cells were purchased from The American Type Culture Collection (ATCC, VA, USA). PEO1 cells were obtained from Dr S. P. Langdon (Cancer Research UK Centre, University of Edinburgh, Edinburg, UK). HEY and CaOV-3 cells were a gift from Prof A. DeFazio (Westmead Millenium Institute, Sydney, Australia). A2780 and A2780cis cells were a gift from Ms R. Harvey (Bill Walsh Cancer Laboratory, Kolling Institute of Medical Research, Australia). OV202 cells were a gift from Dr K. Kalli and Dr C. Conover (Mayo Clinic, Rochester, MN, USA). Characteristics and origin of the cell lines are outlined in Table 2-4.

During the course of this thesis, Domcke and colleagues published data identifying the preferred cell lines to use as models of high-grade serous ovarian cancer (HGSC) based on genomic data [281]. All preferred models, such as OVCAR-3 and CaOV-3, for study were *TP53* mutants. While A2780, HEY and SKOV-3 appeared as less preferred models for the study of HGSC. In agreement with Domcke *et al.*, Ince and colleagues published a paper in June this year, also indicating that the SKOV-3 and A2780 cell lines may not be preferable models for studying HGSC [282]. Substantial work had already been undertaken using these cell lines for this thesis and extensive data on these lines is reported. This is true not only for this thesis, but in the ovarian cancer literature where these cell lines are amongst those most frequently published in studies of HGSC. The A2780 / A2780cis pair are still regarded as excellent matched lines for studying developed drugged resistance. OV202 and PEO1 were not reported by Domcke and colleagues [281].

Table 2-4 Histology, origin and TP53 status of experimental cell lines

Cell line	Histology	Origin	*TP53* status
A2780	Undifferentiated carcinoma	Tumour [283]	Wild-type [281, 282]
A2780cis	Cisplatin resistant cells derived from A2780 cells	Tumour [284]	Wild-type
CaOV-3	Serous adenocarcinoma	Unknown [285]	Mutant [281, 286]
OV202	Serous epithelial ovarian cancer	Tumour [287]	unknown
OVCAR-3	Poorly differentiated papillary epithelial ovarian cancer	Ascites [288]	Mutant [281]
SKOV-3	Serous adenocarcinoma	Ascites [289, 290]	Null [282, 286]
PEO1	Poorly differentiated serous adenocarcinoma	Ascites [291]	Mutant [292]
HEY	Moderately differentiated papillary cystadenocarcinoma	Xenograft of a peritoneal deposit [293]	Wild-type [294]

2.5 Cell line maintenance

2.5.1 Cell line culturing conditions

All cell lines were grown in culture media composed of RPMI 1640 media supplemented with 10% foetal bovine serum (FBS). They were maintained in a humidified incubator at 37°C and 5% CO_2 and routinely cultured in 10 mL of culture media in T75 flasks unless stated otherwise.

2.5.2 Passaging cell lines

Cells were passaged when 80% confluent by washing them in 5 mL of phosphate-buffered saline (PBS) and incubating them in 2 mL of 0.25% trypsin/EDTA for 3 min at 37°C to detach the cells from the flask. Once detached, 5 mL of culture media was added to the flask to neutralise the trypsin and the cell suspension transferred to a 15 mL tube and centrifuged at 300 x g for 3 min. The supernatant was discarded and the cell pellet was resuspended in fresh culture media and replated in T75 flasks. PBS, trypsin/EDTA and culture media were warmed up to 37°C in a water bath prior to use. The cell lines were passaged twice weekly and the splitting ratio range is outlined in Table 2-5.

Table 2-5 Split ratio range for cell lines

Cell line	Split ratio range
A2780	1:20-1:30
A2780cis	1:20-1:30
CaOV-3	1:3-1:5
HEY	1:5-1:10
OVCAR-3	1:3-1:5
OV202	1:3-1:5
PEO-1	1:5-1:10
SKOV-3	1:3-1:5

2.5.3 Routine cell counting

On a routine basis cell number concentrations were determined using a haemocytometer or automatically counted using the Moxi Z.

2.5.4 Cryopreservation of cell lines

All cell lines were cryopreserved in liquid nitrogen. Cells growing in flasks were trypsinised, pelleted by centrifugation and resuspended in an appropriate amount of culture media containing 10% (v/v) of dimethyl sulfoxide (DMSO) to yield a cell concentration of 1 x 10^6 cells / mL. One mL of the cell suspension was aliquoted into a 2 mL cryrovial. Prior to long term storage, the cells in cryrovials were stored at -80°C in cell freezers containing isopropanol for at least 24 h before being transferred to liquid nitrogen tanks for long term storage. Cells were cultured from

liquid nitrogen stocks by defrosting them in a water bath at 37°C, resuspending the cells in culture media, pelleting the cells by centrifugation at 300 x g for 3 min, then resuspending them in fresh media before plating them into culture flasks.

2.5.5 Mycoplasma testing

One mL of conditioned media from cultured cells was routinely monitored for mycoplasma using the MycoAlet™ Mycoplasma detection kit. This assay relies on the conversion of ATP to ADP (by enzymes produced by Mycoplasma) giving a luminescent signal which was read on the Veritas^{TM} Microplate luminometer.

2.5.6 Cell typing

Authenticity of the cell lines was determined by CellBank Australia (Children's Medical Research Institute, Westmead, Australia) using an AmpFLSTR® Identifiler® PCR Amplification Kit.

2.6 Gene expression

2.6.1 RNA isolation

Total RNA was extracted using the RNeasy kit including a DNA digestion step as per the manufacturer's protocol. Briefly, culture media was removed from the cells and the proprietary RNA extraction buffer added directly to the cells. The RNA was precipitated with 70% ethanol, transferred to an RNA extraction column,

cleaned through a series of wash and centrifugation steps and finally eluted from the column with 30 µL of RNase and RNase-free water. DNA was digested on the column in the middle of the wash steps with DNase I in buffer RDD according to the manufacturer's protocol. RNA was stored at -80°C.

2.6.2 RNA and DNA quantification

The quality and quantity of RNA and DNA was determined by measuring absorbance at wavelengths 260 and 280 nm using the NanoDrop ND-1000 spectrophotometer. Two µL of the RNA sample was quantified and a ratio of 1.8-2.1 of A260/A280 was deemed acceptable to carry out qRT-PCR.

2.6.3 Complementary DNA synthesis

RNA was reverse transcribed into complementary DNA using the SuperScript® III First-Strand Synthesis System with oligo(dT) primers according to the manufacturer's protocol. Briefly, 0.2-5 ng of RNA was mixed with 1 µL of oligo(dT) and 1 µL annealing buffer and made up to 8 µL with RNase free water. The annealing reaction mix was incubated in a thermal cycler at 65°C for 5 min and rapidly cooled on ice for at least 1 minute. Ten µL of 2x First-Strand reaction mix and 2 µL SuperScript®III/RNAseOUT Enzyme was added to the annealing reaction mix and incubated for 50 min at 50°C followed by termination at 85°C for 5 min. The cDNA was then diluted 1:5 or 1:10 with RNase free water and stored at -20°C.

2.6.4 Quantitative RT- realtime PCR

Gene expression was determined by quantitative real-time reverse transcriptase PCR (qRT-PCR) using TaqMan gene expression assays. The efficiency of the PCR reaction for each assay was initially determined to be ~90% by absolute quantitation from a standard curve. The qRT-PCR reaction mix consisted of 10 μL of 2x reaction Master mix, 1 μL of 20x Taqman gene expression probe (Table 2-6 for probe details) and 5 μL of cDNA made up to 20 μL in RNase free water. Twenty μL of the reaction mix was pipetted in triplicate for each sample (cDNA) into a 96 well plate using the epMotion 5070 automated pipetting system. Five μl half reactions were prepared when a 384 well plate format was used. The PCR reaction was conducted on the ABI 7900 HT machine with an amplification program of 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min.

Relative gene expression was determined by delta-delta Ct analysis against the *HMBS* (hydroxymethylbilane synthase) reference gene using RQ Manager Software (Life Technologies).

Table 2-6 TaqMan Probes

Assay ID	Gene Symbol	Gene name
Hs00361224_gH	*CGB*	chorionic gonadotropin, beta polypeptide
Hs00751207_s1	*LHB*	luteinizing hormone beta polypeptide
Hs00609297_m1*	*HMBS*	hydroxymethylbilane synthase

*All TaqMan probes were obtained from Life Technologies Australia Pty Ltd, Mulgrave, VIC, Australia. *endogenous reference gene*

2.7 Protein expression

2.7.1 Detection of secreted hCGβ protein using enzyme-linked immunosorbent assay (ELISA)

The level of hCGβ secreted by the cell lines was determined using the Chorionic Gonadotropin beta Human ELISA kit which detects free hCGβ according to the manufacturer's protocol. Briefly, 50 µL of conditioned media and hCGβ standards were incubated overnight at 4ºC in ELISA well strips coated with anti-hCGβ capture antibodies. Excess sample was aspirated from the strips and the wells washed five times with 300 µL of deionized water. One hundred and fifty µL of anti-Chorionic Gonadotropin beta HRP conjugate was added and incubated for 30 min. The strips were then washed five times with 300 µL of deionized water and 100 µl TMB substrate added and incubated for 20 min. One hundred µL stop solution was then added to terminate the reaction. The absorbance was read at 450 nm on a spectrophotometer microplate reader Victor Multilabel Plate Reader. The concentration of protein was calculated based on the standard curve (range 0.25 ng/mL - 50 ng/mL). Refer to Appendix A, Supplementary Figure 1 for standard curve.

2.7.2 Western blotting

Protein expression from cell lysates was determined using western blotting. Cells growing in either flasks or well plates were washed in cold PBS and directly lysed in Laemmli buffer. Lysates were transferred to 1.5 mL Eppendorf tubes and sonicated with a probe for 30 sec to shred the DNA. The lysates were boiled at 95°C for 5 min

and 10-20 μL of the lysates were run against the SeeBlue® Plus protein protein standard (range 4-250 kDa) on precast 4-12% NuPAGE® Novex Bis-Tris gels for 1 h at 180 V in MOPS SDS buffer. Protein from the gel was transferred onto a nitrocellulose membrane for 90-120 min at 100V in cold blotting buffer. The membranes were then blocked in blocking buffer for at least 30 min, followed by incubation with primary antibody overnight at 4°C. Unbound primary antibody was washed off in wash buffer (3 x 10 min washes) and probed with Horseradish Peroxidase coupled (HRP) secondary antibodies in blocking buffer for 1 h before a final wash (3 x 10 min washes). Refer to Table 2-7 for composition of buffers used for western blotting and Table 2-8 for the antibodies used to probe proteins of interest. Bands were detected using chemiluminescence Super Signal ECL Pico, Dura or Femto reagent (in order of increasing sensitivity) on the Fujifilm LAS-4000 imaging system. The intensity of the bands was quantitated using Multi Gauge 3.0 software (Fujifilm Australia, Brookvale, NSW, Australia).

Table 2-7 Composition of buffers used for western blotting

Laemmli buffer		Running buffer		Blotting buffer		Blocking buffer		Wash buffer	
SDS	6% w/v	Tris	50 mM	Tris	25 mM	Tris pH 7.4	40 mM	Tris pH 7.4	20 mM
Sucrose	40% w/v	Glycine	384 mM	Glycine	152 mM	Skim milk	5%	NaCl	150 mM
Tris, pH 6.8	20 mM	SDS	0.1%	Methanol	20% v/v	Tween-20	0.1%	Tween-20	0.1%
Bromophenol blue	0.15% w/v								
β-mercaptoethanol	5% v/v								

Table 2-8 List of antibodies for western blotting

Antibody	Catalogue # and source	Produced in	Molecular weight (kDa)	Dilution
Anti-WAPL	ab109537, Abcam	Rabbit	133	1:50 000
Anti-LIM Kinase 1	ab108507, Abcam	Rabbit	73	1:1000
Anti-SIRT1	ab32441, Abcam	Rabbit	83 and 110 (ubiquitinated)	1:5000
Anti-hCGβ	SAB4500168-100UG, Sigma-Aldrich	Rabbit	17	1:1000
Anti-GAPDH	2118, Cell Signalling	Rabbit	39	1:10 000
Secondary Anti-rabbit IgG – HRP linked	NA934V, GE Healthcare Life Sciences	Donkey	Secondary Antibody	1:2500

2.8 siRNA downregulation using nucleofection

RNA interference (siRNA) was used to downregulate gene expression. In order to minimise off target effects, two siRNA targeting two different regions of the gene were used. siRNAs used are outlined in Table 2-9.

Table 2-9 List of siRNA used for nucleofection

siRNA	Catalogue #	Sequence of probe	Target gene
HS_CGB_4 FlexiTube siRNA	SI00344162	CACCACCATCTGTGCCGGCTA	*CGB*
HS_CGB_5 FlexiTube siRNA	SI03057607	CACCATGACCCGCGTGCTGCA	*CGB*
Hs_CGB_7 FlexiTube siRNA	SI03114580	TCCCTAGCACTGACGACTGA	*CGB*
Hs_SIRT1_2 FlexiTube siRNA	SI00098441		*SIRT1*
Hs_SIRT1_3 FlexiTube siRNA	SI00098448		*SIRT1*
AllStars	SI03650318		non-silencing control

All siRNAs were sourced from Qiagen Pty. Ltd., Chadstone, VIC, Australia. Note: siRNA against CGB do not discriminate against the different CGB genes.

Transfection was conducted by nucleofection using the Amaxa Nucleofector system which is based on cell electroporation. Briefly, cells at 80% confluency were harvested, counted, pelleted and resuspended in 100 µL of the proprietary transfection solution containing 7.5 µl of 20 µM of siRNA. Cells were then

electroporated using a specific program tailored to the cell type being transfected. The cell number, transfection solution and electroporation program for nucleofection of different cell lines is outlined in Table 2-10.

The level of downregulation of the gene transcript was determined 24 h post transfection by qRT-PCR (refer to section 2.6) and at the protein level 48 h post transfection, using either ELISA or western blotting (refer to sections 2.7.1 and 2.7.2, respectively).

Table 2-10 Transfection conditions using the Amaxa Nucleofector system

Cell line	Cell number	Solution	Program
HEY	1.5×10^6	V	U-023
A2780	3×10^6	L	T-020
A2780cis	3×10^6	L	T-020

The protocol to transfect HEY cells using the Amaxa nucleofection system had to be optimised as Lonza did not provide an existing protocol. Optimisation involved transfecting the cells with a green fluorescent protein (GFP) vector provided in the optimisation kit using a range of programs to achieve the highest GFP expression while maintaining cell viability.

HEY cells were passaged and grown to 80% confluence 2 days prior to transfection. Cells were harvested and 1.5×10^6 cells transfected with 2 μg of pmaxGFP vector (part of kit) in 100 μL of Solution V using the following programs: A-020, T-020, T-030, X-001 and X-005. Cells were then seeded into 6 well plates and observed using a florescent microscope 24 h after transfection.

Cell viability was unaffected by the different programs used to transfect the HEY cells using nucleofection; however, GFP expression did vary and the T-020 programme showed the highest level of GFP expression (Figure 2.1). The T-020 program was used for subsequent transfections of HEY cells.

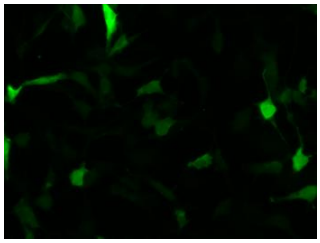
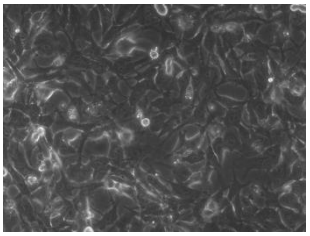
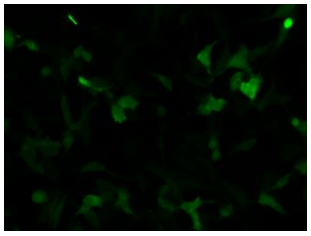
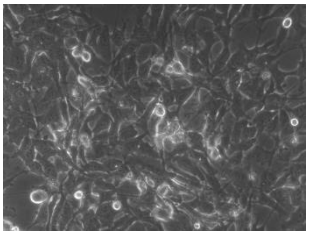
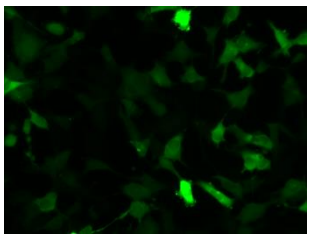
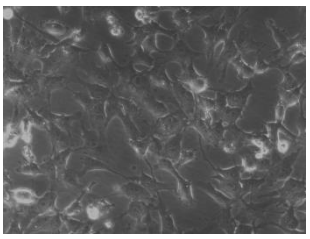
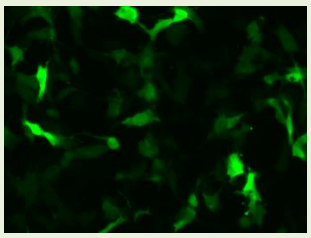
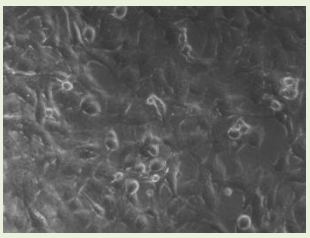
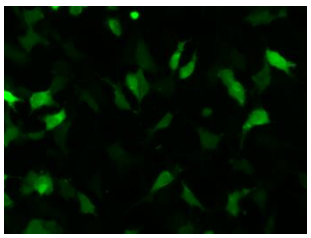
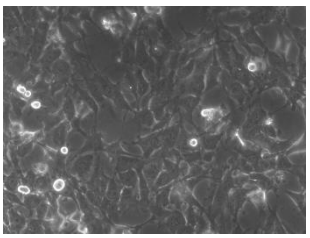
Program	Fluorescence	Phase-contrast
A-020		
T-030		
X-003		
T-020		
X-005		

Figure 2.1 Optimisation of transfection of HEY cells using Amaxa nucleofection

Expression of GFP vector *vs* cell viability 24h post transfection using the following programs: A-020, T-020, T-030, X-001, X-005. T-020 highlighted in green provided the optimum transfection efficiency.

2.9 hCGβ overexpression

hCGβ was transiently overexpressed in cells using plasmid DNA.

2.9.1 Plasmid preparation

2.9.1.1 Bacterial culture

The pCI-neo-hCG plasmid containing an hCGβ insert cloned into the *Xba*I and *Xho*I sites was purchased from Addgene in *E.coli* bacterial cells (Catalogue #16574, Addgene MA, USA). The hCGβ insert (534 base pairs) was generated from human placental cDNA amplified using the 5'−TGTGCTCTAGATCATGACCAAGG-ATGGAGATGTTCCAG−3' and 5'−GCACAGTCTAGATTATTGTGGGAGGAT-CGGG−3 primers and sequenced and cloned in the pCI-neo vector by Clontech, Palo Alto, California [295]. pC1-neo control vector also in *E.coli* were a gift from Dr S. Firth (Hormones and Cancer Laboratory, Kolling Institute of Medical Research, Australia). The stocks were struck out on Lysogeny broth (LB) agar plates (1% w/v NaCl and Tryptone, 0.5% w/v yeast extract, 1% w/v agar, pH 7.5) containing 0.1 mg/mL ampicillin and grown overnight at 37°C. A single colony was then picked and grown in 5 mL of LB broth (1% w/v NaCl and Tryptone, 0.5% w/v yeast extract, pH 7.5) containing 0.1mg/mL ampicillin overnight at 37°C in an orbital shaker.

2.9.1.2 Glycerol stock of bacterial strains

1 mL of the bacterial culture was pelleted, resuspended in 1 mL of LB broth containing 10% glycerol (v/v) and stocked at -80°C.

2.9.1.3 Plasmid extraction and DNA quantification

Fifty to 100 mL of LB broth was inoculated with the bacterial stocks containing plasmids and grown overnight at 37°C in an orbital shaker. Plasmid DNA was extracted from the bacteria using a PureYield™ Plasmid Midiprep System according to the manufacturer's protocol. Briefly, bacterial cells were pelleted and cells lysed with proprietary reagents provided in the kit. DNA was then extracted and purified through a column system and a series of wash (using proprietary reagents provided in the kit) and centrifugation steps. Finally, DNA was eluted from the column with 300 µL of Nuclease-Free Water and quantified using the NanoDrop™ spectrophotometer (section 2.6.2).

2.9.2 Plasmid transfection

One µg of pCI-neo-hCG and pCI-neo control plasmids were transfected into cell lines using a non-lipid based X-tremeGENE 9 DNA Transfection Reagent. Two x 10^5 of HEY cells and 4 x 10^5 of A2780 and A2780cis cells were plated in six well plates in 2 mL of culture media and after 18-24 h, transfection was carried out according to the manufacturer's protocol. Three µL transfection reagent, 97 µL Opti-MEM® I reduced serum medium and 1 µg of plasmid DNA were mixed and incubated for 15 min at room temperature (RT). The reaction mix was added to the 6 well plate containing cells and overexpression of intracellular hCGβ and secreted hCGβ was determined at 48 h using western blotting and ELISA respectively.

2.10 Statistical analysis

Statistical analysis was performed using SPSS Statistics software v 22 (IBM Australia Ltd., St Leonards, NSW, Australia). Statistical significance was determined using the following models:

- *t*-test to compare two sample means
- one-way analysis of variance (ANOVA) with LSD Post Hoc test to compare means of three or more samples
- two-way ANOVA to compare multiple levels of two factors with multiple observations at each level
- repeated measures ANOVA when comparing dose or time response curves

Data was presented as the mean ± Standard error of the mean (S.E.M) from at least three independent experiments. A *P* value of less than 0.05 was considered to be significant.

Chapter 3 Expression of hCGβ and its role in proliferation, adhesion and migration

3.1 Introduction

Presence of the β-subunit of hCG (hCGβ) has been reported in serum, malignant ascites, cyst fluid and tumour tissue from women with ovarian cancer [181, 183-185, 296]. Studies conducted by Ind *et al.* and Vartianen *et al.* showed that high serum levels of hCGβ corresponded to poor survival rate of patients with ovarian cancer [181, 185].

The fact that free hCGβ cannot bind to the receptor LHCGR without being associated with its α-subunit has led to the suggestion that it does not have a biological function and that it may be just a biological marker in epithelial cancers. Contrary to this idea, studies have shown that hCGβ may have effects on biological functions such as proliferation and migration in non-trophoblastic epithelial cancers distinct from the intact hCG heterodimer (Chapter 1, section 1.5). In this chapter, the expression of both *CGB* gene transcript and secreted hCGβ protein was determined in eight commonly studied HGSC cell lines. The role of hCGβ in proliferation, adhesion and migration was determined by transiently down regulating hCGβ by siRNA transfection, as well as overexpression of this protein.

3.2 Materials and Methods

Refer to Chapter 2, section 2.5 for details on how cells were grown, harvested and counted.

3.2.1 Expression of *CCA* and *CGB* transcripts in HGSC cells

Cells were grown until 80% confluent in six well plates in culture media before total RNA was extracted and the *CCA* and *CGB* transcript levels (encoding hCGβ and hCGα proteins) determined by qRT-PCR. Methodological details of RNA isolation, cDNA preparation and determination of the levels of hCGβ transcript by qRT-PCR are described in Chapter 2, section 2.6.

3.2.2 Determining secreted hCGβ protein levels in conditioned media

SKOV-3, HEY, OV202, PEO-1, A2780, A2780cis, CaOV-3 and OVCAR-3 cells were grown until 80% confluent in T25 flasks and serum starved for 18-24 h in 4 mL of RPMI containing 0.1% bovine serum albumin (BSA). Fifty μL of conditioned medium from each cell line was then assayed using the Chorionic Gonadotropin beta Human ELISA kit described in Chapter 2, section 2.7.1.

3.2.3 Downregulation and overexpression of hCGβ

hCGβ was downregulated or overexpressed in HEY, A2780 and A2780cis according to the protocols described in Chapter 2, sections 2.8 and 2.9 respectively.

3.2.4 Proliferation assay

Given that hCGβ has been shown to increase cell proliferation in bladder cancer cells and normal ovarian surface epithelial cells [155, 191], the effect of manipulating hCGβ levels on proliferation of three HGSC cell lines HEY, A2780 and A2780cis was studied.

3.2.4.1 Effect of hCGβ downregulation and overexpression on cell proliferation

Twenty-four hours post transfection with siRNA or plasmid, 1 x 10^5 A2780 and A2780cis cells (in 5 mL culture media) and HEY (in 10 mL of culture media) were seeded into a T25 or T75 flask respectively. Cells were allowed to grow for 3 and 6 days with culture media replenished every 2 days. After day 3 or day 6, cells were harvested, resuspended in 0.5-2 mL of culture media and the cell number determined using a Coulter counter (Ac·T diff Analyzer, Beckman Coulter, Sydney, NSW Australia).

3.2.4.2 Effect of exogenous hCGβ downregulation on cell proliferation determined by MTS viability assay

The effect of exogenous recombinant hCGβ on cell proliferation was determined using the colorimetric CellTiter 96® AQueous One Solution (MTS) cell viability assay over 4 days. Recombinant hCGβ expressed in *Pichia pastoris* was purchased from Sigma-Aldrich. The MTS assay is based on the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay and works on the principle of viable cells converting the active component, a tetrazolium compound called 3-(4,5-

dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) [297], from a yellow colour to soluble formazan which is a deep brown/maroon colour. The absorbance of formazan is measured at 490 nm and is proportional to the number of viable cells.

Compared to cell counts, the MTS assay can underestimate changes in cell number therefore changes in proliferation can go undetected. Furthermore, RPMI media has been shown to reduce MTS which can diminish the results of the assay[298]. However the assay has an advantage over cell counts used in section 3.2.4.1 as it allows for higher throughput and the effect of a range of hCGβ concentrations could be tested at one time. HEY, A2780 and A2780cis cells were seeded in triplicate in 100 µl of culture media into a 96-well plate (Table 3-1 for seeding densities). Six hours after seeding, cells were treated with 100 µl culture media containing hCGβ in 0.1% BSA at 10 X, 25 X and 50 X the basal endogenous concentration of hCGβ expressed by the individual cell lines (Table 3-1 summarises the concentrations of hCGβ used). The culture media of untreated cells contained 0.1% BSA as a vehicle control. The cells were incubated for 4 days at 37 ºC and 5% CO_2. After 4 days, the percentage of viable cells was determined by the addition of the MTS reagent to each well at a dilution of 1:5. After an incubation period of 1-2 h for HEY cells and 2-3 h for A2780 and A2780cis cells at 37 ºC and 5% CO_2, the absorbance was measured at 490 nm on the Wallac Victor 1420 Multilabel Counter. The background absorbance from blank wells was subtracted from the wells that contained cells. The percentage of viable cells indicative of proliferation was calculated as follows:

$$\%\ viability = \frac{absorbance\ of\ hCG\beta\ treated\ cells}{absorbance\ of\ untreated\ cells} \times 100$$

Prior to performing the MTS cell viability assay, the optimal cell number required to produce a reading above the blank reading at 490 nm, was determined. Cells were seeded at a density of 0.5- 5 x10^3 cells per well in triplicate in a 96 well plate and allowed to grow for 96 h (marking the end of the viability assay) at which point the MTS reagent was added. The aim was to achieve a cell number that would give an absorbance reading between 0.1 and 0.5 within 1- 4 h.

Table 3-1 Seeding density of cell lines and concentration of hCGβ for cell proliferation assay

Cell line	Seeding density / per well	Concentration of hCGβ (ng/mL)			
		Basal level	**10 X**	**25 X**	**50 X**
HEY	1 x 10^3	4	40	100	200
A2780	2.5 x 10^3	0.9	9	22.5	45
A2780cis	2.5 x 10^3	0.6	6	15	30

Note: Basal levels were determined in section 3.2.2.

3.2.5 Wound healing assay

The wound healing assay was used to determine cell migration 48 h after siRNA or plasmid transfection. HEY cells were seeded at 5 x 10^4 in 250 µL of culture media in 96-well plates and allowed to form a confluent layer overnight at 37°C in 5%

CO_2. Once the confluent layer was formed, 150µl of condition media was removed from individual wells and reserved in a separate 96-well plate. A scratch was then made to the confluent layer using the Incucyte WoundMaker (Essen Bioscience) tool. Loosened cells were washed off with 200 µL of warm PBS (twice). The remaining cells were replenished with the conditioned media with 5 µM of the cell proliferation inhibitor cytosine β-D-arabinofuranoside. Note that the conditioned media was used in order to take into account secreted levels of hCGβ. The plate was placed into an Incucyte™ FLR Kinetic Imaging System and the Incucyte™ software was programmed to generate phase-contrast photographs of the scratched region at 3 h time intervals for up to 48 h. The Incucyte™ software was then used to automatically determine the percentage of wound closure in individual wells, at a given time point relative to the size of the wound at time zero.

3.2.6 Cell adhesion assay

Cell adhesion was studied in HEY and A2780cis cells using the ECM Cell adhesion Array colorimetric kit according to the manufacturer's protocol. The kit consisted of 12 x 8-well strips percolated with an extracellular matrix (ECM) protein: Collagen I (ColI), Collagen II (ColII), Collagen IV (ColIV), Fibronectin (FN), Laminin (LN), Tenascin (TN), Vitronectin (VN) and BSA as a negative control, as well as Assay, Cell Stain, and Extraction buffers.

Forty-eight hours after siRNA or plasmid transfection, cells growing in a T25 flask were harvested and resuspended into a single cell suspension before being plated onto the ECM matrix proteins. Specifically, cells were washed with warm PBS and detached from the flask by incubating them with 4 mL of Cell Gibco Cell

Dissociation Buffer enzyme-free PBS for 20 min at RT. Cell clumps were broken by gently pipetting the cells up and down with a 5 mL pipette and the cell suspension was transferred to a 15 mL centrifuge tube and cells pelleted by centrifugation at 300 x g for 3 min. The pellet was washed in 2 mL of warm PBS to remove residual serum proteins from the growth media and re-pelleted by centrifugation. This wash step was repeated once more before the cells were resuspended in the Assay buffer, counted and diluted in an appropriate volume of Assay buffer to attain a density of $5x10^5$ and $1x10^6$ cells/mL of HEY and A2780cis cells, respectively. One hundred µl of the cell suspension was added (in duplicate) to ECM coated wells and incubated at 37°C in 5% CO_2 for 1 and 2 h for HEY and A2780cis cells, respectively. After the cells had adhered, the Assay buffer was gently aspirated and unattached cells were washed off with 200 µL of Assay buffer (repeated one more time).

The remaining attached cells were fixed and stained for 5 min at RT with 100 µL of Cell Stain solution. Excess stain was washed off with 200 µL of deionised water (repeated four times) and wells were left to air dry. The cell-bound stain was solubilised by the addition of 100 µL of Extraction buffer to each well and placed on a shaker for 5 min. Absorbance at 560 nm was measured with the Victor Multilabel Plate Reader. The absorbance readings, which were proportional to the amount of cells that had attached to the matrix, were plotted on a bar graph.

Prior to the adhesion assay, the ideal cell number to form a confluent layer and time required for the cells to adhere to the ECM surfaces were optimised. Cell suspensions of 0.5-2 x 10^5 of HEY and A2780cis cells were prepared in Assay buffer as described in the above section and incubated on the ECM matrix proteins to attain a confluent layer within 1-2 h on at least one matrix protein. An optimum cell

number of 5 x 10^4 and 2 x 10^5 and incubation times of 1 and 2 h were determined for HEY and A2780cis cells, respectively.

3.3 Results

3.3.1 Expression of *CGB* transcript and secreted hCGβ in HGSC cell lines

All eight HGSC cell lines: SKOV-3, HEY, OV202, PEO-1, A2780, A2780cis, CaOV-3 and OVCAR-3 expressed hCGβ at both the transcript and protein levels to varying degrees (Figure 3.1). qRT-PCR data normalised to cDNA from SKOV-3 cells showed that HEY cells expressed the highest level of *CGB*, followed by SKOV-3 cells. OV202, PEO1, A2780, A2780cis, CaOV3 and OCAR-3 cells expressed considerably lower levels of *CGB* compared to HEY and SKOV-3 cells (Figure 3.1 A). Notably, the A2780cis cells had much lower *CGB* expression than the parental A2780 cell line.

The level of basal secreted hCGβ determined by ELISA (Figure 3.1 B) from conditioned media revealed a similar relative expression pattern to the transcript levels, specifically SKOV-3 (13.80 ± 0.82 ng/mL) and HEY (3.94 ± 0.34 ng/mL) cell lines produced significantly higher levels of hCGβ compared to OV202 (0.12 ±0.01 ng/mL), PEO1 (1.39 ± 0.57 ng/mL), A2780 (0.95 ± 0.19 ng/mL), A2780cis (0.40 ± 0.02 ng/mL), CaOV-3 (0.23 ± 0.03 ng/mL) and OVCAR-3 (0.15 ± 0.02 ng/mL) cell lines. There were however some notable differences in protein expression levels compared to transcript levels. SKOV-3 cells produced significantly higher levels (more than 4 times) hCGβ protein compared to HEY cells, whereas HEY cells expressed 1.4 times more *CGB* transcript compared to SKOV-3 cells. Although OV202 cells expressed at least 2 times higher levels of *CGB* transcript compared to PEO1, A2780 and A2780cis cells, they secreted lower levels of hCGβ protein.

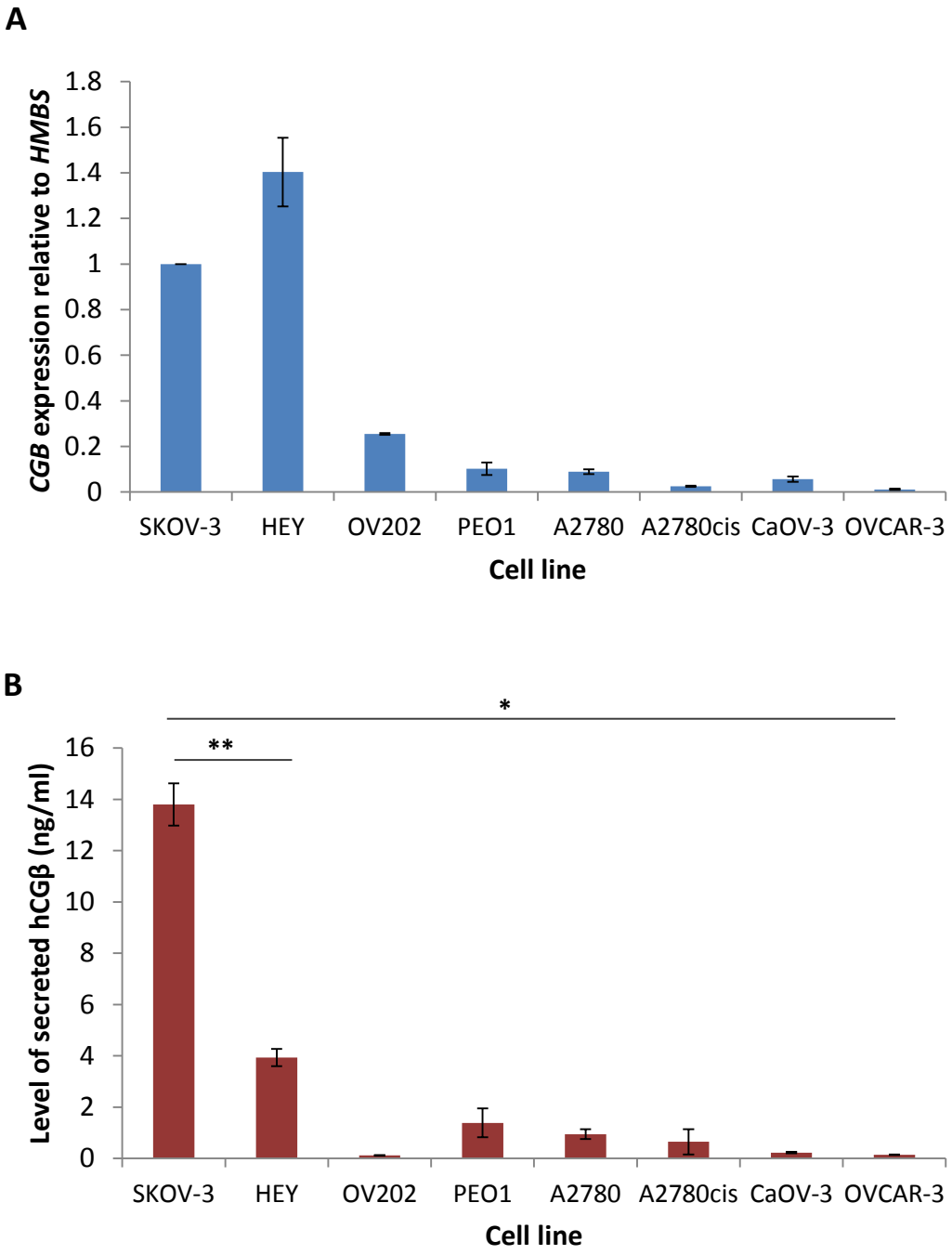


Figure 3.1 Expression of *CGB* transcript and secreted hCGβ in HGSC cell lines

Expression of *CGB* and hCGβ in 8 HGSC cell lines (A) *CGB* transcript level relative to *HMBS*, determined by qRT-PCR normalised to *CGB/HMBS* expression in SKOV-3 cells. (B) Secreted hCGβ protein levels determined by ELISA. Data are expressed as mean ± SEM; (N=3). * $P < 0.05$, ** $P < 0.005$.

3.3.2 Expression of *CGA* in HGSC cell lines

Analysis of expression of *CGA* was also carried out in the eight HGSC cell lines to determine if the cells could potentially produce whole hCG protein that would require the presence of both subunits. All cell lines expressed some level of *CGA* (Figure 3.2). Expression levels of *CGA* in the cell lines were normalised to levels in A2780 cells. *CGA* expression for CaOV-3 cells is not shown on the graph as they expressed 660 times more *CGA* compared to A2780 cells.

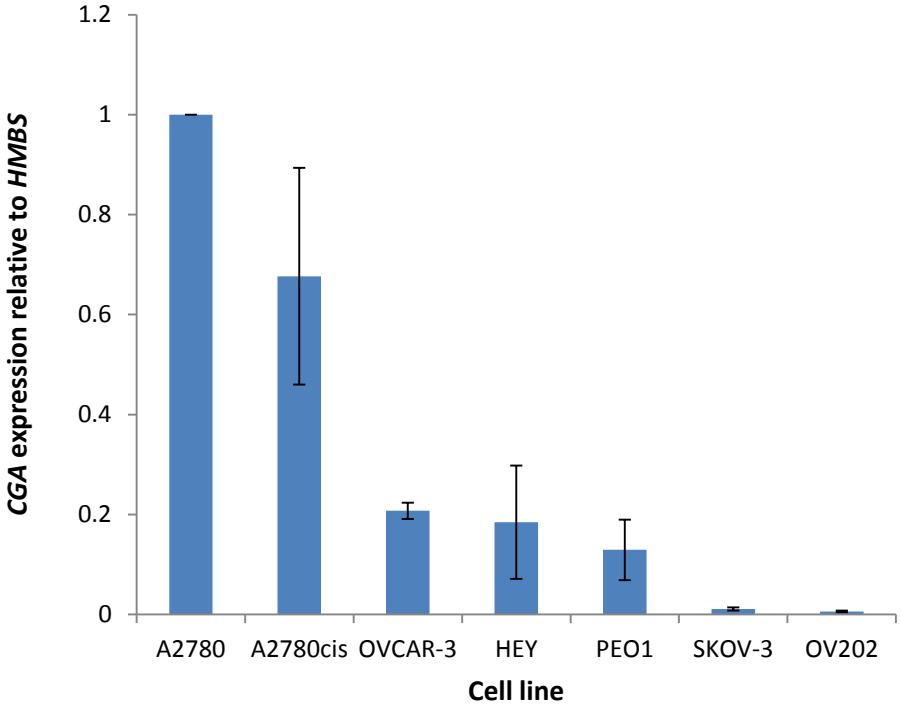


Figure 3.2 Expression of *CGA* in HGSC cell lines normalised to expression in A2780 cells

Expression of *CGA* transcript level relative to *HMBS*, determined by qRT-PCR normalised to *CGA/HMBS* expression in A2780 cells. Data are expressed as mean ± SEM; (N=3). The relative expression value for CaOV-3 cells (not shown) was 660 ± 328.

The degree of expression of *CGA* and *CGB* transcripts in the cell lines was different. The expression level of *CGA* transcript in the order of highest to lowest expression level was: CaOV-3> A2780> A2780cis> HEY> OVCAR-3> PEO1> SKOV-3> OV202 whereas the expression level of the *CGB* in the order of highest to lowest expression level was SKOV-3> HEY> OV202> PEO1> A2780> A2780cis> CaOV-3> OVCAR-3. The most striking differences were that: 1. CaOV-3 cells which expressed one of the lowest levels of *CGB*, expressed the highest level of *CGA;* 2. SKOV-3 cells which expressed the second highest level of *CGB* expressed almost no *CGA*, and 3. HEY cells which expressed the highest level of *CGB* and higher levels compared to the A2780 and A2780cis cells expressed lower amounts of *CGA* compared to A2780 and A2780cis cells. Interestingly, A2780 expressed higher levels of both the *CGA* and *CGB* transcript compared to A2780cis cells.

3.3.3 Downregulation of hCGβ

Twenty-four hours after siRNA transfection, an average reduction of 50-60% in *CGB* transcript was achieved in HEY, A2780 and A2780cis cells using either of two hCGβ targeting siRNAs: CGB_4 and CGB_5 (Figure 3.3 A). CGB_4 siRNA caused a reduction of 65, 55 and 60% of *CGB* in HEY, A2780 and A2780cis cells, respectively. A reduction of 55, 60, and 60% of *CGB* in HEY, A2780 and A2780cis cells, respectively was achieved with the CGB_5 siRNA.

The secreted hCGβ level in conditioned media was determined in HEY cells 48 h post transfection using ELISA, and indicated a 50% downregulation of hCGβ with both CGB_4 and CGB_5 siRNAs (Figure 3.3 B). Due to limited cell number after siRNA transfection, the level of secreted hCGβ in A2780 and A2780cis was below

the detectable thresh-hold of the ELISA assay (as reflected in Figure 3.1 B) hence hCGβ downregulation at the protein level could not be determined. Each transfection yielded 1.5 x 10^6 of A2780 and A2780cis cells and ~ 6 x 10^6 cells were needed to produce secreted hCGβ protein levels which could be detected by the ELISA kit.

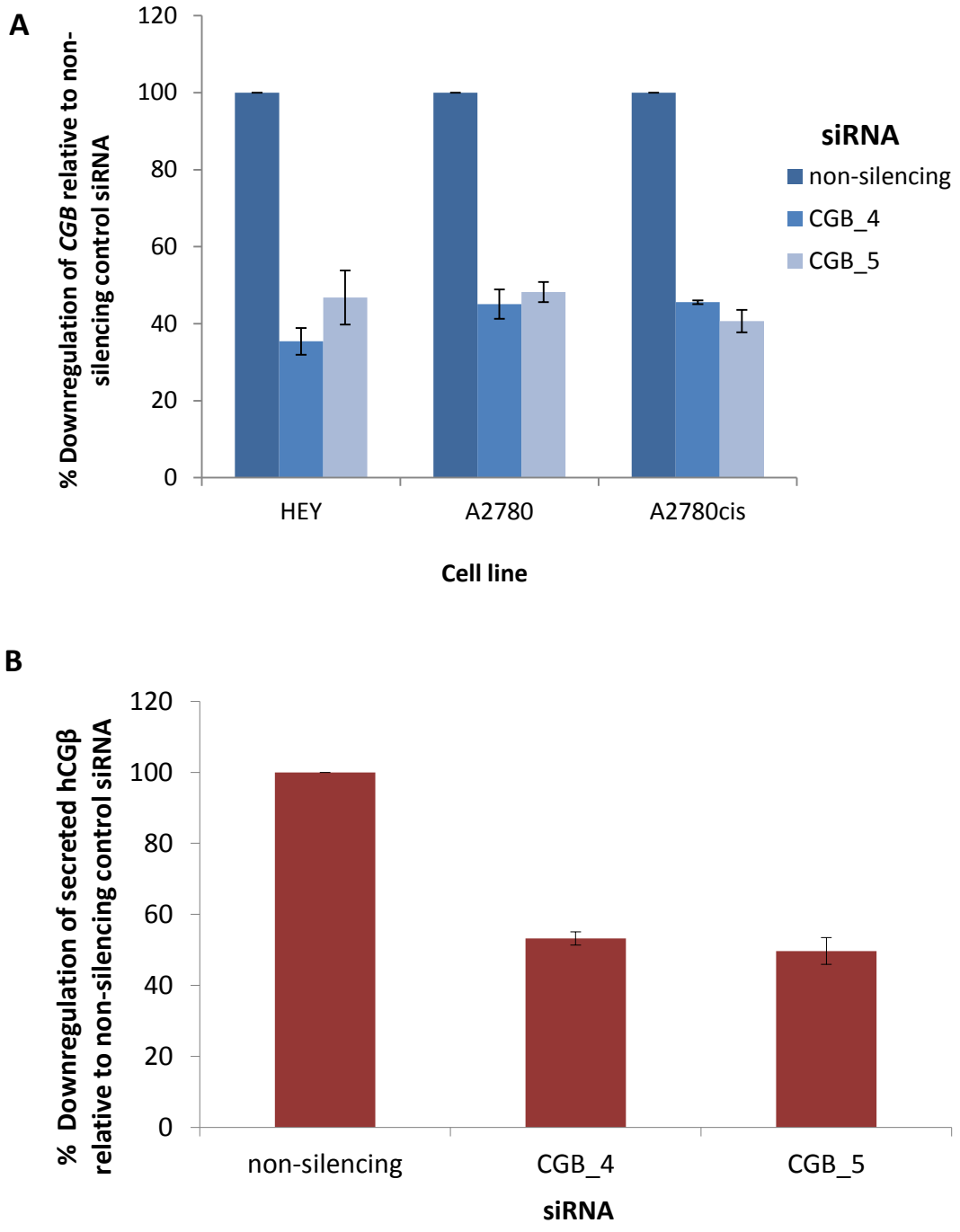


Figure 3.3 Downregulation of *CGB* and secreted hCGβ

CGB transcript level (A) in HEY, A2780 and A2780cis cells determined 24 h post transfection by qRT-PCR and secreted hCGβ protein level (B) in HEY cells, determined 48 h post transfection by ELISA on conditioned media. Secreted hCGβ could not be detected in conditioned media from A2780 and A2780cis cells, likely due to the small number of cells used for these assays. siRNAs: non-silencing control, CGB_4 and CGB_5. Data normalised to negative non-silencing control siRNA and expressed as mean ± S.E.M; N=3.

3.3.4 Effect of hCGβ downregulation on cell proliferation

Cell proliferation was determined following hCGβ downregulation at 3 and 6 days in HEY, A2780 and A2780cis cells. A general trend of reduction of cell proliferation was observed when hCGβ was downregulated (Figure 3.4).

In HEY cells (Figure 3.4 A), there was a significant reduction in cell proliferation following siRNA treatment (ANOVA; $P = 0.013$), and the post hoc test revealed that both CGB_4 and CGB_5 siRNAs caused a significant reduction in proliferation ($P = 0.026$ and $P = 0.005$, respectively). Specifically, a reduction in cell number of 25 and 10% was observed using the CGB_4 siRNA at day 3 and 6 respectively, and a 10% reduction was observed at both day 3 and 6 using the CGB_5 siRNA.

In A2780 cells (Figure 3.4 B), a statistically significant difference in cell proliferation was observed with siRNA treatment ($P = 0.01$) however, the post hoc test revealed that this reduction was statistically significant only when the CGB_5 siRNA was used (ANOVA; $P = 0.004$). Specifically, a reduction in cell number of 40 and 30% at day 3 and 6 respectively was observed using the CGB_5 siRNA.

In A2780cis cells (Figure 3.4 C) there was a significant reduction in cell proliferation following siRNA treatment (ANOVA; $P = 0.01$) and the post hoc test revealed that both CGB_4 and CGB_5 siRNAs showed a significant reduction in proliferation ($P = 0.004$ and $P = 0.016$, respectively). Specifically, a reduction in cell number of 50 and 25% was observed using the CGB_4 siRNA at day 3 and 6, and 40 and 20% at day 3 and 6 respectively using the CGB_5 siRNA.

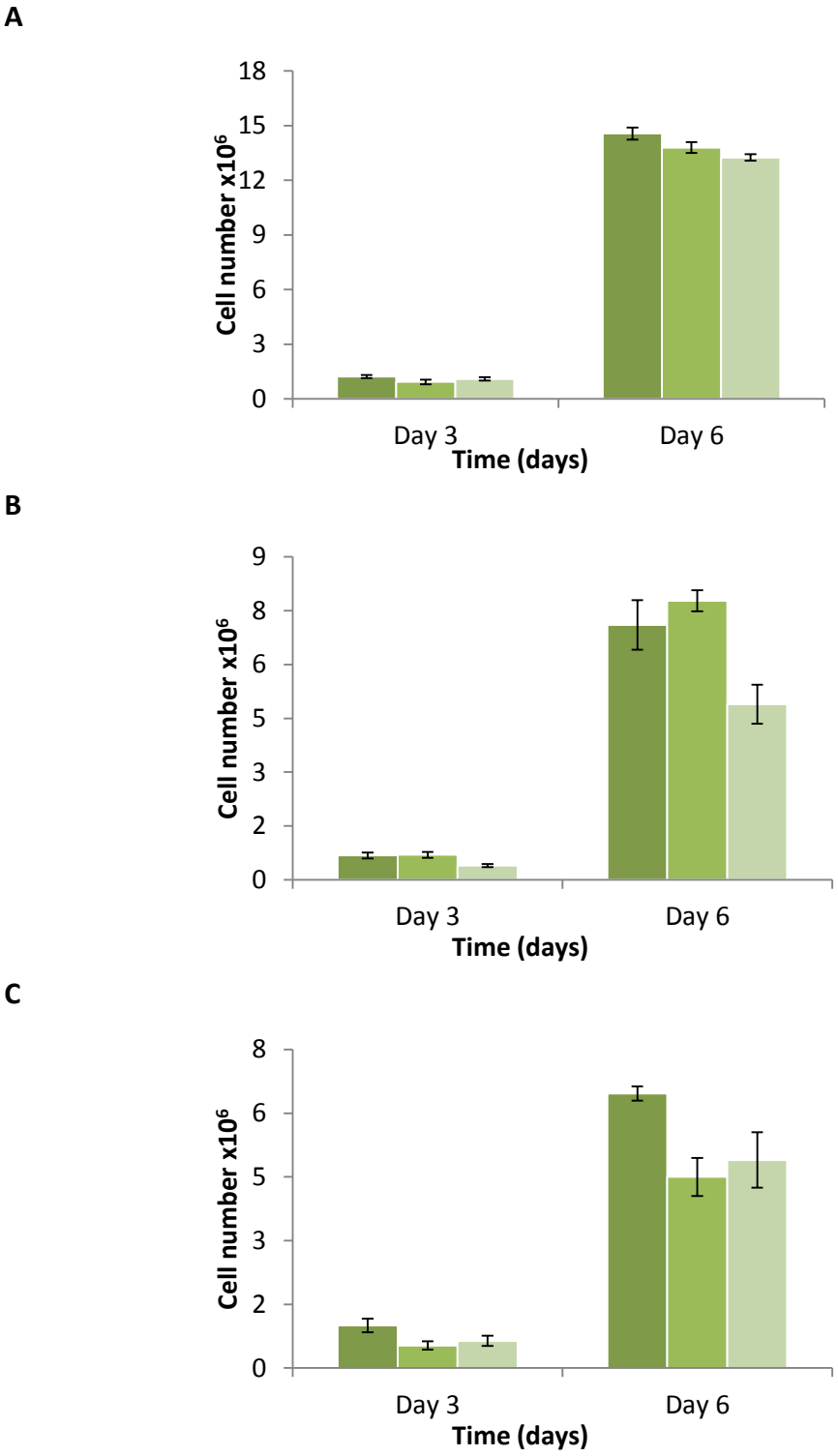


Figure 3.4 Effect of downregulation of hCGβ on cell proliferation

Effect of down regulating hCGβ on proliferation of HEY (A), A2780 (B) and A2780cis (C) cells. siRNAs: negative control non-silencing (■),CGB_4(■), and CGB_5 (■) . Results expressed as mean ± S.E.M; N=3. A significant reduction in cell proliferation following hCGβ downregulation was observed in HEY (P = 0.013) and A2780cis (P = 0.01) using ANOVA.

3.3.5 Effect of hCGβ downregulation on cell adhesion

The effect of downregulation of hCGβ on cell adhesion to plates coated with different matrix proteins was examined in A2780cis and HEY cells. A2780cis did not attach to any of the collagen matrixes hence these matrixes were omitted from further examination. A general increase in cell adhesion was observed in both HEY and, more notably, in A2780cis cells when hCGβ was downregulated (Figure 3.5). In both HEY and A2780cis cells there was no significant difference in cell adhesion between the different ECM matrixes. However, when cell adhesion was analysed across all seven matrixes there was a significant difference ($P < 0.0005$) when hCGβ was downregulated in HEY cells with either CGB_4 or CGB_5 siRNAs.

When the data were normalised to the non-silencing negative control siRNA the differences were more clear than the raw data (Figure 3.6). Cell adhesion increased by 10% on Fibronectin, Laminin and Vitronectin and by 20% on Tenascin in A2780cis when hCGβ was downregulated (Figure 3.6 A). However, the observed increase was not statistically significant due to high variation between experimental runs which could have been due to the high variability in the number of cells that attached to the ECM surfaces between experimental runs and the level of stain taken up by the cells. Statistical analyses were performed using *t*-test, comparing adhesion between cells treated with non-silencing control siRNA and CGB_4 or CGB_5 siRNA. It is possible that increasing the number of replicates may have reached statistically significant differences. The increase in cell adhesion observed in HEY cells (Figure 3.6 B) when hCGβ levels were decreased was more noticeable compared to A2780cis cells but varied between the CGB_4 and CGB_5 siRNA. When CGB_4 was used to downregulate hCGβ the increase in cell adhesion was as

follows: 10% on Collagen I, 30% on Collagen II and IV, Fibronectin, Laminin, and Vitronectin, and a 50% increase on Tenasin. When CGB_5 was used the increase in adhesion was as follows: 40% on Collagen I and II, 80% on Collagen and Tenasin, 50% on Fibronectin and Laminin and 60% on Vitronectin. As was the case for A2780cis cells, these increases did not reach statistical significance.

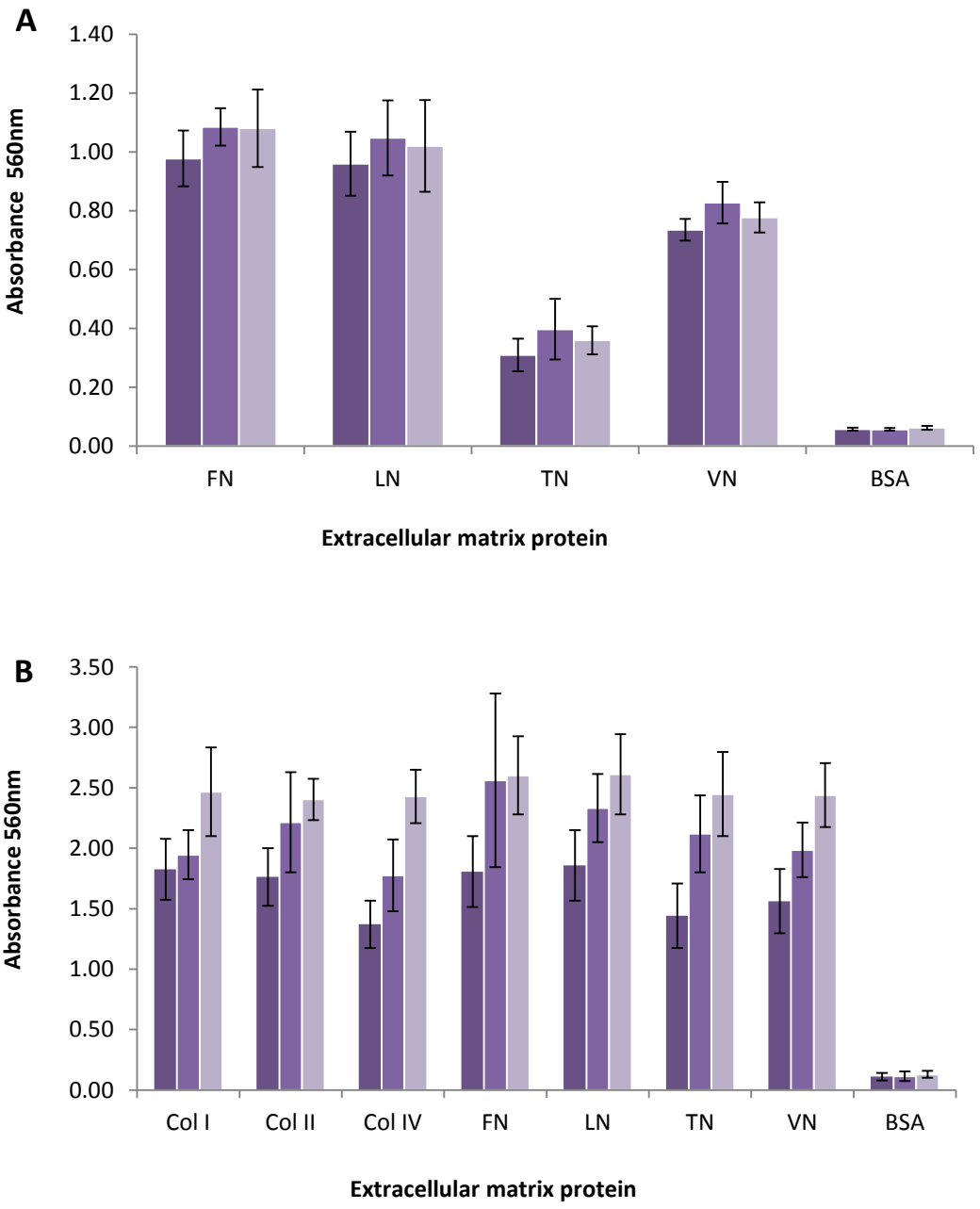


Figure 3.5 Effect of hCGβ downregulation on cell adhesion

Effect of downregulation of hCGβ on cell adhesion to extracellular matrix proteins of A2780cis (A) and HEY (B) cell lines, 48 h post siRNA transfection. siRNAs: non-silencing control (■),CGB_4(■),CGB_5(■). Collagen I (Col I), Collagen II (Col II), Collagen 1 (Col IV), Fibronectin (FN), Laminin (LN), Tenasin (TN), Vitronectin (VN). Results are expressed as mean ± S.E.M; N=3. Statistical test: two-way ANOVA. When cell adhesion was analysed across all seven matrixes in HEY cells there was a significant difference ($P < 0.0005$) when hCGβ was downregulated with either CGB_4 or CGB_5 siRNAs.

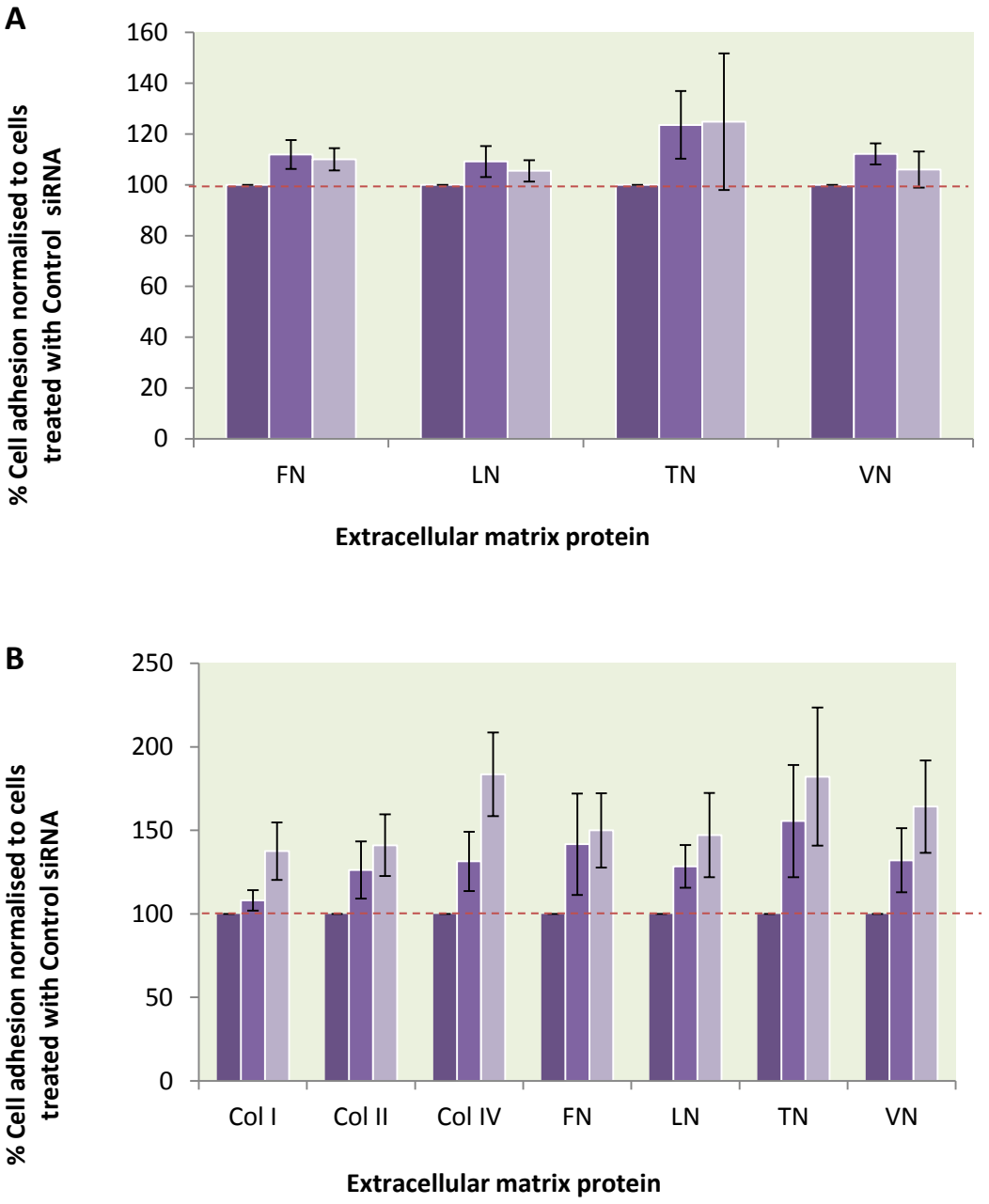
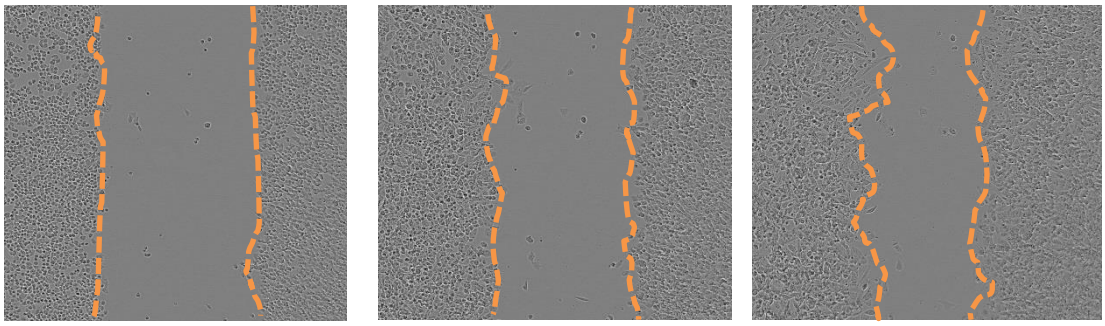


Figure 3.6 Effect of hCGβ on cell adhesion: data normalised to non-silencing negative control

Effect of downregulation of hCGβ, on cell adhesion to extracellular matrix proteins of A2780cis (A) and HEY (B) cell lines, 48 h post siRNA transfection. Data normalised to cells treated with negative control non-silencing siRNA. siRNAs: non-silencing control (■),CGB_4 (■) , CGB_5(■).Collagen I (Col I), Collagen II (Col II), Collagen 1 (Col IV), Fibronectin (FN), Laminin (LN), Tenasin (TN), Vitronectin (VN). Results expressed as mean ± S.E.M; N=3. Red dotted line indicates 100%.

3.3.6 Effect of hCGβ downregulation on cell migration

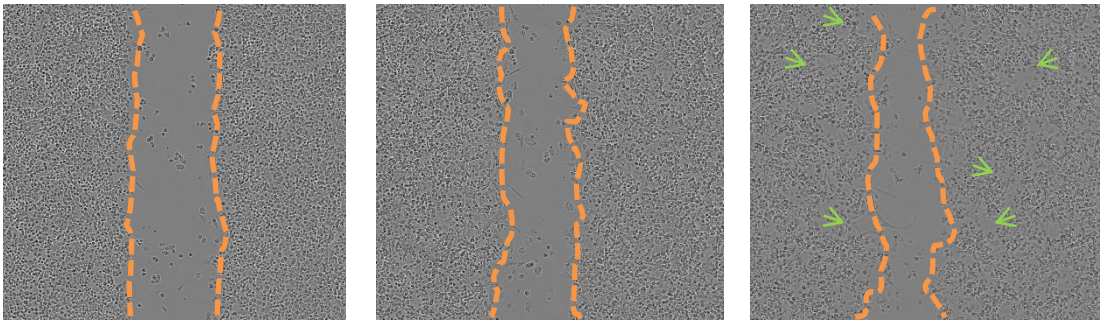
Migration was measured by the rate of closure of a wound made in the cell monolayer. A2780 and A2780cis did not migrate or migrated to a maximum wound closure of 20% during the 48 h period of the assay, after which time they started dying (Figure 3.7 and Figure 3.8, respectively). Due to this limited ability for A2780 and A2780cis cells to migrate despite hCGβ downregulation, migration was studied only in HEY cells.



0 h **24 h** **48 h**

Figure 3.7 Wound closure in A2780 cells over 48 h

Representative images from the Incucyte of wound closure in A2780 cells at 0 h, 24 h and 48 h. Outline of the wound depicted by dashed orange line.



0 h **24 h** **48 h**

Figure 3.8 Wound closure in A2780cis cells over 48 h

Representative images from the Incucyte of wound closure in A2780cis cells at 0 h, 24 h and 48 h. At 48 h cells can be seen to be lifting off the plate forming patches in the confluent layer (indicated by green arrows). Outline of the wound depicted by dashed orange line.

In HEY cells, downregulation of hCGβ did not seem to affect cell migration (Figure 3.9).

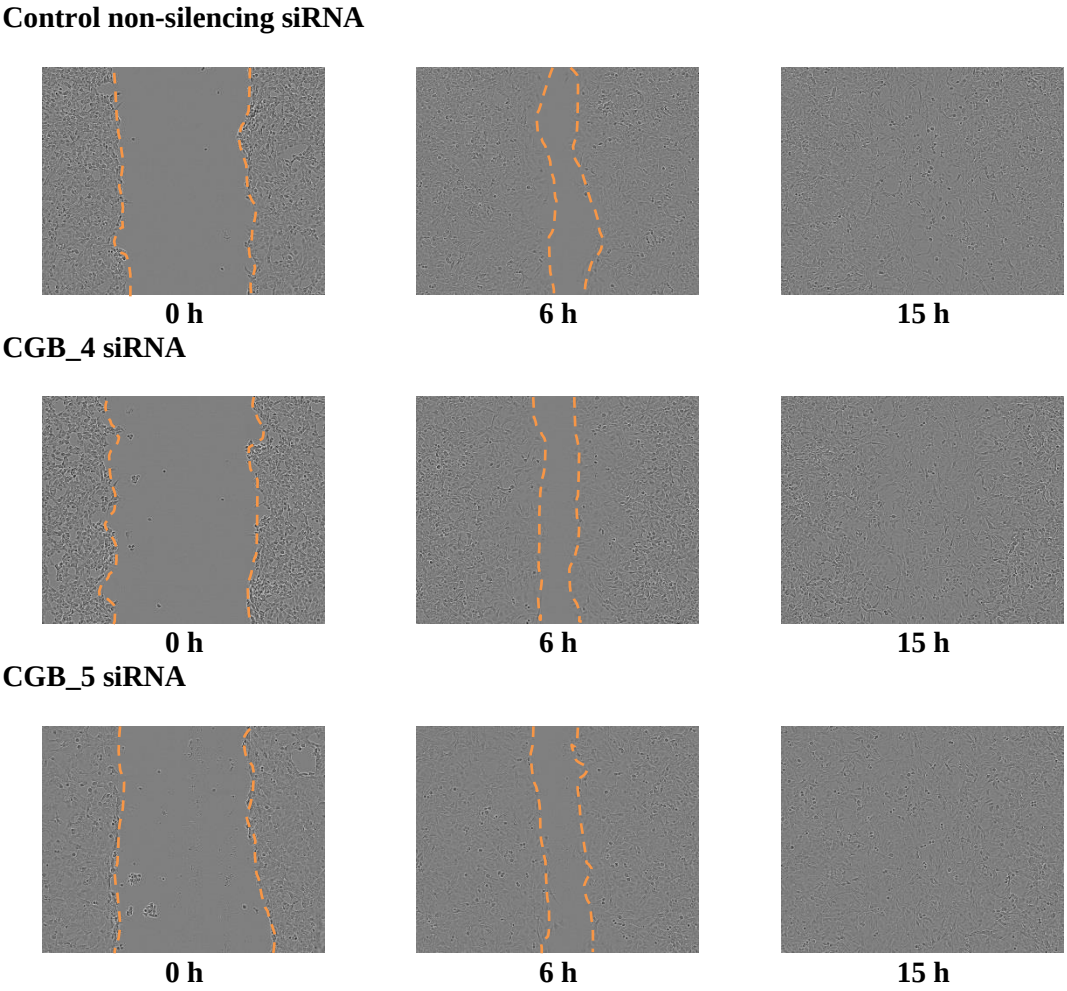


Figure 3.9 Wound closure in HEY cells over 15 h following hCGβ downregulation

Representative images from the Incucyte of wound closure in HEY cells at 0 h, 6 h and 15 h, 24 h post siRNA transfection. Outline of the wound depicted by dashed orange line.

However, a small increase in migration, 4-5% at 10 h, was seen as a result of downregulation of hCGβ following quantitation of wound closure (Figure 3.10). However, this effect did not reach statistical significance.

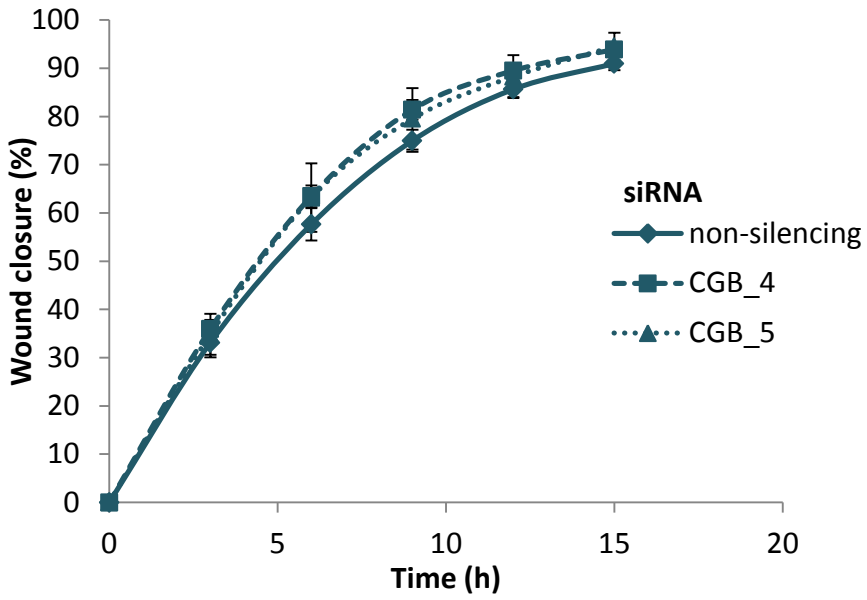


Figure 3.10 Effect of hCGβ downregulation on cell migration in HEY cells

Effect of hCGβ downregulation on migration of HEY cells, 48 h post siRNA transfection. siRNAs: non-silencing control, CGB_4, CGB_5. Results are expressed as mean ± S.E.M; N=4.

3.3.7 Overexpression of hCGβ

hCGβ was overexpressed in HEY, A2780 and A2780cis cells in order to determine if increasing its levels would result in the opposite effect of downregulation with regards to proliferation. The effect of hCGβ overexpression on cell migration was also studied in HEY cells. Protein expression of hCGβ was examined in whole cell lysates and conditioned media 48 h post transfection with an empty vector (pCI-neo) or the vector containing hCGβ insert (pC1-neo+ hCGβ). Intercellular hCGβ was successfully transiently expressed in all three cell lines at levels which could be detected by western blot (Figure 3.11 A). There was an apparent 3, 4 and 6 fold increase in hCGβ in A2780, A2780cis and HEY cells respectively (Figure 3.11 B). The ImageJ program used to quantify the intensity of

the bands will give a reading regardless of whether a band is visible to the naked eye, therefore the extent of overexpression is probably underestimated and it should be noted an hCGβ band is visible only after protein is overexpressed. Background corrections were made by measuring a region of the blot that contained no bands and subtracting it from measured bands. When the level of secreted hCGβ was measured it was found that hCGβ was dramatically increased from 0 ng/mL to 51.84 ng/mL, 53.84 ng/mL and 47.02ng/mL in A2780, A2780cis and HEY cells respectively (Figure 3.11 C).

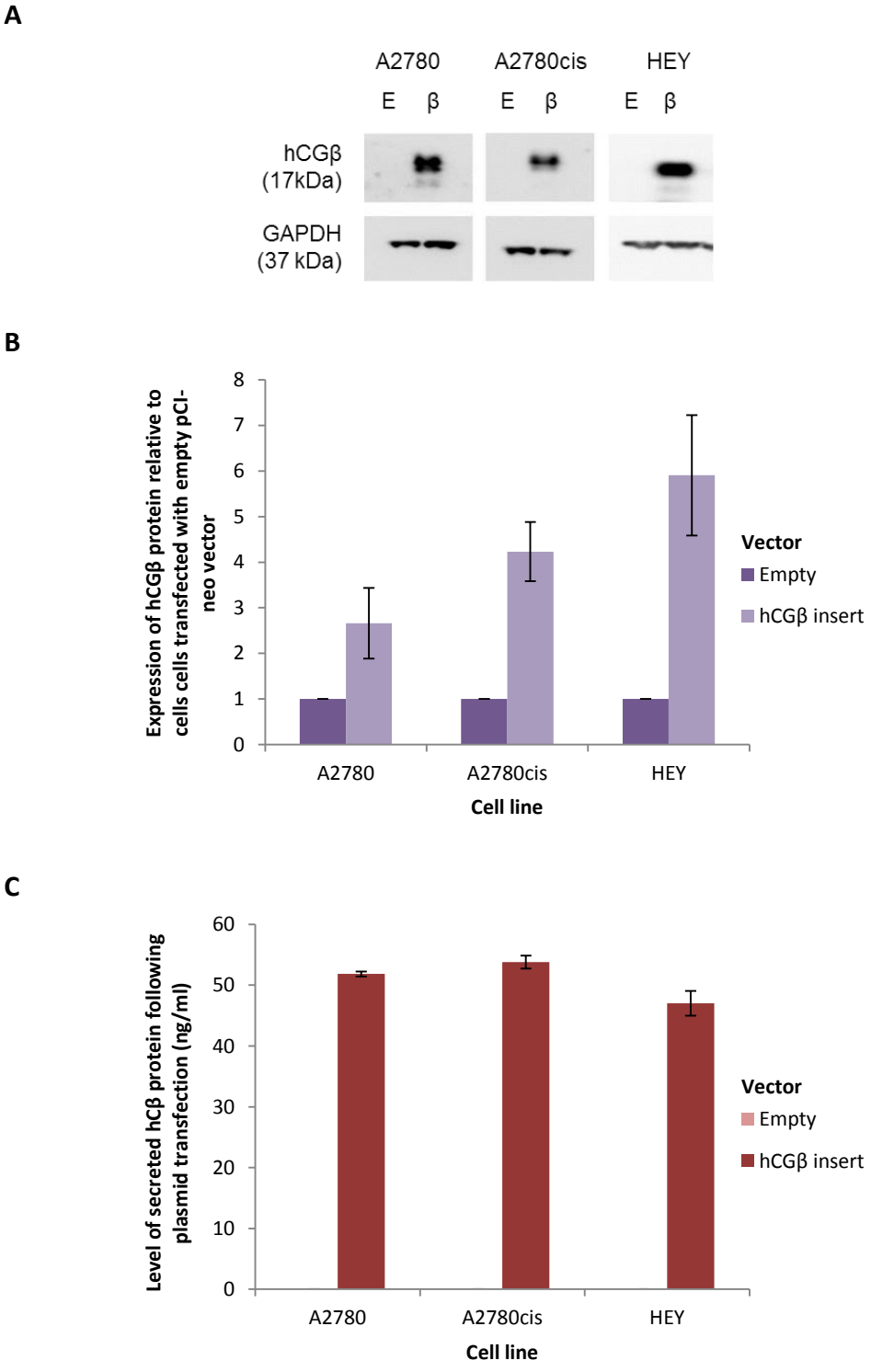


Figure 3.11 Overexpression of hCGβ

Expression of hCGβ 48 h post transfection in A2780, A2780cis and HEY cells (A) Western blot showing expression of hCGβ protein in cell lysates. (B) Measured intensity of bands on western blot and normalised to cells treated with empty pC1-neo vector. (C) Secreted hCGβ measured by ELISA. Plasmid vectors: pC1-neo (E), pC1-neo with hCGβ insert (β). Results expressed as mean ± S.E.M; N=3.

3.3.8 Effect of overexpression of hCGβ on cell proliferation

No significant effect on cell proliferation was observed in A2780, A2780cis and HEY cells when hCGβ was overexpressed (Figure 3.12).

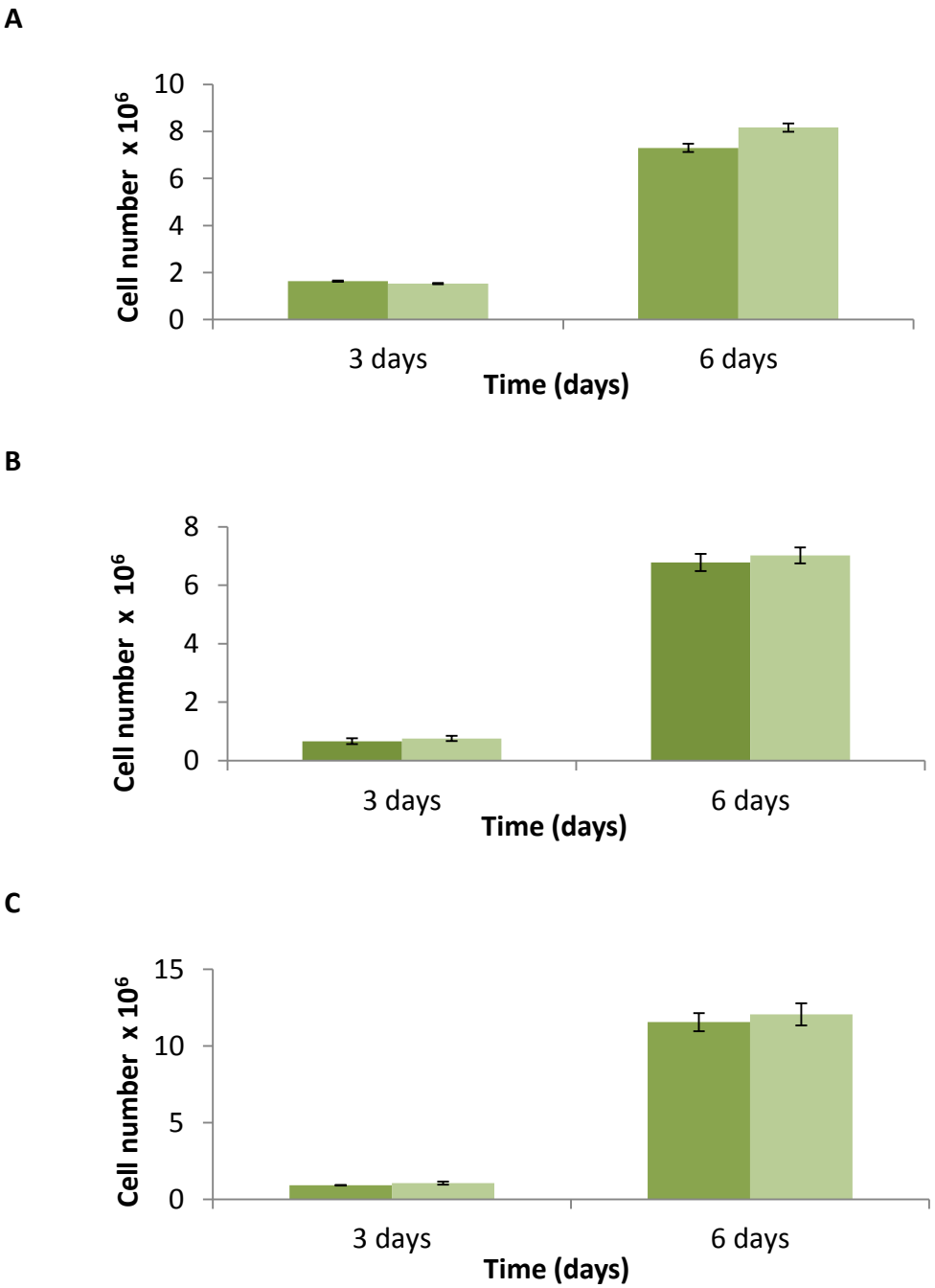


Figure 3.12 Effect of hCGβ overexpression on cell proliferation

Effect of hCGβ overexpression on proliferation of A2780(A), A2780cis (B) and HEY (C) cells. Plasmid vectors: pCI-neo(■) or pCI-neo with hCGβ insert(■). Results expressed as mean ± S.E.M; N=3.

It was possible that the large growth surface area used for the proliferation study (i.e. T75 flask for HEY cells and T25 flasks for A2780 and A2780cis cells) and the presence of serum proteins in the media could have diminished any effects of increased hCGβ. Therefore, to test the effect of cells growing over a smaller growth surface and in serum free conditions the experiment was repeated using 6 and 12 well plates for HEY and A2780 and A2780cis cells, respectively in serum free media. Specifically, 24 h post hCGβ transfection, 1 x 10^5 cells were plated in 6 or 12 well plates, incubated for 6 hours at 37°C in 5% CO_2 after which the media was changed to serum free media (RPMI media containing 0.1% BSA). Cells were then allowed to grow for 4 days. A longer period could not be pursued as the cells were almost 100% confluent by the end of this time period. It was found that a decrease in surface area and serum starvation did not influence proliferation of any of the three cell lines transiently transfected with hCGβ (Figure 3.13).

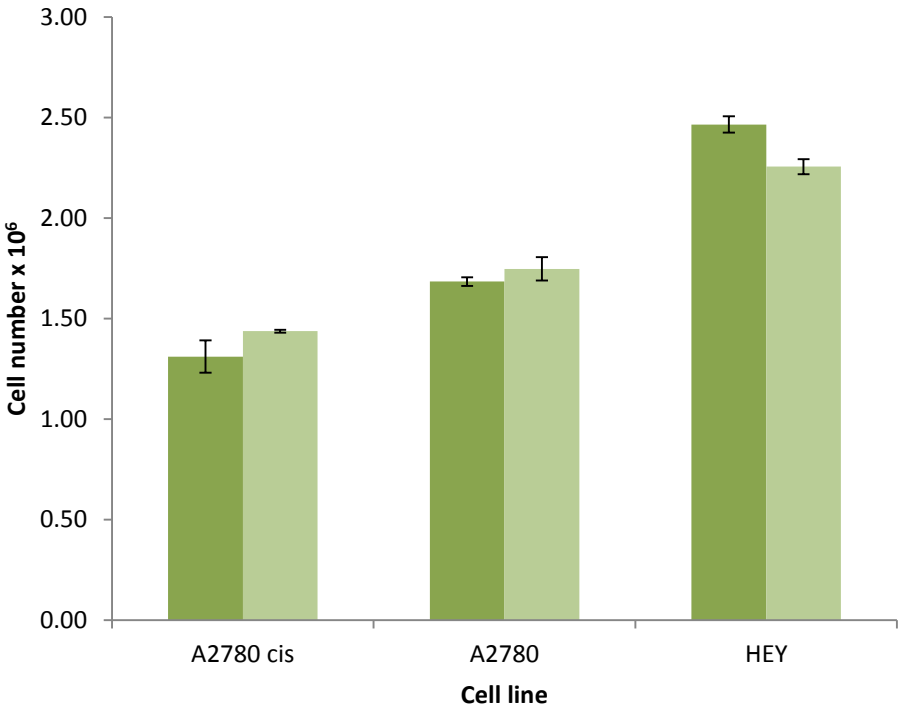


Figure 3.13 Effect of hCGβ overexpression and serum starvation on cell proliferation in a 6 and 12 well format

Effect of hCGβ overexpression on proliferation of A2780, A2780cis and HEY cells under serum starved conditions in a 6 or 12-well plate in 4 days. Plasmid vectors: pCI-neo (■) or pCI-neo with hCGβ insert (■). Results are expressed as mean ± S.E.M; N=3.

3.3.9 Effect of hCGβ overexpression on migration of HEY cells

Overexpression of hCGβ had no effect on migration of HEY cells over a 21 h period (Figure 3.14). Representative images are shown at time points 0 and 21 h (Figure 3.15).

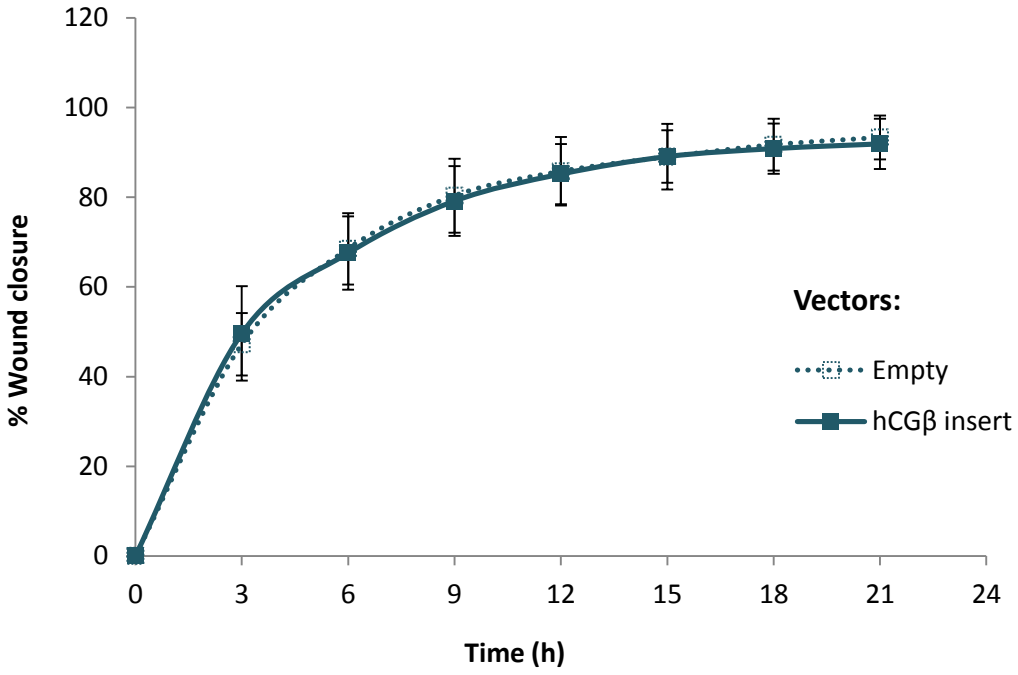
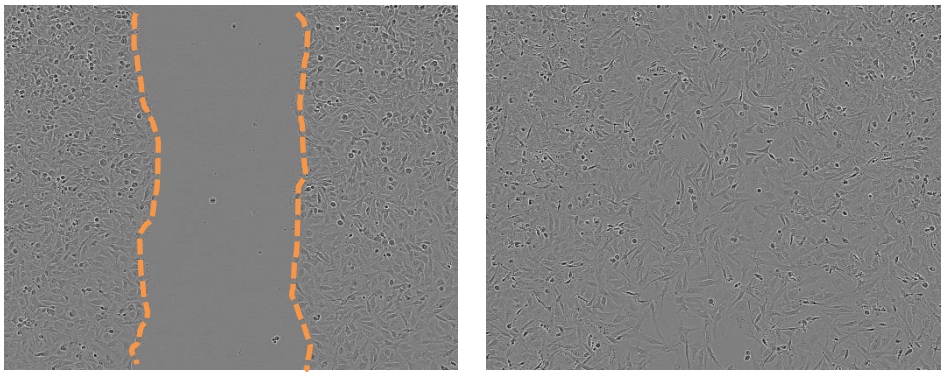


Figure 3.14 Effect of hCGβ overexpression on migration of HEY cells

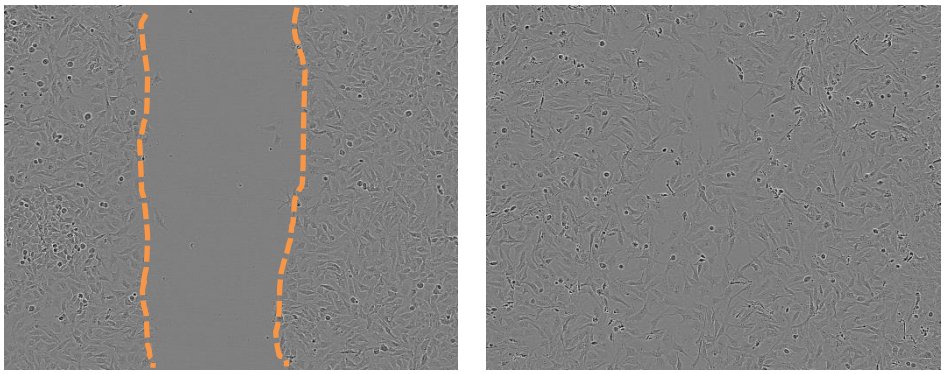
Migration of HEY cells over a period of 21 h, 48 h after transfection. Plasmid vectors: Empty vector pCI-neo, pCI-neo with hCGβ insert. Results are expressed as mean ± S.E.M; N=4.

pC1-neo



0 h **21 h**

pCI-neo with hCG-β insert



0 h **21 h**

Figure 3.15 Wound closure in HEY after overexpression of hCGβ

Representative images from the Incucyte of wound closure in HEY cells at 0 h and 21 h time points. 48 h post transfection. Plasmid vectors: pCI-neo plasmid and pCI-neo with hCGβ insert.

3.3.10 Effect of exogenous hCGβ on cell proliferation

The effect of exogenous recombinant hCGβ on proliferation of HEY, A2780 and A2780cis cells was studied using the MTS cell viability assay. Exogenous hCGβ did not have a significant effect on cell proliferation (Figure 3.16) when delivered at 10, 25 and 50 times the concentration of basal hCGβ levels expressed by the cells (Table 3-1).

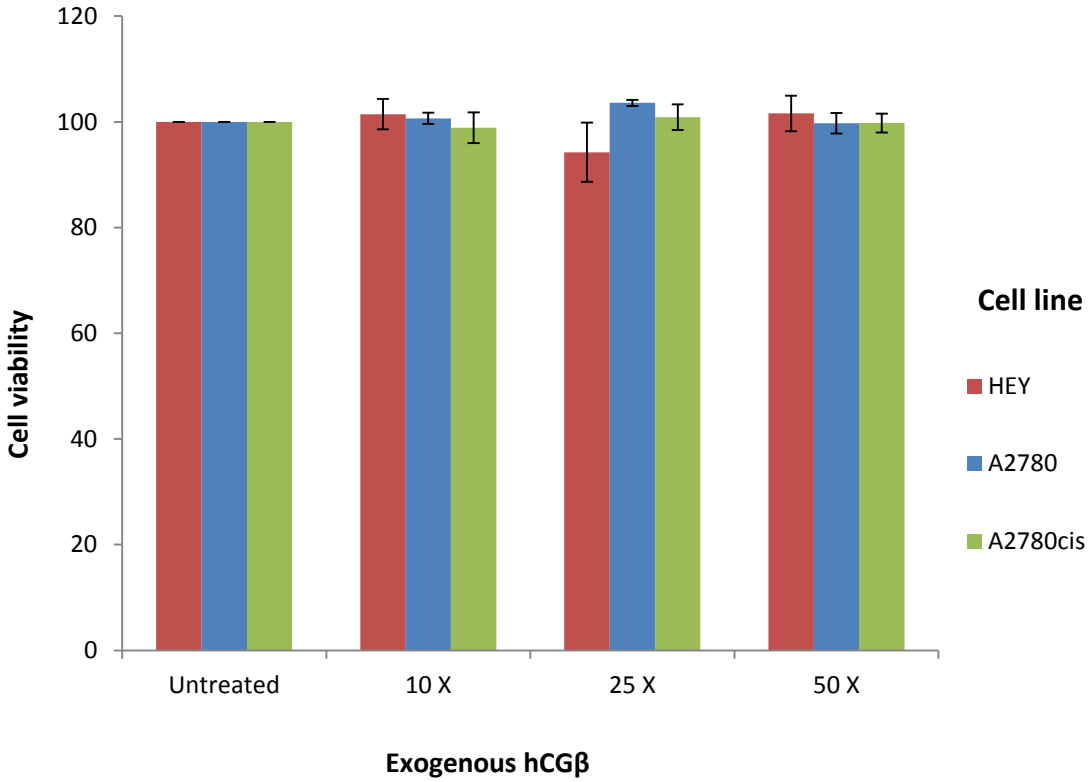


Figure 3.16 Effect of exogenous hCGβ on cell proliferation

Effect of exogenous hCGβ on proliferation of HEY, A2780, A2780cis cells when applied at 10 X, 25 X and 50 X the basal endogenous expression of hCGβ which was 4, 0.9 and 0.6 ng/mL for HEY, A2780 and A2780cis cells, respectively. Untreated cells received 0.1% BSA in RPMI media as the vehicle control. Proliferation was measured 4 days after treatment using the MTS cell viability assay.

3.4 Discussion

3.4.1 Basal expression of *CGB* and secreted hCGβ

All eight HGSC cell lines expressed *CGB* and secreted hCGβ protein to varying degrees with SKOV-3 and HEY cells being the highest expressers. The fact that hCGβ was expressed in the HGSC cell lines agreed with patient data showing that hCGβ is expressed in ovarian cancer [181, 183, 186]. Though the level of hCGβ secreted by the OVCAR-3 cells was below the lowest standard (0.25 ng/mL) in assay, the absorbance was above the blank (media only) and 0 ng/mL standard hence it can be inferred that hCGβ was secreted by the cells.

3.4.2 Downregulation of hCGβ

Three cell line models – HEY, A2780 and A2780cis cells – expressing a varied range of *CGB*/hCGβ, were selected for functional studies. HEY cells were chosen as they expressed the highest level of hCGβ and the pair of cisplatin-sensitive and resistant cells, A2780 and A2780cis, respectively were chosen for drug response studies (investigated in Chapter 4). hCGβ was successfully downregulated in A2780, A2780cis and HEY cells by 50-60% at the transcript level (Figure 3.3 A). A higher level of downregulation was unable to be achieved, despite increasing the amount of siRNA to the highest recommended amount (10 nM). A further increase in the amount of siRNA was not considered as it may have resulted in off-target effects. There are a number of reasons that may explain why a more efficient level of hCGβ downregulation could not be achieved. As mentioned in Chapter 1 section 1.3.3, hCGβ is encoded by 6 different genes. Therefore it is possible that the siRNAs are

differentially targeting the different *CGB* genes so that the reduction observed is a result of preferentially targeting one gene or a subset of the genes. siRNA binding could also be dependent on the which *CGB* gene is being expressed by the cells. If indeed only one or a subset of the genes were targeted, it is possible that other untargeted *CGB* genes could increase their expression in order to compensate for the loss of the other gene. The Taqman assay used to measure *CGB* downregulation does not distinguish between the different *CGB* genes and therefore cannot produce a representation of each individual gene product. We cannot therefore determine which gene(s) are targeted or if there is a compensatory effect. This theory could be tested by using primers that specifically target each transcript. In Chapter 4 section 4.3.8, it was shown that CGB_4 and CGB_5 siRNA differentially targeted the *LHB* gene which encodes for the highly homologous LHβ protein.

It could be argued that even if the different *CGB* genes are targeted it is the protein product which is of functional importance. This is a valid argument; however, hCGβ poses another challenging scenario as hCGβ can be either of two different protein products depending on the gene expressed. The two protein products differ in particular by a single amino acid at position 117: type I genes (*CGB*7) encodes hCGβ with an alanine at position 117, whereas type II genes (*CGB*3, *CGB*5 and *CGB*8) encode a protein product with an aspartic acid at position 117. Therefore if it is assumed that a higher level of gene knockdown could not be achieved due to the inability of the siRNAs to target all *CGB* transcripts then the protein product (type I or type II hCGβ) may be of importance. Differential expression of type I and type II genes can be detected using nested PCR which can detect single nucleotide gene products [165]. To date, there is no antibody assay that is capable of distinguishing between the two hCGβ protein products. However,

Aldaz-Carol *et al*. published a paper in 2015, suggesting they have designed a specific antibody for type II hCGβ [165].

The expression of the α-subunit and β-subunit in HEY, A2780 and A2780cis cells shows the potential that these cells have to produce the heterodimeric hCG protein. This has to be addressed as it would mean effects observed in functional studies involving hCGβ downregulation or overexpression cannot be attributed to, or distinguished from, either whole hCG or free hCGβ. However, a number of studies have investigated hCGβ on its own without regard to expression of the whole protein [156, 187, 191].

The α-subunit of hCG has been shown to be biologically activate in endometrial stromal cells [299-301]. Specifically Blithe and colleagues found that the free α-subunit could act synergistically with progesterone to regulate the differentiation of human endometrial cells *in vitro* [299, 300]. In an attempt to study the effect of hCGβ while discounting the presence of hCGα we used exogenous recombinant hCGβ in experiments to determine its effect on cell proliferation (Figure 3.16). It was found that exogenous hCGβ did not influence cell proliferation. The drawback of the recombinant hCGβ is that its biological activity was unknown. Furthermore, it was produced in yeast cells which may result in varied glycosylation and therefore different stability and activity of the protein compared to the product from mammalian cells. Therefore, downregulation or overexpression of hCGβ was the best approach to ensure that hCGβ produced by the cells had any biological importance.

3.4.3 Role of hCGβ in cell proliferation

Downregulation of hCGβ with both CGB_4 and CGB_5 siRNAs significantly decreased cell proliferation in HEY and A2780cis cells (Figure 3.4 A and C respectively). However, only the use of CGB_5 siRNA caused a significant decrease in cell proliferation of A2780 cells. These data suggest that the role of hCGβ in cell proliferation is cell type dependent which could be in part due to potential differences in the mechanism of action of the two siRNAs in different cell lines. The pro-proliferative role of hCGβ in HEY and A2780cis cells agrees with data shown Gillot *et al.* and Guo *et al*. [154, 191]. Gillot *et al*. showed that exogenous hCGβ could increase cell growth of bladder cancer cell lines [154] and Guo *et al*. showed that overexpression of hCGβ in ovarian surface epithelial cells caused an increase in cell proliferation [191]. Since downregulation of hCGβ had a negative effect on cell proliferation in HEY and A2780cis cells it was expected that overexpression of hCGβ would have the opposite effect. However, overexpression of hCGβ seemed to have no measurable effect on cell proliferation in these cells (Figure 3.12). A possible explanation for this result is that increased expression of hCGβ beyond an endogenous threshold level could not further influence cell proliferation. The influence of exogenous hCGβ on cell proliferation was also investigated in the current study and results showed that exogenous hCGβ did not influence cell proliferation (Figure 3.16). This data is contrary to Gillot's data that showed that exogenous hCGβ could increase cell growth of bladder cancer cell lines [154]. This could be due to the different hCGβ preparations and sources. The recombinant hCGβ sourced from Sigma that was used in the current study had not been confirmed to have biological activity; although Butler *et al*. showed hCGβ from Sigma had biological activity [155]. Given that the actual product number was not stated in this

paper, it is possible that the product used in our study is different and may be biologically less potent or inactive.

3.4.4 Role of hCGβ on cell migration

hCGβ did not have a significant effect on migration of HEY cells irrespective of whether it was downregulated or overexpressed (Figure 3.10 and Figure 3.14). This result is contrary to findings by Wu *et al*. who showed that overexpression of hCGβ in prostate carcinoma cell lines increased cell migration [156]. It is possible that the migratory effect of hCGβ is cell type dependent or that HEY cells express levels of hCGβ that are so high (Figure 3.1) that downregulation of 50-60% or overexpression of up to 6 fold did not have an overall impact on cell migration.

As mentioned in section 3.3.6, A2780 and A2780cis cells did not migrate substantially within a 48h period; however, a 20% difference in wound closure was observed which could be attributed to cell spreading; as cells seemed to be more rounded at time zero perhaps due to the scratching and wash steps, compared to later time points which showed cells to be more spread out.

3.4.5 Role of hCGβ on cell adhesion

The effect of hCGβ on cell adhesion of extracellular matrixes has not been studied before; however the effect of hCGβ on anchorage independent growth has been reported by Guo *et al*. [191]. Specifically, Guo's study showed that overexpression of hCGβ in ovarian surface epithelial cells increased their growth

potential in soft agar. Interestingly, our results showed that downregulation of hCGβ caused a general increased trend in cell adhesion of HEY cells on collagen I, II and IV, fibronectin, vitronectin, tenascin, and laminin (Figure 3.6 B) and A2780cis cells on fibronectin, vitronectin, tenascin, and laminin (Figure 3.6 B). Although cell adhesion and anchorage independent growth are two different assays each indicative of different cellular functions, they are related, as anchorage independent growth demonstrates that cells are capable of growing without having to attach to a surface which is characteristic of anchorage dependent cells.

3.5 Conclusions

All eight HGSC cell lines expressed *CGB* transcript and secreted hCGβ protein to varying degrees with SKOV-3 and HEY cells being the highest expressers. All cells also expressed *CGA* at varying levels with CaOV-3 expressing almost 700 times the amount of A2780 cells which were the next highest expresser of *CGA*. Expression of both the α- and β- subunits of hCG by the cells suggests the whole protein could potentially be expressed; however, specific commercial antibodies targeting hCGβ alone were not available hence the best means of studying the role of hCGβ was to overexpress and downregulate the gene, the means by which the role of hCGβ has been investigated in previously published studies.

Downregulation of hCGβ significantly decreased proliferation of A2780cis and HEY cells suggesting that hCGβ may have a role in cell proliferation. The influence of hCGβ downregulation on cell proliferation of A2780 cells seemed to be dependent on siRNA used to downregulate hCGβ; suggesting that the two siRNA used to target hCGβ may be functioning by different mechanisms. hCGβ may play a role in cell adhesion, as downregulation of hCGβ caused a general increased trend in cell adhesion of HEY cells on collagen I, II and IV, fibronectin, vitronectin, tenascin, and laminin and A2780cis cells on fibronectin, vitronectin, tenascin, and laminin. Migration of HEY cells was not influenced by hCGβ. The effect of hCGβ on the response of HGSC cell lines and differences in the actions of CGB_4 and CGB_5 siRNA are investigated in the next chapter.

Chapter 4 Role of hCGβ in the response to cisplatin and other platinum-based drugs

4.1 Introduction

The role of hCGβ in the response of HGSC cell lines to chemotherapeutic drugs, in particular cisplatin, carboplatin and oxaliplatin, were investigated in this chapter. Two published studies have suggested that hCGβ could be involved in resistance of cancer cells to chemotherapy: 1. Szturmowicz *et al.* observed that patients with small-cell lung cancer who were resistant to chemotherapy and had poor outcomes, had elevated serum levels of hCGβ [180] and 2. Berman *et al.* found that xenografts of small cell bronchial carcinoma which expressed hCGβ were resistant to the chemotherapeutic drug cyclophosphamide [280]. Despite these observational studies, no further work has been published to determine the direct role hCGβ may play in the response of cancer cells to chemotherapy. Furthermore, the suggestion that hCGβ has an anti-apoptotic role in bladder and cervical cancer cells [155, 187], raises the possibility that hCGβ may have a role in determining how cells respond to an apoptotic chemotherapeutic agent.

In order to investigate whether hCGβ plays a role in chemosensitivity (or chemoresistance) in HGSC cells, hCGβ was downregulated in three HGSC cell lines (HEY, A2780 and A2780cis) and the response to cisplatin determined. In addition, sensitivity to other platinum-based drugs, oxaliplatin and carboplatin, and the microtubule targeting agent paclitaxel was investigated. hCGβ was also overexpressed in the cisplatin sensitive and resistant cell lines A2780 and A2780cis

cells respectively, to determine whether high levels of hCGβ could confer decreased sensitivity of the cells to cisplatin.

4.2 Materials and Methods

hCGβ was downregulated (using siRNAs) or overexpressed (using a plasmid construct) in A2780, A2780cis and HEY cells as outlined in Chapter 2, sections 2.8 and 2.9, respectively.

4.2.1 Sensitivity of cells to chemotherapeutics

The response of cells to drug treatment was studied using cell viability and clonogenic (survival) assays. Both assays reflect how cells respond to drug treatment, but work on different principles. The cell viability assay (also known as the cytotoxic assay) is a 3 day colorimetric assay and measures cell viability after drug treatment. The clonogenic assay measures the ability of cells to survive and form colonies (defined as ≥ 50 cells) after the drug has been withdrawn. The clonogenic assay is a longer assay compared to the viability assay, spanning up to 2 weeks.

4.2.1.1 MTS cell viability assay

The MTS cell viability assay (described in Chapter 3, section 3.2.4.2) was used to determine the relative number of viable cells remaining 72 h after drug treatment. The protocol for this assay was based on the methods described by Persons *et al*.,

[302] and O'Toole *et al*., [303] with a few modifications. Twenty-four hours after siRNA or plasmid transfection, HEY, A2780 and A2780cis cells were harvested, counted and seeded in triplicate in 100 µl of culture media into a 96-well plate. The seeding density for HEY cells was 1×10^3 cells /well and 2.5×10^3 cells/well for A2780 and A2780cis cells. Eighteen to 24 h after seeding, the cells were treated with 100 µl of cisplatin, carboplatin, oxaliplatin or paclitaxel in culture media and incubated for 72 h at 37 ºC and 5% CO_2. Table 4-1 lists the final concentration range for each drug used to treat the cells. Wells were set up for blank reading which contained culture media only. After 72 h of drug treatment, the percentage of viable cells was determined as per the protocol described in Chapter 3, section 3.2.4.2. Briefly, the MTS reagent was added to each well and incubated for a period of 1-2 h for HEY cells and 2-3 h for A2780 and A2780cis cells at 37 ºC and 5% CO_2 and the absorbance was measured at 490 nm on the Wallac Victor 1420 Multilabel Counter. The background absorbance from blank wells was subtracted from the wells that contained cells. The percentage of viable cells was then calculated. The IC_{50} was calculated using GraphPad Prism version 6.04 software (GraphPad software, California, USA).

Prior to performing the MTS assay the optimum drug concentration range was determined by treating the cells with 0.125-50 µM cisplatin, 3.12-300 µM carboplatin, 2.5-100 nM oxaliplatin and 2.5-100 nM paclitaxel. From the drug concentration range a maximum dose which killed more than 80% cells (20% viable) and a minimum dose at which cells were unaffected by the drug were chosen, along with several drug doses in the middle of this range (Table 4-1).

Table 4-1 Drug concentration range analysed for cell viability assay

Drug	Range for HEY	Range for A2780	Range for A2780cis
Cisplatin	0.4-6 μM	0.4-12 μM	0.4-12 μM
Carboplatin	5-150 μM	Not tested	5-150 μM
Oxaliplatin	1-50 nM	Not tested	1-50 nM
Taxol	1-50 nM	Not tested	1-50 nM

4.2.1.2 Clonogenic assay

The clonogenic cell survival assay was based on a paper by Gan *et al.* with a few variations [304]. Twenty-four hours after siRNA or plasmid transfection, HEY, A2780 and A2780cis cells were harvested, counted and plated into 6 well plates at a seeding density of 120 cells/well in 1 mL of culture media and allowed to adhere for 5-6 h at 37 ºC and 5% CO_2 before being treated with cisplatin, carboplatin, oxaliplatin and paclitaxel in 500 μl culture media (Table 4-2 for drug concentration range). Seventy-two hours following drug treatment, the medium was replaced with 1.5 mL drug free culture media and HEY cells and A2780 and A2780cis cells were allowed to form colonies over 3 or 8 days, respectively. Media was topped up with 1 mL of fresh media every 3 days. Colonies were then fixed with 0.5% crystal violet in 20% methanol for 5 min and excess stain washed off with water. The stained plates were dried and colonies manually counted. The following equations were used to calculate the plating efficiency and the surviving fraction:

$$\textbf{Plating efficiency} = \frac{number\ of\ colonies\ in\ drug\ free\ media}{number\ of\ cells\ seeded}$$

$$\textbf{Surviving fraction} = \frac{number\ of\ colonies}{(number\ of\ cells\ seeded \times plating\ efficiency)}$$

The IC_{50} was calculated using GraphPad Prism version 6.04 software.

Table 4-2 Drug concentration range for clonogenic assays

Drug	Range for HEY	Range for A2780	Range for A2780cis
Cisplatin	0.25-4 μM	0.13-2 μM	0.5-8 μM
Carboplatin	0.56-25 μM	Not tested	3.75-60 μM
Oxaliplatin	0.63-1 μM	Not tested	0.63-1 μM
Paclitaxel	0.31-5 nM	Not tested	0.31-5 nM

Prior to the clonogenic assay the number of cells and the time required for cells to form colonies was optimised by seeding between 50-500 cells/well into 6 well plates and incubating the plates for 5-14 days at 37 ºC and 5% CO_2, until colonies could be visualised and were not touching each other. It was found that the optimum density was 120 cells/well for HEY, A2780 and A2780cis cells. The number of days required to form colonies was 6 days for HEY and 11 days for A2780 and A2780cis cells.

The drug concentration range was also optimised prior to the clonogenic assay. The highest drug concentration used to treat the cells started at IC_{50} concentration based on the cell viability assay from which 2-fold dilutions were made. The IC_{50} concentrations were chosen as it was assumed that the seeding density for the clonogenic assay ($\geq$10 times less than that used in the viability assay) would require considerably less drug to kill cells. The IC_{50} concentration was an adequate dose to prevent colony formation.

4.3 Results

4.3.1 Sensitivity of cells to cisplatin determined by cell viability assay

The response of A2780, A2780cis and HEY cells to cisplatin, determined by the cell viability (MTS) assay, showed that A2780 cells were the most sensitive to cisplatin, followed by HEY cells and, as expected, A2780cis was the most resistant cell line (Figure 4.1).

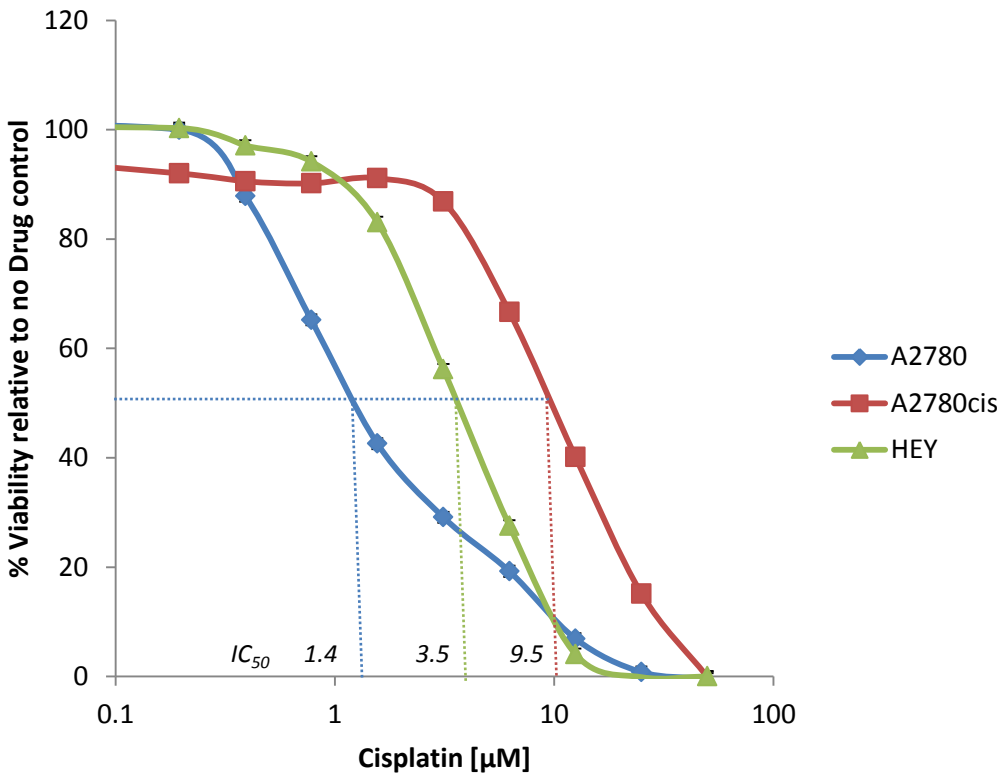


Figure 4.1 Response of A2780, A2780cis and HEY cells to cisplatin treatment

Response of A2780, A2780cis and HEY cells to 72 h treatment with cisplatin, determined by the cell viability (MTS) assay. Data are expressed as mean ± S.E.M; (N=3). The IC_{50} (µM) for cisplatin for all three cell lines is marked on graph.

The derived IC_{50} concentrations showed that A2780cis cells had a significantly higher IC_{50} for cisplatin compared to A2780 cells (6.4 times higher) and HEY cells (2.5 times higher). HEY cells had an IC_{50} cisplatin concentration which was 2.5 times greater than that of A2780 cells (Table 4-3).

Table 4-3 IC_{50} concentration of cisplatin derived from the cell viability assay for A2780, A2780cis and HEY cells

Cell line	IC_{50} [μM]
A2780	1.43 ± 0.04 **
A2780cis	9.23 ± 0.63
HEY	3.5 ± 0.40 **, #

*IC50 concentration of cisplatin derived from the cell viability (MTS) assay for A2780, A2780cis and HEY cells. Data are expressed as mean ± S.E.M; (N=3). ** P < 0.005 when samples were compared to A2780cis; # P < 0.05 when samples were compared to A2780.*

4.3.2 Sensitivity of cells to cisplatin relative to expression of secreted hCGβ

A relationship between the level of *CGB* expression and hCGβ secreted by the cells and their sensitivity to cisplatin (expressed as IC_{50} concentrations derived from the cell viability assay) could not be established (Figure 4.2). The parental cisplatin sensitive A2780 cells expressed 3.5 times more *CGB* and 2 times more secreted hCGβ compared to the cisplatin resistant A2780cis cells and were more sensitive to cisplatin. HEY cells expressed 55 and 10 times more *CGB* and secreted hCGβ respectively compared to A2780cis cells and were more sensitive to cisplatin. In contrast, HEY cells expressed higher levels of *CGB* and hCGβ compared (16 and 4

times more, respectively) to A2780 cells but were less sensitive (up to 2.5 times) to cisplatin.

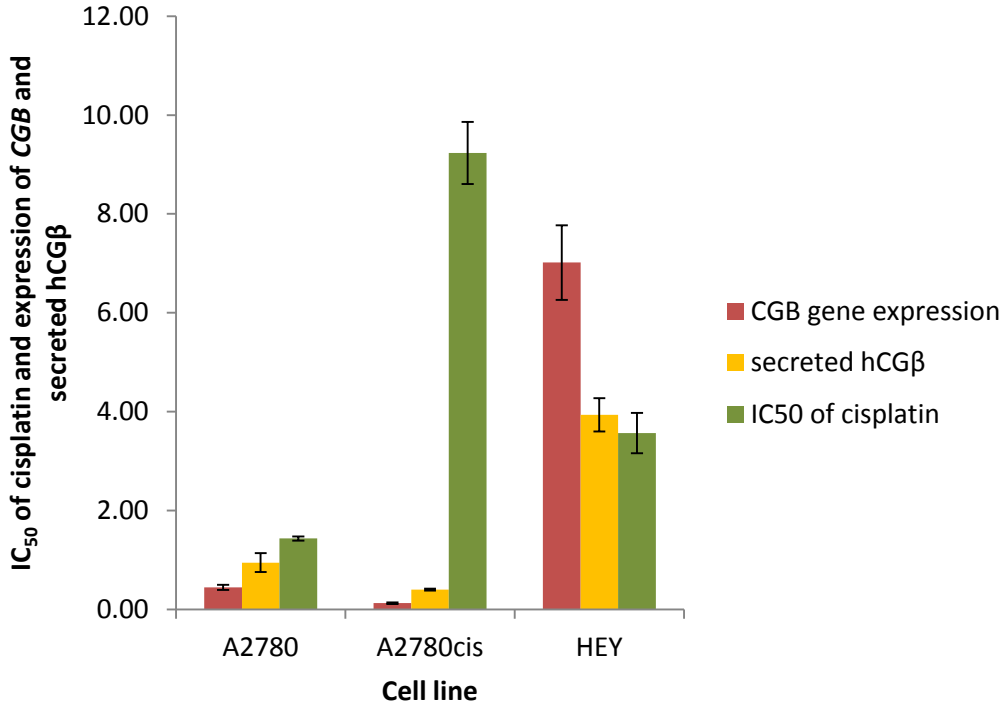


Figure 4.2 Cisplatin sensitivity relative to the expression of *CGB* and secreted hCGβ

IC_{50} of cisplatin (μM) for A2780, A2780cis and HEY cells derived from the cell viability (MTS) assay, relative to *CGB/HMBS* (arbitrary units) and secreted hCGβ (ng/mL) levels. Data are expressed as mean ± S.E.M; (N=3).

4.3.3 Effect of hCGβ downregulation on cell viability following cisplatin treatment

Downregulation of hCGβ had different effects on the response of the cisplatin sensitive A2780 cells and cisplatin resistant A2780cis cells to cisplatin treatment (Figure 4.3). The degree of hCGβ downregulation was previously shown in Chapter 3, Figure 3.3. In A2780 cells, reducing the level of hCGβ by ~60% did not affect the response to cisplatin (Figure 4.3 A). In contrast, downregulation of hCGβ by ~60 % significantly increased the sensitivity of A2780cis cells to cisplatin (Figure 4.3 B). An increase in sensitivity to cisplatin after hCGβ downregulation was also observed in HEY cells (Figure 4.3 C).

Although both hCGβ targeting siRNAs (CGB_4 and CGB_5) downregulated hCGβ to similar levels and both caused increased sensitivity to cisplatin in HEY and A780cis cells, it was evident that the CGB_5 siRNA had a greater effect on how cells responded to cisplatin, suggesting that the two siRNAs did not function in an identical manner.

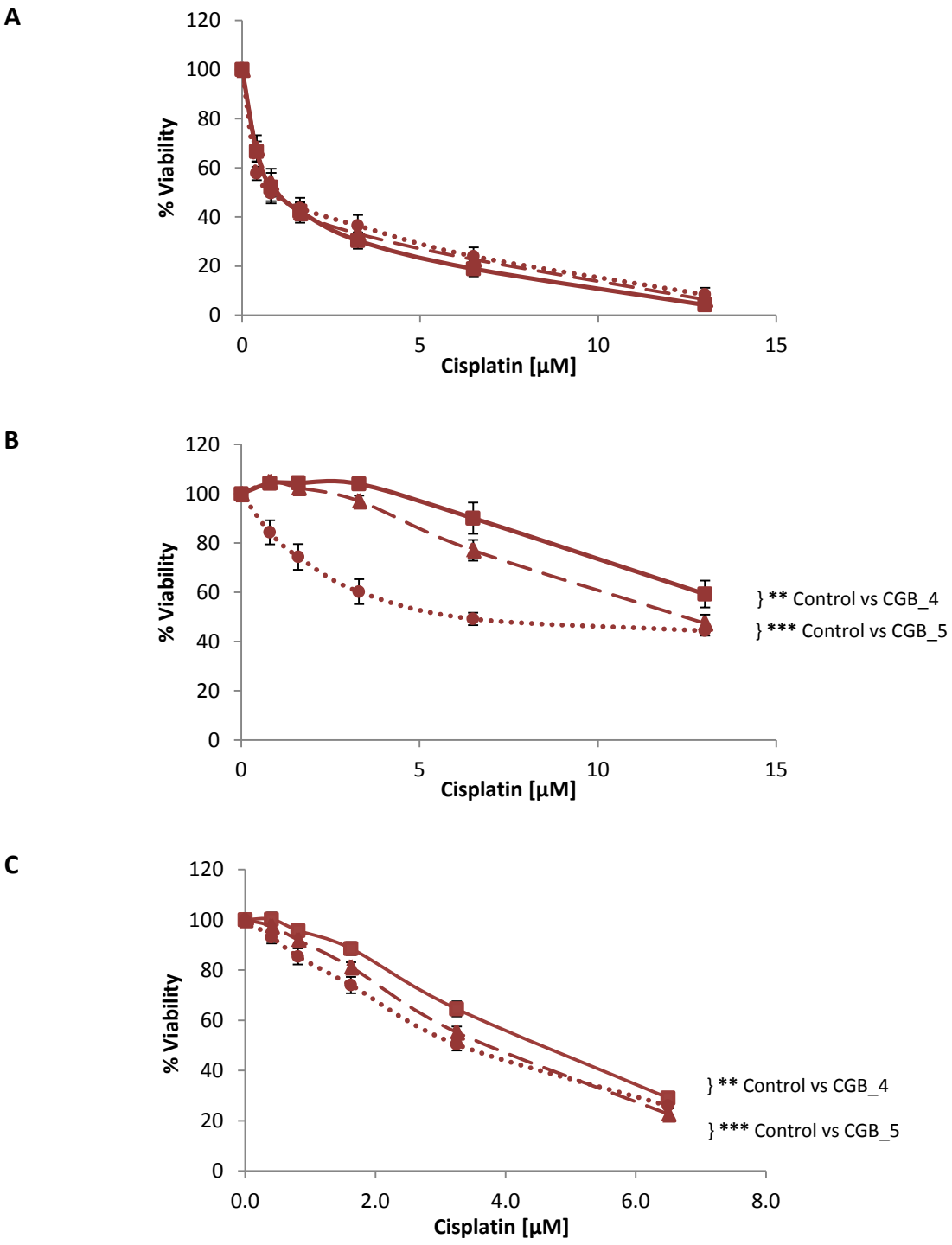


Figure 4.3 Effect of hCGβ downregulation on cell viability following cisplatin treatment

Viability of A2780 (A), A2780cis (B) and HEY (C) cells after cisplatin treatment, 48h post transfection with non-silencing control (—■—), CGB_4 (- -▲- -) or CGB_5(···●···) siRNAs. The MTS viability assay was used to determine cell viability. Data are expressed as mean ± S.E.M; (N=3). ** $P < 0.005$, *** $P < 0.0005$.

4.3.4 Effect of hCGβ downregulation on cell viability following paclitaxel treatment

As A2780 cells did not show any difference in sensitivity towards cisplatin following hCGβ downregulation, from here on drug sensitivity studies following hCGβ downregulation were followed only in HEY and A2780cis cells. Downregulating hCGβ in A2780cis and HEY cells had no significant effect on their sensitivity towards paclitaxel (Figure 4.4). Refer to Appendix B Supplementary Table 1 for IC_{50} concentrations for Paclitaxel.

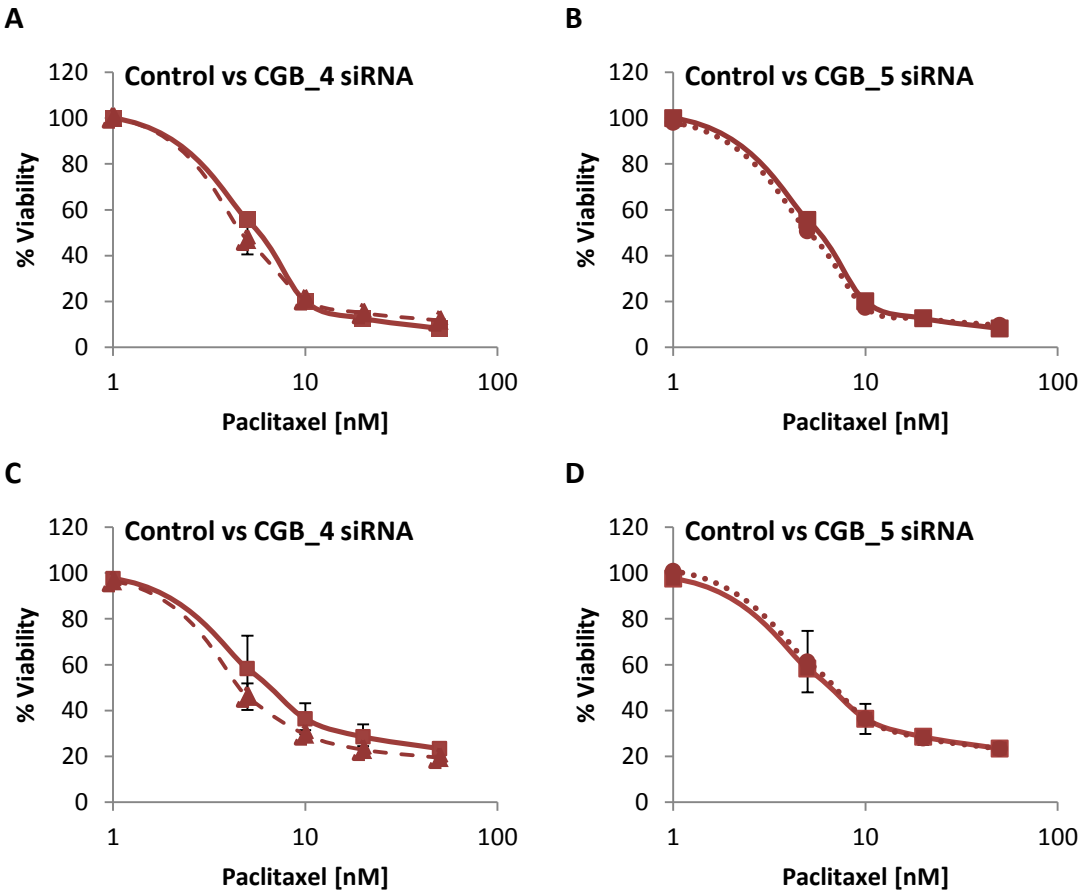


Figure 4.4 Effect of hCGβ downregulation on cell viability following paclitaxel treatment

Viability of HEY (A and B) A2780cis (C and D) cells in response to paclitaxel, 48h post transfection with non-silencing control (—■—), CGB_4 (- -▲- -) and CGB_5 (···●···) siRNAs. The MTS assay was used to determine cell viability. Data are expressed as mean ± S.E.M (N=3).

4.3.5 Effect of hCGβ downregulation on cell viability following treatment with carboplatin and oxaliplatin

The response of A2780cis and HEY cells to other platinum-based drugs, carboplatin and oxaliplatin, was studied to determine whether the increase in sensitivity to cisplatin seen when hCGβ was downregulated, was also seen for these drugs. In A2780cis cells, downregulation with both CGB_4 and CGB_5 siRNAs showed a statistically significant increase in sensitivity to both carboplatin and oxaliplatin (Figure 4.5). Although these trends were reflected in HEY cells, only downregulation with CGB_4 showed a statistically significant increase in sensitivity to carboplatin (Figure 4.6). Refer to Appendix B, Supplementary Table 1 for IC_{50} concentrations for carboplatin and oxaliplatin.

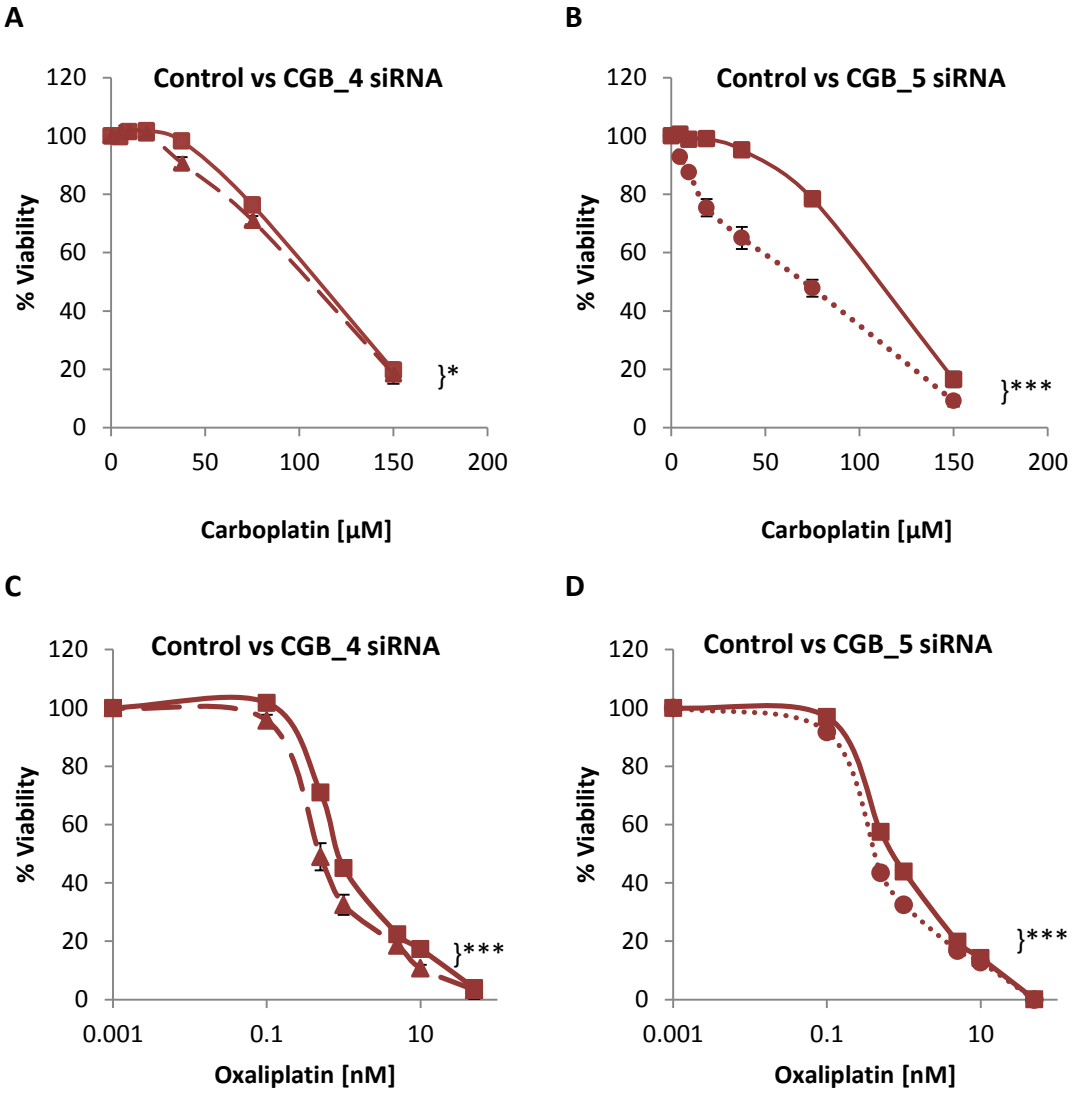


Figure 4.5 Effect of hCGβ downregulation on cell viability of A2780cis cells following treatment with oxaliplatin and carboplatin

Viability of A2780cis cells following treatment with: Carboplatin (A and B), Oxaliplatin (C and D), 48 h post transfection with non-silencing control (—■—), CGB_4 (- -▲- -) and CGB_5 (····●····) siRNAs. The MTS assay was used to determine cell viability. Data are expressed as mean ± S.E.M; (N=3). *P < 0.05, ***P < 0.0005.

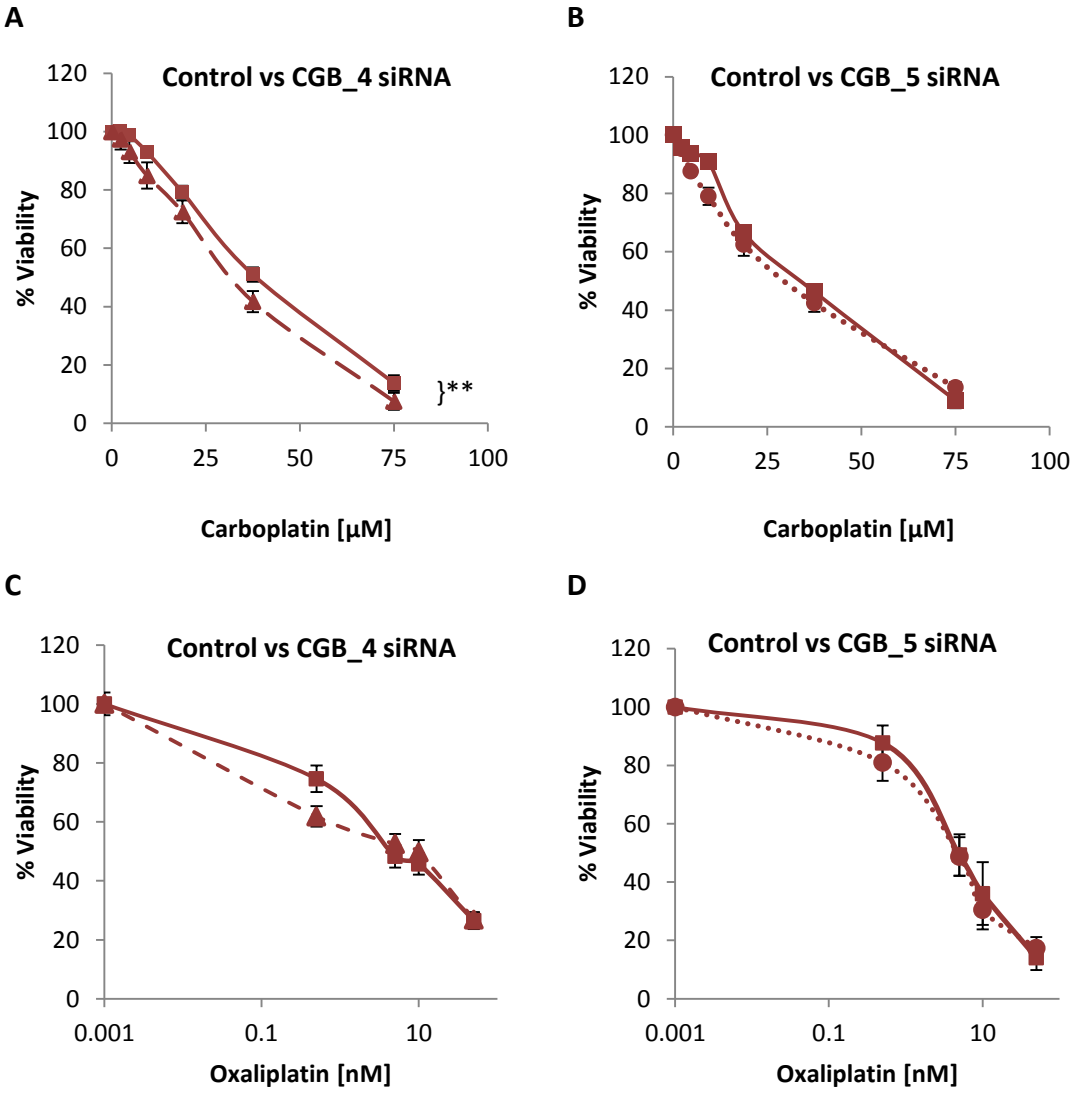


Figure 4.6 Effect of hCGβ downregulation on cell viability of HEY cells following treatment with oxaliplatin and carboplatin

Viability of HEY cells following treatment with: Carboplatin (A and B), Oxaliplatin (C and D), 48 h post transfection with non-silencing control (—■—), CGB_4 (- -▲- -) and CGB_5 (···●···) siRNAs. The MTS assay was used to determine cell viability. Data are expressed as mean ± S.E.M; (N=3). $^{**}P < 0.005$.

4.3.6 Effect of hCGβ downregulation on cell survival following cisplatin, carboplatin and oxaliplatin treatment

The clonogenic assay was used to measure cell survival. Downregulating hCGβ significantly decreased cell survival of A2780cis and HEY cells independently of any drug treatment. The number of colonies formed relative to the number of cells seeded was reduced by ~ 30% in HEY and A2780cis cells (Figure 4.7). Cell survival in the context of hCGβ downregulation was not studied in A2780 cells as the use of CGB_5 siRNA to downregulate hCGβ almost completely prevented colony formation in these cells and therefore subsequent drugs studies could not be pursued.

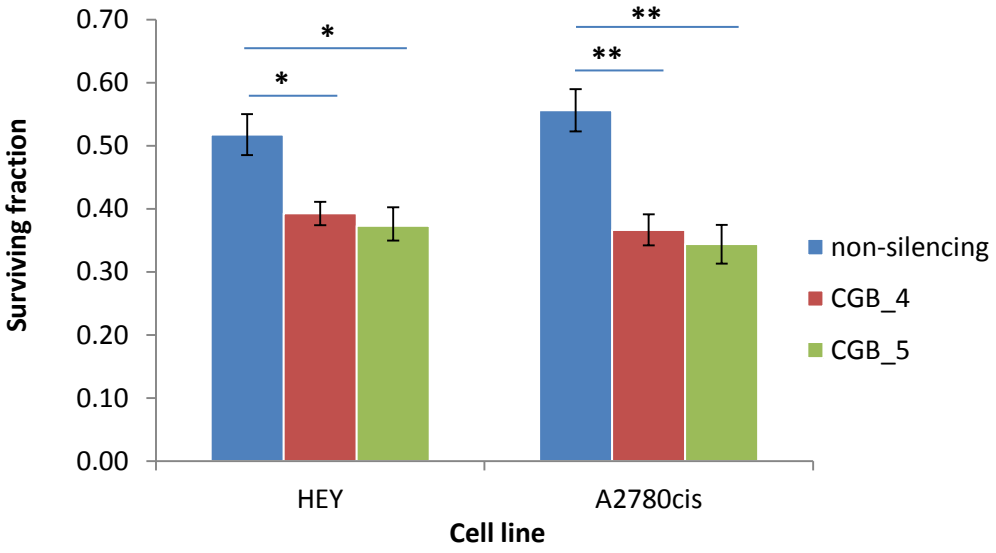


Figure 4.7 Effect of downregulating hCGβ on the ability of HEY and A2780cis cells to form colonies

The surviving fraction which is indicative of cell survival is expressed as the number of cells seeded divided by the number of colonies formed. Data are expressed as mean ± S.E.M; (N=4). * $P < 0.05$, ** $P < 0.005$.

In A2780cis cells (Figure 4.8) there was a general trend towards decreased cell survival following treatment with all three platinum-based drugs independently when hCGβ was downregulated. The use of either CGB_4 or CGB_5 siRNA to downregulate hCGβ caused a significant increase in cisplatin sensitivity (Figure 4.8 A and B). Both hCGβ targeting siRNAs also caused a significant increase in oxaliplatin sensitivity (Figure 4.8 E and F). The use of CGB_5 siRNA also caused a significant increase in carboplatin sensitivity (Figure 4.8 D), but despite CGB_4 siRNA showing a similar increase in sensitivity towards carboplatin (Figure 4.8 C), it did not reach statistical significance (P = 0.079). Refer to Appendix B, Supplementary Table 2 for IC_{50} concentrations for carboplatin and oxaliplatin.

In HEY cells a similar trend in the reduction of cell survival after cisplatin, carboplatin or oxaliplatin treatment was observed with downregulation of hCGβ (Figure 4.9). However, only the use of CGB_5 siRNA showed a significant increase in cisplatin and carboplatin sensitivity (Figure 4.9 B and D). The use of CGB_4 siRNA also showed a similar trend in increased sensitivity to cisplatin, carboplatin and oxaliplatin treatment but these changes were not statistically significant (Figure 4.9 A, C and E, respectively). Refer to Appendix B, Supplementary Table 2 for IC_{50} concentrations for carboplatin and oxaliplatin.

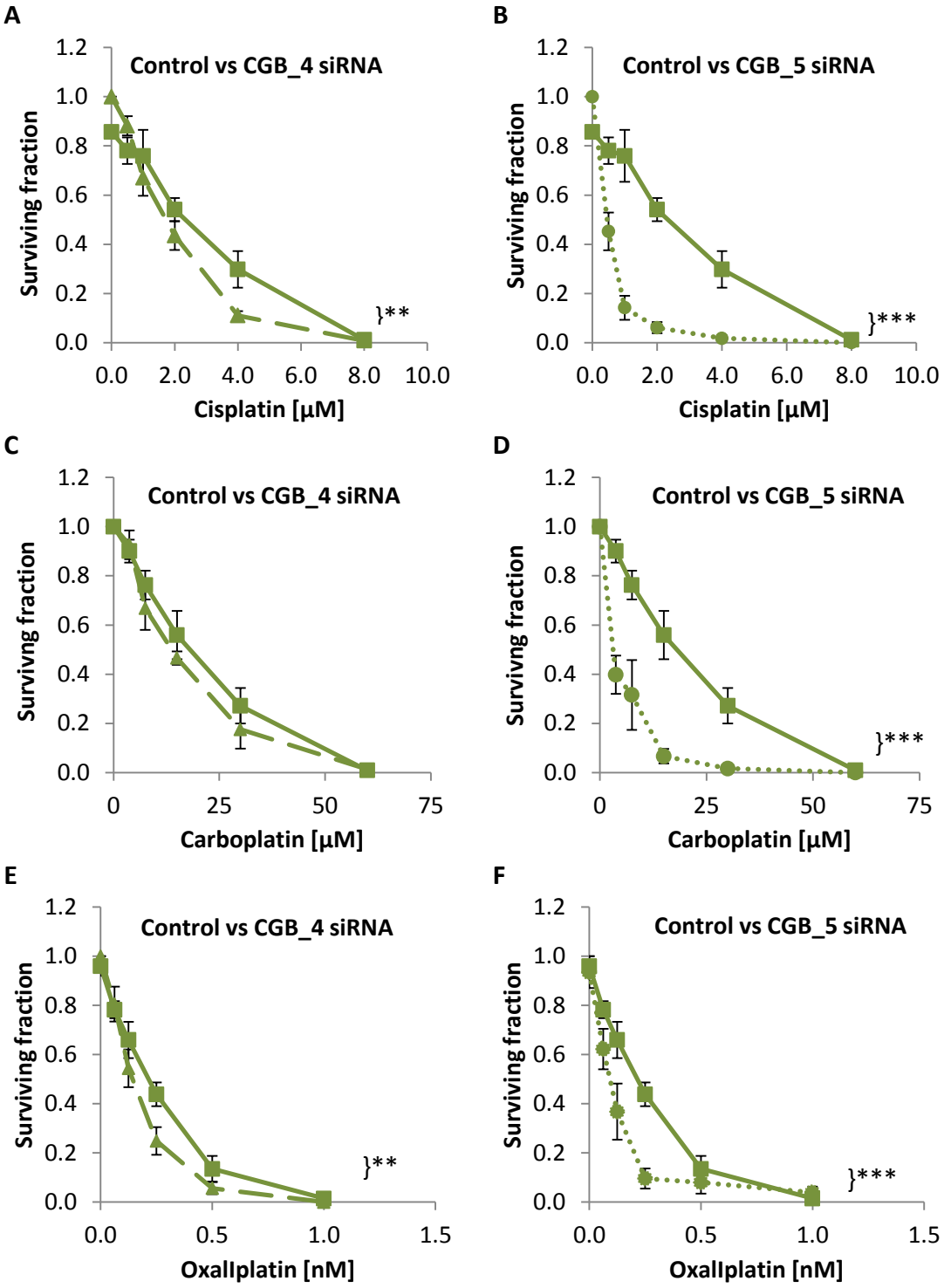


Figure 4.8 Effect of hCGβ downregulation on cell survival of A2780cis cells after treatment with platinum-based drugs

The clonogenic assay was used to measure cell survival in response to: Cisplatin (A and B, N=6), Carboplatin (C and D, N=4) and Oxaliplatin (C and D, N=4), 24 h post transfection with a non-silencing control (—■—), CGB_4 (– ▲ –) or CGB_5 (···●···) siRNAs. Data are expressed as mean ± S.E.M. * $P < 0.05$, ** $P < 0.005$, *** $P < 0.0005$.

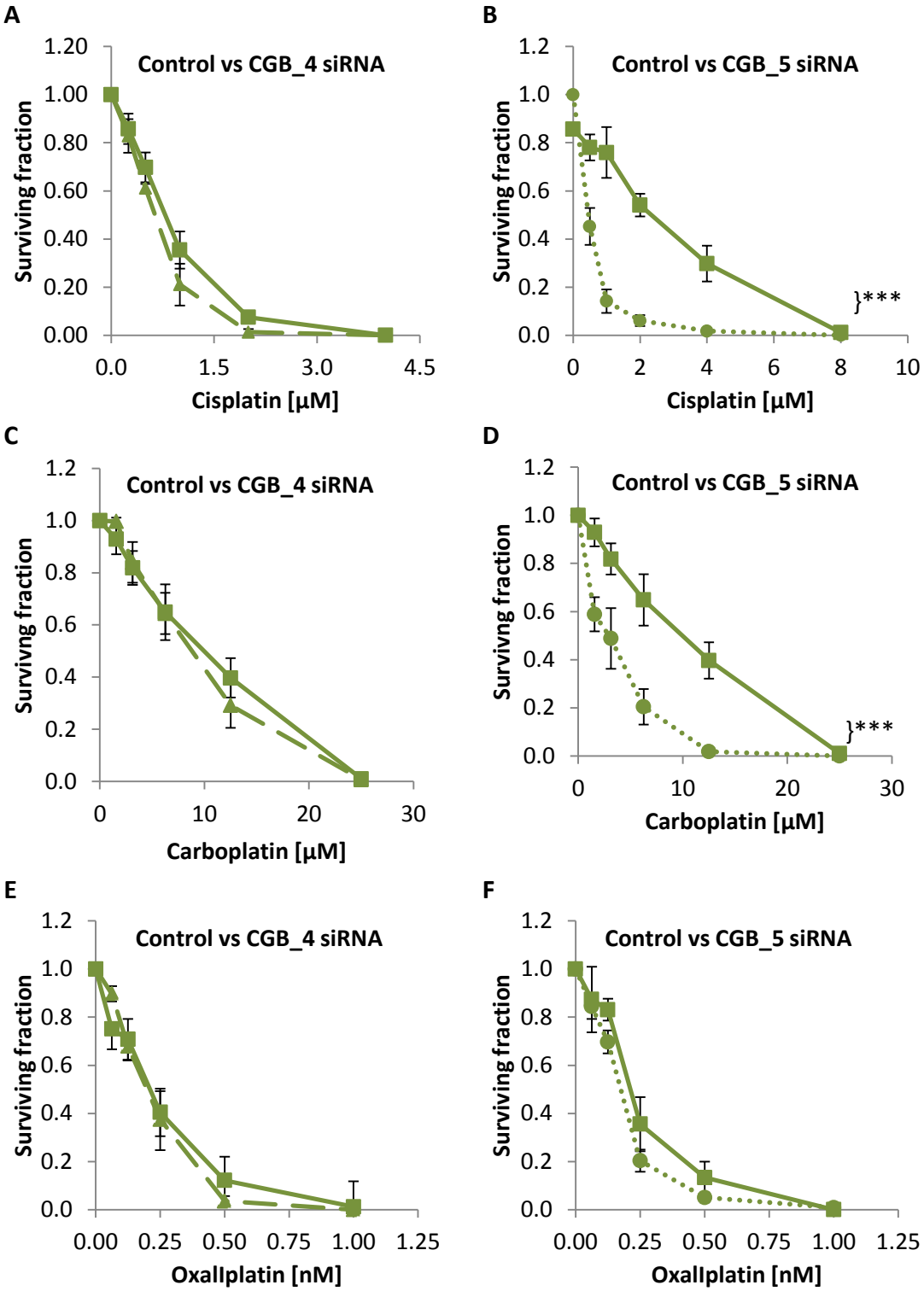


Figure 4.9 Effect of hCGβ downregulation on cell survival of HEY cells after treatment with platinum-based drugs.

The clonogenic assay was used to measure cell survival in response to: Cisplatin (A and B, N=4), Carboplatin (C and D, N=3) or Oxaliplatin (C and D, N=3), 24 h post transfection with a non-silencing control (—■—),CGB_4 (– ▲ –) or CGB_5 (···●···) siRNAs. Data are expressed as mean ± S.E.M. *** $P < 0.0005$.

IC_{50} values for cisplatin, carboplatin and oxaliplatin in A2780cis cells indicated a decrease in the amount of drug required to cause a 50% reduction in cell survival when hCGβ was downregulated (Figure 4.10 A and B). Downregulation of hCGβ with either CGB_4 or CGB_5 siRNAs showed a significant decrease in the IC_{50} for cisplatin and oxaliplatin; however, only CGB_5 significantly decreased the IC_{50} for carboplatin. Despite a general trend in the reduction of IC_{50} for cisplatin, carboplatin and oxaliplatin by both CGB_4 and CGB_5 siRNAs in HEY cells (Figure 4.10 C and D), only CGB_5 siRNA caused a significant decrease in the IC_{50} for cisplatin and oxaliplatin in these cells.

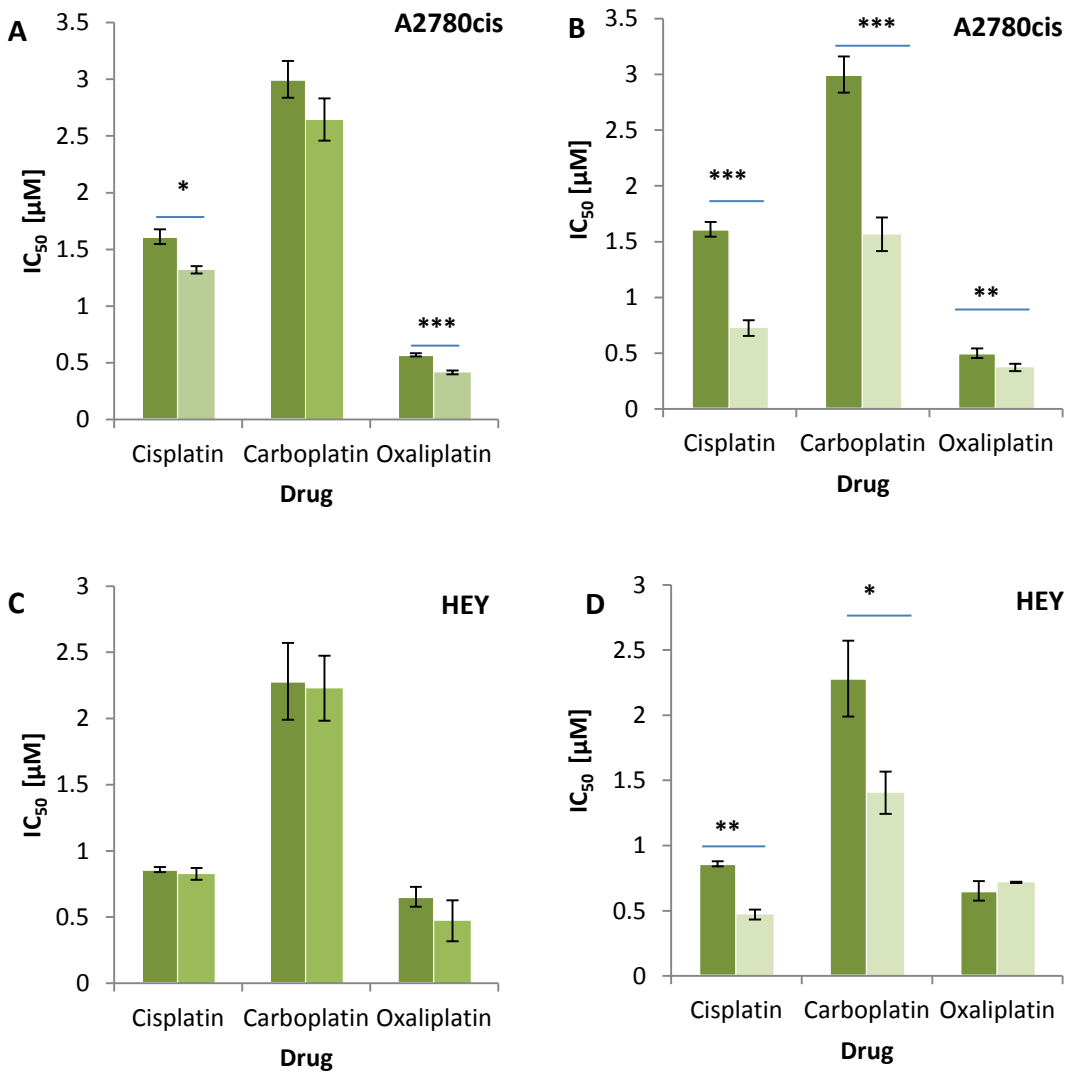


Figure 4.10 IC_{50} of cisplatin, carboplatin and oxaliplatin in A2780cis and HEY cells after hCGβ downregulation based on survival assays

IC_{50} of cisplatin (µM), carboplatin (µM), oxaliplatin (nM) derived from clonogenic assays which were used to measure cell survival, in A2780cis (A and B) and HEY (C and D) cells, 24h post transfection with a non-silencing control (■), CGB_4 (■) or CGB_5 (■) siRNAs. Data are expressed as mean ± S.E.M; (N=4). * $P < 0.05$, ** $P < 0.005$, *** $P < 0.0005$.

4.3.7 Effect of hCGβ downregulation using a third siRNA on cell survival following cisplatin, carboplatin and oxaliplatin treatment

Although the two siRNA targeting hCGβ (CGB_4 and CGB_5) had similar levels (~ 60%) of knockdown in HEY and A2780cis cells (Figure 4.11), and showed similar trends in terms of cell survival and viability in response to cisplatin and carboplatin treatment, there were a few differences that had to be addressed. Therefore a third siRNA, CGB_7, targeting *CGB* was used. This showed a similar level of *CGB* downregulation to CGB_4 and CGB_5 siRNAs (Figure 4.11).

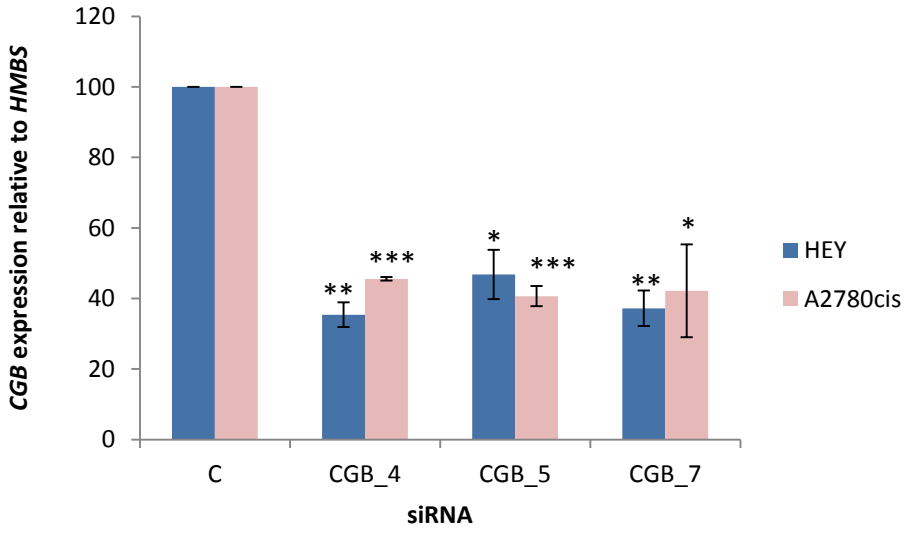


Figure 4.11 Downregulation of *CGB* in HEY and A2780cis using three siRNAs

CGB downregulation in HEY and A2780cis cells measured by qRT-PCR. Expression of *CGB* gene transcript relative to *HMBS*, normalised to *CGB/HMBS* expression in the control non-silencing siRNA (C) 24 h post siRNA transfection. siRNAs: non-silencing control (C), CGB_4, CGB_5 and CGB_7. Data are expressed as mean ± S.E.M. (N=3). Statistical analysis: one sample *t*-test, comparing the different siRNA treatments for each cell line to their respective control siRNA * $P < 0.05$, ** $P < 0.005$, *** $P < 0.0005$.

Although CGB_7 siRNA showed similar levels of hCGβ downregulation (~ 65%) in HEY and A2780cis cells, the resulting response to cisplatin and carboplatin in the two cell lines was different (Figure 4.12). Note that a one sample *t*-test was used for statistical analysis to assess knockdown as the non-silencing control siRNA was normalised to 100%. In A2780cis cells, CGB_7 siRNA caused a small but significant increase in sensitivity to cisplatin and carboplatin (Figure 4.12 A and B). The degree by which CGB_7 siRNA increased cisplatin sensitivity in A2780cis cells was in between the degrees shown by CGB_4 and CGB_5 siRNA (Figure 4.8 A and B). Treatment of HEY cells with CGB_7 siRNA showed no change in cisplatin or carboplatin sensitivity in HEY cells (Figure 4.12 C and D). This result was contrary to the trend in increased in cisplatin and carboplatin sensitivity of caused by treatment of HEY cells with either CGB_4 or CGB_5 (Figure 4.9 A and B).

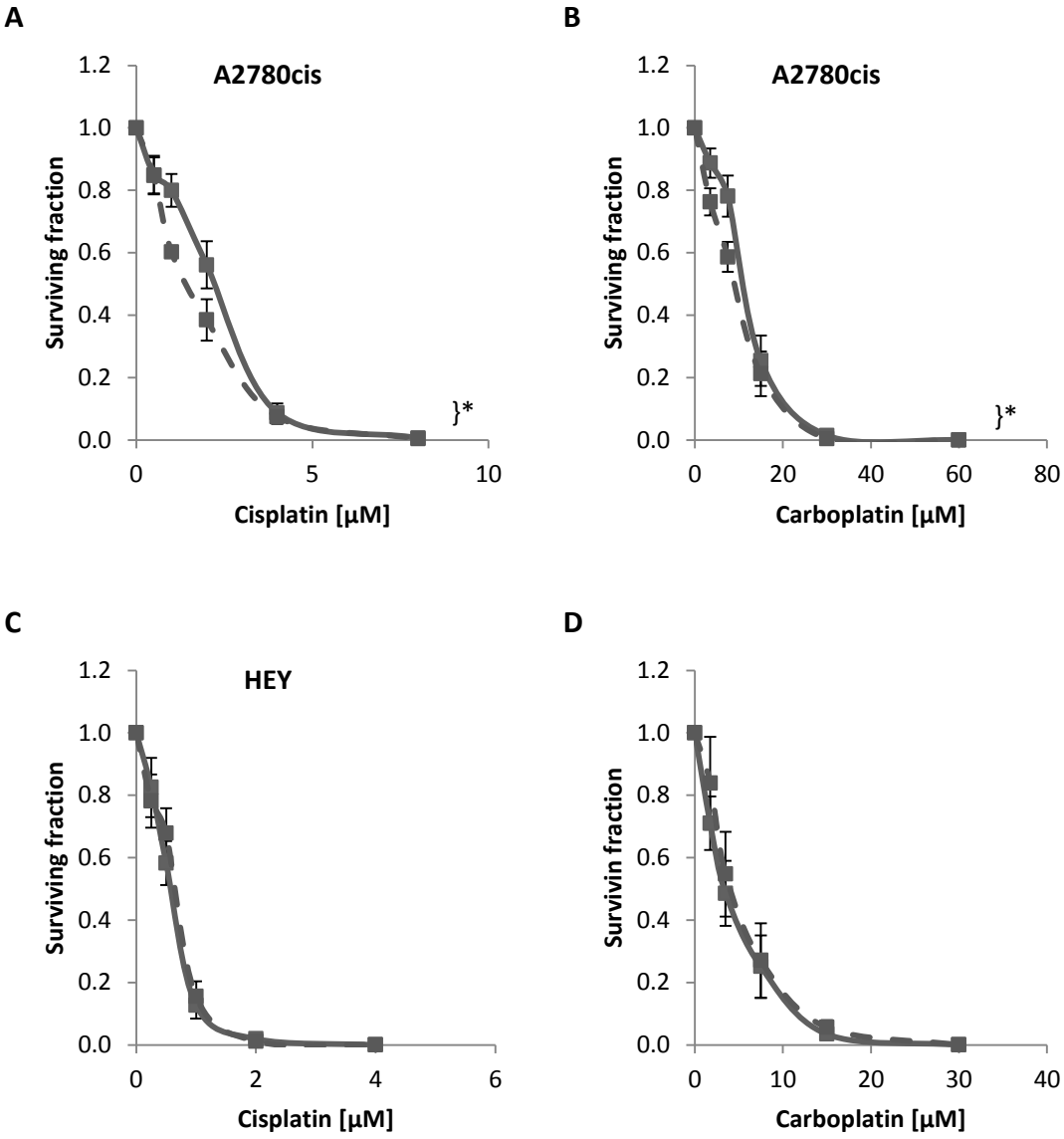


Figure 4.12 Effect of hCGβ downregulation using CGB_7 siRNA on cell survival following cisplatin and carboplatin treatment

The clonogenic assay was used to measure cell survival of A2780cis (A and B) and HEY (C and D) cells in response to treatment with cisplatin and carboplatin, 24 h post transfection with a non-silencing control (—■—) or CGB_7 (- -■- -). Data are expressed as mean ± S.E.M. (N=3). * $P < 0.05$.

4.3.8 Investigation of possible compensatory effects of LHβ on hCGβ downregulation

The β-subunit of the luteinising hormone (LHβ) is highly homologous to hCGβ. Furthermore, the gene encoding LHβ, *LHB*, is positioned on the same gene cluster as the *CGB* genes [130]. For these reasons, a compensatory effect by *LHB* was investigated to determine whether functional differences observed by the siRNAs targeting hCGβ (in particular CGB_4 and CGB_5) could be due to changes in the LHβ levels. Downregulation of *CGB* by CGB_4 siRNA caused ~ 65 % increase in the expression of *LHB* in HEY cells and a 30 % increase in A2780cis cells (Figure 4.13); however, statistical significance was reached only for HEY cells. In contrast, downregulation of hCGβ by CGB_5 siRNA caused a significant decrease of *LHB* (~ 20%) in HEY cells and no change in A2780cis cells. CGB_7 caused a slight reduction of *LHB* expression in both cell lines (Figure 4.13 A) but this was not statistically significant. Endogenous levels of *LHB* were also determined and it was found that HEY expressed 15 times more *LHB* compared to A2780cis cells (Figure 4.13 B). The one sample *t*-test was used for statistical analysis of *LHB* expression levels following *CGB* downregulation as the control siRNA was normalised to 100%.

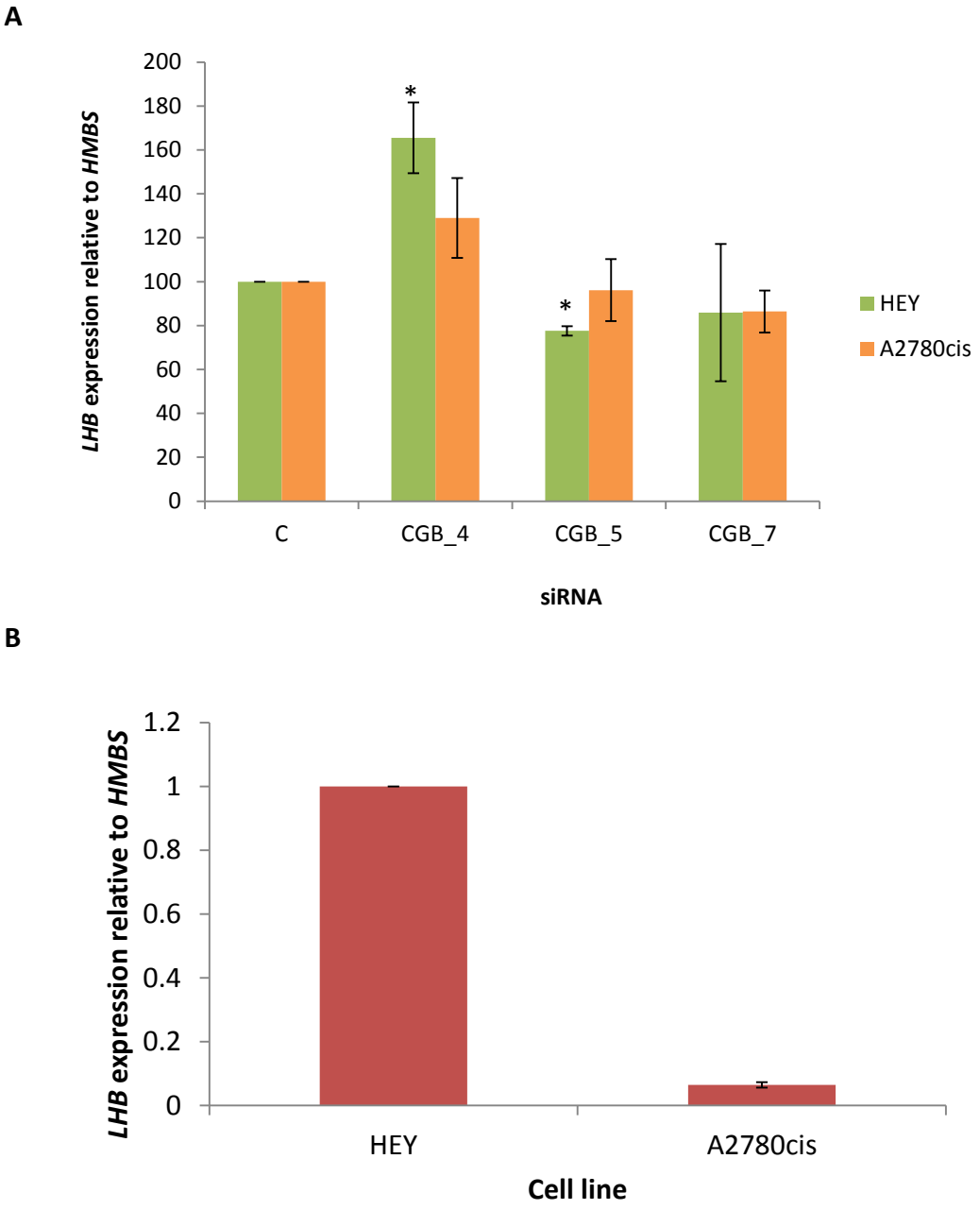


Figure 4.13 Effect of *CGB* downregulation on *LHB* gene expression in HEY and A2780cis cells and endogenous expression of *LHB*

Effect of *CGB* downregulation on *LHB* expression in HEY and A2780cis cells (A) and endogenous expression of *LHB* in HEY and A2780cis cells measured by qRT-PCR (B). siRNAs: non-silencing control (C), CGB_4, CGB_7 and CGB_5. Data are expressed as mean ± S.E.M; (N=3). Statistical analysis: one sample *t*-test. Comparing the different siRNA to their respective control siRNA HEY cells: * $P < 0.05$ (applicable to panel A only).

4.3.9 Effect of hCGβ overexpression on viability and survival of A2780 and A2780cis cells in response to cisplatin treatment

Downregulation of hCGβ showed different effects in terms of how the cisplatin sensitive A2780 cells and cisplatin resistant cells A2780cis responded to cisplatin (Figure 4.3 A and B). Specifically, downregulation of hCGβ in A2780 cells did not affect how the cells responded to cisplatin; however, it did sensitise A2780cis cells to cisplatin. hCGβ was then transiently overexpressed in the two cell lines to determine whether an increase in hCGβ would affect response of A2780 cells to cisplatin and perhaps show a decrease (opposite to effect seen by downregulation of hCGβ) in cisplatin sensitivity in A2780cis. hCGβ was markedly overexpressed following plasmid transfection in A2780 and A2780cis cells as shown previously in Chapter 3 (Figure 3.11). Despite this increase, the response to cisplatin treatment for both cell lines in terms of either cell viability assessed by MTS assay (Figure 4.14) or cell survival assessed by clonogenic assays, (Figure 4.15) was unaffected.

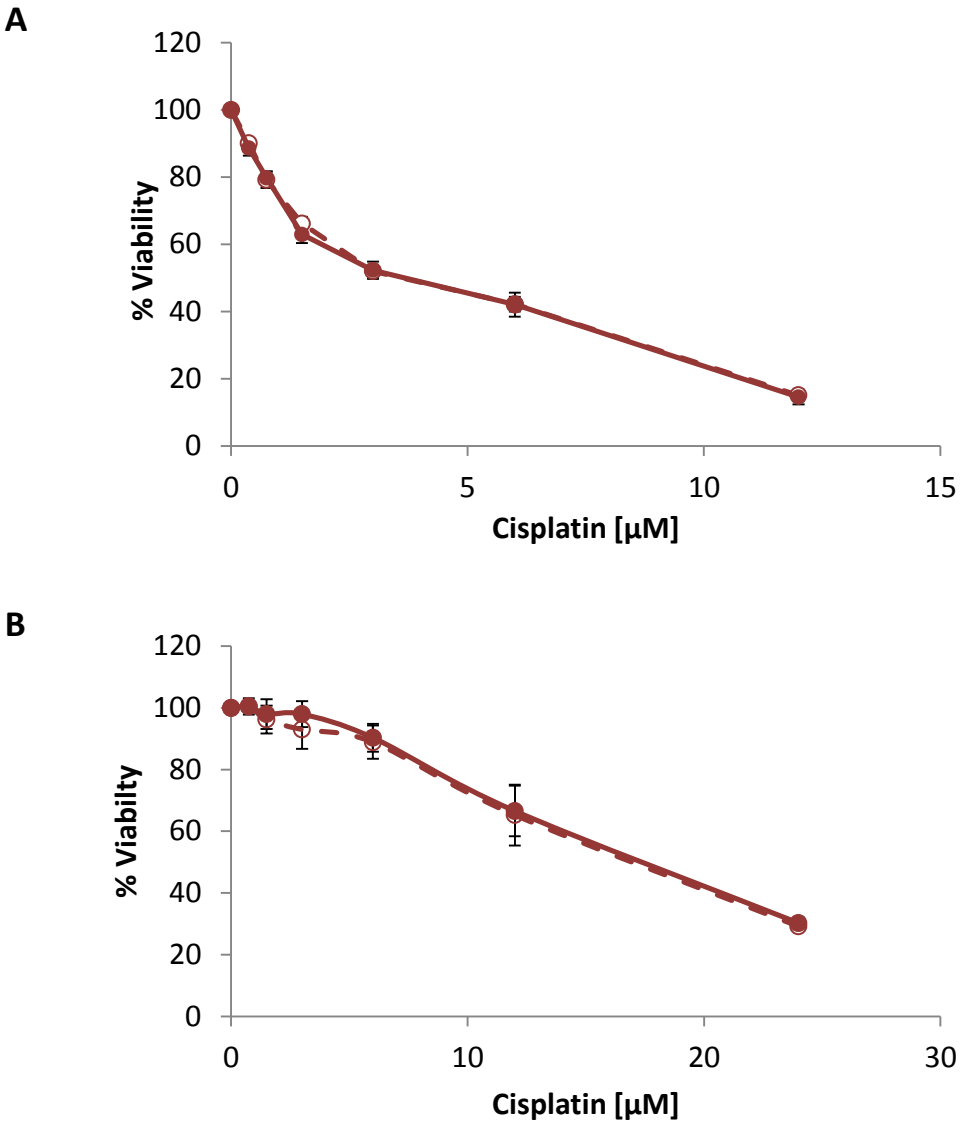


Figure 4.14 Effect of hCGβ overexpression on cell viability following cisplatin treatment

Viability of cells following hCGβ overexpression and treatment with cisplatin. Cells: A2780 (A) and A2780cis (B). Vectors: Empty pCI-neo (—●—), pCI-neo containing hCGβ insert (- -○- -). Data are expressed as mean ± S.E.M; (N=3).

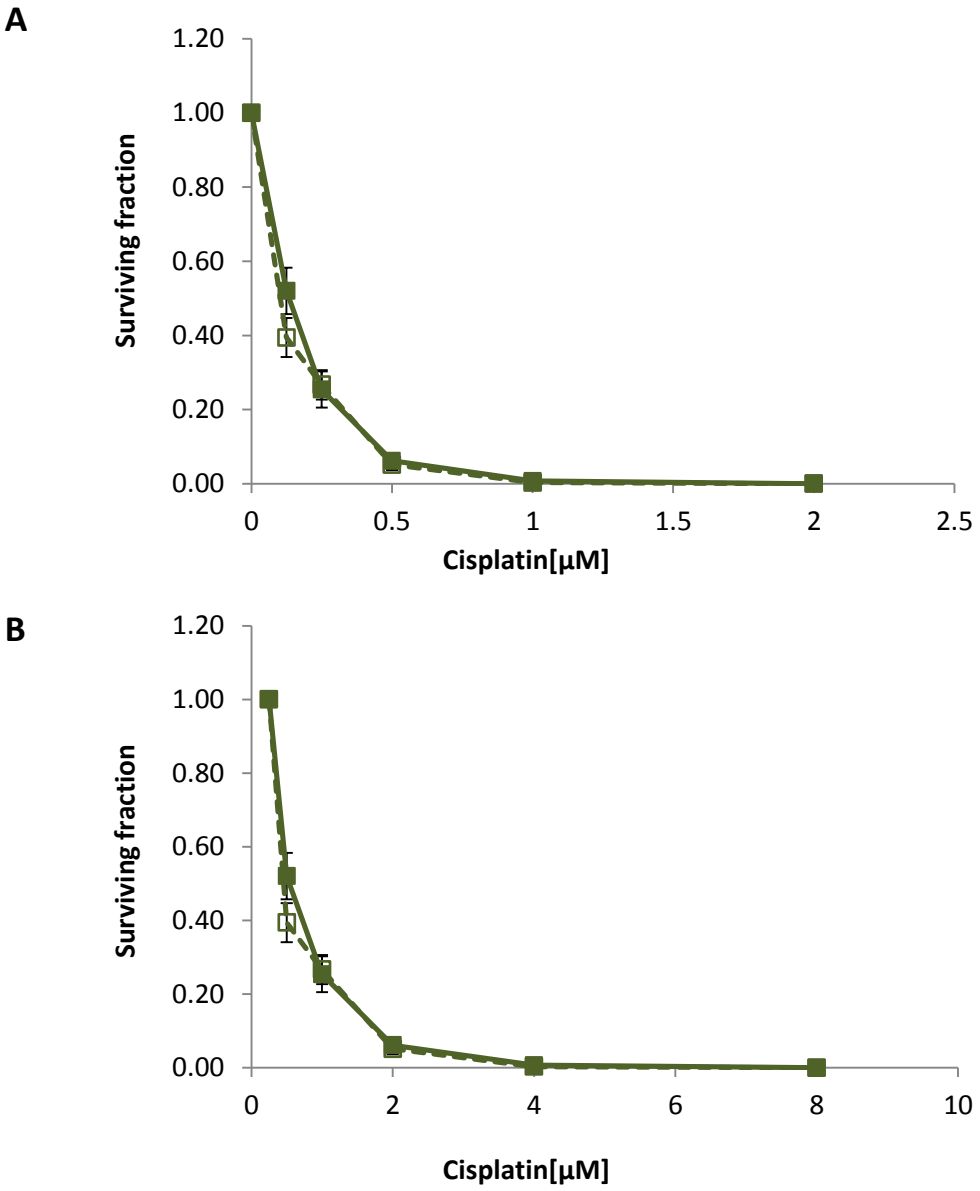


Figure 4.15 Effect of hCGβ overexpression on cell survival following cisplatin treatment

The clonogenic assay was used to measure cell survival of A2780 (A) and A2780cis (B) in response to hCGβ overexpression and cisplatin treatment. Vectors: Empty pCI-neo (- -□- -), pCI-neo containing hCGβ insert (—■—). Data are expressed as mean ± S.E.M; (N=3).

4.4 Discussion

4.4.1 Correlation between levels of secreted hCGβ and cisplatin sensitivity

A clear association between the level of *CGB* and secreted hCGβ (*CGB* and hCGβ) in HEY, A2780 and A2780cis cells and their sensitivity to cisplatin could not be established (Figure 4.2). If only the HEY and A2780 cells were taken into consideration it would appear that higher levels of *CGB*/hCGβ correlate with decreased sensitivity to cisplatin, which would agree with the hypothesis that high expression of hCGβ may be associated with resistance to chemotherapy. However, the question of whether the absolute level of *CGB* and hCGβ expressed by cells could determine sensitivity or resistance to cisplatin arises when the response of HEY and A2780 cells to cisplatin is compared to that of A2780cis cells relative to the levels of *CGB* and hCGβ they express. HEY and A2780 cells expressed higher levels of *CGB* and hCGβ compared to A2780cis cells, and if higher levels of *CGB* and hCGβ correlated with decreased sensitivity to cisplatin then HEY and A2780cis cells should be less sensitive to cisplatin compared to A2780cis which was not the case (Figure 4.2). It should be noted that only three cell lines were used in this experiment therefore conclusive correlative data cannot be inferred; however, there are a few possible reasons why HEY and A2780 cells are more sensitive to cisplatin compared to A2780cis cells that express lower levels of *CGB* and hCGβ. 1. Cisplatin resistance is multifactorial [192], therefore it is likely that the absolute level of *CGB* and hCGβ expression between cell types alone are not able to dictate how cells respond to cisplatin. However, it is possible that the relative level of *CGB* and hCGβ in conjunction with unidentified factors determines hCGβ's role in sensitivity to cisplatin (or other platinum-based drugs); 2. Drug resistance in A2780cis is

independent of *CGB* and hCGβ expression and due to other factors e.g. preventing drug accumulation and increased DNA repair [250, 305]; and 3. It is possible that different isotypes (type I and II) [165] of hCGβ may be expressed at different levels in the different cell lines which may play a varied role in cisplatin response. Whether type I or II hCGβ have different functions remains to be established. The ELISA and gene expression assays used in this study did not have the specificity to determine expression of the different isotypes.

4.4.2 Effect of hCGβ downregulation on cell viability following cisplatin and paclitaxel treatment

Downregulation of hCGβ increased the sensitivity of cisplatin resistant A2780cis cells to cisplatin but did not affect the sensitivity of cisplatin sensitive A2780 cells to cisplatin (Figure 4.3 A and B). From this observation two things can be inferred about the role of hCGβ in cisplatin sensitivity: firstly, hCGβ may play a role in how cells respond to cisplatin, and secondly, hCGβ's role in response to cisplatin is more important in cells that have acquired resistance (or decreased sensitivity) to cisplatin. In agreement with the second proposition, HEY cells which were less sensitive to cisplatin compared to A2780 cells, also displayed increased sensitivity to cisplatin as a result of decreased levels of hCGβ (Figure 4.3 C). These data agree with studies that have shown elevated levels of hCGβ to be associated with chemoresistance [180, 280].

Though downregulation of hCGβ seemed to sensitise A2780cis and HEY cells to cisplatin based drugs it had no effect on how cells responded to paclitaxel (Figure 4.4). One likely explanation is that the paclitaxel and cisplatin have different cellular

targets (microtubules and DNA, respectively) hence different mechanisms of actions. hCGβ may be central to the mechanism of action of cisplatin and not microtubule targets agents. The possible mechanism of action of hCGβ in response to cisplatin treatment is investigated in Chapter 5.

4.4.3 Effect of hCGβ downregulation on cell viability following treatment with other platinum-based drugs

Downregulation of hCGβ significantly increased the sensitivity of A2780cis to carboplatin and oxaliplatin (Figure 4.5) which indicates that the increase in the sensitivity to cisplatin in these cells (discussed in section 4.4.2 above) could cross-over to other platinum-based drugs. Though both CGB_4 and CGB_5 siRNA showed a trend of decreased viability when HEY cells were exposed to carboplatin and oxaliplatin, only the use of CGB_4 siRNA caused a statistically significant increase in sensitivity to carboplatin (Figure 4.6 A). This suggests that the increased sensitivity to cisplatin may not necessarily cross-over to carboplatin and oxaliplatin in HEY cells. It should also be noted that, although the use of CGB_4 siRNA showed a statistically significant increase in cisplatin and carboplatin sensitivity in A2780cis cells, the effect was not as marked as when CGB_5 siRNA was used. A possible reason (involving *LHB*) why the two siRNA were displaying functional differences despite showing similar level of hCGβ knockdown is discussed in section 4.4.6.

4.4.4 Effect of hCGβ downregulation on cell survival

Cell survival was significantly decreased when hCGβ was downregulated in A2780cis and HEY cells (Figure 4.7). This result agreed with data from the cell proliferation studies which showed that downregulation of hCGβ significantly decreased proliferation of HEY and A2780cis cells (Chapter 3, Figure 3.4). Indeed, cell survival and proliferation assays are different assays, but they are both reflective of how the cells are able to survive and proliferate.

4.4.5 Effect of hCGβ downregulation on cell survival following cisplatin, carboplatin and oxaliplatin treatment

Cell survival following hCGβ downregulation and treatment with cisplatin, carboplatin and oxaliplatin showed a trend of decreased sensitivity to the drugs in HEY and A2780cis cells (Figure 4.8 and Figure 4.9, respectively). However, only treatment with CGB_5 siRNA caused a significant increase in sensitivity to cisplatin and carboplatin in both A2780cis (Figure 4.8 B and C) and HEY cells (Figure 4.9 B and C), as well as an increase in sensitivity to oxaliplatin in A2780cis cells (Figure 4.8 E).

Carboplatin has a similar mode of action to cisplatin, supported by the fact that cross-resistance is commonly observed between these two drugs [192, 202, 211, 214, 227]. Therefore it was not surprising that the increase in sensitivity to cisplatin following hCGβ downregulation was also observed following carboplatin treatment; however it was unexpected that cross-sensitivity to oxaliplatin was also observed as this is not often the case [214, 215]. Oxaliplatin forms similar DNA cross-links as

cisplatin however the mechanism of action of oxaliplatin seems to be different to that of cisplatin [215]. It is thought that the bulky 1,2-diaminocyclohexane (DACH) ring of oxaliplatin distorts the DNA in a manner which compromises binding the HMGB and MMR proteins [215]. MMR proteins seem to preferentially recognise and repair cisplatin-DNA adducts over oxaliplatin-DNA and therefore DNA lesions formed by oxaliplatin are bypassed [306]. It has also been suggested that other oxaliplatin interactions other than DNA-interactions may contribute to its mechanism of action [306, 307]. However, the increase in sensitivity following hCGβ downregulation suggests that hCGβ may play a direct or indirect role in common pathway shared by all three drugs which contributes to their cytotoxic effect.

Although CGB_4 siRNA showed a trend of increased sensitivity to the platinum drugs, statistically significant results were achieved only in A2780cis cells after exposure to cisplatin and oxaliplatin (Figure 4.8 A and E). These results indicate that although hCGβ may be involved in sensitivity to platinum-based drugs; it could be more due to the different secondary effects of the siRNAs. A third siRNA targeting hCGβ (CGB_7) was used to test this theory. CGB_7 siRNA knocked down hCGβ at comparable levels to CGB_4 and CGB_5 siRNAs (60-70%) (Figure 4.11); however, this caused a significant decrease in cell survival in only A2780cis cells and not HEY cells following exposure to cisplatin and carboplatin (Figure 4.12). This suggested that the repeated increase in drug (in particular cisplatin and carboplatin) sensitivity observed by the CGB_5 siRNA in A2780cis is not due to a mere secondary effect of the siRNA. This result however, does not explain why HEY cells behaved differently with regards to drug sensitivity depending on the siRNA used to target hCGβ. The effects displayed by CGB_5 siRNA in HEY cells could be due to an off target effect, or perhaps due a compensatory effect of the related *LHB* gene.

The Taqman assay used to measure *CGB* gene downregulation does not distinguish between the different *CGB* genes and therefore cannot provide a representation of each individual gene product. The *LHB* gene could be detected and the results are discussed in below.

4.4.6 Compensatory effect of LHβ subunit following hCGβ downregulation

A compensatory effect of LHβ following hCGβ downregulation was studied in HEY and A2780cis cells in an attempt to explain: 1. the functional differences caused by the two siRNA used to target hCGβ (CGB_4 and CGB_5); and, 2. the different functional effects the siRNAs had on the two cell types (HEY and A2780cis cells). It was found that when *CGB* was downregulated using CGB_4 siRNA, the *LHB* transcript level increased by 65 % and 30 % in HEY and A2780cis cells, respectively. This effect was not seen when CGB_5 siRNA was used (Figure 4.13 A). This result could contribute to the functional differences observed using the two siRNAs. In particular, the significant changes in cell survival and viability after drug exposure observed with the use of CGB_5 siRNA may have been masked by increased *LHB* levels caused by the CGB_4 siRNA. Interestingly, the *LHB* levels decreased following hCGβ downregulation with CGB_5 in HEY cells only. These results suggest that both *LHB* and *CGB* may need to be downregulated concurrently in these cells but not A2780cis cells in order to increase their sensitivity to platinum-based drugs. In agreement with this theory it was observed that the use of CGB_7 siRNA to downregulate hCGβ did not conclusively reduce the levels of the *LHB* gene in HEY cells. This may be a reason why an increase in cisplatin or carboplatin

sensitivity was not observed following hCGβ downregulation with CGB_7 siRNA (Figure 4.12).

A study by Zhang *et al.* showed that *in vitro* exposure to exogenous LH could decrease the sensitivity of ovarian cancer cell lines to cisplatin [308]. As indicated in our data the α-subunit is expressed by both HEY and A2780cis cells (Chapter 3, Figure 3.2) which could potentially bind with the LHβ subunit thus forming the LH heterodimer and contributing to the response to cisplatin. Interestingly, although HEY cells expressed up to 15 times more endogenous *LHB* compared to A2780cis (Figure 4.13 B) they are more sensitive to cisplatin. However it is still possible that when hCGβ is downregulated in HEY cells, *LHB* may contribute to the response of these cells to cisplatin.

4.4.7 Effect of hCGβ overexpression on cell viability and survival following exposure to cisplatin

Overexpression of hCGβ in A2780 and A2780cis cells did not affect their response to cisplatin either in terms of cell viability (Figure 4.14) or cell survival (Figure 4.15). This result was not surprising in A2780 cells given that downregulation of hCGβ did not affect their response to cisplatin (Figure 4.3). It was however hypothesised that increased expression of hCGβ may decrease the sensitivity of A2780cis cells to cisplatin, since downregulation of hCGβ sensitised A2780cis to cisplatin (Figure 4.3). A possible explanation for this result is that increased expression of hCGβ beyond endogenous levels of hCGβ may not have influenced cisplatin sensitivity because a threshold level at which hCGβ has a protective effect to cisplatin had been reached. Another explanation, and one that is further investigated in the next chapter, is that there are additional factors which are altered only when hCGβ is downregulated thus contributing to its mechanism of action.

4.5 Conclusion

Downregulation of hCGβ increased the sensitivity of the HGSC cells A2780cis and HEY to the drugs cisplatin, carboplatin and oxaliplatin, suggesting that hCGβ plays a role in sensitivity of HGSC towards platinum-based drugs. However, the effect may be dependent on several factors including a predisposition to drug resistance (or decreased sensitivity) as cisplatin sensitive A2780 cells did not seem to be affected by hCGβ downregulation. Furthermore, the discrepancies in the results shown by the use of two or three different siRNAs targeting hCGβ suggests that other unidentified factors e.g. LHβ, are required for the response of HGSC towards platinum-based drugs. These additional factors and the mechanism by which hCGβ is involved in the response of HGSC cells to cisplatin are further investigated in Chapter 5.

Chapter 5 Mechanism by which hCGβ may regulate sensitivity of HGSC cells to cisplatin treatment

5.1 Introduction

In the previous chapter it was found that hCGβ may have a role in the response of HGSC cell lines to the platinum-based drugs cisplatin, carboplatin and oxaliplatin. However the mechanism by which hCGβ could be involved in this response needed to be understood. Therefore, further investigation into global protein changes that occur when hCGβ is downregulated following cisplatin treatment needed to be undertaken. To this end, quantitative proteomics using isobaric tags for relative and absolute quantitation (iTRAQ) labelling followed by tandem mass spectrometry (MS/MS) was used. One of the advantages of using iTRAQ is that it allows multiplexing with the ability to analyse and compare 4 (4-plex) or 8 (8-plex) samples in a single MS/MS run.

Briefly, protein samples from A2780cis cells with and without hCGβ downregulation in the absence or presence of cisplatin were extracted, digested with trypsin, iTRAQ labelled, pooled together, separated with liquid chromatography and peptides (correlating to whole proteins) quantified using tandem mass spectrometry.

5.1.1 iTRAQ labelling and quantitation of protein expression

The iTRAQ label or tag consists of three groups: a N-methyl piperazine reporter, a carbonyl balance and a N-hydroxy succinimide ester peptide reactive group shown in Figure 5.1.

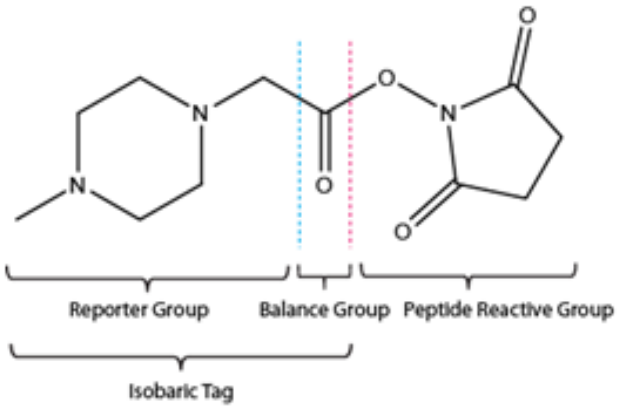


Figure 5.1 Structure of the iTRAQ label

Structure depicts the reporter group (114-117 Da), balance group (28-31 Da) and peptide reactive group.

Figure extracted from [309]

The reporter group has a mass ranging from 114-117 Da and the weight of each reporter group is counter balanced with the balance group from 28-31 Da to yield an isobaric (equal mass) tag with a total mass of 145 Da. The isobaric tag is covalently linked to peptides through an amide bond at the N-terminus of the peptide or side amino group of lysine via the peptide reactive group [310]. Once the peptides are labelled they are pooled together and fractionated by liquid chromatography and then identified and quantified by tandem mass spectrometry. In the first MS round the peptides with different iTRAQ labels will appear as a single peak, however the second MS round will yield the peptide sequences as well as dissociate the reporter

group with a different mass thus allowing the quantitation of peptides from the different samples. The process of iTRAQ from protein extraction to identification and quantitation is outlined in Figure 5.2.

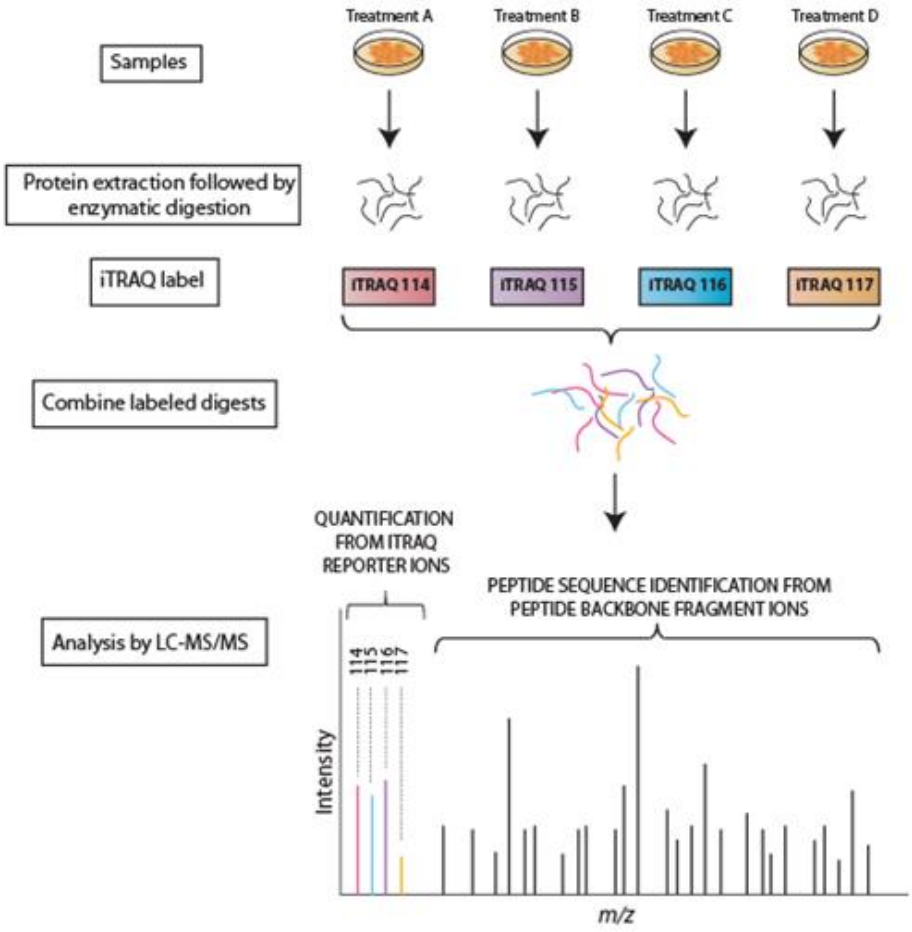


Figure 5.2 iTRAQ coupled with LC-MS/MS for the quantification of global protein changes

Flow of how protein samples are labelled using iTRAQ methodology and quantified using liquid chromatography coupled with tandem mass spectrometry.
Figure extracted from [309]

5.2 Materials and Methods

5.2.1 Downregulation of hCGβ for proteomics

A2780cis cells were used for the proteomic study as these cells showed a consistent differential response to cisplatin treatment after hCGβ was downregulated. CGB_5 was the siRNA chosen to knockdown hCGβ as this was the siRNA that showed significant and the most reproducible results in regards to increasing sensitivity of cells (both HEY and A2780cis cell lines) to cisplatin treatment. Refer to Appendix C Supplementary Figure 2 for quantification of downregulation of *CGB* in A2780cis cells used in the iTRAQ experiment.

hCGβ was downregulated according to the method described in Chapter 2, section 2.8 with a few changes. A large amount of protein (2 mg) was required for the proteomics study hence approximately 1 x 10^7 cells had to be transfected per treatment. Three lots of 3.5 x 10^6 cells were transfected and pooled into a 15 cm petri dish in 15 mL of culture media. The transfections consisted of 2 dishes of cells receiving the negative non-silencing control siRNA, and 2 dishes of cells receiving the CGB_5 siRNA. The media was replaced with fresh media 24 h after transfection.

5.2.2 Treatment with cisplatin for proteomic studies

Forty-eight hours after transfection one plate of cells receiving the non-silencing control and the other receiving CGB_5 siRNA, were treated with 6.5 μM of cisplatin approximating an IC_{50} dose based on the cell viability data (Chapter 4, section 4.3.1), and incubated for 24 h.

5.2.3 Protein extraction for proteomic studies

Twenty-four hours after drug treatment, the cells were washed with PBS twice, followed by the addition of 1 mL of protein lysis buffer (Table 5-1) on ice. Cells were scraped off with a cell scraper and the lysate transferred to a 15 mL tube.

Table 5-1 Components of protein lysis buffer for iTRAQ

Component	Concentration
Sodium dodecyl sulfate	0.15%
HEPES, pH 7.5	20 mM
Sodium chloride	150 mM
Sodium fluoride	10 mM
Sodium orthovanadate	1 mM
EDTA	1 mM
EGTA	1 mM

The lysates were sonicated for 1 min in 30 s bursts, centrifuged for 5 min at 500 g and the supernatant transferred to a 1.5 mL Eppendorf tube. The amount of protein was quantified using the BCA Protein Assay Kit colorimetric assay according to the manufacturer's protocol. Briefly, 25 µl of the protein lysates (diluted 1:5 in lysis buffer) was assayed in duplicate against 25 µl of the BSA standard: 2, 1.5, 1, 0.75, 0.5, 0.25, 0.125, 0.025 and 0 (blank, lysis buffer) µg/mL in a 96 well plate, also in duplicate. Two hundred µl of working reagent (included in the kit) was added to the wells and incubated for 30 min at 37ºC. The absorbance was then measured at 560 nm on the Wallac Victor 1420 Multilabel Counter. A BSA protein standard curve of absorbance vs protein quantity was graphed and the protein concentrations were determined based on this curve.

5.2.4 iTRAQ labelling and proteomic analyses

Once the protein concentrations were established, the samples were sent to the Australian Proteome Analysis Facility (APAF Ltd, Sydney, Australia) for iTRAQ labelling and proteomics analysis. As this process was outsourced on a fee-for-service basis, only a summary of the method for analysis is outlined here. Briefly, protein lysates were buffer exchanged to 0.25 M triethyl ammonium bicarbonate, 0.05% SDS on a Vivaspin 2 5-kDa filter, and 100 μg of protein was then reduced with tris(2-carboxyethyl)phosphine, alkylated with methyl methanethiosulfonate and trypsin digested. The digested samples were isotope labelled using the iTRAQ® Reagent - 4plex system (AB Sciex). Labels assigned to protein samples are summarised (Table 5-2).

Table 5-2 Labels assigned to samples for proteomic studies

Sample/Treatment	Isotope label
Control siRNA	114
Control siRNA + cisplatin	115
CGB_5 siRNA	116
CGB_5 siRNA + cisplatin	117

The labelled samples were washed and fractionated by strong cation exchange high performance liquid chromatography (HPLC) followed by nanoflow liquid chromatography electrospray ionisation tandem mass spectrometry (nanoLC ESI MS/MS) with data acquisition using the Eksigent Ultra nanoLC system (Eksigent)

coupled with a Triple TOF 5600 mass spectrometer (AB Sciex). The data was then processed using ProteinPilot V4.2b (AB Sciex) against the SwissProt 2012 Human database.

Protein changes were reported as iTRAQ ratios. Proteins were considered to be upregulated if the ratio was above 1.2 or downregulated if the ratio was below 0.85. The top 10 upregulated and downregulated proteins of the following comparisons were tabulated (Table 5-3).

Table 5-3 Summary of sample labels and what they measure

Indicated by	Label	Ratio	Measures
A	115:114	control siRNA + cisplatin : control siRNA	Effect of cisplatin
B	116:114	CGB_5 siRNA : control siRNA	Effect of hCGβ downregulation
C	117:114	CGB_5 siRNA + cisplatin : control siRNA	Effect of hCGβ downregulation and cisplatin together
D	117:116	CGB_5 siRNA + cisplatin : CGB_5 siRNA	Effect of cisplatin when hCGβ was downregulated
E	$\frac{115:114}{117:116}$ Where 115:114 is close to 1	$\frac{\text{control siRNA + cisplatin : control siRNA}}{\text{CGB\_5 siRNA + cisplatin : CGB\_5 siRNA siRNA}}$ Where "A" is close to 1 (0.9 > ratio <1.1)	Proteins that were only responsive to cisplatin when hCGβ was downregulated (i.e. were resistant to cisplatin in the presence of hCGβ).

Note that "E" indicates proteins that did not change in "A" (i.e. proteins that did not alter in response to cisplatin measured by a ratio close to 1, 0.9 > ratio <1.1) but

had changed when "A" was divided by "D". This subset of proteins was indicative of proteins that had not changed when cells were treated with cisplatin alone but had changed in a cisplatin-dependent manner in cells where hCGβ was downregulated. In other words, this ratio allowed us to determine in a relative sense, the overall effect of cisplatin on cells where hCGβ was downregulated.

5.2.5 Ingenuity® Pathway Analysis

The protein changes (ratios) from the proteomic data were uploaded into Ingenuity® Pathway Analysis (IPA) software (Qiagen) and differentially regulated networks and pathways were generated. Note: Statistical significance of dysregulated pathways was determined by IPA software.

5.2.6 Validation of proteomic protein changes by western blotting

Changes in protein abundance from the proteomic data, based on "E" (i.e. proteins that became cisplatin-sensitive when hCGβ was downregulated) were validated using western blotting in A2780cis cells, as well as HEY cells. The proteins of interest for validation were chosen from the top 10 downregulated or upregulated proteins, and/or if they played a role in more than one biological pathway.

For western blotting, hCGβ was downregulated in HEY and A2780cis cells by siRNA transfection and 1 x 10^5 or 4 x 10^5 cells, respectively, were plated into

12 well plates. After 24 h the media was replaced with 750 µl of fresh culture media and cells incubated for a further 24 h after which 250 µl of culture media, with or without cisplatin was added to cells. Cells were treated with IC_{50} concentrations (based on cell viability data) of cisplatin: 3.25 µM for HEYs and 6.5 µM for A2780cis, which was added at 4 x the concentration to take into account the dilution factor. After 24 h, protein lysates were extracted blotted and protein expression quantified (Chapter 2, section 2.7.2 for details on western blotting). Note: Protein expression is expressed as changes in the levels of the protein following the different treatments compared to control non-silencing siRNA treatment alone and then divided by the average ratios of protein expression compared to the control GAPDH.

5.3 Results

5.3.1 Global changes after hCGβ downregulation and cisplatin treatment

The changes in proteins from the proteomic study are presented as iTRAQ ratios. The top 10 proteins that were up regulated or downregulated in the following ratios: “A”, “B”, “C” and “E” are presented in Table 5-4 to Table 5-7. All proteins identified by iTRAQ are listed in the supplementary data. Refer to Appendix C Supplementary Figure 3 for the total number of identified proteins and proteins that were up- or down-regulated.

Table 5-4 Top 10 proteins upregulated and downregulated when A2780cis cells were treated with non-silencing siRNA and cisplatin "A"

Protein	A	B	C
60S ribosomal protein L36a	*3.477*	*2.289*	*3.326*
60S ribosomal protein L36a-like	*3.367*	*1.484*	*2.437*
Cytochrome c oxidase subunit 6C	*2.989*	*1.777*	*2.494*
Cytochrome b	*2.809*	*1.857*	*2.657*
SAP30-binding protein	*2.430*	*1.674*	*2.407*
Protein FAM98B	*2.381*	*1.251*	*1.691*
Signal peptidase complex subunit 1	*2.376*	*1.762*	*2.401*
V-type proton ATPase subunit G 1	*2.368*	*1.288*	*1.751*
ADP/ATP translocase 2	*2.279*	*1.244*	*2.091*
U6 snRNA-associated Sm-like protein LSm6	*2.259*	*1.368*	*1.802*
HIG1 domain family member 1A	*0.544*	0.935	*0.797*
E3 ubiquitin-protein ligase ZFP91	*0.528*	*0.797*	*0.697*
Acyl-CoA dehydrogenase family member 9, mitochondrial	*0.519*	*0.645*	*0.421*
Zinc transporter ZIP10	*0.508*	0.976	*0.743*
Uncharacterised protein C10orf46	*0.486*	1.025	1.078
Spermatogenesis-associated protein 5	*0.446*	*0.745*	*0.690*
SH3 domain-binding glutamic acid-rich-like protein 3	*0.439*	*0.841*	*0.800*
VPS33B-interacting protein	*0.391*	*0.649*	*0.649*
Histone H1.2	*0.389*	0.907	*0.274*
PCNA-interacting partner	*0.302*	*0.833*	*0.585*

Data are shown as iTRAQ ratios. "A" is the effect of cisplatin treatment, "B" is the effect of hCGβ downregulation, and "C" is the effect of both treatments combined. Upregulated (■) and downregulated proteins (■).

Table 5-5 Top 10 proteins upregulated and downregulated when A2780cis cells were treated with CGB_5 siRNA alone "B"

Protein	A	B	C
High mobility group protein HMGI	1.078	*3.043*	*3.123*
Histone H2A type 2-A	1.674	*2.841*	*3.908*
Protein S100-A13	1.256	*2.671*	*3.090*
Interstitial collagenase	1.055	*2.448*	*2.398*
Spermatid perinuclear RNA-binding protein	0.912	*2.313*	*1.355*
60S ribosomal protein L36a	*3.477*	*2.289*	*3.326*
Protein phosphatase 1 regulatory subunit 14B	*1.813*	*2.211*	*2.533*
Protein S100-A4	0.940	*2.118*	*1.989*
Phosphate carrier protein, mitochondrial	*1.988*	*1.954*	*2.004*
Cytoskeleton-associated protein 2	0.901	0.552	1.024
G patch domain-containing protein 1	0.990	*0.549*	*0.517*
GC-rich sequence DNA-binding factor 2	*0.546*	*0.537*	*0.568*
Telomeric repeat-binding factor 1	*0.670*	*0.536*	*0.733*
ADP-ribosylation factor 6	1.024	*0.535*	*0.517*
UPF0498 protein KIAA1191	*0.646*	*0.529*	*0.619*
DNA repair protein RAD51 homolog 1	1.129	*0.522*	*0.469*
Argininosuccinate lyase	0.864	*0.518*	*0.776*
Mitochondrial import receptor subunit TOM34	1.084	*0.480*	*0.588*
Branched-chain-amino-acid aminotransferase, mitochondrial	0.940	*0.475*	*0.521*

Data are shown as iTRAQ ratios. "A" is the effect of cisplatin treatment, "B" is the effect of hCGβ downregulation, and "C" is the effect of both treatments combined. Upregulated (■) and downregulated proteins (■).

Table 5-6 Top 10 proteins upregulated and downregulated when A2780cis cells were treated with CGB_5 siRNA and cisplatin "C"

Protein	A	B	C
Histone H2A type 2-A	*1.674*	*2.841*	*3.908*
60S ribosomal protein L36a	*3.477*	*2.289*	*3.326*
Keratin, type I cytoskeletal 10	*1.289*	*1.274*	*3.266*
Keratin, type II cytoskeletal 1	0.953	1.175	*3.181*
High mobility group protein HMGI-C	1.078	*3.043*	*3.123*
Protein S100-A13	*1.256*	*2.671*	*3.090*
Cytochrome b	*2.809*	*1.857*	*2.657*
Protein phosphatase 1 regulatory subunit 14B	*1.813*	*2.211*	*2.533*
Cytochrome c oxidase subunit 6C	*2.989*	*1.777*	*2.494*
60S ribosomal protein L36a-like	*3.367*	*1.484*	*2.437*
ATP-dependent RNA helicase DDX51	*0.656*	*0.750*	*0.548*
Branched-chain-amino-acid aminotransferase, mitochondrial	0.940	*0.475*	*0.521*
ADP-ribosylation factor 6	1.024	*0.535*	*0.517*
G patch domain-containing protein 1	0.990	*0.549*	*0.517*
DNA repair protein RAD51 homolog 1	1.129	*0.522*	*0.469*
52 kDa repressor of the inhibitor of the protein kinase	*0.571*	*0.650*	*0.456*
Probable methyltransferase-like protein 15	*0.697*	0.927	*0.552*
Acyl-CoA dehydrogenase family member 10	*0.820*	*0.698*	*0.430*
Acyl-CoA dehydrogenase family member 9, mitochondrial	*0.519*	*0.645*	*0.421*
Histone H1.2	*0.389*	0.907	*0.274*

Data are shown as iTRAQ ratios. "A" is the effect of cisplatin treatment, "B" is the effect of hCGβ downregulation, and "C" is the effect of both treatments combined. Upregulated ( ) and downregulated proteins ( ).

Table 5-7 Top 10 proteins upregulated and downregulated when A2780cis cells were treated with CGB_5 siRNA and cisplatin compared to cells that received non-silencing siRNA and cisplatin "E"

Protein	A	D	E (A/D)
Cytoskeleton-associated protein 2	0.901	*1.855*	*2.058*
Galectin-related protein	0.910	*1.587*	*1.743*
Glycerophosphodiester phosphodiesterase 1	0.906	*1.563*	*1.724*
Dual specificity tyrosine-phosphorylation-regulated kinase 1A	0.988	*1.701*	*1.721*
CpG-binding protein	0.926	*1.525*	*1.647*
Numb-like protein	1.072	*1.714*	*1.599*
Melanoma-associated antigen D2	0.918	*1.454*	*1.584*
Transmembrane protein 237	1.004	*1.556*	*1.550*
LIM domain kinase 1	0.974	*1.500*	*1.541*
Ras and Rab interactor 1	1.025	0.9221	*1.539*
SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily D member 1	1.028	*0.728*	*0.708*
Wings apart-like protein homolog	0.931	*0.645*	*0.692*
GDP-D-glucose phosphorylase C15orf58	1.007	*0.697*	*0.692*
Glutathione S-transferase theta-1	1.077	*0.743*	*0.689*
5-azacytidine-induced protein 1	1.075	*0.737*	*0.685*
Signal peptidase complex catalytic subunit SEC11C	1.045	*0.703*	*0.672*
UPF0614 protein C14orf102	1.085	*0.706*	*0.651*
Spermatid perinuclear RNA-binding protein	0.912	*0.587*	*0.644*
Collagen type IV alpha-3-binding protein	1.061	*0.631*	*0.594*
Tripeptidyl-peptidase 1	1.067	*0.627*	*0.588*
NAD-dependent deacetylase sirtuin-1	1.099	0.915	*0.833*

Data are shown as iTRAQ ratios. Description of "A", "D" and "E" are shown in Table 5-3. Upregulated ( ) and downregulated proteins ( ). Proteins chosen for validation experiments ( ).

5.3.2 Pathways affected by hCGβ downregulation and cisplatin treatment

The top four to five pathways affected by “A”, “B”, “C” and “E” obtained from IPA analysis are presented in Table 5-8. Refer to Appendix C, Supplementary Figure 4 to Figure 7 which show the top networks.

Cisplatin treatment alone “A” affected the cell death and survival, cell cycle, DNA replication, recombination and repair, and RNA processing pathways. Interestingly, two out of the five top pathways affected in “A” were also affected by hCGβ siRNA treatment alone “B”: the DNA replication, recombination and repair pathway, and the RNA post-translational modification pathway. DNA repair appears to be increased in “A” and “B” however RNA post-transcriptional modification seems to be increased in “A” and decreased in “B”. In addition to these pathways protein synthesis in general seems to be decreased when cells are treated with hCGβ siRNA alone “B”.

The top four pathways affected in “A” and hCGβ siRNA + cisplatin treatment “C” were the same; however, the cellular processes within the pathways and some of molecules associated with the cellular processes were different. For example, though the cell death pathway was affected in “A” and “C” the cellular processes within the pathway were different. In “A” there was an increase in cell death, apoptosis and necrosis where as in “C” neither cell death nor apoptosis seemed to be downregulated or upregulated. Another example is the cell cycle pathway: “A” had an increase in senescence but “C” had a decrease in senescence.

“E” (i.e. pathways that became cisplatin-sensitive when hCGβ was downregulated) showed a decrease in DNA repair and cell cycle progression, and the cellular morphology compared to cisplatin treatment alone “A”.

Table 5-8 Top five pathways affected when A2780cells were treated with "A", "B", "C" or "E"

Pathway	Direction of regulation of cellular processes within the pathway	Associated molecules
Treatment "A"		
Cell death and survival p-value range = $3.95e^{-2}$ to $2.73\ e^{-6}$	anoikis	CDKN2A, EPHA2, FADD, PLK1, SMAD4, SRC
	cell death, apoptosis and survival, necrosis	CDKN2A, BAX, AKT1, CDK2, EPHA2, FADD, PLK1, JAK1, ABL2, H2AFX, HNRNPK, EIF4EBP1, CCNB1
Cell cycle p-value range = $3.95e^{-2}$ to $3.32\ e^{-6}$	G2 phase, mitosis spindle checkpoints	AKT1, SMAD4, CCNB1, BAX, SRC, CDKN2A, SFN, TOP2A
	interphase, M-phase, senescence, cytokinesis	CDKN2A, AKT1, EIF4EBP1, NOTCH3, RELA, CDK2, TOP1, PLK1
DNA replication, recombination and repair p-value range = $3.95e^{-2}$ to $4.63e^{-6}$	DNA repair	POLD1, CDK2, H2AFX, XPC, CDKN2A, PCNA
	damage	BAX, H2AFX, PLK1, TOP1, TOP2A, CDKN2A
RNA Post-transcriptional modification p-value range = $3.95e^{-2}$ to $1.77e^{-16}$	Processing and splicing	RPS17, PABPC4, HNRNPK
Treatment "B"		
Free Radicle Scavenging p-value range = $3.43e^{-2}$ to $4.02e^{-5}$	accumulation, production and synthesis	TXN, HK2, FTL, LIMK1, MAP2K1, SRC
Molecular transport p-value range = $3.43e^{-2}$ to $4.02e^{-5}$	accumulation of oxygen reactive species	CDKN2A, VIPAS39, PPT1
DNA replication, recombination and repair p-value range = $3.43e^{-2}$ to $3.99e^{-5}$	metabolism of DNA, segregation of chromosomes, recombination, aberrations of chromosomes	TOP1, CCNB1, PPT1, CDKN2A, MCL1
	increased repair and checkpoints	CDKN2A, PRKDC, WRN, CCNB1
RNA post-transcription modification P VALUE=$3.43e^{-2}$ to $1.88e5^{-5}$	processing	CDK7
Protein Synthesis p-value range = $2.38e^{-2}$ to $1.17e^{-5}$	translation, expression, proteolysis and catabolism	CDKN2A, FADD, MMP1, AURKA
Treatment "C"		
Cell death and survival p-value range = $3.85e^{-2}$ to $2.92e^{-9}$	cell viability	ADAM17, SRC, CASP3, EPHA2, PRKDC, STAT3, BAX, FADD, KIF11, PAK1, PTK2, SFN, TXN, CDKN2A, MCL1, CCNB1, PCNA
	cell death and apoptosis	ABL2, ADAM17, AX, CAPS3, CDKN2A, CCNB1, BCAP31, HSF1, IKBKG, FADD, CHUK, EPHA2
	anoikis	MCL1, PTK2, CDKN2A, SRC, FADD
Cell cycle p-value range = 4.11^{-2} to $6.12e^{-6}$	mitosis, cytokinesis, M-phase, S-phase	KIF11, FADD, CCNB1, LMNA, RAB35, TXN, SRC, CDKN2A
	senescence	YAP1, RELA, CDKN2A, EIF4EBP1

DNA replication, recombination and repair p-value range = $4.11e^{-2}$ to $3.50e^{-6}$	fragmentation, damage, degradation, replication	FADD, PAK1, BAX, CAPS3, CDKN2A, PPT1, PKT2, PRKDC, NQ01, PCNA, POLD1
	segregation of chromosome	KIF11, LMNA, CCNB1
RNA post-transcription modification p-value range = $3.27e^{-2}$ to $1.28e^{-6}$	processing	CDK7, HNRNPK
p-value range = $3.85e^{-2}$ to $6.12e^{-6}$	organisation of cytoskeleton and cytoplasm	KRT18, STAT3, PAK1, PTK2, KIF11, SRC, CHUCK, CCNB1, EPHA2
	segregation of chromosomes and binding components of chromosomes	KIF11, CCNB1, LMNA
Treatment "E"		
Cell assembly and organisation p-value range = $3.36e^{-2}$ to $6.73e^{-6}$	organisation of cytoskeleton and cytoplasm	NRF1, EP300, LIMK1, VHL, SUN2, MPRIP, SHARPIN, NUMB, MAP9, CHD3, CIT
	microtubule dynamics	NUMB, MAP9, LIMK1, RPS6KB1, CIT, NEDD1, VHL, EP300
Cell cycle p-value range = $3.36e^{-2}$ to $2.69e^{-5}$	cell cycle progression and interphase	AATF, CKS2, SIRT1, EP300, CIT, ERCC1, RPS6KB1, WRN, SUN2, CAMP1, LIMK1, VHL, ORC3, CIT, CDK13, HMOX1 CASP3, MAP9
DNA replication, recombination and repair p-value range = $3.36e^{-2}$ to $2.69e^{-5}$	metabolism, replication	ORC3, SIRT1, WAPAL, CKS2, LIG3, WRN, EP300, NRF1, ERCC1, WRNIP1, GTPBP4
	repair	ERCC1, SIRT1, LIG3, WRN, HMOX1, MRE11A
Cellular morphology p-value range = $3.04e^{-2}$ to $3.76e^{-4}$	autophagy	SIRT1, CAMK1, NAF1, GNAS, RPS6KB1, EP300, MFN2
	morphology	CASP3, LIMK1, SIRT1, ERCC1, EP300, GNAS, GSK3A, SHARPIN, NEDD1, MPRIP

Description of "A", "B", "C" and "E" are shown in Table 5-3. Upregulated ( ) and downregulated cellular processes ( ).

5.3.3 Validation of iTRAQ results: effect of hCGβ downregulation and cisplatin on the protein expression level of WAPAL, LIMK1, and SIRT1

LIM domain kinase 1 (LIMK1), wings apart-like homolog (WAPAL) and sirtuin 1 (SIRT1) were chosen for validation by western blotting. LIMK1 and WAPAL were chosen as they not only appeared in the list of 10 top up- and downregulated proteins in "E" (Table 5-7) but also appeared in at least one pathway that was dysregulated in "E" (Table 5-8). SIRT1 was not part of the top ten downregulated proteins in "E" (although still represented in Table 5-7) but it was chosen for further validation as it appeared to be important in a number of pathways (Table 5-8) hence could be essential in the mechanism by which hCGβ confers cisplatin resistance. Both LIMK kinase and SIRT1 have been previously implicated in ovarian cancer [311-313] as well in response of cancer cells to cisplatin treatment [314-316].

According to the iTRAQ analysis, the levels of WAPAL and SIRT1 were downregulated and LIMK1 levels were upregulated when A2780cis cells were treated with cisplatin after hCGβ downregulation, "E", but were unaffected by cisplatin treatment alone "A" (Table 5-7). In order to validate these results, hCGβ was downregulated in A2780cis as well as HEY cells using CGB_4 and CGB_5 siRNAs, followed by cisplatin treatment (or no treatment for controls). Changes in the level of WAPAL, SIRT1 and LIMK1 were then assessed by western blotting. Western blots representing the levels of WAPAL protein, LIMK1 and SIRT1 in HEY and A2780cis cells after hCGβ downregulation and cisplatin treatment are shown in Figure 5.3. Interestingly, LIMK1 could not be detected by western blot in A2780cis cells despite these cells being used for the iTRAQ study. Quantitative

representations of the levels of WAPAL, SIRT1 and LIMK1 are shown in Figure 5.4, Figure 5.5 and Figure 5.6, respectively.

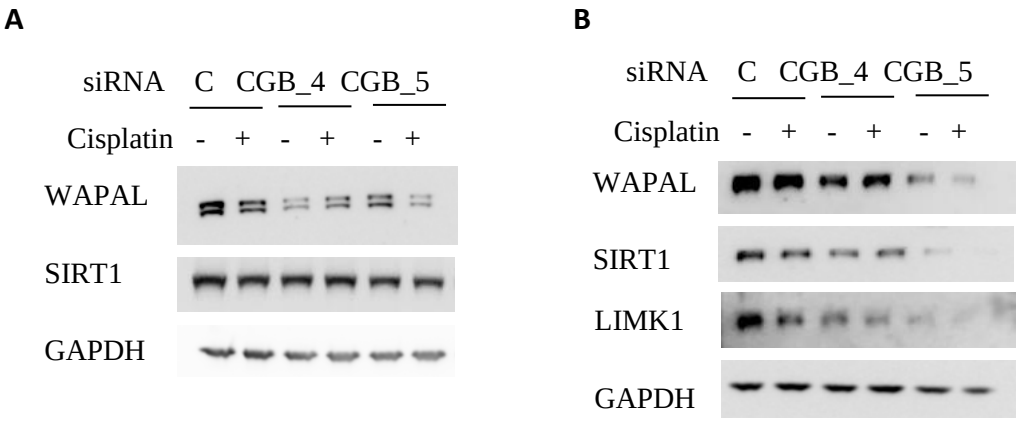


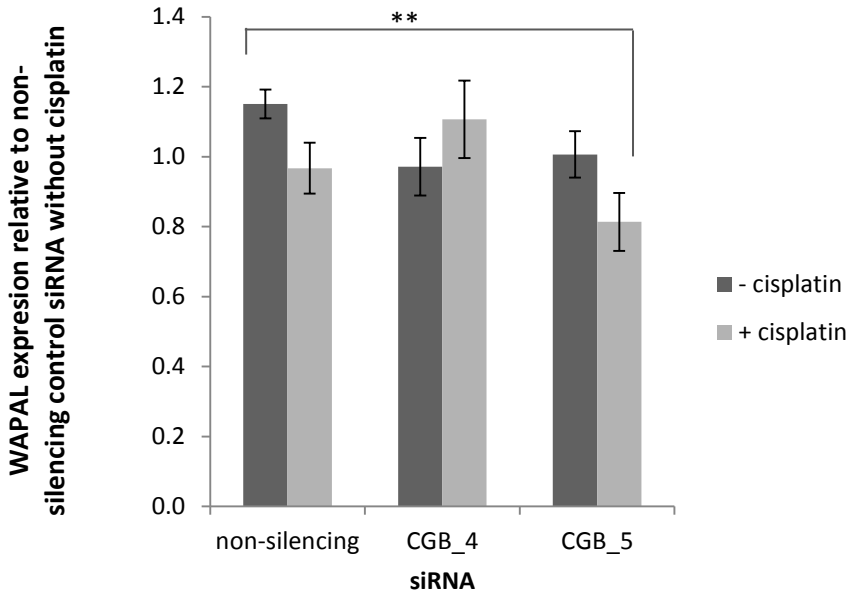
Figure 5.3 Effect of hCGβ downregulation and cisplatin on the expression levels of WAPAL, LIMK1 and SIRT1

Protein levels of WAPAL, SIRT1 and LIMK1 levels in A2780cis (A), or HEY (B) cells following hCGβ downregulation and cisplatin treatment. LIMK1 was undetectable in A2780cis. 48 h post siRNA transfection: non-silencing control (C), CGB_4 and CGB_5.

5.3.3.1 Effect of hCGβ downregulation and cisplatin on WAPAL expression

In contrast to the iTRAQ results, WAPAL levels did not significantly decrease in A2780cis or HEY cells which were treated with cisplatin following hCGβ downregulation "D" compared to cells which were treated with cisplatin when hCGβ was not downregulated "A" (Figure 5.4 A and B, respectively). Treatment with cisplatin following downregulation of hCGβ with CGB_5 siRNA "D" significantly decreased the expression of WAPAL protein compared to the negative non-silencing siRNA, a result which was not shown by cells treated with cisplatin and the non-silencing siRNA "A" as quantitated from western blots (Figure 5.4). Overall, this experiment was unable to confirm WAPAL as a protein whose regulation by cisplatin was dependent on the downregulation of hCGβ, although there was a trend towards this with CGB_5 siRNA in the HEY cells.

A



B

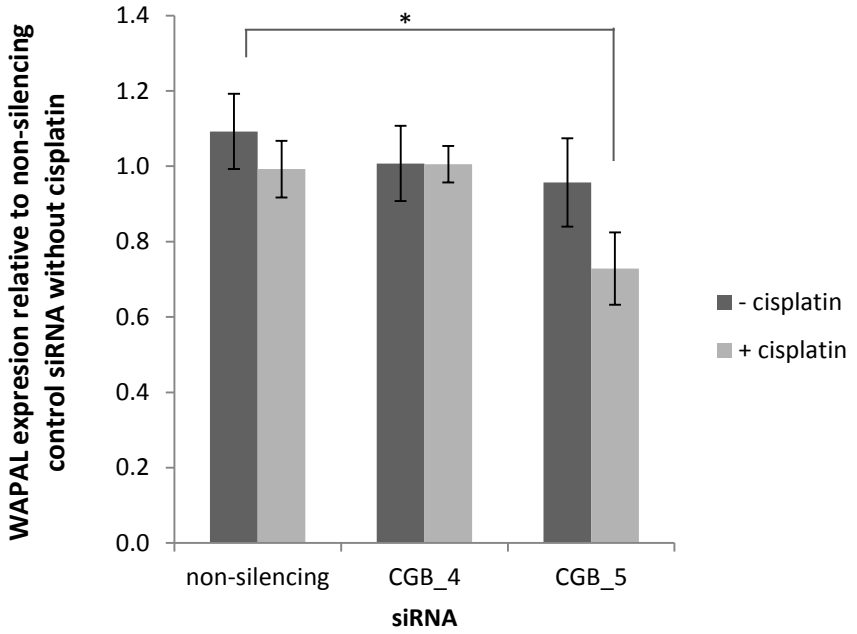


Figure 5.4 Effect of cisplatin treatment following hCGβ downregulation on WAPAL expression

The effect of cisplatin treatment following hCGβ downregulation on the protein levels of WAPAL in A2780cis (A, N=4) and HEY cells (B, N=6), measured by western blotting, 48 h post siRNA transfection. Data are expressed as mean ± S.E.M. Statistical test: one-way ANOVA with LSD post hoc test. Statistical test: one-way ANOVA with LSD post hoc test, * $P < 0.05$, ** $P < 0.005$. Other differences were not significant

5.3.3.2 Effect of hCGβ downregulation and cisplatin on SIRT1 expression

SIRTS levels did not significantly decrease in A2780cis or HEY cells which were treated with cisplatin following hCGβ downregulation "D" compared to cells which were treated with cisplatin when hCGβ was not downregulated "A" (Figure 5.5 A and B, respectively). CGB_5 siRNA treatment alone compared to the non-silencing control siRNA treatment alone caused a near significant (*P=0.061*) reduction of SIRT1 expression in A2780cis cells and a significant decrease in HEY cells (Figure 5.5 A and B, respectively). Overall, similar to WAPAL, this experiment was unable to confirm SIRT1 as a protein whose regulation by cisplatin was dependent on the downregulation of hCGβ although, again, there was a trend towards this with CGB_5 siRNA in the HEY cells.

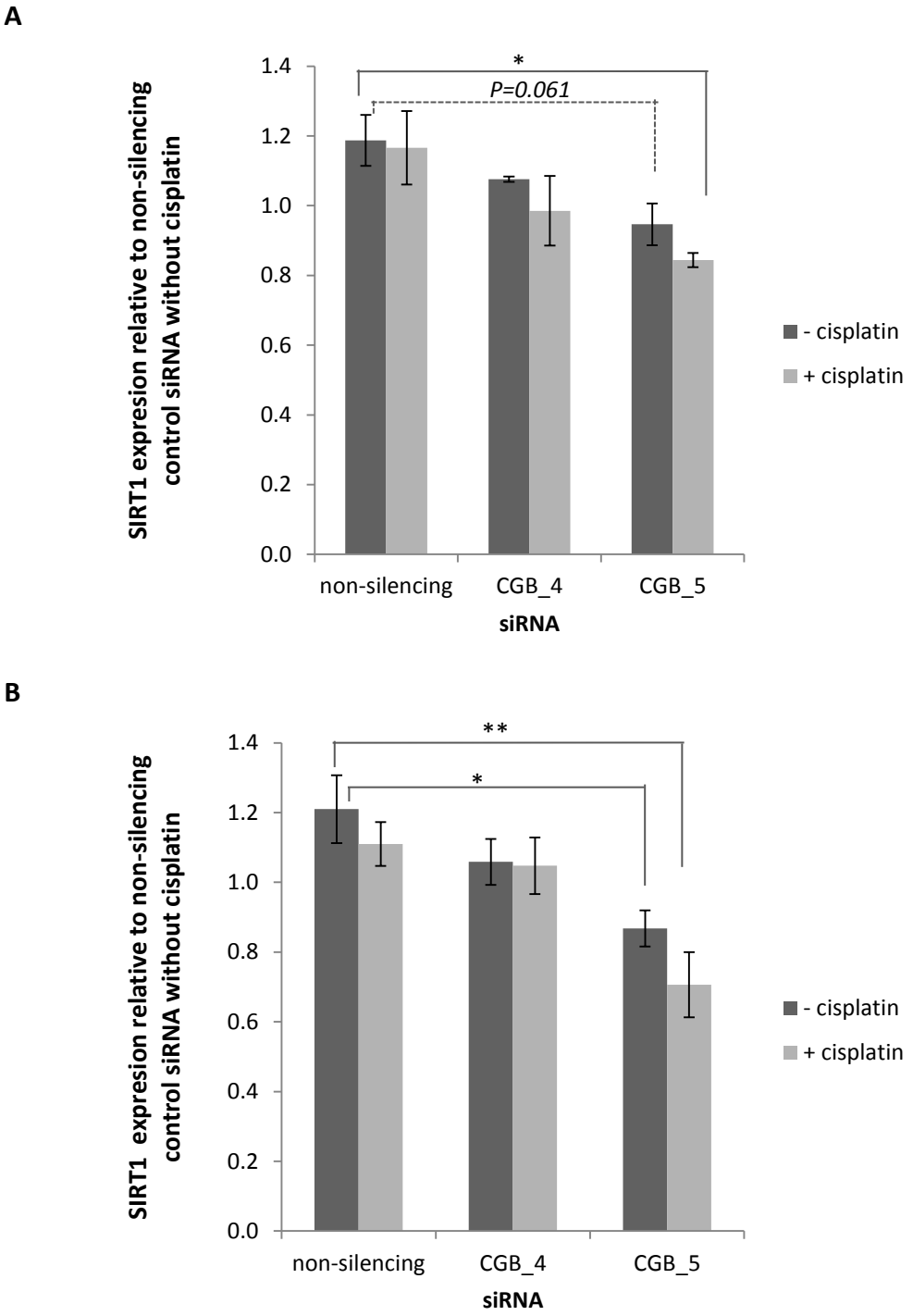


Figure 5.5 Effect of cisplatin treatment following hCGβ downregulation on SIRT1 expression

The effect of cisplatin treatment following hCGβ downregulation on the protein levels of SIRT1 in A2780cis (A) and HEY cells (B) measured by western blotting, 48 h post siRNA transfection. Statistical test: one-way ANOVA with LSD post hoc test, * $P < 0.05$, ** $P < 0.005$.

5.3.3.3 Effect of hCGβ downregulation and cisplatin on expression of LIMK1

In Table 5-7, LIMK1 appeared *upregulated* by cisplatin when hCGβ was silenced. In contrast, in HEY cells the level of LIMK1 was significantly *decreased* in cells which were treated with cisplatin following hCGβ downregulation "D" as well as in cells which were treated with cisplatin alone "A" (Figure 5.6). A significant decrease in LIMK1 was also observed with hCGβ downregulation alone or cisplatin treatment alone. As mentioned in section 5.3.3, LIMK1 could not be detected in A2780cis cells by western blot therefore data on LIMK1 expression following hCGβ downregulation and cisplatin treatment is not presented in Figure 5.6.

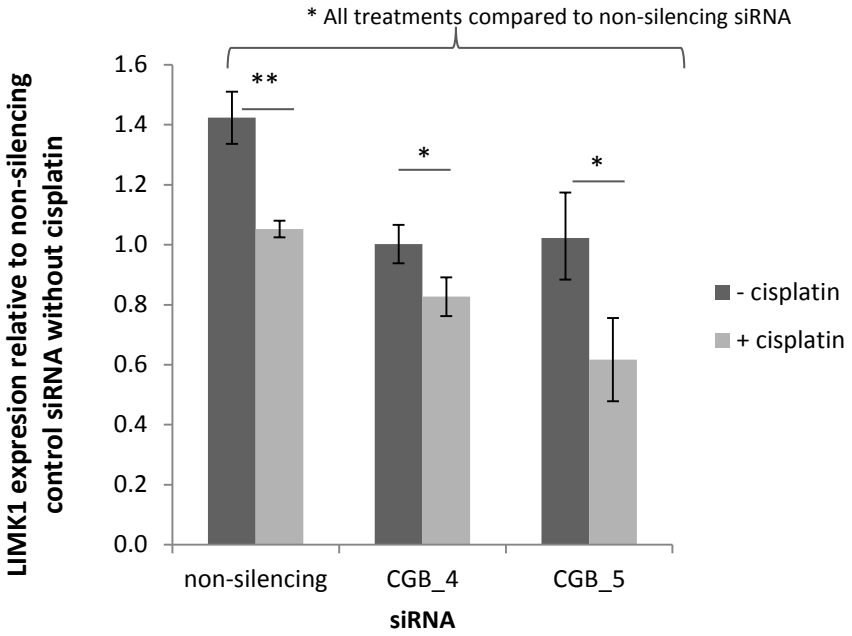


Figure 5.6 Effect of cisplatin treatment following hCGβ downregulation on LIMK1 expression

The effect of cisplatin treatment following hCGβ downregulation on the protein levels of LIMK1 in HEY cells measured by western blotting, 48 h post siRNA transfection. Data are expressed as mean ± S.E.M; (N=4). Statistical test: one-way ANOVA with LSD post hoc test, * $P < 0.05$, ** $P < 0.005$.

5.3.4 Effect of SIRT1 downregulation on cisplatin sensitivity

Of the three proteins validated by western blotting, SIRT1 showed the greatest promise as a protein whose levels might be significantly decreased in A2780cis and HEY cells when the cells were treated with cisplatin following downregulation of hCGβ with the CGB_5 siRNA (Figure 5.5). To further investigate whether a decrease in SIRT1 alone could be a possible mechanism by which CGB_5 or CGB_4 siRNA could sensitise cells to cisplatin, SIRT1 was downregulated in A2780cis and HEY cells by siRNA transfection (Chapter 2, section 2.8) and the response of cells to cisplatin was assessed using the cell viability and clonogenic assays (Chapter 4, section 4.2.1).

5.3.4.1 Downregulation of SIRT1

SIRT1 was downregulated with both siRNAs directly targeting SIRT1 (SIRT1 (2) and SIRT1 (3)) in both A2780cis and HEY cells by up to 90% (Figure 5.7).

A

B

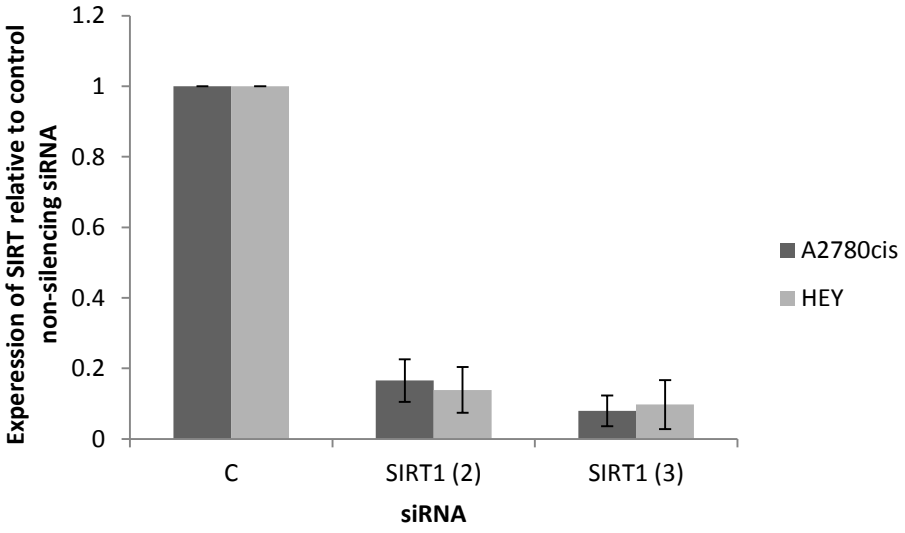


Figure 5.7 Downregulation of SIRT1 in A2780cis and HEY cells

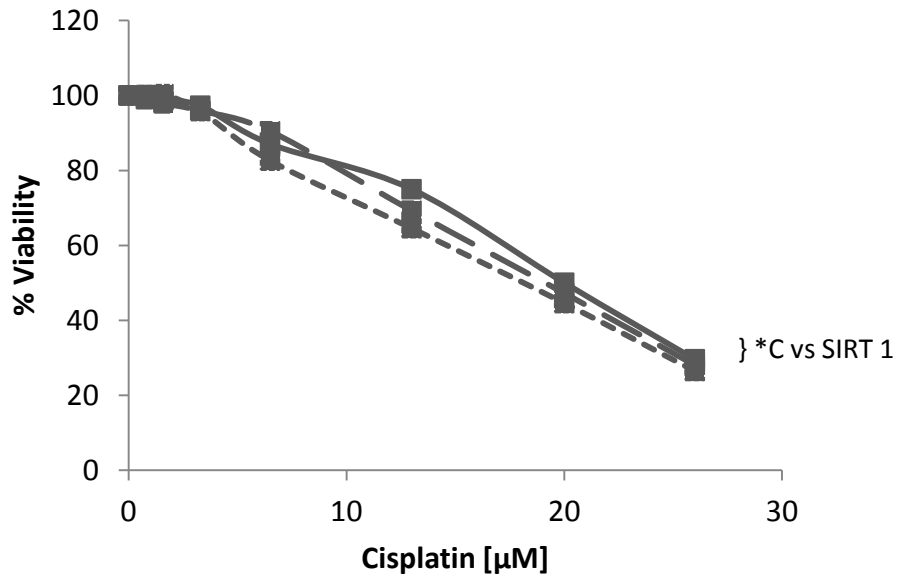
Downregulation of SIRT1 in A2780cis and HEY cells 48h post siRNA transfection. Western blot showing levels of SIRT1 protein (A), quantification of SIRT1 knockdown (B). siRNAs: non-silencing control siRNA (C) two siRNAs targeting SIRT1: SIRT1 (2) and SIRT1 (3). Data are expressed as mean ± S.E.M; (N=3).

5.3.4.2 Effect of SIRT1 downregulation on cisplatin sensitivity determined by cell survival and viability

In A2780cis cells, both siRNAs (SIRT1 (2) and SIRT1 (3)) targeting SIRT1 showed a trend towards increased sensitivity to cisplatin treatment in terms of cell viability (Figure 5.8 A). However, this increase was statistically significant only when the SIRT 1(3) siRNA was used. This trend of increased cisplatin sensitivity was also evident in HEY cells but did not reach statistical significance (Figure 5.8 B).

The clonogenic assay showed that downregulation of SIRT1 in A2780cis cells significantly decreased cell survival following cisplatin treatment using both siRNAs targeting SIRT1 (Figure 5.9 A). However, SIRT1 downregulation in HEY cells did not affect cell survival following cisplatin treatment (Figure 5.9 B).

A



B

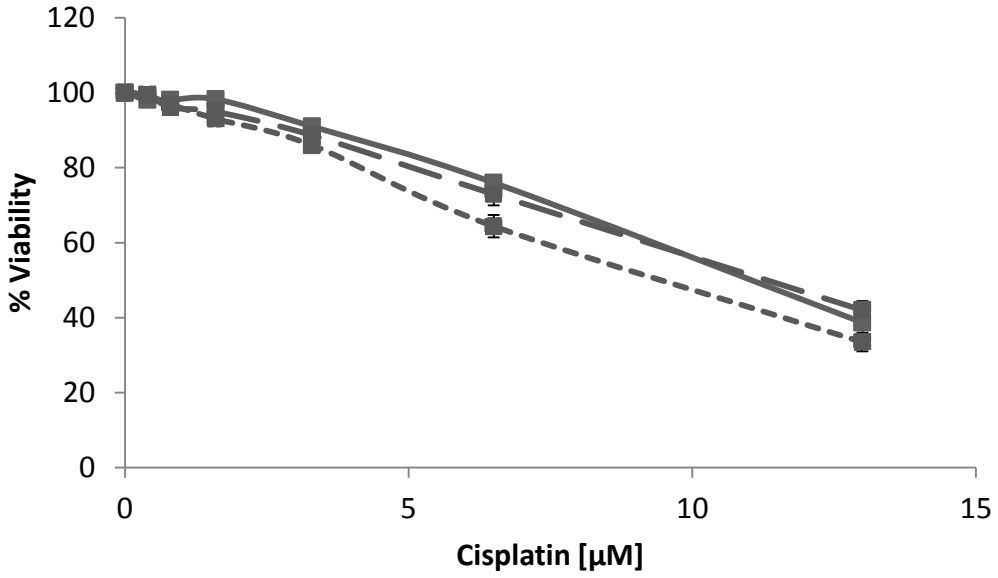
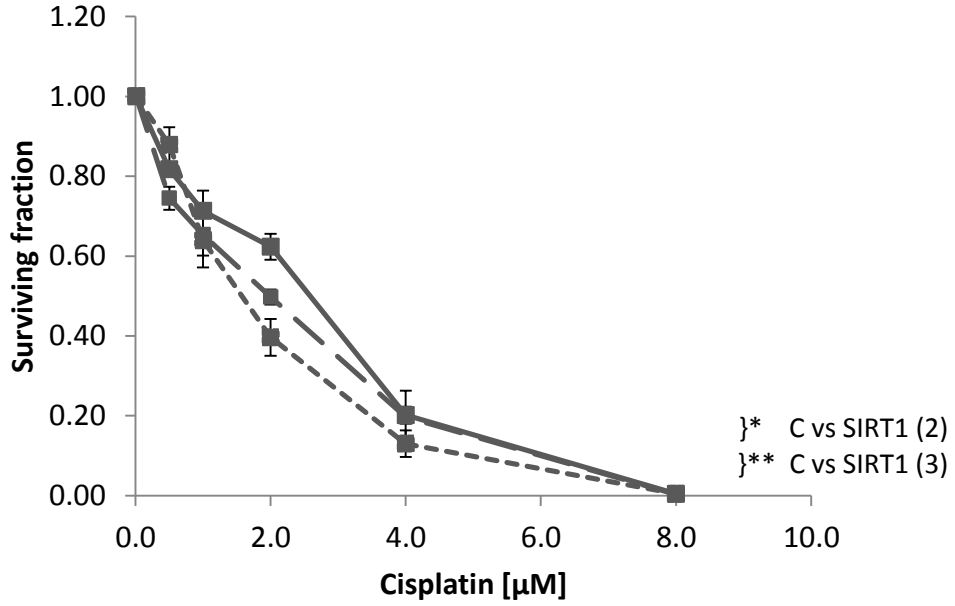


Figure 5.8 Effect of SIRT1 downregulation on the viability of A2780cis and HEY cells following cisplatin treatment

Effect of SIRT1 downregulation on viability of A2780cis (A) and HEY (B) cells measured by the MTS assay in response to cisplatin treatment. 48 h post transfection with siRNAs: non-silencing control (C) (—■—), SIRT1(2) (— ■ — ·) and SIRT1(3) (- - ■ - -). Data are expressed as mean ± S.E.M; (N=3). * *P < 0.05.*

A



B

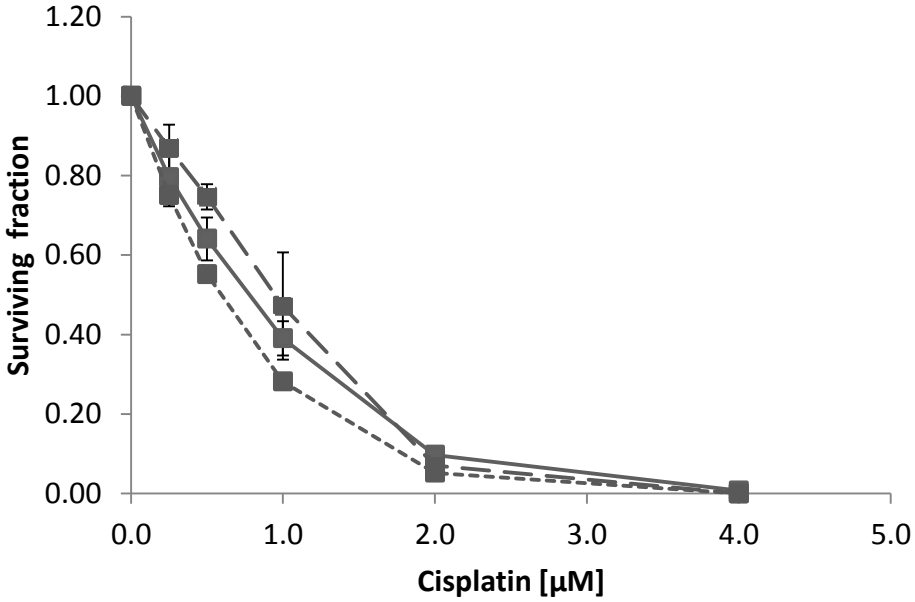


Figure 5.9 Effect of SIRT1 downregulation on the survival of A2780cis and HEY cells following cisplatin treatment

Effect of SIRT1 downregulation on survival of A2780cis (A) and HEY (B) cells measured using the clonogenic assay in response to cisplatin treatment. 24 h post transfection with siRNAs siRNAs: non-silencing control (C) (—■—), SIRT1(2) (— -■— -) and SIRT1(3) (- - -■- - -). Data are expressed as mean ± S.E.M; (N=3). * $P < 0.05$, ** $P < 0.005$.

Interestingly, a query of the online analysis software Kaplan Meier-plotter (KM-plotter) showed that *SIRT1* was a negative prognostic marker of patient survival in ovarian cancer patients who received platinum treatment. Specifically high *SIRT1* levels correlated with a lower rate of progress free survival in ovarian cancer patients who received platinum treatment (Figure 5.10). The KM-plotter is freely available online analysis software that enables the assessment of the prognostic value of a gene based on gene expression and clinical data from the Gene Expression Omnibus (Affymetrix microarrays only), European Genome-phenome Archive and The Cancer Genome Atlas archives [317].

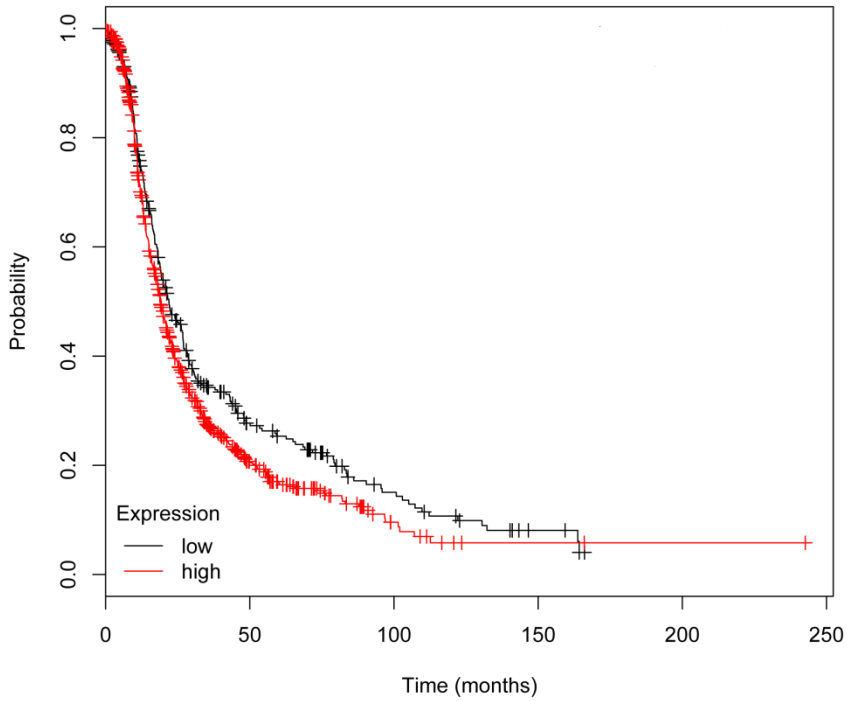


Figure 5.10 Kaplan-Meier Plot of *SIRT1* levels and progress free survival in ovarian cancer patients who received platinum therapy

Assessment of the prognostic value of SIRT1 in ovarian cancer patients who received platinum therapy using data analysed by KM-Plotter. The cohort contained a total of 1185 patients, 320 patients expressed low *SIRT1* and 865 patients expressed high levels of *SIRT1*. Hazard ratio = 1.21 (1.04-1.41) and logrank *P* = 0.013.

5.4 Discussion

5.4.1 iTRAQ protein changes

Downregulation of hCGβ in A2780cis and HEY cells sensitised the cells to cisplatin treatment (Chapter 4, sections 4.3.3 and 4.3.6); however the mechanism by which hCGβ could be involved in the response to cisplatin needed to be understood. In order to do this the global protein changes that occurred when hCGβ was downregulated in A2780cis cells in the absence and presence of cisplatin were studied using iTRAQ. iTRAQ is a quantitative proteomic technique used to determine the relative abundance of specific proteins in one or more samples compared to a reference sample. One of the advantages of iTRAQ, and a reason why it was used in this study, is that it facilitates multiplexing, allowing comparison of up to 8 samples in one run. However the disadvantage of iTRAQ is that variability in labelling efficiencies and protein digestion could lead to discrepancies in the results.

There are a number of other techniques which could have been used to overcome this problem. One example is use of the *in vivo* stable isotope labelling by amino acids (SILAC) technique, whereby "heavy" or "light" isotopes are used to label amino acids under different treatment conditions that are metabolically incorporated into cellular proteins causing a mass shift which can be quantified by MS-based techniques [318]. Specifically, two samples are grown in culture media containing either heavy (e.g. N^{15} and C^{13} L-lysine or Arginine) or light (naturally occurring) amino acids. The proteins are extracted and equal quantities of protein are pooled together before protein digestion and quantitation by MS-based techniques [319]. The disadvantage of SILAC is that it is relatively expensive and only two or three

samples can be compared in a single analysis [320, 321]. For these reasons, iTRAQ was deemed the most suitable technique for the current study.

The 10 top upregulated and downregulated proteins when the A2780cis cells were treated with control non-silencing siRNA with cisplatin "A", CGB_5 siRNA alone "B", or CGB_5 siRNA with cisplatin "C" were different (Table 5-4, Table 5-5, Table 5-6, respectively). Interestingly, when all the protein changes which occurred in "A", "B" and "C" were analysed by the IPA program, a number of pathways overlapped, in particular between "A" and "C" which was an unexpected result (Table 5-8). As mentioned earlier, hCGβ downregulation with CGB_5 siRNA significantly sensitised A2780cis cells to cisplatin; therefore it would be expected that different pathways would be dysregulated in these cells compared to cells which received cisplatin when hCGβ was not downregulated. However, even though the same pathways were affected in "A" and "C" the cellular processes and associated molecules within each pathway were different. For example the cell death pathway was affected in "A" and "C" but in "A" there was an increase in cell death, apoptosis and necrosis whereas in "C" neither cell death nor apoptosis seemed to be downregulated or upregulated. Another example is the cell cycle pathway: "A" had an increase in senescence and "C" had a decrease in senescence. It would be expected that "C" would have contained more cells that had undergone senescence because there seemed to be increased DNA fragmentation, damage and degradation in "C" and decreased DNA repair in "E" which could eventually lead to senescence [322]. A possible explanation is that cisplatin-induced senescence is dependent on the dose of cisplatin used to treat the cells [323]. Berndtsson *et al.* showed that DNA damage triggered by low cisplatin concentrations led to senescence of HCT116 colon carcinoma cells whereas high cisplatin concentrations led to induction of

apoptosis by superoxide production [323]. Therefore it is possible that the cisplatin concentration used to treat the cells following hCGβ downregulation did not induce senescence. However it is also possible that given a longer exposure time these cells would have eventually undergone senescence.

Interestingly DNA repair appears to be increased in "A" and "B". This suggests that downregulation of hCGβ may have led to DNA damage in the cells and therefore the cells initiated DNA repair. Why would hCGβ downregulation lead to increased DNA damage or repair; could it have to do with additional secondary effects brought on by hCGβ downregulation? Indeed, one such effect is the impact of hCGβ downregulation on SIRT1 levels which has been shown to be involved in DNA repair [324] and is further discussed in section 5.4.2.2. It should be noted that the results in regards to either protein or pathway changes are based on a single iTRAQ experiment and therefore validation studies are important. Although the study of pathways affected by the different treatments was beyond the scope of this thesis we addressed some of the protein changes in sections 5.4.2 and 5.4.3.

5.4.2 Validation of iTRAQ results

Three proteins: WAPAL, SIRT1 and LIMK1, were chosen for validation experiments in A2780cis and HEY cells using western blot analysis. These proteins were chosen as they were either up- or downregulated following CGB_5 siRNA treatment in conjunction with cisplatin and not in cells that received non-silencing siRNA in conjunction with cisplatin (Table 5-7). They also appeared in more than one pathway or cellular process and may have had a potential role to play in how cells responded to cisplatin treatment.

5.4.2.1 WAPAL expression following hCGβ downregulation and cisplatin treatment

In A2780cis and HEY cells, cisplatin treatment following downregulation of hCGβ did not significantly decrease the expression of WAPAL protein compared to cisplatin treatment alone, as quantitated from western blots (Figure 5.4). This data disagreed with the iTRAQ data which showed that WAPAL was decreased in a cisplatin-dependent manner in cells where hCGβ was downregulated "E" compared to cells which were treated with cisplatin alone "A" (Table 5-7). In other words the addition of siRNA targeting hCGβ did not increase cisplatin sensitivity. However, the data did show that treatment with cisplatin following downregulation of hCGβ with CGB_5 siRNA significantly decreased the expression of WAPAL compared to the cells treated with the negative non-silencing siRNA alone (Figure 5.4). This difference was not observed when cells were treated with the cisplatin in conjunction negative non-silencing siRNA compared to cells treated with the non-silencing siRNA alone. These data agree with the iTRAQ data which showed WAPAL levels were decreased in cells treated with cisplatin and CGB_5 siRNA "D" compared to cisplatin treatment alone "A" (Table 5-7). Not much is known about the WAPAL protein but it has been shown to be elevated in cervical cancer tissue [325]. Further, overexpression of WAPAL in NIH3T3 cells has been shown to make these cells tumorigenic in mice [325]. WAPAL is thought to be involved in mitosis by binding to cohesin which is involved in the timely separation of sister chromatids during mitosis [326]. Indeed, pathway analysis of the proteomic data suggests that segregation of chromosomes might be decreased when A2780cis were treated with both CGB_5 siRNA and cisplatin, "E", which in turn could be a result of WAPAL (Table 5-8). Contrary to the results shown with the use of the CGB_5 siRNA,

cisplatin treatment following hCGβ downregulation by CGB_4 siRNA had no effect on WAPAL expression in either cell line. The manner in which both CGB_4 and CGB_5 regulate WAPAL in the presence of cisplatin suggest that WAPAL may be a secondary target of CGB_5 and this could account for the differences in the mechanism through which the two siRNAs sensitise cells to cisplatin treatment as seen in Chapter 4 (section 4.3.6). However, CGB_5 siRNA alone did not cause a decrease in WAPAL expression suggesting that WAPAL is not a direct off target effect of CGB_5 siRNA and therefore other downstream effects had occurred only when cells were treated with cisplatin in conjunction with CGB_5 siRNA. A search using the Blast® program confirmed that WAPAL was not a target of CGB_5 siRNA however; it is possible that WAPAL levels in the presence of cisplatin could be indirectly altered due to the siRNA binding to alternative sequences.

5.4.2.2 SIRT1 expression following hCGβ downregulation and cisplatin treatment

SIRT1 levels were not significantly downregulated when A2780cis and HEY cells were treated with cisplatin in conjunction with siRNAs targeting hCGβ compared to cells which were treated with cisplatin alone (Figure 5.5). This data disagrees with the iTRAQ data which showed that SIRT1 was decreased in a cisplatin-dependent manner in cells where hCGβ was downregulated "E" compared to cells which were treated with cisplatin alone "A" (Table 5 7). In other words the addition of siRNA targeting hCGβ did not increase cisplatin sensitivity. However, the data did show that treatment with cisplatin following downregulation of hCGβ with CGB_5 siRNA significantly decreased the expression of SIRT1 compared to the negative non-silencing siRNA, a result which was not shown by cells treated with cisplatin in conjunction with the negative non-silencing siRNA (Figure 5.5). These data agreed with the proteomics data where cisplatin treatment "A" alone did not affect levels of SIRT1 but cells treated with cisplatin and CGB_5 siRNA "D" showed a decrease in SIRT1 (Table 5-7). However, hCGβ downregulation using CGB_4 siRNA had no significant effect on SIRT1 levels in the presence or absence of cisplatin compared to the non-silencing control siRNA without cisplatin treatment Figure 5.5. As with the WAPAL expression levels, the manner in which both CGB_4 and CGB_5 regulate SIRT1in the presence of cisplatin could account for the differences in the mechanism through which the two siRNA sensitise cells to cisplatin treatment as seen in Chapter 4, section 4.3.6. Interestingly, SIRT1 expression was also decreased by CGB_5 siRNA alone in both cell lines which suggests that indeed SIRT1 could be an off target effect of CGB_5 and could be an explanation of why CGB_5 siRNA treatment sensitises cells to cisplatin treatment. A search using the Blast® program however did not show that SIRT1 was a direct

target of CGB_5 siRNA however; it is possible that SIRT1 levels could be indirectly altered due to the siRNA binding to alternative sequences. Alternatively any effect of hCGβ downregulation by CGB_4 may have been offset by a compensatory increase in *LHB* (Figure 4.13). The biological role of SIRT1 on cisplatin sensitivity is further discussed in section 5.4.3.

5.4.2.3 LIMK1 expression following hCGβ downregulation and cisplatin treatment

Changes in LIMK1 expression were detected by iTRAQ in A2780cis cells. However this result could not be validated in A2780cis as LIMK1 could not be detected in these cells, even after increasing the concentration of the LIMK1 primary antibody and using a more sensitive chemiluminescent (ECL) substrate for the HRP enzyme (SuperSignal™ West Femto Maximum). This could be due to low expression of the protein in A2780cis which could only be detected by MS/MS but not by western blot. In HEY cells, LIMK1 expression was decreased with cisplatin treatment alone "A" as well as hCGβ downregulation alone "B", and cisplatin treatment in conjunction with hCGβ downregulation "D". Interestingly, cisplatin treatment following hCGβ downregulation "D" caused a further decrease in LIMK1 expression compared to cisplatin treatment alone "A". These data disagree with the proteomic data attained from A2780cis cells which showed that LIMK1 expression increased with cisplatin and CGB_5 siRNA treatment, "E", but was unchanged when cells were treated with cisplatin alone "A" (Table 5-7). These differences between the two cell lines could be an indication of the different downstream effects of hCGβ downregulation and/or cisplatin treatment in HEY cells and A2780cis and could be a reason for differences in the way the cells respond in regards to response to drug treatment (Chapter 4, section 4.3.6).

LIMK1 is a Serine/Threonine kinase which phosphorylates cofilin, which binds actin filaments and thereby regulates the dynamics of the actin cytoskeleton [327]. LIMK1 has been associated with progression of a range of cancers including ovarian, breast and prostate cancer [311, 328, 329]. Both *in vitro* and *in vivo* data suggests that LIMK1 is mostly involved in invasion and migration of cancers and therefore potentially metastasis of cancer [311, 329-331]. Interestingly, Chen *et al.* showed that downregulation of LIMK1 can sensitise lung cancer cells to cisplatin [314]. In agreement with Chen *et al.* our data shows that the decrease in LIMK1 levels by siRNA targeting hCGβ could be a mechanism by which hCGβ regulates cisplatin sensitivity.

LIMK1 could have been chosen for further analysis to discover whether it had a role to play in cisplatin sensitivity independent of hCGβ and therefore could be a mechanism through which hCGβ confers cisplatin sensitivity; however, as LIMK1 could not be detected in A2780cis cells this was not further investigated. Therefore results still need to be validated in A2780cis. This could be done by immunoprecipitating LIMK1 in order to concentrate it which may make it more visible on a western blot.

5.4.3 Effect of SIRT1 downregulation on cisplatin sensitivity

SIRT1 is a NAD-dependent deacetylase implicated in tumorigenesis and drug resistance [332]. It has a range of substrates including both histone proteins and non-histone proteins. These include transcription factors involved in apoptosis such as p53, and members of the forkhead transcription factors (FOXO) family [333, 334]. SIRT1 levels have been found to be elevated in some epithelial cancers including colon [335, 336], prostate [337], breast [338] and ovarian [312]; however

its function as a tumour suppressor or promoter is still controversial [332]. Interestingly, higher expression of SIRT1 was observed in malignant serous ovarian carcinoma compared to the benign and borderline epithelial tumours, and it was associated with increased survival [312]. SIRT1 has been shown to be involved in inactivating p53 and hence preventing p53 driven apoptosis [339]. *In vitro* studies have shown that SIRT1 can be involved in drug resistance [316, 340]. A study by Zhang *et al*. found that patients with non-small cell lung cancer (NSCLC) whose tumours had high expression levels of SIRT1 were more likely to resist platinum-based chemotherapy compared to patients whose tumours expressed low levels SIRT1. In the same study, it was also found that downregulation of SIRT1 in the NSCLC H292 cell line sensitised these cells to cisplatin treatment [315]. In prostate cancer cell lines it was found that pretreatment of PC3 and DU145 cells with sirtinol, an inhibitor of SIRT1, sensitised them to cisplatin by increasing apoptosis [340]. Chu *et al.* showed that cisplatin resistant IGROV and A2780cis cells had higher levels of SIRT1 compared to the parental chemosensitive cells [316]. Interestingly in this paper they also found that treatment of A2780 cells with a sub-lethal level of cisplatin induced expression of the cell cycle inhibitor p21/WAF1, therefore allowing the cells to adapt and survive the cytotoxic stress. Assessment of the prognostic value of SIRT1 using KM-plotter showed that ovarian cancer patients who received platinum-based therapy had a slightly lower rate of progress free survival when SIRT1 levels were high (Figure 5.10). This suggests that SIRT1 may be an indicator of patients' response to platinum-based chemotherapy.

In agreement with the involvement of SIRT1 in drug resistance, our data showed that downregulation of SIRT1 in A2780cis cells increased their sensitivity to cisplatin treatment (Figure 5.9 A). This result suggests that the decrease in SIRT1

expression following hCGβ downregulation by CGB_5 siRNA could be a mechanism by which hCGβ regulates sensitivity of A2780cis cells to cisplatin. However, CGB_4 siRNA did not cause a significant reduction in SIRT1 levels, again raising the possibility that SIRT1 could be an off target effect of CGB_5 siRNA which results in the increased sensitivity to platinum-based drugs, a result which is not replicated by CGB_4 siRNA. However this does not explain why increased cisplatin sensitivity is not observed when SIRT1 is downregulated in HEY cells (Figure 5.9 B) despite CGB_5 siRNA increasing HEY cells sensitivity to cisplatin. One explanation could be that although downregulation of hCGβ by CGB_5 siRNA decreases levels of SIRT1, it is not the mechanism through which hCGβ confers cisplatin sensitivity/resistance. Another explanation is that CGB_5 siRNA confers cisplatin sensitivity in a SIRT1 dependent manner but the effect of SIRT1 on cisplatin sensitivity is cell type dependent. Lastly, it is possible that the effect of hCGβ on cisplatin sensitivity is independent of SIRT1 levels.

5.5 Conclusions

Global protein changes detected by iTRAQ revealed a number of protein and pathway changes which occurred following hCGβ downregulation by CGB_5 siRNA and cisplatin treatment. Validation of changes in the expression of LIMK1, SIRT1 and WAPAL proteins by western blotting revealed that these proteins may be involved in the response of A2780cis and HEY cells to cisplatin treatment following hCGβ downregulation, but the effect was dependent on the siRNA used to target hCGβ. Although changes in LIMK1 were mirrored by both siRNAs, changes in SIRT1 and WAPAL expression occurred only with the use of CGB_5 siRNA in the presence of cisplatin. This indicated that the two siRNAs worked by different mechanisms to confer cisplatin sensitivity. A possible compensatory effect of *LHB* upregulation by CBG_4 siRNA remains as another reason why the two siRNAs had different effects in some systems.

SIRT1 expression was decreased following CGB_5 siRNA treatment alone. This may be an off target effect of CGB_5 siRNA which could have been responsible for the observed increase in cisplatin sensitivity of A2780cis and HEY cells. Further investigation on the role SIRT1 on cisplatin sensitivity showed that it was indeed involved in cisplatin sensitivity but only in A2780cis cells and not HEY cells. This suggests that the effect of CGB_5 siRNA may be independent of SIRT1 levels and that the actual mechanism by which hCGβ downregulation confers cisplatin sensitivity has not yet been solved. Despite the power of the quantitative proteomic analysis, it raised new questions requiring further exploration.

Chapter 6 General discussion

Ovarian cancer is the most lethal gynaecological disease in the developed world and although there have been great advancements in diagnosis, monitoring disease progress and treatment, it still remains a disease that is hard to fight. One of the main reasons it is such a challenging disease to combat is because even though a high percentage of cancers respond to conventional platinum-taxane based therapy, development of chemoresistance is common, leading to poor survival outcomes. Extensive research has focused on high throughput techniques aimed at understanding ovarian cancer at a molecular level. These techniques include proteomics, and whole genome and transcriptome sequencing which have undoubtedly added a tremendous amount of information to the field, but their impact on how the cancer is diagnosed and treated, and ultimately how this will translate to improved patient outcomes, is yet to be discovered.

6.1 Focus of this thesis

To gain a better understanding of high-grade serous ovarian carcinoma (HGSC) this thesis has taken a basic approach of studying the functional role of a single protein, hCGβ. hCGβ had been proposed as a useful prognostic marker for ovarian cancer [181, 182], but information on its functional role(s), if any, has not been well studied. The aim of this thesis was to determine the expression of hCGβ in HGSC cell lines and to explore the role of hCGβ in cellular processes including proliferation, migration and adhesion as well as in modulating the response of HGSC cells to platinum-based drugs. The power of proteomics was then employed to form

a global picture of the mechanisms by which hCGβ might be involved in the sensitivity and resistance of HGSC cells to cisplatin.

The question may arise as to why we would investigate a protein which may not seem to play any role in ovarian cancer other than as a prognostic biomarker. Firstly, though the functional role of hCGβ has indeed been largely unknown, it is elevated in a number of cancers including ovarian cancer [134, 182-185]. This of course could imply that cancer cells simply produce hCGβ due to aberrant gene expression, but perhaps this is too simple an explanation. Secondly, hCGβ has been shown to have biological implications in terms of proliferation, migration and apoptosis in cancer cell line models other than ovarian cancer [155, 156, 180]. Thirdly, although the level of hCGβ has been shown to be associated with chemoresistance in small-cell lung cancer patients [180], a direct functional role of the molecule is still uncertain. This means that hCGβ has been a protein which has lingered in the background in the context of understanding ovarian cancer and chemoresistance, and therefore its functional significance needed to be investigated.

6.2 Expression of hCGβ and its role in proliferation, migration and adhesion

Firstly it had to be established that hCGβ was expressed in HGSC cell line models before its functional role could be investigated. It was demonstrated that *CGB* transcript and secreted hCGβ protein were expressed in all eight HGSC cell line models (SKOV-3, HEY, OV202, PEO-1, A2780, A2780cis, CaOV-3 and OVCAR-3) to varying degrees. Three cell line models – HEY, A2780 and A2780cis cells – expressing a varied range of *CGB*/hCGβ, were then selected for functional

studies. HEY cells were chosen as they expressed the highest level of hCGβ and the pair of cisplatin-sensitive and resistant cells, A2780 and A2780cis, respectively were chosen for drug response studies. hCGβ was then downregulated with at least two siRNAs, or its cDNA overexpressed with a plasmid vector to determine its effect on proliferation, migration, adhesion and response to platinum-based drugs.

It was found that the effect of hCGβ on cell proliferation was cell type dependent as downregulation of hCGβ significantly decreased proliferation of A2780cis and HEY cells, but its effect on proliferation of A2780 cells was dependent on the siRNA used to downregulate hCGβ. This suggested that the two siRNAs targeting hCGβ may have some differences in their actions. The influence of hCGβ downregulation on cell proliferation should be tested under low serum conditions, as growth factors in the serum may mask the effect of hCGβ. The addition of exogenous hCGβ or overexpression of hCGβ did not seem to affect cell proliferation, perhaps due to a threshold level of hCGβ being reached beyond which hCGβ had no influence. Furthermore, it has to be noted that the biological activity of exogenous recombinant hCGβ could not be easily determined prior to the study.

Downregulation or overexpression of hCGβ did not seem to influence cell migration of the tested HGSC cells, contrary to published data by Wu and colleagues who showed that hCGβ increased the migratory characteristics of prostate cancer cell lines and the human glioblastoma cell line U87MG [156, 189, 190]. A possible explanation is that the influence of hCGβ on cell migration could be cell type dependent. As we only extensively studied cell migration in HEY cells which had a stronger potential to migrate compared to A2780 and A2780cis cells, it may be worthwhile testing for cell migration in a larger set of cell lines.

The role of hCGβ in HGSC cell adhesion has been investigated for the first time in this thesis. Cell adhesion is a means by which cells interact with their extracellular environment and has a number of implications on signalling pathways, which influence cell survival, apoptosis, migration and cancer metastasis [341]. Cell adhesion is regulated by integrins which are heterodimeric molecules composed of non-covalently associated α- and β- transmembrane glycoproteins which interact with extracellular matrix (ECM) proteins. There are up to 18 α- and 8 β-characterised subunits forming 24 different integrin combinations which bind to specific ECM proteins [341]. We found that downregulation of hCGβ resulted in a general increase in cell adhesion of HEY and A2780cis cells onto ECM proteins. This result raises the question of whether hCGβ could be regulating the different integrins within the cell, hence impacting on how the cells interact with ECM proteins. The influence of hCGβ on expression of integrins could be further investigated with integrin arrays, which is a high throughput technique which can determine the expression of a range of integrins on the cells surface [342]. Another possible explanation for the influence of hCGβ on cell adhesion is that it may be blocking cell adhesion, therefore downregulation of hCGβ could be eliminating this inhibitory effect on cell adhesion. The inhibitory effect of hCGβ should be further investigated by determining the impact of hCGβ overexpression or exogenous hCGβ on cell adhesion; however, this was not considered to be a cost-effective line of investigation to pursue for this thesis.

6.3 hCGβ and the cell response to chemotherapeutics

Expression of hCGβ in epithelial cancers is often associated with aggressive disease with poor patient outcomes; however, the impact of hCGβ in chemoresistance has been largely unaddressed. Only two published papers were found to suggest that hCGβ may be involved in chemoresistance [180, 280]. Szturmowicz *et al.* found that patients with small-cell lung cancer who had elevated levels of hCGβ responded poorly to chemotherapy [180]. *In vivo* data published by Berman *et al.* found that small cell bronchial carcinoma xenografts which expressed hCGβ were resistant to the chemotherapeutic drug cyclophosphamide [280]. Both of these published studies were observational; therefore in this thesis, the functional role of hCGβ in the response of HGSC to platinum-based drugs has been a main focus. It was shown that hCGβ does in fact play a role in how HGSC cells respond to the platinum-based drugs cisplatin, carboplatin and oxaliplatin; a response that seemed to be specific to platinum-based drugs and not to the microtubule targeting agent paclitaxel. Downregulation of hCGβ increased the sensitivity of A2780cis and HEY cells to cisplatin; however, it had no influence on the response of cisplatin sensitive A2780 cells to the drug. The result suggests that the effect of hCGβ on drug response is cell type dependent. It could also be suggestive that cells need to have acquired resistance to platinum-based drugs; in this case A2780cis are cisplatin resistant and HEY have higher IC_{50} for cisplatin compared to A2780 cells.

Whether the expression levels of hCGβ in tumour tissue or serum of patients with ovarian cancer (or other cancers) has a direct bearing on how patients respond to chemotherapy has to be further investigated. This could have future implications for determining which patients may benefit from platinum-based chemotherapy. As mentioned in Chapter 4, section 4.4.1 the absolute levels of hCGβ may not positively

correlate to drug response; however, it is possible that it is the absence (or lowered amount) of hCGβ that may determine how cells respond to drugs, particularly in cells that are already drug resistant. If indeed this is the case, then development of neutralising antibodies towards hCGβ, or other mechanisms of downregulating its expression or activity, could be a potential way of combating drug resistance in ovarian cancer.

6.4 Potential role of LHβ in the response to platinum drugs

It was observed that two hCGβ targeting siRNAs (CGB_4 and CGB_5) caused different biological effects in some experimental situations, despite a similar degree of hCGβ downregulation. In order to study whether a compensatory change in *LHB* could be a contributing factor to the different degrees of response of the two hCGβ siRNAs towards cisplatin, the *LHB* levels were determined following hCGβ downregulation. It was found that treatment with CGB_4 siRNA did in fact increase the levels of *LHB* but CGB_5 siRNA decreased its levels. These results were statistically significant in HEY cells compared to A2780cis cells, suggesting that in HEY cells both *LHB* and *CGB* may be an important determinant of the manner in which cells responded to cisplatin. Further to this point, a third siRNA (CGB_7) targeting hCGβ did not significantly affect the levels of *LHB* and did not influence how HEY cells responded to cisplatin treatment. This result demonstrated a potential link between *LHB* and response to cisplatin in some HGSC cells and maybe suggestive of some overlap of biological roles between LHβ and hCGβ. It was difficult to source a siRNA that specifically downregulated *LHB* independent of *CGB* levels. The manufacturer (Qiagen) who supplied our siRNAs, had a number of

siRNAs targeting *LHB* but these targeted identical sequences to *CGB* siRNAs. In future work, the role of *LHB* in response to cisplatin could be investigated by sourcing, or designing, siRNAs that specifically target *LHB* and by overexpressing *LHB* to determine whether it indeed has a role to play in drug response. Similarly, the expression of both LHβ and hCGβ (and/or *LHB* and *CGB*) in tumour tissue or serum from patients with ovarian cancer (or other cancers) could also be studied to determine whether their levels, together or independently, have an influence on how patients respond to chemotherapy.

6.5 iTRAQ and the mechanism of action of hCGβ in response to cisplatin

The quantitative proteomic technique of iTRAQ was used to determine the global protein changes that occurred when A2780cis cells were treated with cisplatin following hCGβ downregulation (with CGB_5 siRNA), in an attempt to understand the mechanism by which hCGβ regulates how HGSC cells respond to cisplatin. In the context of ovarian cancer, iTRAQ has been used to identify potential biomarkers [343] and differences between begin and malignant tumour tissue [344]; however, it had not been used to study chemoresistance. Multiple studies have been used iTRAQ and chemoresistance in two studies on mechanism of chemosensitivity or resistance in cancers such as head and neck carcinoma and colorectal cancer [345-347]; in these two studies, dysregulated proteins discovered by iTRAQ were validated using western blotting and the functional role of candidate proteins in chemosensitivity or resistance was investigated by gene silencing. This approach was followed in this thesis.

Pathway analysis of the protein changes from our iTRAQ study revealed that some pathways that were affected by cisplatin treatment in combination with hCGβ downregulation were also affected by cisplatin or hCGβ downregulation alone; however, the cellular processes and protein molecules involved in the overlapping pathways were different. This result was suggestive of the cells responding differently when treated with both cisplatin and hCGβ targeting siRNA compared to cisplatin or siRNA treatment alone, which was expected.

Due to financial constraints, the iTRAQ experiment was conducted only once; and western blotting used to validate important protein changes. Specifically, changes in the levels of LIMK1, SIRT1 and WAPAL proteins were validated. SIRT1 and WAPAL reflected the iTRAQ data; that is, their cellular abundance was altered by cisplatin treatment when hCGβ levels were decreased by siRNA, but not in the presence of normal hCGβ levels. This suggests that these proteins may be involved in the response of A2780cis and HEY cells to cisplatin treatment following hCGβ downregulation. However the results were dependent on the siRNA used to target hCGβ, indicative of different modes of action of the two siRNAs. Whether the differential responses related to differences in the compensatory induction of LHβ when hCGβ was downregulated, as discussed earlier, remains to be further explored. These findings meant that the actual mechanism by which hCGβ could be involved in drug response was not firmly established, although candidate proteins were identified.

6.5.1 Alternative strategy to iTRAQ

iTRAQ has the advantage of multiplexing samples in a single experimental run; however, the results can be challenging to interpret due to differences in the labelling process of the individual samples. Therefore the list of deregulated proteins generated by iTRAQ in our single run may not provide a comprehensive reflection of the global protein changes that occurred when the cells were treated with cisplatin following hCGβ downregulation. In future work, the iTRAQ experiment could be repeated or used in conjunction with the label free technique Sequential Window Acquisition of all Theoretical Mass Spectra (SWATH) [348]. SWATH is a relatively new technique which allows for both relative and absolute quantitation of proteins in a precise and reproducible manner. It is operated in a data independent acquisition (DIA) mode whereby all precursor ions and product ions are detected and archived allowing for retrospective data analysis. Operation in DIA mode allows for a greater dynamic range, hence is more sensitive for low intensity ions which are often missed by data-dependent acquisition mode (whereby a precursor is selected in order for the product ion to be scanned) [349, 350]. The Australian Proteome Analysis Facility (APAF) has recently started to offer SWATH as an analytical option and in future this may be a more reliable method to study global protein changes.

6.6 Approach of using antibodies to block effect of hCGβ

Expression of both *CGA* and *CGB* by the all the HGSC cell lines suggests the whole hCG protein could be potentially expressed which in turn could contribute to some of the functional effects observed by free hCGβ. Use of an antibody that

specifically targets secreted hCGβ may have been a fruitful approach to studying the role of hCGβ independent of heterodimeric hCG. For this reason, the best means of studying the role of hCGβ was to overexpress and downregulate hCGβ; a means by which the role of hCGβ has been investigated in previously published studies [156, 180, 191]. In future, antibodies against hCGβ could be developed to target free hCGβ allowing the study of hCGβ in culture independent of the whole hCG protein. Alternately, existing hCGβ antibodies designed for western blotting, for example, could also be tested to determine whether they could potentially be used to block hCGβ in culture. However, it is also possible that intracellular hCGβ may have a functional role in its own right which cannot be determined by antibodies directed at secreted hCGβ. Therefore the use of downregulation using siRNA, as employed in this thesis, is clearly a valuable experimental tool.

6.7 Improving hCGβ downregulation

hCGβ was downregulated by 50-60% using the highest amount of siRNA that could be used without theoretically introducing off target effects. Therefore a longer time course following siRNA transfection should be tested to determine whether a higher level of knockdown could be achieved.

6.8 Validation of the on-target effects of hCGβ on response of cells to platinum-based drugs

Validation of the increased sensitivity of the cells to platinum-based drugs following downregulation hCGβ can be done in two ways. 1. Addition of exogenous hCGβ following downregulation 2. Overexpressing hCGβ using a plasmid construct

containing a hCGβ gene insert (containing silent mutations) which is resistant to the siRNAs targeting hCGβ expressed by the cells [351, 352].

6.9 Development of stable knockouts or overexpression

In this thesis the functional role of hCGβ was studied using transient downregulation and overexpression systems. Although a lot of data has been generated using these systems, the development of stable systems could not only facilitate longer experiments but also *in vivo* experiments in animal models. hCGβ could be stably downregulated using short hairpin RNA (shRNA) [353] and overexpressed using the same construct (pCI-neo-hCG) for transient overexpression, but by growing the cells under the selection of the antibiotic G418 [156]. It is possible that shRNA may result in better hCGβ knockdown compared to the use of siRNA. The disadvantage of stable downregulation of growth-regulatory proteins, which involves selection of clones or restricted cell populations, is that other cell markers may also inadvertently be selected, or that the selected populations may have upregulated mechanisms that compensate for the downregulated target protein. These disadvantages might in part be overcome by using an inducible vector to express the shRNA.

6.10 SIRT1 and the response to cisplatin

SIRT1 has been shown to be elevated in a number of cancers including ovarian cancer [312], however its role in tumorigenesis has not been established [332]. Our study shows that SIRT1 is potentially involved in the response to cisplatin of some HGSC cells, as downregulation of SIRT1 increased cisplatin sensitivity in A2780cis

cells but not HEY cells. In future work, the role of SIRT1 in the response to platinum-based drugs could be tested in different cell lines by downregulating or overexpressing SIRT1 or using an inhibitor of SIRT1, e.g. EX-527 (SEN0014196) [354].

Expression of SIRT1 in tumour tissue of patients with ovarian cancer could also be studied to determine whether SIRT1 levels may be a prognostic marker of patients' response to chemotherapy. Interestingly, a preliminary assessment of the prognostic value of SIRT1 using KM-Plotter showed that ovarian cancer patients who received platinum-based therapy had a slightly lower rate of progression free survival when SIRT1 levels were high. This suggests that SIRT1 could be a potentially valuable prognostic marker of patients' response to chemotherapy. Further to this, if it is confirmed that SIRT1 has a functional role in chemosensitivity then a potential outcome could be the use of SIRT1 inhibitors alongside platinum-taxane based therapy for the treatment of ovarian cancer. SIRT1 inhibitors have already been utilised in medical conditions such as diabetes and the neurological condition Huntington's disease [355]. The selective SIRT1 inhibitor, Selisista (6-chloro-2,3,4,9-tetrahydro-1H-carbazole-1-carboxamide, SEN0014196, EX-527) is currently in Phase II clinical trials for the treatment of Huntington's disease [356].

6.11 Changes in the field since undertaking this thesis

One of the major changes in the field since the beginning of this thesis has been the characterisation of existing and new HGSC cell line models. Domcke *et al.* and Ince *et al.* deemed a number of cells lines, including SKOV-3 and A2780 cells that were used in this thesis, as potentially less suitable HGSC models as they did not

contain *TP53* mutations or other genetic or molecular abnormalities characteristic of primary HGSC tumours [281, 282]. Ince *et al.* developed a method to isolate and propagate 25 ovarian cancer cells lines which were considered to be true representative models of the disease [282]. These cell lines not only reflected the histological, genotypic and molecular characteristics of the tumour cells they were derived from but these characteristics were maintained over successive passages.

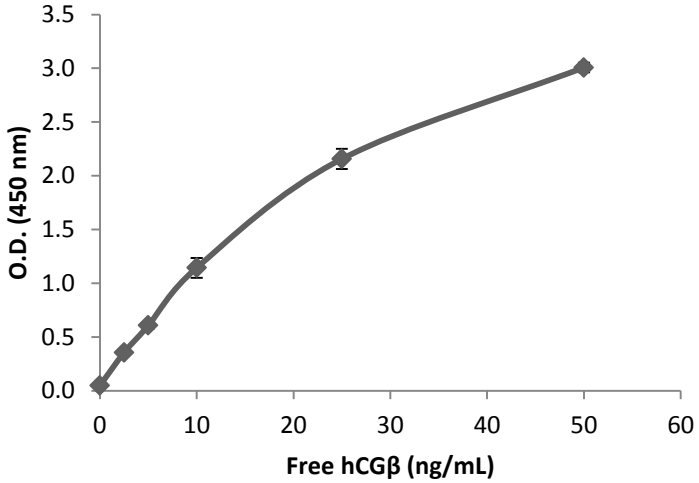
In the future, a panel of HGSC cell lines recommended by Domcke *et al.* and/or developed by Ince *et al.* should be used to confirm the functional role of hCGβ. As mentioned in Chapter 2, the A2780/A2780cis pair is a valuable model for studying drug resistance, however since Domcke *et al.* found that A2780 cells were not an optimum model for HGSC, cell lines resistant to cisplatin, carboplatin, oxaliplatin and paclitaxel could also be developed based on suitable HGSC models to allow the study of drug resistance in the context of hCGβ. This work would best be conducted in follow-up work, as development and testing of drug resistant cell lines can take up to 18 months [357]. Alternately, cell lines established by Ince and colleagues which are already resistant to platinum-taxane treatment could be used for drug based studies [282]. The advantage of using these cell lines is that they may be closer to a true representation of drug resistance as they are derived from chemoresistant tumours as opposed to having been artificially created in the lab.

6.12 Concluding remarks

This thesis has highlighted the potential role of hCGβ in cell proliferation, adhesion and response to platinum-based drugs in ovarian cancer cells. The LHβ, WAPAL and SIRT1 proteins were also discovered to be potentially involved in the

response to cisplatin and could have implications on how chemoresistance is managed in ovarian cancer. Future work on the mechanism by which hCGβ can regulate cellular response to platinum-based drugs needs to be established and may have implications for the management of chemoresistance in ovarian cancer.

Appendix A: Chapter 3 supplementary data



Supplementary Figure 1: Standard curve for detection of hCGβ using the chorionic gonadotropin beta Human ELISA kit from Abam. Standard concentrations included 0, 2.5, 5, 10, 25 and 50 ng/mL of free hCGβ.

Appendix B: Chapter 4 supplementary data

Supplementary Table 1: IC50 concentration of cisplatin derived from the cell viability assay for A2780cis and HEY cells

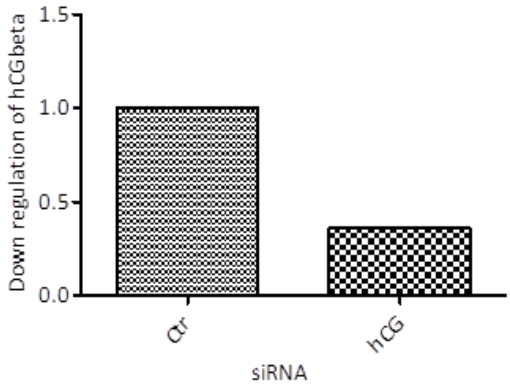
Cisplatin			
	NS control	**CGB_4**	**CGB_5**
A2780cis	12.48 ± 1.14	12.49 ± 1.43	7.46 ± 0.54
HEY	4.65 ± 0.26	4.07 ± 0.13	3.90 ± 0.25
Carboplatin			
	NS control	**CGB_4**	**CGB_5**
A2780cis	99.92 ± 6.60	100.43 ± 7.27	73.10 ± 7.13
HEY	38.36 ± 2.89	37.53 ± 0.81	36.59 ± 0.72
Oxaliplatin			
	NS control	**CGB_4**	**CGB_5**
A2780cis	9.15 ± 0.79	8.03 ± 0.58	7.44 ± 0.38
HEY	15.64 ± 1.41	17.91 ± 0.75	15.69 ± 2.57
Taxol			
	NS control	**CGB_4**	**CGB_5**
A2780cis	14.17 ± 1.12	14.07 ± 0.79	13.73 ± 1.23
HEY	17.84 ± 4.40	15.26 ± 1.00	17.9 5 ± 3.31

Supplementary Table 2: IC50 concentration of cisplatin derived from the cell survival (clonogenic) assay for A2780cis and HEY cells

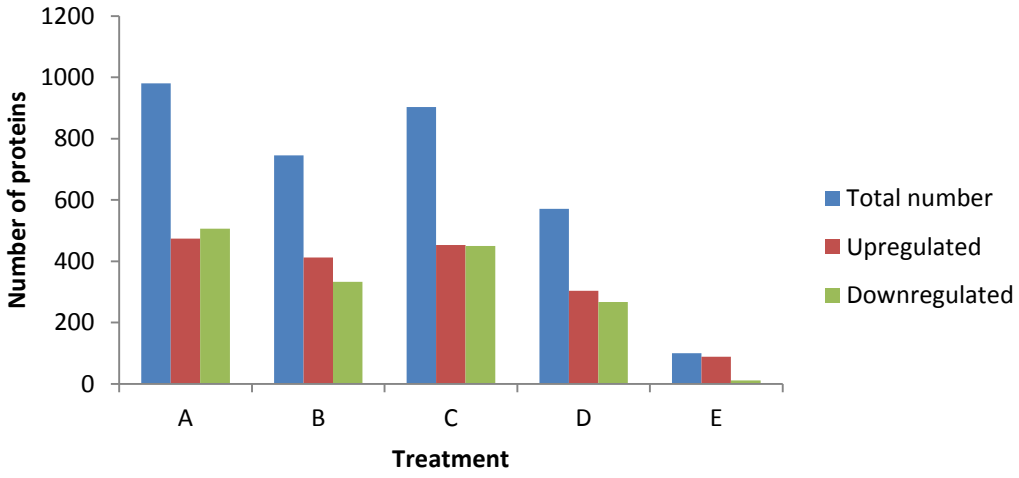
Cisplatin			
	NS control	**CGB_4**	**CGB_5**
A2780cis	2.07 ± 0.35	2.58 ± 0.26	4.34 ± 0.39
HEY	1.28 ± 0.07	1.22 ± 0.18	0.73 ± 0.02
Carboplatin			
	NS control	**CGB_4**	**CGB_5**
A2780cis	23.15 ± 4.57	19.96 ± 4.64	11.85 ± 1.36
HEY	9.94 ± 2.95	9.83 ± 2.45	5.61 ± 1.36
	NS control	**CGB_4**	**CGB_5**
Oxaliplatin			
A2780cis	0.38 ± 0.06	0.28 ± 0.03	0.25 ± 0.05
HEY	0.31 ± 0.07	0.34 ± 0.04	0.35 ± 0.03

Appendix C: Chapter 5 supplementary data

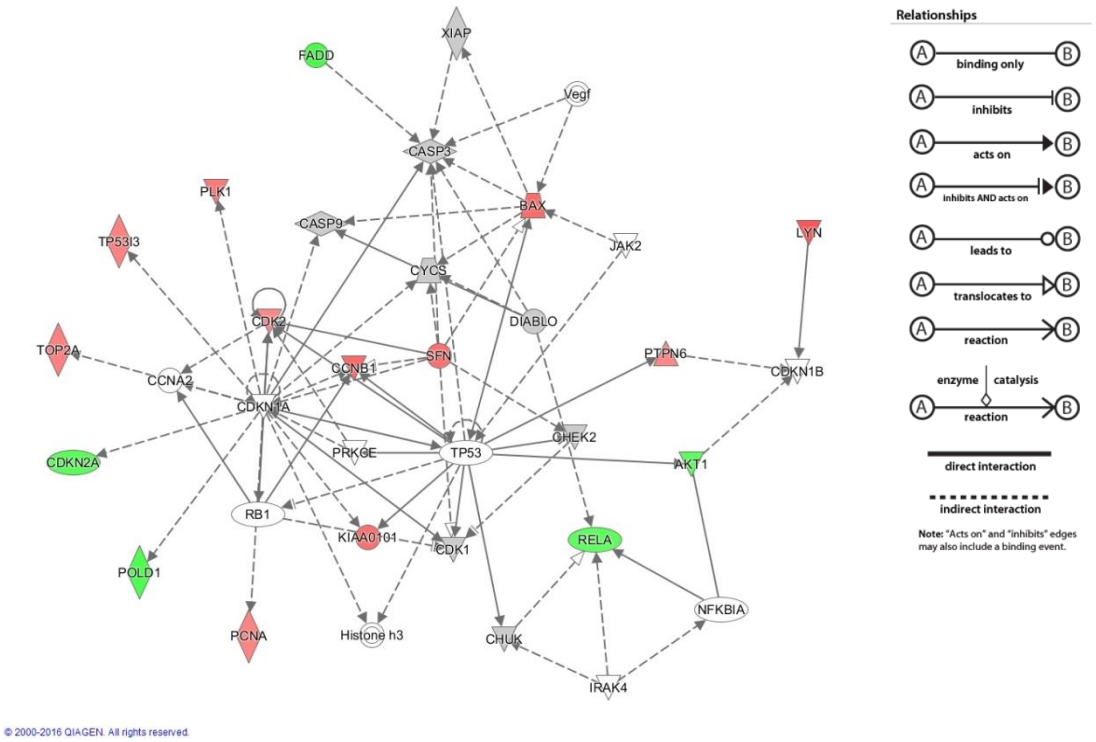
Refer to Compact Disk or attached file for complete iTRAQ data.



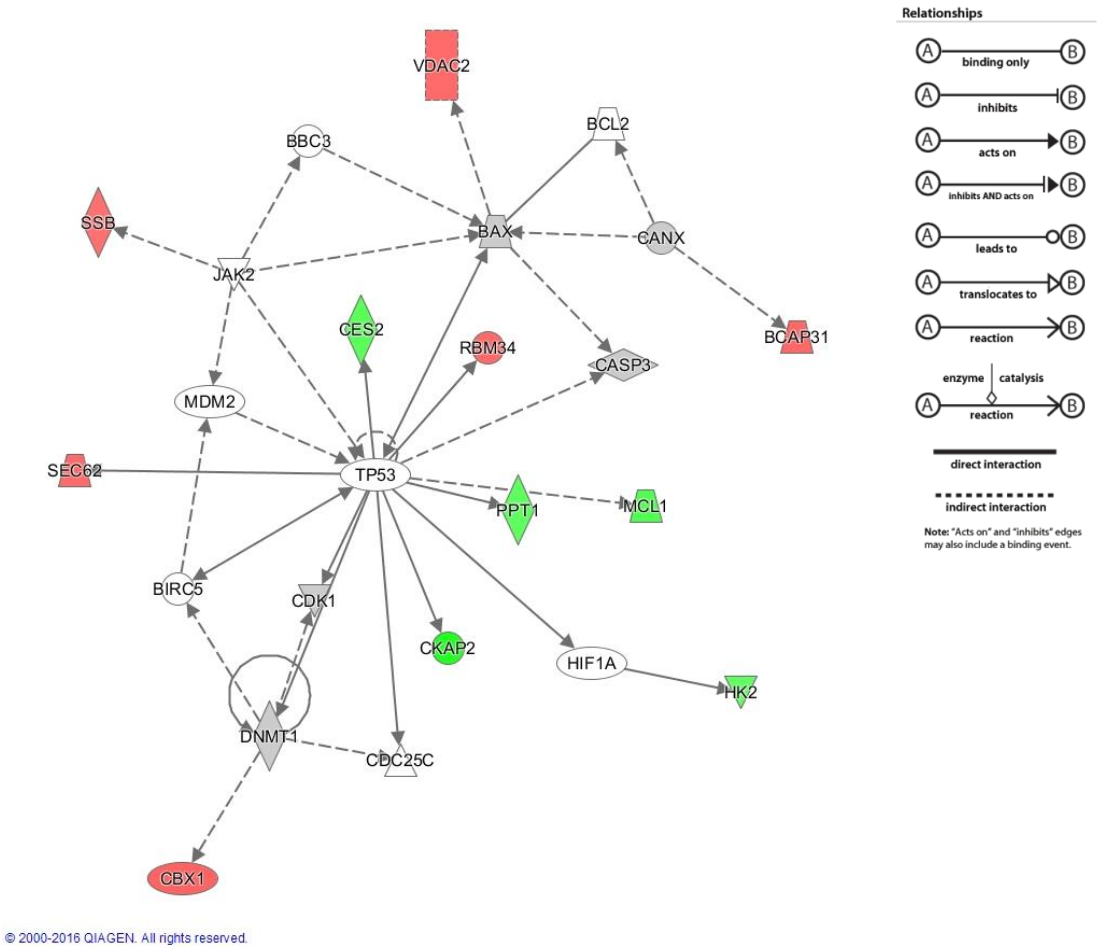
Supplementary Figure 2: Downregulation on *CGB* gene for A2780cis cells undergoing iTRAQ. siRNAs: non-silencing control (ctr), and CGB_5 (hCG). Data normalised to negative non-silencing control siRNA.



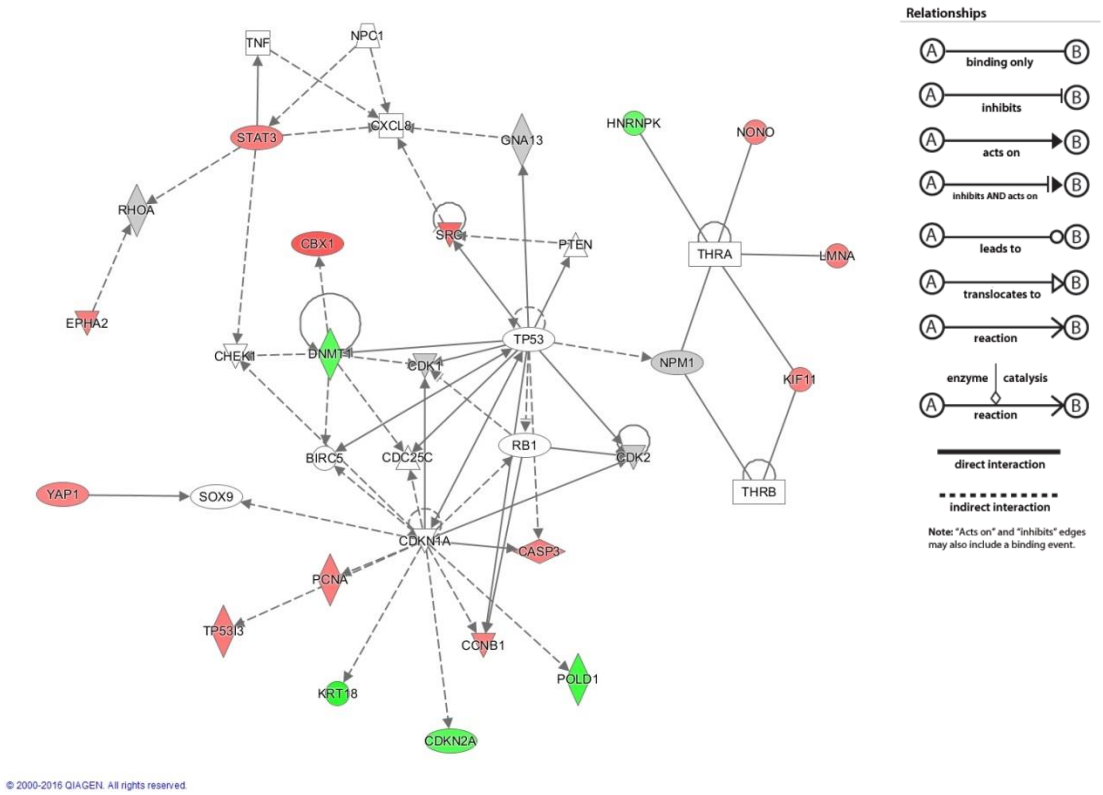
Supplementary Figure 3: "A" is the effect of cisplatin treatment (ratio 115:114), "B" is the effect of hCGβ downregulation (ratio 116:114), "C" is the effect of both treatments combined (ratio 117:114), "D" is the effect of cisplatin when hCGβ was downregulated and "E" are proteins that were only responsive to cisplatin when hCGβ was downregulated ((115:114)/(117:115)).



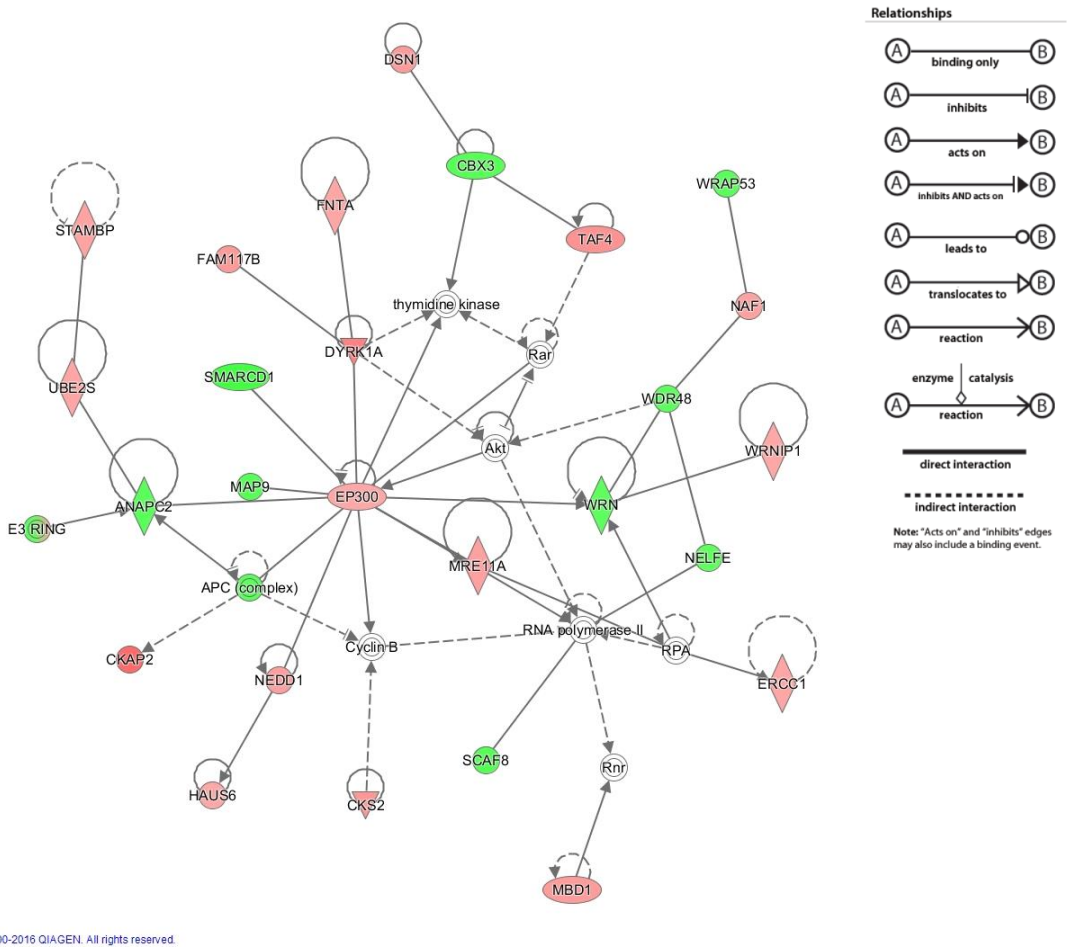
Supplementary Figure 4: Effect of cisplatin treatment (ratio 115:114) on the cell death, survival, DNA replication, recombination and repair network. Indicated colours: RED: User input molecule that is upregulated, GREEN: user input molecule that is downregulated, GRAY: user input molecule that is neither up nor down-regulated, WHITE: molecule that is not user specified, but incorporated into the network through relationships with other molecules. Relationship and colour keys extracted from Ingenuity systems, IPA supporting documentation [358].



Supplementary Figure 5: Effect of hCGβ downregulation (ratio 116:114) on the cell morphology, cell death and survival network. Indicated colours: RED: User input molecule that is upregulated, GREEN: user input molecule that is downregulated, GRAY: user input molecule that is neither up nor down-regulated, WHITE: molecule that is not user specified, but incorporated into the network through relationships with other molecules. Relationship and colour keys extracted from Ingenuity systems, IPA supporting documentation [358].



Supplementary Figure 6: Effect of cisplatin treatment followed by hCGβ downregulation (ratio 117:114) on the cell cycle, DNA replication, recombination and repair network. Indicated colours: RED: User input molecule that is upregulated, GREEN: user input molecule that is downregulated, GRAY: user input molecule that is neither up nor down-regulated, WHITE: molecule that is not user specified, but incorporated into the network through relationships with other molecules. Relationship and colour keys extracted from Ingenuity systems, IPA supporting documentation [358].



Supplementary Figure 7: Effect of a combination of cisplatin and hCGβ downregulation (ratio (115:114)/117:115) on the cell cycle, DNA replication, recombination and repair network. Indicated colours: RED: User input molecule that is upregulated, GREEN: user input molecule that is downregulated, GRAY: user input molecule that is neither up nor down-regulated, WHITE: molecule that is not user specified, but incorporated into the network through relationships with other molecules. Relationship and colour keys extracted from Ingenuity systems, IPA supporting documentation [358].

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