Host cell factors in ovarian cancer influencing efficacy of oncolytic aenovirus dl922-947

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Host Cell Factors in Ovarian Cancer Influencing Efficacy of Oncolytic Adenovirus *dl*922-947

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I, Magdalena Barbara Flak, hereby confirm that the work presented in this thesis is original and is my own. Work undertaken by others in contribution to this thesis has been clearly indicated. Where information has been derived from other sources, this has been acknowledged and appropriately referenced.

Signed ……...

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Abstract

Adenoviral gene therapy holds great potential for cancer treatment, but is limited by a lack of efficient vectors. *dl*922-947, an E1A CR2-deleted adenovirus, replicates selectively within and lyses cancer cells. It is believed that its selectivity depends upon abnormalities in the cell cycle regulatory Rb pathway and subsequent G1/S checkpoint, observed in 90% of human cancers. The cytotoxic efficacy of *dl*922-947 is greater than that of wild-type adenovirus and *dl*1520 (Onyx-015). Nevertheless, sensitivity to *dl*922-947 varies widely among ovarian cancer cell lines, despite similar infectivity. My work aimed to identify host cell factors that influence cytotoxicity and which could be potential biomarkers in clinical trials. Surprisingly, comparison of ovarian cancer lines indicated that cytotoxicity correlated poorly with infectivity, replication and virion production. Immunoblotting suggested correlation between sensitivity to *dl*922-947 and overexpression of p21, p27, Cyclin D, cdk4 and p16. Subsequent experiments confirmed a role for p21 in *dl*922-947 cytotoxic activity. *In vitro* and *in vivo*, Hct116 p21+/+ cells were significantly more sensitive to *dl*922-947 than matched p21-/- cells. p21 knock-down by siRNA in TOV21G and IGROV-1 cells reduced *dl*922-947 cytotoxicity, whilst re-expression in ACP-WAF1 cells increased activity. p21 expression was also greater in sensitive transformed TOSE cells compared to resistant normal IOSE25 cells. Results suggest that p21 promotes *dl*922-947 activity by stabilising Cyclin D thus promoting cell cycle progression. Comparative microarray analysis in TOSE1, 4 and IOSE25 cells and in MRC5 and MRC5-VA cells suggested determinants of *dl*922-947 activity beyond the Rb pathway, which may also prove valuable biomarkers. Moreover, pathways and processes emerged, correlating with sensitivity, and meriting future investigation. Together, my results suggest that a cellular environment conducive for *dl*922-947 function includes mediators of proliferative capacity, amongst which p21 plays a role in enhancing activity of the virus.

A scientist in his laboratory is not a mere technician:
He is also a child confronting natural phenomena that impress him
as though they were fairy tales.
Marie Curie

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ABBREVIATIONS

-/-	Homozygous deletion/knockout
+/+	Wild-type
aa	Amino acids
AAV2	Adeno-associated virus 2
ABCD1	ATP-binding cassette, subfamily D, member 1
Ad	Adenovirus
AFP	α-foetoprotein
ALD	Adrenoleukodystrophy
amp	Ampicillin
APS	Ammonium Persulphate
ATC	Anaplastic thyroid carcinoma
ATP	Adeno-triphosphate
bp	Base pairs
BSA	Bovine Serum Albumin
$CaCl_2$	Calcium Chloride
CAR	Coxsackie and Adenovirus Receptor
CD	Cluster of Differentiation molecule
Cdc	Cell division cycle
cdk	Cyclin-dependent kinase
cDNA	Complementary DNA
CEACAM6	Carcinoembryionic antigen-related cell adhesion molecule 6
cm	Centimetre
CML	Chronic myeloid leukemia
CMV	Cytomegalovirus
CKI	Cdk inhibitor
CPE	Cytopathic effect
CR	Constant region
cRNA	Complementary RNA
ctrl	Control
Δ	Delta (deletion)
d	Days
dH_2O	Deionised water
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
ds	Double stranded
DTT	Dithiothreitol
EC_{50}	Effective concentration (50% effect)
ECL	Enhanced chemiluminescence
EDTA	Ethylene-(2,2)-diamine-tetracetic acid
EGF	Epidermal growth factor
eGFP	Enhanced green fluorescent protein
ERK	Extracellular signal-regulated kinase
EtOH	Ethanol
FACS	Fluorescence-activated cell sorting
FCS	Foetal calf serum
Fig.	Figure
FIGO	International Federation of Gynaecology and Obstetrics
FX	Human coagulation factor X
GDP	Guanosine diphosphate
GEO	Gene expression omnibus
GFP	Green fluorescent protein

GM-CSF	Granulocyte-macrophage colony-stimulating factor
h	Hour
H_2O	Water
H&E	Haematoxylin and eosin (stain)
HDAC-1	histone deacetylase-1
HOXA11	Homeobox A11
HPV	Human papillomavirus
HRP	Horseradish peroxidise
hTERT	Human telomerase reverse transcriptase
HIV	Human immunodeficiency virus
hrs	Hours
HSV	Herpes simplex virus
IC_{50}	Half maximal inhibitory concentration
IgG	Immunoglobulin
IL	Interleukin
i.p.	Intraperitoneal
IOSE	Immortalized ovarian surface epithelial cells
i.t.	Intra-tumoural
ITR	Inverted terminal repeats
i.v.	Intra-venous
JAM	Junctional adhesion molecule
JNK	c-Jun N-terminal kinase
kana	Kanamycin
kb	Kilobases
kDa	Kilo Dalton
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
µ	Micro
m	Milli
MHC	Major histocompatibility complex
MHRA	Medicines and Healthcare products Regulatory Agency
MMP	Matrix-metalloproteinase
MLP	Major late promoter
MLTU	Major late transcription unit
mm	Millimetre
MOI	Multiplicity of infection
mTOR	Mammalian target of rapamycin
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
mRNA	Messenger RNA
n	Nano
NDV	Newcastle Disease Virus
NK	Natural Killer
NF-κB	Nuclear factor kappa B
NO	Nitric oxide
OD	Optical density
ORF	Open reading frame
OTC	Ornithine transcarbamylase deficiency
PAK1	p21-activated kinase 1
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PI3K	Phosphatidyl-inositol-3’ kinase
pfu	Plaque-forming units
p.i.	Post infection
PKR	dsRNA-activated Protein kinase R
PSA	Prostate-specific antigen

qPCR	Quantitative PCR
Rb	Retinoblastoma
RNA	Ribonucleic acid
RNAi	RNAi interference
RU	Relative units
scr	Scrambled
shRNA	Short hairpin RNA
siRNA	Small interfering RNA
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
sec	Seconds
Ser	Serine
ss	Single stranded
SV40 TAg	Simian virus 40 large T antigen
TBS	Tris buffered saline
$TCID_{50}$	50% Tissue culture infective dose
TEMED	Tetramethylethlenediamine
TGF-β	Transforming growth factor-β
Thr	Threonine
TNF-α	Tumour necrosis factor-α
TLR	Toll-like receptor
TOSE	Transformed ovarian surface epithelial cells
TSTA	Two-step transcriptional amplification
UV	Ultraviolet
VLCFA	Very long-chain fatty acids
WT	Wild-type

1 Introduction

Despite decades of research, the majority of women diagnosed with ovarian cancer will die of their disease. There is only limited understanding of ovarian cancer pathogenesis and progression (Cunningham *et al.*, 2009). Further hampering treatment, 75% of patients present with advanced disease, as there is no reliable screening method and symptoms are ambiguous (Saga *et al.*, 2001; Stirling *et al.*, 2005; Yurkovetsky *et al.*, 2006). Less than 25% live more than 5 years (Bankhead, 2004; Fields *et al.*, 2006). Current treatment comprises aggressive debulking surgery followed by platinum-taxane chemotherapy (Morrison *et al.*, 2007). However, even in patients highly responsive to chemotherapy, recurrence of the disease is very common (McGuire *et al.*, 1998). Overall, compared to treatment development in other cancers, survival rates of ovarian cancer patients have improved little over the past three decades (Barnholtz-Sloan *et al.*, 2003). Clearly, development of new, more effective treatments is imperative, based upon a better understanding of the biology of the disease.

1.1 Ovarian Cancer

Ovarian cancer comprises more than thirty types and sub-types of tumours, differing in histopathological features and behaviour (Auersperg *et al.*, 2001).

Different approaches have been tested as a means of diagnosis, as well as to monitor progression of the disease and response to treatment. CA-125, found elevated in the blood of patients with clinically apparent ovarian cancer, has frequently been investigated as a potential diagnostic marker (Bast *et al.*, 1998). However, it is not specific to cancer of the ovaries. Furthermore, CA-125 levels can be increased in various non-cancerous illnesses, as well as during early pregnancy and menstruation (Bast, 2004; Helzlsouer *et al.*, 1993). In an attempt to develop a diagnostic tool sensitive enough to process samples from patients' bodily fluids, aneuploidy of cells in peritoneal fluid was compared by flow cytometry. However, specificity and sensitivity of the assay were suboptimal. Authors concluded that this assay, unless improved, is inappropriate as a sole diagnostic tool for malignant cells (Kehoe *et al.*, 1995).

Prognosis of ovarian cancers is dependent on several factors: disease stage, histological grade and type, as well as molecular markers (Begum *et al.*; Drenberg *et al.*; Lalwani *et al.*). A first line of histological classification distinguishes between three cellular or tissue origins of ovarian malignancies:

A) EPITHELIAL ADENOCARCINOMAS

Approximately 90% of ovarian cancers are derived from the ovarian cell surface epithelium (Herbst, 1994). They are further divided into four subtypes: serous, endometrioid, mucinous and clear cell carcinomas.

a) Serous carcinomas represent the most widespread form of ovarian malignancies. Approximately 50% of serous tumours are of malignant nature, whilst 17% are borderline malignant. Serous carcinomas contain cystic and/or fibrous areas and are filled with clear serous fluid (Auersperg *et al.*, 2001; Scully, 1995).

b) Endometrioid carcinomas make up one fifth of epithelial adenocarcinomas. Generally, 80% are malignant and the remaining 20% tend to show borderline malignancy. Endometrioid carcinomas are associated with endometrial carcinoma in 20% of patients (Auersperg *et al.*, 2001; Jiang *et al.*, 1998). They can contain cystic and solid areas and often resemble benign or malignant endometrial tissue (Schueller *et al.*, 1966).

c) Mucinous carcinomas are relatively rare, constituting approximately 1% of epithelial adenocarcinomas. Mucinous tumours are predominantly benign. They comprise numerous cysts, varying in size. They can also form very large cysts filled with viscous fluid (Auersperg *et al.*, 2001; Collins *et al.*, 1991).

d) Clear cell carcinomas represent only 6% of epithelial carcinomas. However, nearly all of them have been found to be malignant (Auersperg *et al.*, 2001). Tumours contain large cytoplasm-rich epithelial cells and may be highly cystic or solid (Ito *et al.*, 1997).

Recently, evidence emerged that many aggressive epithelial ovarian carcinomas may in fact be derived from the fimbriae of the Fallopian tubes. Studies on ovaries and fallopian tubes removed prophylactically from BRCA-positive women suggested that malignant fimbriae-derived cells metastasise onto the ovaries and the peritoneum. Consequently, these tumours, when detected on the ovaries, are already aggressive late stage tumours (Morgan, 2008). These findings suggest that serous borderline tumours may not be the precursors of serous carcinomas.

A further level of histopathological classification of epithelial adenocarcinomas is based on their degree of histological differentiation. Well-differentiated tumours with distinct glandular features are considered Grade 1. Grade 2 tumours contain glandular areas

but also solid sheets of tumour. Tumours classified as Grade 3 are primarily composed of solid sheets. There appears to be some correlation between grade and clinical aggressiveness of tumours.

B) GERM CELL TUMOURS

These tumours, derived from primordial germ cells within ovaries, occur predominantly in younger women. On average, patients are diagnosed in the early 20s. Despite the tendency to be highly aggressive, they are also very responsive to therapy. Germ cell tumours are generally associated with marker proteins in the blood, such as β-human chorionic gonadotropin (β-HCG) or α-fetoprotein (AFP) (Chobanian *et al.*, 2008).

C) STROMAL TUMOURS

Stromal tumours originate from the sex cord or mesenchyme within the embryonic gonad. They often secrete hormones and contain gonad-related cells and fibroblasts (Appetecchia *et al.*, 2004; Chobanian *et al.*, 2008).

For staging of ovarian cancers, the FIGO (International Federation of Gynaecology and Obstetrics) surgical staging system is widely accepted. Surgical staging of ovarian cancers is based on removal of both ovaries, Fallopian tubes and the uterus, as well as the omentum. In addition, many surgeons also remove pelvic and para-aortic lymph nodes and take random biopsies from throughout the peritoneum. Histopathological assessment of these samples allows classification into stages as listed in Table 1.1.

The identification of prognostic and predictive markers receives much attention in translational research. Prognostic biomarkers provide information about a patient's overall outcome, irrespective of the form of therapy. They are usually based on retrospective analysis of patient data, identifying markers expressed across a patient subpopulation that responded well or poorly to treatment. Prognostic biomarkers, such as age, tumour grade or stage, allow an estimate of a patient's overall survival (Oldenhuis *et al.*, 2008).

Stage	Characteristics
I	**Cancer is restricted to the ovaries.**
IA	- cancer is restricted to one ovary - outer ovarian capsule is intact not ruptured - no tumour on the external surface of the ovary - no ascites and/or pelvic washings are negative
IB	- cancer is present in both ovaries - outer capsule is not ruptured - no tumour on external surface - no ascites and pelvic washings are negative
IC	- cancer is either Stage IA or IB level - *but* capsule is ruptured - *or* tumour on the ovarian surface - *or* malignant cells are present in ascites or pelvic washings.
II	**Cancer involves one or both ovaries with spread to other pelvic organs or surfaces.**
IIA	- extension or implants onto uterus and/or fallopian tube - no ascites and pelvic washings are negative
IIB	- extension or implants onto other pelvic tissues - no ascites and pelvic washings are negative
IIC	- pelvic extension or implants like Stage IIA or IIB - *but* positive pelvic washings
III	**Cancer spread beyond the pelvis to the abdominal area, including metastases to liver surface.**
IIIA	- tumour predominantly confined to the pelvis - *but* microscopic peritoneal metastases beyond pelvis to abdominal peritoneal surfaces or the omentum
IIIB	- same as IIIA - *but* macroscopic peritoneal or omental metastases beyond pelvis <2cm in Size
IIIC	- same as IIIA - *but* peritoneal or omental metastases beyond pelvis >2cm - *or* lymph node metastases to inguinal, pelvic, or para-aortic areas
IV	**Metastases or spread to the liver or outside the peritoneal cavity to distant areas such as the chest or brain.**

Table 1.1 FIGO (International Federation of Gynaecology and Obstetrics) surgical staging system. *Listed are stages and sub-stages and underlying characteristics, as determined by histopathological analyses (adapted from http://imaging.ubmmedica.com/cancernetwork).*

Increasingly, epigenetic alterations, particularly methylation, are studied as prognostic biomarkers in cancers (Kim *et al.*). Comparing methylation status of 71 genes in ovarian cancer and normal cells identified methylation of Homeobox A11 (HOXA11) gene to correlate with ovarian cancer (Fiegl *et al.*, 2008).

Similarly, post-translational modification patterns may be useful prognostic markers. Phosphorylation of p21-activated kinase 1 (PAK1) on threonine 212 (Thr^{212}) has been reported to predict for poor overall and disease-free survival of ovarian cancer patients. Also, an increase in nuclear PAK1 Thr^{212} was detected in poorly differentiated ovarian cancer (Siu *et al.*, 2009).

Another approach to detect early stage ovarian cancer is based on group biomarkers. Each group biomarker comprises a set of single biomarkers, the expression pattern of which has found to be the same across ovarian cancers of a specific stage and consistently changes to a different pattern in cancers of a different stage. Three such group biomarkers have been identified: one comprising genes LCN2, WNT7A and ITGB4, the second WFDC2, MUC1 and MSLN, the third KLK8, KLK7, MLSN. All three showed promising pre-clinical results detecting early-stage ovarian cancer using blood samples (Tchagang *et al.*, 2008). Although a plethora of candidates have been described, they have not yet proven sufficiently reliable to establish a staging system, similar to that of FIGO.

As in breast cancer, mutations of BRCA1 and BRCA2 have been found to predict increased risk of developing ovarian cancer (Miki *et al.*, 1994; Milne *et al.*, 2008), although patients with germline BRCA1/2 mutations account for only approximately 5% of all ovarian cancers in the UK. However, Ashkenazi Jewish patients with germline mutations in either BRCA1 or 2 have a better prognosis stage for stage than patients with sporadic ovarian cancer (Chetrit *et al.*, 2008).

Another prognostic marker correlating with ovarian cancer grade is p53. Mutations of p53 are very frequent in high-grade serous carcinomas, present in nearly 100% of tumours (Ahmed *et al.*, 2010). In low-grade serous carcinomas, p53 mutations are detected less frequently and they are very rare in serous borderline tumours (Marks *et al.*, 1991; Salani *et al.*, 2008; Teneriello *et al.*, 1993). In contrast, comparative analysis of activating KRAS and BRAF mutations in serous borderline tumours and serous carcinomas showed such mutations in only 12% of the latter, but in 60% of borderline tumours (Singer *et al.*, 2003).

Unlike prognostic biomarkers, predictive markers aim to foretell response to a given therapeutic agent in a patient expressing that marker (Oldenhuis *et al.*, 2008). Identification of predictive markers generally begins in a small model system, where its role is validated before expanding the testing of its potential to other systems. A bottom-up approach, from the molecular to the systemic level, is applied to elucidate and take advantage of the underlying mechanisms (Liu, 2005).

To date, loss of BRCA1 function is the only true predictive marker for chemotherapy in ovarian cancer (Quinn *et al.*, 2009). However, as novel therapeutic agents emerge, cancer therapy may well be standing on the brink of a new era of patient/treatment stratification. Development of new therapeutics, such as oncolytic viruses, is strongly based on molecular mechanisms. Many of the cellular targets may be suitable as predictive biomarkers (Tchagang *et al.*, 2008).

Members of the E2F family of transcription factors involved in cell cycle regulation have recently been described as predictive biomarker candidates. Whilst they may also be indicators of prognosis, Reimer et al. found correlations between low expression of E2F-7 and platinum resistance. The authors suggest that down-regulation of E2F-7 may promote such resistance, rendering the transcription factor a valuable predictive factor (Reimer *et al.*, 2007).

Establishing predictive biomarkers is a challenging task. Unlike for prognostic markers, which can be correlated with response and survival without being directly involved in tumourigenic mechanisms, identification and application of predictive markers requires understanding of their molecular role.

1.2 Stratified Medicine

Currently, most therapeutics are being prescribed empirically, based on maximum benefit for the largest population of patients found in clinical trials. In contrast, the strategy behind “stratified” or “personalised medicine” is the matching of therapies with specific patient population characteristics (Trusheim *et al.*, 2007). This approach aims at increasing both effectiveness and safety of treatment. Based on biomarkers, a given patient can be matched with a similar patient cohort, shown to respond particularly well to a certain drug regime (Acharya *et al.*, 2008). In cancer treatment, prioritising of anti-cancer drugs based on biomarkers is common. The first example of a more personalised therapy followed the recognition of the potential of tamoxifen as an anti-

oestrogen (Jordan, 1976). Tamoxifen is able to bind to oestrogen receptors (ERs) in oestrogen-dependent tumours. It competes with and blocks endogenous oestrogen but has no activating effects on ERs in the breast, itself (TANAKA *et al.*, 1978). The ER status of breast cancer tumours is routinely tested to devise the optimal treatment (Jorgensen, 2008). Imatinib (Gleevec), an inhibitor of bcr-abl kinase activity is very effective in chronic myeloid leukaemia (CML) (Capdeville *et al.*, 2002). However, patients carrying a point mutation in the bcr-abl kinase are resistant to imatinib (Nardi *et al.*, 2004). For these patients, two alternative bcr-abl inhibitors have been developed, nilotinib (AMN107) and dasatinib (BMS-354825) (Shah *et al.*, 2004; Weisberg *et al.*, 2006). Over-expressed epidermal growth factor receptor 2 (HER2) is an important stratification factor for treatment with monoclonal antibody trastuzumab (Lesko, 2007). By binding to the mediator of proliferation, trastuzumab blocks its function, resulting to G1 cell cycle arrest (Sumikawa *et al.*, 2008). In HER2 over-expressing breast cancer patients, trastuzumab treatment in combination with chemotherapy has proven very effective (Burstein, 2005). In contrast, panitumumab and cetuximab, drugs targeting the EGFR receptor, were discovered to be of low efficacy in patients with KRAS mutations (Amado *et al.*, 2008; Cunningham *et al.*, 2004; Van Cutsem *et al.*, 2007). The marker for poor response was found after retrospective re-analysis of clinical trial data. Several years later, the FDA has issued the recommendation for genetic testing prior to onset of therapy with panitumumab or cetuximab (Hughes, 2009).

Identifying biomarkers earlier, possibly before entering clinical phase trials, could speed up and potentiate cohort-specific treatment and expand the clinical application of stratified medicine (Hughes, 2009; Trusheim *et al.*, 2007).

1.3 Gene Therapy

Gene therapy, sometimes specified as somatic gene therapy, is a promising therapeutic approach for a wide range of diseases. Originally devised to treat hereditary single gene disorders, the underlying idea was to insert genes into patient's cells, to replace a mutant allele with a functional version.

In recent years, there have been a multitude of encouraging pre-clinical and early-phase clinical trials. Although many clinical trial phase II results have not met expectations, several examples demonstrating major therapeutic benefits have emerged.

Several gene therapy trials have been completed that involved children suffering from severe combined immunodeficiency-X1 disease (SCID-X1). The disease is caused by a mutant Interleukin-2 receptor gamma chain (IL2RG) gene, blocking differentiation of T and Natural Killer cells (Noguchi *et al.*, 1993). Therapy is generally based on *ex vivo* transduction of lymphocytes with a retrovirus-derived vector containing the correct version of the mutant gene, before cells were introduced into patients. Major therapeutic success was seen in two clinical trials involving very similar retroviral vectors (Cavazzana-Calvo *et al.*, 2000; Gaspar *et al.*, 2004; Hacein-Bey-Abina *et al.*, 2003b). However, 4 out of 9 patients in one, and 1 out of 10 patients in the other trial later developed clonal T cell acute lymphoblastic leukaemia (T-ALL) (Hacein-Bey-Abina *et al.*, 2003a; Howe *et al.*, 2008). Close investigation confirmed anti-sense vector integration upstream of the proto-oncogene LIM domain only 2 (LMO2), resulting in its over-expression. However, leukaemia may have been caused by the combination of insertional mutagenesis and other genetic abnormalities unrelated to vector integration (Howe *et al.*, 2008).

A second example of successful gene therapy is the treatment of two children with the fatal neurodegenerative disease X-linked adrenoleukodystrophy (ALD). In these patients, mutation of ATP-binding cassette, subfamily D, member 1 (ABCD1) gene results in the loss of function of a transporter protein responsible for the transfer of fatty acids into peroxisomes (Mosser *et al.*, 1993). Instead, saturated very long-chain fatty acids (VLCFA) accumulate in the tissues, leading to progressive neuronal demyelination and adrenal failure (Moser HW *et al.*, 1989; Moser *et al.*, 1981).
Ex vivo transduction of haematopoietic cells by a lentiviral vector containing the wild type ABCD1 gene and re-introduction into the patient successfully halted progression of the disease (Cartier *et al.*, 2009).

Similarly, in patients with progressive vision-deteriorating type 2 Leber congenital amaurosis, treatment with an adeno-associated virus-based vector containing wild type RPE65 cDNA led to sustained improvement in vision in all patients with nearly complete restoration of vision in one 8-year old patient (Maguire *et al.*, 2009). The disease is caused by lack of functional RPE65, a protein required for isomerohydrolase activity of retinal pigment epithelium (den Hollander *et al.*, 2008). The vector was injected directly into the sub-retinal region of the eye (Maguire *et al.*, 2009).

1.3.1 Oncolytic gene therapy

The work presented here lies in the field of gene therapy for cancer. A slight adaptation of the gene replacement strategy discussed above, this type of gene therapy is aimed at inducing cell death and encompasses several potential strategies: molecular chemotherapy, replacement of tumour suppressor genes, immunotherapy, inhibition of oncogenes and selectively replicating viruses (Raki *et al.*, 2006). In the latter case, the oncolytic virus can be equipped with a suicide gene, resulting in increased cell death after exposure to and metabolism of a prodrug (Tang *et al.*, 2007).

The use of viruses as oncolytic agents has intrigued scientists for over a century. Exactly one hundred years ago, at the International Cancer Congress in Paris, the Italian clinician De Pace reported the regression of a large cervical carcinoma after the patient had been –accidently- infected with rabies (Sinkovics *et al.*, 2008). Several clinical trials with oncolytic viruses were conducted in the mid-20th century: In 1952, 34 patients with advanced neoplastic disease were treated with Egypt 101 virus (Southam *et al.*, 1952). In 1956, 30 cervical carcinoma patients received injections of adenovirus adenoidal pharyngeal-conjuctival virus (APC) (Georgiades *et al.*, 1959); and in 1974, 90 patients with various terminal cancers were intentionally infected with mumps virus (Asada, 1974). Many other viruses were screened for efficacy. There was some response to these treatments, such as localised necrosis, tumour regression and even sporadic cases of complete remission. Yet, success after intentional viral infection was too rare and unpredictable to outweigh the often severe side effects (Kelly *et al.*, 2007). Thus, viruses could not fulfil the promise of reliable, safe anti-cancer drugs. Lack of knowledge of virus molecular structures and mechanisms made it impossible to develop this therapeutic approach further (McCormick, 2001). It wasn't until molecular biology reached the stage of recombinant DNA technology that viruses could be specifically altered and used as a tool for cancer treatment (Kelly *et al.*, 2007).

Today, several viruses are known to possess natural oncolytic functions (Roberts *et al.*, 2006). Nevertheless, a large portion of oncolytic virus research is devoted to the engineering of viral mutants, specifically tailored to mechanisms and features of cancers (Vaha-Koskela *et al.*, 2007). Currently, the most prominent limitation of gene therapy is the need for a non-toxic, yet efficient gene delivery system (Matthews *et al.*, 2009). Furthermore, the route followed in a specific therapeutic approach depends highly on the type of disease targeted. Treatment of inherited monogenic disorders, such as cystic fibrosis or Duchenne muscular dystrophy, may require a different

approach than cardiovascular disease or cancer (Young *et al.*, 2006). However, for the sake of brevity, I will limit my discussion to selectively replicating viruses.

1.3.2 Oncolytic viruses in cancer therapy

Selective replication is achieved by using a virus (a) that is deleted for a gene essential for replication in normal cells but expendable in cancer cells, or (b) whose replication depends on pathways naturally overactive in human cancers. Such a virus, after infection, will replicate selectively in a cancer cell and kill it. Ideally, the viral progeny are released and infect neighbouring cells, resulting in self-perpetuating anti-tumour effects. This may be accompanied by viral induction of cytokine and tumour antigen release, leading to activation of a host immune response against the tumour (Biederer *et al.*, 2002).

Several examples exist of viruses that are naturally oncolytic, amongst them measles virus, picornavirus, Adeno-associated virus 2, Newcastle disease virus and reovirus. Three examples, Adeno-associated virus 2, Newcastle disease virus and reovirus are described in more detail below.

1.3.2.1 Adeno-associated virus 2

Adeno-associated virus 2 (AAV2) is a small, non-pathogenic single-stranded DNA (ssDNA) virus, which does not illicit strong immune responses in the host (Coura Rdos *et al.*, 2007). For gene expression the virus is dependent on the machinery of a helper virus, such as adenovirus or Human Papilloma Virus. But even in the absence of such a helper, AAV2 is able to infect cells, where it integrates into host DNA at a specific site on 19q and remains latent until secondary infection with a helper virus (Jurvansuu *et al.*, 2005; Kotin *et al.*, 1992; Kotin *et al.*, 1990). The potential of AAV2 for cancer therapy is based on its inherent ability to disrupt cell cycle progression of the infected cell and subsequently, in p53-deficient cells, to mediate cell death. This intrinsic oncolytic character is particularly favourable, as more than fifty percent of tumours lack p53-function (Bullock *et al.*, 2001). In high grade serous ovarian carcinomas, the incidence of p53 mutations is as high as 97% (Ahmed *et al.*, 2010). The mechanism of action is based on AAV2 inducing host DNA damage response, which arrests the cell in G2, the cell cycle phase following DNA replication. Cells deficient in p53 fail to sustain the arrested state and undergo prolonged, incomplete mitosis, resulting in over-duplication of centromeres and finally succumb to mitotic catastrophe. Promising for therapeutic application was the observation that pre-treating cells with UV increased ability of AAV2 to induce G2 arrest (Jurvansuu *et al.*, 2005). A multitude of phase I and

II clinical trials have been performed with AAV2-based vectors to treat various indications. Generally, however, the purpose was gene replacement in chronic disease (Carter, 2005; Kaplitt *et al.*, 2007). To my knowledge, there is no clinical trials with AAV vectors for cancer therapy completed or recruiting.

1.3.2.2 Newcastle disease virus

Newcastle disease virus (NDV) is a ssRNA avian virus, potentially harmful to birds, but causing mild influenza-like symptoms and conjunctivitis in humans, at most. Its negative-sense single stranded RNA genome comprises 6 genes and additional products are synthesised after RNA splicing. NDV has been shown successfully to infect most human cells (Krishnamurthy *et al.*, 2006; Sinkovics *et al.*, 2000). Similar to AAV2, NDV possesses intrinsic oncolytic and immunostimmulatory attributes. These properties are based on three fundamental mechanisms:

(1) oncolytic activity
(2) supply of danger signals, such as viral mRNA, to trigger innate immune responses via Toll-like receptor activation
(3) further activation of innate and acquired immune response, namely of natural killer (NK) cells, monocytes, macrophages and T-cells through induction of cytokine release (Janke *et al.*, 2007).

NDV strains can be classified as lytic or non-lytic. Whilst both types are able to kill cancer cells, lytic strains replicate faster and induce cell death more efficiently. Following replication, they lyse the plasma membrane of the infected cell without induction of apoptotic pathways (Polos *et al.*, 1981). In contrast, non-lytic NDV is thought to kill its host cell by inducing apoptosis. Both lytic and non-lytic NDV have been used to generate anti-cancer vaccines and as oncolytic agents (Schirrmacher, 1999). The first application of wild type NDV to treat cancer dates back to 1965, when a cervical carcinoma patient received intra-tumoural injections of live virus (Cassel *et al.*, 1965). However, in the following decades only anecdotal responses emerged (Kelly *et al.*, 2007). More recently, studies with recombinant viruses have been performed. In a trial involving live attenuated MTH-68/H, promising effects were reported. Four patients with advanced high grade glioblastoma were treated with MTH-68/H. In all patients, the treatment highly improved quality of life and extended survival rates of 5-9 years were achieved (Csatary *et al.*, 2004). Similarly, a Phase I clinical trial of PV701 in patients with advanced solid tumours indicated some response after intra-venous injections. Resulting progression-free survival varied between 4-31 months (Pecora *et al.*, 2002). In 11 glioblastoma patients, variant NDV-HUJ showed some effect, with transient complete remission in one patient. However, all patients eventually developed

progression (Freeman *et al.*, 2006). Interestingly, investigating molecular mechanisms of NDV-HUJ *in vitro* revealed that the virus is able to overcome apoptotic resistance of Livin-over-expressing advanced melanoma primary cultures. Livin is a member of the inhibitor of apoptosis (IAP) family. Livin-negative melanoma primary cultures were resistant to NDV-HUJ (Lazar *et al.*). A recombinant form of NDV, encoding granulocyte/macrophage colony-stimulating factor (GM-CSF), produced some evidence of clinical activity. Tumour cells infected with such modified NDV were superior in their ability to induce anti-neoplastic by-stander effects *in vivo*, as compared to wild type NDV. This was accompanied by significantly augmented levels of IFN-γ and initiation of immunological response cascades (Janke *et al.*, 2007).

1.3.2.3 Reovirus

Reovirus is a double-stranded RNA virus, hence it replicates exclusively in the cytoplasm. So far, reoviral infection in humans has not been linked to disease (Pandha *et al.*, 2009). Replication is believed to be restricted to cells with Ras signalling pathway deregulations (Strong *et al.*, 1998). This abnormality is common in human cancers (Bos, 1989). Completed and ongoing clinical trials so far demonstrated safety of reovirus-based cancer treatment (Comins *et al.*, 2008). Its therapeutic efficacy, possibly in combination with chemotherapy, remains to be proven. *In vitro* and *in vivo* studies indicated synergistic effects of reovirus and several different chemotherapeutic agents (Pandha *et al.*, 2009). Phase I clinical trials of reovirus/chemotherapy combinations are ongoing (Comins *et al.*, 2008).

1.3.3 Deletion mutants for cancer therapy

Early viral constructs specifically engineered for cancer gene therapy were based on replication-deficient viruses, used to deliver tumour suppressor genes or pro-drug activating genes or to induce anti-tumoural immune responses (Gahery-Segard *et al.*, 1997; Raki *et al.*, 2006; Tang *et al.*, 2007).

Preclinical data on replacement of mutant p53 by the wild-type protein, for example, were encouraging. Constructs of non-replicating adenoviruses encoding human wild-type p53 under CMV-promoter control achieved expression of the tumour suppressor and anti-proliferative effects in ovarian cancer cell lines (Mujoo *et al.*, 1996; Santoso *et al.*, 1995). Equally, *in vivo* experiments in xenograft mouse models showed promising results after intra-tumoural and intraperitoneal injections of virus (Mujoo *et al.*, 1996). A phase I clinical trial was conducted with a replication-deficient adenovirus containing human p53 cDNA in patients with ovarian cancer. The virus was delivered via an

intraperitoneal catheter. No severe toxicity was observed and one patient showed stable disease (Wolf *et al.*, 2004). The phase I/II clinical trial of another p53-encoding non-replicating adenovirus, both alone and in combination with platinum-based chemotherapy, showed promising results. In fallopian tube, peritoneal or ovarian cancer patients with mutant p53-expressing tumours, dose limiting toxicity was not reached. The virus was generally well-tolerated, but significant responses were only seen in combination with chemotherapy (Buller *et al.*, 2002a). Long-term follow-up showed that median survival in patients treated with the adenoviral vector was better than in patients treated with chemotherapy, alone (Buller *et al.*, 2002b). However, the randomised phase III trial of Ad-p53 given in conjunction with platinum-based chemotherapy following initial debulking surgery was abandoned early, due to increased toxicity in the Ad-p53 arm and has never been formally reported (Zeimet *et al.*, 2003). Other such therapeutic vectors revealed a loss of effect relatively soon after transfection, due to host immune reactions, which made re-administration of the same virus impossible (Blau *et al.*, 1995; Jooss *et al.*, 1998).

A more potent class of gene-attenuated virus was engineered based on replication-competent herpes virus and adenovirus. These oncolytic viruses are designed to replicate selectively in cancerous cells by specifically deleting a gene required for replication in normal, but not in cancer cells (Bischoff *et al.*, 1996; Fueyo *et al.*, 2000; Kirn, 2000; Kucharczuk *et al.*, 1997). The unique advantage of this treatment is its self-perpetuating nature: the virus multiplies in a cancer cell before lysing it and spreading to adjacent cancer cells (Heise *et al.*, 2000). Deletion mutants engineered for cancer treatment are derived from different virus families.

1.3.3.1 Herpes simplex virus

G207 is a multi-mutant based on Herpes simplex virus (HSV) with deleted $_{gamma1}$34.5 (ICP34.5) genes and inactivated ribonucleotide reductase ICP6. In wild type HSV, ICP34.5 is a major virulence gene; its deletion significantly reduces neurovirulence (Campbell *et al.*, 2007). Tumour selectivity of G207 is also based on the lack ICP6 activity, which in normal cells leads to virus replication inability. The ICP6 gene encodes the large subunit of HSV ribonucleotide reductase, a key enzyme for synthesis of viral DNA in non-dividing cells. In many dividing cells, however, this particular enzymatic activity has been found dispensable (Goldstein *et al.*, 1988). Cancer cells, for example, can complement this mutation (Fukuhara *et al.*, 2005). Preclinical studies conducted with viral mutant G207 were very successful and phase I clinical trials brought satisfactory results (Markert *et al.*, 2009; Markert *et al.*, 2000) .

Yet, its therapeutic efficiency was not ideal: in a mouse model G207 was less potent than its wild type parental virus and another HSV derivative (Markert *et al.*, 2000).

Another promising phase I clinical trial was conducted to assess safety and biological activity of a GM-CSF-encoding HSV, OncoVEX$^{GM\text{-}CSF}$. Expression of GM-CSF has been shown to induce differentiation and proliferation of myeloid progenitors, rendering them more susceptible to therapy and also enhances activity of dendritic cells (Bennett *et al.*, 2001; Welte *et al.*, 2006). In addition to the ICP34.5 deletion of G207, viral protein US11 is expressed early, rather than late during infection, further augmenting replication advantage and tumour selectivity (Mulvey *et al.*, 1999). Finally, OncoVEX$^{GM\text{-}CSF}$ is derived from a clinical isolate rather than an established strain and carries an ICP47 deletion. The viral protein normally prevents antigen presentation on the infected cell. Its absence is thought to increase anti-tumoural immune response (Mohr *et al.*, 1996). Thirty patients with malignant melanoma, breast, head and neck or gastrointestinal cancer received single- or multidose intra-tumoural injections into cutaneous or subcutaneous lesions. In general, the virus was well-tolerated, with main side effects being fever and skin rashes. Local inflammatory response was dose limiting. GM-CSF expression, viral replication and HSV antigen-induced tumour necrosis were detected. Overall, OncoVEX$^{GM\text{-}CSF}$ had some anti-tumoural effect. Whilst almost all injected tumours developed some inflammatory response, tumour necrosis or apoptosis occurred in 14 patients. Three patients showed disease stabilisation and 6 patients had tumour regression of both injected and non-injected sites. Inflammation of injected and non-injected lesions was observed in a further 4 patients (Hu *et al.*, 2006).

1.3.3.2 Vaccinia virus

Almost ten years ago, preliminary clinical trials were conducted with a Vaccinia virus mutant, JX-594. Replication selectivity of JX-594 is based on activation of epidermal growth factor receptor (EGFR)-Ras pathway in cancer cells. In addition, the virus encodes granulocyte-macrophage colony-stimulating factor (GM-CSF). Expression of the transgene induces vascular shut-down in tumours and elicits anti-tumoural immune responses (Park *et al.*, 2008). In cutaneous melanoma patients, JX-594 produced local responses after intra-tumoural injection (Mastrangelo *et al.*, 1999). More recently, another phase I clinical trial with the same virus was conducted in patients with primary or metastatic liver cancer. Side effects induced by intra-tumoural injection of the virus were flu-like symptoms, thrombocytopenia and dose-limiting hyperbilirubinaemia. Overall, safety of JX-594 was acceptable. Anti-tumoural effects were observed in both injected and non-injected tumours. In most patients either partial response or stable disease were observed (Park *et al.*, 2008).

1.4 Adenoviridae

A multitude of cancer gene therapy approaches rely on recombinant adenoviral vectors. Their ability to infect dividing and non-dividing cells and exceptional efficiency *in vivo* gene transfer make them more suitable than many other oncolytic viruses (Tsutsumi *et al.*, 1999). Also, they are easily manipulated and possess high cloning capacity amongst viral vectors (Kozarsky *et al.*, 1993). Also, they can be produced in high titres (Rein *et al.*, 2006). In order to provide understanding of how this virus works and why it may be a suitable base for development of oncolytic vectors, the following section describes basic adenovirus biology, as well as adenoviral structure, genome organisation and life cycle.

Adenoviridae are a family of infectious agents typically causing mild infections of the upper respiratory tract (Tsutsumi *et al.*, 1999). They have been in the focus of medical and microbiological research for many decades, making them one of the best-studied families of viruses (Greber *et al.*, 2007). In fact, it was in adenovirus that alternative RNA splicing and introns were first discovered (Berget *et al.*, 1977). The family of adenoviridae is subdivided into 54 serotypes and 7 groups (A-G), based on serological cross-reactions in haemagglutination assays (see Table 1.2) (Ebner *et al.*, 2005; Martin *et al.*, 2007).

Groups	Serotype
A	12, 18, 31
B	3, 7, 11, 14, 16, 21, 34, 35, 50
C	1, 2, 5, 6
D	8, 9, 10, 13, 15, 17, 19, 20, 22-30, 32, 33, 36-39, 42-49, 51, 53, 54
E	4
F	40, 41
G	52

Table 1.2 Classification of human adenoviridae. *To date, the adenovirus family comprises 7 groups and 54 serotypes. Adapted from (Martin et al., 2007).*

Currently, most gene therapy vectors derived from adenovirus are based on serotypes 2 and 5 (Braithwaite *et al.*, 2001; Lopez-Campos *et al.*, 2007). Correspondingly, the characterisation below is based on these two serotypes.

1.4.1 Structure and Genome

Adenovirus consists of a non-enveloped icosahedral capsid formed by hexon and penton proteins, to which fibre proteins are attached (see Figure (Fig.) 1.1). The capsid encloses a double stranded linear DNA genome, 36 kilobases (kb) in length with inverted terminal repeats (ITRs) and a terminal protein at each 5' end and surrounded by core proteins (Braithwaite *et al.*, 2001; Robinson *et al.*, 1979). The ITRs form hairpins which act as primers for a primase-independent synthesis of the second DNA strand (Bohenzky *et al.*, 1988). This phenomenon, called self-priming, facilitates efficient amplification of the viral genome (Davison *et al.*, 2003). Although viral replication and transcription of viral genes are highly dependent on the host transcription machinery, expression is orchestrated by viral proteins and processes (Miller *et al.*, 2007). A wild type adenovirus 5 genome map is shown in Fig. 1.2.

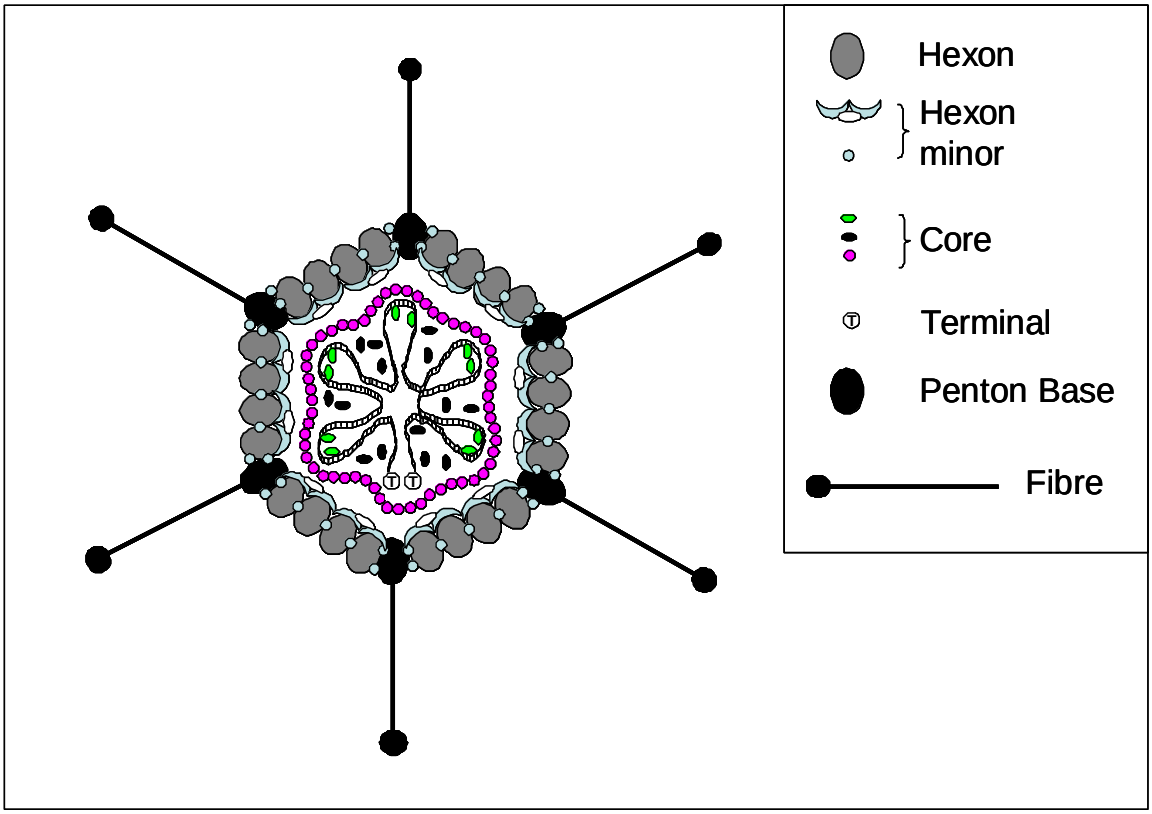


Figure 1.1 Adenovirus particle structure. *The non-enveloped icosahedral capsid consists of hexon and penton proteins. Fibres mediate adhesion to cellular surface receptors. Terminal proteins are associated with 5' ends of the linear DNA. Core proteins are associated with and form a layer around the DNA . Adapted from (Glasgow et al., 2006).*

The eight transcription units on the adenoviral genome are transcribed by cellular RNA polymerase II. Gene transcription of the virus follows a strictly regulated temporal pattern, allowing distinction of early, intermediate and late genes (Braithwaite *et al.*, 2001). Alternative RNA splicing leads to synthesis of multiple transcripts from each transcription unit and, consequently, of multiple protein products (Muhlemann *et al.*, 2000). Early genes regulate expression of viral genes and replication. Intermediate and late genes encode structural components of the virus particle (Braithwaite *et al.*, 2001).

1.4.2 Early genes

Expression of the early genes on the adenoviral genome is critical for its propagation (Miller *et al.*, 2007).

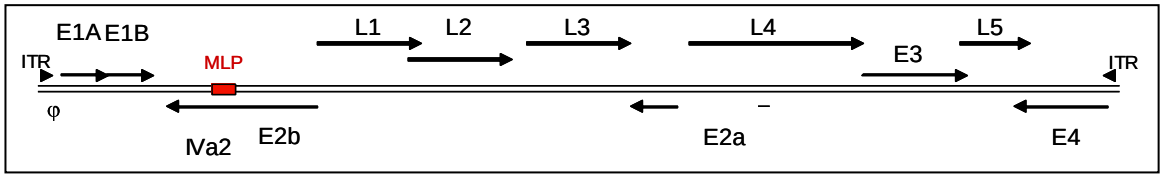


Figure 1.2 Map of wild type adenovirus 5 genome. *Early genes are marked as E1-E4; late, structural genes are marked as IVa2 and L1-L5. ITR=Inverted terminal repeats; MLP= major late promoter;* φ *= packaging signal.*

Early region 1A:

Once the virus has successfully infected its target cell, E1A is the first gene to be transcribed (Fields *et al.*, 1996). Transcription is activated by the binding of a cellular nuclear factor EF-1A and transcription factor E2F to multiple binding sites within and upstream of the E1A enhancer region (Bruder *et al.*, 1991; Bruder *et al.*, 1989). In addition, E1A proteins autoregulate their own transcription (Hearing *et al.*, 1985).

From the primary E1A transcript, five mRNAs products are produced by alternative splicing (Perricaudet *et al.*, 1979; Stephens *et al.*, 1987; Ulfendahl *et al.*, 1987). Major E1A proteins are 243R and 289R, translated from two mRNA transcripts of 12S and 13S, respectively. Both transcripts are derived from two exons, with the second exon being shared by both (Felsani *et al.*, 2006). Within the first 139 amino acids (aa) of most E1A proteins lie two highly conserved regions, CR1 (aa 44-72) and CR2 (aa 115-137) (Avvakumov *et al.*, 2004). By interacting with numerous host cell factors, E1A proteins exert a plethora of effects, ranging from induction of quiescent cells to enter and progress through the cell cycle, inhibition of differentiation, immortalisation and, when paired with another oncogene, transformation (Dyson *et al.*, 1992; Mymryk, 1998;

Peeper *et al.*, 1993; Shenk *et al.*, 1991). But E1A has also been described as anti-oncogenic, inhibiting tumourigenesis, metastasis and inducing apoptosis (Chinnadurai, 1992; Liu *et al.*, 1996; Mymryk, 1996). The interaction crucial for the work discussed here is that between E1A and pRb, a cell cycle regulator discussed in more detail, below . Binding and inactivation of pRb proteins by E1A is based on the two highly conserved regions (Felsani *et al.*, 2006).

Formation of E1A-pRb complexes, as found in co-immunoprecipitation experiments (Whyte *et al.*, 1988), involves a so-called B-domain, or B-box, of the pRb pocket region. E1A binds the B-domain through a specific LxCxE motif in its CR2 region (aa 122-126) (Felsani *et al.*, 2006). In fact, all proteins known to target the pRb B-box, such as Cyclin Ds, HDAC-1 or BRG-1, contain the LxCxE motif (Morris *et al.*, 2001; Singh *et al.*, 2005). By displacing regulatory cellular proteins bound to the B-domain, such as E2F family members, E1A alters the function of the pocket protein (Felsani *et al.*, 2006).

However, E1A also binds to pRb via its CR1 region, albeit with a 10-fold lower affinity than CR2 (Dyson *et al.*, 1992). CR1 binding of pRb occurs within the same pocket region and with a comparable affinity as E2F-binding (Fattaey *et al.*, 1993). Based on these observations, a two-step model was developed for E1A-provoked disruption of E2F-pRb complexes:

1. Based on the high affinity of CR2, E1A associates with E2F-pRb to form a ternary complex. As a consequence, concentration of CR1 around the pRb-E2F interaction site is elevated.
2. CR1 competes directly with E2F for binding pRb, resulting in dissociation of E2F from the complex (Felsani *et al.*, 2006).

As the cumulative affinity of CR1 and CR2 is higher than that of E2F, it tips the balance towards dissociation of E2F from pRb (Fattaey *et al.*, 1993; Ikeda *et al.*, 1993). Therefore, E1A-binding of hypo-phosphorylated pRb liberates E2F, permitting E2F-initiated transcription and subsequent passage into S phase (Ghosh *et al.*, 2003).

E1A function regulates a broad range of processes. It induces transcription of down-stream viral genes, thus promoting progression of the viral replication cycle (Wong *et al.*, 1994). Furthermore, it counteracts cellular senescence caused by stress and telomere shortening (Ben-Porath *et al.*, 2005; Ben-Porath *et al.*, 2004). Its regulatory role is extended further, as it may also orchestrate pRb protein activity indirectly by altering various cellular mediators: E1A binds p300 and CBP, multifunctional proteins

known to interact with numerous transcription factors (Arany *et al.*, 1995). Furthermore, p300/CBP exert histone acetyltransferase (HAT) activity (Iyer *et al.*, 2004). E1A stimulates acetylation of pRb, by recruiting the latter to p300 (Chan *et al.*, 2001). Also, by altering p300/CBP activity, E1A influences expression and function of other pocket proteins (Frisch *et al.*, 2002; Magenta *et al.*, 2003; Martelli *et al.*, 1994). Also, E1A-binding of p300/CBP interferes with TGF-β transcription and DNA-damage-induced transcription of p21 (Datto *et al.*, 1997; Steegenga *et al.*, 1996). Additionally, E1A has been described to interact directly with and inactivate p21 and p27, abrogating inhibition of Cyclin E-cdk2 complexes, leading to pRb phosphorylation (Felsani *et al.*, 2006; Mal *et al.*, 2000; Mal *et al.*, 1996). On the other hand, 13S E1A, but not 12S E1A is able to induce p21 expression in cancer cells (Najafi *et al.*, 2003).

Early region 1B:

Transcripts of E1B are also subject to alternative splicing, giving rise to important regulators of induced cell death. One splice variant is E1B-19K, a homologue of Bcl-2. It is capable of inhibiting premature cell death induced by E1A or p53 by inhibiting pro-apoptotic Bax protein (Cuconati *et al.*, 2002; Farrow *et al.*, 1995; Rao *et al.*, 1997). E1B-55K, another E1B protein, functions as an inactivator of the p53 pathway (Yew *et al.*, 1992). It is able to bind p53 via a transcription-repressing domain. Owing to this potent domain, E1B-55K silences expression of p53-regulated genes without displacing the transcription factor from its DNA binding site (Yew *et al.*, 1994). As a result, growth arrest and apoptosis of the infected host cell are prevented. Furthermore, E1B-55K binds viral E4orf6 protein and cellular proteins to form an E3 ubiquitin ligase complex, which induces proteasomal degradation of p53 (Harada *et al.*, 2002; Querido *et al.*, 1997; Querido *et al.*, 2001b). E1B-55K is also responsible for shutting off of host protein synthesis (Babiss *et al.*, 1984). In addition E1B-55K/E4orf6 complexes hinder DNA damage response by degrading cellular DNA repair proteins (Carson *et al.*, 2003). Finally, mRNA transport and late protein synthesis during infection depend on E1B-55K/E4orf6 (Dobner *et al.*, 2001; Flint *et al.*, 2003).

Early region 4:

E4 is situated at the right end of the adenoviral genome. Unlike the previously described early genes, it is transcribed leftwards. It contains several open reading frames (ORFs) which, in combination with alternative splicing, lead to synthesis of a multitude of proteins (Virtanen *et al.*, 1984). E4 proteins regulate a wide range of processes:

ORF 4 product: regulates phosphorylation states of various transcription factors, by mediating protein phosphatase 2A activity (Muller *et al.*, 1992)

ORF 6/7 product: associates with E2F and drives accumulation of E2F mRNA in the cell.

ORF 6 product: binds p53 and inhibits transcription activation function of the tumour suppressor (Dobner *et al.*, 1996; Querido *et al.*, 1997).

ORF 3 product: responsible for retargeting of cellular factors into viral replication domains within the nucleus (Doucas *et al.*, 1996).

Knowledge of E4orf1 and E4orf2 protein functions remains limited.

ORF 1 product: interacts with a group of scaffolding proteins involved in assembly of cell signalling complexes (Tauber *et al.*, 2001). It has been described to signal via the phosphatidylinositol 3'-kinase (PI3K) pathway to activate serine-threonine kinase Akt, mammalian target of rapamycin (mTOR) and S6 ribosomal protein kinase (p70 S6K), which regulate protein synthesis and cell survival (Frese *et al.*, 2003). Interestingly, deletion of E4orf1 enhanced oncolytic effects of E1B55K-deleted ONYX-015, suggesting that presence of E4orf1 limits therapeutic efficacy of oncolytic virus with such a deletion (Thomas *et al.*, 2009). This includes H101, which is being used for cancer therapy in China (Garber, 2006; Yu *et al.*, 2007).

ORF 2 product: has never been observed in complex with any cellular protein. It appears to be a soluble cytoplasmic component in infected cells (Dix *et al.*, 1995). As deletion has no visible effect it may exert late lytic functions (Tauber *et al.*, 2001).

Early region 3:

E3 region encodes for approximately nine overlapping mRNAs produced from pre-mRNA by alternative splicing (Bhat *et al.*, 1985; Craig *et al.*, 1977). The E3 region constitutes 10% of the entire viral genome, suggesting an important role of its protein products. Surprisingly, deletion of the gene does not abort viral replication (Toth *et al.*, 2003). However, mice infected with an E3-deleted adenoviral mutant mounted a significantly increased inflammatory response. This indicates a role for E3 in modulating the host immunological response (Braithwaite *et al.*, 2001; Ginsberg *et al.*, 1989). E3gp-19K protein is known to reduce expression of class I major histocompatibility complex (MHC I) antigens on the surface of infected cells (Toth *et al.*, 2005). Two other important E3 proteins, E3-10.4K (also known as RIDα) and E3-14.5K (RIDβ) proteins, have anti-apoptotic function. They modulate expression of Fas, a member of the tumour necrosis factor (TNF) receptor family, and down-regulate epidermal growth factor receptor (EGF-R) expression (Shisler *et al.*, 1997; Toth *et al.*,

2002). Similarly, the 6.7K protein is involved in down-regulation of two death receptors for tumour necrosis factor-related apoptosis inducing ligand (TRAIL). As a consequence, infected cells are less susceptible to TRAIL-mediated apoptosis (Benedict *et al.*, 2001). Furthermore, E3-14.7K protein has been shown to inhibit ligand-mediated internalisation of TNF-receptor 1 (TNFR1). This prevents formation of the death-inducing signalling complex (DISC), a crucial step in the TNF-induced pro-apoptotic cascade (Schneider-Brachert *et al.*, 2006). Another product of the E3 region is 11.6K, the adenovirus death protein (E3/ADP), which may be required for efficient killing of infected cells. During early infection stages, only small amounts of E3/ADP are synthesised. In contrast, large amounts are produced at late stages of infection. E3/ADP is thought to mediate release of viral progeny (Tollefson *et al.*, 1996). Interestingly, however, absence of E3-11.6K in the adenoviral genome appears not to alter mode or extent of cell death in cancer cells (Baird *et al.*, 2008).

Early region 2:

Protein products of the E2 gene control viral DNA synthesis (Braithwaite *et al.*, 2001). Once their concentration reaches a critical threshold, synthesis of viral DNA commences. This marks progression into the late stage of infection and results in activation of the late transcriptional program (Miller *et al.*, 2007).

Late proteins are encoded by the major late transcription unit (MLTU) regions L1 to L5. Their expression from the major late promoter (MLP), followed by differential splicing and polyadenylation, produces structural proteins (Akusjarvi, 2008). Despite its name, however, the MLP is active early after infection and some proteins from MLTU regions are already expressed concomitantly with early region proteins, as are intermediate gene products IVa2 and IX, responsible for enhancing MLTU expression during the transition from early to late phases. During early post infection phases, levels of late proteins are low. Although understanding of the role of late proteins throughout the viral life cycle is far from complete, they have been linked to regulation of the host cell splicing machinery, orchestration of structural and non-structural late and intermediate gene expression (Akusjarvi, 2008; Morris *et al.*, 2009). Highest levels of MLTU expression are reached during late stages of infection, allowing production of the full array of structural proteins (Morris *et al.*, 2009).

1.4.3 Life cycle of Adenovirus

Adenovirus gains entry into its target cell by interacting with specific receptors on the cell surface. For all groups of adenovirus other than group B, the knob of the

adenovirus fibre protein first binds the Coxsackievirus and adenovirus receptor (CAR). Recent evidence suggests that CAR is dispensable for adenovirus infection (Nicklin *et al.*, 2005; Waddington *et al.*, 2007). However, internalisation requires a second interaction of the adenovirus penton base with integrins, such as $\alpha_v\beta_3$ or $\alpha_v\beta_5$, on the cell surface (Hidaka *et al.*, 1999). Bound adenovirus is then quickly endocytosed into a clathrin-coated vesicle. Subsequently, the virus particle can escape into the cytosol, where it uses microtubules and microtubule-dependent motors for transport to the nuclear membrane. After docking at nuclear pore complexes, it enters the host nucleus (Martin-Fernandez *et al.*, 2002).

According to Greber, *et al*., successful infection is dependent on an orchestrated disassembly programme of the viral protein capsid, which takes place during endocytosis. It is believed that the first disassembly step, shedding of fibre proteins, occurs immediately after cell adsorption (Greber *et al.*, 1993). This event is probably promoted by penton base - $\alpha_v\beta_3/\beta_5$ receptor contact. This particular interaction has also been shown to regulate adenovirus-mediated cell signalling, virus endocytosis and endosomal transport (Greber, 2002; Rauma *et al.*, 1999). The second disassembly step is penton base dissociation, which occurs simultaneously with escape of the virus from the endocytotic vesicle (Blumenthal *et al.*, 1986). Once in the cytosol, interaction between microtubule motor cytoplasmic dynein and hexon facilitates translocation of the partially disassembled adenovirus to the nuclear pore complex, along microtubules (Bremner *et al.*, 2009). In the meantime, protein IX, another structural protein that adheres hexon proteins, is removed (Martin-Fernandez *et al.*, 2002). When the adenoviral particle reaches the nucleus and binds to the nuclear pore complex, the final step of disassembly occurs: loss of the hexon component. The viral genome is released and it is now small enough to enter the host nucleus, where it can initiate viral replication (Greber *et al.*, 1997; Martin-Fernandez *et al.*, 2002).

Once adenovirus has manipulated the host cell to produce vast amounts of viral DNA and viral protein, its propagation requires correct assembly of the new infectious particles. Exactly how packaging of adenoviral particles is controlled is yet unclear. Ostapchuk and Hearing proposed the following model: viral assembly begins with the formation of a procapsid from structural and scaffolding proteins, competent for packaging of viral DNA. They hypothesize that recognition of this preliminary capsid structure by viral genome is mediated by binding of proteins to a packaging domain on adenovirus DNA. Ensuing internalisation of the genome through a so-called “portal” in

the procapsid is driven by an ATP-dependent motor. Following encapsidation, the portal is sealed and final maturation of the viral particle occurs (Ostapchuk *et al.*, 2005). An illustration of the proposed model can be seen below, in Fig. 1.3.

Another enigma in the viral life cycle is the manner of cytolysis and subsequent release of adenoviral progeny. Until recently, the predominant opinion has been that adenovirus-induced cell death resembles classical apoptosis (Hall *et al.*, 1998). This presumption was based on findings of E1A inducing apoptosis in many cell systems and of E4orf4-prompted caspase-dependent cell death (Rao *et al.*, 1992; Robert *et al.*, 2002a). However, more recent studies have aimed to monitor the entirety of interactions and factors involved in virus-induced cell lysis, rather than assessing function of single genes (Baird *et al.*, 2008). This approach bore surprising results: El Hassan *et al*. described the character of virus-induced cell death as “necrosis-like programmed cell death” (Abou El Hassan *et al.*, 2004). In another study, autophagy also appeared to play a crucial role (Ito *et al.*, 2006).

A study conducted by our group found replicating adenoviruses not to cause cell death via classical apoptosis. Pure necrosis or autophagy were equally excluded as cytopathic pathways. Although there was some indication of autophagy induction, this appeared to be a survival mechanism of the cell. Morphology did show certain signs for apoptosis, but mitochondria –mediators of classical apoptosis and some forms of necrosis – did not contribute to cell death. The conclusion drawn from our group is that replicating adenoviruses kill host cells by a virus-regulated non-classical cell death. Elucidation of the exact lytic pathway requires further investigation (Baird *et al.*, 2008).
Release of viral progeny has been shown to be enabled by break down of the intermediate filament network. Cellular filaments, particularly cytokeratins, are proteolysed by adenoviral proteinase L3. This destruction appears to be promoted by viral shutoff of host cell translation. Indeed, preventing viral inhibition of host translation resulted in a several-hundredfold decrease of viral release (Zhang *et al.*, 1994).

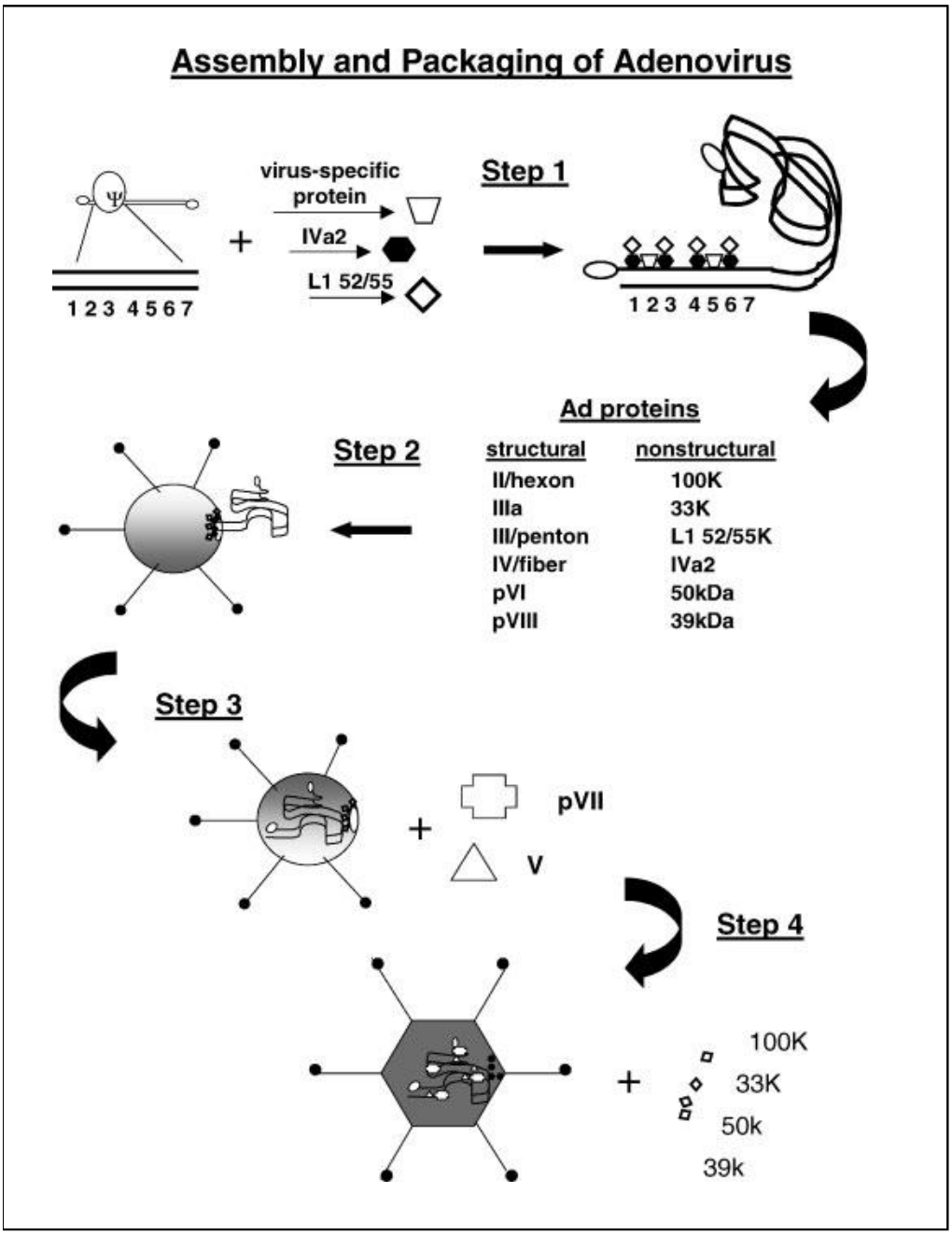


Figure 1.3 Model for packaging/assembly of adenovirus. *Step 1: Formation of a procapsid from structural and scaffolding proteins, competent for packaging of viral DNA. Step 2: Recognition of the procapsid capsid by the viral genome (mediated by binding to an adenovirus DNA packaging domain). Step 3: ATP-driven internalisation of the genome through a "portal" in the procapsid. Step 4: Sealing of the portal and final maturation of the viral particle (Ostapchuk et al., 2005).*

1.5 Oncolytic adenoviruses

Among adenovirus-based anti-tumoural agents, *dl*1520, also called ONYX-015, was the first to be submitted to clinical trials (Kirn, 2001). Similar to previous gene therapy strategies, the targeted mutation in cancer cells was thought to be p53 (Zeimet *et al.*, 2003). But, rather than restoring p53 wild type activity, as attempted earlier, a new strategy exploited the lack of functional p53 (Heise *et al.*, 1997). As described earlier, adenoviral E1B-55kD gene encodes a p53-inhibitory protein and is involved in viral mRNA transport and shut-off of host cell protein synthesis (Heise *et al.*, 2000). Lack of region E1B-55kD in the *dl*1520 genome was thought to restrict replication of the virus to cells that lack p53 activity (Kirn, 2001). According to Heise *et al*., epithelial and endothelial cells with normal p53 function showed a high degree of resistance to replication and cytotoxicity (Heise *et al.*, 1997). The subsequent clinical trials, however, brought deflating results. Auspiciously, the virus could infect most types of tumours included in the clinical trial – colorectal, head-and-neck and pancreatic cancer, although not ovarian – irrespective of the route of administration. But although some tumour-selective replication was detected, results were transient at best, such as in head-and-neck cancer (Harada *et al.*, 2002; Heise *et al.*, 2000). In other tumours, no responses were detectable (Mulvihill *et al.*, 2001; Vasey *et al.*, 2002). In general, replication of *dl*1520 in tumours was profoundly attentuated, whilst the virus was able to replicate in p53-positive cells (Kirn, 2001). Although no further clinical trials with *dl*1520 followed, data from these trials were very helpful. Most importantly, toxicity profiles collected from more than 200 patients were very promising and an encouragement to develop more efficiently replicating adenoviral vectors (Kirn, 2001; Lockley *et al.*, 2006). Secondly, observations on host immune responses and side effects are likely to be expandable to other adenoviruses, thus providing valuable background knowledge for virus-based therapy. Another interesting finding of these studies was a synergistic anti-tumour activity of a combination of *dl*1520 in combination with cisplatin or 5-fluorouracil. Some insight to the lack of clinical response to *dl*1520 was gained from subsequent *in vitro* studies. These revealed incomplete correlation between mutant p53 and replication efficiency. Investigating this observation led to two major findings: first, loss of $p14^{arf}$ in tumours can mediate replication of *dl*1520, even in cells with wild type p53 (Ries *et al.*, 2000). In a normal cell, p14 negatively regulates Mdm2, itself an inhibitor of p53. Therefore, a lack of p14 in a cell disrupts the p53 pathway (Fang *et al.*, 2000; Momand *et al.*, 1992). More importantly, it has been shown that the likely true mechanism determining *dl*1520 selective replication in tumours is not loss of functional p53. Instead, it is based on a loss of late viral RNA export,

another function of E1B55K (Dobbelstein *et al.*, 1997; O'Shea *et al.*, 2004; O'Shea *et al.*, 2005).

After purchasing the patent for ONYX-015, the Chinese company Shanghai Sunway Biotech Co., Ltd produced a nearly identical virus under the name H101. The latter contains a slightly larger deletion in E3 than ONYX-015 (Garber, 2006). In 2005, the Chinese State Food and Drug Administration was the first to approve a replicating adenoviral oncolytic vector, H101, in combination with chemotherapy, to treat head and neck cancer (Garber, 2006).

1.5.1 Second Generation Adenoviral Vectors

To overcome the limited efficiency in clinical results of first generation replicating therapeutic viruses, new strategies were developed to target lytic viruses to cancer cells.

1.5.1.1 E1A constant region mutations

A mutation in constant region 1 of E1A (E1A-CR1) in *dl*1101 renders the adenovirus unable to bind p300, a transcriptional co-activator deregulated in many types of tumours. In cancer cells, virus replication occurs despite the lack of E1A-p300 interaction. However, *dl*1101 showed disappointing results, as it was attenuated in both normal and tumour cells. Heise *et al.* described a 10 to 100-fold lower potency compared to *dl*922-947, the E1A-CR2-deleted oncolytic virus my work focuses on and which will be discussed in more detail in the next section (Heise *et al.*, 2000). More promising data were obtained with another E1A-CR2 mutant, pm928. pm928 carries a single point mutation within the pRb binding site of CR2 (Kraus *et al.*, 1992). The inability of CR2 to bind pRb prevents viral replication in normal cells, while in cancer cells this regulatory step is bypassed. The phenotype mediated by pm928 is very similar to *dl*922-947. Nevertheless, *dl*922-947 is the more potent oncolytic virus of the two (Heise *et al.*, 2000).

An example for another E1A-mutation based virus is CRAd5-Δ24RGD, which carries a 24bp-deletion within its E1A-CR2 region. This deletion conveys replication selectivity as described for *dl*1101. In addition, CRAd5-Δ24RGD expresses an arginine-glycine-aspartate (RGD) sequence motif in its fibre knob. *In vitro*, this motif directs the virus to target $\alpha_v\beta$ cell surface integrins, broadly expressed on most cancer cells, resulting in increased infectivity (Abou El Hassan *et al.*, 2004). This upgrade of the viral properties

of CRAd5-Δ24RGD was expected to add to the advantages of *dl*922-947, proven in pre-clinical models of ovarian carcinoma (Heise *et al.*, 2000). Nevertheless, side effects could not be eliminated. Injection of Ad5-Δ24RGD in rats caused mild peritonitis. Toxicity was tolerable, but elevation of liver function and hepatic toxicity were detected (Page *et al.*, 2007). Thus, bio-distribution and toxicity were generally not better than with *dl*922-947.

1.5.1.2 Transcriptional control

CV706 and CG7870 are two adenoviral constructs in which E1A expression is initiated via prostate-specific antigen (PSA) promoter/enhancer elements. In clinical trials for treatment of hormone-refractory prostate cancer, which is characterised by high levels of PSA transcription, toxicity profiles were satisfactory and some response was detected (DeWeese *et al.*, 2001; Small *et al.*, 2006). Similarly, AdPSAE1 is a replication-competent adenovirus construct in which the E1 region is expressed under the control of a PSA promoter. Oncolytic activity of AdPSAE1 was highly efficient in prostate cancer cell lines, but undetectable in other human cancer cell lines. In xenograft murine models with prostate tumours, intra-tumoural injection of the virus reduced tumour size, whilst it had no effect on bladder cancer xenografts in mice (Chang *et al.*, 2004). A more complex construct is the prostate-targeted two-step transcriptional amplification (TSTA) oncolytic adenovirus, described by Sato *et al*.. In this virus, enhanced expression of viral genes under strict prostate-specific control was achieved by combining two regulatory instances: expression of E1A and E1B under the control of strong activator Gal4VP16 and a PSA promoter controlling activity of Gal4VP16, itself. The resulting prostate-specific oncolytic adenovirus was able to replicate in prostate cancer cells, but not in non-prostate cancer cell lines, such as HeLa or A549 (Sato *et al.*, 2006).

1.5.1.3 E2F1 promoter control

ONYX-411 is an oncolytic adenovirus in which E1A and E4 expression is controlled by the E2F1 promoter. The latter is pRb responsive and contains four autoregulatory E2F binding sites. Its oncolytic potential is similar to that of wild-type adenovirus, both in colon, lung, pancreatic, cervical, bone and head-and-neck cancer cell lines and after systemic administration *in vivo* (Johnson *et al.*, 2002). ONYX-411 was also efficient in killing multiple anaplastic thyroid carcinoma (ATC) cell lines *in vitro*. ATCs are very aggressive and highly resistant to radio- and chemotherapy (Chiacchio *et al.*, 2008).

Similarly, in xenograft mouse models of ATC, ONYX-411 treatment led to reduced tumour growth (Reddi *et al.*, 2008).

1.5.2 *dl*922-947

One of the major limitations of cancer therapy with some oncolytic viruses is their reduced potency due to gene deletions (Heise *et al.*, 2000). Clinical trials of many therapeutic viruses concluded that replication of genetically attenuated mutants is less efficient than of respective wild type counterparts (Bischoff *et al.*, 1996; Kirn, 2000; Kucharczuk *et al.*, 1997; Martuza *et al.*, 1991; Mineta *et al.*, 1995). Comparison of therapeutic efficiencies revealed a small list of vectors outperforming the general bulk of oncolytic virus constructs. Among them was *dl*922-947, the adenoviral mutant at the centre of this study (Heise *et al.*, 2000).

*dl*922-947 is an adenovirus serotype 5 (Ad5) mutant. E1A constant regions CR1 (amino acids 30-60) and CR2 (amino acids 120-127) are vital for binding pRb (Kim *et al.*, 2007). *dl*922-947 carries a 24bp-deletion within E1A-CR2 (Heise *et al.*, 2000). This alteration confers selectivity to the virus, based on induction of viral replication specifically in cells with abnormalities in the retinoblastoma protein pathway (Rb pathway) (Fueyo *et al.*, 2000).

1.5.3 *dl*922-947 in cancer cells

In most cancers, including ovarian carcinomas, the Rb pathway is disrupted; the cells contain constant pools of free E2F (Sherr *et al.*, 2002). These free levels of E2F make E1A-CR2 activity redundant for *dl*922-947. The viral mutant can replicate efficiently and initiate cell death (Heise *et al.*, 2000). In cells with intact G1/S-phase checkpoint viral replication is disabled, as in quiescent cells, there is no free E2F. An illustration of these processes in normal and cancer cells is shown below (Fig. 1.4).

A previous study by our group showed that in ovarian cancer cells, *dl*922-947 induces S phase entry more rapidly and replicates more efficiently than ONYX-015 and wild type adenovirus. In all tested ovarian cancer cells, *dl*922-947 was also more efficient in lysis induction than wild type adenovirus and ONYX-015. Cytopathic effects in normal cells, on the other hand, were very low. In murine xenograft models, *dl*922-947, again, was superior to ONYX-015 in terms of response and survival rates, causing a 4-fold increase in median survival. However, hepatotoxic effects were observed in some mice (Heise *et al.*, 2000; Lockley *et al.*, 2006).

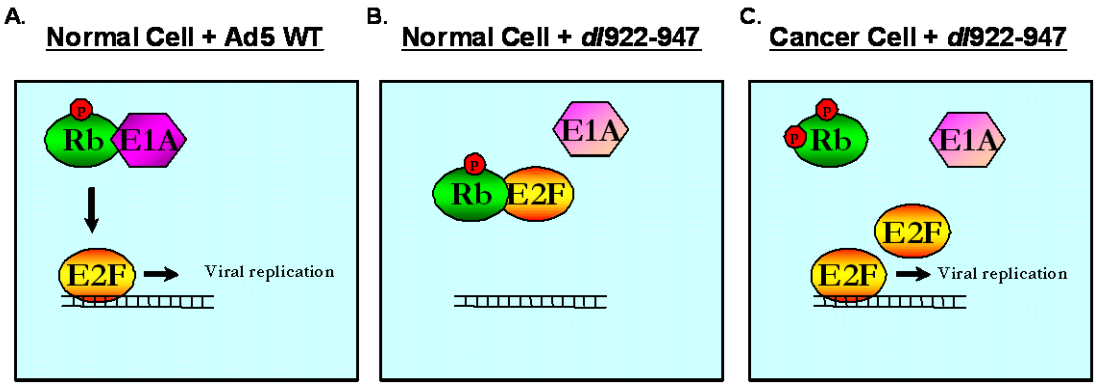


Figure 1.4 Mechanisms of selective replication of *dl*922-947. *Interactions between wild type and dl*922-947*-derived E1A in normal and cancer cells are shown.* A. *In normal cells, wild type E1A binds pRb and forces release of E2F.* B. *In normal cells, dl*922-947*-derived mutant E1A (deleted in CR2) is unable to associate with pRb. E2F remains bound to pRb and cell cycle entry is inhibited.* C. *In cancer cells, Rb pathway dysfunction is accompanied by high levels of free E2F, making E1A-pRb binding redundant for cell cycle progression.*

Another group constructed an E1A-CR2 deleted mutant, Δ24, which is almost identical to *dl*922-947. In most human glioma cells infected with Δ24 at a multiplicity of infection (MOI) of 10 plaque-forming units per cell (pfu/cell), lysis occurred within two weeks following infection. Again, normal fibroblasts, as well as cancer cells with restored Rb pathway remained resistant to the oncolytic virus (Fueyo *et al.*, 2000).

1.5.4 Immunogenicity of Adenoviral vectors

Immunogenicity of adenoviruses is considered a serious impediment to the efficacy of oncolytic viral therapy (Chen *et al.*, 2000). Not only does the majority of the adult population possess neutralising antibodies against adenoviruses (Chirmule *et al.*, 1999). Also, the inflammatory response can be a dose limiting factor in the clinic (Engler *et al.*, 2004). Systemic administration of adenoviral vectors is met by innate and adaptive immune responses of the host, elicited by interactions of the virus with host factors, such as integrins or CAR (Di Paolo *et al.*, 2009a; Shayakhmetov *et al.*, 2004). This has been shown to lead to chemokine and cytokine induction, resulting in inflammatory effects via MAPK, PI3K or NFkB signalling pathways (Vorisek *et al.*, 1976).

Surprisingly, pre-existing immunity against adenovirus 5 in syrian hamsters has been shown to have no detrimental effects on efficacy of an oncolytic

adenovirus INGN007. In contrast, the presence of Ad5-specific neutralising antibodies prevented liver toxicity and vector leakage (Dhar *et al.*, 2009). This suggests that, should a similar phenomenon be seen in humans, the apprehension towards immunogenic effects of oncolytic adenoviruses may be less justified than originally believed.

1.5.5 Further limitations of *dl*922-947 and other adenoviruses

So far, as there are no data from clinical trials, it is difficult to estimate the safety of therapeutic application of *dl*922-947 and other novel oncolytic adenoviruses. In murine ovarian cancer models, signs of hepatotoxicity were observed after intraperitoneal injection of the virus. Mouse livers appeared necrotic and showed eosinophilic degeneration, albeit to a lesser degree than after injection with the wild type equivalent (Lockley *et al.*, 2006).

Another possible risk factor is infection of normal haematopoietic stem cells. As they are highly replication-active cells, it was feared *dl*922-947 might be able to replicate within them, subsequently causing lysis. However, a study conducted in hematopoietic stem cells showed high resistance of these cells to adenovirus infection. Replication and infection was only detected at MOI 100pfu/cell (Medina *et al.*, 1999). This dose exceeds MOIs applied *in vivo* (Heise *et al.*, 2000). Further attributes of viral gene therapy potentially dangerous to the host are immune responses to and systemic distribution of *dl*922-947. Wild type adenoviruses often cause strong inflammatory effects in humans. On the one hand, this can be a dangerous side effect to oncolytic virus therapy, as happened in the case of Jesse Gelsinger (Hollon, 2000). Gelsinger suffered from an X-linked defect of the urea cycle, ornithine transcarbamylase deficiency (OTC). At the age of 18, he took part in a clinical trial during which he was given a dose of approximately 3.8×10^{13} particles of an E1A-deleted OTC-encoding adenovirus. After four days, Gelsinger died of multiple organ failure, most likely due to a systemic inflammatory response (Hollon, 2000). However, the host immune response is not necessary a limitation to viral therapy. In fact, it could be an important contribution to the anti-cancer effect of the oncolytic virus. Thus, understanding host response mechanisms through *in vivo* and clinical studies may allow us to exploit rather than prevent immune reactions (Kasuya *et al.*, 2007).

As described earlier, the classical model of adenovirus 5 infection is based on CAR/fibre knob binding and interactions between penton base RGD-motif and $a_v\beta_{3/5}$

integrins (Kirby *et al.*, 2000; Wickham *et al.*, 1993). This poses another challenge for the application of oncolytic adenoviruses. On the one hand, CAR is expressed in a relatively ubiquitous manner across tissues and cells in the human body (Carlisle *et al.*, 2009; Lamba *et al.*, 2004). This may result in the undesired uptake of oncolytic virus into non-target cells, when applied systemically. On the other hand, downregulation of CAR has been frequently described in tumours and primary cancer cell lines (Anders *et al.*, 2009; Jee *et al.*, 2002; Matsumoto *et al.*, 2005; Mikami *et al.*, 2001; Rauen *et al.*, 2002). Loss of CAR receptor can severely impede infection efficacy of cancer cells (Rein *et al.*, 2006). However, an increasing number of reports identifies alternative cell surface receptors facilitating binding and entry of adenovirus 5 into target cells, such as cell adhesion molecule 1 or heparan sulfate proteoglycans (Chu *et al.*, 2001; Dechecchi *et al.*, 2001). Equally, integrin interaction of adenovirus 5 does not seem to be restricted to $\alpha_v\beta_{3/5}$. It has been shown that for internalisation, the virus can also use $\alpha_5\beta_1$, $\alpha_v\beta_1$, $\alpha_M\beta_2$ or $\alpha_3\beta_1$ (Davison *et al.*, 1997; Huang *et al.*, 1996; Li *et al.*, 2001; Salone *et al.*, 2003).

Intensive research of the hepatotoxicity accompanying adenovirus infection has revealed that liver uptake of the virus is mediated by coagulation factors, amongst which FX appears to play the principal role (Shayakhmetov *et al.*, 2005; Waddington *et al.*, 2008). The difficulty in ameliorating this side effect is that recent evidence suggests FX is not only facilitating entry into hepatocytes, but may also mediate tumour transduction (Gimenez-Alejandre *et al.*, 2008). Therefore, ablating the underlying FX/hexon interaction may have detrimental effects on therapeutic effects of oncolytic adenoviruses (Waddington *et al.*, 2008).

1.5.6 Future of virus-based therapy in cancer

Clinical data obtained during clinical trials and after approval of oncolytic deletion virus H101 in head and neck cancer indicated the potential of gene therapy with oncolytic adenoviruses (Lu *et al.*, 2004). Intra-tumoural administration of virus was well-tolerated, dose limiting toxicity or serious adverse effects were not observed. Mild side effects and no apparent leakage from intra-tumoural injections into other body compartments suggested high levels of safety in application (Yuan *et al.*, 2003). These data are also promising for future virotherapy in other cancer types and with other vectors (Crompton *et al.*, 2007).

Nevertheless, for adenoviruses to be broadly applied in cancer therapy, there remain some crucial hurdles to be overcome. Firstly, it is fundamental to maximise infectivity

and selective efficacy of oncolytic viruses (Kirn, 2001). Extensive research has been invested into by-passing the limiting effects of CAR receptor downregulation and liver tropism described above. Infectivity can be increased by altering the viral coat (Romanczuk *et al.*, 1999). Introduction of modified receptors, for example, can retarget virus binding to host cell surface receptors other than CAR. Specifically retargeted oncolytic virus may also improve intra-tumoural spread and -where required- systemic delivery, particularly, if coupled with host immuno-suppression (Kirn, 2001). Very promising results were obtained with a recombinant adenovirus in which an $\alpha_v\beta_6$-binding motif was introduced into the fiber knob domain. $\alpha_v\beta_6$ is commonly over-expressed on cancer cells, but not in normal human tissues (Ahmed *et al.*, 2002; Bates, 2005; Elayadi *et al.*, 2007). This virus showed increased CAR-independent infectivity of $\alpha_v\beta_6$ expressing cell lines, compared to control virus with wild-type fiber knob. Interestingly, reduced liver uptake was observed *in vivo* (Coughlan *et al.*, 2009). Other recombinant adenoviruses, expressing RGD-4C nonapeptide or polylysine in their fibers, indicated augmented $\alpha_v\beta_{5/6}$-mediated infectivity of CAR-negative cells (Dmitriev *et al.*, 1998; Nagel *et al.*, 2003). The protein transduction protein (PTD) of HIV is a small peptide mediating protein transfer across cell membranes (Nagahara *et al.*, 1998). Incorporation of PTD into the adenoviral fiber knob has been shown to enhance infectivity in cell lines expressing both, low and high levels of CAR. Similar effects were seen in xenograft mouse models *in vivo* (Han *et al.*, 2007).

To better predict efficacy and safety of oncolytic adenoviruses, development of immuno-competent cancer models is indispensable. So far, data published on adenoviral therapy effects in animal tumour models are derived almost exclusively from immuno-compromised mouse human xenograft models (Kirn, 2001). Although, in 2003, Wang *et al.* described an interesting approach to investigate effects of E3 deletions on immuno-competent host responses to selectively replicating adenoviruses. Immuno-competent tumour mouse models were generated, using murine carcinoma cell lines permissive for viral activity of wild-type human adenovirus 5, as well as E3B-deleted *dl*309 and E3gp-19K-deleted *dl*704. Intra-tumoural injection of *dl*309 elicited stronger immune responses than wild-type adenovirus or *dl*704, highlighting the importance of the E3B region for prevention of premature clearance and strong toxicity of oncolytic adenoviruses. The model provided valuable information on host responses to such viral vectors and for the design of novel, more potent oncolytic adenoviruses (Wang *et al.*, 2003b). However, replication of wild-type human adenovirus and many oncolytic adenoviral vectors is strongly attenuated or completely blocked in murine cells (Eggerding *et al.*, 1986; Ginsberg *et al.*, 1991; Silverstein *et al.*, 1986). Therefore, the

higher degree of viral replication in more permissive tumours may elicit more profound effects.

Alternative, immuno-competent animal models exist, such as the Syrian hamster or the cotton rat, and have provided valuable insights into adenoviral effects on the immuno-competent organism (Thomas *et al.*, 2008; Toth *et al.*, 2005). However, as they are less widely applied in research, molecular tools available are far more limited than for murine models (Robinson *et al.*, 2009).

1.6 The Rb pathway and cell cycle control

Imperative for the application of oncolytic adenoviruses is also a more precise understanding of which cancer cell genes promote and predict for viral efficacy, which is the aim of my study. As *dl*922-947 function is believed to be largely dependent on the Rb pathway status of the infected cell, a major part of my work focuses on this pathway.

Rb pathway abnormalities are almost universal in human cancers, making the Rb pathway a very important target for treatment. This section explains the pathway and its components in normal cells, then describes their implications in cancer and how they relate to the activity of *dl*922-947.

1.6.1 Rb pathway and cell cycle regulation

The mammalian cell cycle is divided into different phases: G1, S, G2, and M phase, as well as the G0 phase. The latter comprises cells in their quiescent or senescent stage. A normal quiescent cell cannot re-enter the cell cycle unless it receives a stimulating mitogenic signal. Upon stimulation, the cell proceeds to the G1 phase (Vaillant *et al.*, 1995). During this phase, a crucial decision is made: will the cell continue DNA replication and division, or will it (re-)enter the quiescent state? This so-called restriction point is the checkpoint beyond which a cell becomes committed to advance through the entire cycle, a process that is self-regulated and not dependent on any further stimulating signals (Sherr, 1996). If, however, DNA damage or defects are detected, downstream checkpoints are activated and the cell cycle is delayed or halted (Stevens *et al.*, 2003).

The importance of the G1 restriction point is mirrored by the strict and complex regulatory network it is subjected to. This network, the so-called retinoblastoma pathway, comprises several components:

- Rb family: pRb, p107 and p130,
- Cyclin D and Cyclin E,
- Cyclin-dependent kinase cdk4, cdk6 and cdk2,
- E2F family of transcription factors,
- the INK4 and CIP/KIP families of cdk inhibitors (CKIs), including p15, p16, p18, p19, p21 and p27 and p57 (Genovese *et al.*, 2006).

Among these components, pRb acts as a “molecular guard”. It prevents progression past the restriction point, after which the cell is irreversibly committed to enter and complete the cell cycle (Lomazzi *et al.*, 2002). The main steps of the Rb pathway are pictured in Fig. 1.5. Upon induction by mitogenic growth factors -or stimulation by adenovirus and other DNA viruses, Cyclin D and cdk4 or cdk6 form complexes with kinase activity, which phosphorylate pRb. As the cell passes through G1, Cyclin E-encoding genes are activated. Subsequently, Cyclin E-cdk2 complexes further phosphorylate pRb. Hyper-phosphorylation of pRb leads to dissociation of the pRb-repressor complex. Released, active E2F is able to induce transcription of S-phase genes, allowing progression from G1 to S-phase (Tonini *et al.*, 2002; Viallard *et al.*, 2001). Simultaneously, transcription of genes required for DNA replication begins.

However, pRb regulates cell cycle entry not only by inhibiting E2F. Specific binding sites on the pRb protein allow the hypophosphorylated protein to recruit chromatin modifying and remodelling enzymes, such as histone deacetylase-1 (HDAC-1) and -2, BRG-1 and BRM, which render DNA inaccessible for transcription activators (Kadam *et al.*, 2003; Sherr *et al.*, 2002). Furthermore, it has been found that pRb is also involved in DNA repair. By regulating transcription factors of DNA repair pathways, pRb protects cells from double-strand breaks (Genovese *et al.*, 2006).

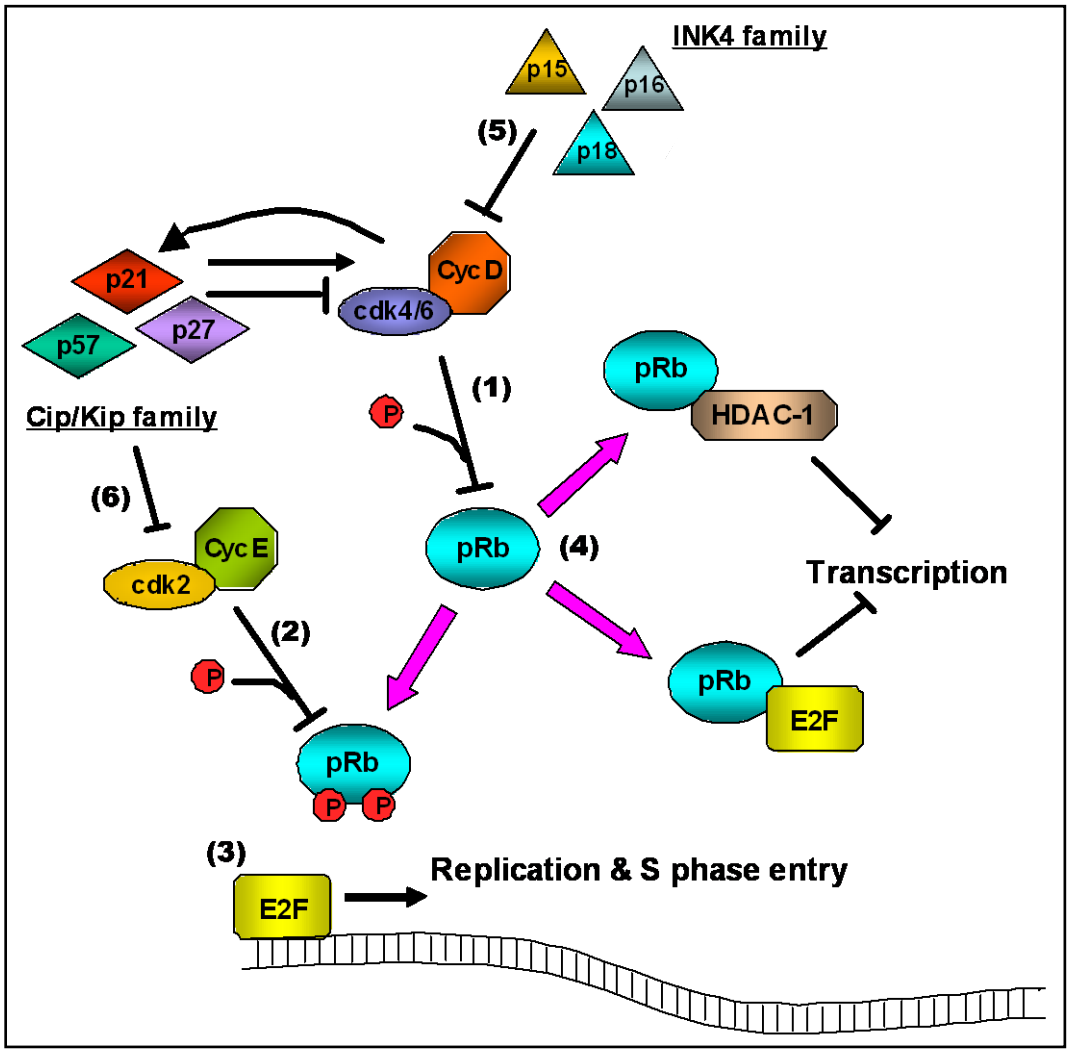


Figure 1.5 Rb pathway. *Shown are main cell cycle regulatory steps.* **(1)** + **(2)** *Sequential phosphorylation by Cyclin-cdk complexes forces pRb to release E2F.* **(3)** *E2F binds its target promoters facilitating replication.* **(4)** *Non-phosphorylated pRb inhibits transcription by binding E2F and recruitment of HDAC-1.* **(5)** *INK4 family inhibits cdk4/6.* **(6)** *Cip/Kip family inhibits or promotes Cyclin-cdk complex activity and in turn, is inhibited or stabilised by cdk4/6-CycD.*

1.6.1.1 Rb family

This protein family of transcription repressors and tumour suppressors has three members: pRb (p150), p130 (pRb2) and p107 (Paggi *et al.*, 1996). The three proteins, also referred to as “pocket proteins”, due to the structure of their functional domain, are able to bind and inhibit E2F. Such prevention of E2F activity interferes with gene transcription, cell cycle progression, and also inhibition of apoptosis and DNA damage repair (Genovese *et al.*, 2006).

Generally, pRb, p107 and p130 are involved in controlling events of the cell cycle, such as clonal expansion, terminal exit from the cell cycle and maintenance of the post-mitotic state. They also induce transcription of tissue-specific genes and play a regulatory role in programmed cell death (Classon *et al.*, 2001). Although pRb, p107 and p130 share a strong homology, particularly within their "pocket region", and their activities are very similar, sometimes even overlapping, they are by no means identical (Wang, 1997). Expression studies revealed that each Rb family member shows a distinct profile of expression depending on cell status:

p130 expression is highest in G0 phase, thus in quiescent and differentiated cells. As soon as a quiescent cell is stimulated to enter the cell cycle, p130-levels drop rapidly.
p107 is present at low levels at a stage of terminal differentiation. Expression levels rise upon growth factor stimulation (Classon *et al.*, 2001). Interestingly, both p107 and p130 are able to interact with Cyclin A/cdk2 and Cyclin E/cdk2 complexes, inhibiting their kinase activity, thus preventing phosphorylation of cdk-inhibitor p27. As a consequence, p27 is not marked for proteasomal degradation but accumulates in the cell (Genovese *et al.*, 2006).
pRb, in contrast, is moderately expressed in both quiescent and cycling cells (Classon *et al.*, 2001).

Findings from *in vitro* studies support the functional discrimination of pocket proteins. It was shown that T98G human glioblastoma cells were insensitive to pRb and p107. Exposure to p130, on the other hand, resulted in suppression of proliferation (Claudio *et al.*, 1996; Claudio *et al.*, 1994). Unlike pRb and its role in inhibiting cell cycle entry, p107 and p130 were shown to be involved in governing cell growth and maintenance of the extracellular matrix (Black *et al.*, 2003).

Furthermore, all three pocket proteins have been found to interact with members of the E2F family of transcription factors. Again, binding partners differ, depending on cell cycle stage and particular pocket protein. p130 binds to E2F-4 and -5 during G0. In G1, it is mainly p107 that binds E2F-4. In S phase E2F-4 is bound by pRb. Such fluctuations of protein associations throughout the cell cycle suggest a sequential activity of the Rb family to prevent S phase progression (Ginsberg *et al.*, 1994; Sardet *et al.*, 1995). On the whole pRb binds to E2F proteins involved in activation of cell cycle genes, while p107 and p130 interact with E2Fs which repress transcription (Black *et al.*, 2003).

Distinctive roles of pRb, p130 and p107 were also observed in a study by Classon *et al*. Therefore, cells lacking pRb and/or either of the other two pocket proteins showed truncated G1, but elongated S phase, compared to wild type cells or those lacking only p107 or p130. The conclusion drawn from this study was that derepressing E2F target genes drives cells to enter S phase earlier. But, as other factors needed to synthesise DNA are still sparse, synthesis is slowed down (Classon *et al.*, 2000).

Although all members of the Rb family are in some way involved in the cell cycle, the Rb gene is the only one of the three frequently found to be mutated in tumours (Weinberg, 1995). This is particularly unexpected in light of the high degree of sequence homology between them (Wang, 1997).

1.6.1.2 E2F

E2F proteins constitute a family of transcription factors regulating expression of a wide range of genes, many of them vital for regulation of cell cycle progression (Attwooll *et al.*, 2004; Dimova *et al.*, 2005). The E2F family comprises eight members. Five E2F proteins, E2F3b, E2F4, E2F5, E2F6 and E2F7 have repressor function. Three members, E2F1, E2F2 and E2F3a, are activators of gene expression during late G1 phase (Dyson, 1998). They are preferentially bound and inhibited by pRb (Cobrinik, 2005).

E2F target genes encode, for example, Cyclins E, A and D1, cell division cycle 2 (Cdc2) (also called cdk1), cdc25A, enzymes required for DNA synthesis, proteins involved in replication, but also apoptotic proteins, including apoptotic protease activating factor-1 (Apaf-1) and p73. E2F-1, E2F-2 and E2F-3 are able to activate S phase entry (Dyson, 1998; Moroni *et al.*, 2001). In addition, E2F-3 is able to induce apoptosis in a p53-dependent and -independent manner (Ziebold *et al.*, 2003). Its activity was detected in response to DNA damaging agents as well as T-cell development (Nip *et al.*, 2000). Regulation of E2F is carried out in a cell cycle-dependent fashion, via temporal binding of pRb, p107 and p130. Pocket protein function, in turn, is controlled via phosphorylation by cdks (Stevens *et al.*, 2003).

Unlike E1A and other pRb-binding proteins, E2F proteins do not contain an LxCxE sequence motif. Instead, they bind pocket proteins with a specific 18 aa-motif within their transactivation domain, close to the C-terminus (Helin *et al.*, 1992; Shan *et al.*, 1996). Although this binding motif is also located within the pRb pocket region, it is

distinct from the LxCxE-binding groove (Kirn, 2001). In fact, the two functionally significant binding grooves face one another within the pRb pocket.

1.6.1.3 New findings on pRb-E2F interactions

It has been known for some time that by binding E2F, pRb is able to regulate negatively both cell cycle entry and apoptosis. A recent study by Julian *et al*. showed that, even when the apoptosis-blocking domain of pRb is lost, the protein can still block cell cycle progression. Further experiments revealed a novel manner of regulation: dual pRb function appears to be based on two distinctive types of contact between the same types of molecules. According to Julian and colleagues, control of E2F-induced S-phase entry and apoptosis are not only based on timing of pRb-E2F binding, but also on the physical nature of interaction. This stands in contrast to past publications, according to which interaction between proteins, per se, is sufficient for a positive or negative functional outcome. Julian *et al*., on the other hand, have been able to identify separate E2F docking sites on pRb: one binding all members of the E2F family. The other is an E2F-1-specific site, responsible for regulation of apoptosis (see Fig. 1.6). The latter interaction involves a domain of E2F-1 not found in other E2F proteins (Dyson, 1998; Julian *et al.*, 2008).

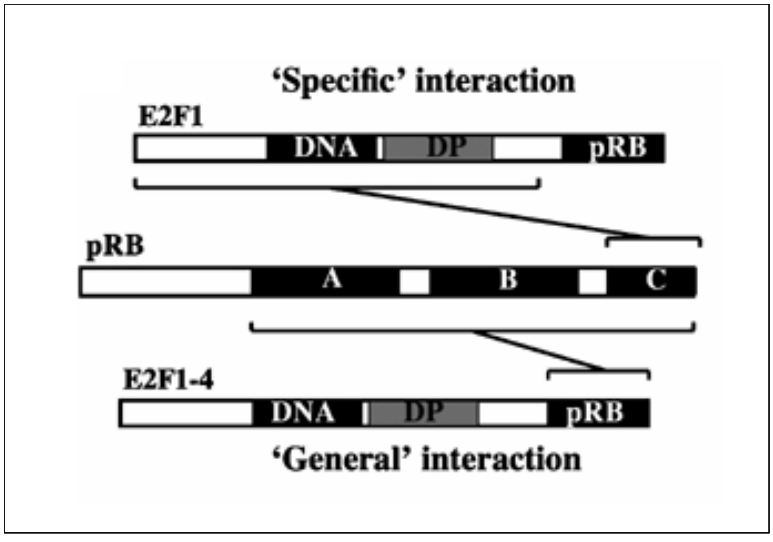


Figure 1.6 Diagrams of the pRb and E2F ORFs. *Shown are domains that mediate the 'specific' pRb–E2F1 interaction as well as the 'general' interaction between pRb and all E2F members (Julian et al., 2008).*

1.6.1.4 Cyclins and cyclin-dependent kinases

Cyclin-dependent kinases are serine and threonine phosphorylating entities, which, in their enzymatic activity, depend on another group of molecules: cyclins, whose levels within a cell are regulated via protein synthesis and degradation (Sherr *et al.*, 1999).

In mammalian cells, each cdk is subject to control by multiple cyclins. This is partially based on overlap of cyclin function (Fisher *et al.*, 1996). However, activity of a given cyclin is ultimately unique, providing the regulatory system with a refined control mechanism. Various regulatory routes confer specificity of cyclin-cdk interactions. Cyclin expression and maintenance of stable levels occur in a cell cycle-dependent manner, based on differentially induced transcription and proteolysis. Cyclin activity is also sensitive to inhibitory phosphorylation. In fact, it is phosphorylation that initiates DNA synthesis and at the same time ensures DNA replication is limited to one round per cycle. Furthermore, cyclin proteins possess regulative domains, such as nuclear localisation sequences, destruction boxes mediating degradation or hydrophobic patches facilitating substrate interactions (Barral *et al.*, 1995). In general, cyclin specificity follows a common scheme: a late-functioning cyclin is kept inactive by its inhibitor. An early-functioning cyclin inactivates this particular inhibitor, thus abrogating repression of the late cyclin (Bloom *et al.*, 2007).

1.6.1.5 Cyclin D1/D2/D3 and cdk4/6

Different cyclins are involved in regulation of different stages of the cell cycle. In the context of this study, the focus lies on Cyclins D and E, with regard to their crucial role in G1 entry and G1/S transition (Takaki *et al.*, 2005; Viallard *et al.*, 2001). Firstly, Cyclins D1/D2/D3 associate with cdk4 or cdk6. The kinase function of the active complex partially phosphorylates and inactivates pRb (Sherr *et al.*, 1999). Cdk4 and cdk6 have been found virtually identical in their biochemical properties. Their specific roles are believed to be regulated by differential expression (Malumbres *et al.*, 2004). Although their activity may be redundant in cell cycle control, cdk4 and cdk6 have been shown to have unique roles. *In vitro* studies for E2F-pRb interaction proved it irrelevant whether pRb phosphorylation was cdk4- or cdk6-derived. In contrast, in murine astrocytes, ectopic expression of cdk6 led to morphological changes, whilst ectopic expression of cdk4 caused no alterations (Ericson *et al.*, 2003). Another study involving cdk4 and cdk6 knock-out mice revealed phenotypical differences between the two (Kozar *et al.*, 2004). Interestingly, if pRb lacks its functional region, it cannot be phosphorylated by cdk6, while ckd4-mediated phosphorylation still occurs (Takaki *et al.*, 2005).

1.6.1.6 Cyclin E and cdk2

G1/S transition is regulated by two parallel, cooperating cascades: the Rb and the Myc pathway. The point of convergance of this interaction lies in the regulation of Cyclin E-cdk2 activity, mirroring its crucial role in S phase entry (Bartek *et al.*, 2001). Cyclin E-cdk2 was found preferentially to phosphorylate a different pRb serine than Cyclin D-cdk4/6 (Kitagawa *et al.*, 1996; Smartt *et al.*, 2007). Cyclin E-cdk2 is thought to initiate chromosome and centromere duplication. The complex is also believed to play an essential role in S phase entry. Inhibitors of cdk2, such as p21 and TGF-β, were found to block G1/S progression (Roberts *et al.*, 2003). Similar conclusions were drawn from a Drosophila study: Cyclin E-cdk2 was required to form the replication initiation complex, and was thus indispensable for a cell to proceed into S phase (Brehm *et al.*, 1998). Surprisingly, neither Cyclin E nor cdk2 are essential for S phase entry in humans. Similarly, cdk2-deleted mice were viable and developed normally (Sherr *et al.*, 1999).

1.6.1.7 New findings on cdk function

In the mammalian genome, at least 12 loci encode cdks (Malumbres *et al.*, 2005). According to a majority of publications, a minimum of four cdks must be activated to allow a mammalian cell to enter and proceed through the first stages of the cell cycle: cdk2, 4 and 6. A fourth mammalian cyclin-dependent kinase, cdk1, drives the cell through mitosis. In recent years, more and more evidence has been found indicating that, in many murine cells, proliferation can occur despite multiple deletions of cdk2, 4 and/or 6 (Malumbres *et al.*, 2004). Santamaria *et al.*, observed that loss of up to three cdks did not prevent organogenesis and embryos developed to the mid-gestation stage. Closer examination revealed ability of cdk1 to bind all members of the Cyclin family. Such interaction resulted in pRb phosphorylation and transcription of E2F-regulated genes, albeit less efficiently than in wild type cells. Slower life cycles of cells derived from these embryos were ascribed to imperfect inactivation of pRb. These findings suggest that cdk1 may be the only essential cell cycle cdk and that its kinase activity is sufficient for all events vital for proliferation. Indeed, upon serum-stimulation, mouse $cdk2^{-/-}cdk4^{-/-}cdk6^{-/-}$ cells entered the cell cycle and accomplished all steps necessary for S phase transition. In contrast, knock-down of cdk1 prevented S-phase entry. Accordingly, *in vivo* analysis of $cdk1^{-/-}$ mice revealed impaired cell division during embryogenesis (Santamaria *et al.*, 2007). These data challenge the general perception of cdk1 as a mitosis-specific kinase (Fourest-Lieuvin *et al.*, 2006; Stumpff *et al.*, 2004).

1.6.1.8 Inhibitors of cyclin-dependent kinases

Cyclin-dependent kinase inhibitors (CKIs) execute negative regulation of Cyclin-cdk activity (Sherr *et al.*, 1999). They comprise two families:

(1) INK4 proteins: p16, p15, p18, which inhibit cdk4 and cdk6 catalytic subunits
(2) Cip/Kip family: p21, p27 and p57. Their action is more general, regulating function of kinases dependent on Cyclin D, Cyclin E and Cyclin A (Bartek *et al.*, 2001; Polyak, 2006; Sherr *et al.*, 1999).

The magnitude of inhibition by these proteins was demonstrated by infecting cells with normal pRb function, such as MT1A2, with adenoviral vectors carrying CKI genes. Ectopic expression of p16, p18, p21 or p27 resulted in arrested cell growth, aborted DNA synthesis and failure of pRb phosphorylation. Infection of pRb-deficient cells with the same adenoviral vectors, on the other hand, did not halt replication or induce cell growth. *In vitro* application of the CKI encoding adenoviruses resulted in delayed tumour formation in a mouse breast cancer model, the effect being strongest upon ectopic p27 expression. This was in accordance with previously described maximal occurrence of both p27 expression and cyclin-cdk complex activity (Schreiber *et al.*, 1999).

1.6.1.9 p21

The 165 aa protein p21, also referred to as WAF1/CIP1 and encoded by *CDKN1A*, is generally perceived as a regulator of proliferative effects. It was first described as a mediator of p53-induced cell cycle arrest (el-Deiry *et al.*, 1993). In the meantime, a plethora of functions has been ascribed to p21, both p53-dependent and -independent (Brugarolas *et al.*, 1995; Deng *et al.*, 1995; Gartel *et al.*, 2000). The primary pathway of p21-mediated growth inhibition is based on inhibition of cdk1 and cdk2, by disrupting their direct interaction with proteins that bind to cyclin/cdk complexes, such as members of the pRb family or cdc25C (Abbas *et al.*, 2007; Mandal *et al.*, 1998; Saha *et al.*, 1997; Shiyanov *et al.*, 1996; Smits *et al.*, 2000; Zhu *et al.*, 1995; Zhu *et al.*, 2005). The latter, a phosphatase, de-phosphorylates cdk1 bound to Cyclin B, thus allowing entry into mitosis (Saha *et al.*, 1997). Furthermore, it has recently been shown that cdk1/2-Cyclin A and cdk1-Cyclin B are both de-phosphorylated and activated by cdc25A. Cdc25B, on the other hand, can only activate cdk1-Cyclin B (Ray *et al.*, 2008).

p21 also exerts cell cycle-arresting function in a cdk-independent manner by inhibition of PCNA. By this mechanism, p21 interferes with PCNA-dependent DNA polymerase activity, preventing DNA replication and modulating PCNA-regulated DNA repair (Moldovan *et al.*, 2007; Mortusewicz *et al.*, 2005). However, the regulatory activity of

p21 goes beyond protein-protein interactions. Gene array studies conducted by Chang et al. indicated that high levels of p21 correlate with the suppression of cell cycle regulatory and senescence-associated genes (Chang *et al.*, 2000). Although, to some extent, this will be a consequence of cdk inhibition, p21 also acts more directly as transcription regulator. It is able to bind and inhibit transcription factors E2F, STAT3 and Myc (Coqueret *et al.*, 2000; Delavaine *et al.*, 1999; Kitaura *et al.*, 2000). Equally, it mediates p53-dependent repression of cdc25C, cdk1, chk1, Cyclin B1, TERT and survivin expression (Lohr *et al.*, 2003; Shats *et al.*, 2004).

On the other hand, p21 functions as an activator of transcription by inducing transcriptional co-activators p300/CBP (Snowden *et al.*, 2000). This mechanism is also the basis of a positive feedback loop, by which p21 perpetuates its own expression (Ait-Si-Ali *et al.*, 1998). Furthermore, p21 has both positive and negative effects on DNA repair. Inhibiting cell cycle progression allows more time for repair processes. On the other hand, by binding PCNA, p21 prevents the association of other PCNA-binding proteins involved in DNA repair (Moldovan *et al.*, 2007; Mortusewicz *et al.*, 2005; Walsh *et al.*, 2006). Also, direct interaction of p21 with PCNA has been show to inhibit PCNA-dependent mismatch repair and base excision (Tom *et al.*, 2001; Umar *et al.*, 1996).

Apoptosis is another process partially modulated by p21. However, the roles attributed to the CKI are conflicting. Various publications conclude that p21 acts as an inhibitor of apoptosis. In some cancer cell lines, downregulation of p21 sensitised them to apoptosis after exposure to genotoxic stress (Detjen *et al.*, 2003; Mahyar-Roemer *et al.*, 2001; Tian *et al.*, 2000). This observation may be based on senescence-preventing effects of p21 loss, as in cell cycle-arrested cells, apoptosis is not induced(Han *et al.*, 2002). Moreover, p21 binds and inhibits apoptotic pathway members, such as pro-caspase 3, caspases 8 and 10, stress-activated protein kinases (SAPKs) and mitogen-activated protein kinase kinase kinase 5 (MAP3K5) (Dotto, 2000; Roninson, 2002; Suzuki *et al.*, 2000). On a transcriptional level, p21 suppresses expression of several pro-apoptotic genes by inhibiting their transcription factors Myc and E2F, and it induces upregulation of anti-apoptotic genes (Dotto, 2000; Roninson, 2002).

In contrast, Qin et al. have reported enhanced cisplatin-induced apoptosis in various cancer cell lines when over-expressing p21 (Qin *et al.*, 2001). Whilst Gartel *et al*. conclude that p21 may promote apoptosis in a p53-dependent and –independent manner by upregulating pro-apoptotic BAX and activating TNF-death receptor family

members. Also, p21 effects on DNA damage repair may enhance apoptosis (Gartel, 2005). Yet, the underlying mechanisms remain unclear.

Effects of p21 are mediated by its N-terminal domain, which is necessary and sufficient for interaction with and inhibition of Cyclin/cdk activity. This domain is common to all three members of the Cip/Kip family (Watanabe *et al.*, 1998). Furthermore, p21 possesses a unique C-terminal domain, facilitating PCNA-binding and consequences thereof (Sherr *et al.*, 1999).

Activity of p21 is regulated on transcriptional and post-translational level. A multitude of stimuli induce p21 upregulation via transcription factors like SP1, SP3 or STATs (Bellido *et al.*, 1998; Chin *et al.*, 1996; Matsumura *et al.*, 1997; Xiao *et al.*, 1999). Expression of *CDKN1A* can be controlled in a p53-dependent and –independent fashion. In response to cellular stress, the primary line of p21 regulation is via p53 (O'Reilly, 2005). *CDKN1A* possesses two highly conserved p53-specific binding sites in its promoter region (el-Deiry *et al.*, 1995). Cellular stress, comprising not only DNA damage, but also hypoxia or nutrient depletion, induces p53 activity (Hammond *et al.*, 2002; Nelson *et al.*, 1994; Zhan *et al.*, 1993): Stress-activated ataxia telangiectasia mutated (ATM) kinase phosphorylates p53 and enhances its transcriptional potential further by promoting the association of p300, a known co-activator of *CDKN1A* expression (Banin *et al.*, 1998; Canman *et al.*, 1998; Helt *et al.*, 2001). In normal, non-immortalised cells, p53-independent activation of p21 expression by Ras is mediated by E2F and via the HRAS-RAF-MAPK pathway (Gartel *et al.*, 2000; Gartel *et al.*, 1999; Woods *et al.*, 1997). As described earlier, co-activator p300/CBP induces transcription of *CDKN1A* downstream of various stimuli, amongst which is p21 itself (Ait-Si-Ali *et al.*, 1998; Gartel *et al.*, 1999).

Post-translational control of p21 is equally important as transcriptional regulation (Abbas *et al.*, 2009). In cycling cells p21 is very unstable, with a half life between 20-60 minutes. Newly synthesised protein is protected from the proteasome by binding of heat shock protein 90 (HSP90) (Jascur *et al.*, 2005). At specific stages during the normal cell cycle, proteolysis of p21 is mediated by E3 ubiquitin ligase complexes (Bornstein *et al.*, 2003; Sarmento *et al.*, 2005; Wang *et al.*, 2005; Yu *et al.*, 1998). However, these complexes mark p21 for proteasomal degradation only if it is bound to PCNA, Cyclin E/cdk2, Cyclin A /cdk2, Cyclin A/cdk1 or Cyclin B/cdk1 (Abbas *et al.*, 2008; Amador *et al.*, 2007; Kim *et al.*, 2008; Nishitani *et al.*, 2008). Unbound p21, at least in some cell lines, is targeted for ubiquitin-independent degradation by direct

binding to a subunit of the proteasome, other than the ubiquitin-binding subunit (Chen *et al.*, 2007; Li *et al.*, 2007b; Sheaff *et al.*, 2000; Touitou *et al.*, 2001). Stabilisation of p21 can be conferred by binding of Cyclin D/cdk4 or cdk6 complexes (Coleman *et al.*, 2003). Ubiquitination of p21 is inhibited by signalling molecules such as TGF-β, BMP2 or JNK1 (Beck *et al.*, 2007; Fan *et al.*, 2007; Gong *et al.*, 2003). It has been shown that, in order to reach the high p21 protein levels required for DNA damage-induced cell cycle arrest, stabilisation of the protein is required. Transcriptional upregulation alone is not sufficient (Jascur *et al.*, 2005).

Stabilisation, as well as cellular localisation, of p21 is controlled by phosphorylation through various kinases (Child *et al.*, 2006). A table correlating the phosphorylation sites on p21 with the kinases targeting them and the consequences of phosphorylation is shown below (Table 1.3).

Phosphorylation site	**Kinase**	**Effects**	**Ref.**
Thr57	GSK3-β	in serum-stimulated endothelial cells: degradation of p21	(Rossig *et al.*, 2002)
Thr145	AKT1	disruption of PCNA binding; cytoplasmic accumulation	(Li *et al.*, 2002; Rossig *et al.*, 2001; Zhou *et al.*, 2001)
Ser130	Cyclin E /cdk2	marking for ubiquitination and proteolysis	(Bornstein *et al.*, 2003)
Ser146	AKT1	stabilisation of p21 and cell survival	(Li *et al.*, 2002)
Ser146	PKC	stabilisation of p21 or: degradation of p21 (probably dependent on cellular context)	(Oh *et al.*, 2007; Scott *et al.*, 2002)

Table 1.3 Phosphorylation sites on p21. *Along with the phosphorylation site, the table shows the kinases targeting it and the consequences of phosphorylation at this specific site.*

Localisation of p21 is crucial for its functions. Whilst growth-inhibitory activities require nuclear localisation, anti-apoptotic and proliferative functions, for example, are commonly linked to cytoplasmic accumulation (Child *et al.*, 2006). In the cytoplasm, p21 binds and inhibits apoptotic proteins and promotes proliferation by stabilising Cyclin D/cdk4 complexes and alleviating cdk2 and PCNA inhibition in the nucleus (Dotto, 2000; LaBaer *et al.*, 1997; Roninson, 2002; Suzuki *et al.*, 2000).

In this context, cytoplasmic p21 is frequently found in human cancers and correlates with aggressiveness and poor prognosis (Abbas *et al.*, 2009).

1.6.1.10 Viruses and p21

Interestingly, viruses commonly modulate p21 stability and activity in order to regulate progression of the cell cycle and apoptosis. Human papilloma virus (HPV) E6 protein and SV40 TAg from simian virus 40 downregulate p21 in a p53-dependent manner to enhance apoptosis (Lane *et al.*, 1979; Werness *et al.*, 1990). This may be by targeting transcriptional co-activators p300/CBP for degradation (Zimmermann *et al.*, 1999). Similarly, adeno-associated virus 2 (AAV2) promotes p21 down-modulation in HPV-infected cells. However, AAV2 prevents further progression through S phase to maximise its own replication (Alam *et al.*, 2006). Hepatitis C virus (HCV) is also able to inhibit p21. Surprisingly, the virus did not alter mRNA levels or degradation patterns of p21. This suggests that inhibition takes place on a post-transcriptional level (Yoshida *et al.*, 2001).

Regulation of p21 expression by adenovirus can be both promotive and repressive. By binding p300/CBP, E1A prevents TGF-β expression and, subsequently, DNA damage-activated p21 expression (Datto *et al.*, 1997; Steegenga *et al.*, 1996). In cancer cells, however, p21 expression is known to be induced by the 13S isoform of adenovirus E1A (Najafi *et al.*, 2003).

1.6.1.11 Role of p27 in the Rb pathway

p27 may be a means to fine-tune events during cell cycle progression (Smartt *et al.*, 2007). As with pRb, p27 exerts tumour suppressor function by impeding S phase entry. It does so by binding to Cyclin D/cdk4/6 and Cyclin E/cdk2 complexes, thereby inhibiting cdk kinase activity (Aleem *et al.*, 2005). Various findings imply a close link between pRb and p27. A phenotypical study of p27-deficient mice described development of tumours in the pituitary gland, thus bearing resemblance to pRb-deficient mice (Polyak, 2006). Polyack *et al*. interpreted this as an indication of

negative regulatory effect of p27 over pRb. However, in a more recent publication, Park *et al*. suggest that cell cycle control through pRb and p27 may be of mutual nature. Double knock-out mice, lacking pRb and p27, suffer from earlier and more aggressive pituitary tumours than animals lacking either one of the tumour suppressors. The same phenomenon was observed in thyroid c cell carcinomas. It is possible that pRb and p27 prevent cell cycle progression via a partially overlapping, albeit not identical, pathway (Park *et al.*, 1999). p27 shares functions, pathways, structural features and regulators with p21 (Coqueret, 2003; Lacy *et al.*, 2005; Sharma *et al.*, 2005). Like its fellow Cip/Kip family member, p27 is induced in a p53-dependent manner by cellular stress but also other signals, such as progesterone (Green *et al.*, 2001; Hsu *et al.*, 2008). Both p21 and p27 stabilise Cyclin D/cdk4 complexes at low levels (Cheng *et al.*, 1999; LaBaer *et al.*, 1997). Both are targeted for degradation by the same ligases (Sarmento *et al.*, 2005). 12S E1A binds and inhibits p21 and p27 (Chattopadhyay *et al.*, 2001; Mal *et al.*, 1996). They have been described as mutual negative prognostic markers in rectal cancer (Schwandner *et al.*, 2002). Yet, there are examples of differential regulation of the two CKIs. In hepatocyte and keratinocyte differentiation, p27, unlike p21, has been shown to have no effects on differentiation (Di Cunto *et al.*, 1998; Ilyin *et al.*, 2003). In contrast to p21, mitogenic stimulation resulted in down-modulation of p27 in several cell types, whilst levels increased after anti-mitogenic signalling (Agrawal *et al.*, 1995; Nourse *et al.*, 1994). Moreover, although all three Cip/Kip family members have some functional structures, such as the N-terminal domain, the ability to bind pRb described for p21 and p57 is absent p27 (Nakanishi *et al.*, 1999; Polyak *et al.*, 1994a; Poon *et al.*, 1995).

1.6.2 Rb pathway and cancer

Considering the strong regulatory impact of each protein associated with the Rb pathway on cell cycle progression, there is reason to expect that deregulation of any such factor could lead to tumourigenesis (Viallard *et al.*, 2001; Yamasaki, 2003).
Indeed, as listed below, altered function of most Rb pathway components has been found associated with cancer:

1.6.2.1 pRb

pRb is the ultimate guard of S phase entry and DNA replication. It was the first tumour suppressor to be cloned (Weinberg, 1995) and was the model system for Knudson's "Two-Hit Hypothesis", where in the case of tumour suppressors two mutational events are required for tumourigenesis to occur (Knudson, 1971). In keeping with this

hypothesis, it has been shown that hereditary mutations in one pRb allele predispose an individual to the development of different types of cancer (Kleinerman *et al.*, 2005). Indeed, disruption of pRb function has been found in nearly all tumours (Malumbres *et al.*, 2008). In most cases, disruption is caused by malfunctioning of the protein, rather than a loss of the wild type gene, *per se* (Bosco *et al.*, 2007). Interestingly, low-level pRb expression was found to increase proliferation only during early ovarian tumourigenesis. At later stages carcinomas seemed to have become independent of pRb expression (D'Andrilli *et al.*, 2004).

1.6.2.2 Cyclins, cdks and their inhibitors

Deregulation of cyclins and cdks is very common in human cancers (Deshpande *et al.*, 2005).Their overexpression induces non-scheduled proliferation and division, eventually resulting in further genomic instability (Malumbres *et al.*, 2008). Gene mutations often cause structural modifications on the protein-level, eliminating interactions with inhibitors, and thus leading to permanent activation of kinase function (Stevens *et al.*, 2003). In many solid tumours, Cyclin D1 overexpression emerged as a marker for poor prognosis (Viallard *et al.*, 2001). Overexpression of Cyclin D is believed to play a critical role in ovarian tumourigenesis, alongside that of cdk4 (D'Andrilli *et al.*, 2004). Abnormally high levels of cdk4, as well as activating mutations of the protein, are frequently found in melanomas and malignant gliomas and approximately 15% of ovarian cancers (Bartkova *et al.*, 1996; Muthusamy *et al.*, 2006). While overexpressed Cyclin E is commonly found in malignancies, particularly in ovarian and breast tumours, its functional partner, cdk2, is dysregulated in only 6% of ovarian carcinomas (D'Andrilli *et al.*, 2004). Among CKIs, p16, p21 and p27 are most frequently altered kinase inhibitors in cancers. Solid tumours and leukaemias often feature p16 gene deletions. p21 overexpression has been regularly identified in various types of tumours, such as ovarian, gastric, tonsillar, pancreatic or bladder cancers (Hafkamp *et al.*, 2009; Kim *et al.*, 2003a; Koff, 2006; Korkolopoulou *et al.*, 2000; Ralhan *et al.*, 2000). Equally, p21 down-modulation has been frequently detected in cancers (Bukholm *et al.*, 2000; Edmonston *et al.*, 2000; Ogino *et al.*, 2006; Polyak *et al.*, 1996). Furthermore, p21 can be a marker of both good and poor prognosis. The difference is likely to arise from the heterogeneity in intracellular localisation of p21, tumour cell type and expression of other oncogenes.

Altered p27 expression occurs in many tumours (Koff, 2006; Ozkara *et al.*, 2004; Yamamoto *et al.*, 2009). Although somatic mutations of the cdk inhibitor are rare, hemizygous or post-transcriptional loss are driving factors in tumourigenesis (D'Andrilli

et al., 2004). Usually, during tumour development, mRNA levels of p27 are low, yet not fully ablated (Koff, 2006; Malumbres *et al.*, 2008). In colon carcinomas reduced protein levels of p27 are known to predict poor patient clinical outcome (Smartt *et al.*, 2007). In contrast, in rectal cancer, expression of p27, alongside p21, has been described as a prognostic marker of late clinical stage and high incidence of distant metastases (Schwandner *et al.*, 2002). Again, the specific role of the CKI may be context-dependent.

The vast range of mutations in the multitude of cell cycle regulatory components complicate treatment with current technologies. It is therefore crucial to clarify mechanisms involved in normal and abnormal cell proliferation, in order to turn these hurdles into points of attack for novel therapeutic approaches.

1.6.3 E2F and the Rb pathway in ovarian cancer

Cell cycle deregulation is a major contributor to cancer development (Hanahan *et al.*, 2000). A microarray-based study in high-grade serous ovarian carcinomas and lower-grade serous borderline tumours indicated over-expression of E2F and its target genes. The authors suggested this was a result of global cell cycle deregulation in high-grade serous carcinomas (De Meyer *et al.*, 2009).

Many studies have been conducted, investigating prognostic potential of Rb pathway members in ovarian cancer. In many cases, there are conflicting reports on the prognostic nature of these cell cycle regulators. In one study, abnormalities in pRb were described as rare, whilst loss of p16 protein was found to occur in 20% of tumours. In this case, the loss of p16 appeared to be based on methylation, as gene mutations and deletions were not commonly detected (Hashiguchi *et al.*, 2001). In contrast, another study in late-stage epithelial ovarian carcinomas suggested low p16 and pRb levels to be independent markers of poor prognosis (Kommoss *et al.*, 2007). Dong et al., on the other hand, claimed that in early stage ovarian tumours, low p16 levels correlate with high pRb abundance, whilst most advanced tumours express both proteins at low levels (Dong *et al.*, 1997). Comparison of 165 patients with ovarian cancer indicated a correlation between high levels of p21 expression and early tumour stages (Schmider-Ross *et al.*, 2006). Interestingly, another study found p21 to be an independent poor prognostic marker in p53-negative ovarian carcinomas. At the same time, p21 appeared to predict survival advantage in ovarian tumours with wild-type p53. The authors suggest such dual identity may be dependent on p21 expression levels (Rose *et al.*, 2003). In keeping with their findings, in stage 1-2 endometrioid

ovarian cancer, a combination of increased survivin and p53 and low p21 levels appeared to be a poor prognostic marker (Steinbakk *et al.*, 2009). Comparison of clear cell and serous carcinomas of the ovary shows that the prognostic nature of p21 is also dependent on the ovarian cancer subtype. In the latter, low p21 expression is perceived as a poor prognostic marker (Buchynska *et al.*, 2007). In contrast, clear cell carcinomas, known to be more resistant to chemotherapy than other ovarian carcinomas, and generally associated with poorer prognosis, express high levels of p21 .

Schmider-Ross, et al., described p27 as a positive marker of disease-free and overall survival (Schmider-Ross *et al.*, 2006). However, another report suggests that the intracellular localisation of p27 may be crucial. Therefore, predominantly cytoplasmic localisation of p27 predicts poorer prognosis, particularly in late-stage ovarian cancer (Rosen *et al.*, 2005). In epithelial ovarian carcinomas, over-expressed Cyclin E and cdk2, combined with the loss of p27 function, has been found to correlate significantly with malignancy, as compared to borderline or benign tumours (Sui *et al.*, 2001). Aberrant expression of another Cyclin D1, was also significantly related to aggressiveness and predicted poor prognosis in epithelial ovarian cancer (Bali *et al.*, 2004). In addition, a combination of low levels of p21 and p27 with high p53 and Cyclin D1 expression predicted poor clinical outcome in serous carcinomas (Bali *et al.*, 2004).

Overall, it may be necessary to consider cell cycle regulators in combination, rather than as single markers. Also, in view of post-translational regulation, functionality of factors should be studied on mRNA and protein level.

1.7 Aims of this study

- Identification of biomarkers in ovarian cancer that predict cytotoxic efficacy of oncolytic adenovirus *dl*922-947.

- Evaluation of the Rb pathway, as a whole, as well as of individual Rb pathway members, as potential predictive biomarker candidates for response to the oncolytic adenovirus *dl*922-947.

- Identification of further biomarkers of response to *dl*922-947 beyond the Rb pathway and cell cycle control.

2 Materials and Methods

2.1 Cell lines

2.1.1 Human ovarian cancer cell lines

A2780 and A2780CP were kindly provided by Dr Aris Eliopoulos (University of Birmingham, UK) and IGROV-1 by Dr M Ford (Glaxo Wellcome Research and Development, Stevenage UK). CAOV3 and OVCAR5 were obtained from the American Type Culture Collection (ATCC). OVCAR4 were obtained from Dr R. Camalier (NCI-Frederick, MD, USA). SKOV3ip1 were kindly provided by Dr Janet Price (University of Texas-MD Anderson Cancer Center, Texas). SKOV3 cells were obtained from Cancer Research UK Cell Services (Clare Hall, South Mimms, Hertfordshire, UK). TOV21G cells came from Prof Fran Balkwill (Centre for Cancer and Inflammation, Institute of Cancer, Barts and The London School of Medicine and Dentistry, London, UK). Ovarian cancer cell lines were maintained in Dulbecco's modified Eagle medium plus 10% heat-inactivated foetal calf serum and 100µg/ml penicillin/streptomycin (10% FCS E4).

2.1.2 Other human cell lines

JH293, 293, MRC5 and MRC5-VA cells were obtained from the Cancer Research UK Central Cell Services (Clare Hall, Hertfordshire, UK) and were maintained in 10% FCS E4. hTERT-immortalised human ovarian surface epithelial cells IOSE20, IOSE21 and IOSE25, as well as pre-transformed TOSE1 and TOSE4, were also kindly provided by Prof F Balkwill and were maintained in NOSE-CM medium, supplemented with human epidermal growth factor (10ng/ml), hydrocortisone (0.5µg/ml), insulin (5µg/ml), bovine pituitary extract (4µl/ml), and 15% FCS (Li *et al.*, 2004).

2.2 Adenoviral mutants

*dl*922-947 was provided by Dr. D. Kirn (Kirn Oncolytic Consulting, Mill Valley, USA) and is deleted in the region encoding amino acids 122-129 of the E1A CR2 domain, as well as in E3 (Heise *et al.*, 2000). Wild type adenovirus 5 (Ad5 WT) was kindly provided by Dr. W.S. Wold (St. Louis University, St. Louis, MO, USA). In *dl*922-947, the 745bp deletion in E3 lies between base pairs 30,005 and 30,750 and is substituted for by a 642bp non-coding DNA fragment. Ad-CMV-GFP (Ad-GFP) was kindly provided by V. Stoll (Cancer Research UK, Charterhouse Square, London, UK) and is deleted in E1 and E3B with green fluorescent protein (GFP) in the E1 position under the control of the cytomegalovirus (CMV) immediate early promoter.

2.3 Virus amplification

For virus amplification, 293 cells, grown in 10% FCS E4 on a 15cm-plate, were infected with 150µl concentrated virus stock. When clear cytopathic effect was observed 48-72 hours (hrs) later, cells were harvested in their own medium after pipetting up and down to ensure detachment of all cells and then subjected to three rounds of freeze/thawing (liquid nitrogen/37˚C). After centrifugation at 1500xg to remove cell debris, this viral seed stock was used to infect 293 cells in 10% FCS E4 in 20 15cm-plates. To each plate, 1ml viral seed stock was added once 293 cells were 80% confluent. The virus infection was maintained in 5% FCS E4 until cytopathic effect emerged 48-72 hrs later. Cells were harvested as above and centrifuged for 10 minutes (min) at 2000xg after which the supernatant was discarded and the pellet resuspended in 2ml ice-cold 0.1M Tris pH 8.0 per 15cm plate. After three cycles of snap freezing in liquid nitrogen, followed by immediate thawing at 37°C, the virus suspension was centrifuged for 10min at 6000xg prior to separation on caesium chloride (CsCl) gradients.

CsCl gradients were prepared by placing 11.4ml of a 1.25g/ml CsCl solution into an ultra-clear, 25 x 89mm centrifuge tube (Beckman Coulter Ltd, Bucks, UK) and carefully under-layering 7.6ml of a 1.4g/ml CsCl solution. The virus solution was then layered onto the gradient and centrifuged at 25,000rpm for 2hrs at 15°C using a Beckman SW28 swing-out rotor in an Optima LE-80K ultracentrifuge (Beckman Coulter Ltd, Bucks, UK). After the ultracentrifugation, 3 bands were visible; an upper band of cellular debris, a central band of empty virus particles and a lower band of successfully packaged viable adenoviral particles. The ultracentrifuge tube was placed in a clamp and pierced using a 19-gauge needle fitted to a 5ml syringe. The lower virus band was aspirated and layered onto 3ml of a 1.35g/ml CsCl solution in a 31 x 51mm ultra-clear centrifuge tube (Beckman Coulter Ltd, Bucks, UK). The tubes were centrifuged at 40,000rpm for 15hrs at 15°C using a Beckman SW55 swing out rotor in an Optima LE-80K ultracentrifuge (Beckman Coulter Ltd, Bucks, UK). The virus band was then removed with a 19-gauge needle as described above, made up to a total volume of 5ml with TSG (90ml Solution A [150mM NaCl, 1mM Na_2HPO_4, 5mM KCl, 30mM Tris Base], 450µl Solution B [200mM $MgCl_2$, 180mM $CaCl_2$] and 38.5ml Glycerol) and then injected into a 3-12ml Slide-a-Lyzer cassette (molecular weight cut off 3500Da [Pierce Biotechnology Inc., Il., USA]). This cassette was then placed in the float provided and placed in a 5l beaker containing 2l of dialysis solution (10mM Tris pH 7.4, 1mM $MgCl_2$, 150mM NaCl, 10% Glycerol in distilled water). The beaker was then placed on a

magnetic stirrer in a cold room and the virus left to dialyse for 24hrs. Following dialysis, the virus was removed using a syringe, aliquotted and stored at –80°C.

2.4 Viral particle count

For viral particle count determination, viral lysis buffer (0.1% SDS, 10mM Tris pH 7.4 and 1mM EDTA in distilled water) was used. 100µl of virus stock was added to lysis buffer to make a total volume of 200µl. The samples were incubated at 55°C for 10min and then 300µl of distilled water was added. Absorbance of the sample was read at 260nm after a mixture of 200µl lysis buffer and 300µl of distilled water was used to blank the machine. The number of viral particles was then calculated using the following formula:

$$\text{Particles/ml} = \frac{\mathbf{OD_{260}} \text{ x dilution factor (in this case 5)}}{\mathbf{9.09 \times 10^{-13}}}$$

The coefficient factor 9.09 x 10^{-13} was calculated from measurements of virion total protein and OD_{260} (assuming a molecular mass of adenovirus (Ad5) DNA of 2.3×10^7 D and also that 87% of the dry weight of Ad5 is protein) (Mittereder *et al.*, 1996).

2.5 Viral titration by $TCID_{50}$ assay

Infectious virions were quantified by the limiting dilution titration assay, $TCID_{50}$ (tissue culture inhibitory dose, 50%). JH293 cells were seeded in 96-well plates, at least three plates for each sample, at 1×10^4 cells per well in 200µl medium per well. 24hrs after plating the cells, adenovirus test samples were diluted in DMEM and 22µl was added to each well of the second row of each plate. Serial dilutions within the same plate were made, by taking 10% of the volume from each well of the second row (22µl) and adding it to the third row and so on until the final row. The top row of each plate was left uninfected. The JH293 cells were then scored for cytopathic effect (CPE) 11 days post infection. The observed number of wells displaying CPE at each dilution were counted and the $TCID_{50}$ calculated with an established formula (O'Reilly *et al.*, 1994).

$$\mathbf{TCID_{50} = 10^1 - d\ (S - 0.5)}$$

where **d**= Log 10 of the dilution at which CPE is first seen

S= the ratio of the number of wells per row with CPE : number without CPE

This gives a value for 22µl of virus and is divided by 0.022 to give $TCID_{50}$/ml.
Since $TCID_{50}$ overestimates viral titre by 0.7 log compared to plaque assay, $TCID_{50}$/ml is converted to pfu/ml using the following formula:

$$\mathbf{Pfu/ml = 10^{n-0.7}}$$

where **n**=log$TCID_{50}$/ml

The titration is only valid if the lowest dilution gives greater than 50% CPE, the highest dilution gives less than 50% CPE and the negative controls show none.

2.6 Virion detection in serum samples by $TCID_{50}$

In order to quantify infectious virion titres in murine plasma samples, JH293 cells were seeded onto 96-well plates at 10^4 cells per well in 10%FCS E4 medium. The following day. Serum samples were diluted 1:10 in 0.1M Tris, pH8.0, prior to use for limiting dilutions on JH293 cells and average viral titres/ml assessed as above.

2.7 Sensitivity of cells to adenoviral cytotoxicity

Survival of human cell lines following viral infection was estimated with an MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. When MTT (Sigma Chemicals Co., Dorset, UK) is dissolved, its tetrazolium ring can be cleaved to form an insoluble purple formazan by dehydrogenase enzymes such as those found in the mitochondria of living cells (Mosmann, 1983). This converted formazan dye can be solubilised with DMSO (dimethyl sulfoxide; *Fisher Scientific,* Loughborough, UK) and the absorbance of the purple solution measured at 560nm on a plate reader gives an estimate of cell number.

Cell lines were plated on 24-well plates at 1 x 10^4 cells per well. 24hrs later, cells were infected in triplicate with *dl*922-947 at a range of MOIs in 500µl serum-free medium. Three hours later, cells were refed by adding 500µl of 10% FCS -containing medium. Up to 144hrs post transfection, 100µl of a 5mg/ml solution of MTT in PBS was added to each well. After 3hrs incubation at 37°C the medium was discarded and the crystals dissolved in 0.5 - 1ml DMSO per well. Absorbance at 560nm was measured on a Victor^3 Wallac 1420 multilabel counter (PerkinElmer LAS (UK) Ltd., UK) or

alternatively, 100µl per well of the crystal solution from each well were transferred to a well on a 96-well plate and the absorbance measured at 560nm with an Opsys MR microplate reader (Dynex Technologies Ltd, West Sussex, UK). The IC_{50} (inhibitory concentration, 50%), or concentration at which 50% of cells survived, was calculated using GraphPad Prism version 3 (GraphPad Software, San Diego, CA, USA).

2.8 Infectability with adenoviral vectors

2.8.1 Flow cytometry

Flow cytometric analysis was used to evaluate infectability of cells. $5x10^5$ cells were plated on 6cm-plates. 24hrs later, cells were infected with Ad-GFP, an E1 deleted adenovirus type 5 containing the green fluorescent protein gene driven by the cytomegalovirus (CMV) immediate early promoter at a multiplicity of infection (MOI) of either 5 or 50 pfu/cell. 24hrs post infection the cells were trypsinised, re-suspended in medium containing 10% FCS to inactivate the trypsin. Cell suspensions were centrifuged for 5min in an ALC multispeed PK121 centrifuge (ThermoScientific, Waltham, MA, USA) at 2000 rpm. After removal of the supernatant, the cell pellet was re-suspended in phosphate buffered saline (PBS) and then centrifuged for a further 5min at 2000 rpm. The cells were washed once more with PBS and re-suspended in 500µl PBS per sample. Samples were processed in a BD FACSCalibur™ cytometer (BD Biosciences, San Jose, CA, USA) using CellQuest software (Becton Dickinson, Oxford, UK) or FlowJo software 8.8.4 (Tree Star, Ashland, OR, USA) and GraphPad Prism version 3 (GraphPad Software, San Diego, CA, USA). Infectivity was determined after 10,000 total events were recorded. The percentage of GFP-positive cells was determined from the total event count. All experiments were carried out in triplicate.

2.8.2 Quantitative PCR

For quantitative PCR (qPCR) of viral DNA for assessment of infectability with *dl*922-947, $5x10^5$ cells were plated on 6cm-plates. 24hrs later, cells were infected with *dl*922-947 at MOI 10pfu/cell in serum-free medium. Two hours later, cells were scraped into 500µl cold PBS and frozen at -70°C. DNA was extracted from the collected cell samples using a QIAamp DNA Blood Mini Kit (Qiagen, Crawley, West Sussex, UK). To create a standard curve, the concentration of DNA from undiluted wild type adenovirus (provided by Dr Michelle Lockley, Centre of Molecular Oncology and Imaging, Institute of Cancer, Barts and The London, UK) was measured with a NanoDrop® ND-1000 spectrophotometer (Wilmington, DE, USA). The standard curve for genomic DNA was created using wild type adenoviral DNA, according to instructions provided by Applied

Biosystems (Cheshire, UK). Briefly, the mass of DNA per haploid genome was calculated using the following formula:

$$m = n \times 1.096e\text{-}21$$

where: **m**= mass of haploid DNA
n= genome size in base pairs

The concentration of genomic DNA needed to achieve the copy number of interest was calculated as follows:

$$\text{concentration required (C2)} = \frac{\text{copy number of interest} \times \text{mass of haploid DNA}}{\text{volume to be used per sample}}$$

From the initial, measured (C1) and required (C2) DNA concentrations, and assuming a final volume (V2) of 100µl, the volume of stock DNA (V1) needed to obtain the highest concentration of DNA for the standard curve was calculated according to the following formula:

$$C1 \times V1 = C2 \times V2$$

Serial dilutions of this stock were prepared by 1:10 dilutions in nuclease free water (Ambion, Cambridgeshire, UK) as shown in Fig. 2.1.

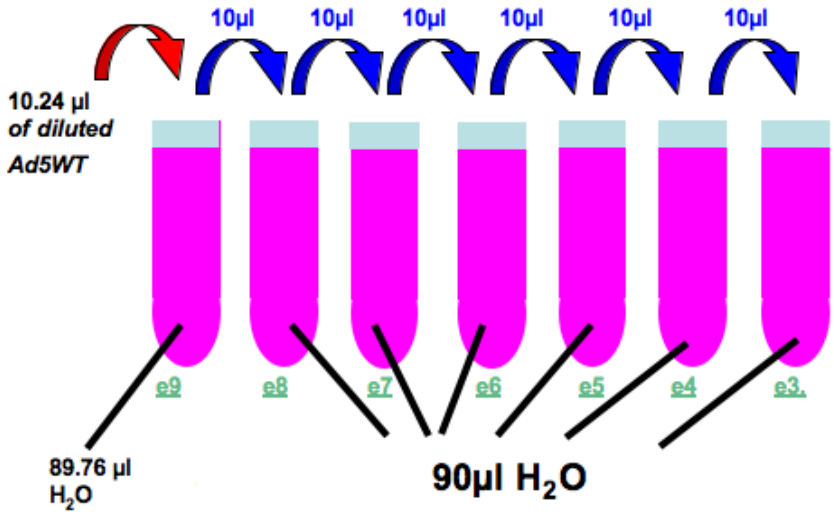


Figure 2.1 Serial dilution of qPCR standard curve template DNA stocks.

The DNA samples from infected cells were quantified using the NanoDrop® ND-1000 spectrophotometer and diluted to a concentration of 100ng/µl in nuclease free water (Ambion, Cambridgeshire, UK). Primers and probes were purchased from Applied Biosystems (Cheshire, UK) and are listed in Table 2.1 below. Probes bind DNA between the two primers. Each probe is labelled with a reporter fluorophore at its 5' end, as well as a quencher fluorophore at the 3' end. The proximity of the two fluorophores quenches emitted fluorescence. During the PCR, polymerase extends DNA from the primers, fluorophores are cleaved off and the quencher effect is lost. Hence, a fluorescence signal is emitted, which can be detected by the PCR equipment.

Variability due to pipetting errors were kept at a minimum by quantifying E1A and hexon DNA simultaneously in each sample. 2µl template (viral DNA or DNA standard) and non-template control were mixed with 2X reaction buffer (TaqMan Universal PCR Master Mix, Applied Biosystems, Cheshire, UK) and 5µM each of forward primers, reverse primers and probes for both E1A and hexon. Samples were plated in triplicate on a 96-well-plate (Applied Biosystems, Cheshire, UK). The plate was then covered with an optical adhesive lid and the samples centrifuged briefly to remove air bubbles.

A.

	5’ Primer sequence
E1A	5’-CCACCTACCCTTCACGAACTG-3’
Hexon	5’-AGCGCGCGAATAAACTGCT-3’

	3’ Primer sequence
E1A	5’-GCCTCCTCGTTGGGATCTTC-3’
Hexon	5’-AGGAGACCACTGCCATGTTGT-3

B.

Dye	Probe Sequence
VIC	5’-ATGATTTAGACGTGACGGCC-3’
6-FAM	5’-CCGCCGCTCCGTCCTGCA-3’

Table 2.1 Primers (A.) and probes (B.) used for quantitative PCR.

The PCR reaction was carried out on a 7500 Real Time System (Applied Biosystems, Cheshire, UK) using the following program:

50°C for 2 min
95°C for 10 min followed by:
95°C for 15 seconds
60°C for 60 seconds for 40 cycles.

Light emission from the relevant probe was detected by the 7500 System SDS Software (Applied Biosystems, Cheshire, UK). This is quantified as the cycle threshold (CT), or the number of PCR cycles needed for emission to reach a pre-determined level. The software then constructs a curve for the DNA standards in which CT is plotted against log DNA copy number. A slope of -3.3 indicates 100% PCR efficiency, in which there is a tenfold increase in PCR product every three cycles. Slopes within the range -3.1 to -3.6, representing PCR efficiency of greater than 90%, are acceptable according to publications in the field (http://www.dorak.info/ genetics/glosrt.html). From this standard curve, the software quantified DNA copy number for each sample.

2.9 Protein expression

$5x10^5$ cells were seeded onto 6cm-plates. For viral protein, cells were either mock-infected or infected at MOI 10pfu/cell with the relevant virus after 24hrs. Up to 144hrs later, cells were washed twice with cold PBS and then scraped into 200µl per plate of Western lysis buffer (150mM NaCl, 50mM Tris Base, 0.05% SDS, and 1% Triton X 100). Samples were sonicated, incubated on ice for one hour, then centrifuged at 6000rpm for 5min and the supernatants stored at –20°C.

2.9.1 Protein concentration evaluation

The protein concentration of cell lysates was measured using the DC protein assay (Bio-Rad, CA, USA). Dilutions of protein ranging from 0.1mg/ml to 1.0 mg/ml were made using bovine serum albumin (BSA, Sigma Chemicals Co., Dorset, UK) as positive protein standard controls. Protein lysates were first diluted 1:5 with Western lysis buffer. Reagent A' was prepared by adding 20µl Bio-Rad reagent S to 1ml Bio-Rad reagent A. To each 5µl of protein standard or cell lysate, 25µl A' and 200µl Bio-Rad reagent S was added. A total of 230µl of this mixture was added per well of a 96-well plate, with protein standards being measured in duplicate and protein lysates in triplicate. The plate was incubated at room temperature for 10 min and then the absorbance measured at 630nm with an Opsys MR microplate reader (Dynex Technologies Ltd, West Sussex, UK). The absorbance of the protein standards was used to construct a standard curve and the equation for this line was used to calculate the protein concentrations of the cell lysates.

2.9.2 Western blot analysis (Immunoblotting)

10% to 15% denaturating SDS polyacrylamide gel electrophoresis (SDS PAGE) gels were poured as shown in Table 2.2. Once set, a stacking gel was poured on top (Table 2.2). In separate 1ml tubes, up to 30µg/lane of the protein lysates were mixed with dH_2O and 2x loading buffer (50mM Tris, 4% SDS, 10% Glycerol, 5% Mercaptoethanol and 0.01% Bromophenol Blue) to a total volume of 20-30µl/lane. Samples were boiled on a heating block at 100°C for 5 min, then transferred to ice and centrifuged briefly before loading. Gels were prepared with a Hoefer SE-245 Western blot system (FisherScientific, Pittsburgh, PA). Electrophoresis took place at 110 -120V for 90 – 120 min in 1X running buffer diluted from a 10X stock (30.2g Tris Base, 94g Glycine and 100ml 10% SDS in dH_2O at total volume of 1 litre). Molecular weight rainbow markers (Amersham Biosciences, Bucks, UK) were loaded alongside the protein samples to allow size comparison of bands. Proteins were transferred onto nitrocellulose

membranes (Amersham Biosciences, Bucks, UK) using a semi-dry transfer system (Trans-Blot, Bio-Rad, CA, USA), set at 20V for 45 -60 min, in the presence of transfer buffer (39mM Glycine, 48mM Tris Base and 20% Methanol in dH_2O at total volume of 1 litre).

Membranes were blocked with a 4% solution of non-fat milk protein in a solution of 0.1% Tween 20 (Sigma Chemicals Co., Dorset, UK) in PBS (0.1% Tween/PBS) for at least one hour at room temperature (RT) or over night (o/n) at 4°C. The primary antibody was diluted between 1:1000 and 1:2000 in a 1.5% solution of BSA (Sigma Chemicals Co., Dorset, UK) in 0.1% Tween/PBS and incubated with the membrane at least 2hrs at RT or at 4°C overnight. Primary antibody was removed by three sequential washes with 0.1% Tween/PBS for 5, 10 and 15 min. Horseradish peroxidase-conjugated secondary antibody was diluted 1:2000 with a 1.5% solution of BSA (Sigma Chemicals Co., Dorset, UK) in 0.1% Tween/PBS and incubated with the membrane for one hour at RT. The three wash steps were repeated after removal of the secondary antibody. Details of the antibodies used are listed in Table 2.3, below.

Reagent	**10%**	**15%**	**Stacking gel**
Water	4.0ml	2.3ml	3.4ml
30% Proto-Gel (Kimberley Research Hull UK)	3.3ml	5.0ml	0.83ml
1.5M Tris (pH 8.8) (Cancer Research UK, Clare Hall, UK)	2.5ml	2.5ml	-
1M Tris (pH 6.8) (Cancer Research UK, Clare Hall, UK)	-	-	0.63ml
10% SDS (Bio-Rad, CA, USA)	0.1ml	0.1ml	0.05ml
10% Ammonium persulphate (Sigma Chemicals Co., Dorset, UK)	0.1ml	0.1ml	0.05ml
TEMED (N,N,N',N'- Tetramethylethylenediamine) (Sigma Chemicals Co., Dorset, UK)	0.004ml	0.004ml	0.005ml

Table 2.2 Components of SDS PAGE resolving (10%, 15%) and stacking gels. *Reagent, suppliers and volumes are listed*.

The chemiluminescent detection of horseradish peroxidase-conjugated secondary antibodies was performed by ECL Plus Detection reagent (Amersham Biosciences, Bucks, UK) according to the manufacturer's instructions. Visualisation of signal was achieved with BioMaxMR film (Kodak, UK).

Antibody	**Primary/ Secondary**	**Species**	**Supplier**
E1A	Primary	Mouse	Oncogene Research Products, CA, USA
Penton	Primary	Rabbit	Kindly provided by Dr P Freimuth, Brookhaven National Laboratory Upton, NY, USA
Hexon	Primary	Goat	Accurate Chemical and Scientific Corp., New York, USA
Ad5 structural proteins	Primary	Goat	Abcam, Cambridge, UK
Actin	Primary	Goat	DakoCytomation, Denmark
Anti-mouse HRP	Secondary	Rabbit	DakoCytomation, Denmark
Anti-rabbit HRP	Secondary	Goat	DakoCytomation, Denmark
Anti-goat HRP	Secondary	Rabbit	DakoCytomation, Denmark
Retinoblastoma Protein (pRb)	Primary	Mouse	BD PharMingen, Oxford, UK
p16	Primary	Mouse	BD PharMingen, Oxford, UK
Cdk4	Primary	Mouse	BD PharMingen, Oxford, UK
Cyclin D1/D2/D3	Primary	Mouse	BD PharMingen, Oxford, UK
Cdk2	Primary	Mouse	BD PharMingen, Oxford, UK
Cyclin E	Primary	Mouse	BD PharMingen, Oxford, UK
p27	Primary	Mouse	BD PharMingen, Oxford, UK
p21	Primary	Mouse	BD PharMingen, Oxford, UK
Actin	Primary	Goat	DakoCytomation, Denmark
phospho - pRb	Primary	Mouse	BD PharMingen, Oxford, UK

Table 2.3 Antibodies used to detect viral and cellular proteins. *Antibodies, species and suppliers. Antibodies were diluted 1:1000–1:3000 in a 1.5% solution of BSA in 0.1% Tween/PBS. All murine antibodies were monoclonal, and the others were polyclonal.*

2.9.3 Densitometric analysis

Densitometric analysis of band intensity in Western blot experiments was performed on scanned films using ImageJ software (Collins, 2007) and GraphPad Prism version 3 (GraphPad Software, San Diego, CA, USA). Ratios were calculated using loading

controls (actin, Ku70, GAPDH), in order to correct for any variation in the loading, and are shown as relative units (RU) in bar graphs.

2.10 Viral replication

2.10.1 $TCID_{50}$ assays

To quantify intracellular production of infectious viral particles, $1x10^5$ cells were seeded in 6-well plates in appropriate medium. Cells were infected 24hrs later with *dl*922-947 at MOI 10 pfu/cell in serum-free medium and re-fed 2hrs later with serum-containing medium. Up to 72 hours post infection, cells were washed twice with PBS and then scraped into 0.5ml 0.1M Tris pH 8.0. The collected samples were freeze/thawed three times and replicating virus titre (expressed as plaque forming unit (pfu)/ml) determined 11 days later by limiting dilution $TCID_{50}$ assay as described above. Viral titre in pfu/ml can be converted to pfu/cell as below:

$$\text{pfu/cell} = \frac{\text{pfu/ml x volume harvested}}{\text{initial number of cells infected}}$$

In this case the volume harvested was 0.5ml and the initial cell number was $2x10^5$, assuming that the $1x10^5$ cells had doubled over night.

2.10.2 Quantitative PCR

To quantify viral DNA replication in infected cells, $1x10^5$ cells were infected with *dl*922-947 at MOI 10pfu/cell. 24, 48 and 72hrs later, cells were harvested, DNA was extracted using a QIAamp DNA Blood Mini Kit (Qiagen, Crawley, West Sussex, UK) and the DNA concentration measured with a NanoDrop® ND-1000 spectrophotometer (Wilmington, DE, USA). Samples were diluted to 100ng/µl prior to analysis by qPCR.

2.11 Transient knock-down of p21, p16 and Cyclin D1, D2 and D3 using small interfering RNA

For transient knock-down of p21, p16 and Cyclin D1, D2 and D3, IGROV-1 and TOV21G cells were treated with Dharmacon SMARTpool siRNA (ThermoFisher Scientific, Lafayette, CO, USA) targeting the gene of interest or Scrambled (scr) Control Non-Targeting siRNA pool as a control (ThermoFisher Scientific, Lafayette, CO, USA). DharmaFECT_1 was used as transfection reagent (ThermoFisher Scientific, Lafayette, CO, USA).

2.11.1 Optimisation of siRNA doses

To determine the optimal dose of siRNA for knock-down, cell were transfected with 20pmol, 60pmol or 200pmol of siRNA. First, $3x10^5$ cells/well were plated on 6-well plates. 24 hours later, transfection reactions were prepared as described in Table 2.4.

	A		B	
siRNA dose	**siRNA stock (2µM)**	**Serum-free medium**	**DharmaFECT_1**	**Serum-free medium**
20pmol	10µl	10µl	0.6µl	19.4µl
60pmol	30µl	30µl	2µl	28µl
200pmol	100µl	100µl	6µl	194µl

Table 2.4 Transfection reactions for siRNA knock-down. *Components of transfection reactions for siRNA as well as amounts used are listed. Components in column pairs A and B were mixed in a first step, then combined in a second step.*

First, for each dose of siRNA, siRNA stock or DharmaFECT_1 were mixed with serum-free medium in separate tubes (column pairs A and B). After 5min incubation at RT, contents from mix A were combined with respective mix B, followed by incubation at RT for 20min. Next, the volume of each tube of reaction mix was increased to a total of 1ml by adding complete medium (penicillin/streptomycin-free 10% FCS E4). Contents of each tube were added dropwise to cells whilst swirling the plate, before adding a further 1ml of complete medium to each well. The optimum dose of siRNA, i.e. the lowest dose effectively knocking down the protein of interest, was identified by immunoblotting, and was used for subsequent experiments.

2.12 Transfection of cells with pEGFP-p21 and control vector

For transfection of SKOV3ip1 and A2780CP cells to increase intracellular p21 levels, plasmid pEGFP-p21 was used. pEGFP-p21 was a kind gift from Dr Sally Wheatley, University of Sussex, UK. It had been constructed by inserting wild type p21 cDNA into the EGFP-N1 vector (Clontech), as described in (Cazzalini *et al.*, 2003). The resulting p21-eGFP fusion protein is expressed under the control of the human CMV promoter.

SKOV3ip1 and A2780CP cells were seeded onto 6-well plates at a density of $5x10^6$ cells/well. The following day, transfection mixes were prepared from FuGENE 6 and DNA at FuGENE 6:DNA ratios of 3:1 using pEGFP-p21 or pCMV-GFP. One mix per

cell line was prepared without DNA for mock-transfections. Cells were transfected as described above and cultured in selective growth medium containing 1mg/ml G-418 in 10%FCS E4.

2.13 Generation of p21 over-expression cells using pCEP-WAF1

SKOV3ip1 and A2780CP cells were transfected with a p21-encoding plasmid to increase intracellular levels of the protein. For controls, both cell lines were also transfected with a plasmid expressing GFP under the control of a CMV promoter. Plasmid pCEP-WAF1 (Addgene, Cambridge, MA, USA) encodes p21 under transcriptional control of a human cytomegalovirus (CMV) immediate early promoter. It also confers resistance to hygromycin in eukaryotic cells.

The plasmid used to create control cell lines was pCMV-GFP (Stratagene, La Jolla, CA, USA). The plasmid contains a neo-cassette, which imparts resistance to the antibiotic G-418 in eukaryotic cells. SKOV3ip1 and A2780CP cells were seeded onto 6-well plates at a density of 5x 10^5 cells/well. The following day, transfection mixes were prepared from plasmid DNA and FuGENE 6 according to the manufacturer's protocol and at FuGENE 6:DNA ratios 3:1 and 3:2. After the final incubation step, each transfection mix was added dropwise cells whilst swirling the plate. One well per cell line was mock-transfected with FuGENE 6 alone. After 48 hours, cells from each well were transferred to two T25 flasks and grown in10% FCS E4 medium containing 500µg/ml or 1mg/ml G-418 to determine the minimum required antibiotic concentration for selective growth of transfected cells. Subsequently cells were cultured in medium containing the higher concentration of antibiotic, which was lethal to mock-transfected but not to plasmid-transfected cells.

2.14 Inhibition of proteasomal degradation after infection

Six hours prior to harvest, cells on 6cm-plates were refed with medium containing 50µM proteasome inhibitor MG132. Cell lysates for total protein samples were harvested 24 and 48hrs p.i.. In parallel, cell lysates from infected but non-treated cells were harvested at the point of re-feeding (0h), as well as 24 and 48 hours p.i.

2.15 Induction of genotoxic stress by X-irradiation

To assess effects of genotoxic stress on cellular p21 levels, 10^6 cells were seeded per 6cm-plate. After allowing 2 hrs for attachment, cells were exposed to 5 Gray (Gy) X-

irradiation, using the Hs-X-Ray System (A.G.O. Installations Ltd., Reading, UK). Protein samples for Western blotting were harvested 6 hrs post irradiation.

2.16 *In Vivo* studies

Six-week-old female CD1 or BALC/c nu/nu mice were purchased from Harlan (Blackthorn, Bicester, UK) or Charles River Laboratories, Inc. (Margate, UK). All animal studies were conducted under suitable UK Home Office Personal and Project Licence authority.

2.16.1 Efficacy study with Hct116 xenografts

Female CD1 nu/nu mice received subcutaneous injections of $5x10^6$ Hct116 p21+/+ or p21-/- cells were. The animals were left until tumours reached approximately $150mm^3$, then treated with intra-tumoural injections of 10^{10} particles of dl922-947 or control virus Ad-CMV-GFP in 50µl PBS on three occasions. Tumours were measured twice weekly with callipers. Tumour volumes were calculated with the following formula:

$$\text{Volume} = \frac{(L \times L \times W)}{6} \times \pi$$

where L = largest length

W = perpendicular width

Mice were killed once Home Office limits were reached. Studies were ended 42 or 84 days after first treatment, killing all remaining animals.

2.16.2 Efficacy study with ACP-WAF1 and –GFP xenografts

Female BALB/c nu/nu mice received intraperitoneal (i.p.) injections of ACP-WAF1 or ACP-GFP cells. Injections were given into both sides of the peritoneum, with a total inoculum of $5x10^6$ in 200µl PBS per animal. Preparation of cells for injections was performed as follows: cells were trypsinised and rescued into 10%FCS E4 medium. After counting, cells were centrifuged for 5 min at 1500rpm in an ALC multispeed PK121 centrifuge (ThermoScientific , Waltham, MA, USA) and pellets resuspended in 40ml PBS. Cells were washed twice in PBS by centrifugation. Final pellets were resuspended in PBS to obtain $2.5x10^7$ cells/ml. For injections, 1ml syringes and 25-gauge needles were used. Starting either day 2 or day 6 after injections of cells, mice

received i.p. injections of virus on 5 consecutive days. Ten mice from the ACP-WAF1 and the ACP-GFP group were treated with $5x10^9$ viral particles of *dl*922-947 in 400µl 20% icodextrin (Innovata plc, Nottingham, UK). The remaining ten animals from each group were treated with $5x10^9$ particles of non-replicating control virus Ad-GFP in 400µl 20% icodextrin. Each animal was injected with 200µl into each flank using 1ml syringes and 25-gauge needles. Animals were monitored daily and killed when reaching Home Office limits.

2.16.3 Replication study

To detect viral activity in BALB/c nu/nu mice bearing ACP-WAF1 or ACP-GFP xenografts, 10 female animals were injected IP with either $5x10^6$ ACP-WAF1 or ACP-GFP as before on day 1. Mice were left for 10 days to allow tumours to establish. On days 11-13, mice were injected with 10^{10} viral particles of *dl*922-947 in 400µl icodextrin as described above. 24 and 48hrs after the last virus treatment, blood samples were taken from each mouse via the tail-vein or by terminal cardiac puncture. Blood samples were left to coagulate on ice, centrifuged in an Eppendorf PK121 benchtop centrifuge (Eppendorf UK Limited, Cambridge, UK) at 1500xg for 5min to separate serum and cellular fraction. Serum was transferred to fresh eppendorf tubes and stored at -70ºC until further use. 48hrs after the last treatment, animals were killed and livers and tumours harvested into 10% neutral phosphate buffered *formalin (Fisher Scientific,* Pittsburgh, PA) or snap frozen on dry ice and ethanol, then stored at -70ºC until further use.

2.16.4 Isolation of viral DNA from blood and organs for qPCR

After diluting 50µl of serum from each sample in PBS to yield a total volume of 200µl and centrifugation for 15 min at 1500xg in an eppendorf PK121 benchtop centrifuge (Eppendorf UK Limited, Cambridge, UK), supernatants were transferred to fresh tubes and volumes adjusted to 200µl with PBS. Viral DNA was extracted using a Qiagen QIAmp DNA minikit (Qiagen UK, Crawley, West Sussex, UK) according to manufacturer's protocol. As carrier DNA, 5µg of sheared denaturated salmon sperm DNA were added. Finally, extracted DNA was eluted in 100µl elution buffer (10µM Tris pH8.0) and stored at -20ºC until analysis by qPCR.

2.16.5 Virion detection in plasma samples by qPCR

To quantify virus genome copy number in murine plasma samples, qPCR analysis was performed as described above. 5µl from each sample of extracted DNA were added to the reaction mix/well.

2.16.6 Visualisation of viral gene expression in tissue samples by immunohistochemistry

Tumours and livers harvested from mice bearing ACP-WAF1- and ACP-GFP-derived tumours, stored in 10% neutral phosphate buffered *formalin (Fisher Scientific,* Pittsburgh, PA), were processed in the Molecular Pathology Lab (Centre for Molecular Oncology and Imaging, Institute of Cancer, Barts and The London School of Medicine and Dentistry, London, UK). Tissues were embedded in paraffin, 4µm slides prepared from each tissue sample and stained for E1A and counterstained with haematoxylin. In addition, slides from tumours were stained for p21 using a mouse-on-mouse kit (Millipore, Billerica, MA, USA). For antibodies used, see Table 2.5. Images were acquired with a Nikon DXM1200 digital camera (Nikon, Tokyo, Japan) mounted on a Zeiss Axiophot microscope (Carl Zeiss, Inc., USA).

Antibody	**Primary/ Secondary**	**Species**	**Supplier**
E1A 13S-5	Primary	Rabbit	Santa Cruz Biotechnology, CA, USA
p21	Primary	Mouse	BD PharMingen, Oxford, UK
Anti-mouse HRP	Secondary	Rabbit	GeneTex, Irvine, CA, USA
Anti-rabbit HRP	Secondary	Rabbit	GeneTex, Irvine, CA, USA

Table 2.5 Antibodies used in immunohistochemistry staining. *Antibody, species and supplier.*

2.17 Cell Cycle Flow Cytometric analysis

Cells were seeded onto 6-well plates with $5x10^6$ cells in 5ml medium per well. The following day, cells were infected with *dl*922-947 at MOI10 pfu/cell in 3ml serum-free medium or mock-infected with serum-free medium, alone. Cells were refed after 3hrs by adding 3ml 10% FCS E4 to each well. Cells were harvested 48hrs post-infection by trypsinising and rescue with complete medium. Cells were centrifuged 5min at 1200rpm in an ALC multispeed PK121 centrifuge (ThermoScientific, Waltham, MA,

USA). Pellets were washed twice in cold PBS and ultimately fixed by resuspending in 1ml ice-cold 70% EtOH whilst vortexing. Prior to staining, cells were spun 5min at 2000rpm in an ALC multispeed PK121 centrifuge (ThermoScientific, Waltham, MA, USA), supernatants were discarded and cell pellets resuspended in 5ml PBS, followed by another centrifugation step for 5min at 1500rpm. After aspirating supernatants, 250µl of RNase solution (200µg/ml in PBS) and 250µl propidium iodide (PI, Invitrogen, Paisley, UK) solution (100µg/ml in PBS) were used to resuspend each cell pellet. Cell suspensions were incubated in the dark at RT for 30 min. Cell cycle analysis was carried out on a BD FACSCalibur™ cytometer (BD Biosciences, San Jose, CA, USA) using CellQuest software (Becton Dickinson, Franklin Falls, NJ, USA) and FlowJo software 8.8.4 (Tree Star, Ashland, OR, USA).

2.18 Microarray analysis of gene expression

2.18.1 Microsatellite genotyping of MRC5 and MRC5-VA cells

To confirm the isogenic character of the MRC5 and MRC5-VA cell line pair, microsatellite genotyping was carried out by the Genome Centre (William Harvey Research Institute, Barts and The London School of Medicine and Dentistry, London, UK). Microsatellites are short tandem repeats of approximately 3-6 nucleotides, generally found within non-coding regions of the genome (Hamada *et al.*, 1982; Stringer, 1985). As their length and sequence vary between genomes of different individuals, but should be highly similar between two copies of the same genome, they can serve as molecular markers to test for isogenicity between cells (Choudhary *et al.*, 1993). The microsatellite genomic fingerprint of MRC5-VA cells, which are transformed MRC5 cells, was expected to overlap well with that of parental MRC5 cells. Analysis was performed on DNA extracted from both cell lines by the phenol-chloroform method: Cells were grown on 15cm-plates until approximately 80% confluent, washed twice with cold Tris-buffered saline (TBS) and scraped into 1ml digestion buffer (1% SDS, 0.5mg/ml proteinase K, 50mM Tris-Cl pH9.0, 200mM NaCl). For all subsequent steps an eppendorf 5417R centrifuge was used. After incubation at 55ºC for 15hrs, lysates were transferred to 15ml-tubes and left to cool to room temperature. 1ml of phenol was added to each tube prior to incubation at RT for 3hrs, which was followed by centrifugation for 20min at 6000xg and RT. The top (aqueous) phase was transferred to a fresh 15ml tube and 1ml phenol/chloroform/isoamyl alcohol was added. Suspensions were vortexed and centrifuge in the same centrifuge as before for 10min at 12000xg at RT. Again, the aqueous layer was transferred to a fresh tube and 1ml chloroform added, followed by vortexing and centrifuging at 1700xg for 2min. After

transferring the top (aqueous) layer to a new tube and adding, first, 500µl 3M sodium acetate, then 1.5ml ice-cold isopropanol, a centrifugation step was carried out at 1700xg for 2min. Pellets were left to dry at RT, before DNA was dissolved in up to 1ml 100mM Tris-Cl pH8.0, 5mM EDTA, 100mM NaCl ($T_{100}E_5N_{100}$) plus 1µg/ml RNase A (34mg/ml, Sigma Chemicals Co., Dorset, UK). Next, the volume in the tubes was doubled by adding equal amounts of phenol/chloroform/isoamyl alcohol, suspensions were vortexed and centrifuged for 10min at 12000xg. The chloroform extraction step was repeated as above. Then, the top (aqueous) layer was transferred to a new tube, before adding 2ml of 100% EtOH and centrifuging for 2min at 12000xg. After air-drying, the DNA pellet was dissolved in dH_2O.

For the analysis, three separate panels of fluorescently labelled oligonucleotide primers (ABI) were used in the amplification of 14 of loci on 6 chromosomes before electrophoresis and detection using the Applied Biosystems 3700 DNA analyzer (Applied Biosystems Inc., Foster City, CA, USA).

2.18.2 Extraction and preparation of RNA from cultured cells

Cells were grown on 10cm-plates until 80-90% confluent, when they were trypsinised, transferred to 15ml falcon tubes and washed twice in PBS. RNA was extracted by the phenol-chloroform method. Each cell pellet suspended in 1ml TRIzol (Invitrogen, Paisley, UK) and incubated at RT for 5 min. Addition of 200µl chloroform and vigorous shaking for 15 seconds was followed by another incubation at RT for 2-3 min. For adequate phase separation, tubes were centrifuged 10 min at 12000xg and 4ºC. The aqueous upper phases were transferred to fresh tubes. To precipitate the RNA, 500µl isopropanol were added to each tube, before incubating at RT for 10min. Then the RNA was pelleted by centrifugation for 10 min at 12000xg and 4ºC. RNA was washed by removing supernatant, vortexing pellets in 1ml 75% EtOH and centrifuging 5 min at 7500xg and 4ºC. Finally, RNA pellets were briefly air-dried, re-dissolved in 100µl RNase-free H_2O and incubated for 10 min at 55-60ºC. A clean-up step to remove contaminating DNA was performed using a QIAamp RNeasy Mini Kit (Qiagen, Crawley, West Sussex, UK). First, 350µl of RLT buffer were added to each RNA suspension, followed by 250µl 100% ethanol. Samples were transferred onto RNeasy Mini spin columns and centrifuged at 8500xg for 15 seconds. Flow-through was discarded and the columns washed with 350µl Buffer RW1 by repeating the centrifugation steps. Again, flow-through was discarded. To ensure removal of all contaminating DNA, 10µl RNase-free DNase I (2.7 units/µl) in 70µl Buffer RDD were pipetted onto each membrane. Incubation at RT for 15min was followed by a wash step

with 350µl Buffer RW1 and repeating the centrifugation as above. Next, columns were washed with 500µl Buffer RPE and centrifugation at 8500xg for 15 seconds. This washing step was repeated, centrifuging for 2min. After discarding the flow-through, membranes were dried further by centrifuging for an additional minute at maximum speed. Elution of RNA was achieved by pippetting 30µl RNase-free water onto each membrane and centrifuging at 8500xg for 1min.

2.18.3 Microarray analysis

Microarray analysis was carried out using GeneChip Affymetrix System (Qiagen, Crawley, West Sussex, UK). The Chip format was U133 Plus 2.0. Unless stated otherwise, buffers and compounds used were part of the GeneChip kit. The thermal cycler used for all PCR steps was a BioRad/MJ Research DYAD (PTC-0220) (Bio-Rad, Hertfordshire, UK). As the starting amount of total RNA was 7µg, serial dilutions of Poly-A RNA Control stock were prepared as shown below (Fig. 2.2).

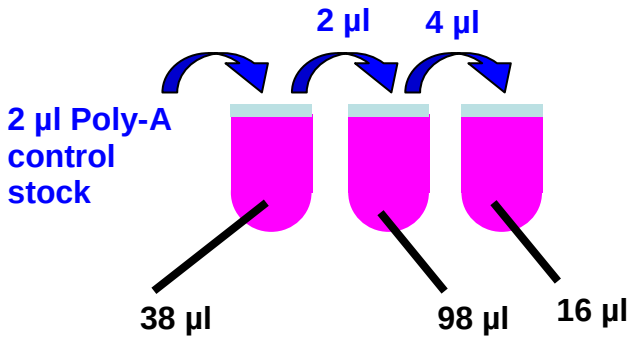


Figure 2.2 Dilution steps for Poly-A Controls spike-in solution. *To obtain the final dilution of 1:5000, a three-step serial dilution was performed.*

For the first-strand cDNA synthesis, 7µg of total RNA were mixed with 2µl diluted Poly-A controls and 7µl T7-oligo(dT) primer (50µM) in a 0.5ml PCR reaction tube. The volume of each reaction mix was adjusted to 11µl with RNase-free water. Tubes were placed in the thermal cycler and the following programme was used for first-strand cDNA synthesis:

70ºC - 10min
4ºC - 5min
42ºC - 2min
42ºC - 1h
4ºC - hold

After 10 min at 70ºC and 2 min at 4ºC 7µl of master-mix (MM) were added to each tube. The MM contained 4µl 5x 1st Strand Reaction Mix, 2µl DTT (0.1M) and 1µl dNTP (10mM) per reaction tube. After incubation for 2 min at 42ºC, 1µl SuperScript II (reverse transcriptase enzyme) were pipetted into each PCR tube. Once the end of the programme was reached, second-strand cDNA synthesis was set up. The thermal cycler programme used for this step was:

16ºC - 2hrs
4ºC - 5min
16ºC - 5min
4ºC - hold

A second-strand MM was prepared from 91µl RNase-free H_2O, 30µl 5X 2nd Strand Reaction Mix, 3µl dNTP (10mM), 1µl *E.coli* DNA Ligase, 4µl *E.coli* DNA Polymerase I and 1µl RNase H per reaction. Upon mixing the MM with the solutions of cDNA from the previous step, the total volume was divided into two 0.5ml PCR reaction tubes, so as not to exceed the maximum volumes permitted for this particular thermal cycler. During the first 4ºC-step of the reaction, 1µl T4 DNA Polymerase were pipetted into each tube. Once this second synthesis step was finished, corresponding sample pairs were pooled and mixed with 10µl 0.5M EDTA to inactivate enzymatic activity. For clean-up of the synthesised double-stranded cDNA, each sample was transferred to a fresh 1.5ml eppendorf tube. After adding 600µl cDNA Binding Buffer and vortexing, 500µl of each suspension were pipetted onto a cDNA Cleanup Spin column. Columns were centrifuged for 1min at 8000xg, the flow-through was discarded and the remaining suspension added to the respective column. Upon centrifugation for 1min at 8000xg and discarding of flow-through, 750µl cDNA Washing Buffer were added. Centrifugation was repeated 1min at 8000xg, again discarding flow-through. Finally, membranes were dried by spinning for 5min at maximum speed with open column lids. Elution was achieved by adding 14µl cDNA Elution Buffer, leaving for 1 min to incubate at RT, then centrifugation for 1 min at maximum speed.

For synthesis of biotin-labelled cRNA, a mastermix was prepared of the following components per sample: 8µl RNase-free H_2O, 4µl 10X IVT Labelling Buffer, 12µl IVT Labelling NTP Mix and 4µl IVT Enzyme Mix. To 28µl MM, 12µl template cDNA obtained in the previous step were added. The reactions mixes were incubated in the thermal cycler at 37ºC for 17-20hrs. Clean-up of the resulting biotin-labelled cRNA was carried out in a similar fashion as before. Firstly, 60µl RNase-free H_2O and 350µl IVT cRNA Binding Buffer were added to each tube, vortexing after each step. Next, 250µl 100% EtOH was mixed in by pipetting. Each suspension was loaded into a cRNA Cleanup Spin Column and centrifuged 15sec at 8000xg. After washing with 500µl IVT cRNA Wash Buffer, columns were centrifuged for another 15sec at 8000xg, followed by 500µl 80% EtOH and repeating the spinning step. To dry the membranes, columns were centrifuged 5 min at maximum speed with open lids. For elution, 11µl RNase-free H_2O were loaded onto the membranes before centrifuging for 1min at maximum speed. This was followed by another elution step with 10µl RNase-free H_2O and centrifugation. Before continuing with the fragmentation of the labelled cRNA, the adjusted cRNA yield had to be calculated. This is required when using total RNA as starting material for microarray analysis, in order to take into account the carry-over of unlabelled total RNA in the samples. To this end, each sample underwent spectrophotometric analysis using was measured with a NanoDrop® ND-1000 spectrophotometer (Wilmington, DE, USA). To determine adjusted cRNA yields the following formula was applied:

$$\textbf{Adj. cRNA yield} = \textbf{RNA}_m - (\textbf{total RNA}_i) \times y$$

where

$\textbf{RNA}_m$ = amount of cRNA after IVT (µg)
$\textbf{total RNA}_i$ = starting amount of total RNA (µg)
y = fraction of cDNA used in IVT.

Based on these calculations, a fragmentation mix was prepared for each sample: 20µg adjusted cRNA were combined with 5x Fragmentation Buffer and volumes adjusted with RNase-free H_2O to a total of 40µl. These mixes were placed in the thermocycler and incubated at 95ºC for 35min. To confirm successful fragmentation, electrophoresis was performed with 2µl of each fragmented cRNA sample in 8µl loading buffer (1mM EDTA pH8.0, 50% glycerol (*Fisher Scientific,* Pittsburgh, PA, USA) 0.25% bromophenol blue (Sigma Chemicals Co., Dorset, UK), 0.25% xylene cyanol (Sigma

Chemicals Co., Dorset, UK) in H_2O) and 1µl non-fragmented cRNA in 9µl loading buffer as a negative control. The 1% agarose gel was prepared by dissolving 1g agarose (Invitrogen, Paisley, UK), in 100ml 1x Tris/Borate/EDTA (TBE) buffer and adding 5µl GelRedTM Nucleic Acid Gel Stain (Biotium, Hayward, CA, USA) once it was hand-warm. Electrophoresis was performed in 1X TBE buffer at 100V for 90 min. Bands were visualised under UV light.

Remaining samples of fragmented cRNA were used to prepare the eukaryotic target hybridisation cocktail to be mounted onto the probe array (gene chip). Per array, 30µl (equivalent to 15µg) fragmented cRNA were mixed with 5µl Control Oligonucleotide B2 (3nM), 15µl 20X Eukaryotic Hybridisation Controls (bioB, bioC, bioD, cre), 3µl BSA (50mg/ml), 30µl DMSO, 64µl RNase-free H_2O and 150µl 2X Hybridization Buffer. Hybridization (16h) and scanning of the arrays were performed by Tracy Chaplin (Centre for Medical Oncology, Institute of Cancer, Barts and The London School of Medicine and Dentistry, London, UK). Data analysis was carried out by Dr Claude Chelala (Centre for Molecular Oncology and Imaging, Institute of Cancer, Barts and The London School of Medicine and Dentistry, London, UK) using Bioconductor libraries within the freely available R statistical environment (http://www.r-project.org). After generating log-transformed images from the *.CEL. files, hybridization quality was checked. Array chips were independently normalized by the quantiles method (Bolstad *et al.*, 2003). For background correction robust multi-array analysis (RMA) was used (Irizarry *et al.*, 2003). Probe level data were summarised by median polishing, resulting in log2 scale transformed data. Subsequently, the data quality was assessed using boxplots, histograms and quantile plots to ensure a Gaussian-like distribution. Differentially expressed genes were identified by the Welch two sample t-test, with p value correction using the false discovery rate (FDR) method (Capurso *et al.*, 2006). Lists were generated of differential genes with an FDR corrected p value <0.05.

In addition, TOSE cell data were subjected to a more comprehensive analysis of gene expression. This analysis was performed by Dr Probir Chakravarty (Bioinformatics & Biostatistics, Lincoln's Inn Fields, London, UK.) Statistically enriched pathways or processes were identified by applying the adjusted p value as a statistical filter. Metacore pathway analysis tool (GeneGo Inc., St. Joseph, MI, USA) (Ekins *et al.*, 2006; Ekins *et al.*, 2007) was used for hypergeometric testing with a p value =0.05.

2.19 *In silico* comparative analysis of NCI-60 microarray data

To identify differentially expressed genes correlated with sensitivity in ovarian cancer cell lines, analysis of published NCI-60 ovarian cancer data (GEO accession numbers: GSM35955 (IGROV-1), GSM35956 (OVCAR3), GSM35957 (OVCAR4), GSM35958 (OVCAR5), GSM35960 (SKOV3)) (Blower *et al.*, 2007; Shankavaram *et al.*, 2007) was performed using Bioconductor (BioConductor) packages within the open source R statistical environment (Blower *et al.*, 2007). Following intra-array loess normalization, Limma (Smyth GK, 2004) was employed for differential expression analysis. Genes differentially regulated in the most sensitive line (GSM35955 IGROV-1) compared to the others were identified. P-values were obtained from the distribution of the moderated t-statistic (the-lower-the-better). B-values are the empirical Bayes logg-odds of differential expression (the-higher-the-better).

2.20 Cloning of SV40 large T antigen expression vector.

An SV40 TAg expression plasmid, pCMV-SV40, was to be generated. For this purpose, SV40 TAg was digested out of the pX8 plasmid (a kind gift from Prof George Tsao, Hong Kong University) by *Eco*RI digest and inserted into the *Eco*RI site of the pCMV-Script vector backbone (Stratagene, La Jolla, CA, USA), which contains a neo-cassette and imparts resistance to antibiotic G-418. Digestion mixes (see Table 2.6 below) were incubated for 2h at 37ºC. After 1h the second dose of enzyme was added.

	DNA	**Buffer**	**H_2O**	**Enzyme**
pX8	10µg	EcoRI buffer 5µl	ad 50µl	EcoRI 3µl + 2µl after 1h
pCMV	5µg	EcoRI buffer 5µl	ad 50µl	EcoRI 3µl + 1µl after 1h
pX8 – undigested ctrl.	5µg	EcoRI buffer 5µl	ad 50µl	-
pCMV - undigested ctrl.	5µg	EcoRI buffer 5µl	ad 50µl	-

Table 2.6 Digestion mixes to obtain components of pCMV-SV40 as well as control digests are shown. *Buffers and enzymes are from New England Biolabs (UK) Ltd., Hertfordshire, UK.*

Prior to ligation of vectors and inserts, in order to prevent religation of linearised plasmid DNA, vector DNA was de-phosphorylated by adding 5 units/µg of calf intestinal alkaline phosphatase (CIP, New England Biolabs (UK) Ltd., Hertfordshire, UK) in CIP buffer (also New England Biolabs (UK) Ltd.) and incubating at 37°C for 45min. Electrophoresis of vector and insert DNA followed on 0.8% TAE agarose gel. Bands containing fragments of interest were detected by ethidium bromide (Sigma Chemicals Co., Dorset, UK) staining. After excision of the relevant areas of the gel, fragments were eluted using the QIAquick Gel Elution Kit (Qiagen, Crawley, West Sussex, UK). Concentrations of plasmid DNA were measured using a NanoDrop® ND-1000 spectrophotometer (Wilmington, DE, USA). Ligation reactions were set up with 50ng vector DNA at an insert:vector ratio of 3:1. For this purpose, the molarity of ends was calculated for each fragment, where

Molarity of ends (nM) = [(**C**)/ (base pairs x 650 daltons)] x 2 ends

and **C** = concentration of plasmid DNA sample in µg/µl.
Once volumes of samples for the 3:1 ratio had been determined, ligation reactions (see Table 2.7) were prepared and fragments ligated at 25ºC overnight.

	DNA	**10X Ligase buffer**	**H_2O**	**T4 DNA ligase**
Insert alone	121ng	2µl	ad 20µl	1µl
Vector alone (phosphorylated)	50ng	2µl	ad 20µl	1µl
Vector alone (de-phosphorylated)	50ng	2µl	ad 20µl	1µl
SV40-pCMV (de-phosphorylated) **3:1**	insert: 121ng vector: 50ng	2µl	ad 20µl	1µl
SV40-pCMV (de-phosphorylated) **3:1**	insert: 242ng vector: 50ng	2µl	ad 20µl	1µl

Table 2.7 Ligation reactions for cloning of pCMV-SV40. *Components of reaction mixes to ligate vector and insert as well as control reactions are shown. Ligase buffer and enzyme are from New England Biolabs (UK) Ltd., Hertfordshire, UK.*

For amplification of newly synthesised plasmids, ligation products were transformed into chemocompetent TOP10 *E.coli* cells (Stratagene, La Jolla, CA, USA) as follows: To each tube of bacterial cells, 10µl of ligation reaction were added, followed by 30min of incubation on ice. After a heat shock step of 30sec at 42ºC, tubes were again left on ice for 5-10min. 250µl SOC medium (0.5% Yeast extract, 2.0% tryptone, 10mM NaCl, 2.5mM KCl, 10mM $MgCl_2$, 20mM $MgSO_4$, 20mM glucose) were pipetted into each tube prior to incubation in a New Brunswick Innova 4000 Incubator Shaker (New Brunswick Scientific, Edison, NJ, USA) at 37ºC for 1h whilst shaking at 250rpm. Finally, 50µl transformed *E.coli* were streaked onto kanamycin-containing (40µg/ml) agarose plates and grown in an incubator overnight at 37ºC.

The following day, five clones from each plate were picked, grown as pre-cultures and miniprepped using a Qiagen Plasmid Mini Kit (Qiagen, Crawley, West Sussex, UK), according to the manufacturer's protocol. Concentrations of plasmid DNA in samples were measured with a NanoDrop® ND-1000 spectrophotometer (Wilmington, DE, USA). Test digests using 500ng plasmid DNA, enzymes EcoRI, XhoI and BamHI, and gel electrophoresis were performed as described above to confirm successful ligation of vector and insert as well as to determine the orientation of the insert in each miniprep sample. Two samples, pCMV-SV40-F (containing insert in forward orientation) and pCMV-SV40-R (insert in reverse orientation), were further amplified and purified using a Qiagen Plasmid Maxi Kit (Qiagen, Crawley, West Sussex, UK). The test digest described above was repeated to confirm that plasmids contained inserts of the right size and in the correct orientation.

2.20.1 Generation of new SV40-transformed and control cells lines

Plasmids pCMV-SV40-F and pCMV-SV40-R were transfected into IOSE20 and IOSE25 cells using FuGene6 transfection reagent (Roche). In parallel, cells from both cell lines were transfected with empty pCMV vector as a control. For that purpose, 10^5 cells were seeded per well on 6-well plates in antibiotic-free complete medium. The following day, cells were transfected as described earlier, at FuGENE 6:DNA ratios 3:1 and 3:2. One well per cell line was mock-transfected with FuGENE 6 without plasmid DNA. The maximum tolerable antibiotic concentration for selective growth of transfected cells was determined as 1mg/ml G-418 in NOSE medium.

2.20.2 Transfection of cells with pSV3neo

In a second attempt to create SV40 TAg expressing IOSE20 and IOSE21 cells, plasmid pSV3neo was used for transfection. The plasmid was a kind gift from Prof Guy Whitley (St Andrew's University, London, UK), originally created by Southern and Berg (Southern *et al.*, 1982). pSV3neo contains the SV40 TAg gene a neomycin (G-418) resistance gene for selective growth in eukaryotic cells. Following amplification of the received plasmid and purification using a Qiagen Plasmid Maxi Prep Kit (Qiagen, Crawley, West Sussex, UK), samples of maxiprepped DNA and of the original plasmid were test-digested with NcoI. After separation on a 0.8% agarose gel, all lanes contained identical triplicates of bands (Fig. 2.3), suggesting that the amplified DNA corresponded to the original plasmid.

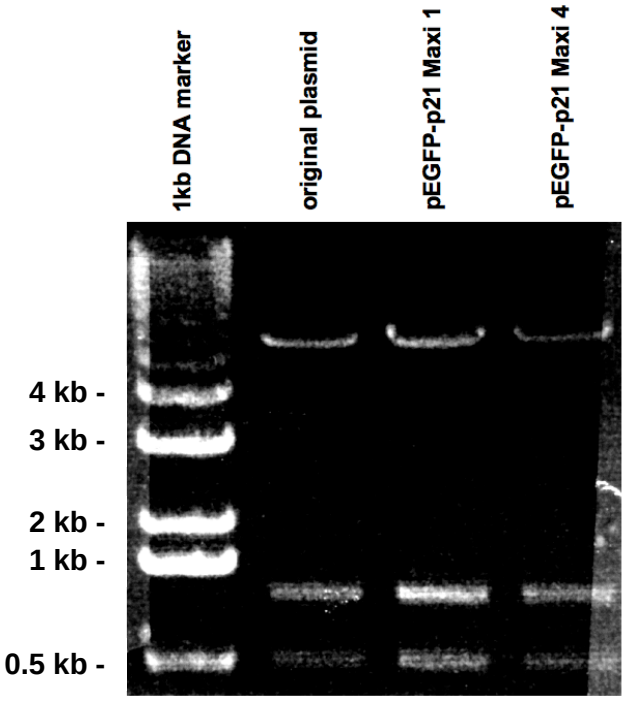


Figure 2.3 Gel electrophoresis of test-digested pSV3neo plasmid. *Original pSV3neo plasmid (lane 2), as well as maxiprepped DNA (lanes 3 and 4) were run on a 0.8% agarose gel following digestion with restriction enzyme NcoI.*

Transfections of IOSE20 and IOSE21 cells were conducted using FuGENE 6 as described above. The maximum tolerable antibiotic concentration for selective growth of transfected cells was determined to be 1mg/ml G-418 in NOSE medium.

2.21 shRNA-mediated knock down

2.21.1 RNAi library

With the aim to create a readily accessible small-scale RNAi library containing shRNAs targeting 42 transcripts, we used a Human RNAi (Hannon) library to "copy" (duplicate) constructs of interest onto a 96-well-plate format. Access to the full-size Human RNAi (Hannon) library (Babraham Bioincubator, MRC geneservice, Cambridge, UK) was kindly granted by Professor Ian Hart, Centre for Tumour Biology, Institute of Cancer, Barts and The London School of Medicine and Dentistry, London, UK). The library had been created by Dr Gregory Hannon (Paddison *et al.*, 2004), and copies for distribution produced by Dr Troy Moore (Open Biosystems, Huntsville, USA). The Human RNAi (Hannon) library consists of 18,825 shRNA constructs based on human miR30 microRNA incorporated into pSHAG-MAGIC 2 (pSM2) expression vectors (Silva *et al.*, 2005). The latter are based on mouse stem cell virus (MSCV), a retrovirus. However, in the work presented here, they were used as non-packaged plasmids. The vectors convey chloramphenicol and kanamycin resistance in bacteria and allow selection for transfection stability in eukaryotic cells via resistance to puromycin. Expression of hairpins lies under the control of the human U6 promoter. Plasmids are amplified using PIR1 *E.coli*. Pools of the bacterial cells containing shRNA expression plasmids for specific genes are stored in kanamycin-containing (50µg/ml) 8% glycerol (Fisher Scientific, Pittsburgh, PA, USA) Luria Bertani (LB) broth (1% bacto-trypone, 0.5% yeast extract, 1% NaCl in H2O, pH 7.5) on 96-well plates at -80°C.

2.21.2 Creation of mini-RNAi library

The following steps were performed in collaboration with the services of the Genome Centre (William Harvey Research Institute, Barts and The London School of Medicine and Dentistry, London, UK). Using the Biomek FX (Beckman) robotic liquid handler, wells of a deep-well 96-well sterile growth plate were filled with 1.2ml LB broth containing 50µg/ml kanamycin. Bacterial glycerol stocks were thawed. The Biomek FX mixed each stock by pipetting and transferred 5µl into a well on the deep-well growth plate. After inoculation with all desired construct-containing bacterial clones, the growth plate was covered with gas-permeable seals and incubated in a New Brunswick Innova 4000 Incubator Shaker (New Brunswick Scientific, Edison, NJ, USA) at 37°C, shaking at 300rpm for 17hrs. Following incubation, the Biomek FX liquid handler prefilled wells on two round-bottom 96-well plates with 40µl autoclaved 15% glycerol (Fisher Scientific, Pittsburgh, PA, USA). Next, 80µl of each bacterial culture from the growth plate were transferred by the robot into the destination well on the round-bottom 96-

well plate. This step was repeated with the second destination plate. Both plates were covered with an air-tight seal tolerant for freezing and stored at -80°C. A table of contents of the newly created mini-RNAi library is shown in Table 2.8.

Well ID	Gene targeted by shRNA
A1	Homo sapiens v-akt murine thymoma viral oncogene homolog 3
A2	Homo sapiens v-akt murine thymoma viral oncogene homolog 3
A3	Homo sapiens MAGE family testis and tumor-specific protein (MAGEB5)
A4	Homo sapiens phosphoinositide-3-kinase, catalytic, beta polypeptide
A5	Homo sapiens phosphoinositide-3-kinase, catalytic, alpha polypeptide
A5	Homo sapiens phosphoinositide-3-kinase, catalytic, beta polypeptide
A7	Homo sapiens APR-1 protein (MAGEH1)
A8	Homo sapiens cyclin-dependent kinase inhibitor 1B (p27, Kip1)
A9	Human retinoblastoma related protein (p107)
A10	Homo sapiens cyclin E2 (CCNE2), transcript variant 3
A11	Homo sapiens melanoma antigen, family A, 11 (MAGEA11)
A12	Homo sapiens melanoma antigen, family A, 9 (MAGEA9)
B1	Homo sapiens cyclin E2 (CCNE2), transcript variant 3
B2	Homo sapiens melanoma antigen, family A, 2 (MAGEA2)
B3	Homo sapiens melanoma antigen, family A, 9 (MAGEA9)
B4	Homo sapiens cyclin E2 (CCNE2), transcript variant 3
B5	Homo sapiens cyclin E1 (CCNE1), transcript variant 1
B6	Homo sapiens melanoma antigen, family D, 1 (MAGED1)
B7	Homo sapiens melanoma antigen, family A, 4 (MAGEA4)
B8	Homo sapiens melanoma antigen, family B, 1 (MAGEB1)
B9	Homo sapiens melanoma antigen, family A, 4 (MAGEA4)
B10	Homo sapiens AKT1 substrate 1 (proline-rich) (AKT1S1)
B11	Homo sapiens melanoma antigen, family B, 3 (MAGEB3)
B12	Homo sapiens melanoma antigen, family B, 1 (MAGEB1)
C1	Homo sapiens AKT1 substrate 1 (proline-rich) (AKT1S1)
C2	Homo sapiens melanoma antigen, family B, 3 (MAGEB3)
C3	Homo sapiens similar to Melanoma-associated antigen E1 (MAGE-E1)
C4	Homo sapiens v-akt viral oncogene homolog1 (AKT1),
C5	Homo sapiens phosphoinositide-3-kinase, regulatory subunit,
C6	Homo sapiens phosphoinositide-3-kinase, catalytic, delta
C7	Homo sapiens beclin1(coiled-coil, myosin-like BCL2 interacting protein)
C8	Homo sapiens melanoma antigen, family E, 1, cancer/testis specific
C9	Homo sapiens CyclinD1(PRAD1:parathyroid adenomatosis1) (CCND1)
C10	Homo sapiens CyclinD1(PRAD1:parathyroid adenomatosis1)(CCND1)
C11	Homo sapiens cyclin-dependent kinase inhibitor 1C (p57s Kip2)
C12	Homo sapiens cyclin-dependent kinase 4 (CDK4)
D1	Homo sapiens cyclin-dependent kinase inhibitor 1C (p57s Kip2)
D2	Homo sapiens phosphoinositide-3-kinases class 2, alpha polypeptide
D3	Homo sapiens phosphoinositide-3-kinases class 2, beta polypeptide
D4	Homo sapiens cyclin D2 pseudogene (CCND2P) on chromosome 11.
D5	Homo sapiens phosphoinositide-3-kinases class 3 (PIK3C3), mRNA.
D6	Homo sapiens similar to Melanoma-associated antigen E1 (MAGE-E1)

Table 2.8 Contents of mini-RNAi library. *Listed are genes targeted by shRNA encoded by the respective plasmid and the position of the clone on the library 96-well-plate (Well ID).*

2.21.3 Miniprep and verification of hairpin-containing pSM2 plasmids

Selected of the remaining bacterial cultures on deep-well plate were used to inoculate 10ml kanamycin-containing LB broth (50µg/ml). A list and their position on the plate are shown in Table 2.9. Bacterial cultures were incubated in a New Brunswick Innova 4000 Incubator Shaker (New Brunswick Scientific, Edison, NJ, USA) o/n at 37°C, shaking at 250rpm. The following day, miniprep of plasmid DNA was carried out using a Qiagen Plasmid Mini Kit (Qiagen, Crawley, West Sussex, UK). At the final step, plasmid DNA was resuspended in TE buffer (10mM Tris-Cl, pH8.0; 1mM EDTA). No plasmid was obtained of hairpins targeting p107, p57, Cyclin D2, Cyclin E1 and one of the two Cyclin E2-specific constructs, as overnight cultures had not grown

Clone with shRNA targeting...	**Position on 96-well plate**	**Successfully purified**
p27	A10	Yes
p57	C11	No
p107	A9	No
Cyclin D1	C9	Yes
Cyclin D1	C10	Yes
Cyclin D2	D4	No
Cyclin E1	B5	No
Cyclin E2	A10	No
Cyclin E2	B4	Yes
cdk4	C12	Yes

Table 2.9 Bacterial clones used to purify shRNA encoding pSM2 plasmids. *Listed in column on the right are clones used to inoculate o/n cultures for purification of plasmids encoding shRNA annotated for specific genes for test digests. Middle column shows their position on the 96-well mini-RNAi library plate. Column on the right indicates whether plasmids could be successfully purified (Yes) or not (No).*

Presence of the correct inserts in plasmids in library clones was verified by test-digesting mini-prepped constructs as described earlier using enzymes XbaI and HindIII (New England Biolabs (UK) Ltd., Hertfordshire, UK).

2.22 Stable knock-down of Rb pathway components

For stable knock-down of p27, Cyclin D1, Cyclin E2 and cdk4 expression, plasmids amplified and purified from the mini-RNAi library were transfected into TOV21G and IGROV-1 cells.

2.22.1 Amplification and purification of RNAi library plasmids

10ml of kanamycin (50µg/ml) and chloramphenicol (25µg/ml) containing LB broth were inoculated with glycerol stock of RNAi bacterial clones specific for p27, Cyclin E1, Cyclin E2, Cyclin D1,Cyclin D2, or cdk4. These pre-cultures were incubated in a New Brunswick Innova 4000 Incubator Shaker (New Brunswick Scientific, Edison, NJ, USA) at 37°C, shaking at 250rpm for 8hrs. Of each bacterial culture 100µl were used to inoculate 400ml of kanamycin and chloramphenicol containing LB broth (see above), followed by incubation in a New Brunswick Innova 4000 Incubator Shaker (New Brunswick Scientific, Edison, NJ, USA) at 37°C, shaking at 250rpm overnight. The following day, plasmids were purified using a Qiagen Plasmid Maxi Prep Kit (Qiagen, Crawley, West Sussex, UK) according to manufacturer's protocol. Recovered DNA was resuspended in TE buffer (10mM Tris-Cl, pH8.0; 1mM EDTA).

2.22.2 Transfection of TOV21G and IGROV-1 cells with RNAi library plasmids

Purified plasmids were transfected into TOV21G and IGROV-1 cells as described earlier, at a FuGENE 6:DNA ratio of 3:1. In some cases, two plasmids are annotated to target the same gene, thus were used for transfection in combination. Table 2.10 shows genes, which constructs used were targeting and amounts used for transfection. Per well, a total of 10µg plasmid DNA was used. Selection of successfully transfected cells was carried out by culture of cells in 10%FCS E4 with 100µg/ml puromycin.

shRNA-pSM2 construct targeting:	Amount of DNA (µg) per well
p27	10µg
cdk4	10µg
Cyclin D1	5µg+ 5µg
Cyclin D2	10µg
Cyclin E1	10µg
Cyclin E2	5µg + 5µg

Table 2.10 Genes targeted for knockdown by transfection with respective shRNA-pSM2 plasmids*. Listed are target genes of mini-RNAi library plasmids and amounts of DNA used per well. If two plasmids targeting the same gene were used in combination, equal amounts of respective DNA were transfected.*

2.23 Statistical analysis

Statistical analysis was conducted using GraphPad Prism version 3 (GraphPad Software, San Diego, CA, USA). Unless otherwise stated, all results are presented as mean +/- standard deviation, n=3 and all statistical analyses are unpaired, two-tailed Student's *t* test, where $p<0.05$ is considered statistically significant.

3 Results: Determinants of oncolytic virus efficacy

3.1 Introduction

Various publications have demonstrated the efficacy of *dl*922-947 in selectively lysing cancer cells (Heise *et al.*, 2000; Lockley *et al.*, 2006). Although it has been shown that cancer cells are significantly more susceptible to its oncolytic effects than normal cells (Heise *et al.*, 2000), differences exist between sensitivities of cancer cell lines. This chapter describes the efforts taken to identify factors of the infected cell that contribute to maximum oncolytic efficiency of *dl*922-947. In the immediate future, such biomarkers would be a valuable asset to stratify patients taking part in phase I clinical trials of *dl*922-947 in ovarian cancer, allowing identification of patients who are more likely to respond to treatment. Already, cancer treatment is moving more and more into the field of stratified medicine (Hughes, 2009; Kantarjian *et al.*, 2007; Ueno *et al.*, 2007). In the future, patients may routinely be pre-screened to devise the most appropriate therapy (Mullenders *et al.*, 2009; Nemunaitis *et al.*, 2007). In this light, knowledge of biomarkers enhancing *dl*922-947 efficacy could identify those patients who would benefit from such treatment, once it has been licensed by the regulatory agencies (Medicines and Healthcare products Regulatory Agency (MHRA)).

3.2 Methods

The study presented here was set up to compare host cell factors in sensitive and more resistant cell lines to reveal those influencing oncolytic efficacy of *dl*922-947. To this end, the major steps of the adenoviral life cycle were scrutinised, first:

- infectivity of the host cell,
- viral protein expression, and
- replication.

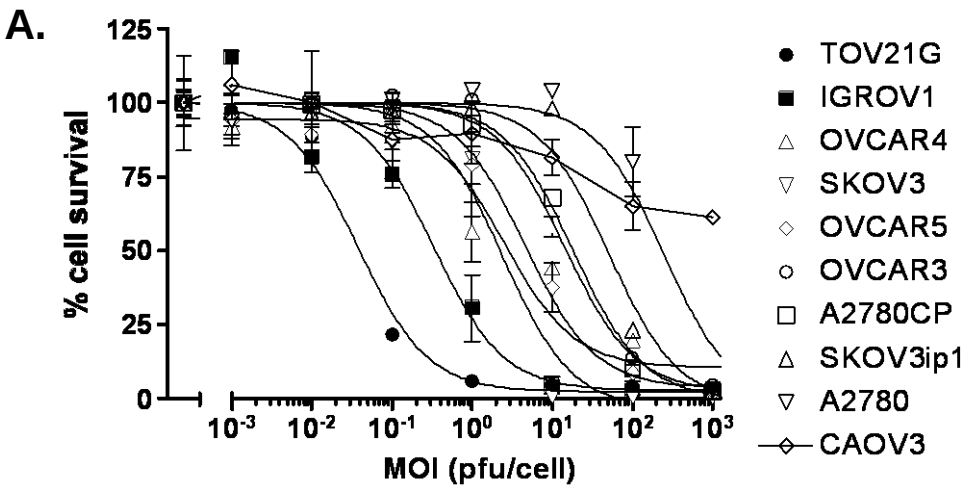
Furthermore, selectivity of *dl*922-947 is believed to rely upon a dysregulated Rb pathway (Heise *et al.*, 2000). As was learnt from the early-generation oncolytic adenovirus ONYX-015 (*dl*1520), this belief may be incorrect. ONYX-015 was designed to selectively replicate in and lyse p53-deficient cells by deleting E1B-55K in the virus. However, subsequent investigation revealed that not only was its function independent from the p53 status (Rothmann *et al.*, 1998). Rather, the deletion it contained led to loss of late RNA export. Only those tumour cells with altered RNA export mechanisms allow ONYX-015 to overcome this deficiency (O'Shea *et al.*, 2004). To test whether selectivity of *dl*922-947 is indeed based on an abnormal Rb pathway, experiments with the virus were carried out in an isogenic cell pair with a normal or a dysregulated Rb

pathway. Finally, a much closer look was taken at individual components of the Rb pathway in cell lines with different sensitivities to *dl*922-947.

3.3 Sensitivity of Cancer Cells to *dl*922-947

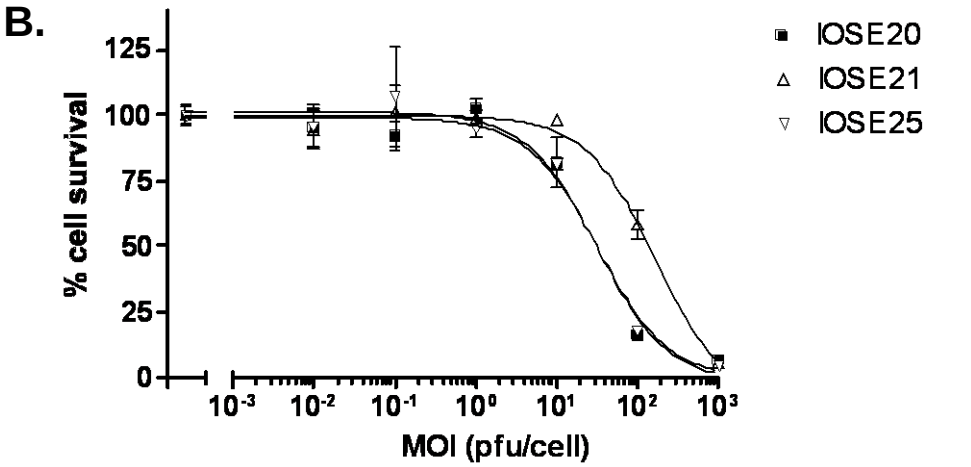
In order to compare sensitivity of different cell lines to *dl*922-947, I infected the ovarian cancer cell lines IGROV-1, OVCAR-4, TOV21G, CAOV3, SKOV3ip1, A2780 and A2780CP with *dl*922-947, as well as hTERT-immortalised ovarian surface epithelial cells IOSE20, IOSE21 and IOSE25, as non-cancerous controls. Survival was determined 120 hours later by MTT assay. Dose response curves and half maximal inhibitory concentration (IC_{50}) values for each cell line were obtained using Graph-Pad Prism software (Fig. 3.1).

Of the cancer cell lines studied, two cell lines were highly sensitive: TOV21G (IC_{50}=0.038pfu/cell) and IGROV-1 (IC_{50}=0.33pfu/cell). SKOV3, OVCAR4 and OVCAR5 cells were less sensitive to oncolysis by *dl*922-947, with IC_{50} values of 2.4, 2.5 and 4.6pfu/cell, respectively. Three cell lines were more resistant: OVCAR3 (14pfu/cell), A2780CP (18pfu/cell) and SKOV3ip1 (51pfu/cell). Two lines were defined as non-sensitive: A2780 (IC_{50}= 250pfu/cell) and CAOV3 (IC_{50}>10,000pfu/cell). Compared to the ovarian cancer cells, the three hTERT-immortalised cells had IC_{50} as follows: IOSE20: 33pfu/cell, IOSE25: 32pfu/cell IOSE21: 173pfu/cell.



	TOV21G	IGROV-1	SKOV3	OVCAR4	OVCAR5
IC_{50}	0.038	0.33	2.4	2.5	4.6

	OVCAR3	A2780CP	SKOV3ip1	A2780	CAOV3
IC_{50}	14	18	51	250	>10,000



	IOSE20	IOSE21	IOSE25
IC_{50}	33	170	32

Figure 3.1 Cytotoxicity of *dl*922-947. *Dose response curves of* **A.** *cancer cells and* **B.** *control cells IOSE20, IOSE21 and IOSE25 graphed relative to log MOI pfu/cell of dl*922-947*. Points represent mean +/- standard deviation. A table below each graph shows IC_{50} values (pfu/cell).*

3.4 Infectivity of cell lines

Infectivity of the host cell will have a major influence on lytic success of *dl*922-947. Quantitative PCR (qPCR) and flow cytometric analyses were used in parallel to evaluate and compare infectivity of ovarian cancer and control cell lines. For the flow cytometry-based approach, cells were infected with non-replicating adenoviral vector encoding enhanced green fluorescent protein (Ad-GFP) at multiplicity of infection (MOI) 5pfu/cell. Twenty-four hours later, flow cytometric analysis was performed, Percentages of GFP-expressing, that is infected, cells are shown in Fig. 3.2. qPCR analysis probing for adenoviral DNA in the E1A and hexon regions allowed quantification of viral genomes present in a host cell two hours after infection with *dl*922-947 at MOI 10pfu/cell. At this timepoint at least 50% of maximal uptake of virus particles should have occurred (Yotnda *et al.*, 2001), while it is too early for viral replication to have begun. Thus, only internalised genomes are detected. Log copy numbers of viral genomes are shown in Fig. 3.2. This experiment was performed in a selection of representative cancer cells: the highly sensitive TOV21G and IGROV-1 cells, as well as the less sensitive SKOV3ip1 and A2780CP cells.

Flow cytometric analysis indicated that TOV21G cells were the most infectable of all assayed cancer cell lines; OVCAR-4 and A2780CP cells were also very infectable. IGROV-1 and SKOV3ip1 showed lower rates of infectivity. A2780 were very difficult to infect, while CAOV3 could generally not be infected at all with MOI 5pfu/cell. Percentage of GFP-positive cells in control cell lines IOSE20, IOSE21 and IOSE25 cells was far lower.

qPCR analysis confirmed TOV21G to be the most infectable cancer cell line. Viral genome copy numbers in SKOV3ip1 cells were higher than in A2780CP cells, indicating they can be more easily infected. This contrasted with GFP-flow cytometry results. A similar discrepancy was found in the control cell group. IOSE21 cells showed considerably lower levels of GFP-expression, while viral genome copy numbers were higher than in SKOV3ip1 and A2780CP cells. Comparatively lower GFP-expression and copy numbers in the other control cell lines were consistent.

In general, both methods proved that, apart from CAOV3 cells, all investigated cell lines could be infected. The divergence can be explained by the different factors each method measures. Flow cytometry results quantify the number of cells expressing GFP

above a pre-determined threshold, whilst qPCR results show the pure number of viral genome copies per infected cell.

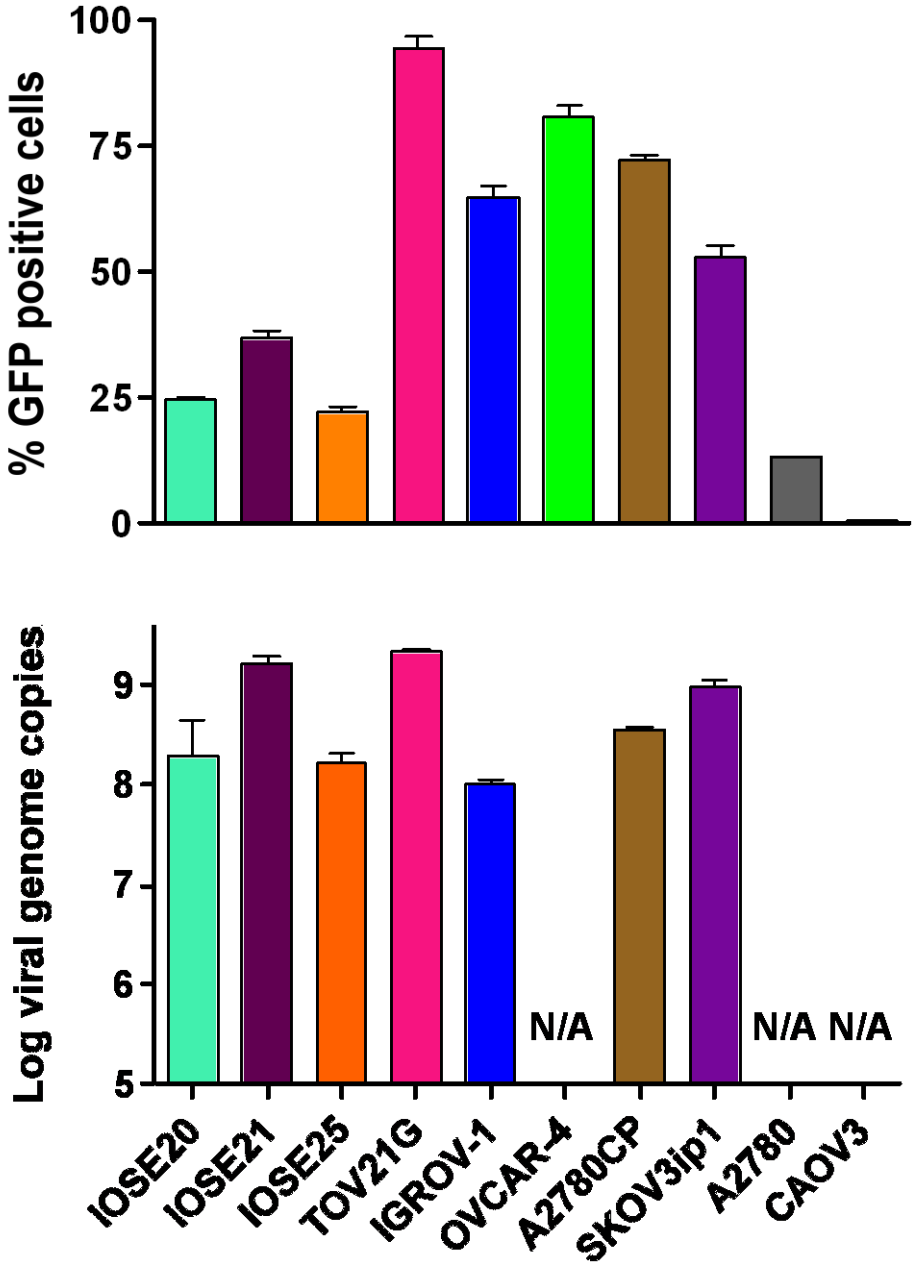


Figure 3.2 Infectivity of cell lines with adenovirus 5 mutants. *Percentages of cancer and control cells expressing GFP after infection with Ad-GFP at MOI 5pfu/cell and measured by flow cytometry* **(top)**. *Log copy number of viral genomes in host cells 2 hours after infection with dl*922-947 *quantified by qPCR* **(bottom)**. *Error bars indicate mean +/- standard deviation.* **N/A** = *not assessed.*

3.5 Expression of viral protein

The difference in viral cytotoxic efficacy between cells could be also based on the inability of a host cell to synthesise all viral components required for assembly of a functional virus or complete shut-down of host and viral protein translation due to anti-viral IFN response (Naik *et al.*, 2009; Reichel *et al.*, 1985; Samuel, 1993; Thomis *et al.*, 1993). To assess expression of both early and late viral proteins, IGROV-1, TOV21G, SKOV3ip1, A2780CP, A2780, CAOV3 and OVCAR-4 cells were infected with *dl*922-947 at MOI 10pfu/cell. Protein extracted up to 72 hours after infection was separated via SDS-PAGE gel electrophoresis, then immunoblotted expression of E1A, as well as late structural proteins. Protein expression was visualised using enhanced chemiluminescence (Fig. 3.3).

All investigated cancer cells synthesised early protein E1A as well as later expressed, structural proteins hexon, penton and fiber. There was a difference in the onset of E1A expression. It appeared earliest in TOV21G cells, followed by IGROV-1 cells. In A2780CP and SKOV3ip1 cells, E1A was detected at later timepoints after infection.

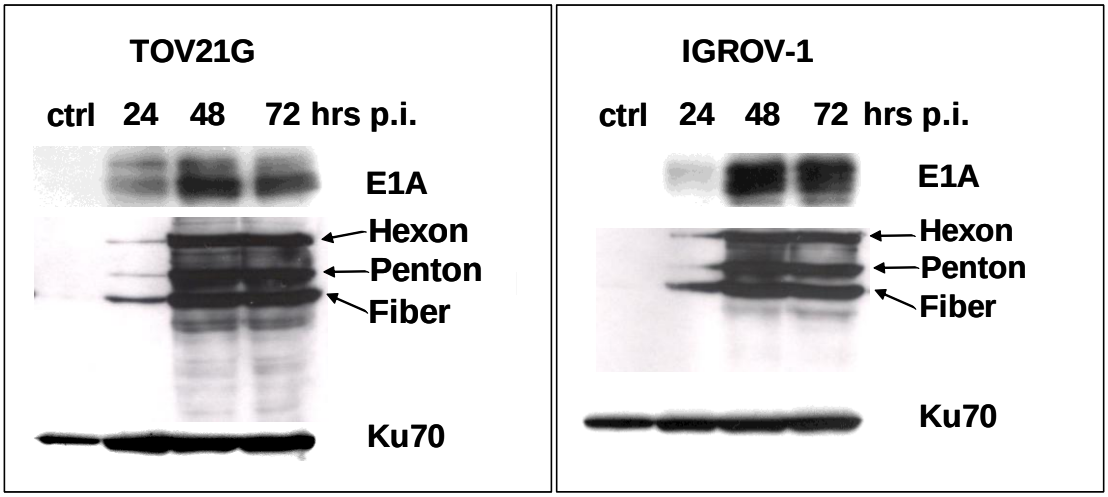


Figure 3.3.a Viral protein expression. *Expression profiles of viral protein E1A, and structural adenovirus proteins (hexon, penton, fiber) over time in TOV21G and IGROV-1 infected with dl*922-947 *analysed by Western Blotting. Ku70 served as a loading control.*

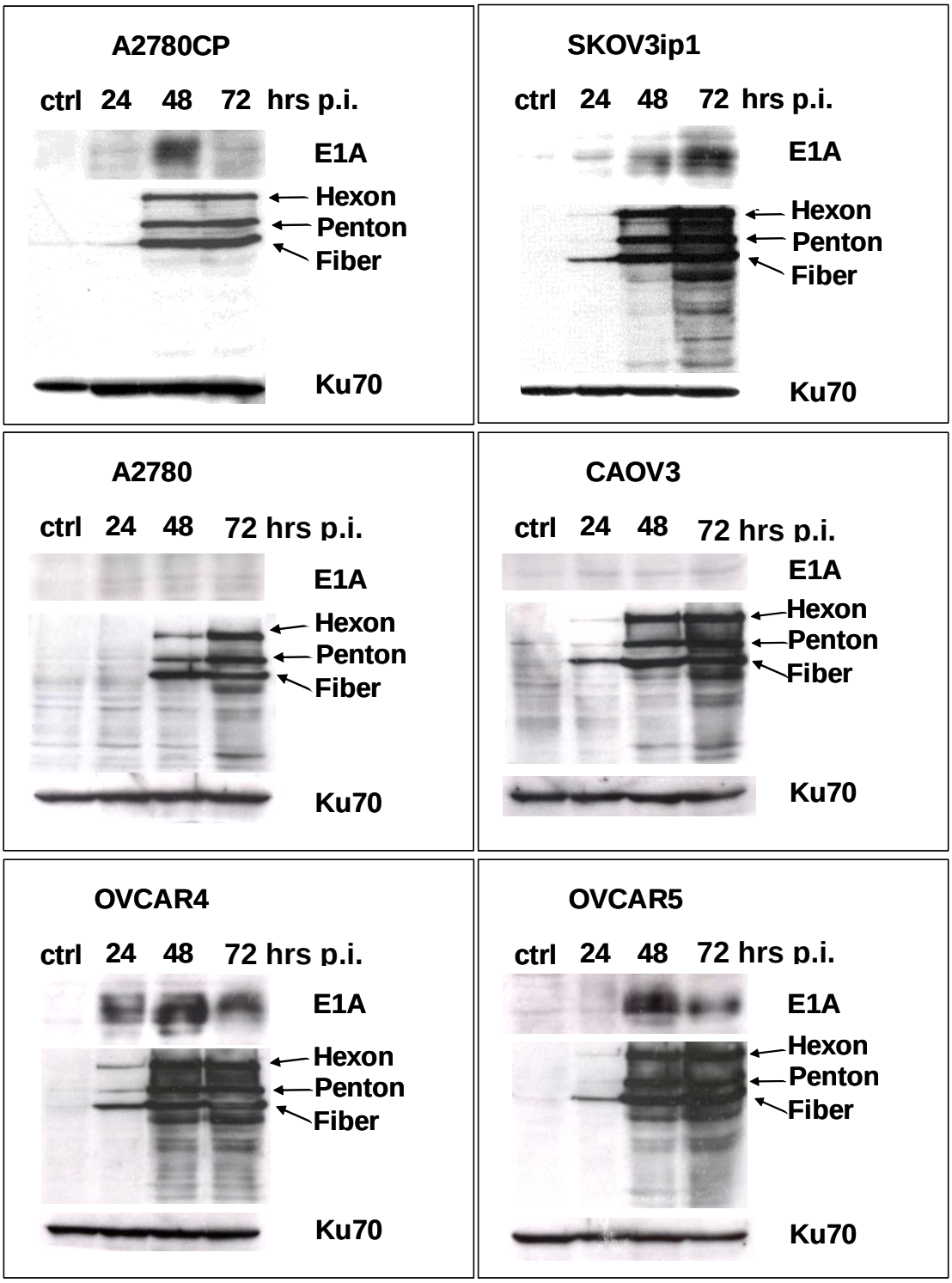


Figure 3.3.b Viral protein expression. *Expression profiles of viral protein E1A, and structural adenovirus proteins (hexon, penton, fiber) over time in A2780CP, SKOV3ip1, A2780, CAOV3, OVCAR4 and OVCAR5 cells infected with dl*922-947 *analysed by Western Blotting. Ku70 served as a loading control.*

3.6 Replication

The rate at which a virus replicates within a host cell would be expected to have a major impact on its cytotoxicity. I compared replication of *dl*922-947 in a representative panel of cell lines via tissue culture infective dose 50% ($TCID_{50}$) assays, commonly used to measure viral replication. In addition, samples were analysed for genome copy number by quantitative PCR technology (qPCR).

For $TCID_{50}$ assays, cancer cells TOV21G, IGROV-1, A2780CP and SKOV3ip1, as well as control cells IOSE20, IOSE21 and IOSE25, were infected with *dl*922-947 at MOI 10pfu/cell. Cell samples were collected 24, 48 and 72 hours after infection. Eleven days after titrating the lysates onto JH293 cells on 96-well plates, cytopathic effect (CPE) was assessed and titres of virus were calculated. The number of plaque forming units per cell produced by each cell line over time are plotted in Fig. 3.4. Overall, no conclusive correlation across all cell lines was found between measured pfu/cell and sensitivity to *dl*922-947. Seventy-two hours post-infection, TOV21G and SKOV3ip1 cells had produced the highest number of plaque forming units per cell, closely followed by IGROV-1 cells. A2780CP and IOSE20 control cells had produced fewest plaque forming units. Interestingly, levels of pfu/cell increased quickly between 24 and 48 hours and more or less plateaued after 48hours, with the exception of SKOV3ip1 and IOSE20.

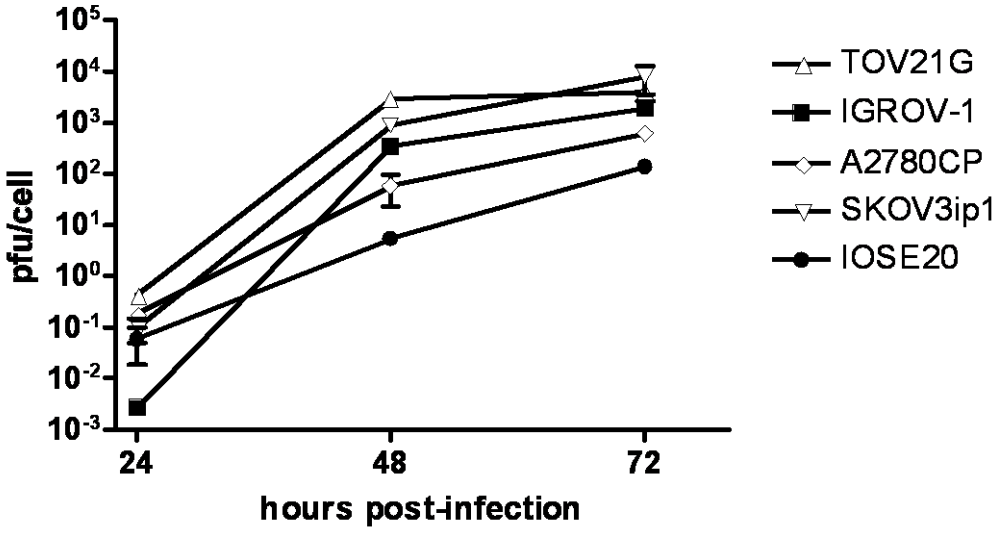


Figure 3.4 Virus replication assessed by $TCID_{50}$. *Infectious virions produced in cancer and control cells 24, 48 and 72 hours after infection with dl*922-947 *at MOI 10pfu/cell. Error bars indicate mean +/- standard deviation.*

For qPCR analysis, I infected IGROV-1, TOV21G, SKOV3ip1, A2780CP, IOSE20, IOSE21 and IOSE25 with *dl*922-947 at MOI 10pfu/cell; in addition, one sample per cell line was mock-infected. Again, cell samples were harvested 24, 48 and 72 hours post infection. DNA extracted from these samples was used to quantify the copy number of viral genomes per µg of total DNA loaded at a given time after infection, using primers and probes for E1A and hexon regions. After subtracting the background inferred from mock-control samples, replication profiles of *dl*922-947 were determined and are shown below (Fig. 3.5). Curves for hexon copy numbers were identical to E1A and not shown.

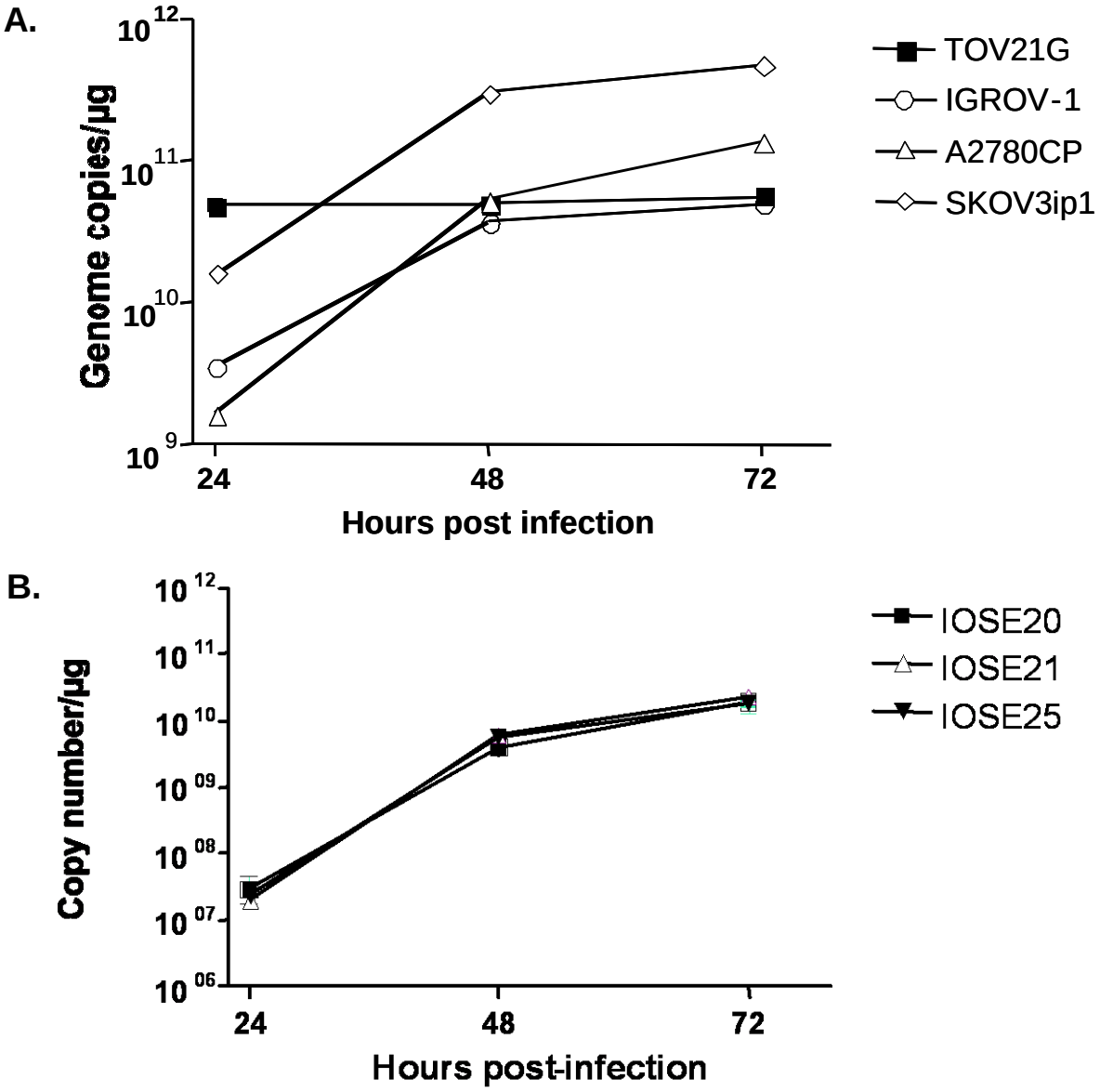


Figure 3.5 Virus replication assessed by qPCR. *Copy number of viral genomes per µg DNA 24, 48 and 72 hours after infection with dl*922-947 *at MOI 10pfu/cell recorded in* **A.** *cancer cell lines and* **B.** *control cell lines. Error bars indicate mean +/- standard deviation.*

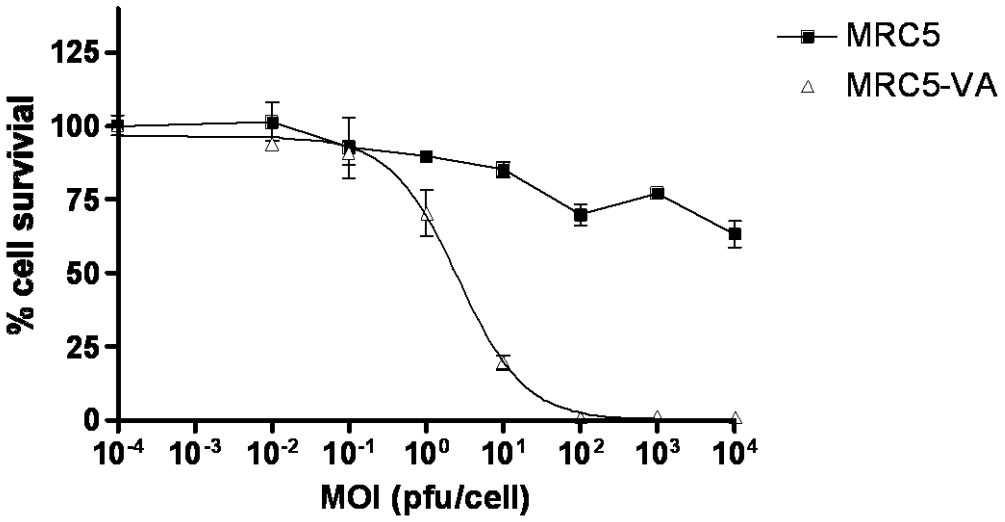
Twenty-four hours following infection, TOV21G, the most sensitive cell lines, contained the greatest number of viral genomes, but there was no real increase over the next 48 hours. Surprisingly, both 48 and 72 hours after infection, the cell line with the highest number of virus genomes was SKOV3ip1, with A2780CP second. IGROV-1 and TOV21G cells contained more or less identical amounts of viral genome at this point. These results back up the data from $TCID_{50}$ experiments and suggest that there was no complete correlation between viral genome replication and viral cytotoxicity.

3.7 Rb pathway and sensitivity

Results earlier in this chapter suggested that, although ovarian cancer cells that were more sensitive to *dl*922-947 were generally more infectable, the differences in infectivity cannot explain the large range in sensitivity. Determining factors and events must therefore lie beyond the infection step. Furthermore, the oncolytic selectivity of *dl*922-947 is believed to be based on an aberrant Rb pathway (Heise *et al.*, 2000). To examine whether the latter is the case, experiments were performed using MRC5 and MRC5-VA cells. As described earlier, MRC5-VA cells are derived from cell line MRC5, but have been transformed by SV40 large T antigen (SV40 TAg). In addition to interfering with p53 function, SV40 TAg disrupts the normal Rb pathway by binding to pRb and forcing it to release transcription factor E2F, resulting in entry into the cell cycle (DeCaprio *et al.*, 1988; Linzer *et al.*, 1979; Nevins, 1992). Any difference in viral efficacy between the cell pair is likely to be due to Rb pathway status as *dl*922-947 encodes wild-type E1B-55K, which inactivates p53 (Yew *et al.*, 1992). Furthermore, TOV21G cells possess wild-type p53 genes, yet are highly susceptible to *dl*922-947, implying functional p53 does not impede *dl*922-947 activity (Bunz *et al.*, 1998; Mizuarai *et al.*, 2009).

3.8 Sensitivity of MRC5 and MRC5-VA cells to *dl*922-947

Cell viability assays were performed to compare sensitivity of MRC5 and MRC5-VA cells to *dl*922-947. Survival curves are shown in Fig. 3.6., below. Whilst MRC5-VA cells were very sensitive to *dl*922-947, with an IC_{50} of 9.9pfu/cell, the majority of MRC5 cells could not be lysed even with the highest MOI.



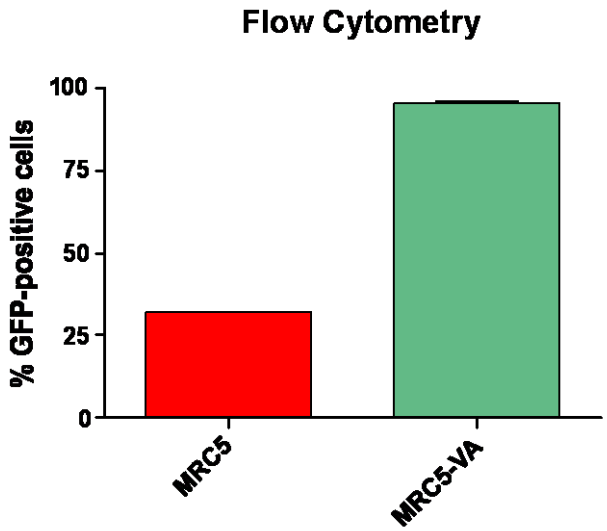
	MRC5	MRC5-VA
IC_{50}	>10,000	2.6

Figure 3.6 Sensitivity of MRC5 and MRC5-VA cells to *dl*922-947. *Dose response curves of MRC5 and MRC5-VA cells graphed relative to log MOI pfu/cell of dl*922-947*. Points represent mean +/- standard deviation. A table below each graph shows IC_{50} values (pfu/cell).*

3.9 Infectivity of MRC5 and MRC5-VA cells

Infectivity of the cell pair was measured by two methods, flow cytometry and qPCR, as described above. Based on flow cytometric analysis (Fig. 3.7), MRC5-VA cells were more infectable than MRC5 cells. Surprisingly, results obtained by qPCR suggested that levels of infection were more similar than suggested by the flow cytometry results, (Fig.3.7). Again, this discrepancy is due to the different entities each method measures and is discussed below.

A.



B.

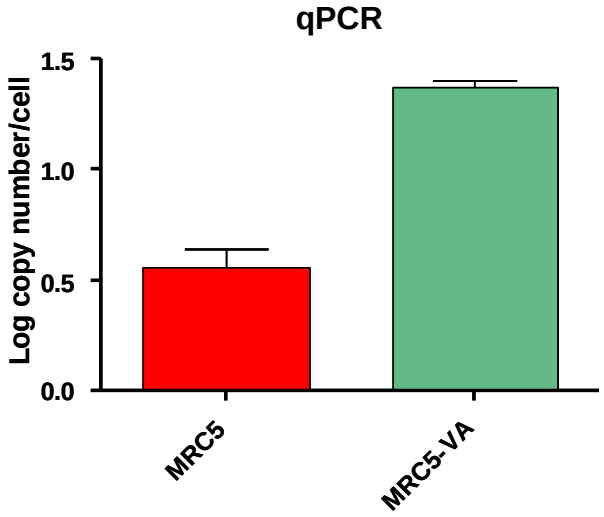


Figure 3.7 Infectivity of MRC5 and MRC5-VA cells with adenovirus 5 mutants. A. *Ratios of GFP-expressing MRC5 and MRC5-VA cells after infection with Ad-GFP at MOI 5pfu/cell and measured by flow cytometry.* **B.** *Copy number of viral genomes in host cells 2 hours after infection with dl*922-947 *quantified by qPCR. Error bars indicate mean +/- standard deviation.*

3.10 Replication of *dl*922-947 in MRC5 and MRC5-VA cells

To assess replication of *dl*922-947 in MRC5 and MRC5-VA cells, the qPCR method was applied for quantification of virus genome copy number per microgram DNA. MRC5 and MRC5-VA cells were infected with *dl*922-947 at MOI 10pfu/cell. Cells were harvested 24, 48 and 72 hours post infection. DNA from the cells was used for qPCR using primers and probes for E1A and hexon. After normalising for background copy number, replication profiles of *dl*922-947 were generated and are shown in Fig. 3.8. Curves for hexon copy numbers were identical to E1A (data not shown). At every timepoint assessed, viral copy numbers in MRC5-VA cells were higher than in MRC5 cells.

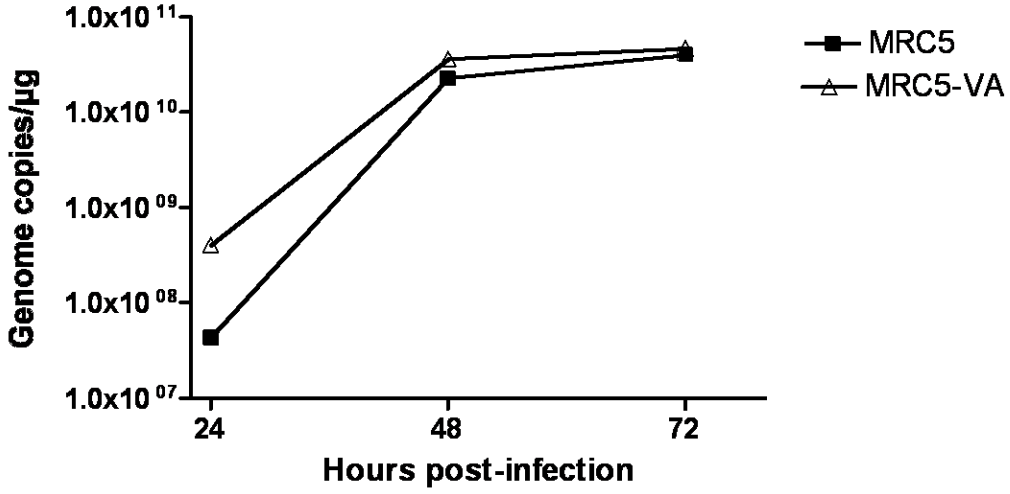


Figure 3.8 Virus replication assessed by qPCR. *Copy number of viral genomes per µg DNA measured in MRC5 and MRC5-VA cells 24, 48 and 72 hours after infection with dl*922-947 *at MOI 10pfu/cell. Error bars indicate mean +/- standard deviation.*

3.11 Rb pathway

The Rb pathway plays a major role in cell cycle control (Genovese *et al.*, 2006). Also, its functionality is believed to be the determinant for selective replication and oncolysis mediated by *dl*922-947 (Heise *et al.*, 2000). Furthermore, experiments in MRC5 and MRC5-VA cells supported a role for Rb pathway dysregulation in high *dl*922-947 efficiency. Therefore features of the host cell that affect sensitivity at the post-infection stage were to be investigated more closely, and in particular the Rb pathway. Experiments were performed using the four representative ovarian cancer cell lines IGROV-1, TOV21G, SKOV3ip1 and A2780CP, as they can all be infected with *dl*922-947 but their sensitivity varies from 0.038 to 51pfu/cell. Furthermore, I employed three control cell lines IOSE20, IOSE21 and IOSE25.

To investigate whether altered expression of one or more components of the Rb pathway correlated with host cell sensitivity, Western blot assays of the four ovarian cancer cell lines and three control cell lines were performed, immunoblotting for pRb, p16, p21, p27, Cyclin D1/D2/D3, Cdk4, Cyclin E and cdk2. Patterns of protein levels are shown in Fig. 3.9.

For pRb, Cyclin E and cdk2, no correlation could be established between their expression levels in a cancer cell line and cell sensitivity to *dl*922-947. However, results did suggest possible correlation between sensitivity and expression of p21, p16, p27, Cyclin D1/D2/D3 and to some extent cdk4. The first four proteins showed highest levels in TOV21G cells, the most sensitive cell line, somewhat lower levels in IGROV-1 cells, which are slightly less sensitive to *dl*922-947. Amounts of protein were lowest in A2780CP and SKOV3ip1 cells, which have been shown to be the least sensitive to the virus. The profile for cdk4 differed slightly, as levels in IGROV-1 cells appeared slightly higher than in TOV21G cells. However, the correlation between expression of these proteins and sensitivity to *dl*922-947 did not emerge for hTERT-immortalised control cells.

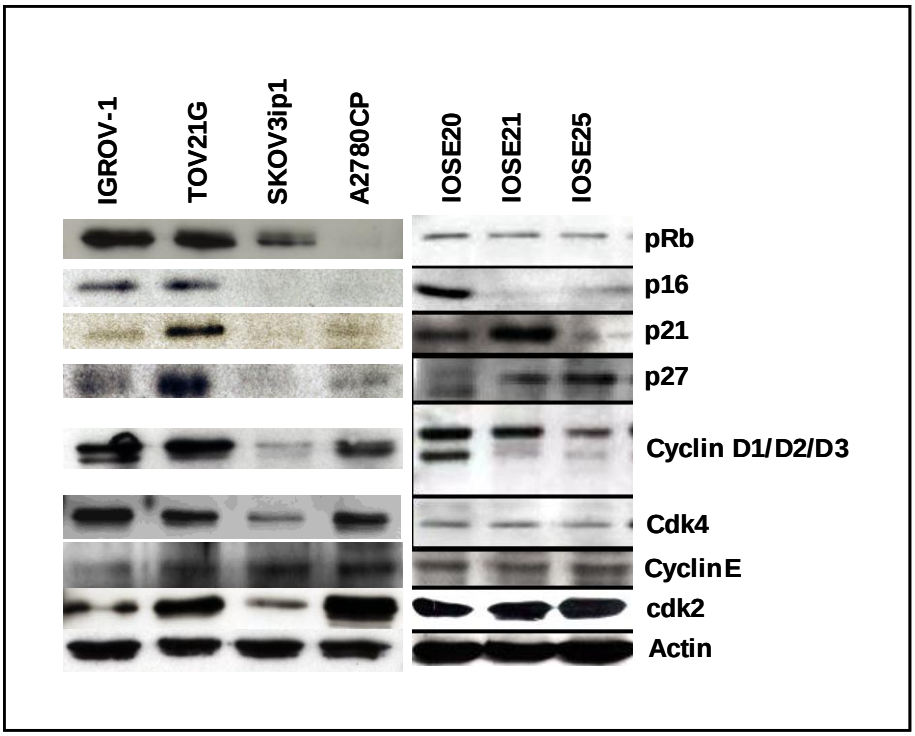


Figure 3.9 Expression of Rb pathway components. *Profiles of Rb pathway component expression in cancer cell lines and control cell lines analysed by Western Blotting. Actin served as a loading control.*

3.12 Expression analysis of cells in NCI-60 panel

In silico analysis was performed comparing gene expression in the sensitive ovarian cancer cell line IGROV-1 and four less sensitive ovarian cancer cell lines OVCAR3, OVCAR4, OVCAR5 and SKOV3. The study was carried out by Dr Claude Chelala (Centre for Molecular Oncology and Imaging, Institute of Cancer, Barts and The London School of Medicine and Dentistry, London, UK). To identify potential host cell genes associated with sensitivity to *dl*922-947, published NCI-60 ovarian cancer data (GEO accession numbers: GSM35955 (IGROV-1), GSM35956 (OVCAR3), GSM35957 (OVCAR4), GSM35958 (OVCAR5), GSM35960 (SKOV3)) (Blower *et al.*, 2007; Shankavaram *et al.*, 2007) were analysed using BioConductor (BioConductor) packages within the open source R statistical environment (Blower *et al.*, 2007). After intra-array lowess normalization, Limma (Smyth GK, 2004) was used for differential expression analysis. Genes differentially regulated in the most sensitive line (GSM35955 IGROV-1) versus the others were identified. A list of the top 100 differentially expressed genes found is shown in the appendix (Appendix Table 1). The two most up-regulated cell cycle-related genes were p21 and Cyclin D2 (Table 3.1).

ID reference	Name	Log_2-fold change	P-value	B-value	Rank
3580	CDKN1A (p21)	2.42	0.0002	1.06	67
5994	CCDND2 (Cyclin D2)	3.56	0.0005	0.253	97

Table 3.1 Comparative *in silico* analysis of the NCI-60 cell panel. *Shown are the two cell cycle regulatory genes most differentially expressed in IGROV-1 versus OVCAR3, OVCAR4, OVCAR5 and SKOV3 cells. Also listed are gene ID reference numbers, their Log_2-fold change in expression,as well as the rank in the top 100 list of differentially regulated genes. P-value is obtained from the distribution of the moderated t-statistic (the-lower-the-better). B-value is the empirical Bayes log-odds of differential expression (the-higher-the-better).*

3.13 Discussion

Comparison of a number of ovarian cancer and hTERT-immortalised ovarian surface epithelial cell lines revealed great variation between sensitivities to *dl*922-947. Among cancer cells, IC_{50} values ranged from 0.038pfu/cell to 250pfu/cell in A2780 cells. CAOV3 cells appeared even more resistant to the virus; however, these cells are very difficult to infect and do not express CAR (Lockley, 2007). As expected, hTERT-immortalised IOSE cells were less susceptible to the virus, with IC_{50} values in the same range as in non-sensitive cancer cells, SKOV3ip1 and A2780.

It is undisputable that infectivity is an important determinant of sensitivity. In primary ovarian and other tumours, low expression of CAR has frequently been described as a limiting factor for oncolytic adenoviral therapy (Cripe *et al.*, 2001; Vanderkwaak *et al.*, 1999). Comparison of ovarian cancer cell lines and primary ovarian carcinoma cells from 3 patients indicated relatively moderate to low levels of CAR in the latter. Also, primary cells expressed high levels of $\alpha_v\beta_3$, but little $\alpha_v\beta_5$ (Kelly *et al.*, 2000). However, the largest series of primary ovarian cancer studied indicated that 97% (36 of 37) expressed CAR, with the majority on the cell surface. In addition, 62% (23 of 39) expressed $\alpha_v\beta_3$ and 67% (24 of 37) $\alpha_v\beta_5$ (Zeimet *et al.*, 2002). This implies that loss of adenovirus receptors may not be as significant a problem in ovarian cancer as some have suggested. Corroborating this, ongoing GFP flourescence assays in our lab, involving primary ascitic cells from 6 ovarian cancer patients, indicated that cells from 4 patients were readily infectable.

Clearly, high infectability of TOV21G cells will render them more susceptible to lysis by adenovirus than the largely non-infectable CAOV3 cells. Nevertheless, my results imply infectivity cannot account for the full extent of variation in *dl*922-947 oncolytic efficacy. For instance, IGROV-1 and A2780CP cells are very similar in their infectability, measured by qPCR and GFP-flow cytometry, respectively. Nonetheless, IC_{50} of A2780CP cells is almost 16 times higher than that of IGROV-1 cells. In addition, TOV21G cells are 1300 times more sensitive than SKOV3ip1 cells, yet are only 20% more infectable, based on GFP-flow cytometry results.

The discrepancy found between the two methods assaying for infectivity itself merits discussion: interestingly, whilst both assays identified TOV21G cells as most infectable, SKOV3ip1 cells were more infectable than A2780CP cells by qPCR, but GFP-FACS suggested the opposite. However, these two methods measure different events. Flow cytometric analysis detects cells which express GFP after series of steps has occurred: binding of the adenovirus vector to the cell surface, internalisation, nuclear transport and expression from a CMV promoter. Meanwhile, the qPCR-based assay measures only the outcome of the first two steps: binding and internalisation. This is particularly important in light of a recent publication by Wang *et al*. In pancreatic cell lines, over-expression of a member of the immunoglobulin superfamily, carcinoembryonic antigen-related cell adhesion molecule 6 (CEACAM6) had no effect on binding and endocytosis of adenovirus. However, it was found to hamper adenovirus trafficking to the nucleus by inhibiting the Src signalling pathway and downregulating proteins of the cancer cell cytoskeleton. CEACAM6 knock-down led to significant increase in oncolytic effect of *dl*309 (Wang *et al.*, 2009). That binding and endocytosis of adenovirus is required but not sufficient for delivery of viral genes has been reported several years ago (Xia *et al.*, 2000).

Human cells have developed a range of anti-viral mechanisms (Castelli *et al.*, 1997; Lecellier *et al.*, 2005; Sanders *et al.*, 1998). One example is induction of protein kinase R (PKR) by interferon-γ (IFN-γ). Activated PKR phosphorylates and inhibits the α-subunit of translation initiation factor eIF-2, resulting in shut-down of protein translation in the cell (Naik *et al.*, 2009; Reichel *et al.*, 1985; Samuel, 1993; Thomis *et al.*, 1993). Adenovirus encodes two mRNAs, VAI and VAII, which are able to interfere with PKR activation and counteract this anti-viral mechanism (Kaufman, 1985; Kaufman *et al.*, 1987; Kitajewski *et al.*, 1986; Mori *et al.*, 1996). Obviously, should the VAI and VAII response of *dl*922-947 fail, total protein translation shut-down in the host cell would also prevent production of functional virus particles. However, Western blot analyses

provided evidence that all cell lines supported expression of viral proteins. Clearly, probing for a subset of viral proteins is not representative for expression of the entire genome. Yet, as both early and late gene products were found, it suggests that a lack of sensitivity is not caused by a general block in viral protein expression. The immunoblots suggested a correlation between onset of E1A expression and sensitivity, with earliest expression detected in TOV21G cells. E1A is the first viral gene to be expressed after infection and its transcription is entirely induced by transcriptional activators of the host cell (Bruder *et al.*, 1991; Bruder *et al.*, 1989). This implies that any host cell factor that promotes E1A expression will profoundly affect overall virus function.

At present, definitive knowledge of the mechanism of virus-induced cell death is limited. For a long time, it was generally assumed that death occurred upon lysis caused by the viral load within the cell (Fields *et al.*, 1985). To assess viral replication, two approaches were taken: numbers of genome copies were assessed by qPCR and of functional virus particles by $TCID_{50}$ assay. There was some link between sensitivity and $TCID_{50}$, especially early after infection. However, there were exceptions, especially SKOV3ip1 cells, which produced 2nd highest amounts of pfu/cell, despite being the least sensitive of the cancer cell lines. A very different picture emerged from qPCR analysis: 48 and 72 hours p.i., highest genome copy numbers were found in the two least sensitive cancer cell lines, SKOV3ip1 and A2780CP cells. This implies that these cells are able to generate high levels of viral DNA that is not packaged into functional virions and which does not trigger a death response. Overall, while some correlation may exist between E1A expression, viral replication and sensitivity to *dl*922-947-induced cytotoxicity, the relationship is far from complete. Moreover, my results strongly suggest that cells do not simply die once a threshold level of intracellular virus or viral DNA is reached. Thus, there must be other factors intrinsic to the host cell that define how readily that cell will die.

Western blot analysis of G1/S phase checkpoint components suggested that a number of Rb pathway members were associated with increased sensitivity, among them Cyclin D and cdk4, promoters of G1/S phase progression (Albrecht *et al.*, 1999; Depoortere *et al.*, 1998). A link between their over-expression and sensitisation in cancer cell lines is conceivable, as it could increase viral DNA replication. Other candidates found, however, where surprising: p21, p27 and p16 are generally perceived as inhibitors of entry into the cell cycle (Harper *et al.*, 1993; Serrano *et al.*, 1993; Wang *et al.*, 1996). Therefore, their over-expression would be expected to

prevent efficient viral replication. The outcome of this immunoblot assay was confirmed, in part, by *in silico* comparison of published expression array data, where p21 and Cyclin D emerged as the cell cycle genes most differentially expressed between sensitive and non-sensitive ovarian cancer cell lines of the NCI-60 panel (Blower *et al.*, 2007; Shankavaram *et al.*, 2007). In contrast, the pattern of protein expression in ovarian cancer cells could not be confirmed in hTERT-immortalised IOSE cells, which have normal Rb pathway function (Chapman *et al.*, 2006; Li *et al.*, 2007a). This suggests that the profile seen in sensitive TOV21G and IGROV-1 cells (high p21, high Cyclin D) is specific for malignant cells.

The isogenic MRC5/-VA cell line pair provides a very useful model to compare *dl*922-947 function in cells with a normal versus abnormal G1/S phase check-point. Yet, MRC5-VA cells are SV40 TAg-transformed and alterations of the cell cycle checkpoint mediated by SV40 TAg may be different to those present in ovarian cancers. Although there is growing evidence that many cancers are virus-induced (McBride, 2008), ovarian cancer is not one of them. Furthermore, MRC5 cells are derived from male foetal lung fibroblasts (Jacobs *et al.*, 1970). Biomarker candidates found in ovarian cancer cells may be very different to those in MRC5-VA cells, in particular within the context of the Rb pathway. The complexity of G1/S checkpoint control suggests that there are likely to be several mechanisms by which cells can be rendered sensitive to the effects of *dl*922-947. Experiments in the MRC5/-VA model may provide insight into such alternative pathways. For the stepwise dissection of the mechanisms underlying *dl*922-947 efficacy in ovarian cancer, however, normal and cancerous ovarian cell lines are the most suitable models.

In conclusion, the data presented in this chapter indicate that, although infectivity and viral replication do have some impact on cytotoxic efficacy of *dl*922-947, they are not the sole determinants. Equally, experiments in MRC5/-VA cells strongly support the hypothesis that Rb pathway dysregulation plays a role in enhancing sensititvity to the virus. Surprisingly, loss of this cell cycle checkpoint appears to go hand in hand with an upregulation of both cell cycle inhibitors (p21, p16, p27) as well as promoters (Cyclin D and E, cdk4). This phenomenon has previously been described by Black *et al*.: gene expression arrays in pRb- and p130/p107-knockout mice revealed that loss of the checkpoint proteins concurred with upregulation for example of Cyclins A and B, cdk2 and PCNA, but also CKIs p57 and p18 (Black *et al.*, 2003). From these observations, two questions arose: is the expression pattern of Rb pathway members found in sensitive ovarian cancer cells no more than a prognostic marker, a pattern that

happens to coincide with sensitivity to *dl*922-947? Or are these Rb pathway components genuine predictive markers that directly influence *dl*922-947 activity? Experiments in the following chapter were designed to address these questions.

4 Results: The role of p21 and other Rb pathway components in *dl*922-947 virus function

4.1 Introduction

Results discussed in the previous chapter suggested several potential biomarkers for *dl*922-947 cytotoxicity: p21, Cyclin D, p27, p16 and cdk4. This chapter describes the evaluation of these host cell factors.

4.1.1 p21

Upregulated p21 is a known marker of poor prognosis in various cancers, amongst them ovarian cancer (Gimenez *et al.*, 2009; Hu *et al.*, 2008; Werness *et al.*, 1999; Winters *et al.*, 2003).

The idea that p21 could be a promoter of *dl*922-947 oncolytic function is counter-intuitive. It is generally perceived as a cell cycle inhibitor by suppressing Cyclin/cdk activity and thus mediating cell cycle arrest (Brugarolas *et al.*, 1999; Bunz *et al.*, 1998). Also, p21 has been shown to prevent PCNA-mediated replication (Mattock *et al.*, 2001). Both effects would appear detrimental to viral propagation. However, there is increasing evidence for another dimension of p21 regulatory function. Various reports indicate that a profound distinction has to be made: cell cycle inhibitory effects of p21 result from high intracellular levels of the protein. In contrast, at basal levels, it has the opposite effect: early on in the cell cycle, p21 enhances kinase activity of Cyclin D/cdk4 complexes by binding to them and stabilising their interaction. This also sequesters p21 away from Cyclin E/cdk2 complexes. Moreover, p21 mediates nuclear targeting of cdk4 and Cyclin D and inhibits export of the latter (Alt *et al.*, 2002; LaBaer *et al.*, 1997). In turn, association with Cyclin D has been found to have stabilising effects on p21, itself (Coleman *et al.*, 2003). Later, transiently phosphorylated p21 facilitates Cyclin B/cdk1 complex formation (Dash *et al.*, 2005). These results define p21 as a promoter of both G1/S and G2/M phase progression. In addition, by complexing with co-activators CREB binding protein (CBP) and p300, p21 has been implicated in enhancing their function. The result is increased activation of target transcription factors, such as E2F, activator protein-1 (AP-1) and nuclear factor κB (NFκB) (Horvai *et al.*, 1997; Perkins *et al.*, 1997; Snowden *et al.*, 2000).

Finally, p21 has been reported to modulate apoptosis although published data are contradictory. On one hand, a decrease in p21 in various types of cancer cell lines has been described to sensitise them to apoptosis after exposure to cytotoxic drugs, γ-irradiation and IFN-γ (Detjen *et al.*, 2003; Mahyar-Roemer *et al.*, 2001; Tian *et al.*, 2000). Also, p21 was found to complex with pro-caspase 3, inhibiting Fas-mediated

apoptosis (Suzuki *et al.*, 2000), whilst in apoptotic cells caspase 3 cleaved p21 (Jin *et al.*, 2000). Other findings, however, imply that over-expressing p21 in glioma, liver and ovarian cancer cell lines leads to enhanced apoptosis after treatment with cisplatin (Qin *et al.*, 2001). A study on sodium butyrate (NaB)-induced apoptosis suggested that p21 may be expendable for cell cycle arrest but strictly required for induction of apoptosis (Chopin *et al.*, 2004).

In view of the effects of p21 in promoting cell cycle progression and potentially augmenting cell death, an enhancing effect of p21 on *dl*922-947 function appears less abstruse.

4.1.2 p27, Cyclin D, cdk4, Cyclin E and p16

Amongst the other candidates for *dl*922-947-enhancing roles, p27 appears to be the most similar to p21, with regards to structure, function and involved pathways (Sherr *et al.*, 1995). The prevailing conception of p27 is that of a cdk inhibitor (Polyak *et al.*, 1994b). By binding to cdk4/6 p27 can inhibit Cyclin D/cdk4/6 complex formation (Ray *et al.*, 2009). Both p21 and p27 are marked for degradation through phosphorylation by Cyclin E/cdk2 kinase activity. More importantly, like p21, p27 has been reported to promote Cyclin D/cdk4 complex formation (Cheng *et al.*, 1999). The switch from inhibitor to promoter function of p27 may be based on a specific phosphorylation (Ray *et al.*, 2009). Cheng et al. used a knock-out model in mouse embryonic fibroblasts (MEFs) to investigate effects of p21 and p27 loss on Cyclin D/cdk4 complex formation and cellular localisation. They found that loss of p21 or p27 led to reduction of total Cyclin D protein and Cyclin D/cdk4 complexes. In cells lacking both p21 and p27 even less Cyclin D was detected and Cyclin D/cdk4 complexes appeared completely lost. In the latter case, the effect superseded that of mere reduction of Cyclin D protein. Finally, just like p21, by associating with Cyclin D/cdk4 complexes, p27 targets them to the nucleus. Although neither CKI is required for Cyclin D nuclear import, their presence strongly increases Cyclin D levels in the nucleus. In view of the similarities between p21 and p27, it would be interesting to see not only whether they are true determinants of *dl*922-947 cytotoxic function, but also if they have additive or synergistic effects (Cheng *et al.*, 1999).

A *dl*922-947 enhancing role for Cyclin D, cdk4 and, in view of its functional proximity, also Cyclin E, is easily conceivable. All three facilitate cell cycle progression, proliferation and, ultimately, replication of host and viral DNA (Akli *et al.*, 2003; Grana *et al.*, 1995; Reed, 1997). In the presence of mitogenic signals, Cyclin D and cdk4

accumulate and form complexes in the cytoplasm. Next, they translocate to the nucleus, where they undergo phosphorylation (Ensslen *et al.*, 1976). In turn, the now active holoenzymes phosphorylate pRb (Kato *et al.*, 1993). Among the target genes repressed by pRb inactivation is Cyclin E, whose activity is necessary for S phase entry (Geng *et al.*, 1996; Matsumoto *et al.*, 2004).

Cyclin levels are known to fluctuate throughout the cell cycle, constituting a limiting factor in Cyclin/cdk kinase activity. In contrast, levels of cdks remain more stable (Hengstschlager *et al.*, 1999). In that context, higher levels of cdk4 may not predict higher *dl*922-947 cytotoxicity. The ability of cdk4 to complex with Cyclin D is dependent on the proteins associated with that particular cdk4. Cdk4 bound to Hsp90/Cdc37 and p16, although stable, does not bind Cyclin D. However, cdk4 is enzymatically active only when in complex with Cyclin D (Cheng *et al.*, 1999). Although an increase in cdk4 alone may not promote *dl*922-947 function, it might if coupled to higher levels of Cyclin D.

In contrast, a role for p16 in promoting oncolytic effects of *dl*922-947 seems more difficult to perceive. As explained in Chapter 1, p16 was originally identified as an inhibitor of cdk4 and cdk6, and consequently, of pRb phosphorylation and G1/S phase progression (Serrano *et al.*, 1993). Unlike p21 and p27, no additional, cell cycle promoting functions for p16 have been reported to date. Mice with complete knock-out for p16 are viable and normal, except for increased tendency to develop cancer (Krimpenfort *et al.*, 2001; Sharpless *et al.*, 2001). It is possible that correlations between levels of p16 and sensitivity may be a consequence of the upregulation of the other cell cycle promoters.

4.2 Methods

To validate or discard a role for p21 in cytotoxic effect of *dl*922-947, I evaluated isogenic matched p21 +/+ and -/- cells. In addition, siRNA was used to knock down p21 protein in TOV21G and IGROV-1 cells. To obtain a pair of ovarian cancer cells with high and low p21 expression, A2780CP cells were transfected with a vector encoding p21. Cells were tested for alterations in sensitivity to virus, infectivity, production of viral progeny and viral protein expression. To validate *in vitro* results, xenograft mouse models were established. Subcutaneous Hct116 p21+/+ or p21-/- tumours and intraperitoneal (i.p.) A2780CP tumours with high and low p21 expression were treated with *dl*922-947 to compare responses to treatment.

The potential role of Cyclin D1/D2/D3, p27, cdk4, Cyclin E and p16 was tested by RNAi knock-down and assessing changes in sensitivity to and production of *dl*922-947. To this end, a small-scale RNAi library, containing shRNA-encoding vectors, was generated. Subsequently, TOV21G cells were transfected with plasmids from the library to stably knock down p27, Cyclin D1, cdk4 or Cyclin E.

4.3 Hct116 p21+/+ and p21-/- cells

Hct116 is a human colorectal carcinoma cell line. In recent years, several knock-out cell pairs have been generated. On such pair, consisting of the wild-type p21 expressing (Hct116 p21+/+) and the p21 knock-out cell line, was kindly provided by Dr. Bert Vogelstein. The cell pair constitutes a pure system of two nearly isogenic cell lines, differing only in the expression of p21. It therefore allowed a comparison of effects of p21 on *dl*922-947 in a system with very little background noise.

As a preliminary step, to confirm basal levels of p21 in one and absence thereof in the other cell line, Western blot analysis on total protein samples was performed by Katrina Pirlo (Centre for Molecular Oncology and Imaging, Institute of Cancer, Barts and The London School of Medicine and Dentistry, London, UK). Hct116 p21+/+ samples provided a strong specific band for p21 protein. In contrast, in Hct116 p21-/- cells the stable knock-out resulted in complete abrogation of p21 protein, as can be seen in the immunoblot in Fig. 4.1.

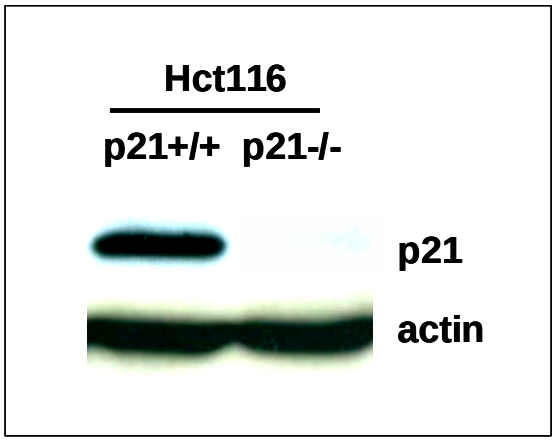
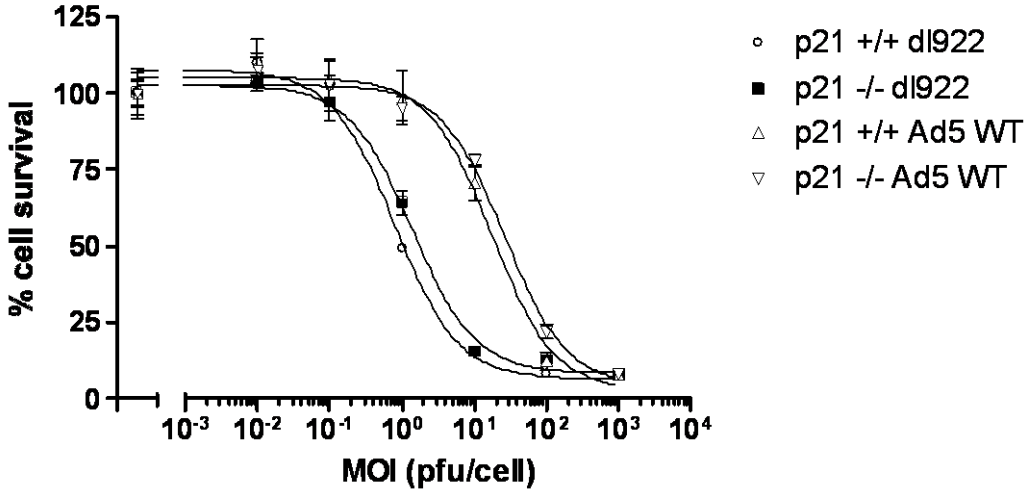


Figure 4.1 p21 protein in Hct116 p21+/+ and p21-/- cells. *Western blot analysis was performed on cell lysates probing for p21. Actin levels served as a loading control.*

4.4 Sensitivity of Hct116 cells to *dl*922-947

Cell viability assays were carried out to compare sensitivity to adenoviruses *dl*922-947, wild-type adenovirus 5 (Ad WT) and *dl*309 in p21 expressing and non-expressing Hct116 cells. Cell survival was assessed up to 120 hours post infection.

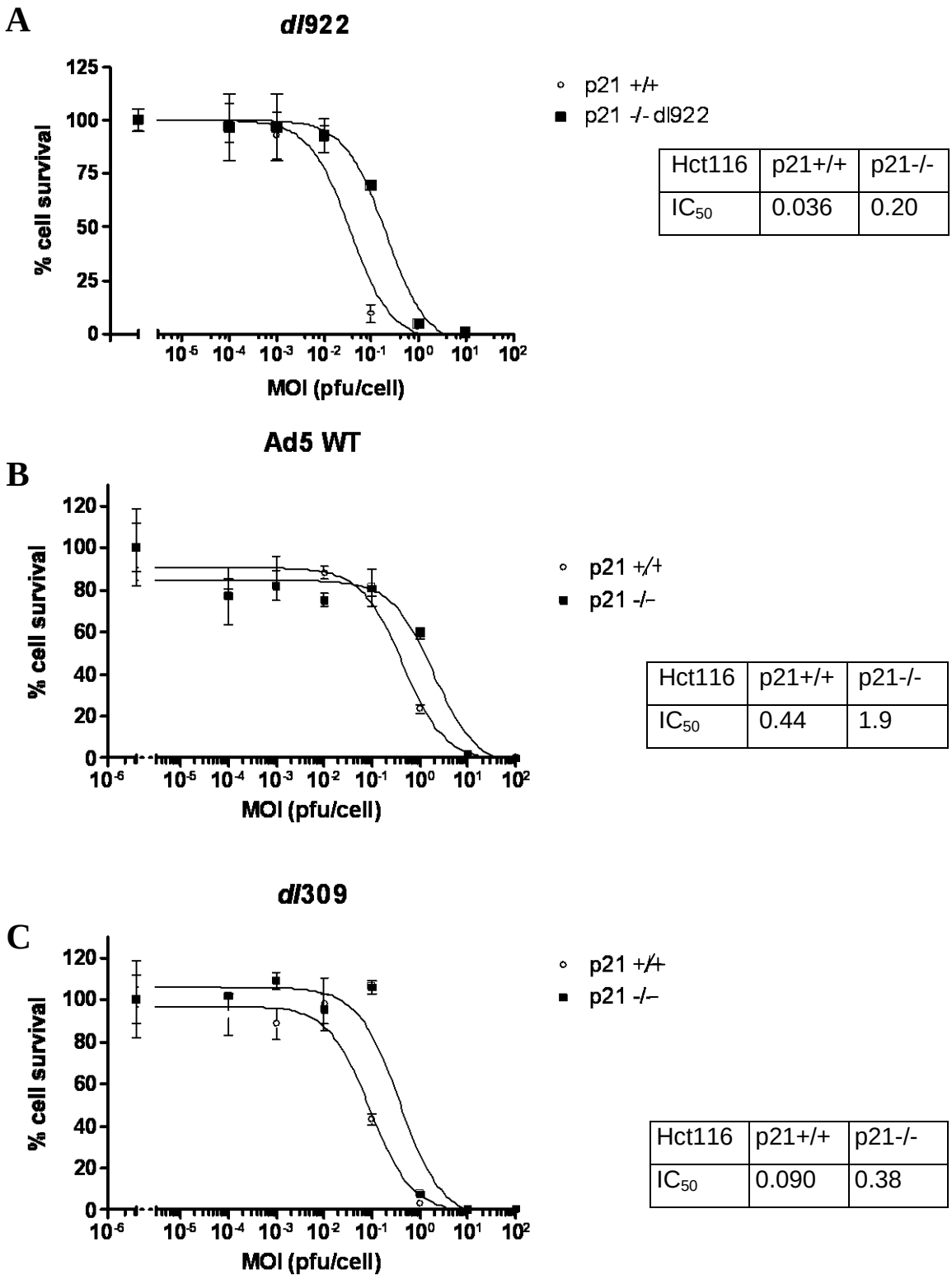
At 72 hours p.i. with *dl*922-947 or Ad WT, there was no difference in sensitivity between Hct116 p21+/+ and p21-/- cells (Fig. 4.2). There was, however, a notable difference regarding cytotoxicity between the two viruses.



	*dl*922-947		Ad WT	
Hct116	p21+/+	p21-/-	p21+/+	p21-/-
IC_{50}	0.79	1.4	17	26

Figure 4.2 Sensitivity of p21+/+ and p21-/- cells to *dl*922-947 and Ad WT. *Dose response curves of Hct116 p21+/+ and p21-/- cells 72 hours p.i. with dl922-947 or Ad WT graphed relative to MOI pfu/cell of dl*922-947*. Points represent mean +/- standard deviation.*

At 120 hours p.i., Hct116 p21-/- cells were strikingly less senstitive to *dl*922-947 than their p21-positive counterparts. A similar phenomenon was seen with Ad WT and *dl*309 (Fig. 4.3).



Hct116	p21+/+	p21-/-
IC_{50}	0.036	0.20

Hct116	p21+/+	p21-/-
IC_{50}	0.44	1.9

Hct116	p21+/+	p21-/-
IC_{50}	0.090	0.38

Figure 4.3 Sensitivity of p21+/+ and p21-/- cells to different adenoviruses. *Dose response curves of Hct116 p21+/+ and p21-/- cells 120 hours p.i. graphed relative to MOI pfu/cell of* **A.** *dl*922-947, **B.** Ad WT *and* **C.** *dl*309*. Points represent mean +/- standard deviation.*

4.5 *In Vivo*: Efficacy of *dl*922-947 in p21 expressing and non-expressing tumours in mice

In a pilot study, $5x10^6$ Hct116 p21+/+ or p21-/- cells were injected subcutaneously into CD1 nu/nu mice female mice. Once tumours reached approximately $150mm^3$ (p21+/+ tumours: $196mm^3$; p21-/- tumours: $161mm^3$), mice were treated, in groups of four, with intra-tumoural injections of 10^{10} *dl*922-947 particles in 50µl PBS on days 18, 22 and 26. Tumours were measured twice weekly with callipers.

The study was ended 42 days after the initial virus injection and remaining animals killed. Tumour sizes in the two groups are shown in Fig. 4.4. Whilst Hct116 p21+/+ tumours responded well to treatment with *dl*922-947, Hct116 p21-/- tumours increased in volume at a higher rate. However, the differences did not reach statistical significance.

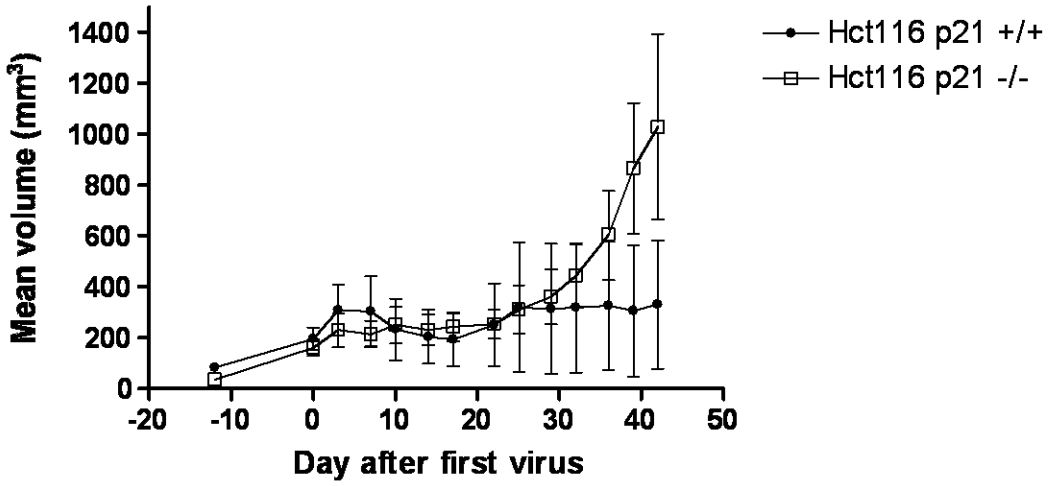


Figure 4.4 *In vivo* response of mice bearing p21 expressing and non-expressing xenograft tumours to *dl*922-947. *Mice with Hct116 p21+/+ or p21-/- -derived sub-cutaneous tumours were treated with 3 intra-tumoural injections of 10^{10} particles dl*922-944*. Points represent mean tumour volumes +/- standard error.*

The study was repeated, however, using 20 CD1 nu/nu mice. Five mice per each group were treated with three injections of *dl*922-947 and the other five mice with non-replicating control virus Ad-CMV-GFP. As before, tumour volumes were recorded twice weekly and mice killed once Home Office limits were reached. 84 days after first treatment the study was ended. Fig. 4.5 shows tumour sizes over time in the four groups. Overall, tumours treated with control virus showed continuous and rapid growth and mice had to be killed approximately 30 days after the first virus injection In contrast, *dl*922-947 treated p21+/+ tumours responded well, with only 1 mouse with visible tumour at the end of the experiment. p21-/- tumours treated with *dl*922-947 initially decreased in size. Later, however, tumours grew steadily until the study was terminated. p21+/+ tumours were significantly smaller than p21-/- tumours at late timepoints following *dl*922-947 injection.

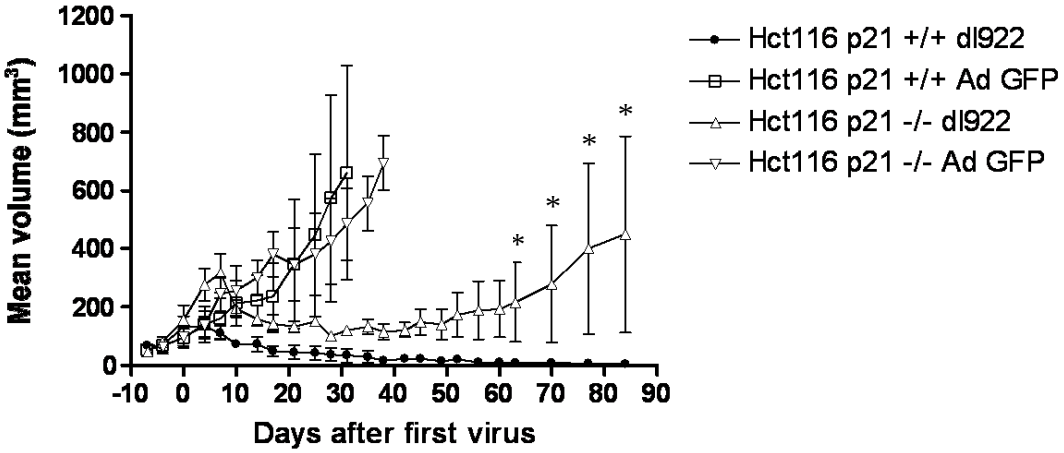


Figure 4.5 *In vivo* response of mice bearing p21 expressing and non-expressing xenograft tumours to *dl*922-947. *Mice with Hct116 p21+/+ or p21-/- -derived sub-cutaneous tumours were treated with dl*922-947 *or control virus Ad-GFP. Points represent mean tumour volumes +/- standard error. *p<0.05, one-tailed unpaired student's t-test.*

4.6 Knock-down of p21 in IGROV-1 and TOV21G cells

To examine whether a loss of p21 in TOV21G and IGROV-1 cells would bring about a decrease in *dl*922-947 cytotoxic efficacy, p21 was knocked down by siRNA. As is evident from Western blot analysis and densitometry shown in Fig. 4.6, using Dharmacon SMARTpool siRNA specific for p21, efficient knock-down of p21 protein levels in IGROV-1 and TOV21G cells was achieved. The effect persisted for at least 96 hours post transfection.

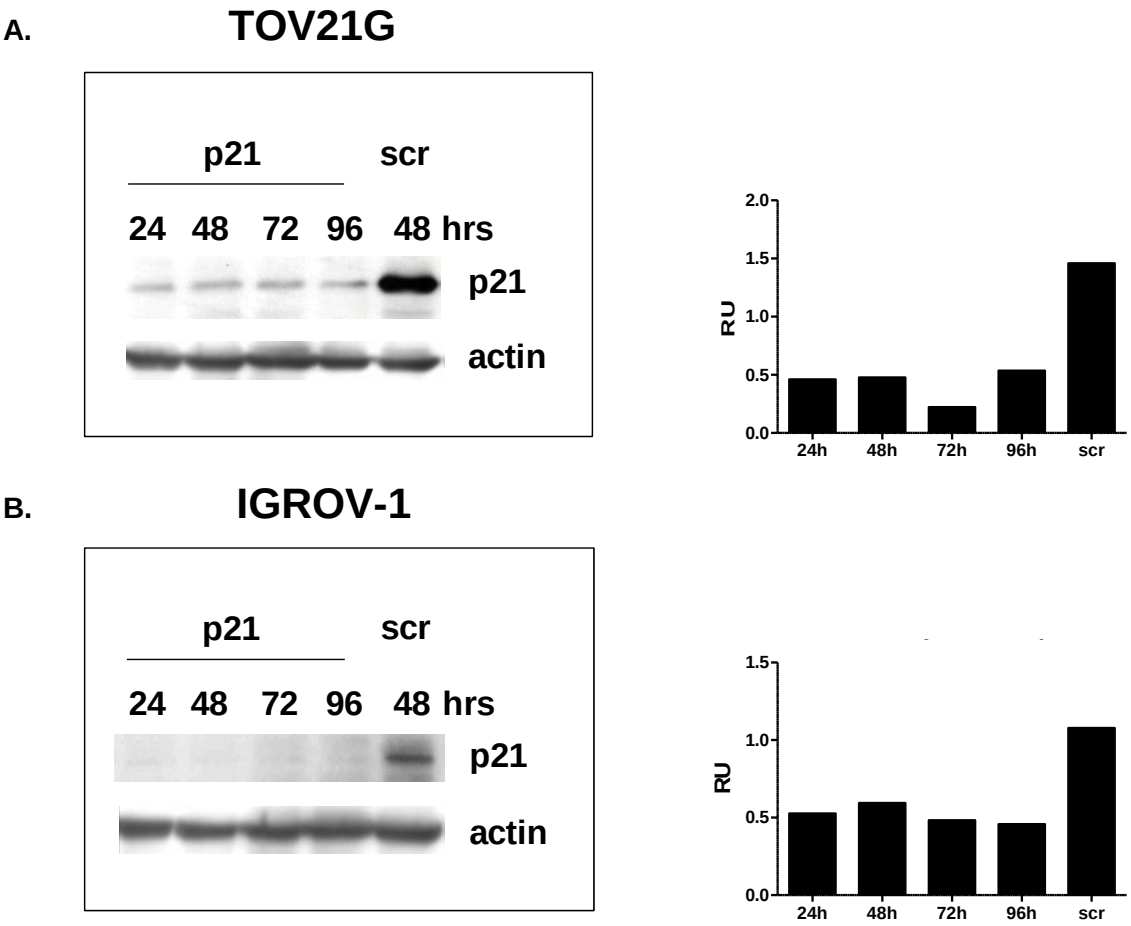


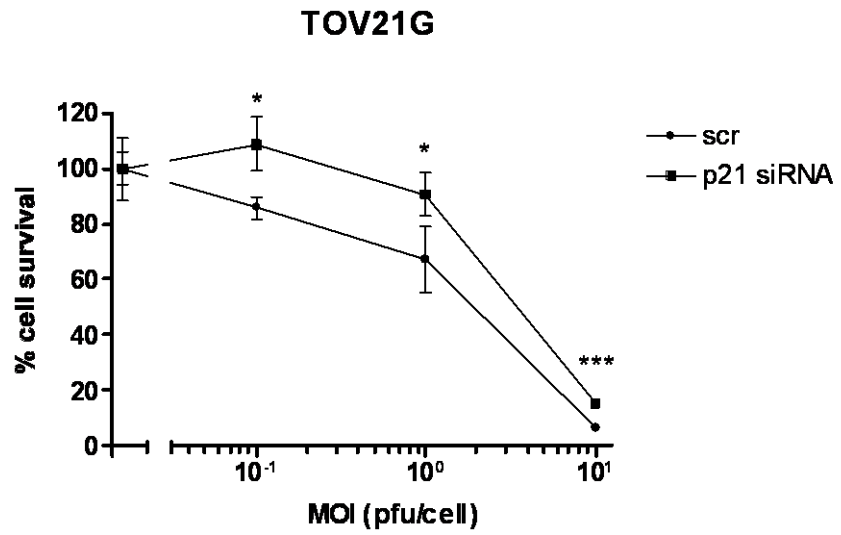
Figure 4.6 Knock-down efficiency of p21 siRNA in IGROV-1 and TOV21G cells. *Western blot analysis of p21 protein levels in* **A.** *TOV21G cells (**left**) with corresponding densitometric analysis (**right**) and* **B.** *IGROV-1 cells (**left**) with corresponding densitometric analysis (**right**). Actin was used as a loading control and for normalisation in densitometry. RU=relative units.*

4.7 Loss of p21 and sensitivity to *dl*922-947

To investigate further whether p21 expression played a role in cell sensitivity to *dl*922-947, cell survival experiments were performed on the two cell lines after transient knock-down of p21. Eight hours post transfection with p21 siRNA or control scrambled

(scr) siRNA, cells were infected with *dl*922-947 at MOIs between 100 and 0.01pfu/cell. Cell viability was quantified 96 hours later by MTT assay (Fig. 4.7). siRNA-mediated loss of p21 in TOV21G cells resulted in a significant decrease in sensitivity to *dl*922-947 ($p \leq 0.05 – 0.001$) at all three MOIs applied, as compared to cells transfected with scrambled control. Similarly, sensitivity of IGROV-1 cells was affected by the loss of p21. However, the decrease in sensitivity was statistically significant only at MOI 1 and 100pfu/cell.

A.



B.

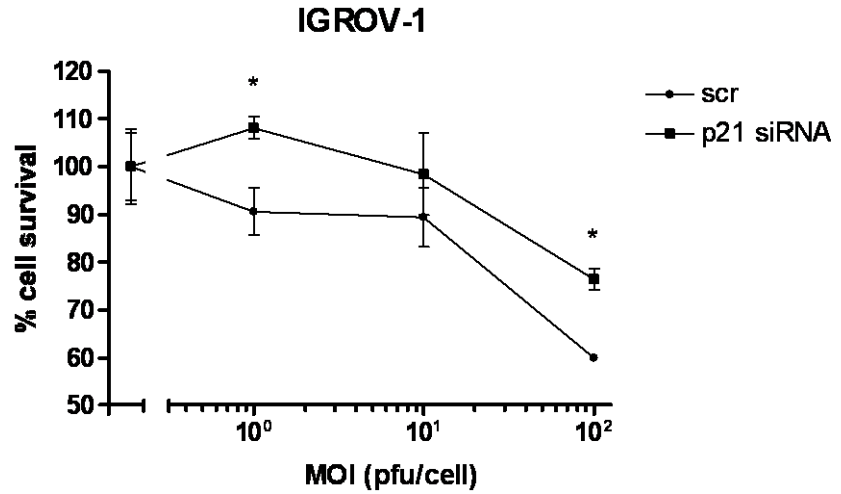


Figure 4.7 Cytotoxicity of *dl*922-947 after k.d. of p21. *Dose response curves of* **A.** *TOV21G and* **B.** *IGROV-1 cells transfected with p21 or scrambled control siRNA graphed relative to MOI pfu/cell of dl*922-947*. Points represent mean +/- standard deviation. * p=0.05, *** p = 0.001*

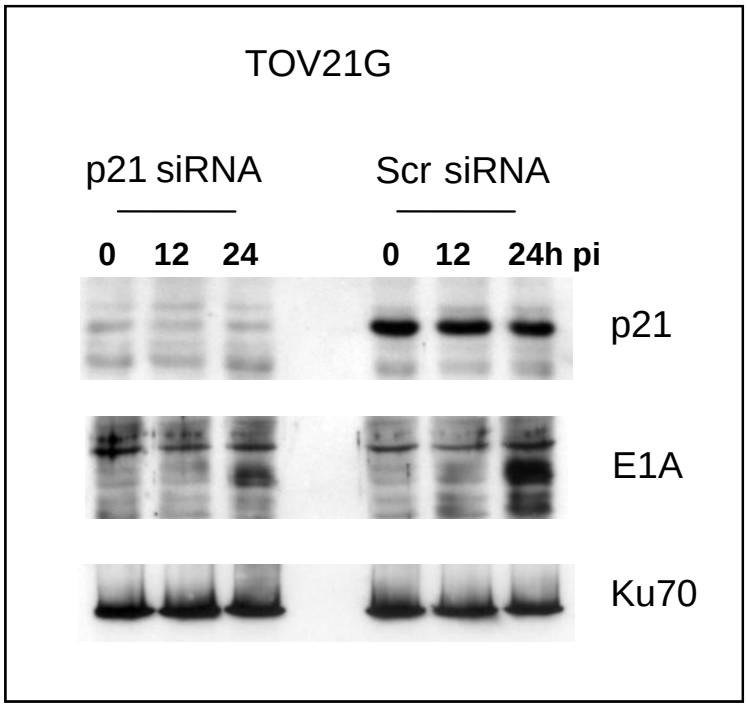
4.8 p21 and viral gene expression

Results in Chapter 3 suggested that cells sensitive to *dl*922-947 supported earlier expression of adenoviral proteins, especially E1A, the first viral gene to be expressed, and imperative to adenoviral replication (Bruder *et al.*, 1991; Bruder *et al.*, 1989). To assess the effects of altering intracellular p21 levels on viral gene expression, two approaches were taken. Firstly, reduction of the protein was achieved by siRNA-mediated knock-down in TOV21G cells before comparing E1A expression levels by Western blot. Secondly, expression levels were compared in p21 over-expressing cells, ACP-WAF1, and their low-p21 counterpart, ACP-GFP.

4.9 E1A expression after p21 knock-down in TOV21G cells

TOV21G cells on 6-well plates were transfected with siRNA targeting p21 or non-targeting scrambled siRNA. 24 hours later, cells were infected with *dl*922-947 at MOI 10pfu/cell. 12 and 24 hours later, protein was collected and Western blots performed probing for p21 and E1A. An image of the results is shown in Fig. 4.8. Immunoblots for E1A revealed a weak but specific band at 12 hours p.i. and a strong band at 24 hours p.i. in scrambled controls. In contrast, in p21 knock-down cells, no band was detectable at 12 hours p.i. A signal was visible at 24 hours p.i., but it was weaker than in scrambled controls.

A.



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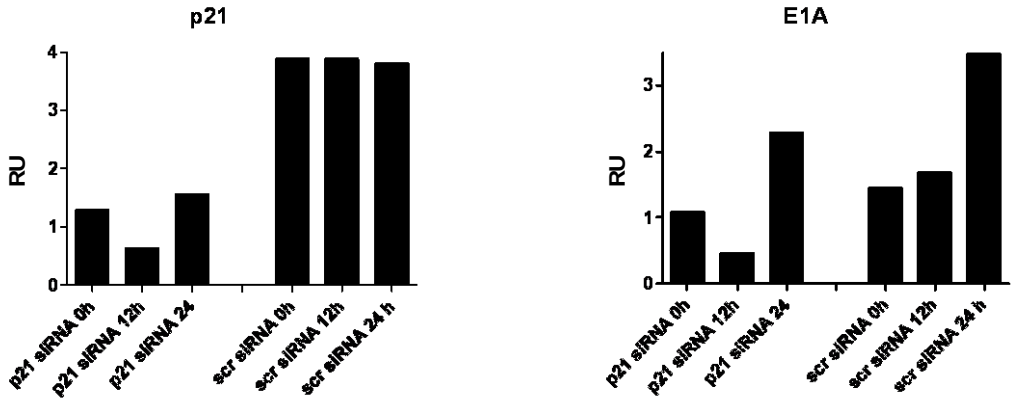


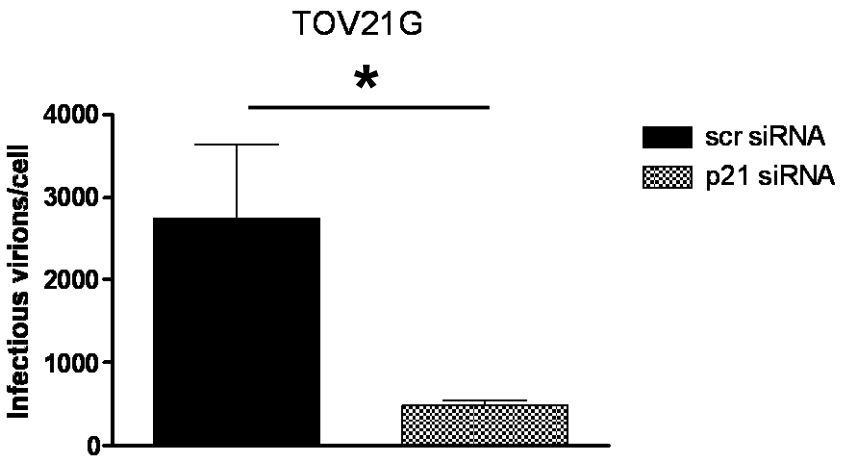
Figure 4.8 Expression of p21 and Cyclin D in TOV21G cells after p21 knock-down. A. *Image of the immunoblots for p21 and E1A in TOV21G cells treated with p21-specific or scrambled (Scr) siRNA. Staining for Ku70 served as a loading control.* **B.** *Quantification of results by densitometric analysis. Values of p21 or E1A signal are normalised against the respective Ku70 control and shown in relative units (RU).*

4.10 Loss of p21 and virion production

To gain a deeper understanding as to how p21 loss may affect cytotoxic potential of *dl*922-947, I examined whether transient knock-down of the protein might have repercussions on production of functional virions. Again, 8 hours after transfecting IGROV-1 and TOV21G cells with p21 or scrambled control siRNA, cells were infected

with *dl*922-947 (MOI 10pfu/cell). Cells were harvested 48 hours p.i. and analysed by $TCID_{50}$ assay. Infectious virion titres per cell in TOV21G cells after p21 knock-down were significantly (p=0.02) lower than in their scrambled control-treated counterparts (Fig. 4.9.A). Similarly, loss of p21 in IGROV-1 cells resulted in lower, albeit not significantly, numbers of infectious virions produced per cell compared to scrambled control cells (Fig. 4.9.B).

A.



B.

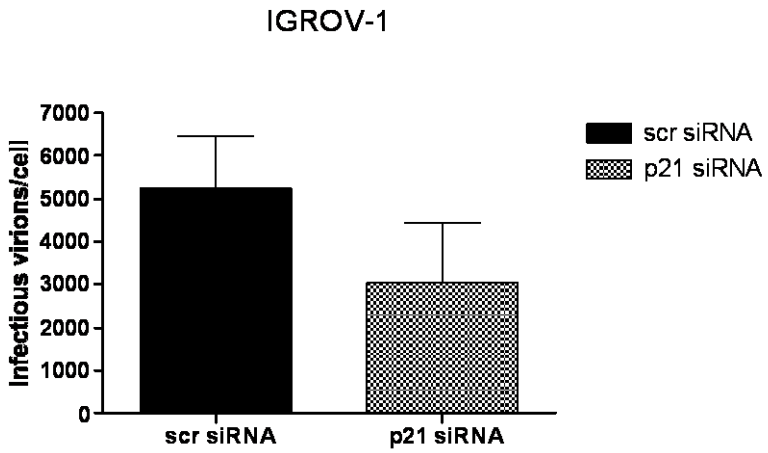


Figure 4.9 Virus replication upon transfection with p21 siRNA. *Infectious virions produced in* **A.** *TOV21G and* **B.** *IGROV-1 cells after transfection with p21 or scrambled control siRNA and 48 hours p.i. with dl*922-947 *quantified by* $TCID_{50}$ *assay. Bars represent mean +/- standard deviation. *p=0.02*

4.11 Overexpression of p21 in A2780CP and SKOV3ip1 cells

To investigate further the effects of p21, I attempted to generate A2780CP and SKOV3ip1 cells stably expressing increased levels of p21.

4.11.1 Transfection of SKOV3ip1 and A2780CP cells

SKOV3ip1 and A2780CP cells were transfected with pEGFP-p21 and selected in G-418. As control, both cell lines were transfected with pCMV-GFP. Approximately 80% of transfected A2780CP cells grown in selective media were positive for green fluorescence, as assessed by microscopy. After several passages, cells were FACS-sorted to enrich the cell pools for GFP-positive cells. These cell pools, referred to as ACP-p21 and ACP-GFP cells, were used for subsequent experiments. In contrast, although some transfected SKOV3ip1 cells were able to grow in antibiotic-containing medium, they failed to emit a green fluorescence signal under UV light, as assessed by microscopy.

4.11.2 Expression of EGFP-p21 in transfected cells

Transgene expression was assessed by Western blot. The expected GFP band (30kDa) was detected in ACP-GFP cells, as well as a faint band corresponding to low level endogenous p21 (Fig. 4.10). The latter was also found in A2780CP and ACP-p21 cells. However, there was no 50kDa band corresponding to the GFP-p21 fusion protein in the ACP-p21 cells. Instead, there was a strong double band of approximately 30kDa on GFP staining and no detecateble band on p21 staining. Experiments using ACP-p21 cells were discontinued.

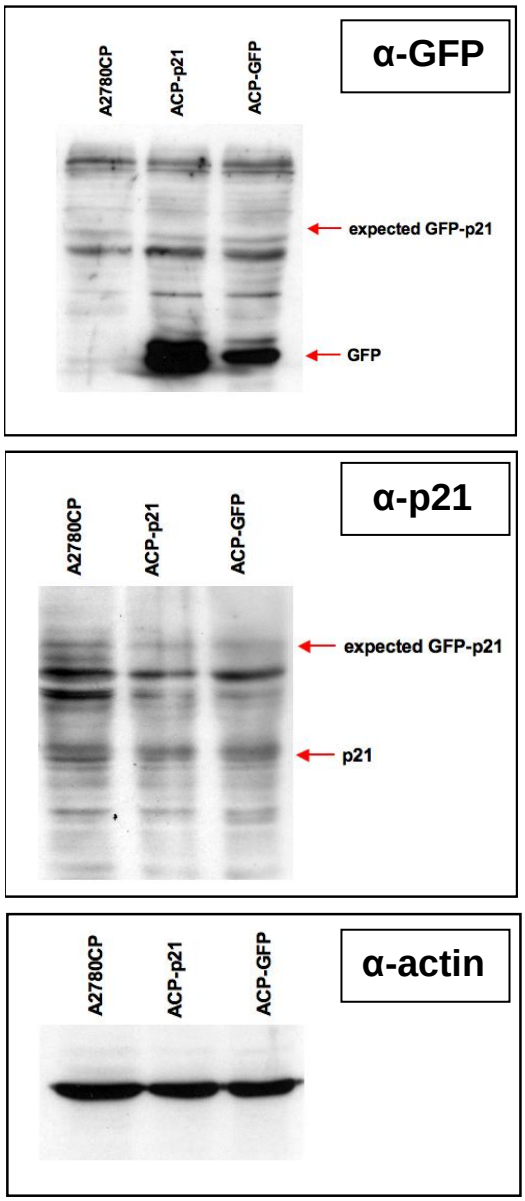
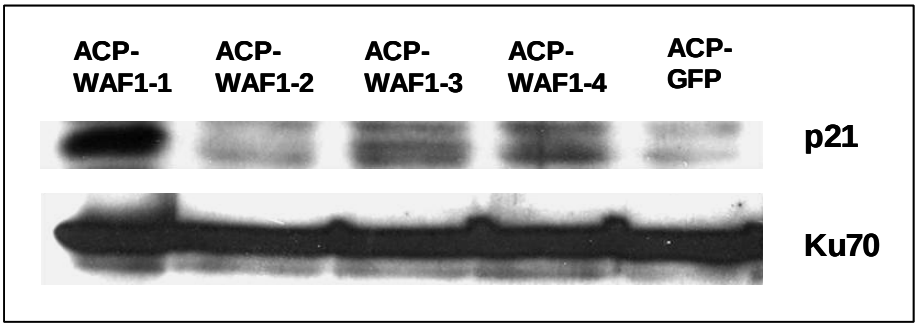


Figure 4.10 Detection of GFP-p21 and GFP and p21 expression in A2780CP, ACP-p21 and ACP-GFP cells. *Western blot analysis was performed on cell lysates to assess transgene expression. Membranes were blotted for GFP (**α-GFP**), p21 (**α-p21**) or actin (**α-actin**). Actin levels served as a loading control.*

4.11.3 Transfection of A2780CP and SKOV3ip1 cells with pCEP-WAF1

In a second attempt to increase levels of p21 in A2780CP and SKOV3ip1 cells, plasmid pCEP-WAF1 was used, which encodes human p21 under the control of the CMV promoter, as well as a hygromycin resistance gene for selection. Transfected SKOV3ip1 cells were not viable in antibiotic-containing medium. However, four pools of pCEP-WAF1-transfected A2780CP cells were generated, which are referred to as ACP-WAF1. After two passages, p21 protein expression in the four pools was assessed by Western blot analysis (Fig. 4.11).

A.



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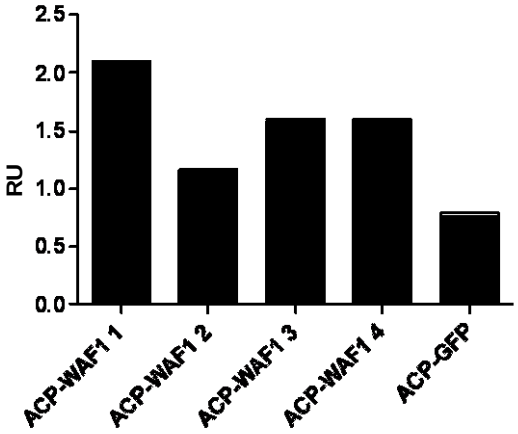


Figure 4.11 Expression of p21 protein in transfected A2780CP cells. A. *Image of the immunoblot for p21 protein in pools 1-4 of ACP-WAF1 cells as well as control cells ACP-GFP. Staining for Ku70 served as loading control. Staining for Ku70 served as loading control.* **B.** *Quantification of results by densitometric analysis. Values of p21 signal are normalised against the respective Ku70 control and shown in relative units (RU).*

Of the four ACP-WAF1 pools, only pool 1 expressed detectable p21 and these cells were used in all subsequent experiments.

4.12 Proliferation rates of ACP-WAF1 and ACP-GFP cells

Over-expression of a cell cycle inhibitor, such as p21, in a cell line may result in a lowering of the growth rate. Were that the case in ACP-WAF1 cells, any increase in sensitivity and virion production could be due to fewer cells being exposed to virus, as compared to ACP-GFP cells. This could lead to false positive results. To compare growth rates of the cell line pair, a proliferation assay was set up. Fig. 4.12. shows that expression of p21 has no effect on cell growth over 96 hours.

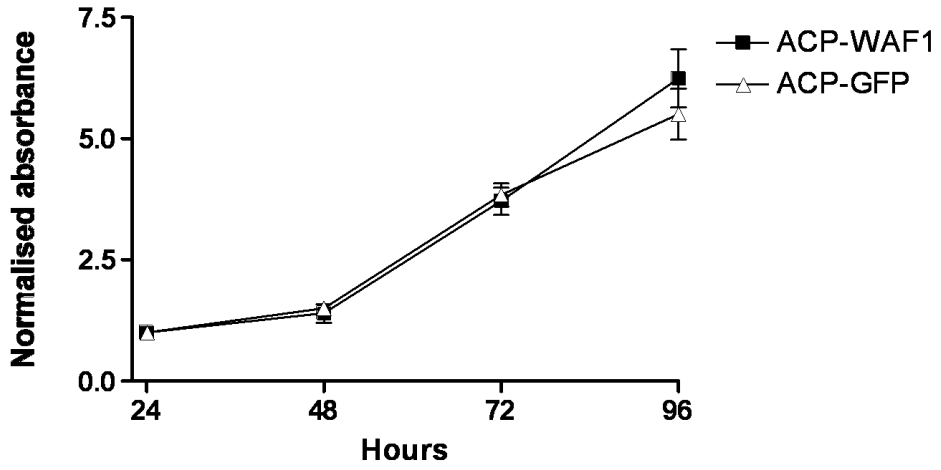
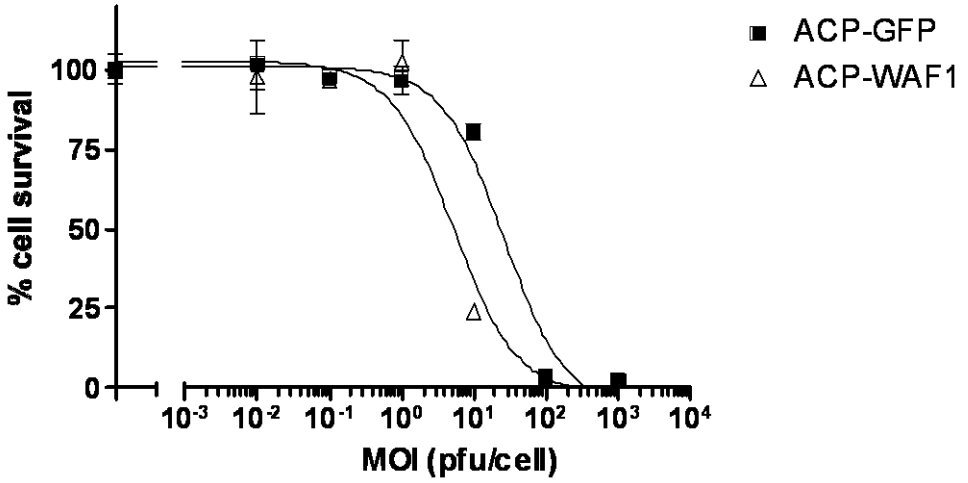


Figure 4.12 Growth curve of ACP-WAF1 and ACP-GFP cells. *Increase of cell numbers per well over a period of 96 hours is shown. Curves have been normalised against absorption at 24h. Points represent mean +/- standard deviation.*

4.13 Over-expression of p21 and sensitivity to *dl*922-947

ACP-WAF1 and ACP-GFP cells were infected with *dl*922-947 (MOI 0.01-1000pfu/cell) or mock-infected. Cell survival was quantified 144 hours p.i. Experiments were repeated three times. Fig. 4.13 shows representative curves and IC_{50} values. ACP-WAF1 cells had an IC_{50} of 5pfu/cell compared to 26pfu/cell in ACP-GFP controls.



	ACP-WAF1	ACP-GFP
IC_{50}	5.8	27.8

Figure 4.13 Sensitivity of ACP-WAF1 and ACP-GFP to *dl*922-947. *Dose response curves of ACP-WAF1 and ACP-GFP cells 144 hours p.i., graphed relative to MOI pfu/cell of dl*922-947*. Points represent mean +/- standard deviation. The table below shows IC_{50} values (pfu/cell).*

4.14 Infectivity of ACP-WAF1 and ACP-GFP cells

In order to investigate whether the increase in sensitivity to *dl*922-947 in ACP-WAF1 cells was based on increased virus uptake, their infectivity was compared to that of parental A2780CP cells using the GFP fluorescence assay (Fig. 4.14). Interestingly, particularly after infection with the lower MOI, p21 over-expressing cells were significantly less infectable than their parental counterpart.

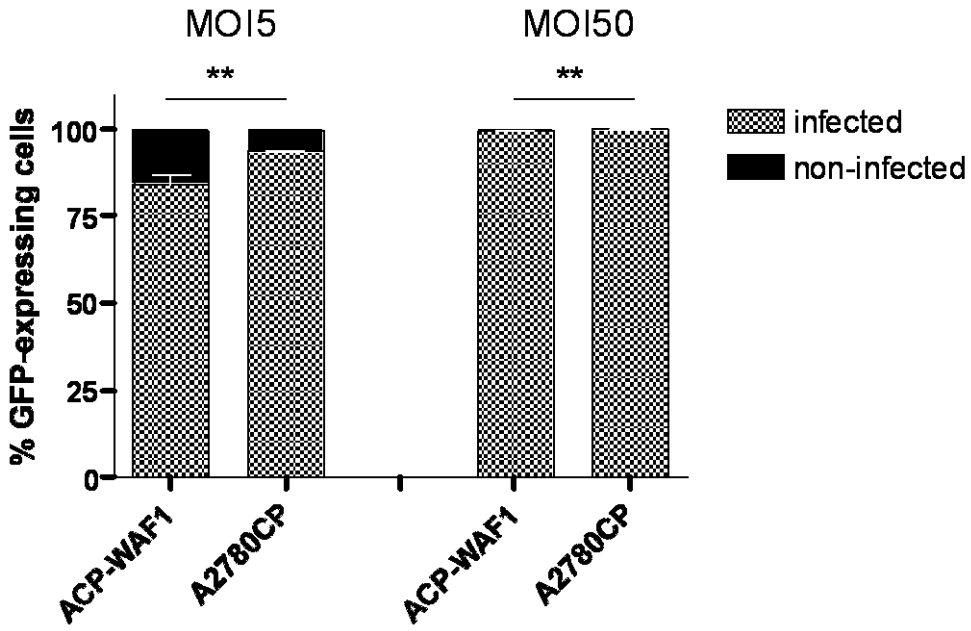


Figure 4.14 Infectivity of ACP-WAF1 and A2780CP cells. *Percentages of GFP-expressing ACP-WAF1 and A2780CP cells compared to non-infected cells after infection with Ad-GFP at MOI 5pfu/cell and 50 pfu/cell measured by flow cytometry. Bars represent mean +/- standard deviation. **<0.007.*

4.15 E1A expression in p21 over-expressing cells

ACP-WAF1 and ACP-GFP cells were infected with *dl*922-947 (MOI 10pfu/cell). Protein was harvested up to 48h p.i.. Fig. 4.15. shows images of immunoblots for E1A. Although both cell lines supported E1A expression, this expression was evident earlier in the ACP-WAF1 cells.

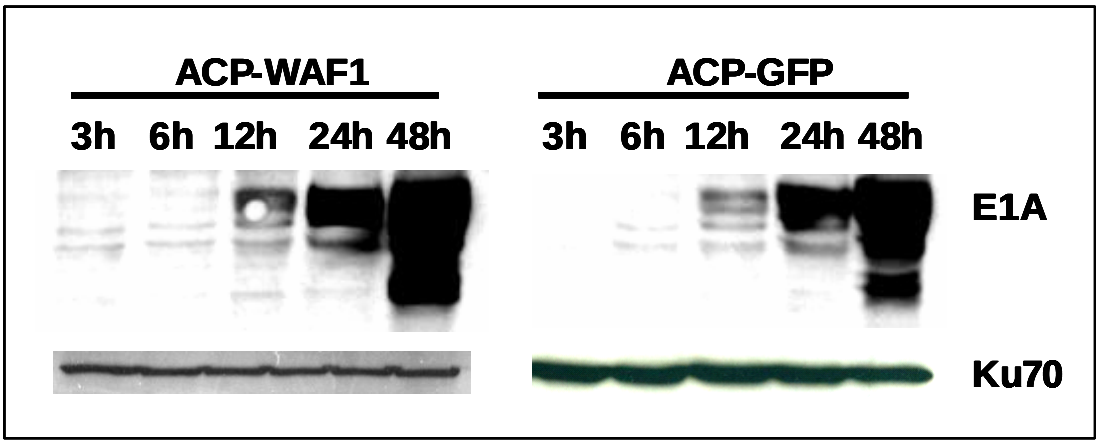


Figure 4.15 Expression of E1A in ACP-WAF1 and ACP-GFP cells after infection. A. *Image of the immunoblots for E1A in ACP-WAF1 and ACP-GFP cells 3-48 hours after infection with dl*922-947 *at MOI 10pfu/cell. Staining for Ku70 served as a loading control.*

4.16 Virion production in p21 over-expressing cells

To explore effects of p21 on virion production in A2780CP cells, $TCID_{50}$ assays were performed in *dl*922-947-infected ACP-WAF1 and ACP-GFP cells. Both supernatants and cell lysates were harvested 48 and 72 hours p.i. (Fig. 4.16). At both timepoints, higher amounts of functional virus were produced and released from ACP-WAF1 cells than control cells. These differences reached statistical significance in supernatants at 48 hours (p=0.02) and in intracellular samples at 72 hours (p=0.001) p.i..

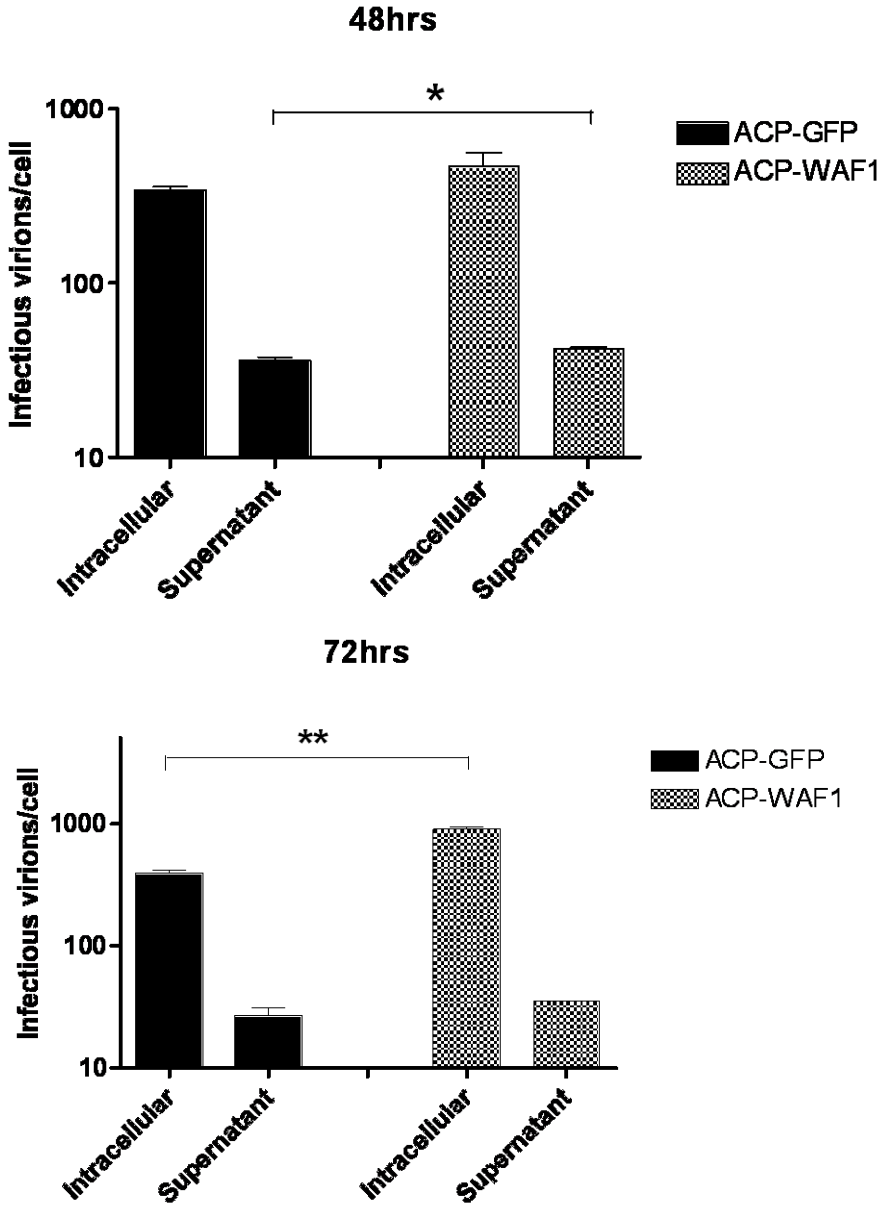


Figure 4.16 Virion production after p21 over-expression. *Infectious virions detected by $TCID_{50}$ assay in cell lysates or supernatants from ACP-WAF1 and ACP-GFP cells 48 and 72 hours post infection with dl*922-947*. Bars represent mean +/- standard deviation. *p=0.04, **p=0.002.*

4.17 *In vivo* I: Replication study of *dl*922-947

In an attempt to reproduce *in vivo* the beneficial effects of p21 over-expression on *dl*922-947 oncolytic activity seen *in vitro*, 10 female BALB/c nu/nu mice were injected i.p. with $5x10^6$ ACP-WAF1 or ACP-GFP cells. Each animal received injections of $5x10^9$ particles of *dl*922-947 on three successive days starting from day 8. Blood samples were taken and serum isolated from the animals 24 and 48 hours after the last treatment. The mice were killed 48 hours after last treatments and livers and tumours were harvested and fixed for further analysis, as described below.

4.17.1 Viral titres in serum

Virus titers in serum were assessed by $TCID_{50}$ assay (Fig. 4.17). Titres of infectious virions in serum of mice with ACP-WAF1 tumours rose from 24 to 48 hours post treatment and, at both timepoints, were higher than in serum from ACP-GFP mice. In the latter group, there was no increase in viral titres between 24 and 48 hours post treatment. However, the differences between ACP-WAF1 and ACP-GFP did not reach statistical significance (24 hrs: p=0.16, 48 hrs: p=0.17).

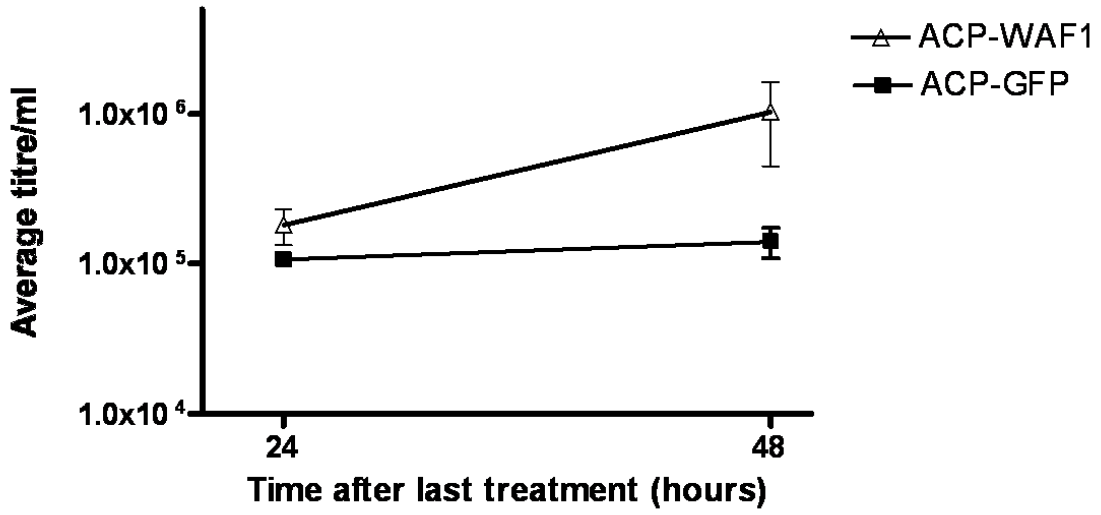


Figure 4.17 Viral titres in serum from mice bearing high- and low-p21 xenograft tumours. *Serum taken 24 and 48 hours after last treatment from mice with ACP-WAF1 or ACP-GFP i.p. tumours and treated with dl*922-947 *was titred onto JH293 cells. Points represent mean +/- standard deviation.*

4.17.2 Immunohistochemistry for E1A and p21 in tissues

Tumours derived from ACP-WAF1 and ACP-GFP cells and harvested from mice after treatment with *dl*922-947 were fixed in 10% buffered formalin, then embedded in paraffin and slides cut. Staining was carried out with anti-E1A primary antibody, followed by anti-rabbit secondary antibody. Images of stained sections are shown in Fig. 4.18.

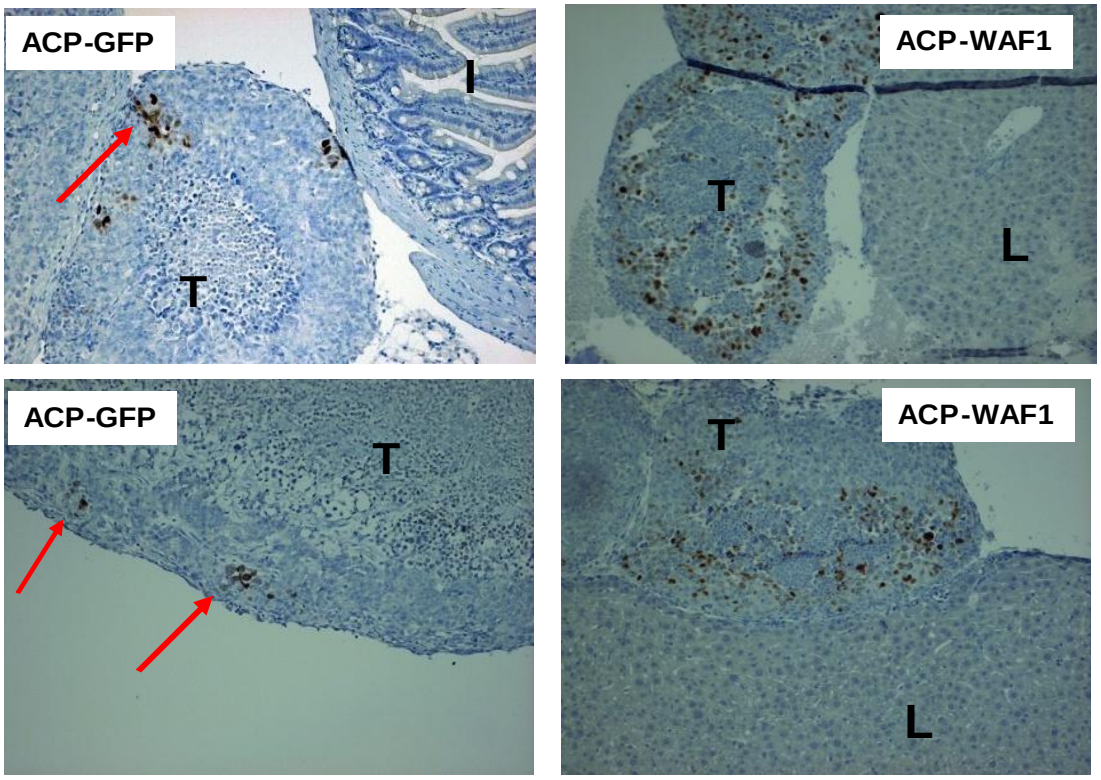


Figure 4.18 Immunohistochemistry in ACP-WAF1 and ACP-GFP tumours. *Staining for E1A in sections of ACP-GFP tumours (**left**) and ACP-WAF1 tumours (**right**). Arrows point to positive foci in GFP tumours. T= tumour, I= intestine, L= liver.*

Slides show tumour deposits (T) on murine small intestines (I) and livers (L). In the case of E1A, no staining was found in normal intestinal or liver tissues. Some spots of E1A-positive staining were present in ACP-GFP tumours (arrows) suggesting expression of early viral genes. However, levels of E1A staining were far more prominent in tumours from ACP-WAF1 mice. Staining for p21 on consecutive slides of the same tissues showed high degrees of non-specific background staining (data not shown) and requires further optimisation.

4.18 *In Vivo* II: Efficacy of *dl*922-947 in p21 over-expressing tumours in mice

$5x10^6$ ACP-WAF1 or ACP-GFP cells were injected intraperitoneally (i.p.) into 40 female BALB/c nu/nu mice on day 1. On days 5 to 9 inclusive, 10 mice per group were injected with either *dl*922-947 or non-replicating control virus Ad-GFP (5×10^9 particles/day in 400 µl 20% icodextrin i.p.). Mice were monitored daily and killed once Home Office limits were reached. ACP-WAF1 and ACP-GFP derived tumours were of such aggressive nature, that all mice had to be killed within less than 30 days after injections. There was no significant difference in survival between the 4 groups (not shown). The experiment was repeated with virus injections commencing on day 2. As before, animals were monitored daily and killed upon reaching Home Office limits. Kaplan-Meier curves tracing survival of the four groups were generated and are shown in Fig. 4.19. Again, no difference in median survival was found.

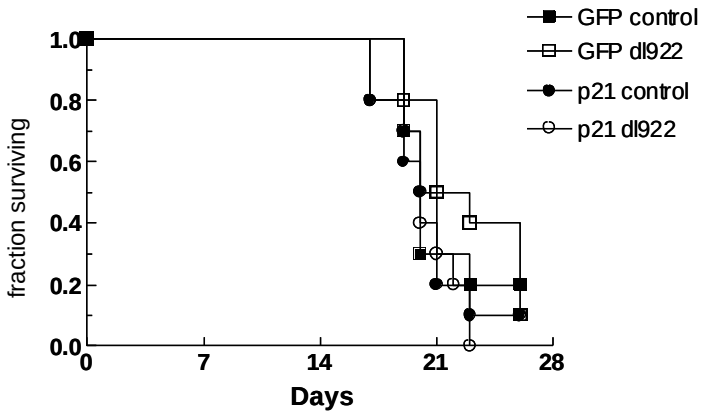


Figure 4.19 *In vivo* response of mice bearing high- and low-p21 xenograft tumours to *dl*922-947. *Mice with ACP-WAF-1 or ACP-GFP-derived i.p. tumours were treated with dl*922-947 *or control virus Ad-GFP. Kaplan-Meier survival curves are presented.*

4.19 *dl*922-947 and the cell cycle post infection

Adenoviruses manipulate the cell cycle of the infected cell to promote their own replication and propagation. To investigate the effect of *dl*922-947 on cell cycle progression and a potential role for p21 in afftecting this, in cells with high and low basal levels of p21 were infected with *dl*922-947 (MOI 10pfu/cell) and harvested 48 hours later, fixed in 70% EtOH, stained with propidium iodide (PI) and analysed by

flow cytometry. The experiment was performed in triplicates. One representative from each triplicate of cell cycle profiles is shown in Fig. 4.20.a and 4.20.b.

A.

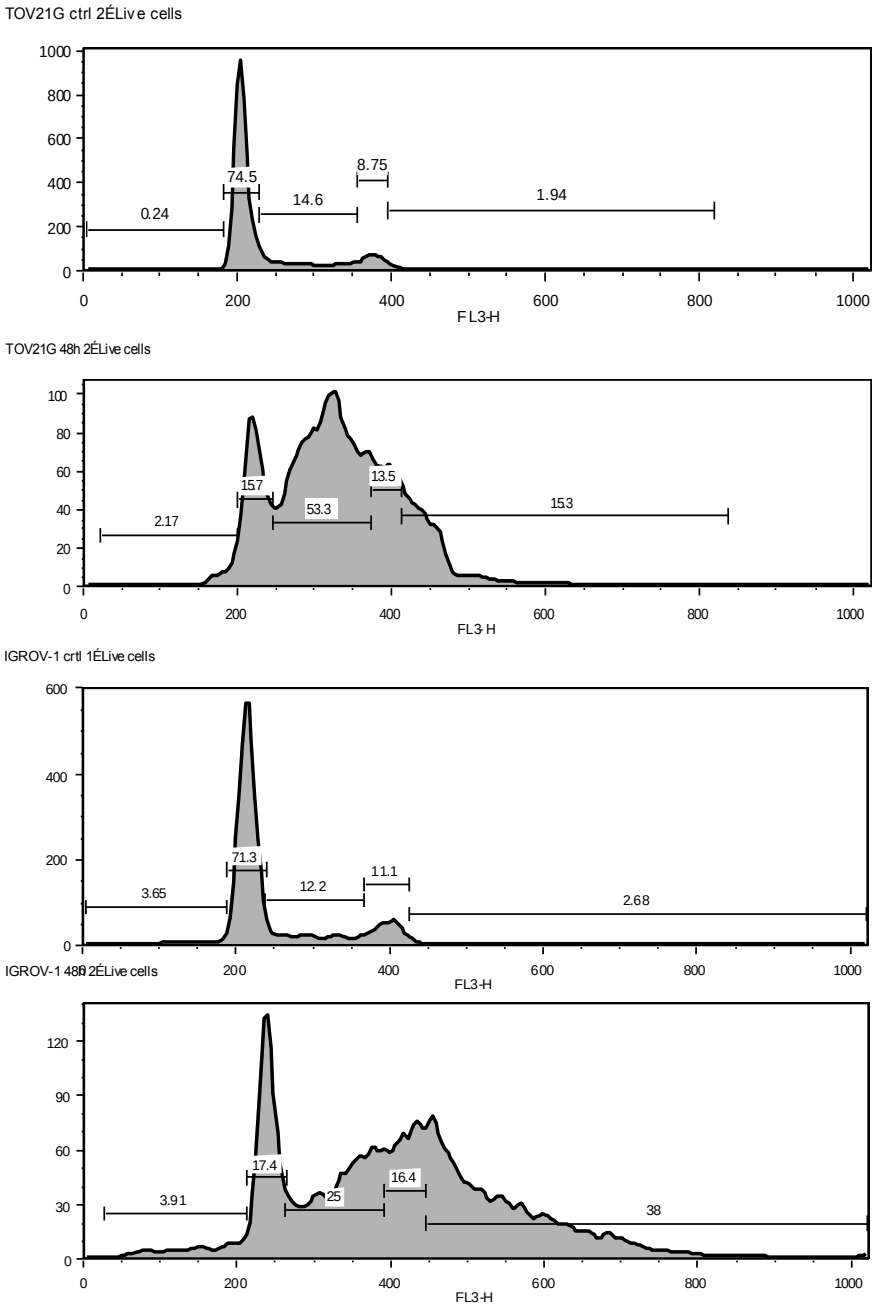
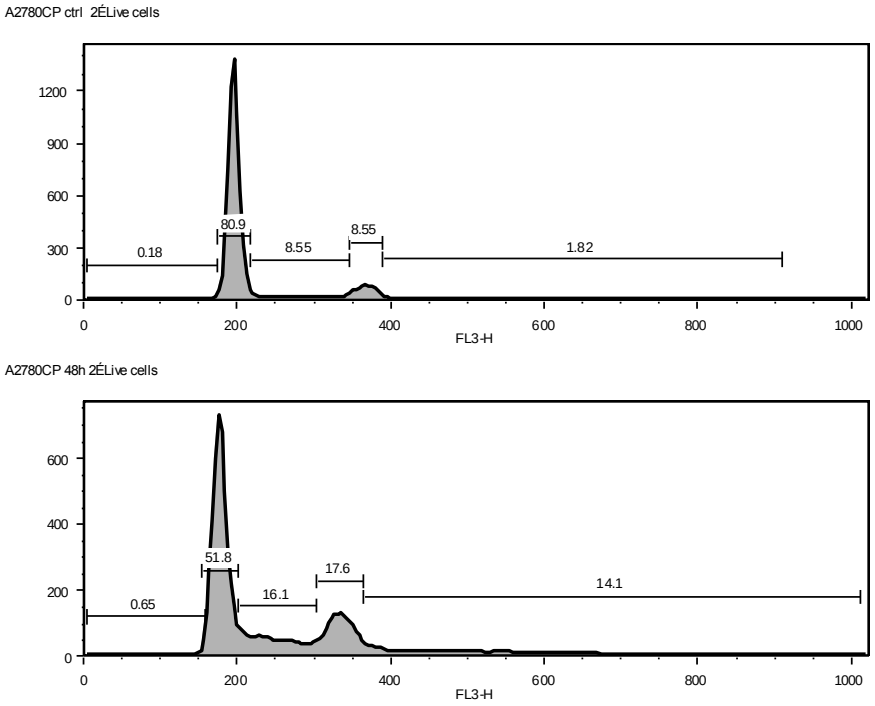


Figure 4.20.a Cell cycle profiles of mock- or virus-infected ovarian cancer cell lines. *Flow cytometric analysis was performed on* **A.** *TOV21G and* **B.** *IGROV-1 cells that had been mock-infected (**upper**) or infected with dl*922-947 *at MOI 10pfu/cell (**lower**) followed by fixation and PI-staining after 48 hours. Experiments were performed in triplicates. Representative profiles are shown.*

C.



D.

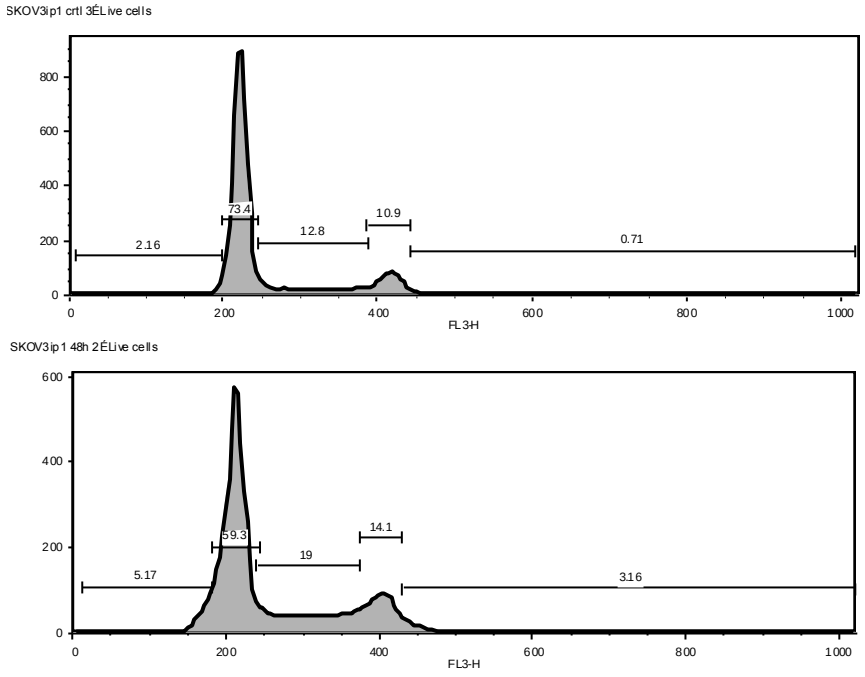


Figure 4.19.b. Cell cycle profiles of mock- or virus-infected ovarian cancer cell lines. *Flow cytometric analysis was performed on* **C.** *A2780CP and* **D.** *SKOV3ip1 cells that had been mock-infected (**upper**) or infected with dl*922-9497 *at MOI 10pfu/cell (**lower**) followed by fixation and PI-staining after 48 hours. Experiments were performed in triplicates. Representative profiles are shown.*

Comparing cell cycle fractions (Fig. 4.21), sensitive TOV21G and IGROV-1 cells had a significantly higher resting S phase fraction (16 and 13%) than non-sensitive A2780CP cells (9%) (A2780CP versus TOV21G: p=0.006, A2780CP versus IGROV-1: p=0.01). In SKOV3ip1 cells, 12% cells resided in S phase. Following infection, all cells progressed through the cell cycle. All changes in cell cycle fractions were statistically significant. However, the degree of change was greater in sensitive cells, approximately 80% of which had >2n DNA by 48 hours p.i. The >2n population in A2780CP and SKOV3ip1 was much lower, adding up to 40-50%.

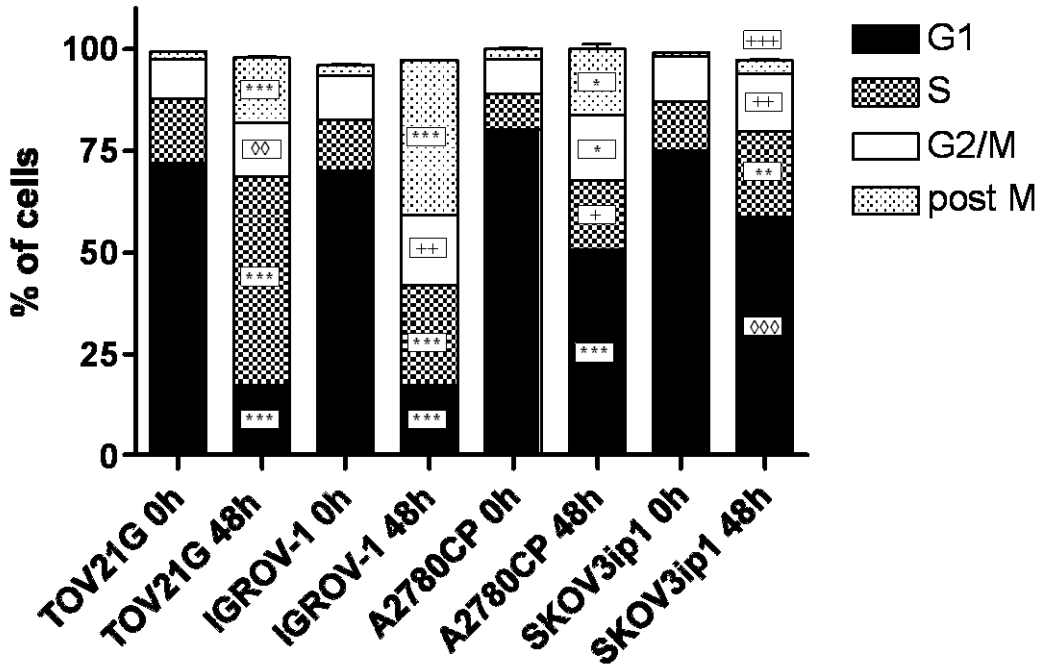


Figure 4.21 Virus infection-induced changes in cell cycle populations.
*Percentages of mock (0h)- and virus-infected (48h) TOV21G, IGROV-1, A2780CP and SKOV3ip1 cells residing in G1 phase, S phase, G2/M phase and post M phase, are compared. Bars represent mean +/- standard deviation. ***p<0.0004, +++p=0.001, ◊◊◊p=0.002, **p=0.004, ++p=0.01, ◊◊p=0.02, *p=0.01, +p=0.05*

4.20 *dl*922-947 and the cell cycle in ACP-WAF1 and –GFP cells

The experiment was repeated in ACP-WAF1 and ACP-GFP cells, to investigate whether a similar change in cell cycle profiles occurs in the model. Each experiment was carried out in triplicate. Fig. 4.22 shows representative cell cycle profiles, whilst percentages of cells in respective phases are compared in separate graphs in Fig. 4.23. In the latter case, numbers represent the mean of three samples.

Graphing proportion of cells per cell cycle phase after infection with *dl*922-947 or mock-infection showed far less prominent changes between the two treatment groups.

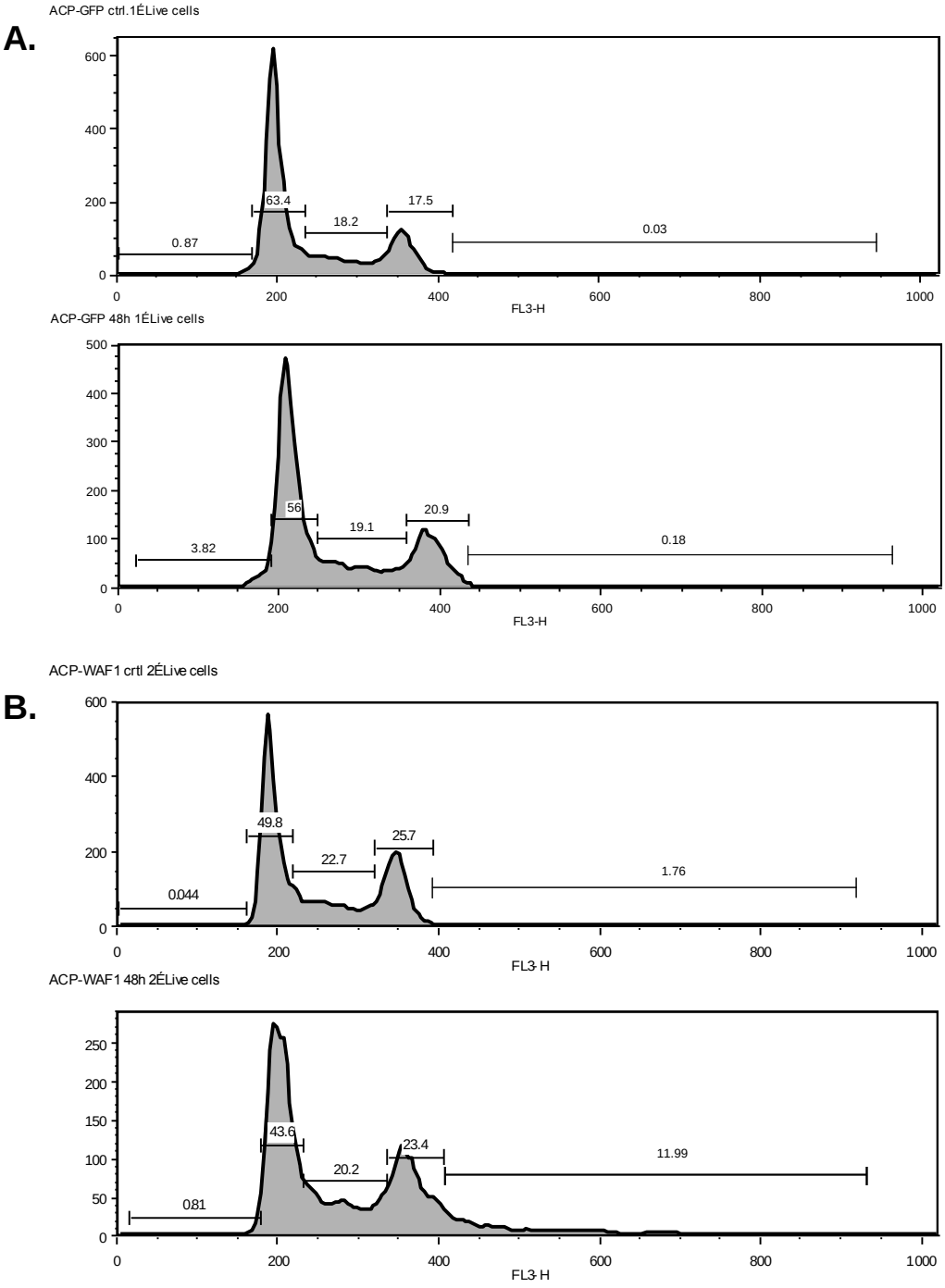


Figure 4.22 Cell cycle profiles of mock- or virus-infected ACP-GFP and ACP-WAF1 cells. *Flow cytometric analysis was performed on* **A.** *ACP-GFP, and* **B.** *ACP-WAF1 cells that had been mock-infected (upper) or infected with dl*922-947 *at MOI 10 pfu/cell (lower) followed by fixation and PI-staining after 48 hours.*

As Figure 4.23 shows, expression of p21 significantly increased the basal S phase fraction in ACP-WAF1, compared to ACP-GFP cells (p=0.002). However, following infection, apart from in the post M fractions (p=0.02), there was no significant change in profile in ACP-WAF1 cells. Nevertheless, still more of them contained >2n DNA than ACP-GFP.

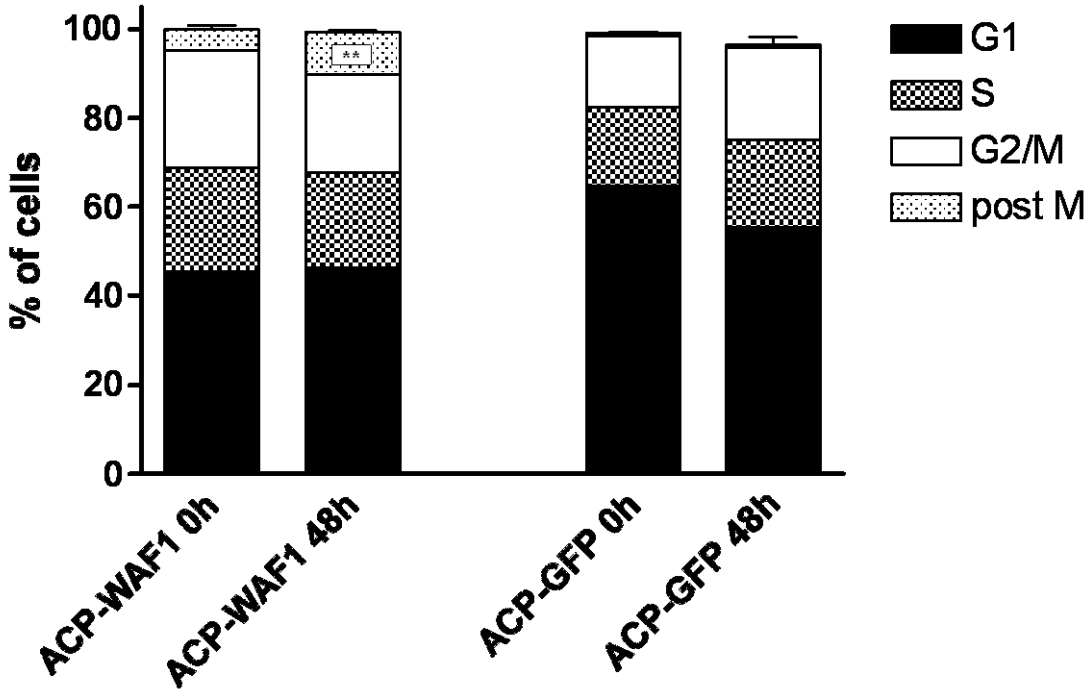


Figure 4.23 Virus infection-induced changes in cell cycle populations. *Percentages of mock (0h)- and virus-infected (48h) ACP-WAF1 and ACP-GFP cells residing in G1 phase, S phase, G2/M phase and post M phase, are compared. Bars represent mean +/- standard error. **p=0.01*

4.21 p21 protein levels over time post infection

Intracellular levels of p21 in ovarian cancer cells were monitored over time after infection with *dl*922-947 (Fig. 4.24). In the two sensitive lines, p21 expression diminished and was no longer detectable by 72 hrs (TOV21G) and 96 hours (IGROV-1) post-infection. In the A2780CP and SKOV3ip1 cells, p21 expression appeared to increase over time, especially in the more resistant SKOV3ip1 cells.

A.

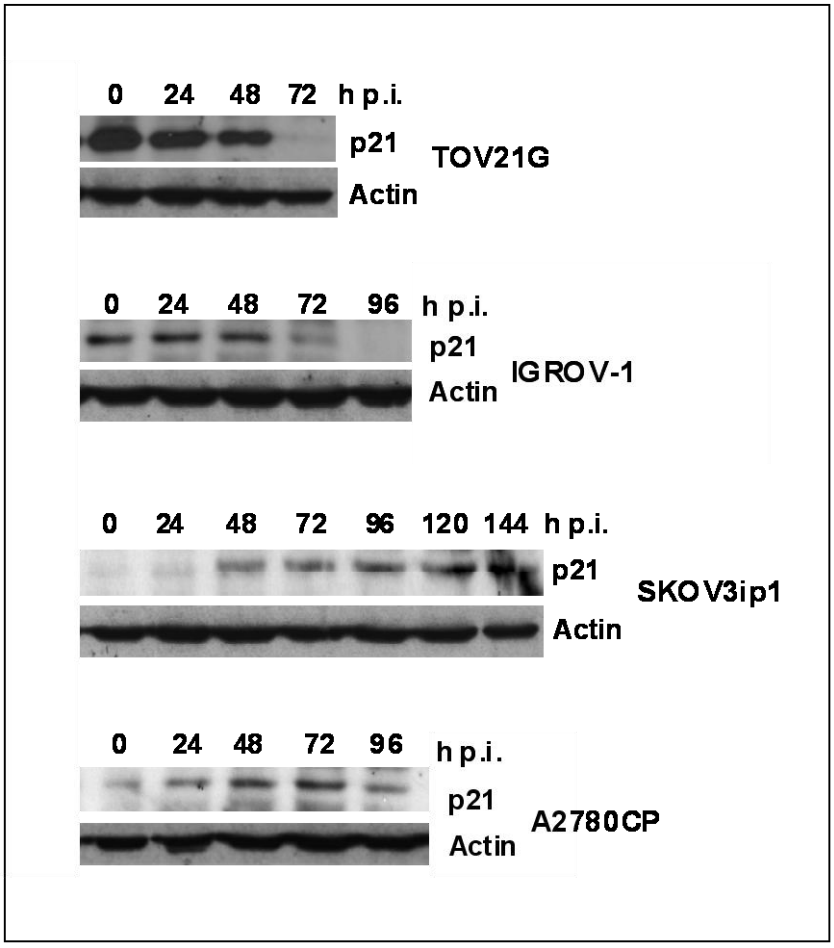


Figure 4.24.a Expression of p21 over time in ovarian cancer cells p.i. with *dl*922-947. A. *Images of immunoblots for p21 protein of samples harvested from IGROV-1, TOV21G, SKOV3ip1 and A2780CP cells over a period of up to 144 hours after infection with dl*922-947*. Staining for actin served as loading control.*

B.

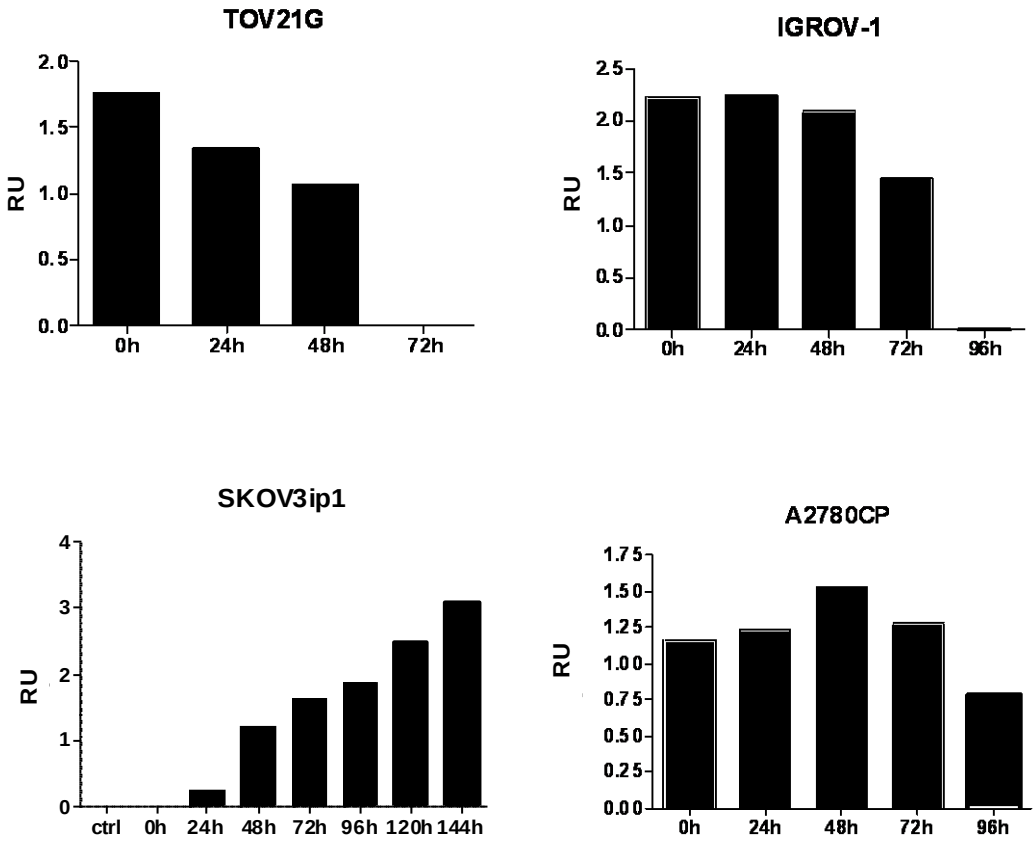


Figure 4.24.b Expression of p21 over time in ovarian cancer cells p.i. with *dl*922-947. B. *Quantification of results by densitometric analysis. Only lanes containing actin are shown. Values of p21 signal are normalised against the respective actin control and shown in relative units (RU).*

To asses wether changes in p21 expression were also seen in transfected cells, ACP-WAF1 cells were infected with *dl*922-947 (MOI 10pfu/cell) and harvested up to 48 hours later. Western blot analysis of these samples resulted in the patterns shown in Fig. 4.25. p21 expression appeared to diminish 3 hours post infection but rose abruptly at 24 hours

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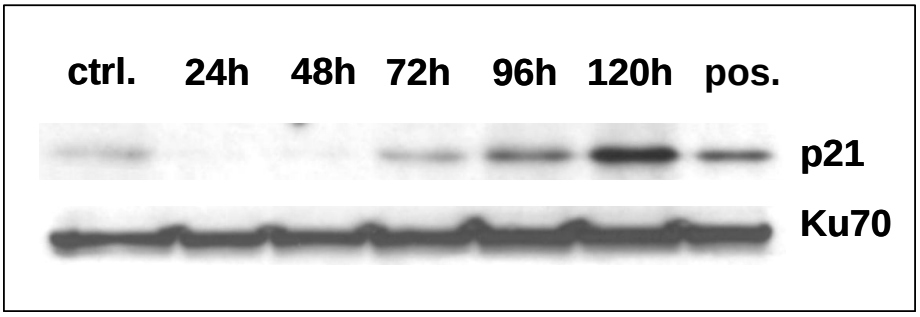
A.



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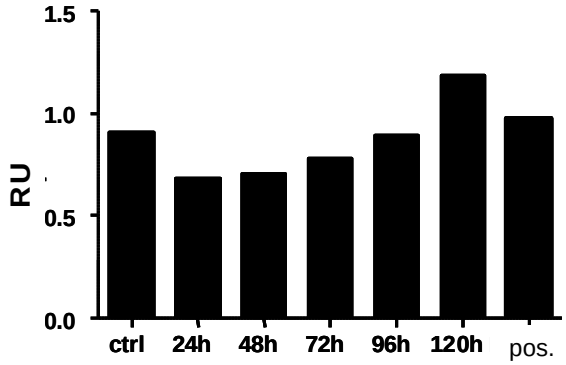


Figure 4.25 Expression of p21 over time in ACP-WAF1 cells p.i. with *dl*922-947. A. *Immunoblot for p21 protein on samples harvested from ACP-WAF1 cells over a period of 120h hours after infection with dl*922-947. IGROV-1 cells were used as a positive control (pos.) **B.** *Quantification of results by densitometric analysis. Values of p21 signal were normalised against the respective Ku70 control and are shown in relative units (RU).*

4.22 Mechanism of p21 downregulation by *dl*922-947

In order to investigate the mechanism of p21 loss following infection, Hct116 p21+/+ cells were infected with *dl*922-947 (MOI 10pfu/cell) and treated with the proteasome inhibitor MG132 (50µM) 6 hours prior to harvest. Lysates from inhibitor-treated cells were collected at 24 and 48 hours. This experiment was performed by Claire Connell (Centre for Molecular Oncology and Imaging, Institute of Cancer, Barts and The London School of Medicine and Dentistry, London, UK). Results of Western blot analysis for p21 are shown in Fig. 4.26, below. As seen in TOV21G cells, above, p21 levels decreased 24 and 48 hours post infection with the virus. However, inhibition of the proteasome prevented this loss, suggesting that p21 is targeted for proteasomal degradation following adenovirus infection.

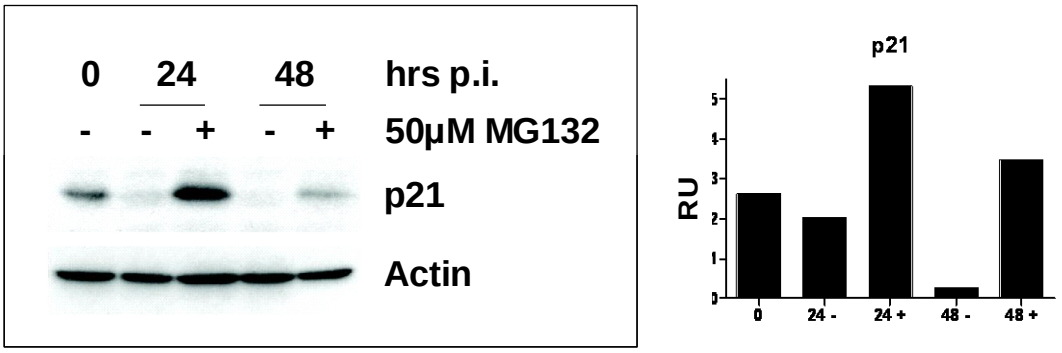


Figure 4.26 Levels of p21 protein post infection and proteasome inhibition. *Western blot analysis of p21 protein levels in Hct116 p21+/+ cells after infection with dl*922-947*. Lanes 3 and 5 contain samples from cells treated MG132. Actin was used as a loading control (**left**). Quantification of results by densitometric analysis. Values of p21 signal are normalised against the respective actin control and shown in relative units (RU) (**right**).*

4.23 Effects of genotoxic stress on p21

Adenovirus infection is known to induce a DNA damage response. To test if Hct116 p21+/+ cells are able to mount such a DNA damage response, normally accompanied by p21 induction, they were exposed to 5Gy X-irradiation and harvested 6 hours later. These experiments were performed at the University of Sussex Genome Damage and Stability Centre by Claire Connell. Western blotting (Fig. 4.27) indicated that irradiation cause a marked increase in p21 expression, suggesting that Hct116 cells respond differently to different forms of genotoxic stress.

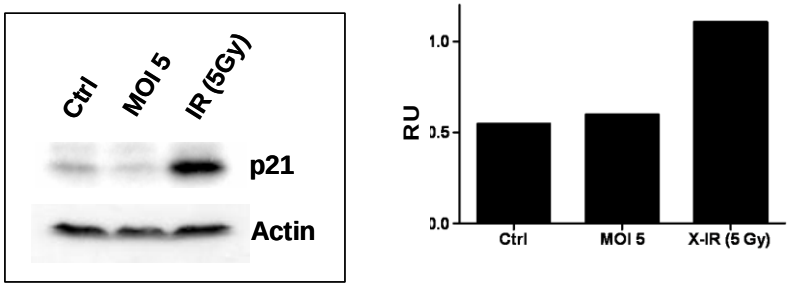
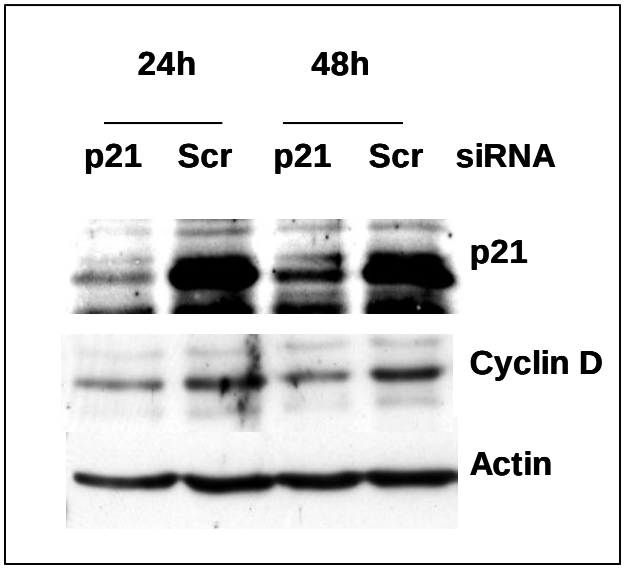


Figure 4.27 Levels of p21 protein post infection or X-irradiation. *Western blot analysis of p21 protein levels in non-treated Hct116 p21+/+ cells (**Ctrl**), after infection with dl922-947 at MOI 5pfu/cell or exposure to 5 Gy X-irradiation (**X-IR**). Actin was used as a loading control (**left**). Quantification of results by densitometric analysis. Values of p21 signal are normalised against the respective actin control and shown in relative units (RU). An artefact in the MOI 5-actin band skews the p21/actin ratio (**right**).*

4.24 Interplay between p21 and Cyclin D

Because p21 can function to stabilise Cyclin D, Western blot analysis was performed after knock-down of p21 in TOV21G cells showed that not only was there a reduction in p21 protein, but also in Cyclin D protein. An image of the immunoblot is shown in Fig. 4.28, along with densitometric analysis of the results.

A.



B.

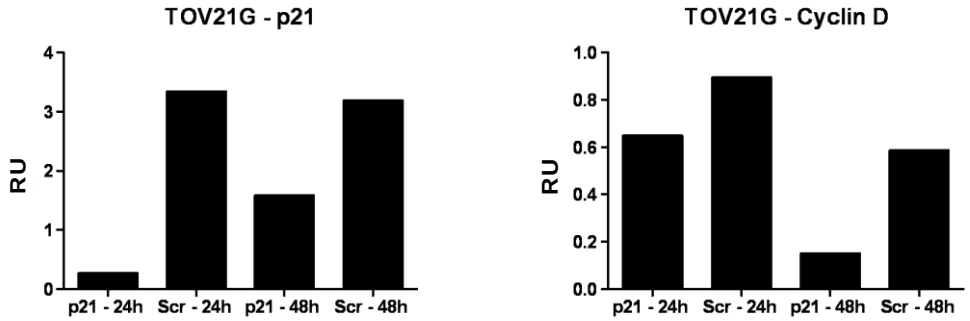


Figure 4.28 Expression of p21 and Cyclin D in TOV21G cells after p21 knock-down. A. *Image of the immunoblots for p21 and Cyclin D in TOV21G cells treated with p21-specific or scrambled siRNA. Staining for actin served as a loading control.* **B.** *Quantification of results by densitometric analysis in ACP-WAF1. Values of p21 and Cyclin D signal are normalised against the respective actin control and shown as relative units (RU).*

In addition, Cyclin D levels were compared in ACP-WAF1 and ACP-GFP cells (Fig. 4.29). In keeping with the previous experiment, in p21 over-expressing ACP-WAF1 cells Cyclin D levels were increased, compared to controls.

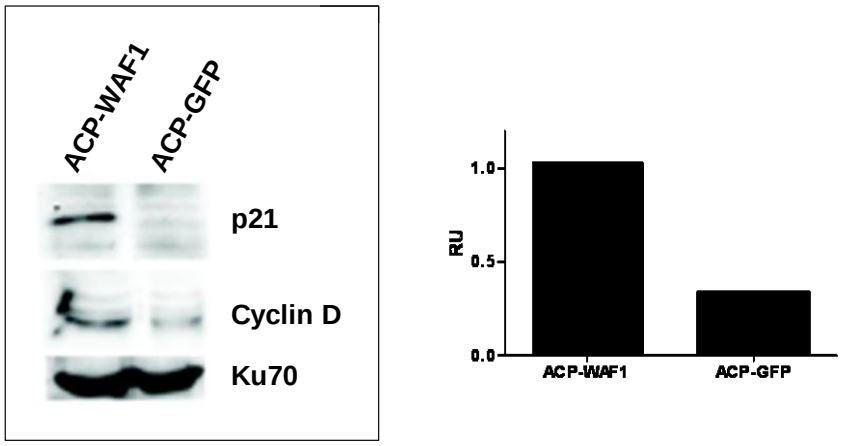
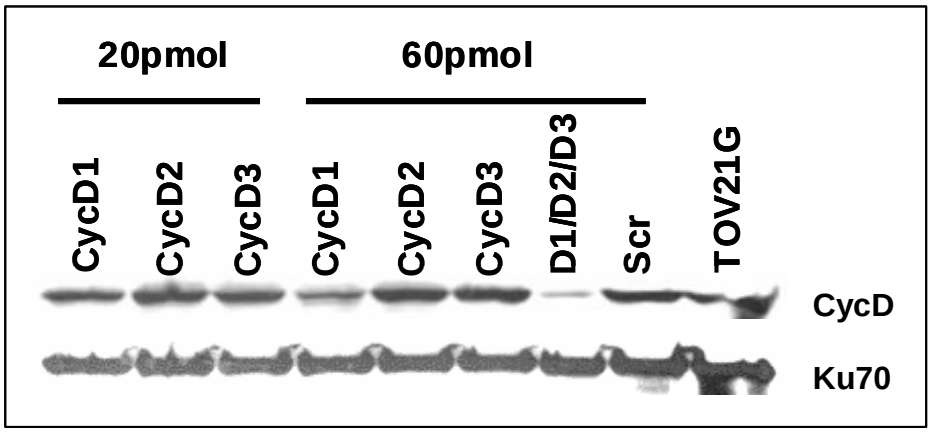


Figure 4.29 Expression of p21 and Cyclin D in ACP-WAF1 and ACP-GFP cells. *Image of the immunoblots for p21 and Cyclin D in ACP-WAF1 and ACP-GFP cells. Staining for Ku70 served as a loading control (***left***). Quantification of results by densitometric analysis in ACP-WAF1. Values of p21 and Cyclin D signal are normalised against the respective Ku70 control and shown as relative units (RU) (***right***).*

4.25 Effects of Cyclin D knock-down on *dl*922-947 function

To investigate the link between p21 and Cyclin D further, the effects on Cyclin D knock-down were assessed. In an initial experiment, optimal amounts of siRNA specific for Cyclin D1, D2 and D3 were determined. TOV21G cells were transfected with siRNA pools directed against all three isoforms individually or together. Protein expression was assessed 48 hours later (Fig. 4.30). When applied separately, only siRNA targeting Cyclin D1 (CycD1) appeared to have any knock-down effect. Profound knock-down was only achieved by transfecting cells with a combination of 20pmol of each siRNA SMARTpool.

A.



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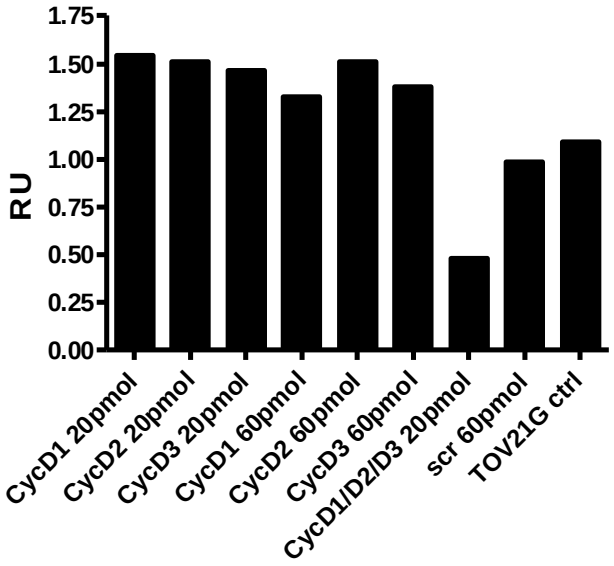


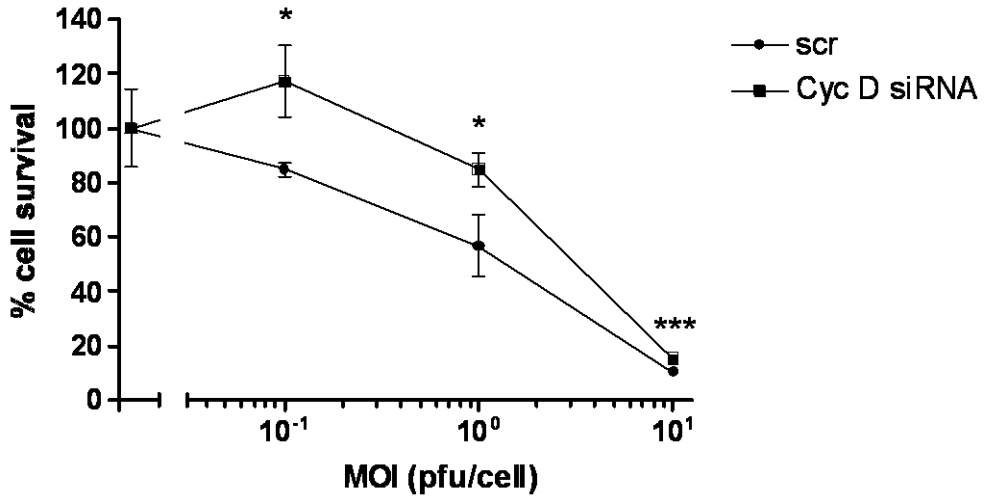
Figure 4.30 Knock-down efficiency of Cyclin D siRNAs in TOV21G cells. A. *Western blot analysis of Cyclin D protein levels in TOV21G cells. Ku70 was used as a loading control.* **B.** *Densitometric analysis of results normalised for Ku70 protein and shown in relative units (RU).*

4.26 Sensitivity of TOV21G cells to *dl*922-947 after Cyclin D knock-down

The effects of Cyclin D knock-down on dl922-947 cytoxicity were then assessed. TOV21G cells were transfected with all three siRNA SMARTpools or scrambled siRNA. 24 hours later, cells were infected in triplicate with *dl*922-947 (MOI 0.01 – 100pfu/cell). Cell survival was quantified 96 and 120 hours later (Fig. 4.31.A). In parallel, cells on 6-cm plates were treated with the same siRNA combination or scrambled siRNA per plate. Protein samples were harvested at 24 hours p.i. to confirm efficiency of Cyclin D

knock-down by Western blotting (see Fig. 4.31.B). Knock-down of Cyclin D resulted in a significant de-sensitisation of TOV21G cells with significantly greater survivial at all three MOIs.

A.



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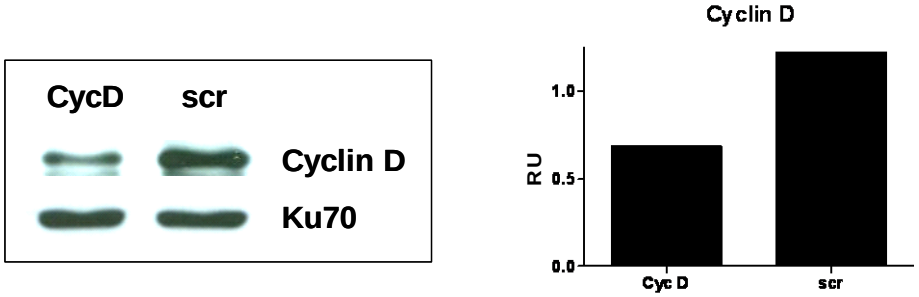


Figure 4.31 Sensitivity of TOV21G cells to *dl*922-947 after k.d. of Cyclin D1/D2/D3.
A. *Percentages of live TOV21G cells transfected with Cyclin D-targeting (CycD) or scrambled control (scr) siRNA 96 hours p.i. with dl*922-947 *graphed relative to MOI pfu/cell of the virus. Points represent mean +/- standard deviation. *p=0.05, ***p=0.001*
B. *Western blot (**left**) and densitometric analysis (**right)** of Cyclin D protein levels in TOV21G cells after transfection with Cyclin D-targeting (CycD) or scrambled control (scr) siRNA. Ku70 served as a loading control and for normalisation. RU= relative units.*

4.27 Other Rb pathway members as potential biomarkers

Limited analysis of Rb pathway members described in Chapter 3 had suggested p21 and Cyclin D as candidate host cell factors to influence *dl*922-947 function. However, these were not the only Rb pathway components showing a different expression in sensitive and non-sensitive cell lines. Other candidates were p27, cdk4 and p16. I attempted to assess these candidates by shRNA- or siRNA-mediated knock-down.

4.27.1 shRNA-mediated knock down of Rb pathway members

To create a readily accessible small-scale RNAi library, I used the Human RNAi (Hannon) library as a starting point (Babraham Bioincubator, MRC Geneservice, Cambridge, UK). Access to the full-size library was granted by Prof Ian Hart (Centre for Tumour Biology, Institute of Cancer, Barts and The London School of Medicine and Dentistry, London, UK). The mini-RNAi library contained shRNAs to 44 target genes of interest to our group. The process of copying bacterial clones containing the hairpin-encoding pSM2 plasmids of choice onto 96-well plates is described in detail in Chapter 2, as well as a table of contents of the new mini-RNAi library. Plasmids to be used immediately for transfection were test-digested with restriction enzymes XbaI and HindIII, followed by gel electrophoresis. Bands obtained suggested that plasmids contained inserts of the appropriate size. As we have no records of the shRNA sequences, themselves, verification by sequencing was not possible. pSM2-shRNA plasmids targeting members of the Rb pathway (p27, cdk4, Cyclin D1, Cyclin E1 and E2) were amplified. TOV21G cells were transfected with each pSM2-shRNA followed by selection in puromycin. None of the cells transfected with pSM2-shRNA targeting Cyclin E1 and E2 survived even lowest concentrations of the antibiotic. Pools of the other cells were grown to confluence. Immunoblotting was performed to assess degree of knockdown (Fig. 4.32). However, staining with the relevant antibodies revealed no apparent knock-down.

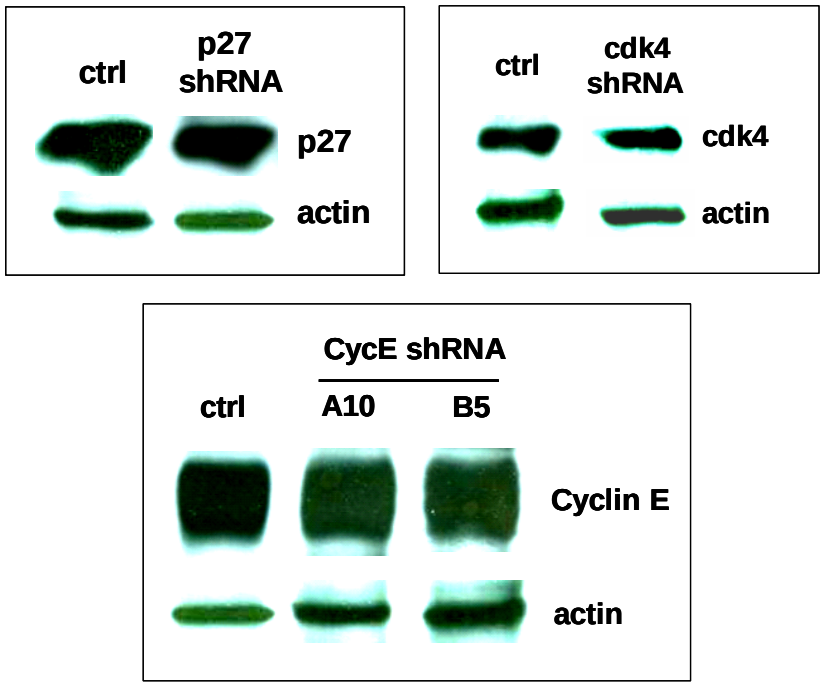


Figure 4.32 Expression of RNAi-targeted proteins. *Western blot analysis was performed on cell lysates from cells transfected with RNAi library plasmids and non-treated controls to assess knock-down efficiency in TOV21G cells. Actin levels served as a loading control.*

4.27.2 Effects of p16 knock-down on *dl*922-947 efficacy

As described in Chapter 3, expression p16 was increased in sensitive cells and thus might also act as a marker for cell sensitivity. siRNA was used to knock down p16 expression in IGROV-1 and TOV21G cells.

IGROV-1 and TOV21G cells were transfected with an siRNA SMARTpool targeting p16, with equal quantities of scrambled siRNA as control. Efficacy of p16 knock-down by this method was evaluated by immunoblotting (Fig. 4.33). In TOV21G cells, noticeable knock-down occurred only 48 hours after transfection. In IGROV-1 cells, p16 protein levels were reduced 24 and 48 hours after transfection. At 72 hours, p16 levels appeared strongly increased. In contrast, p16 protein was also very low at 96 hours post transfection. It is possible that transfection was ineffective in the well containing the 72h-sample.

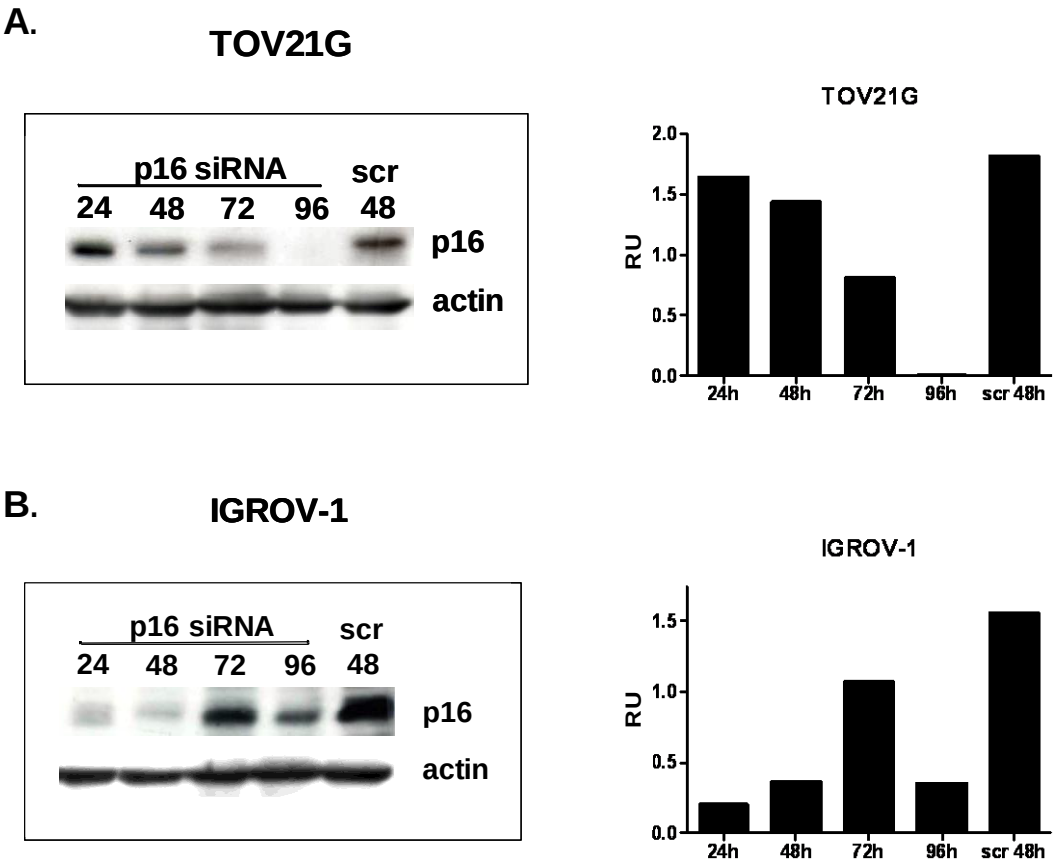
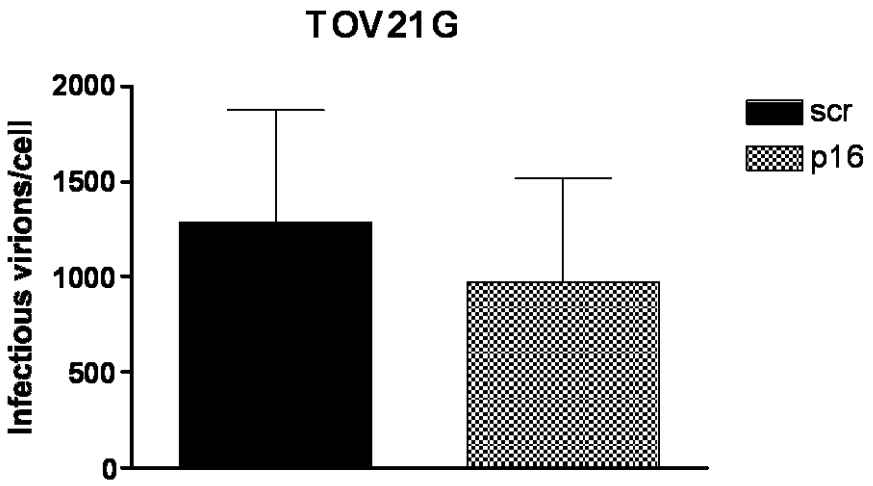


Figure 4.33 Efficiency of siRNA-mediated p16 knock-down. *Shown are images of Western blot analyses of p16 protein levels over 96hrs post transfection in* **A.** *TOV21G and* **B.** *IGROV-1 cells transfected with p16 siRNA or scrambled control siRNA (scr). Actin served as a loading control. Densitometric analyses are shown below each immunoblot after normalisation for actin. RU= relative units.*

IGROV-1 and TOV21G cells were then infected with *dl*922-947. 24 hours after transfection with p16 siRNA. Cells were harvested 48 hours post-infection and intracellular virus titred by $TCID_{50}$ assay (Fig. 4.34). In IGROV-1 cells, there was no change in virion production after p16 knockdown. In TOV21G cells, there was a small reduction in virion production in p16 knock-down cells (p16 siRNA: 970 pfu/cell; Scr siRNA. 1300 pfu/cell), but this did not reach statistical significance.

A.



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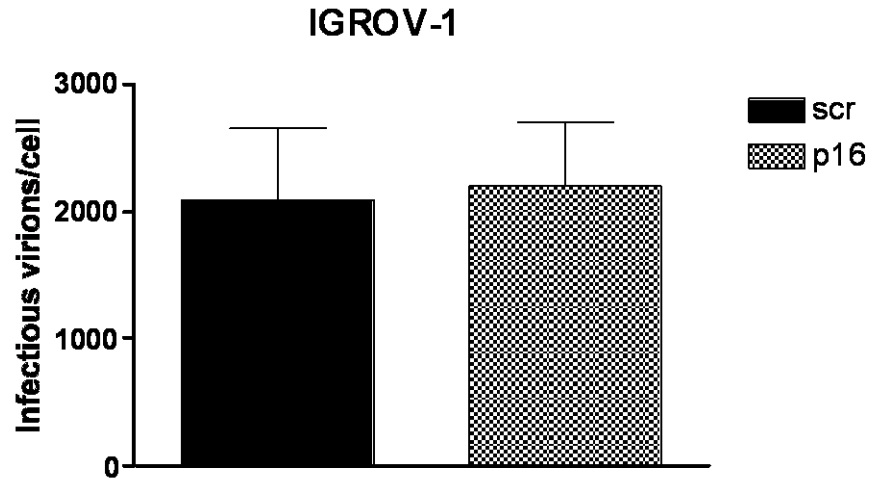


Figure 4.34 Virion production after k.d. of p16 assessed by $TCID_{50}$. *Infectious virions produced in* **A.** *IGROV-1 and* **B.** *TOV21G cells transfected with p16 siRNA 48 hours after infection with dl922-947 at MOI 10pfu/cell. Bars indicate mean +/- standard deviation.*

Finally, both TOV21G and IGROV-1 cells were transfected with p16 siRNA, followed 24 hours later by infection with *dl*922-947 at two MOIs: 0.1 and 10pfu/cell for TOV21G; 1 and 10pfu/cell for IGROV-1. Cell survival was assessed up to 96 hours later by MTT assay. No significant decrease in sensitivity to *dl*922-947 was detected after p16 knock-down in TOV21G (Fig. 4.35) or IGROV-1 (not shown).

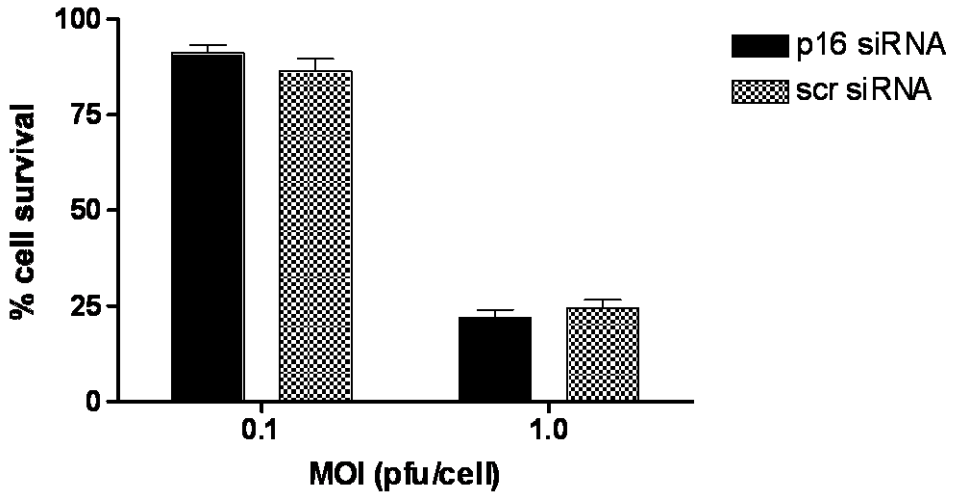


Figure 4.35 Cytotoxicity of *dl*922-947 after p16 knockdown. *Shown are percentages of p16- or scr siRNA-treated cells alive 96 hrs p.i. with dl*922-947 *(MOI 0.1 and 1.0pfu/cell). Bars represent mean +/- standard deviation.*

4.28 Discussion

Manipulation of p21 protein levels in sensitive and non-sensitive cell lines confirmed a role for this cell cycle regulator in the efficiency of *dl*922-947. Loss of p21 in cancer cell lines, either by knock-out, as in Hct116, or by siRNA, as in TOV21G, resulted in significant loss of sensitivity to *dl*922-947. Equally, p21 knock-down caused a reduction in functional viral particles produced per cell. The decrease was statistically significant in TOV21G, although did not reach significance in IGROV-1 cells. This may be due to the difference in basal p21 levels between the two cell lines. As untreated TOV21G cells contain more of the protein than IGROV-1 cells, siRNA knock-down may cause a greater relative change in p21 expression, mirrored by higher inhibition of virus production. It was noticable that, although correlation between sensitivity and replication of functional virions was not complete when comparing a panel of different ovarian cancer cell lines, it does appear to exist when comparing within the same cell line.

Generating ACP-WAF1, a cell line in which p21 levels were increased compared to parental A2780CP and control ACP-GFP cells, allowed me to assess further the role of p21. The initial attempt to over-express p21 in A2780CP and SKOV3ip1 cells was not successful. Immunoblotting of ACP-p21 protein failed to show a band of the expected p21-GFP fusion protein (Cazzalini *et al.*, 2003). Instead, a double band emerged at the same height as from GFP in ACP-GFP cells, but even higher in intensity. This explains why ACP-p21 cells did emit a green signal under the fluorescence microscope, but not the lack of the fusion protein. A reason could be inappropriate post-transcriptional alterations, such as mRNA processing (Yarus *et al.*, 1997). However, transfection with pCEP-WAF1 was successful in A2780CP cells, although only one of five cell pools expressed meaningful levels of p21 protein. In contrast, no SKOV3ip1 cells over-expressing p21 could be generated. It is possible, that SKOV3ip1 cells cannot tolerate higher levels of p21 without undergoing cell cycle arrest. Increased expression of p21 in A2780CP resulted in increased sensitivity to *dl*922-947 as well as production and release of functional virus particles. Interestingly, higher levels of p21 in ACP-WAF1 cells made them less infectable than their parental cell line, as assessed by GFP-flow cytometric analysis.

At first, these findings seem counter-intuitive, in view of the cell cycle inhibitory function of p21. However, they may be explained by p21's additional roles: high levels of p21 prevent G1/S phase progression, whilst low/basal levels have the opposite effect. They

enhance Cyclin D/cdk4 and Cyclin B/cdk1 complex formation and stability. My data are consistent with this function of p21 as a stabiliser of Cyclin D and promoter of cell cycle progression. In TOV21G, IGROV-1 and ACP-WAF1 cells p21 levels appear to promote cell cycle progression, but levels are evidently not high enough to induce cell cycle arrest. Ultimately, enhanced progression appears to increase cytotoxic function of *dl*922-947. Results from experiments on Cyclin D strengthen this hypothesis. Knock-down of p21 in TOV21G cells led to reduction in Cyclin D protein levels, whereas Cyclin D levels were increased in ACP-WAF1 cells. In turn, siRNA-mediated knock-down of Cyclin D reduced TOV21G cells sensitivity to *dl*922-947 oncolysis.

A role of p21 for *dl*922-947 was partially verified *in vivo*. In a subcutaneous Hct116 p21+/+ and p21-/- tumour model in CD1 nude mice, p21-/- tumours responded less well to direct tumoural *dl*922-947 than p21+/+ tumours in two experiments. These results were very encouraging. In Balb C nude mice bearing ACP-WAF1 i.p. xenografts, greater serum virus levels were seen following i.p. *dl*922-947 than in ACP-GFP bearing animals, along with greater E1A expression within tumours. However, neither efficacy study could reproduce the shift in sensitivity to *dl*922-947 seen *in vitro* and the Hct116-tumour *in vivo* study. This lack of efficacy may simply reflect the highly aggressive nature of A2780CP xenografts, such that the increase in virus replication may not have been strong enough to impact overall toxicity. Certainly, other members of our group have failed to demonstrate any anti-tumour efficacy with *dl*922-947 when using A2780CP-derived tumours.

Sensitive TOV21G and IGROV-1 cells had in addition to demonstrable p21 expression, a higher fraction of cells in S phase in asynchronous populations and also supported earlier and greater E1A expression following dl922-947 infection. Similarly, more ACP-WAF1 cells resided in S phase, compared to ACP-GFP controls, whilst low-p21 A2870CP cells showed lower S phase fractions than TOV21G and IGROV1 cells. This stands in line with the hypothesis that in these cells p21 is a facilitator of G1/S phase progression (LaBaer *et al.*, 1997). However, in asynchronous SKOV3ip1 cells, the S phase fraction was similar in size to IGROV-1 cells. Also, E1A protein appeared from 24 hours post infection. Despite these parallels, SKOV3ip1 cells are resistant to *dl*922-947. This implies that in SKOV3ip1 cells, induction of cell death, as seen in sensitive cell lines, is blocked. Whether the lack of p21 or another mechanism is responsible, remains unclear. The failure to generate p21-expressing SKOV3ip1 clones made further investigation difficult.

Following infection, *dl*922-947 drives cells the cell cycle. This phenomenon was greater in sensitive, but was also detectable in non-sensitive cells. However, it was not observed in ACP-WAF1 cells. Compared to ACP-GFP cells and parental A2780CP, exogenous expression of p21 seems to already have induced such progression in non-infected cells. The underlying mechanism is unclear. However, a comparison of ACP-WAF1 and ACP-GFP cell cycle profiles with those of A2780CP cells may not be possible. Correlation between two sets of flow cytometric experiments is difficult, due to variability in set-up and gating (Maecker *et al.*, 2006). This may also explain the discrepancy between profiles of A2780CP and ACP-GFP cells.

In all p21-expressing cells, levels of the protein fell following infection. Adenovirus infection is associated with production of host cell genomic DNA double strand breaks (Cuconati *et al.*, 2003; Nichols *et al.*, 2009), a form of DNA damage normally associated with increased p21 expression and cell cycle arrest. This was confirmed in Hct116 p21+/+ cells, which showed a marked increase in p21 expression following X-irradiation, a potent inducer of DNA double strand breaks. A cell cycle arrest would act to block productive adenovirus activity, which relies upon host cell cycle progression. Proteasome inhibition prevented loss of p21 protein in Hct116 p21+/+ cells, suggesting that the virus targets p21 for destruction either directly or indirectly. It has been shown that adenoviral E1B-55K and E4orf6 form part of an E3 ubiquitin ligase complex targeting various host cell proteins for destruction. It is possible that p21 is also targeted for proteasomal degradation by the complex (Querido *et al.*, 2001a). Several publications describe an increase in apoptosis induced by cytotoxic drugs or γ-irradiation of cells after downregulation of p21 (Detjen *et al.*, 2003; Han *et al.*, 2002; Mahyar-Roemer *et al.*, 2001; Tian *et al.*, 2000). It is possible that the downregulation of p21 seen in TOV21G, IGROV-1 and Hct116 p21+/+ cells promotes cytotoxic effects of *dl*922-947, whilst in A2780CP and SKOV3ip1 cells rising p21 levels inhibit virus-induced death. The absence of a definitive mode or mechanism of adenovirus-induced cell death makes further investigation of this difficult.

I was unable to confirm a role for p27, cdk4 and Cyclin E in promoting *dl*922-947 oncolytic efficacy, as shRNA-mediated knock-down was not achieved. In the case of Cyclin E, none of the cells was resistant to antibiotics, suggesting failure of transfection. For vectors encoding p27 and cdk4 shRNAs, a number of explanations are possible. Generally, knock-down effects mediated by shRNA tend to be less efficient as those achieved by siRNA. One possibility is a faulty plasmid. RNAi libraries are known to expel their hairpins of interest, as vectors are inherently prone to

spontaneous recombination of LTR regions (Chakiath *et al.*, 2007). However, test digests of amplified plasmid DNA prior to transfection (data not shown) suggested that hairpin-containing vectors were still intact. However, no direct sequencing of plasmids was performed. Another possible cause would be dysfunctional RNAi machinery in the transfected cell. siRNA-mediated transient knock-down of p21, Cyclin D and p16 in TOV21G cells was successful. However, siRNA needs no further processing before it can mediate degradation of target mRNA (Ahlquist, 2002). RNA hairpins, on the other hand, require Dicer enzymatic activity (Bernstein *et al.*, 2001). Reduced Dicer and Drosha function in ovarian cancer has recently been reported. Comparison of Drosha and Dicer mRNA in invasive epithelial ovarian cancer specimens indicated that 50% of tumours expressed reduced levels of Drosha. Expression of Dicer was downregulated in 60% patients. In 40% of cases, mRNA levels of both enzymes were decreased (Merritt *et al.*, 2008). However, there have been previous reports of successful shRNA-mediated expression knock-down in TOV21G cells has been reported (Bartz *et al.*, 2006).

p16 could not be validated as a predictive marker. siRNA-mediated knock-down, albeit less pronounced than p21-knock-down, was efficient. Yet, no significant change in viral replication or sensitivity to *dl*922-947 was detected. This implies that p16 has no regulatory effect on *dl*922-947 activity. In cervical cancer, p16 is used as a marker of HPV positivity (Queiroz *et al.*, 2006). Western blots of Rb pathway members in Chapter 3 suggested a correlation between sensitivity and p16 expression. It would be interesting to see, by analysing past or future clinical trial data, whether p16 could be used as a prognostic marker in ovarian cancer.

In summary, data presented in this chapter suggest that p21 may promote an intracellular environment favourable for adenoviral E1A expression and ultimately, *dl*922-947 efficacy. The mechanism by which this is achieved may be through stabilisation of Cyclin D and enhanced S phase activity in cells at the time of infection.

5 Results: The Quest for Further Candidates

5.1 Introduction

Results in Chapters 3 and 4 suggested a number of candidates which may promote oncolytic effects of *dl*922-947. This chapter describes the approaches taken to identify further host cell factors influencing the cytotoxic efficacy of *dl*922-947 outside the obvious Rb pathway, utilising large scale techniques: whole genome expression screening by microarray on established cell lines as well as attempts to generate new cell lines for such screening.

5.1.1 Methods

The first approach was to return to the matched cell pair of MRC5 and MRC5-VA cells. From these cells, total RNA was isolated and used for Affymetrix Microarray analysis. With the aim to confirm the candidates that had emerged from MRC5/-VA cells, I attempted to generate paired cell lines consisting of IOSE20 or IOSE21 cells and their SV40 TAg-transformed counterparts. Furthermore, I obtained TOSE1 and TOSE4 cells from Professor Fran Balkwill (Centre for Cancer and Inflammation, Institute of Cancer, Barts and The London School of Medicine and Dentistry, London, UK). These two cell lines emerged, when Kyra Archibald in the Balkwill lab attempted to transform IOSE25 cells by treating them with TNF-α. The cytokine has been shown to induce DNA damage (Babbar *et al.*, 2007). Surprisingly, some of the non-treated control cells, later named TOSE, acquired the ablilty to grow as colonies in soft agar, but do not form tumours in nude mice, and will be referred to as pre-transformed. Microsatellite sequencing confirmed that TOSE originated from IOSE25 cells (oral communication with Kyra Archibald, Centre for Cancer and Inflammation, Institute of Cancer, London, UK). After characterisation of TOSE cells with regards to their behaviour when infected with *dl*922-947, basal gene expression in cycling non-infected cells was compared between the three cell lines by microarray analysis. As these cells represent a very interesting and highly suitable model for my quest for biomarker candidates, they will be described first.

5.2 Infectivity of TOSE cells

Infectability of IOSE25, TOSE1 and TOSE4 cells was assessed by GFP flow cytometry, as before (Fig. 5.1). The least infectable cell line was IOSE25. At MOI 5pfu/cell, 10% of these cells were positive for green fluorescence, increasing to 47% at MOI 50pfu/cell. TOSE4 cells appeared the most infectable. After infection at MOI 5pfu/cell, 36% of cells were GFP-positive and 85% at MOI 50pfu/cell. Infectivity of TOSE1 cells lay in-between with 18% cells expressing GFP after infection at MOI 5pfu/cell and 83% at MOI 50pfu/cell.

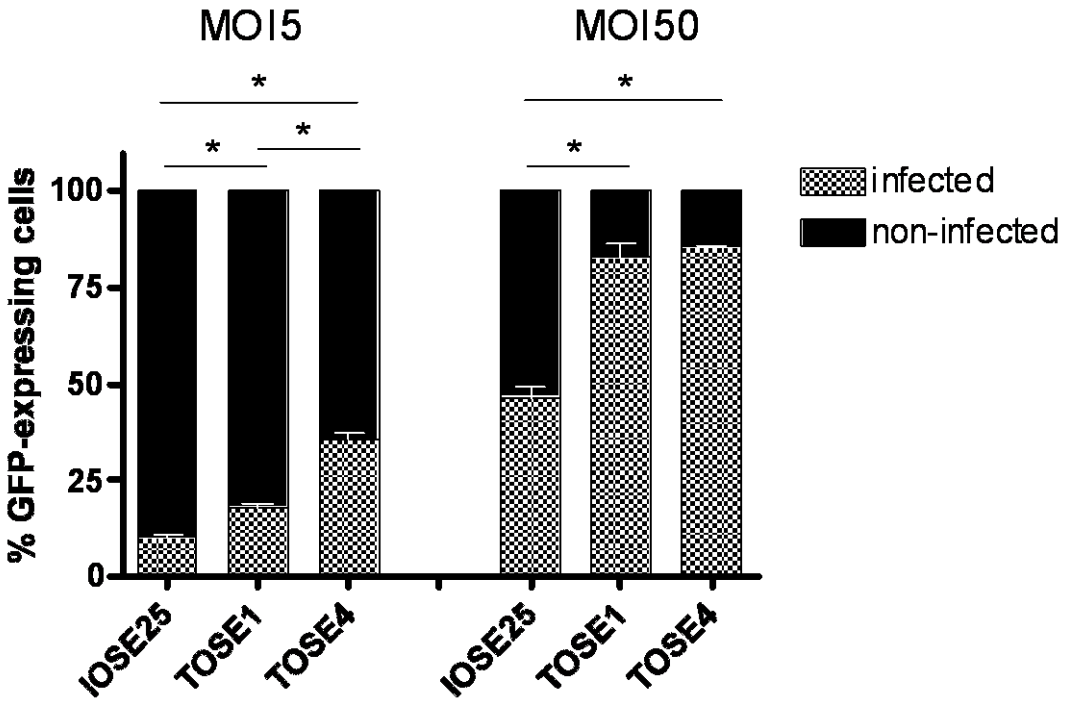
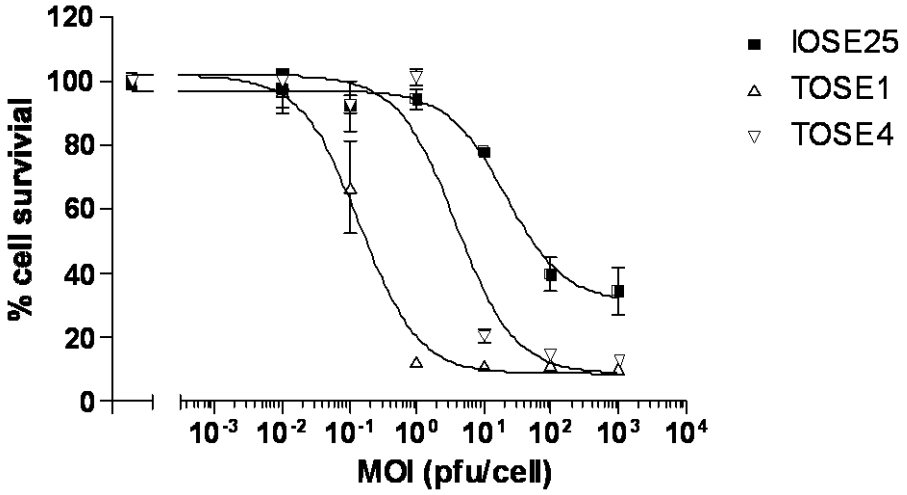


Figure 5.1 Infectivity of TOSE1, TOSE4 and IOSE25 cells. *Mean percentages of GFP-positive cells after infection with Ad-GFP at MOIs 5pfu/cell (**top**) or 50pfu/cell (**bottom**). Bars represent mean +/- standard deviation. *p≤0.01*

5.3 Sensitivity of TOSE cells to *dl*922-947

Sensitivity to dl922-947-mediated cytotoxicity was next assessed (Fig. 5.2). Control cells IOSE25 were the least sensitive (IC_{50} 21.4pfu/cell). TOSE4 cells were more sensitive (IC_{50}s 3.9pfu/cell), whilst TOSE1 were the most sensitive, with an IC_{50} of 0.14pfu/cell.



	IOSE25	TOSE1	TOSE4
IC_{50}	21	0.14	3.9

Figure 5.2 Sensitivity of IOSE25 and TOSE cells to *dl*922-947. *Dose response curves of IOSE25, TOSE1 and TOSE4 cells 144 hours p.i. graphed relative to MOI pfu/cell of dl*922-947*. Points represent mean +/- standard deviation. Table shows IC_{50} values (pfu/cell).*

5.4 Expression of p21 in TOSE cells

Expression of p21 was assessed in TOSE1, TOSE4 and IOSE25 cells by Western blot. As Fig. 5.3 shows, p21 protein levels were markedly increased in TOSE cells, as compared to IOSE25 controls, with slightly more p21 in TOSE1 than TOSE4 cells, confirming the data from ovarian cancer cells.

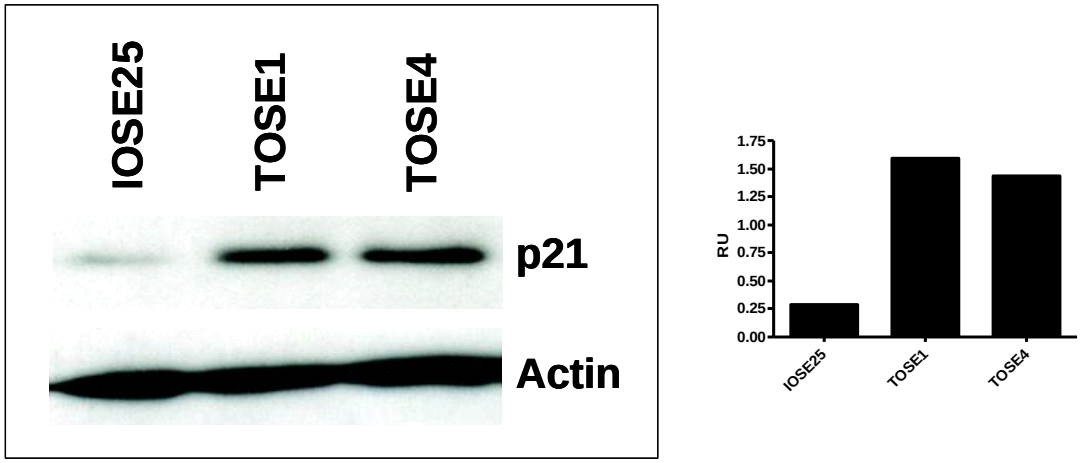


Figure 5.3 Western blot analysis in TOSE cells. *Protein levels of p21 in cell lysates of TOSE1, TOSE4 and IOSE25 cells are shown with respective densitometric values in relative units (RU) (**below**). Actin was used as a loading control and for normalisation.*

5.5 Gene expression profiling in TOSE cells

Unlike MRC5-VA cells, TOSE1 and TOSE4 cells have acquired their sensitising genetic changes very recently, which implies that the number of additional alterations should be limited. Equally importantly, TOSE cells have not been SV40 large T antigen (SV40 TAg) -transformed, so should be more relevant to primary ovarian cancer. Thus they represent a very suitable model for whole genome expression analysis. Microarray-based gene expression profiling was undertaken to compare basal gene expression profiles in proliferating TOSE1, TOSE4 and IOSE25 cells. Raw results from this analysis were analysed by Dr Claude Chelala (Centre for Molecular Oncology and Imaging, Institute of Cancer, Barts and The London School of Medicine and Dentistry, London, UK) and Dr Probir Chakravarty (Bioinformatics & Biostatistics, Cancer Research UK London Research Institute, London).

5.5.1 Microarray analysis in IOSE25, TOSE1 and TOSE4

Total RNA was extracted from 80% confluent cells growing in triplicate on 6-cm plates. Next, RNA was reverse-transcribed into double-stranded cDNA, then into biotin-labelled cRNA. This was followed by fragmentation into cRNA pieces 35-200 bases in length. To verify successful fragmentation, a fraction of the fragmented samples was run on a 0.8% agarose gel alongside a non-fragmented control. An image of the electropherogram is shown in Fig. 5.4, below.

Whilst the lane containing the non-fragmented sample shows a relatively compact band of a large size, the other lanes lack distinct bands. This absence of bands in fragmented samples is due to the smaller size and heterogeneity of cRNA fragments. As a consequence, the GelRed signal emitted lies under the threshold detectable by UV imaging.

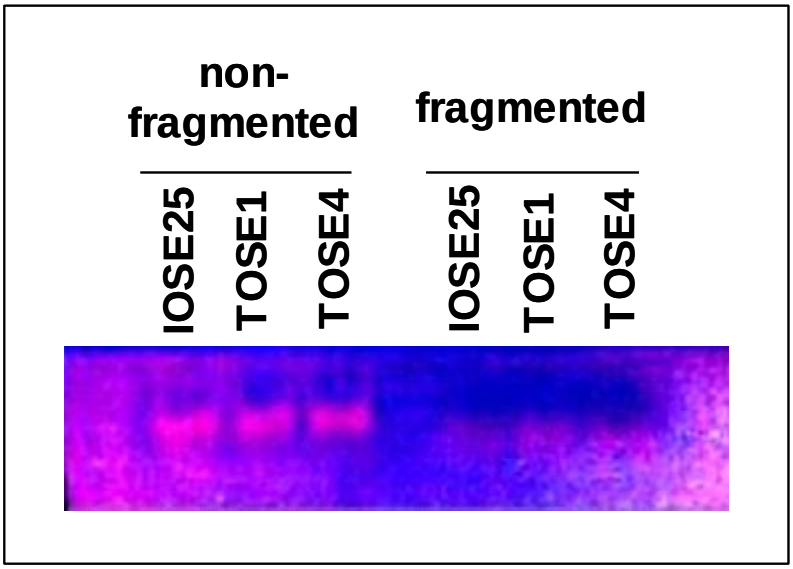


Figure 5.4 Fragmented biotin-labelled cRNA. *Shown is a 0.8% agarose gel after electrophoretic fractionation of fragmented biotin-labelled cRNA from IOSE25, TOSE1 and TOSE4 cells. Lanes 1-3 contain non-fragmented cRNA from IOSE25, TOSE1 and TOSE4 cells, as a control. Nucleic acid was visualised by adding GelRed to the agarose gel prior to setting, and UV radiation.*

Next, hybridisation cocktails were prepared from fragmented RNA. These were passed on to Tracy Chaplin (Centre for Medical Oncology, Institute of Cancer, Barts and The London School of Medicine and Dentistry, London, UK) alongside Human Genome U133 Plus 2.0 chips. Hybridisation, scanning of the arrays and preliminary quality

control were carried out by Tracy Chaplin. As the latter was highly satisfactory, raw data gained from the array were processed and analysed by Dr Claude Chelala.

5.6 Gene expression profiling in IOSE25, TOSE1 and TOSE4

A list of genes significantly up- or downregulated in TOSE1 and TOSE4, as compared to IOSE25 cells, was generated. The cut-off threshold of fold-change was set at 2 log. Lists of top 100 most differentially expressed genes are shown in the appendix (Appendix Tables 2-5). Tables 5.1-5.6, below, show the 20 genes most significantly up- or downregulated in TOSE1 and TOSE4 compared to IOSE25 cells. Ranking is based on fold-change in TOSE1 cells. Furthermore, genes from these lists were clustered into pathways and processes based on their functions. Table 5.7 shows the number of these 20 up- or downregulated genes involved in a given pathway or process.

Gene	Fold change		Function
	TOSE1 compared to IOSE25	TOSE4 compared to IOSE25	
chitinase 3-like 1 (cartilage glycoprotein-39) x2	9.01 7.87	5.40 4.15	promoter of proliferation in synovial, foetal lung fibroblast and skin cells; at low levels, has synergistic effects with Insulin-like growth factor-1 (Kawada *et al.*, 2007); induces activation of AKT and other signalling pathways (Recklies *et al.*, 2002)
selenoprotein P, plasma, 1	8.13	6.01	extracellular glycoprotein involved in oxidant defence and selenium transport (Olson *et al.*, 2007)
amylase, alpha 1A (salivary)	7.48	1.47	catalyser of starch/glycogen breakdown (Robert *et al.*, 2002b) and inhibitor of cAMP-dependent protein kinase (Furusawa *et al.*, 2002)
melanoma antigen family A, 12	7.14	7.46	normal function: probably in cell cycle regulation, particularly during germ line differentiation, (Ohman Forslund *et al.*, 2001), , MAGE-A12 normally expressed in testis (Mollaoglu *et al.*, 2008)
sodium channel, nonvoltage-gated 1 alpha	7.12	6.35	epithelial sodium ion channel (Meisler *et al.*, 1994), interacts with NEDD4, NEDD4L and ubiquitin C (Farr *et al.*, 2000)
major histocompatibility complex, class II, DP alpha 1	6.87	4.76	antigen-presenting cell surface molecule, adaptive immune system (Monaco, 1993; Ramachandra *et al.*, 1999)

Table 5.1 Twenty most significantly upregulated genes in TOSE cells as compared to IOSE25 cells. *Fold change in mRNA levels as detected by Affymetrix microarray. x2 / x3 = Appeared twice / three times on the top 20 list.*

Gene	Fold change		Function
	TOSE1 compared to IOSE25	TOSE4 compared to IOSE25	
melanoma antigen family A, 11	6.79	6.26	normal function: probably in cell cycle regulation, particularly during germ line differentiation (Ohman Forslund *et al.*, 2001), MAGE-A11 normally expressed in testis & placenta(Mollaoglu *et al.*, 2008)
haemoglobin, epsilon 1	6.68	4.85	oxygen transporter (Southworth *et al.*, 1926)
melanoma antigen family A, 3	6.49	6.76	MAGE genes: almost exclusively expressed in tumours; normal function: probably in cell cycle regulation, during germ line differentiation (Ohman Forslund *et al.*, 2001),
melanoma antigen family A, 6	6.41	6.75	normal function: probably in cell cycle regulation, particularly during germ line differentiation, {Ohman Forslund, 2001 #90, MAGE-A6 normally expressed in Testis
major histocompatibility complex, class II, DR alpha	6.28	1.87	antigen-presenting cell surface molecule, adaptive immune system (Monaco, 1993; Ramachandra *et al.*, 1999)
melanoma antigen family B, 2	6.07, 5.72	5.06, 6.97	normal function: probably in cell cycle regulation, particularly during germ line differentiation (Ohman Forslund *et al.*, 2001),MAGE-B2 normally expressed in testis, placenta
TIMP metallopeptidase inhibitor 3	6.07	5.44	inhibitor of MMP(Woessner, 2001); its overexpression results in apoptosis in lung cancer cells (Finan *et al.*, 2006), upregulated in virus-transformed fibroblasts, acts as a growth factor and can alter cell adhesion (Yang *et al.*, 1992)

Table 5.2 Twenty most significantly upregulated genes in TOSE cells as compared to IOSE25 cells. *Fold change in mRNA levels as detected by Affymetrix microarray. x2 / x3 = Appeared twice / three times on the top 20 list.*

Gene	Fold change		Function
	TOSE1 compared to IOSE25	TOSE4 compared to IOSE25	
haemoglobin, gamma G	6.03	3.86	oxygen transporter (Southworth *et al.*, 1926)
G1 to S phase transition 2	5.96	5.64	GTP-binding protein involved in G1 to S phase progression (Le Goff *et al.*, 2002)
major histocompatibility complex, class II, DR beta 1 x2	5.84 5.84	1.54 1.74	antigen-presenting cell surface molecule, adaptive immune system (Monaco, 1993; Ramachandra *et al.*, 1999)
secretory leukocyte peptidase inhibitor	5.84	4.48	inhibitor of serine proteases in epithelial cells; broad spectrum of antibiotic effects; repressed by IL-1 and TNFa (King *et al.*, 2002)
alpha-2-glycoprotein 1, zinc-binding	5.81	1.99	inducer of lipolysis (Russell *et al.*, 2004)
lipopolysaccharide-induced TNF factor	5.80	5.70	promoter/activator of TNF-α expression (Myokai *et al.*, 1999)
placenta-specific 8	5.71	6.99	Marker of plasmacytoid dendritic cells(Colonna *et al.*, 2004); enhances proliferation (Li *et al.*, 2006); confers resistance to apoptosis and loss of G2/M checkpoint; Inhibitor of differentiation (Rogulski *et al.*, 2005, Huang, 2006 #345); viral clearance in primates (Lanford *et al.*, 2007)

Table 5.3 Twenty most significantly upregulated genes in TOSE cells as compared to IOSE25 cells. *Fold change in mRNA levels as detected by Affymetrix microarray. x2 / x3 = Appeared twice / three times on the top 20 list.*

Gene	Fold change		Function
	TOSE1 compared to IOSE25	TOSE4 compared to IOSE25	
collagen, type I, alpha 2 x2	-8.96 -7.60	-8.86 -7.36	required for osteogenesis (Byers *et al.*, 1991); role in proliferation and migration of bladder cancer (Mori *et al.*, 2009)
chemokine (C-X-C motif) ligand 6 (granulocyte chemotactic protein 2)	-8.96	-8.97	Neuronal migration (chemoattractant); embryogenesis/olfactory development, (Edman *et al.*, 2008)
microsomal glutathione S-transferase 1 x3	-7.52 -6.64 -6.06	-8.31 -8.09 -7.69	protect against anti-oxidative stress (Maeda *et al.*, 2005)
pregnancy-associated plasma protein A, pappalysin 1 x4	-7.50 -7.28 -7.12 -6.68	-7.53 -7.36 -7.24 -6.81	matrix metalloproteinase, cleaves insulin-like growth factor binding proteins (Laursen *et al.*, 2001); role in proliferation and bone remodelling (Kumar *et al.*, 2005; Tanner *et al.*, 2008)
chemokine (C-X-C motif) ligand 12 (stromal cell-derived factor 1)	-7.19	-7.35	Neuronal migration; embryogenesis/olfactory development, acts together with CXCR4 (Schwarting *et al.*, 2006)
CD9 molecule	-6.73	-6.73	transmembrane proteins on stromal cells; crucial for osteoclast development (Tanio *et al.*, 1999)
leucine rich repeat containing 17	-6.46	-6.34	negative regulator of osteoclast-differentiation induced by receptor activator NF-κB -ligand (RANKL)(Kim *et al.*, 2009)
periostin, osteoblast specific factor	-6.44	-8.66	osteoblast differentiation (Litvin *et al.*, 2004), cardiac development, repair and remodelling; cell adhesion (Blanchard *et al.*, 2008), induces proliferation (Kuhn *et al.*, 2007); ligand for various $\alpha_v\beta_{3/5}$ integrins (Gillan *et al.*, 2002)

Table 5.4 Twenty most significantly downregulated genes in TOSE cells, compared to IOSE25 cells. *Fold change in mRNA levels detected by Affymetrix microarray ("-"= downregulation). x2 / x3 = Appeared twice / three times on the top 20 list.*

Gene	Fold change		Function
	TOSE1 compared to IOSE25	TOSE4 compared to IOSE25	
prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)	-6.28	-7.28	also called Cox-2; promoter of prostaglandins, which are involved in cell growth (Chen *et al.*, 2008), role in differentiation and expansion of bone cells (Xie *et al.*, 2008)
versican x2	-6.23 -5.72	-8.04 -6.49	extracellular matrix proteoglycan; involved in adhesion (Zimmermann *et al.*, 1989)
regulator of G-protein signalling 4 x2	-6.20 -6.07	-5.38 -5.17	inhibitor of G-protein via its GTPase-accelerating protein activity (Chasse *et al.*, 2003; De Vries *et al.*, 2000)
EGF-like repeats and discoidin I-like domains 3	-6.18	-6.76	mediator of angiogenesis, possibly vessel remodelling & development (Rezaee *et al.*, 2002); ligand for $\alpha_v\beta_3$ (Hidai *et al.*, 1998)
CD200 molecule	-6.13	-6.04	membrane glycoprotein, member of immunoglobulin family; negatively regulates macrophage and myeloid lineage ; induced by IFN-γ & TNF-α , induces IL-6 release and cell death (Chen *et al.*, 2009), inhibitor of osteoclast differentiation (Cui *et al.*, 2007)
Neurotrimin	-5.93	-5.85	adhesion molecule (Struyk *et al.*, 1995); role in development of neuronal projections (Gil *et al.*, 1998); restricted to post-mitotic neurons; expression peaks in during first post-natal week (Struyk *et al.*, 1995)
gamma-aminobutyric acid (GABA) B receptor, 2	-5.82	-6.05	inhibitory nervous system transmembrane receptor (Bowery, 2006); promoter of development (Dzitoyeva *et al.*, 2005); associated with G protein for intracellular signalling (Bowery, 2006)

Table 5.5 Twenty most significantly downregulated genes in TOSE cells as compared to IOSE25 cells. *Fold change in mRNA levels detected by Affymetrix microarray ("-"= downregulation). x2 / x3 = Appeared twice / three times on the top 20 list.*

Gene	Fold change		Function
	TOSE1 compared to IOSE25	TOSE4 compared to IOSE25	
ceramide kinase-like	-5.79	-5.90	lipid kinase; prevents ROS-induced apoptosis (Tuson *et al.*, 2009)
ATPase, class I, type 8B, member 1	-5.72	-4.01	activator of the farnesoid X receptor (Frankenberg *et al.*, 2008), which has tumour suppressor functions (Chiang *et al.*, 2000; Modica *et al.*, 2008)
neuronal cell adhesion molecule	-5.67	-5.85	cell-cell adhesion; induces neuronal outgrowth (Doherty *et al.*, 1996), reduction of the receptor increased metastasis in pancreatic cancer (Perl *et al.*, 1999)
Myocardin	-5.65	-5.80	transcriptional co-activator of serum response factor (SRF) (Wang *et al.*, 2003c); promotes differentiation of fibroblasts; frequently downregulated during malignant transformation, resulting in increased proliferation (Milyavsky *et al.*, 2007)
SAM domain, SH3 domain and nuclear localization signals 1	-5.64	-7.41	signal adapter protein (Uchida *et al.*, 2001); downregulated in malignant cells (Rimkus *et al.*, 2006)

Table 5.6 Twenty most significantly downregulated genes in TOSE cells as compared to IOSE25 cells. *Fold change in mRNA levels detected by Affymetrix microarray ("-"= downregulation). x2 / x3 = Appeared twice / three times on the top 20 list.*

Function / Process / Pathway		TOSE	
		Up	Down
Differentiation & Development	P	0/20	9/20
	I	5/20	2/20
Cytoskeletal regulation		0/20	0/20
Adhesion/Cell surface molecules & signalling /	P	5/20	8/20
Extracellular matrix	I	2/20	0/20
Anti-viral & Stress-induced mechanisms		7/20	1/20
Cell cycle promotion / Proliferation / Tumour growth	P	8/20	4/20
	I	0/20	3/20
Cell death/survival	P	1/20	1/20
	I	0/20	1/20
Transcription (other*)		0/20	0/20
Translation		0/20	0/20

Table 5.7 ***Clustering of most significantly deregulated genes according to function, pathway and process.*** *Shown are ratios of upregulated (**Up**) or downregulated (**Down**) genes to total of genes included in the clustering. ; * = involved in other pathways/processes than those listed. P=promoting effects, I=inhibiting effects.*

In addition, raw data were subjected to a more comprehensive analysis of gene expression. This analysis was performed by Dr Probir Chakravarty (Bioinformatics & Biostatistics, Lincoln's Inn Fields, London, UK.) Lists of top 30 statistically enriched pathways or processes were generated by applying the adjusted p value as a statistical filter. Metacore pathway analysis tool (GeneGo Inc., St. Joseph, MI, USA) (Ekins *et al.*, 2006; Ekins *et al.*, 2007) was used for hypergeometric testing with a p value of 0.05. The resulting list was separated into up- and down regulated lists (see Tables 5.8-5.14). In this context, the term ”pathway” represents a uni-directional flow of information, normally from the exterior of the cell to the nucleus of the cell. A “process” is a set of genes or proteins that act together, such that the end result is a recognised biological event or a series of events.

A multitude of pathways and processes was found to be enriched in TOSE cells. Their relevance is reviewed in the discussion at the end of this chapter.

Pathway enrichment – genes upregulated in TOSE1

1	Cytoskeleton remodeling_Cytoskeleton remodelling	7.05E-10	56/96
2	Cell adhesion_Chemokines and adhesion	2.19E-08	52/93
3	Cytoskeleton remodeling_TGF, WNT and cytoskeletal remodelling	1.40E-07	56/107
4	Cell cycle_The metaphase checkpoint	2.16E-06	24/36
5	Cell adhesion_Integrin-mediated cell adhesion and migration	2.43E-06	28/45
6	Cytoskeleton remodeling_Role Activin A in cytoskeleton remodelling	2.61E-06	16/20
7	Cell cycle_Chromosome condensation in prometaphase	2.61E-06	16/20
8	Transcription_CREB pathway	5.15E-06	23/35
9	Cytoskeleton remodeling_Role of PKA in cytoskeleton reorganisation	6.51E-06	21/31
10	Cytoskeleton remodeling_Regulation of actin cytoskeleton by Rho GTPases	8.26E-06	17/23
11	Cytoskeleton remodeling_Fibronectin-binding integrins in cell motility	1.76E-05	19/28
12	Development_TGF-beta induced EMT via SMADs	2.38E-05	22/35
13	Cytoskeleton remodeling_Integrin outside-in signalling	6.18E-05	26/46
14	Normal wtCFTR traffic / ER-to-Golgi	6.68E-05	23/39
15	Development_TGF-beta induced EMT via MAPK	7.62E-05	25/44
16	Regulation of CFTR activity (norm and CF)	1.15E-04	23/40
17	Immune response_MIF - the neuroendocrine-macrophage connector	1.42E-04	19/31
18	Development_EGFR signaling via small GTPases	1.70E-04	18/29
19	Muscle contraction_ GPCRs in the regulation of smooth muscle tone	2.46E-04	28/54
20	Cell cycle_Spindle assembly and chromosome separation	2.57E-04	19/32
21	Apoptosis and survival_BAD phosphorylation	3.62E-04	20/35
22	Cell adhesion_Endothelial cell contacts by non-junctional mechanisms	5.42E-04	15/24
23	Cardiac Hypertrophy_NF-AT signaling in Cardiac Hypertrophy	6.15E-04	29/59
24	Cell cycle_Role of Nek in cell cycle regulation	6.71E-04	17/29
25	Development_WNT signaling pathway. Part 1. Degradation of beta-catenin in the absence WNT signalling	7.54E-04	13/20
26	Cytoskeleton remodeling_FAK signalling	9.02E-04	24/47
27	DNA damage_ATM/ATR regulation of G1/S checkpoint	9.20E-04	18/32
28	Signal transduction_Calcium signalling	9.20E-04	18/32
29	Cell cycle_ESR1 regulation of G1/S transition	9.20E-04	18/32
30	Cell adhesion_Histamine H1 receptor signaling in the interruption of cell barrier integrity	9.55E-04	20/37

Table 5.8 ***Pathway and process enrichment analysis of differentially expressed genes.*** *Shown are enriched upregulated pathways, their fold-upregulation and ratios of proteins/genes differentially expressed in a given pathway versus total amount of proteins/genes involved in that pathway.*

Process enrichment – genes upregulated in TOSE1

1	Cell cycle_Mitosis	1.84E-13	93/177
2	Cell adhesion_Integrin-mediated cell-matrix adhesion	1.81E-12	103/209
3	Cell cycle_G2-M	1.44E-10	96/202
4	Cytoskeleton_Actin filaments	1.26E-09	85/178
5	Cytoskeleton_Spindle microtubules	2.43E-09	58/108
6	Cytoskeleton_Regulation of cytoskeleton rearrangement	2.71E-09	86/183
7	Development_Regulation of angiogenesis	4.87E-07	81/186
8	Cell adhesion_Cell junctions	1.21E-06	67/149
9	Signal transduction_WNT signalling	1.56E-06	74/170
10	Cell cycle_Core	1.88E-06	54/114
11	DNA damage_Checkpoint	3.23E-06	57/124
12	Cell cycle_S phase	3.54E-06	65/147
13	Cell cycle_G1-S	5.35E-06	70/163
14	Cell adhesion_Amyloid proteins	9.06E-06	71/168
15	Cell cycle_G1-S Growth factor regulation	1.51E-05	74/179
16	DNA damage_DBS repair	2.43E-05	49/108
17	Signal Transduction_TGF-beta, GDF and Activin signalling	4.53E-05	61/145
18	Cell cycle_Meiosis	5.15E-05	46/102
19	Inflammation_Amphoterin signalling	7.97E-05	50/115
20	Cell adhesion_Integrin priming	8.49E-05	44/98
21	Cell adhesion_Cadherins	9.01E-05	70/175
22	Cytoskeleton_Cytoplasmic microtubules	1.72E-04	49/115
23	Cardiac development_Wnt_beta-catenin, Notch, VEGF, IP3 and integrin signalling	2.30E-04	55/134
24	Inflammation_Protein C signalling	2.39E-04	41/93
25	Cell adhesion_Attractive and repulsive receptors	2.64E-04	66/168
26	Signal transduction_Androgen receptor signaling cross-talk	3.82E-04	28/58
27	Development_Hemopoiesis, Erythropoietin pathway	4.56E-04	51/125
28	Cytoskeleton_Intermediate filaments	4.65E-04	36/81
29	Signal transduction_ERBB-family signalling	5.18E-04	32/70
30	Signal transduction_NOTCH signalling	6.32E-04	82/223

Table 5.9 ***Pathway and process enrichment analysis of differentially expressed genes.*** *Shown are enriched upregulated processes, their fold-upregulation and ratios of proteins/genes differentially expressed involved a given process versus total amount of proteins/genes involved in that process.*

According to enrichment analysis, no pathway was significantly downregulated in TOSE1 cells.

Process enrichment – genes downregulated in TOSE1

1	Translation_Translation initiation	4.14E-14	102/163
2	Translation_Elongation-Termination	2.52E-12	91/147

Table 5.10 ***Pathway and process enrichment analysis of differentially expressed genes.*** *Shown are enriched downregulated processes, their fold-downregulation and ratios of proteins/genes differentially expressed involved a given process versus total amount of proteins/genes involved in that process.*

Pathway enrichment – genes upregulated in TOSE4

1	Cytoskeleton remodeling_Cytoskeleton remodelling	3.17E-13	54/96
2	Cell adhesion_Chemokines and adhesion	5.60E-12	51/93
3	Cytoskeleton remodeling_Integrin outside-in signalling	3.00E-09	29/46
4	Development_TGF-beta induced EMT via SMADs	5.99E-09	24/35
5	Cytoskeleton remodeling_TGF, WNT and cytoskeletal remodelling	1.41E-08	50/107
6	Development_TGF-beta induced EMT via MAPK	1.35E-07	26/44
7	Cytoskeleton remodeling:Role of PKA in cytoskeleton reorganisation	5.33E-07	20/31
8	Cell adhesion_Integrin-mediated cell adhesion and migration	1.20E-06	25/45
9	Cytoskeleton remodeling_Regulation of actin cytoskeleton by Rho GTPases	1.70E-06	16/23
10	Transcription_CREB pathway	8.05E-06	20/35
11	Cytoskeleton remodeling_Slit-Robo signalling	9.04E-06	18/30
12	Cytoskeleton remodeling_Fibronectin-binding integrins in cell motility	1.30E-05	17/28
13	Cardiac Hypertrophy_NF-AT signaling in Cardiac Hypertrophy	1.62E-05	28/59
14	Cell adhesion_Role of tetraspanins in the integrin-mediated cell adhesion	2.48E-05	20/37
15	Transcription_Androgen Receptor nuclear signalling	4.54E-05	21/41
16	Muscle contraction_ GPCRs in the regulation of smooth muscle tone	7.71E-05	25/54
17	Cell adhesion_Histamine H1 receptor signaling in the interruption of cell barrier integrity	1.00E-04	19/37
18	Cell adhesion_Endothelial cell contacts by non-junctional mechanisms	1.43E-04	14/24
19	Apoptosis and survival_BAD phosphorylation	1.49E-04	18/35
20	Transcription_PPAR Pathway	1.59E-04	19/38
21	Cytoskeleton remodeling:Reverse signaling by ephrin	2.01E-04	16/30
22	Normal wtCFTR traffic / ER-to-Golgi	2.46E-04	19/39
23	Regulation of lipid metabolism_ACM stimulation of Arachidonic acid production	2.48E-04	22/48
24	Cytoskeleton remodeling_Role Activin A in cytoskeleton remodelling	3.03E-04	Dec-20
25	Development_WNT signaling pathway. Part 2	3.57E-04	22/49
26	Cardiac Hypertrophy_Ca(2+)-dependent NF-AT signaling in Cardiac Hypertrophy	3.66E-04	18/37
27	Regulation of CFTR activity (norm and CF)	3.72E-04	19/40
28	wtCFTR and delta508-CFTR traffic / Generic schema (norm and CF)	3.72E-04	19/40
29	Delta508-CFTR traffic / ER-to-Golgi in CF	4.00E-04	Sep-13
30	Signal transduction_PTEN pathway	7.98E-04	19/42

Table 5.11 ***Pathway and process enrichment analysis of differentially expressed genes.*** *Shown are enriched upregulated pathways, their fold-upregulation and ratios of proteins/genes differentially expressed in a given pathway versus total amount of proteins/genes involved in that pathway.*

Process enrichment – genes upregulated in TOSE4

1	Cell adhesion_Integrin-mediated cell-matrix adhesion	3.85E-17	97/209
2	Cytoskeleton_Actin filaments	2.27E-13	80/178
3	Development_Regulation of angiogenesis	3.42E-11	78/186
4	Cytoskeleton_Regulation of cytoskeleton rearrangement	3.77E-11	77/183
5	Cell adhesion_Amyloid proteins	1.76E-10	71/168
6	Cell adhesion_Cadherins	5.65E-10	72/175
7	Development_Blood vessel morphogenesis	4.01E-08	77/208
8	Cell adhesion_Cell junctions	1.09E-07	59/149
9	Cardiac development_Wnt_beta-catenin, Notch, VEGF, IP3 and integrin signalling	1.92E-07	54/134
10	Signal Transduction_TGF-beta, GDF and Activin signalling	2.40E-07	57/145
11	Signal transduction_WNT signalling	2.85E-07	64/170
12	Development_Neurogenesis:Axonal guidance	1.63E-06	65/181
13	Cell adhesion_Attractive and repulsive receptors	2.25E-06	61/168
14	Development_Skeletal muscle development	2.81E-06	54/144
15	Cell adhesion_Cell-matrix interactions	5.26E-06	70/205
16	Cell adhesion_Platelet aggregation	9.53E-06	52/142
17	Signal transduction_NOTCH signalling	1.80E-05	73/223
18	Cardiac development_FGF_ErbB signalling	2.41E-05	45/121
19	Signal transduction_Androgen receptor signaling cross-talk	3.39E-05	26/58
20	Cardiac development_Role of NADPH oxidase and ROS	3.87E-05	45/123
21	Development_Ossification and bone remodeling	5.42E-05	52/150
22	Cell adhesion_Integrin priming	8.68E-05	37/98
23	Reproduction_FSH-beta signaling pathway	9.80E-05	52/153
24	Signal transduction_Leptin signalig	1.44E-04	33/86
25	Reproduction_Feeding and Neurohormones signaling	1.76E-04	65/206
26	Inflammation_Amphoterin signaling	3.55E-04	40/115
27	Cell adhesion_Platelet-endothelium-leucocyte interactions	3.77E-04	55/172
28	Cytoskeleton_Cytoplasmic microtubules	7.42E-04	39/115
29	Inflammation_TREM1 signaling	1.39E-03	41/126
30	Inflammation_Neutrophil activation	2.21E-03	57/192

Table 5.12 ***Pathway and process enrichment analysis of differentially expressed genes.*** *Shown are enriched upregulated processes, their fold-upregulation and ratios of proteins/genes differentially expressed involved a given process versus total amount of proteins/genes involved in that process.*

Pathway enrichment – genes downregulated in TOSE4

1	Oxidative phosphorylation	5.31E-08	48/95
2	Development_WNT signaling pathway. Part 1. Degradation of beta-catenin in the absence WNT signaling	1.77E-04	13/20

Table 5.13 ***Pathway and process enrichment analysis of differentially expressed genes.*** *Shown are enriched downregulated pathways, their fold-downregulation and ratios of proteins/genes differentially expressed in a given pathway versus total amount of proteins/genes involved in that pathway.*

Process enrichment – genes downregulated in TOSE4

1	Translation_Translation initiation	2.37E-21	95/163
2	Translation_Elongation-Termination	1.41E-14	78/147
3	Translation_Translation in mitochondria	1.15E-09	53/103
4	Translation_Regulation of initiation	9.80E-08	52/111
5	Transcription_Nuclear receptors transcriptional regulation	1.13E-06	74/187
6	Transcription_mRNA processing	4.54E-04	57/159
7	Transcription_Transcription by RNA polymerase II	7.01E-04	56/158
8	Transcription_Chromatin modification	2.93E-03	44/125
9	Proteolysis_Ubiquitin-proteasomal proteolysis	3.02E-03	56/167

Table 5.14 ***Pathway and process enrichment analysis of differentially expressed genes.*** *Shown are enriched downregulated processes, their fold-downregulation and ratios of proteins/genes differentially expressed involved a given process versus total amount of proteins/genes involved in that process.*

5.7 Microarray analysis in MRC5 and MRC5-VA cells

In a previous chapter, experiments on MRC5 and MRC5-VA cells had confirmed that a dysregulated Rb pathway increases cell sensitivity to *dl*922-947. MRC5-VA cells showed a large increase in sensitivity to *dl*922-947 compared to the parental non-transformed MRC5 cells. Given their genetic proximity, this cell pair represents another suitable system for comparative gene expression profiling to identify host cell genes responsible for sensitisation to *dl*922-947. Furthermore, comparing array results of TOSE and MRC5-VA cells could highlight the most potent general determinants of *dl*922-947 efficacy. Overlap of certain differentially expressed genes and pathways between the two models would emphasise their influence on cell sensitisation to *dl*922-947.

5.7.1 Microsatellite sequencing of MRC5 and MRC5-VA cells

Prior to microarray analysis, genomes of MRC5 and MRC5-VA cells underwent microsatellite sequencing to assess their proximity in genetic background (Choudhary *et al.*, 1993). Microsatellite sequencing was performed by the Genome Centre (William Harvey Research Institute, Barts and The London School of Medicine and Dentistry, London, UK) using 100ng genomic DNA from each cell line. A panel of 14 microsatellites on 6 chromosomes was compared in both cell lines. A table of chromosomes and ratios of consistent or inconsistent markers is shown in Table 5.15. Most markers assessed emerged as inconsistent. It is thus possible that MRC5 and MRC5-VA are unrelated cells lines. On the other hand, according to oral communication with Charles Mein (Genome Centre, William Harvey Research Institute, Barts and The London School of Medicine and Dentistry, London, UK) , a tendency of multiple alleles per locus was detected in the microsatellite sequencing. This is likely to be due to multiple copies of different lengths per cell (Fu *et al.*, 1991; Vogelstein *et al.*, 1988). Another possibility is that alleles are different between the cells within a pool. Either could indicate microsatellite instability (Wolman *et al.*, 1992). The cell pair was established in 1983 (Huschtscha *et al.*, 1983). Thus, passaging of the cells has undoubtedly introduced genomic changes in the two cell lines, in particular, as SV40 TAg-transformation renders cells more prone to genomic instability (Barbanti-Brodano *et al.*, 2004).

Chromosome	Ratio of consistency
Chr 03	1/3 markers consistent, 2/3 inconsistent
Chr 04	1/1 markers inconsistent
Chr 05	3/3 markers inconsistent
Chr 06	1/1 markers inconsistent
Chr 17	1/1 markers inconsistent
Chr 18	2/2 markers inconsistent

Table 5.15 ***Microsatellite sequencing in MRC5 and MRC5-VA cells.*** *Shown are ratios of consistent or inconsistent markers between MRC5 and MRC5-VA cells. Chr = chromosome.*

5.8 Gene expression profiling in MRC5 and MRC5-VA cells

For whole genome basal expression analysis in MRC5 and MRC5-VA cells total RNA was processed the same way as for TOSE cells. Again, biotin-labelled cRNA was compared using the Affymetrix Human Genome U133 Plus 2.0 microarray platform. Below, Tables 5.16-5.20 show the 20 genes most significantly up- or downregulated in MRC5-VA compared to MRC5 cells. A list of the top 100 differentially expressed genes is shown in the appendix (Appendix Table 6 and 7). In addition, genes from the top 20 lists were clustered into pathways and processes. Table 5.21 shows how many of these 20 up- or downregulated genes were involved in a given pathway or process. Clustering was based on functions attributed to each gene. It was to serve as a starting point to identify further biomarkers for sensitivity to *dl*922-947.

Gene	Fold change compared to MRC5 cells	Function
placenta-specific 8	7.82	marker of plasmacytoid dendritic cells(Colonna *et al.*, 2004); enhances proliferation (Li *et al.*, 2006); confers resistance to apoptosis and loss of G2/M checkpoint; Inhibitor of differentiation (Rogulski *et al.*, 2005, Huang, 2006 #345); viral clearance in primates (Lanford *et al.*, 2007)
brain expressed, X-linked 1	7.38	promoter of proliferation and inhibitor of differentiation by interacting with p75 (Vilar *et al.*, 2006)
protein phosphatase 2 (formerly 2A), regulatory subunit B, beta isoform (PP2, also known as PP2A)	6.71	Ser/Thr phosphatase, involved in broad range of cellular functions; target proteins include Raf, MEK, AKT (Dworakowska *et al.*, 2009); suppression of PP2 results in inhibition of Akt by phosphorylation and increased cell death (Mao *et al.*, 2005; Ory *et al.*, 2003; Yin *et al.*, 2006)
death-associated protein kinase 1	6.54	mediator of apoptosis, induced by IFN-γ (Deiss *et al.*, 1995)
ELOVL family member 7, elongation of long chain fatty acids (yeast)	6.48	promoter of cancer growth and cell survival (Tamura *et al.*, 2009)
nuclear factor I/B x2	6.42 6.23	Binds promoters of cellular and viral promoters and origin of replication of Ad2; activator of cellular and viral transcription and replication; involved in development and tumourigenesis (Gronostajski, 2000; Nagata *et al.*, 1982)
hypothetical protein	6.36	N/A
F11 receptor	6.14	also called junctional adhesion molecule (JAM)) (Ong *et al.*, 2009); adhesion protein for reovirus (Stehle *et al.*, 2004); structural similarities to CAR (Goosney *et al.*, 2003; Walters *et al.*, 2002)

Table 5.16 Twenty most significantly upregulated genes in MRC5-VA cells as compared to MRC5 cells. *Fold change in mRNA levels as detected by Affymetrix microarray. x2 / x3 = Appeared twice / three times on the top 20 list.*

Gene	Fold change compared to MRC5 cells	Function
transcription factor AP-2 alpha (activating enhancer binding protein 2 alpha)	6.10	cell-type-specific stimulation of proliferation and the suppression of terminal differentiation during embryonic development (Eckert *et al.*, 2005); growth inhibitory effects in tumours, when cytoplasmic (Eckert *et al.*, 2005); the p21-promoter possesses an AP-2 binding site (Anttila *et al.*, 2000)
radical S-adenosyl methionine domain containing 2	5.95	endoplasmic reticulum-associated, IFN-induced antiviral protein, induced by CMV (Chin *et al.*, 2001)
carboxypeptidase A3 (mast cell)	5.88	up-regulation in prostate cancer leads to differentiation and apoptosis, induced by histone deacetylase inhibitors; p21 transactivation is required for CBA3 induction (Huang *et al.*, 1999)
odz, odd Oz/ten-m homolog 2 (Drosophila)	5.82	cell surface and transmembrane receptor, promotes cell-cell adhesion (Tucker *et al.*, 2006), translocates to PMLs (Bagutti *et al.*, 2003)
zinc finger protein 236	5.77	target for tumour growth inhibition; transcription factor activity; glucose-induced expression (Holmes *et al.*, 1999)
ELAV (embryonic lethal, abnormal vision, Drosophila)-like 2 (Hu antigen B)	5.74	binds to poly-A tails of mRNA; required for cellular differentiation (Akamatsu *et al.*, 1999); regulates poly-adenylation, mRNA stabilisation and translation (Yannoni *et al.*, 1999)
leucine rich repeat neuronal 1	5.68	transmembrane protein; expression restricted to early development of central nervous system, later downregulated (Carim-Todd *et al.*, 2003)
plakophilin 2	5.67	scaffold for adhesion and signalling (Bass-Zubek *et al.*, 2009)

Table 5.17 Twenty most significantly upregulated genes in MRC5-VA cells as compared to MRC5 cells. *Fold change in mRNA levels as detected by Affymetrix microarray. x2 / x3 = Appeared twice / three times on the top 20 list.*

Gene	Fold change compared to MRC5 cells	Function
ISL1 transcription factor, LIM/homeodomain, (islet-1)	5.57	transcription factor in neuronal differentiation and cardiac and pancreatic cell lineage development (Cai *et al.*, 2003; Sun *et al.*, 2007), marker of self-renewal capacity/pluripotency (Bu *et al.*, 2009; Lin *et al.*, 2007; Moretti *et al.*, 2006)
pleckstrin homology domain containing, family A member 7	5.57	promotes E-cadherin, α- and β-catenin in zona adherens; involved in tethering microtubules to zona adherens in epithelial cells (Meng *et al.*, 2008)
neurexin 3	5.43	cell adhesion and receptor molecule in nervous system (Kelai *et al.*, 2008)
radical S-adenosyl methionine domain containing 2	5.25	cytomegalovirus-induced gene 5 protein; virus inhibitory protein, interferon-inducible (Chin *et al.*, 2001; Hinson *et al.*, 2009)

Table 5.18 Twenty most significantly upregulated genes in MRC5-VA cells as compared to MRC5 cells. *Fold change in mRNA levels as detected by Affymetrix microarray. x2 / x3 = Appeared twice / three times on the top 20 list.*

Gene	Fold change compared to MRC5 cells	Function
matrix metallopeptidase 1 (interstitial collagenase)	-7.84	breaks down collagen (Seltzer *et al.*, 1989)
collagen, type VI, alpha 3	-7.15	extracellular matrix protein, cell growth (Miner *et al.*, 1994)
peroxiredoxin 2	-7.02	antioxidant enzyme; implications with anti-viral mechanism in T-cells (Geiben-Lynn *et al.*, 2003); promoting cell death (Fang *et al.*, 2007)
collagen triple helix repeat containing 1	-7.00	inhibitor of collagen expression, promoter of cell migration (Pyagay *et al.*, 2005)
heat shock 70kDa protein 1A	-6.78	stress-inducible protein (Daugaard *et al.*, 2007); induced by E1A and large tumour antigen genes (Milarski *et al.*, 1986); under normal conditions promotes G1 to S phase progression (Milarski *et al.*, 1986)
cadherin 11, type 2, OB-cadherin (osteoblast)	-6.36	downregulation decreases adhesion to other cadherin 11 proteins (Chu *et al.*, 2008)
Decorin x3	-6.29 -6.06 -5.90	proteoglycan involved in assembly of extracellular matrices and regulation of proliferation (Krusius *et al.*, 1986); downregulation of decorin may be due to loss of Rb function, as re-introducing pRb into knock-out cells upregulated decorin levels (Rohde *et al.*, 1996)
adipose differentiation-related protein	-6.28	marker for adipocyte differentiation (Jiang *et al.*, 1992)
forkhead box F2	-6.08	transcription activator involved in development of epithelia (Wang *et al.*, 2003a)
prostaglandin-endoperoxide synthase 1 (prostaglandin G/H synthase and cyclooxygenase)	-6.06	enzyme promoting production of prostaglandins, which in turn are involved in cell growth; inhibition of cox-1 results in cell cycle arrest in tumour cells (Robertson *et al.*, 1998); pro-inflammatory role (Garcia-Bueno *et al.*, 2009)

Table 5.19 Twenty most significantly downregulated genes in MRC5-VA cells as compared to MRC5 cells. *Fold change in mRNA levels as detected by Affymetrix microarray (minus before values indicates downregulation). x2 / x3 = Appeared twice / three times on the top 20 list.*

Gene	Fold change compared to MRC5 cells	Function
thyrotropin-releasing hormone degrading enzyme	-5.99	induce growth-hormone secretion (Schally, 1978)
microtubule-associated protein 1A	-5.93	in non-differentiated neurons associated with mitotic spindle; protein levels increase during differentiation (Vaillant *et al.*, 1995)
G protein-coupled receptor 37 (endothelin receptor type B-like)	-5.78	associated with differentiation of tumour cells (Hoshi *et al.*, 2009), loss of the protein disrupts differentiation of cell lineages in nervous system (Nataf *et al.*, 1996)
insulin-like growth factor binding protein 4	-5.74	inhibitor of insulin-like growth factor, thus preventing proliferation and differentiation (Durai *et al.*, 2007)
spondin 2, extracellular matrix protein	-5.69	required for T cell priming by dendritic cells and for normal morphogenesis of respiratory tract and limbs; involved in Wnt-signalling (Blaydon *et al.*, 2006)
heat shock protein, alpha-crystallin-related, B6	-5.64	role in muscle function (Dreiza *et al.*), indirectly affects actin cytoskeleton (Seit-Nebi *et al.*, 2009)
HIV-1 Tat interactive protein 2, 30kDa	-5.60	transcriptional co-factor for Tat-mediated transcription from HIV promoter (Xiao *et al.*, 1998)
eukaryotic translation initiation factor 1A, Y-linked	-5.58	important translation initiation factor, required for stabilisation and binding of ribosomal 43S complex to the 5' end of capped mRNA (Chaudhuri *et al.*, 1997; Pestova *et al.*, 1998)
transient receptor potential cation channel, subfamily A, member 1	-5.53	stress sensor (Bang *et al.*, 2009; Garcia-Anoveros *et al.*, 2007); role in keratinocyte differentiation and mediating acute inflammatory pain (Atoyan *et al.*, 2009)
CUG triplet repeat, RNA binding protein 2	-5.49	inhibits translation of COX-2 mRNA (Sureban *et al.*, 2007)

Table 5.20 Twenty most significantly downregulated genes in MRC5-VA cells as compared to MRC5 cells. *Fold change in mRNA levels as detected by Affymetrix microarray (minus before values indicates downregulation). x2 / x3 = Appeared twice / three times on the top 20 list.*

Function / Process / Pathway		MRC5-VA	
		Up	**Down**
Differentiation & Development	P	2/20	6/20
	I	5/20	2/20
Cytoskeletal regulation		1/20	1/20
Adhesion / Cell surface molecules & signalling /	P	4/20	3/20
Extracellular matrix	I	0/20	2/20
Anti-viral & Stress-induced mechanisms		3/20	4/20
Cell cycle promotion / Proliferation / Tumour growth	P	5/20	4/20
	I	1/20	2/20
Cell death / survival	P	2/20	1/20
	I	2/20	0/20
Transcription (other*)		2/20	1/20
Translation		1/20	1/20

Table 5.21 ***Clustering of most significantly deregulated genes according to function, pathway and process.*** *Shown are ratios of upregulated (**Up**) or downregulated (**Down**) genes to total of genes included in the clustering. ; * = involved in other pathways/processes than those listed. P=promoting effects, I=inhibitory effects.*

5.9 Generation of ovarian cell lines with Rb pathway abnormalities

Microsatellite sequencing indicated many inconsistencies in microsatellites between MRC5 and MRC5-VA cells. To obtain a more relevant model system for my work, I wanted to establish matched cell pairs of ovarian cells, comprising one cell line with a normal Rb pathway and an isogenic cell line in which the pathway is deregulated. IOSE20 and IOSE21 cells are human normal ovarian surface epithelial (OSE) cells induced to overexpress human telomerase reverse transcriptase (hTERT) by retroviral transduction. This results in their immortalisation without disrupting normal Rb pathway function (Li *et al.*, 2007a). I attempted to transform both cell lines by transfection with a plasmid encoding SV40 TAg. As explained earlier, SV40 TAg is able to bind and inhibit both p53 and pRb, leading to a loss of function of the cell cycle checkpoint. Ovarian-specific Cre/Lox-mediated excision of both genes in a murine model was observed to result is a phenotype very similar to that of high grade serous ovarian cancer (Flesken-Nikitin et al., 2003). Thus, SV40 TAg- transformed cells could represent an ideal model to study in more detail the implications of Rb pathway deregulations for cytotoxic function of *dl*922-947 in ovarian cells. Furthermore, I planned to carry out comparative microarray analysis in these cell line pairs, taking advantage of their isogenic nature.

5.9.1 Cloning of plasmid pCMV-SV40

The first step towards generating SV40-transformed cells was the cloning of a suitable plasmid encoding SV40 TAg. I had access to a plasmid containing the SV40 TAg gene, pX8 (kindly received from Prof George Tsao, University of Hong Kong, China) (Tsao *et al.*, 2002). However this vector did not contain a selectable marker. The latter is not necessary for transformation of normal cells, as only transfected cells will continue to proliferate. To transfect IOSE cells, on the other hand, an additional tool for growth selection in required, as these cells already are immortalised. Thus, the SV40 TAg gene was subcloned from pX8 into a vector containing an antibiotic resistance gene. Cloning steps were performed by or under close supervision of Dr Carin Ingemarsdotter in our group as described in Material and Methods. In brief, the SV40 TAg-encoding sequence was excised from pX8 by EcoRI digestion. After gel purification, the EcoRI fragment was ligated into pCMV-Script (Stratagene, La Jolla, CA, USA) which contains a neomycin-resistance gene.

Test digests were performed to distinguish between plasmids containing the insert in forward and reverse orientation, the latter representing an insert-containing control. Subsequently, two plasmids, one containing the insert in forward orientation (pCMV-

SV40-F) and one with the insert in reverse orientation (pCMV-SV40-R), were further amplified and purified before transfection into IOSE cells.

5.9.2 Transfection of IOSE cells with pCMV-SV40

IOSE20 and IOSE21 cells were each transfected with plasmids pCMV-SV40-F, pCMV-SV40-R and, to obtain further control cell lines, empty vector pCMV-Script. The transfection reagent used was FuGENE 6 (Roche). Transfected cells were selected in G-418 (1mg/ml). A list of the newly generated cell pools as well as the plasmids they contain is shown in Table 5.22. After several passages, protein was extracted from all cell pools and protein levels of intracellular SV40 TAg assessed by Western blot.

Cell pool	Parental cell line	Transfected plasmid
I20-SV40-F1	IOSE20	pCMV-SV40-F
I20-SV40-F2	IOSE20	pCMV-SV40-F
I20-SV40-R1	IOSE20	pCMV-SV40-R
I20-SV40-R2	IOSE20	pCMV-SV40-R
I20-CMV	IOSE20	pCMV-Script
I21-SV40-F1	IOSE21	pCMV-SV40-F
I21-SV40-F2	IOSE21	pCMV-SV40-F
I21-SV40-R1	IOSE21	pCMV-SV40-R
I21-SV40-R2	IOSE21	pCMV-SV40-R
I21-CMV	IOSE21	pCMV-Script

Table 5.22 Transfected cell pools. *Listed are newly generated pools, their parental cell lines and the plasmid transfected into them.*

5.9.3 SV40 TAg expression in transfected cells

Western blot analysis was performed to assess levels of SV40 TAg in transformed cells. As a positive control, total protein harvested from MRC5-VA cells was also run. The experiment was repeated three times. Representative images of the Western blots are shown in Fig. 5.5. I could not detect SV40 TAg protein in any of the samples from pCMV-SV40 transfected cells. Although the GAPDH control is over-exposed and loading may not be fully equal, this does not alter the overall conclusion.

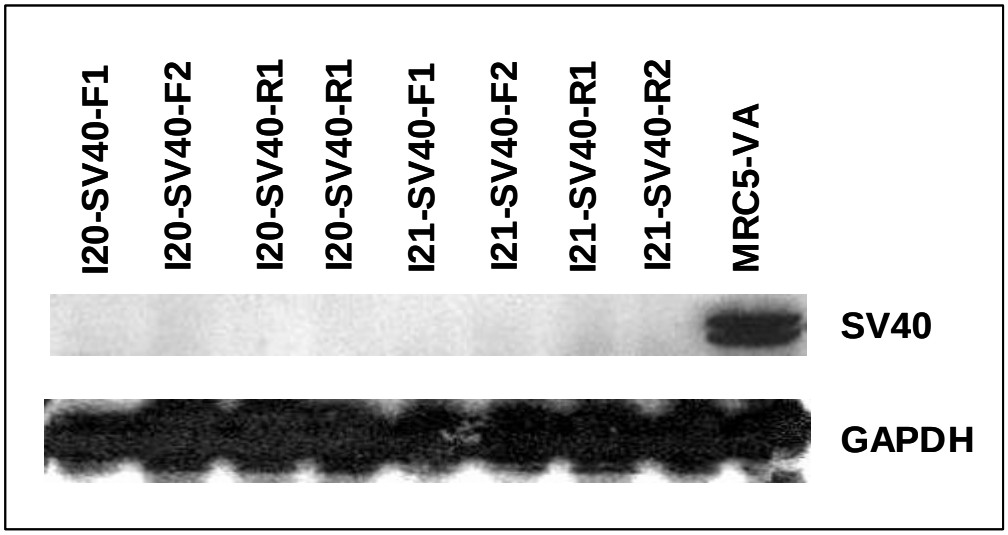
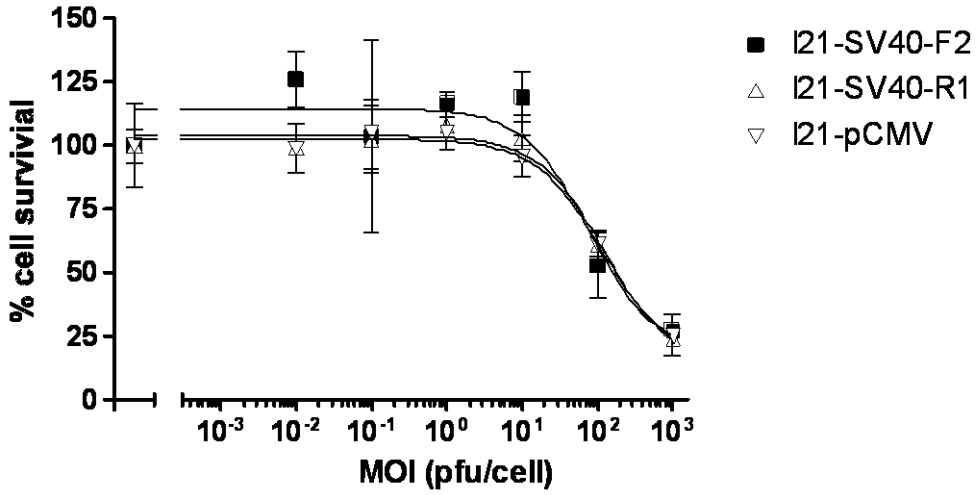


Figure 5.5 Expression of SV40 TAg in transfected cells. *Western blot of protein samples from transfected I20/I21-SV40 cells probed for SV40 TAg. As a loading control GAPDH was used.*

5.9.4 Sensitivity of I20/I21-SV40 cells to *dl*922-947

In parallel to processing I20-SV40-F, I20-SV40-R, I21-SV40-F and I21-SV40-R for Western blot analysis, cell survival assays were set up to compare sensitivity of these cells to *dl*922-947 120 hours p.i.. Representative survival curves and IC_{50} values in I21-SV40-F, I21-SV40-R and I21-pCMV cells are shown in Fig. 5.6. In keeping with the Western blot results, there was no difference in IC_{50} values between the three cell pools.



	IOSE21	I21-SV40-F2	I21-SV40-R2
IC_{50}	110	78	120

Figure 5.6 Sensitivity of pCMV-SV40 transfected cells to *dl*922-947. *Dose response curves of I21-CMV, I21-SV40-F2 and I21-SV40-R2 120hrs p.i.. Percentage of cell survival is graphed relative to log MOI pfu/cell of dl*922-947*. Points represent mean +/- standard deviation. Table shows IC_{50} values (pfu/cell).*

Considering the lack of detectable SV40 Tag protein in any of the relevant cell lines, further experiments on these cells were discontinued.

5.9.5 Transfection of IOSE20 and IOSE21 cells with pSV3neo

As transfection of IOSE20 and IOSE21 cells neither with pCMV-SV40-F nor with pCMV-SV40-R resulted in detectable expression of SV40 TAg, I wanted to repeat the transfection using a published plasmid encoding SV40 TAg, pSV3neo. The plasmid was generated by Peter J Southern and Peter Berg (Southern *et al.*, 1982) and kindly donated by Prof Guy Whitley (University of St Andrews, UK). Apart from the sequence for SV40 TAg, pSV3neo also contains the neomycin resistance gene to allow positive selection of transfected cells. IOSE20 and IOSE21 cells were transfected with pSV3neo plasmid using FuGENE 6 and cultured in selective G-418 containing medium as described earlier.

5.9.6 Detection of SV40 TAg protein in pSV3neo-transfected cells

Western blot analysis was performed in order to assess SV40 TAg expression in the transfected IOSE20 and IOSE21 cells (Fig. 5.7). Total protein from cell clones I20-pSV3neo 1, I20-pSV3neo 2, I21-pSV3neo 1 and I21-pSV3neo 2 was harvested after several passages in G-418 (1mg/ml) in NOSE medium. Protein samples were also obtained from control cells I20-pCMV and I21-pCMV. Whilst a strong signal could be detected in the positive control, no bands of the correct size (approximately 82kDa) emerged in any of the other lanes.

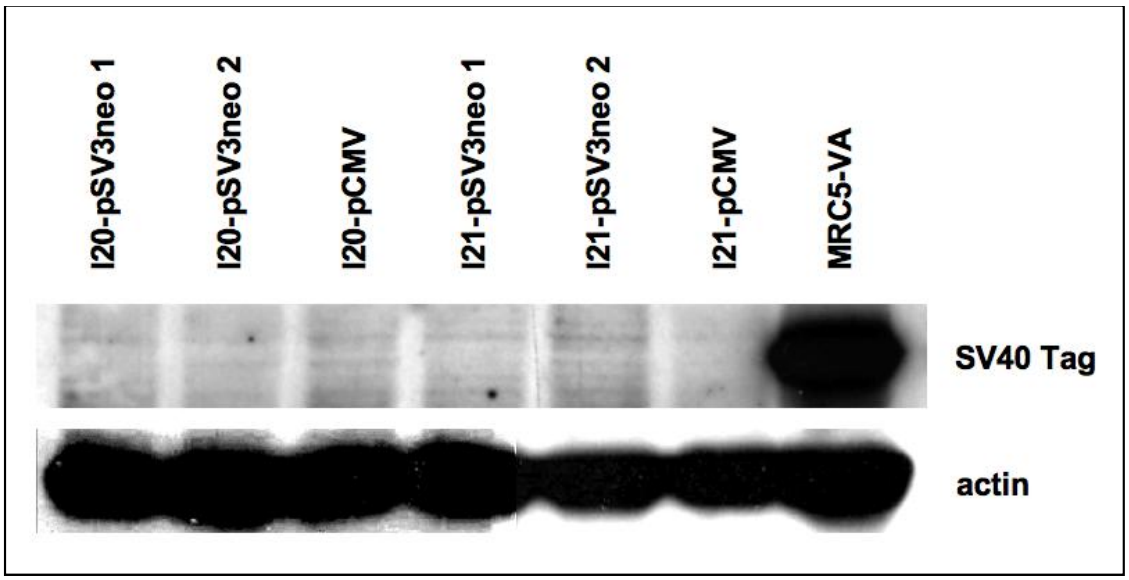


Figure 5.7 SV40 TAg expression in pSV3neo-transfected cells. *Western blot analysis was performed on lysates from I20-pSV3neo 1, I20-pSV3neo 2, I20-pCMV, I21-pSV3neo 1, I21-pSV3neo 2, I21-pCMV and positive control MRC5-VA using an antibody against SV40 TAg. Actin levels serve as a loading control.*

As, again, no SV40 TAg expression was induced and, consequently, there was no difference in sensitivity to *dl*922-947 (data not shown), experiments were discontinued.

5.10 Discussion

Experiments in pre-transformed TOSE cells produced interesting results. Compared to IOSE25 parental cells, TOSE1 and TOSE4 were much more sensitive to dl922-947-induced cytotoxicity and also expressed markedly more p21. This is in keeping with previous results of high basal p21 levels in TOV21G, IGROV-1, Hct116 p21+/+ and ACP-WAF1 cells correlating with higher sensitivity. In TOSE1 cells the IC_{50} was at least 150-fold and in TOSE4 5-fold lower than in parental cells. In TOSE4 cells, this enhanced sensitivity may be partially due to the small increase in infectivity compared to IOSE25 cells. However, in TOSE1, increase in sensitivity is greater than 2 logscales, which is far greater than the increase in infectivity. This confirms that infectivity is not the sole determinant of viral efficacy, a point raised in Chapter 3. In view of these results, TOSE cells represented a suitable system to search for new biomarker candidates by microarray analysis. In addition, the matched pair of MRC5 and MRC5-VA cells underwent the same analysis.

Comparing broad gene expression by microarray produced a list of genes differentially transcribed in TOSE versus IOSE25 cells. Firstly, the twenty most up- and downregulated genes were clustered into common pathways and processes. In agreement with previous results, this small scale strategy for comparison showed that many of the differentially expressed genes are involved in cell cycle progression, proliferation and tumour growth. Although some of the genes that promote these processes were downregulated, the majority were over-expressed. In addition, none of the inhibitors of cell cycle, proliferation or growth was upregulated, whilst expression of three inhibitors was reduced. This pattern of cell cycle and proliferation enhancing gene expression is typical for cancer cells, underlining the pre-transformed character attributed to TOSE cells. Furthermore, as shown and discussed in previous chapters, enhanced S phase entry and proliferation can promote *dl*922-947 cytotoxic function, including in TOSE cells.

Despite higher p21 protein levels in TOSE cells, transcription of its gene *CDKN1A* was not upregulated. As shown in Chapter 4, p21 protein levels are dependent on protein stability rather than gene expression alone (Abbas *et al.*, 2009). Thus, high basal p21 levels in TOSE cells may result from enhanced stabilisation, which could be assessed using cycloheximide treatment. Furthermore, certain post-translational modifications of p21 and complex formation with other proteins regulate stability and degradation (Chen *et al.*, 2007; Li *et al.*, 2007b; Sheaff *et al.*, 2000; Touitou *et al.*, 2001). Assessing the

extent of such modifications by Western blot analysis or association with other proteins by pulldown experiments could reveal differences in the post-translational fate between cell lines.

Cytotoxic efficacy of *dl*922-947 may also be further promoted by increased expression of cell death promoting gene TIMP-3 (Finan *et al.*, 2006) and decreased expression of an anti-apoptotic gene ceramide kinase-like (Tuson *et al.*, 2009). On the other hand, these effects may be counteracted by downregulation of another pro-cell death gene, CD200 (Chen *et al.*, 2009). TOSE cells also showed increased transcription of a high number of genes involved in mechanisms usually induced by stress, in response to virus-infection or in pro-inflammatory pathways (King *et al.*, 2002; Lindqvist *et al.*, 2005; Oliva *et al.*, 2001; Papi *et al.*, 2000). This stands in accordance with the well-established correlation between cancer and inflammation (Balkwill *et al.*, 2001; Coussens *et al.*, 2002).

Interestingly, the highest numbers of genes deregulated in TOSE cells, as compared to parental IOSE25 cells, are of those involved in differentiation and development. Amongst the top 20 genes down-regulated in TOSE cells, nine were promoters of differentiation. This suggests that, compared to IOSE25 cells, expression patterns in TOSE cells may promote a status of de-differentiation. A similar phenomenon was found in MRC5-VA cells after clustering the 20 genes most up- and downregulated, compared to parental MRC5 cells. Again, a considerable fraction of over-expressed genes had anti-differentiation function. This was coupled with reduced transcription of a series of differentiation-promoting genes.

Many of the most differentially expressed genes are those implicated in regulation of cell cycle, proliferation and tumour growth. Although four genes promoting these processes were downregulated, alongside one inhibitor being over-expressed, they are out-numbered by over-expressed cell cycle promoters and downregulated inhibitors. Taken together, the gene expression profile in MRC5-VA cells appears to be promoting cell cycling and proliferation.

Similar to TOSE cells, in MRC5-VA cells there was both up-and downregulation of genes promoting and inhibiting cell surface molecules and signalling, adhesion and extracellular matrix. And, as shown in Chapter 3, MRC5-VA cells are more infectable than their parental cell line. Like TOSE cells, MRC5-VA cells are able to grow in soft agar (Huschtscha *et al.*, 1983).

Microarray data obtained from TOSE1, TOSE4 and IOSE25 cells were subjected to comprehensive pathway and process enrichment analysis. As described earlier, in the context of this study, a pathway is a uni-directional flow of information, from the cell exterior to its nucleus. A process is a set of genes or proteins acting together, resulting in a certain biological event. The meta-analysis revealed some surprising results. Compared to IOSE25 cells, in both TOSE1 and TOSE4 cells most of the 30 most highly upregulated pathways are involved in cytoskeleton remodelling. In TOSE1 cells, this category is also represented several times among the top 30 upregulated processes, but less often in TOSE4 cells. In the small scale clustering analysis described above, two genes involved in cytoskeletal regulation were found deregulated in TOSE1 cells. Transcription of one of them was reduced, the other was over-expressed. In TOSE4 cells, none of the 20 most differentially expressed genes was implicated with the cytoskeleton. However, the term cytoskeleton remodelling is broad and networks and pathways involved may be far too extensive to be encompassed entirely by the limited scope of the first clustering analysis.

In TOSE1 cells, the second largest group of upregulated pathways was that of cell cycle regulation. This was also the most prominent group of upregulated processes and confirms the importance of this upregulation found in the previous, small-scale analysis. Equally, several pathways regulating cell adhesion were represented in the top 30 list of upregulated genes in TOSE1 cells and were, in fact, the second largest group among over-active processes.

In the initial cluster analysis of TOSE cells, many genes implicated in differentiation and development were deregulated. On the list of upregulated pathways of the meta-analysis, they appear four times, whilst among the 30 most downregulated pathways, none regulates differentiation and development. This result apparently stands in contrast to the previous analysis. The list of the 30 most upregulated processes in TOSE1 cells contained none labelled “development”. However, in this list, two of the processes assigned to “signal transduction” are known to be involved in differentiation and development: WNT signalling and Notch signalling (Hayward *et al.*, 2008; Sekiya *et al.*, 2007). This is an example of the disparities between the initial small-scale and the meta-analyses. Clearly, differences in clustering parameters can produce highly varied results. This also emphasises the value in performing different types of analyses on the same microarray data set.

Surprisingly, according to enrichment analysis, no pathway was significantly downregulated in TOSE1 cells, and only two processes. Both of them have very broad functions: translation initiation and elongation termination. In view of the number of down-modulated genes expressed in TOSE1 cells, the lack of downregulated pathways and processes was unexpected. It is possible that downregulated genes are so varied in function, that enrichment does not meet the cut-off levels of statistical significance.

The number of significantly downregulated pathways was also very low in TOSE4 cells: oxidative phosphorylation and, interestingly, development. The latter was specifically regulating degradation of beta-catenin in the absence of Wnt signalling. This finding implies that, in TOSE4 cells, beta-catenin is stabilised. The resulting prolonged activity is a well-described cancer-promoting feature (Chang *et al.*, 2009; Morin *et al.*, 1997; Taipale *et al.*, 2001).

In this cell line, more than 30 processes were significantly downregulated. Among the top 30, the most frequent processes are implicated in translation and transcription. These results are too general to predict mechanisms of enhancement of *dl*922-947 function. Furthermore, inflammation and immune response were also large groups of downregulated processes. This stands in disagreement with the previous, small-scale analysis, where anti-viral, pro-inflammatory and stress-induced mechanisms were frequently over-expressed. Three processes involved in development are downregulated in TOSE4 cells. But in addition, another process, under the term signal transduction, may be involved in development and differentiation, further specified at “WNT signalling”. However, its effects may be neutralised by the abovementioned downregulation of beta-catenin degradation. Despite the loss of Wnt signalling, beta-catenin would not be degraded. Western blot analysis of WNT and beta-catenin in TOSE4 and IOSE25 cells could elucidate, whether this is the case.

Meta-analysis in TOSE4 cells confirmed upregulation of cell adhesion, as seen in the initial clustering. Similarly, deregulation of cytoskeleton remodelling, again, appeared in form of several pathways and processes. Several upregulated pathways involved in CFTR signalling. This is a transmembrane ion channel (Mansoura *et al.*, 1998). How it may be correlated to tumourigenesis or efficacy of *dl*922-947 is unclear. Surprisingly, no pathway or process involved in cell cycle regulation appeared on the lists for upregulated expression.

It is important to consider the limitations of gene expression arrays: probe sets on the array are known to provide a certain number of false-negative and false-positive results (Pounds *et al.*, 2003; Slonim, 2002). Several transcripts can bind to the same probe (Harbig *et al.*, 2005). High background noise can obscure detection of genuine signals (Robinson *et al.*, 2007). Validation of gene array results by other methods such as qRT-PCR or Northern blotting commonly shows that it is difficult to reproduce findings (Maxwell *et al.*, 2003; Mitchell *et al.*, 2009; Vawter *et al.*, 2006). Using published microarray data can lead to misleading results. Datasets published by different labs are based on a broad range of procedures and varying quality of starting material (Huttenhower *et al.*, 2006). Addressing this problem, the Minimum Information About a Microarray Experiment (MIAME) standard was established to standardise published gene array data (Brazma *et al.*, 2001). Reporting microarray data according to the MIAME standard is expected to increase their reproducibility and their usefulness beyond the studies they originate from (Quackenbush, 2009). As discussed above, in my experiments heterogeneity of samples was kept at a minimum by using isogenic cell lines, identical storage conditions and processing of samples. Therefore the absence of cell cycle regulators in lists of differentially expressed genes may be due to altered protein translation, stability or post-translational modification (Cham *et al.*, 2003; Garavelli *et al.*, 2001; Jensen *et al.*, 2002).

Nevertheless, data from these gene expression arrays are far from meaningless. The deregulated genes, pathways and processes provide a starting point to identify further determinants of *dl*922-947 efficacy, beyond the Rb pathway. The first step would be validating differential expression of potential candidates by qRT-PCR. Then, knock-down of upregulated and over-expression of down-regulated candidates could further validate their role in *dl*922-947 efficacy. In the case of MRC5-VA cells, meta-analysis, as performed on TOSE data, will provide a broader view on deregulated pathways and processes.

Among the 20 most upregulated genes in TOSE cells, a family of antigens was represented by several members: five genes encoding different melanoma antigen (MAGE) proteins were highly over-expressed in TOSE cells. In fact, albeit not strongly enough to rank within the top 20 list, another 8-10 MAGE genes were detected to be overexpressed among the top 100 upregulated genes (see Appendix Tables 8 and 9). Interestingly, MRC5-VA cells also over-express at least three different MAGE genes, compared to parental MRC5 cells. Again, the difference in expression is not large enough for the top 20 list (see Appendix Table 10). Although the family of MAGE

proteins has received increasing attention over the past years, knowledge about its functions remains limited. They appear to play a role in proliferation by inhibiting p53 and apoptosis inhibitory proteins XIAP and ITA (Jordan *et al.*, 2001; Jungbluth *et al.*, 2005). MAGE genes were first isolated from melanomas (van der Bruggen *et al.*, 1991). In the meantime, they have been described in many different cancers. Apart from cancer cells, MAGE genes have only been found expressed in the germ line. In all other normal cells, expression of MAGE genes is completely switched off. They are thus considered tumour-specific and are very promising targets in cancer treatment. In humans, the MAGE family comprises several classes of antigens. Of those, members of the MAGE-A class have been found most highly represented among upregulated genes in TOSE and MRC5-VA cells. Although some MAGE genes are expressed in the female germ line, after birth MAGE-A members are limited to male germ cells (Barker *et al.*, 2002; Gjerstorff *et al.*, 2007). This class is also implicated in regulation of development and differentiation in germ and cancer cells (Ohman Forslund *et al.*, 2001). MAGE-A1 mRNA was detected in low to moderately differentiated, but downregulated in moderately to highly differentiated cell lines of hepatocarcinoma (Xiao *et al.*, 2005). Similarly, in normal germ cells, MAGE-A1 was detected immediately after induction of sexual differentiation. From then on, expression levels decreased continuously and terminated at the time of birth (Gjerstorff *et al.*, 2007). However, none of the microarray analyses detected upregulation of MAGE-A1, the most extensively characterised gene of the MAGE-A class. However, it is possible that members of the same gene family or even class may have similar functions.

This pattern of high expression during early differentiation stages followed by downregulation as cells proceed to later stages, has been reported for other genes strongly up-regulated in my small scale array analysis. “ISL1 transcription factor” is involved in regulation of early differentiation of neuronal, pancreatic and cardiac cell lineages. The marker of pluripotency is downregulated in later developmental stages. “Leucine rich repeat neuronal 1 (LERN1)”, over-expressed in MRC5-VA cells, is a transmembrane protein. Its expression is restricted to early development of the central nervous system (Carim-Todd *et al.*, 2003). Intriguingly, both LERN1 and two members of the MAGE family, neurotrophin-receptor-interacting MAGE homologue (NRAGE) and necdin, interact with p75, a receptor for neurotrophins. p75 is a promoter of differentiation, cell cycle arrest and mediates apoptosis. A third gene highly over-expressed in MRC5-VA cells and interacting with p75 is “brain expressed, X-linked 1”, an inhibitor of differentiation and promoter of proliferation.

The only gene present in the top 20 most differentially expressed genes in both TOSE and MRC5-VA cells is overexpressed “placenta-specific 8 (PLAC8)”. The marker of plasmacytoid dendritic cells, also called onzin, is highly expressed in various organs involved in immune responses (Colonna *et al.*, 2004; de Heer *et al.*, 2004; Reeves *et al.*, 2008; Rogulski *et al.*, 2005). Increased expression has been detected after infection with HBV in primates, as an adaptive immune response for viral clearance (Lanford *et al.*, 2007). Roles for PLAC8 have been reported in apoptotic resistance and proliferation (Li *et al.*, 2006; Rogulski *et al.*, 2005). Also, it appears to act as an inhibitor of differentiation (Huang *et al.*, 2006). Moreover, overexpression of PLAC8 has been implicated in overriding G2/M checkpoint control and in tumourigenic transformation (Rogulski *et al.*, 2005).

Several other differentially expressed genes can be functionally linked to results of previous chapters and represent promising biomarker candidates. Protein phosphatase 2 (PP2), overexpressed in MRC5-VA cells, is a promoter of Raf and MEK, in turn, promoters of cell cycle progression. Acute activation of Raf/MEK/Erk increases p21 expression. Furthermore, the Ras pathway has been shown to promote p21/Cyclin D complex formation (Coleman *et al.*, 2003). Whether this is relevant to *dl*922-947 function is doubtful, as SV40 TAg mediates p21 degradation. In fact, upregulation of PP2 may be an attempt of MRC5-VA cells to counteract this SV40 TAg effect.

A very direct enhancing effect on *dl*922-947 oncolytic function could be attributed to NFI/B. In MRC5-VA cells, overexpressing this transcription factor of cellular but also viral genes, could increase expression and activity of important *dl*922-947 genes. F11 receptor, also referred to as JAM-1, is structurally similar to CAR and is the attachment protein for reovirus (Goosney *et al.*, 2003; Stehle *et al.*, 2004). It may also allow binding of adenovirus, thus its upregulation in MRC5-VA cells might enhance adenoviral infection and possibly spread to neighbouring cells (Walters *et al.*, 2002).

SV40 TAg transformation is frequently linked to microsatellite instability, as DNA damage-induced checkpoints are inactivated (Barbanti-Brodano *et al.*, 2004). More importantly, although microsatellites are generally found within non-coding sequences, their amplification could be indicative of gene amplification in MRC5-VA cells. Also, chromosome aberrations could disrupt genes. The resulting upregulation or loss of wild type transcripts from affected genes could contribute to sensitisation to *dl*922-947. Identification of amplified regions and genes, for instance with whole genome SNP arrays, could provide further biomarker candidates.

Although genomic instability might contribute to *dl*922-947 efficacy, it makes detection of genes relevant for its oncolytic function more difficult. Disappointingly, attempts to generate another model system for gene expression arrays, based on IOSE20 and IOSE21 cells, were not successful. Cells transfected with pCMV-SV40 did not express any SV40 TAg. Most likely, the exogenous protein was toxic to the cells and either was immediately degraded, or cells were not viable. Equally, transfection of IOSE20 and IOSE21 cells with pSV3neo did not increase sensitivity to *dl*922-947 either, as once again, none of the cell pools expressed SV40 TAg. Transformation of cells by exogenous SV40 TAg expression is commonly difficult to achieve. Various publications state low transfection efficiencies (Haas *et al.*, 1997; Novak *et al.*, 1992; Risser *et al.*, 1974). In view of the model systems described above, I did not further pursue the attempt to generate such a model.

In summary, experiments described in this chapter provide a valuable starting point to identify further biomarkers of *dl*922-947 efficacy. Some of the top 20 most significantly up- or downregulated genes appear to be very promising candidates. Trends that emerged from pathway and process enrichment were changes in gene expression affecting cell cycle control and proliferation, cytoskeleton remodelling, cell surface, immunomodulation and cell death and several pathways involved in differentiation. Small-scale clustering of most differentially expressed genes in TOSE and MRC5-VA cells recaptured most of these trends. A microarray-based study of wild-type adenovirus-induced changes of host gene expression in human foreskin fibroblasts revealed that more than 2000 genes were deregulated. The authors described the transcriptional alterations as “unexpectedly complex”. Most prominent changes were linked to cell proliferation and reversal of quiescence (Miller *et al.*, 2007). The results in this chapter confirm that deregulation of these pathways in transformed cells leads to an environment conducive to the activity of *dl*922-947.

6 Final Discussion

The present standard treatment of ovarian cancer, surgical de-bulking followed by platinum-based chemotherapy, is of limited success, at best (Morrison *et al.*, 2007). There remains an chronic need for novel treatment strategies (Matthews *et al.*, 2009). The oncolytic virus *dl*922-947 has high therapeutic potential for the treatment of ovarian cancer (Lockley *et al.*, 2006). However, variability exists between its oncolytic efficacy across different ovarian cancer cell lines. This phenomenon is likely to translate into the clinic. The main objective of my work was to identify host cell factors which influence the oncolytic efficacy of *dl*922-947 in ovarian cancer. Such factors could serve as biomarkers in clinical trials of *dl*922-947, and potentially other oncolytic adenoviruses, in patients with relapsed ovarian cancer.

6.1 Infectivity and *dl*922-947 activity

Various steps of the viral life cycle may limit its success. Clearly, initial infection of any cell with *dl*922-947 is necessary for all subsequent steps. Cells that are non-infectable even at high MOIs, such as CAOV3 cells, are resistant to the virus. Low infectivity owing to downregulation of CAR expression is a major obstacle, for instance in hypoxic tumours (Kuster *et al.*; Legendre *et al.*, 2009; Rein *et al.*, 2006). Lack of this important cellular receptor of adenovirus has been reported in many human cancers and considerably hampers effective infection with oncolytic adenoviral vectors (Douglas *et al.*, 2001; Li *et al.*, 1999; Miller *et al.*, 1998; Okegawa *et al.*, 2000; Rauen *et al.*, 2002; Rein *et al.*, 2006). Similarly, loss of $a_v\beta_{3/5}$ integrins would hinder virus uptake into cancer cells (Bruning *et al.*, 2001; Turturro *et al.*, 2000; Wickham *et al.*, 1993). A growing body of evidence suggests that adenoviruses can use alternative cell surface proteins for infection (Davison *et al.*, 1997; Huang *et al.*, 1996; Li *et al.*, 2001; Nicklin *et al.*, 2005; Salone *et al.*, 2003; Waddington *et al.*, 2007). Recent work in our lab on primary ascitic cells demonstrated that ovarian cancer cells from 4 out of 6 patients were readily infectable with *dl*922-947 at MOI 50pfu/cell (data not shown). This is an encouraging finding, suggesting good infection potential of the oncolytic virus in patients with advanced ovarian cancer. Yet my results indicate that infectivity alone cannot account for the full extent of variation in cytotoxicity. For instance, TOSE4 cells were more infectable, yet less susceptible to *dl*922-947 than TOSE1 cells. This stands in line with Wang *et al*. recently reporting that binding and endocytosis of adenoviruses is required but not sufficient for delivery of viral genes (Wang *et al.*, 2009).

6.2 E1A expression and S phase fraction are markers of sensitivity

Comparison of sensitive and non-sensitive cell lines established that a large S phase fraction and early onset of E1A expression were conducive to *dl*922-947 activity. E1A is the only adenoviral gene entirely dependent on host cell factors for its expression (Bruder *et al.*, 1991; Bruder *et al.*, 1989), containing EF-1A and E2F binding sites in its promoter region. Transcription of all other viral genes requires viral factors (Bruder *et al.*, 1991; Bruder *et al.*, 1989; Nevins, 1990). Therefore, it is the host cell environment at the time of infection that will dictate initial levels of E1A expression. The size of the S phase fraction may reflect the degree of G1/S checkpoint deregulation and could be a marker of a cellular environment enhancing E1A expression and possibly subsequent viral replication.

6.3 Viral replication and cell death

My experiments indicated that rates of viral replication, both at the level of DNA as well as functional virion particles, cannot fully predict for cytotoxic efficacy, when comparing different cell lines. It would be wrong to completely separate the processes of replication and death. As seen in ACP-WAF1 cells, within a given cell line, any increase in virion production is accompanied by increased cytotoxicity. But comparing replication across different cell lines points to a partial separation of the two. Virion production in SKOV3ip1 is nearly as high as in TOV21G cells, even surpassing the latter at 72 hours post infection. Yet, SKOV3ip1 cells are resistant to *dl*922-947, indicating that a larger viral load does not necessarily increase lysis. Interestingly, TOV21G cells support early virus production at a high level, but then production plateaus. This could be a result of onset of cell death. Alternatively, in TOV21G cells viral replication may rapidly reach the maximum capacity of the host cell. At this high level, all available host cell machinery may occupied and production cannot be further increased. That the mode of cell death following infection by *dl*922-947 is actively regulated by the virus has already been described by our group. We reported that viruses not only activate multiple survival mechanisms, the actual cause of death is based on a novel programmed cell death mode that is also significantly controlled by the virus itself (Baird *et al.*, 2008). Therefore, *dl*922-947 oncolytic function must depend on more than merely high degree of replication. Cell cycle analyses show that *dl*922-947-infected cells are driven to proceed beyond G1 phase. Our group also reported that cells that proceed into irregular mitoses, reconstitute an interphase nucleus and re-initiate replication. This results in increased viral replication, production

of functional viral particles and oncolytic effects (Connell *et al.*, 2008). Cell lines in which *dl*922-947 is particularly efficient at doing so would therefore be more sensitive, such as is seen in TOV21G and IGROV-1 cells.

According to another publication, by Varmark *et al.*, Hct116 cells continuing progression through G2/M after DNA damage, succumbed to cell death three days after exiting mitosis. In fact, proceeding through mitosis enhanced cell death after DNA damage, which was independent from caspase and p53 activity. It was, however, accompanied by chromatin de-condensation and phosphorylation of H2AX (Varmark *et al.*, 2009). Could *dl*922-947 kill its host cells by a similar mechanism? Wild type adenovirus is known to elicit a DNA damage response (Hart *et al.*, 2007). Expression of E1A induces H2AX phosphorylation even when apoptosis is inhibited (Cuconati *et al.*, 2003). Therefore, *dl*922-947 could induce DNA damage response, then drive the cell through the cell cycle, including G2/M. Based on the Varmark paper, this should lead to enhanced cell death.

6.4 p21 enhances *dl*922-947 efficacy

The work presented here established that p21 is at least a marker of an environment favourable for the activity of *dl*922-947, as well as wild type adenovirus and, potentially, other adenoviral vectors. However, my results suggest that p21 is more than a simple marker of deranged cell cycle control – rather, p21 itself appears capable of promoting virus activity. The multiple functions of p21 could promote oncolytic virus effects by different pathways. It possesses cell cycle promoting activity at basal levels via stabilisation of Cyclin/cdk complexes (LaBaer *et al.*, 1997). Underpinning this, Cyclin D emerged as another potential biomarker for *dl*922-947 activity. Taken together, high basal levels of p21 and stabilised Cyclin D/cdk4 complexes should result in increased pRb phosphorylation on S780 (LaBaer *et al.*, 1997; Mohamedali *et al.*, 2003). Surprisingly, I did not find this to be the case, suggesting that in the ovarian cancer cells investigated, free E2F levels are independent from the phosphorylation status of pRb. One explanation may be that several other regulatory proteins are capable of binding pRb (Ewen *et al.*, 1993; Nakanishi *et al.*, 1995). p21 itself, for example, associates with pRb in the same A/B pocket region as E2F, and with a higher affinity. Co-expression of p21 in cells containing E2F, pRb and Cyclin D1 has been found to displace and free E2F from pRb (Nakanishi *et al.*, 1999). In this case hyper-phosphorylation of the latter would become unnecessary. SV40 TAg associates with pRb in the same region as E2F and,

consequently, as p21 (Hu *et al.*, 1990; Kaelin *et al.*, 1990). The function of p21 and SV40 TAg in freeing E2F is therefore potentially interchangeable. Hence, MRC5-VA cells do not require high levels of p21 to increase *dl*922-947 efficacy, as indeed seen in Western blot analysis. Fig. 6.1 illustrates effects of pRb association with p21 or SV40 TAg.

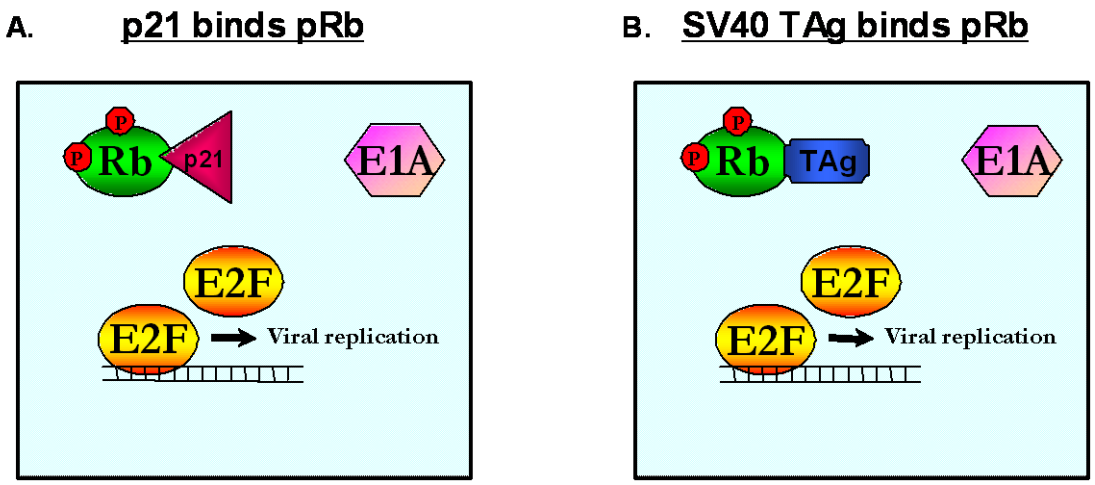


Figure 6.1 De-repression of E2F by p21 or SV40 TAg binding. *When* **A.** *p21 or* **B.** *SV40 TAg (TAg) associate with pRb via its A/B pocket, E2F is released. Inability of CR2-deletion-bearing E1A is overcome.*

Furthermore, Nakanishi *et al.* observed that co-expressing p21 with pRb and Cyclin D/cdk4 complexes prevented hyper-phosphorylation of pRb by Cyclin D/cdk4 (Nakanishi *et al.*, 1999). This may explain the pRb phosphorylation profile found in the four representative ovarian cancer cell lines. In SKOV3ip1 cells, which express barely detectable levels of p21, pRb was phosphorylated on S780 (Knudsen *et al.*, 1997). This was not the case in TOV21G, IGROV-1 or A2780CP cells, suggesting that even the low levels of p21 generated in A2780CP cells were enough to prevent hyper-phosphorylation. Nakanishi *et al.* also found that excess levels of Cyclin D/cdk4 prevented p21 binding to pRb and resulted in hyperphosphorylation of the latter (Nakanishi *et al.*, 1999). Thus, a fine balance must be maintained between levels of p21 and Cyclin D/cdk4 to prevent pRb hyperphosphorylation and increase free E2F levels.

6.5 Relationship between p21 and E1A

A correlation between p21 and E1A became evident as knock-down of p21 in infected TOV21G cells led to a decrease in E1A. In ACP-WAF1, higher levels of p21 were accompanied by more E1A expression post infection than in ACP-GFP cells. These data are consistent with the observed differences in virion production and cytotoxic efficacy. As discussed above, p21 may contribute to E1A expression by increasing levels of free E2F, which, in turn acts as a transcription factor for E1A expression (Bruder *et al.*, 1991; Bruder *et al.*, 1989).

In a recent publication (Shiina *et al.*, 2009), authors argue that expression of p21 has negative effects on Delta-24, a virus carrying the same deletion in the E1A CR2 domain as *dl*922-947 (Fueyo *et al.*, 2000). However, there seem to be several caveats in these data: dose response experiments to measure cytotoxicity were performed at 72 hours post infection, only. In contrast, in our hands, meaningful differences in cell survival do not emerge until 120 to 144 hours post infection. Furthermore, Shiina et *al.*, used MOIs of 1.0pfu/cell or more to infect cells. TOV21G and Hct116 p21+/+ cells have IC_{50} values lower than 0.1pfu/cell and that of IGROV-1 is lower than 0.5pfu/cell. To obtain a meaningful dose response curve, cell have to be infected with lower viral doses. During RNAi experiments, cells were replated after transfection with siRNA targeting p21. This is likely to alter cell cycle-related gene expression, irrespective of siRNA-mediated knock-down. Also, sensitivity of cells to the virus after siRNA-transfection was assessed using a single dose of virus, rather than a range. This is not the method generally accepted in the field. Finally, results were not followed up *in vivo*.

6.6 p21 regulates differentiation

A growing body of evidence indicates an additional role for p21 in the regulation of differentiation (Devgan *et al.*, 2006). The notion that a progenitor-like state may constitute an optimal environment for *dl*922-947 cytotoxic function emerged from microarray analyses in TOSE and MRC5-VA cells. In addition to the roles already described above, it has been found that in keratinocytes, p21 expression promotes initial commitment of stem cells to differentiate (Topley *et al.*, 1999). It has also been observed to contribute to differentiation-associated growth arrest (Missero *et al.*, 1996). However, in normal developing keratinocytes, following an incipient increase in p21, protein levels are down-regulated. Devgan *et al.* showed that if p21 levels are sustained in keratinocyte progenitors, cells fail to proceed through terminal

differentiation (Devgan *et al.*, 2006). The same downregulation of p21 during later differentiation was noted in other tissues. Equally, over-expression of p21 repressed terminal differentiation in them (Bellosta *et al.*, 2003). These findings suggest that p21 must be switched off in order for differentiation to proceed (Di Cunto *et al.*, 1998). It may also explain the aggressive nature of clear cell carcinoma of the ovaries (Shimizu *et al.*, 1999). These tumours produce large amounts of the cell cycle inhibitor. However, rather than inducing growth arrest, p21 perpetuates a semi-differentiated, highly proliferative state (Devgan *et al.*, 2006).

6.7 Virus-mediated downregulation of p21 enhances cell death

After infection of sensitive cell lines TOV21G and IGROV-1, downregulation of p21 occurred. In fact, this may be an important step in augmenting cell death, as described in publications on increased drug- and irradiation-induced apoptosis after p21 down-modulation (Detjen *et al.*, 2003; Han *et al.*, 2002; Mahyar-Roemer *et al.*, 2001; Tian *et al.*, 2000). In ACP-WAF1 cells, p21 is also downregulated. In fact, as seen in proteasome inhibitor-treated Hct116 cells, infection can induce further p21 expression, but the p21 is targeted for destruction. Adenovirus infection can induce host cell DNA damage response via phosphorylation of histone H2AX (Nichols *et al.*, 2009). Upregulation of p21 may be part of this response, normally leading to cell cycle arrest. To prevent arrest and for cell cycle progression to continue, the virus must downregulate p21 levels.

In addition, *dl*922-947-induced down-modulation of p21 protein levels, may drive cells into terminal differentiation and maximise death. Whether and how driving a cell into differentiation increases cytotoxicity of *dl*922-947 remains unclear. There is indication that progenitor cells are less susceptible to induced cell death, thus induction of differentiation may sensitise them (Bhatt *et al.*, 2003; Fan *et al.*, 2006; Haraguchi *et al.*, 2006; Szotek *et al.*, 2006; Woodward *et al.*, 2007). According to Devgan et *al.*, the mechanism by which p21 regulates differentiation in keratinocytes is cell cycle-independent. Instead, it involves activation of insulin-like growth factor-1 (IGF-1) (Devgan *et al.*, 2006). Interestingly, one of the genes found highly upregulated by microarray analysis was chitinase 3-like 1 (Chi3L1). In lung fibroblasts and skin cells, Chi3L1 acts in synergy with IGF-1 (Kawada *et al.*, 2007). The latter is a potent inhibitor of programmed cell death (Geier *et al.*, 1995; Geier *et al.*, 1992). Therefore, downregulation of p21 and, consequently, IGF-1 activity would render a cell more susceptible to death.

6.8 Beyond the Rb pathway

Microarray results point at pathways and processes affecting *dl*922-947 efficacy other than the Rb pathway. Many differentially expressed genes in sensitive cells are part of other cell cycle regulating pathways. Other aspects that are worthy of further investigation are cell death and anti-viral mechanisms, changes in the cytoskeleton and cell surface and in collagen synthesis. These alterations could affect *dl*922-947 activity by enhancing infectivity, intra-cellular trafficking, entry into and possibly exit from the cell or virus-induced cell death (Li *et al.*, 1998; Smith *et al.*, 2002). As mentioned above, there is some indication that a semi-differentiated, progenitor-like state may constitute an environment conducive for *dl*922-947 activity. In most cases, it will be necessary to untangle these pathways down to single genes, in order to identify potential biomarkers. Some interesting candidates were among the most deregulated genes, such as PLAC8, MAGE, F11 receptor or NFI/B.

The emergence of PLAC8 and MAGE genes underscores the notion of a progenitor-like state representing an optimal environment for *dl*922-947 cytotoxic function. PLAC8, the only gene present in the top 20 list of over-expressed genes in both MRC5-VA and TOSE cells, is a facilitator of tumourigenic transformation and a known inhibitor of differentiation (Rogulski *et al.*, 2005, Huang, 2006 #345).

Expression of a number of genes from the MAGE family is required during early stages of differentiation. However, for terminal differentiation to proceed, their expression must be down-modulated (Xiao *et al.*, 2005). Over-expression of MAGE genes may therefore keep cells in a semi-differentiated state. Remodelling of the cell surface frequently accompanies transformation and can be used to define specific grades of tumour development (Harsha *et al.*, 2009; Houghton *et al.*, 1988; Kaul-Ghanekar *et al.*, 2009; Morrison *et al.*, 1993; Nelson *et al.*, 2006). However, it has also been commonly described in the context of de-differentiation of tumours (Aust *et al.*, 1997; Zalik *et al.*, 1972). Equally, pronounced changes in cytoskeletal architecture have been observed in connection with de-differentiation (Kim *et al.*, 2003b; Timmers *et al.*, 1998). How would *dl*922-947 benefit from such a poorly differentiated cellular environment? Unlike stem cells and differentiated cells, which are generally slowly cycling or quiescent, progenitor cells are highly proliferative (Hoffmann *et al.*, 2008; Radtke *et al.*, 2005). De-differentiation “rewinds” differentiated cells to a progenitor-like status, with rapid cell cycling (Grafi *et al.*, 2004). Such reversed differentiation has been observed in various cancers as they progress and, in fact poorly differentiated tumours are

generally more aggressive than the well-differentiated (Draisma *et al.*, 2006; Gabbert, 1985; Oku *et al.*, 2008; Rufini *et al.*, 2006; Sell *et al.*, 1994; Soper *et al.*, 1984; Sutton *et al.*, 1986). Rapid cell cycling is accompanied by high rates of replication, both of cellular and viral genes and may increase *dl*922-947 virion production.

6.9 Conclusion

The quest for host cell factors influencing *dl*922-947 efficacy in ovarian cancer has brought some interesting insight into the mechanisms underlying *dl*922-947 function. I have been able to establish p21 and Cyclin D as potential biomarkers for efficient oncolytic activity. My work suggests that the virus is at least partially dependent on high basal levels of p21, resulting in large S phase populations, early onset of E1A expression and, to some extent virion production. These levels of p21 may be indicative of a progenitor-like state of sensitive cells. I have shown that *dl*922-947 down-regulates p21. This may prevent cell cycle arrest in response to the presence of virus and host cell DNA damage.

Microarray analysis has produced a large amount of data which will need to be validated. The single genes and pathways that emerged from these experiments represent a valuable starting-point for the identification of further biomarkers for *dl*922-947 efficacy in treating ovarian cancer.

6.10 Immediate future experiments

Among the most immediate steps to be taken is the optimisation of immunohistochemical p21 staining of tumours. Generation of a Tet-on or Tet-off system for conditional p21 expression may prove useful in the future, to study in greater detail implications of p21 presence or absence at specific points before and after the time of infection. Efficient knock-down of p27, cdk4 and Cyclin E by RNAi could confirm or disprove their role as enhancers of *dl*922-947 activity. Validation of biomarker candidates found by microarray analysis is a necessary step before investigating their role more closely. It would be interesting to see whether sensitive ovarian cancer cells possess progenitor-like status and whether *dl*922-947 can induce their differentiation. The ability to test *dl*922-947 behaviour in an immunocompetent animal model would be invaluable for predicting its effects in humans. Finally, verification of p21 as a predictor for response to *dl*922-947 in clinical trials would be an exciting step for its establishment as a genuine biomarker in the future.

6.11 Future of adenoviral gene therapy

Adenovirus-based vectors in gene therapy are particularly useful a tool when the ultimate aim is cell death. Whilst normal infection is usually mild, they are able to infect dividing and non-dividing cells very efficiently (Tsutsumi *et al.*, 1999). Their efficient replication in *in vitro* systems facilitates the large-scale production required for pharmaceutical purposes, and their exceptional cloning capacity allows insertion of large sequences of interest (Rein *et al.*, 2006). Encouraging results of pre-clinical studies suggest high levels of safety and therapeutic potential of most current generation oncolytic adenoviruses (Matthews *et al.*, 2009). Yet, two major obstacles remain for application in humans: poor efficacy and potential toxicity. Many of the promising oncolytic adenoviruses show only limited success in human trials (Matthews *et al.*, 2009). H101 is very potent in combination with cisplatin, but is not efficient as a monotherapy (Garber, 2006; Yu *et al.*, 2007). A similar pattern was seen with prostate cancer-specific adenovirus CV787, which, on its own, showed limited oncolytic activity. Combination with docetaxel or paclitaxel resulted in synergistic effects (Yu *et al.*, 2001). Many trials report poor vertical spread of the virus and its progeny throughout solid tumours (Sauthoff *et al.*, 2003). Yet, there is hope in clinical trials with novel adenoviral vectors soon to begin or already underway. *In vitro*, *dl*922-947 has been shown to be more efficient in killing cancer cells than *dl*1520, the original version of H101 (Heise *et al.*, 2000; Lockley *et al.*, 2006). Oncolytic effects may be augmented further by arming the virus. One example is AdsiSurvivin. In addition to its intrinsic cytotoxic nature, it has been converted to deliver siRNA targeting anti-apoptotic survivin (Yang *et al.*). Oncolytic viruses in combination therapy have frequently been proven highly potent, synergising with the chemotherapeutic agent by sensitising even resistant cells (Takakura *et al.*). However, monotherapy would be ideal, reducing unnecessary exposure to chemotherapy and the burden of side effects for the patient. The ability to stratify patients based on biomarkers, prior to onset of therapy may limit superfluous treatment of resistant patients. The growing understanding of cancer development and intra-tumoural processes provides novel characteristics to be considered, and creates new targets for intervention. In addition to the genomic heterogeneity between patients, it may be necessary to distinguish between different fractions of tumours within one patient. The importance of killing tumour-associated fibroblasts has been discussed for many years (Francia *et al.*, 2009; Schuler *et al.*, 2003). It is well-established that tumours can feature complex architecture: a hypoxic core, accompanied by low CAR expression, surrounded by outer layers expressing different sets of markers (Gabril *et al.*, 2005; Kocher *et al.*, 1995; Takahashi *et al.*,

2007; Williamson *et al.*, 2009). Metastases can exhibit other characteristics than the primary tumour (Gomez-Roca *et al.*, 2009). Different grades of differentiation within one patient can complicate treatment (Elsasser *et al.*, 1992). Taken together, this implies that, stratification may be called for within the patient, to devise a therapy of a combination of highly specific drugs. This emphasises the need for complex biomarker identification.

Cancer stem cells are an obstacle recognised only relatively recently. As they are slow cycling and resistant to many common anti-cancer treatments, their eradication, though crucial, remains difficult (O'Brien *et al.*, 2009). Excitingly, adenoviral deletion vectors have been shown to be effectively infecting cancer stem cells, replicating within them and inducing their death (Short *et al.*, 2009). Clearly, these considerations are secondary to the most urgent need: Any highly efficient oncolytic virus that can be used for cancer therapy without causing profound side effects. The latter refers to toxicity expected from administration of oncolytic adenovirus. In mice, intravenous injection of adenoviral vectors is followed by rapid uptake into spleen and liver, causing severe liver toxicity (Lieber *et al.*, 1997). Intensive efforts have revealed that adenovirus is taken up into Kupffer cells, with profound contribution from coagulation factor X (Parker *et al.*, 2006; Wolff *et al.*, 1997). Apart from the detrimental renal complications, this process also sequesters oncolytic adenoviruses away from their tumour target (Shashkova *et al.*, 2009). In recent years, major advances have been made, not only in understanding the underlying mechanisms, but also in devising strategies to re-target oncolytic adenoviruses away from the liver and towards the tumour (Di Paolo *et al.*, 2009b). A further potential obstacle of adenoviral gene therapy is the host immune response, particularly in immuno-competent patients. Apart from the risk of sepsis and complications induced by exposure to high doses of virus, neutralising antibodies may restrict repetitive administration of adenovirus-based vectors (Parker *et al.*, 2009; Zaiss *et al.*, 2009). The vast majority of the population have been exposed to and have developed antibodies against adenoviruses, at some point in their life (Chirmule *et al.*, 1999; Harvey *et al.*, 1999; Tsai *et al.*, 2004). Predictions, how the host immune system will affect oncolytic activity of adenoviruses are largely speculative (Chen *et al.*, 2000; Tsai *et al.*, 2004). To date, there are few good immuno-competent mouse models for oncolytic human adenoviral vectors (Bischoff *et al.*, 1996; Dhar *et al.*, 2009; Heise *et al.*, 1997; Robinson *et al.*, 2009). Human adenovirus is unable to replicate efficiently in murine cells (Eggerding *et al.*, 1986; Ginsberg *et al.*, 1991; Silverstein *et al.*, 1986). Xenograft models with human tumours are based on immuno-compromised mice (Heise *et al.*, 2000; Johnson *et al.*,

2002; Ramesh *et al.*, 2006). Alternative, immuno-competent animal models include the Syrian hamster and the cotton rat (Thomas *et al.*, 2008; Toth *et al.*, 2005). However, being less well established model systems, they lack most of the molecular tools available for mouse models (Robinson *et al.*, 2009). This is a clear deficiency in our ability to evaluate pre-clinically evaluate novel adenoviruses for gene therapy, and will hopefully be eradicated, soon. Even then, murine models cannot fully predict effects of a therapeutic agent in humans (Bolton, 2007), and work is ongoing to generate better models, such as tissue and organ cultures, computer simulation (Geschwind *et al.*, 2009; Lutolf *et al.*, 2009; Varani *et al.*, 1998).

Overall, after severe set-backs in the development of adenoviral vectors for cancer gene therapy, recent years have brought about some long-sought achievements and a plethora of promising novel strategies for the future.

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8 APPENDIX