Evaluation of focal adhesion kinase as a novel radiosensitising target

Kathryn Graham
BSc, MB ChB, MRCP

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University of Glasgow
Faculty of Medicine

and

Beatson Institute for Cancer Research
Garscube Estate
Switchback Road
Bearsden
Glasgow, G61 1BD

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Abstract

Focal adhesion kinase (FAK) is a cytoplasmic tyrosine kinase that is upregulated in a variety of human cancers. While there is evidence that FAK is implicated in a wide range of crucial cellular processes that are perturbed in malignancy including proliferation, cell cycle, adhesion and invasion, there is limited information regarding the role of FAK in radiation survival. We aimed to evaluate whether FAK is a novel radiosensitising target by studying clonogenicity in wt p53 FAK +/+ versus FAK -/- squamous cell carcinoma (SCC) cell lines generated in this laboratory. Surprisingly, the absence of FAK was associated with increased radioresistance. In this particular context, FAK indirectly inhibits p53 mediated transcriptional regulation of p21 in response to ionising radiation. Why FAK should repress the pro-survival function of p53 is unclear, but this data indicates that inhibition of FAK in combination with radiation may not always be advantageous in the clinical setting and contributes to an increasing body of literature highlighting a close interaction between FAK and p53.

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Author's declaration

I declare that the work in this thesis was performed personally unless stated otherwise. No part of this work has been submitted for consideration as part of any other degree or award.

Abbreviations

α	alpha
ATM	ataxia telangectasia mutated
ATP	adenosine 5'triphosphate
ATR	ataxia telangectasia and Rad3 related
β	beta
BSA	bovine serum albumin
CAKβ	cell adhesion kinase beta
CDK	cyclin dependent kinase
cDNA	complementary DNA
Chk1	checkpoint kinase 1
Chk2	checkpoint kinase 2
CT	computed tomography
CTV	clinical target volume
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco's Modified Eagle's Medium
DMBA	dimethylbenzanthracene
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
DNA-PK	DNA-dependent protein kinase
DSB	double strand break
E	glutamic acid
ECL	enhanced chemiluminescence
ECM	extracellular matrix
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ERK	extracellular regulated kinase
EMT	epithelial to mesenchymal transition
F	phenylalanine
FACS	fluorescence activated cell sorting
FAK	focal adhesion kinase
FAT	focal adhesion targeting
FBS	foetal bovine serum
FERM	functional ezrin radixin myosin
FIP200	FAK interacting protein of 200kDa

FGFR	fibroblast growth factor receptor
FRNK	FAK related non kinase
γ	gamma
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GAPs	GTPase-activating proteins
GDIs	guanine nucleotide dissociation inhibitors
GEFs	guanine nucleotide exchange factors
GFP	green fluorescent protein
Grb2/7	growth factor receptor bound protein 2 and 7
GTP	guanosine 5'-triphosphate
GTV	gross tumour volume
Gy	Gray
H&E	haematoxylin and eosin stain
H-Ras	Harvey Ras
HR	homologous recombination
HUVEC	human umbilical vein endothelial cells
IB	immunoblotting
IF	immunofluorescence
IGFR	insulin growth factor receptor
IGRT	image guided radiotherapy
IHC	immunohistochemistry
ILK	integrin linked kinase
IMRT	intensity modulated radiotherapy
IP	immunoprecipitate
K14	keratin 14
K5	keratin 5
KD	kinase dead
K-Ras	Kirsten Ras
mAb	monoclonal antibody
MAPK	mitogen activated protein kinase
MEF	mouse embryo fibroblast
MEM	Modified Eagle's Medium
MMP	matrix metalloproteinase
MTT	thiazolyl blue tetrazolium bromide
mTOR	mammalian target of rapamycin
NER	nucleotide excision repair
NHEJ	non-homologous end joining

N-Ras	neuroblastoma Ras
OER	oxygen enhancement ratio
4-OHT	4-hydroxy-tamoxifen
PARP	poly(ADP-ribose)polymerase
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDGF	platelet derived growth factor
PE	plating efficiency
PET	positron emission tomography
PI	propidium iodide
PI3K	phosphatidylinositol 3 kinase
PIP_2	phosphatidylinositol 4,5 bisphosphate
PKC	protein kinase C
PLC	phospholipase C
PMSF	phenylmethylsulphonyl fluoride
PRE	p53 responsive element
PtdIns	phosphatidylinositol
PTEN	phosphatase and tensin homologue
PTK	protein tyrosine kinase
PTP	protein tyrosine phosphatase
PTV	planning target volume
Pyk2	protein tyrosine kinase 2
qRT-PCR	quantitative reverse transcriptase – polymerase chain reaction
R	receptor
RAFTK	related focal adhesion tyrosine kinase
RNA	ribonucleic acid
RNAi	RNA interference
RSV	Rous sarcoma virus
RT-PCR	reverse transcriptase – polymerase chain reaction
S	serine
SCC	squamous cell carcinoma
SD	standard deviation
SDS	sodium dodecyl sulphate
SEM	standard error of the mean
SF	surviving fraction
SFK	Src family kinase
SH	Src homology

Shc	Src homolgous and collagen like protein
siRNA	short interfering RNA
SSB	single strand break
SSRs	site specific recombinases
TPA	12-*o*-tetradecanoyl-13-phorbol acetate
TNF-α	tumour necrosis factor alpha
TSC	tuberous sclerosis complex
UV	ultra violet
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor
WB	western blotting
wt	wild type
Y	tyrosine

Chapter 1

Introduction

1 Introduction

1.1 Radiotherapy in cancer management

1.1.1 History of radiotherapy

Radiotherapy, the medical application of ionising radiation, has been in clinical use for over a century, since the discovery of x-rays in 1895 (Rontgen 1895). The rapid development of normal tissue injuries in the first generation of scientists exposed to x-rays suggested that this form of radiation could have deleterious effects at the cellular level and prompted interest in manipulating this novel form of radiation in order to treat malignant lesions. Initial attempts at radiotherapy were extremely limited by the rather primitive nature of early x-ray generators, which produced radiation that varied in energy and intensity, making reproducible administration of radiation impossible. Moreover, the low x-ray energies available limited radiotherapy to the treatment of superficial lesions and the low dose rates necessitated protracted treatments. Anecdotal reports of cancer patients successfully treated with radiation began to emerge, however, and were sufficient to encourage development of the modality, but the limitations of the technology precluded routinely curative therapy.

The field began to grow significantly in the early 1900s, largely due to Marie Curie and her groundbreaking work on the radioactive elements radium and polonium. Radium, in particular, was utilised in various forms for several decades until cobalt (^{60}Co) and caesium (^{137}Cs) units came into use. This method of radiotherapy delivery was relatively reliable and the machinery simple to maintain. However, the advent of megavoltage linear accelerators, which generate high energy therapeutic x-rays, in the 1950s, revolutionised radiotherapy treatment. By obviating the need for an external radioactive source, treatment was much safer for both patient and medical staff. This means of therapy also allowed a much higher and better directed radiation dose. Clinical (or radiation) oncology then rapidly evolved as a separate medical specialty.

1.1.2 Applications of radiotherapy

Although radiotherapy can be useful in certain benign conditions, such as dysthyroid eye disease or pituitary adenomas, its predominant application is in the management of cancer and it has been estimated that 50% of all cancer patients will receive radiotherapy at some point in their care (http://info.cancerresearchuk.org/cancerstats/). While radiation undoubtedly has a major role in the palliation of cancer symptoms, for example the treatment of painful bone metastases, it also has a valuable role as an adjunct before or after surgery. Most importantly, radiotherapy can be used as first line management with radical (curative) intent, alone or in combination with chemotherapy, in a range of solid tumours, particularly cancers of the head and neck, cervix, prostate, and lung.

1.1.3 Delivery of radiotherapy

Radiotherapy can be administered by several routes: external beam radiotherapy (which usually implies the use of x-rays or γ-rays from a megavoltage unit); brachytherapy (use of a sealed radioactive source); and systemic radioisotope therapy (use of an unsealed radioactive source). The specific type of radiotherapy selected largely depends on tumour type and location. Brachytherapy, for example, can be very useful in the management of intra-cavitary or intra-luminal malignancies whereas systemic radioisotope therapy may provide symptomatic relief in patients with disseminated bone metastases. External beam radiotherapy is the most commonly applied form of radiation treatment and unless otherwise specified the term radiotherapy refers to this form of treatment.

Radiotherapy treatment requires careful planning in order to ensure not only adequate tumour coverage and dose, but also to minimise toxicity to normal tissue. Currently, most centres in the UK employ computed tomography (CT) based planning where the patient is simulated in the treatment position, which usually requires some form of immobilisation so that the same position can be maintained during the entire course of treatment.

The target volume is then delineated on the CT-acquired images, first taking into account visible macroscopic disease, which is referred to as the gross tumour volume (GTV). A margin of normal tissue must be added to this volume to allow for subclinical tumour spread that is not visible on diagnostic imaging; this is known as the clinical target volume (CTV). Finally, a further margin is required to compensate for potential movements relating to respiration and/or digestion. Also, despite careful immobilisation, small errors in patient set-up are likely to occur from day to day, usually no more than a few millimetres, but this must be taken into account.

The resultant planning target volume (PTV) can then be taken forward to beam direction and dose distribution studies. This is generally performed on dedicated computers using specialised planning software, and input is required from trained medicial physicists and dosimetrists. Depending on the radiation delivery method and target volume, it is likely that several x-ray beams will be required to deliver a uniform prescription dose to the tumour bed and minimise dose to surrounding healthy tissues. The clinical oncologist must ensure that the delivery parameters of a prescibed dose are satisfactory and, where practicable, minimise the irradiation of critical structures, such as the spinal cord. Examples of planning target volumes and a dose distribution profile are shown in **Figure 1** and **Figure 2**, respectively.

The amount of radiation used in radiation therapy is measured in gray (Gy), and varies depending on both the type and stage of cancer being treated. While lymphoma can be effectively treated with just 20 to 40 Gy, the radical dose required for a solid epithelial tumor ranges from 55 to 80 Gy. The total dose is fractionated, typically 2Gy per day, which means that a course of treatment is extended over a number of weeks. One of the most important reasons for fractionation is that it allows normal cells time to recover between radiation doses. In some cancer types, however, prolongation of the treatment schedule over too long a period of time can permit repopulation of cancer cells, and for these tumour types, including squamous cell carcinomas of the head and neck and cervix, radiation treatment is preferably completed within a limited time frame.

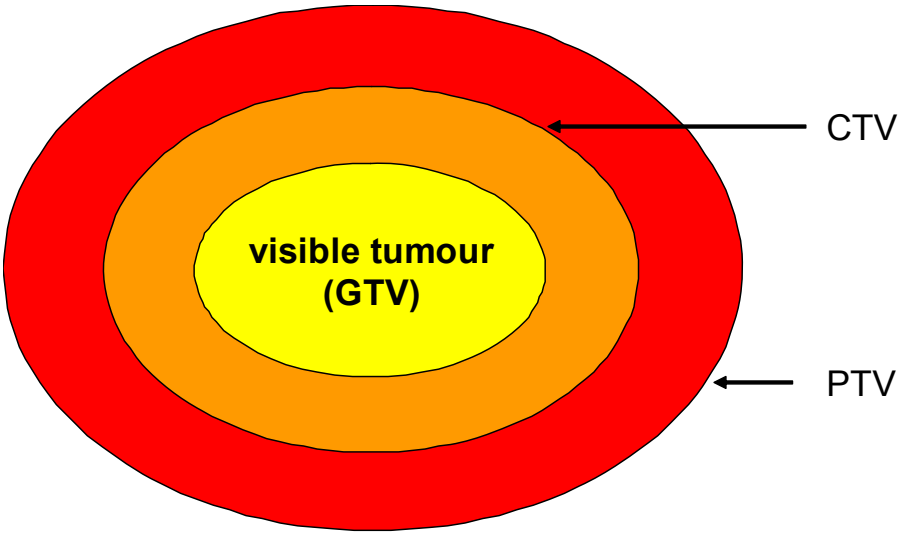


Figure 1 - Delineation of radiotherapy target volumes
The visible tumour or gross tumour volume (GTV) seen on diagnostic imaging is marked in yellow. A margin is added to allow for sub-clinical tumour spread into surrounding tissue (orange). These combined areas are known as the clinical target volume (CTV). Finally, a margin is applied to allow for day to day set-up errors and organ motion (red). Beams can then be directed at the resultant planning target volume (PTV).

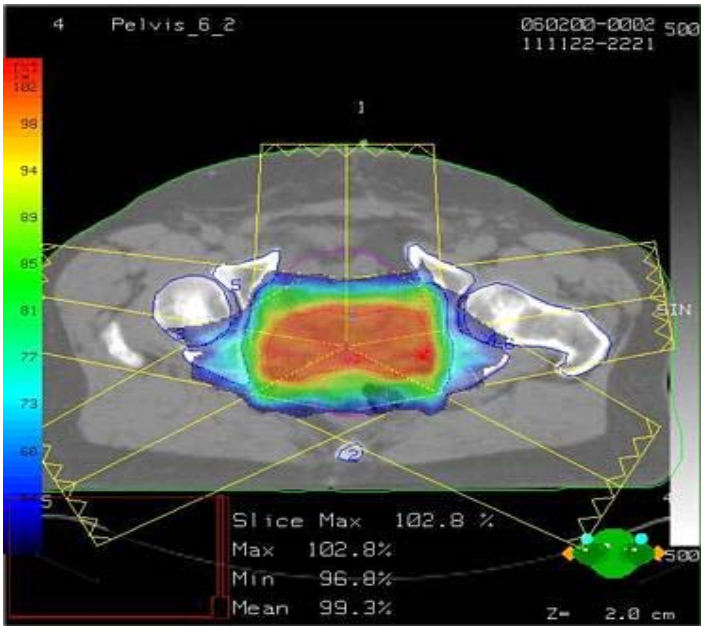


Figure 2 - Radiotherapy dose distribution profile
The image depicted above represents the dose distribution profile for a patient being treated with radical radiotherapy for prostate cancer. Five beams are directed at the pelvis to encompass the PTV (shown in red). Ideally this area should receive 95-104% of the prescribed dose. Surrounding tissues receive a fall-off dose (green – blue) which reduces as distance from the target volume increases.

1.1.4 Radiotherapy related toxicity

The majority of patients will experience some toxicity during radiotherapy treatment; this varies depending on the applied dose and area of treatment. In general, most of the acute effects are manifested in rapidly dividing epithelial tissues and can range from erythema to moist desquamation in the skin and mucous membranes, or diarrhoea if the gut is in the radiation field. These symptoms usually settle rapidly following treatment, often within a few weeks. More concerning is the potential for radiation to induce late effects in normal tissue. This is largely due to damage to connective tissue and small blood vessels and can result not only in cosmetic effects due to fibrosis, but also more problematic sequelae such as ulceration, fistula formation, and organ dysfunction. With modern planning techniques, it is possible to minimise but not eliminate the likelihood of these late effects and much of this is dependent on the size and position of the tumour.

It is clear that some individuals are more susceptible to normal tissue damage. One of the reasons for this is the tendency to mount an excessive inflammatory response. Ionising radiation triggers the production of inflammatory cytokines in a number of cell types, such as macrophages, epithelial cells, and fibroblasts. This contributes to increased collagen production and the development of fibrosis. In some cases, the inflammatory reaction is more pronounced, leading to the expression and maintenance of a perpetual cytokine cascade, and ultimately increasing the severity of the late reaction. Transforming growth factor-beta-1 (TGF-β1) is one such cytokine that has been implicated in mediating radiation related fibrosis (Anscher, Kong et al. 1997; Anscher, Marks et al. 2001). It is likely that the regulation of cytokines, including TGF-β1, in response to radiation is controlled at the genetic level. Hence, there is currently interest in trying to use inflammatory and/or genetic profiling in order to identify those individuals at greater than normal risk of toxicity (reviewed by (Okunieff, Chen et al. 2008)). If so, at some point in the future it may be possible to consider limiting the total radiation dose in these patients and/or attempt to modulate their immune response in some way in order to minimise the late effects of radiotherapy treatment.

As well as the effects of radiation on tissue integrity and organ function, it is important to note that ionising radiation can itself induce cancer. The risk of malignancy from radiation exposure is well documented. However, this data is largely based on atomic bomb survivors (Little 2001) and it is difficult to accurately extrapolate the relative risks in this cohort to those of medically exposed individuals. Nonetheless, a treatment course of radiotherapy does induce a risk of second malignancy (Curtis 2006). This risk is typically very small and it can take many years for such cancers to develop.

1.2 Radiobiology

1.2.1 Radiation induced subcellular damage

It has been widely recognised for decades that ionising radiation exerts its effect by damaging cellular DNA. While the ability of radiation to induce DNA damage is unquestionable, the field of radiobiology has grown extensively over the past 10 – 15 years as it is becoming clear that the cellular response to ionising radiation is extremely complex. Indeed, it has already been mentioned that radiation can trigger an inflammatory response. More importantly, it has now been acknowledged that radiation can actively encourage cell preservation mechanisms. This ability of radiation to promote cell survival will be discussed in later sections. At this point, the traditional view of radiation induced DNA damage will be discussed in more detail.

Cellular DNA damage following exposure to radiation is sustained as a result of either direct or indirect ionisation. The latter is due to the ionisation of water, which forms free radicals, notably hydroxyl radicals, which are highly toxic. In the most common forms of radiation therapy, including traditional external beam radiotherapy, most of the damage is induced indirectly by free radicals, as opposed to direct ionisation. However, the vast majority of these molecules do not produce significant injury. The effect of ionisation is strongly influenced geographically by proximity to DNA and biologically by free-radical scavenging processes and cellular repair abilities. In addition, some lesions are more important than others and although radiation can cause a wide range of DNA damage as outlined in **Table 1**, radiation lethality appears to correlate most

significantly with the number of residual, unrepaired double-strand breaks (DSB) present several hours after radiation. The precise location and extent of these DSBs has a significant impact on whether a cell lives or dies following a radiation insult.

Table 1 - Types and frequency of radiation induced damage

Type of damage	Number per Gy per cell
DNA double-strand breaks	40
DNA single-strand breaks	1000
DNA – protein crosslinks	150
DNA – DNA crosslinks	30
Base damage	2000
Sugar damage	1500

This table was adapted from (Steel, 2002).

1.2.2 Radiation induced cell kill

An important product of DNA damage is observed as chromosome aberrations when cells are irradiated in the G1 phase of the cell cycle or chromatid aberrations when cells are irradiated after the DNA replication phase. The amount of chromosome or chromatid damage observed at the first mitosis after irradiation is perhaps the subcellular endpoint that correlates best with cell kill for most cell types. This has encouraged the view that the major cause of cell death after irradiation is physically aberrant mitosis leading to uneven distribution of chromsomes fragments, known as mitotic catastrophe. Such large scale loss of DNA probably leads to metabolic imbalance that is incompatible with further proliferation.

Not all irradiated cells enter mitosis before they begin to degenerate and in this case, apoptosis appears to be a significant cell death mechanism. Interestingly, radiation induced apoptosis is not necessarily explained by effects on DNA as it has been shown that ionising radiation can effect changes in the cell membrane that trigger apoptosis (reveiwed by (Verbrugge, de Vries et al. 2008)). The role that each form of cell death contributes to clinical radiosensitivity has been the

subject of much discussion for many years and is still controversial (reviewed by (Eriksson and Stigbrand 2010)).

1.2.3 Clonogenicity

To complicate matters, cells often do not die rapidly in response to radiation but typically sustain damage that impedes further proliferation after several cell divisions. Indeed, it is this loss of reproductive integrity or clonogencity that underpins the biological effects of ionising radiation. In normal tissues the key determinant of growth is the stem cell population which forms the proliferative driving force due to an unlimited ability to divide. Similarly, in tumours there appears to be a subset of cells that make the major contribution to tumour growth – whether this population is composed of "cancer stem cells" is open to debate. It is essential to sterilise this subset of cells during a course of radiotherapy in order to minimise the risk of local or distant treatment failure.

The evaluation of the reproductive capacity of these cells in the laboratory is best made using clonogenic assays, where the surrogate of a stem cell is the clonogenic cell. This is defined as a cell that will produce a colony of 50 cells or more under defined experimental conditions. This threshold represents the ability to undergo 5 – 6 cell divisions and is chosen in order to exclude cells that have a limited growth potential as a result of sublethal radiation damage, or having embarked on differentiation. The basic experimental procedure is to prepare a single cell suspension, irradiate the cells with a range of radiation doses, and allow them to proliferate in appropriate growth conditions for a suitable period of time (usually 7 – 21 days). The number of visible colonies are then scored and the plating efficiency of each group determined by simply calculating the ratio of the number of colonies formed and the number of cells seeded. The efficacy of each radiation dose is then related to the untreated cells by the surviving fraction, which is defined by:

$$\text{Surviving fraction (SF)} = \frac{\text{Plating efficiency of treated cells}}{\text{Plating efficiency of untreated cells}}$$

Radiation induced cell kill is exponential so the surviving fractions are plotted on semilogarithmic axes to produce cell survival curves, examples of which are shown in **Figure 3**. For some very radiosensitive cell types, for example lymphoma or seminoma, the cell survival curve is linear. However, for most cell types there is either a shoulder on the curve (that is, a region at low dose where an increment of radiation dose has little or no effect on cell kill) or the curve may be continuously bending. Several mathematical equations exist to describe these curves, including the multi-target equation, the multi-target with single hit equation, and the linear quadratic equation. As the latter most accurately describes the data from collective experiments on both normal tissue and human tumour cells, this is the most commonly used model.

For the purposes of laboratory studies, it is important to carefully consider the endpoint when designing radiation experiments. While it is acceptable to perform short term assays of cell survival or viability, it is good practice to compare these results against the more laborious and time consuming, but generally more reliable, clonogenic assay. Similarly, due to the controversy over apoptosis and whether the level detected truly reflects cellular radiosensitivity, apoptosis assays should be carried out in parallel with an assessment of clonogenicity. Often, surrogates of cell death are used such as the comet assay, which is designed to quantify the number of strand breaks, and can be adapted to measure either single- or double-strand breaks. However, it is important to point out that this assay does not take into account either the rate or fidelity of DNA repair. To some extent, this can be assessed by the appearance of phosphorylated histone H2AX (γH2AX) foci formation in the nucleus and their subsequent clearance. Phosphorylation on the serine 139 site of the histone γH2AX occurs in response to ionising radiation (Rogakou, Pilch et al. 1998) and is generally considered to be a reliable surrogate of DSB repair. Although these DNA damage based assays can provide useful information on the extent of DNA damage and the kinetics of DNA repair, it is essential to support the findings from these assays with clonogenicity experiments.

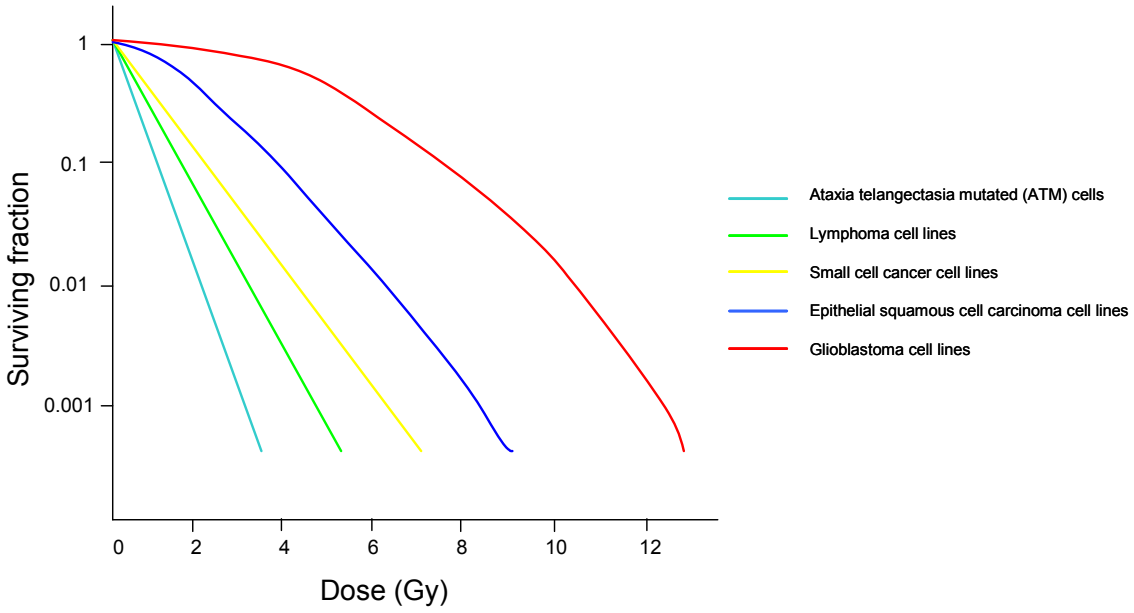


Figure 3 - Radiation survival curves vary in different cell lines

1.2.4 Radiosensitivity

The efficacy of a course of fractionated radiotherapy is determined by both the physical and technical aspects of radiation delivery, and the biological effects on the tumour and normal tissue. There are five factors that are highly influential in dictating the biological response – the "5Rs of radiotherapy". Two of these, recovery and repopulation, lead to a decrease in response when treatment is prolonged, while two, reassortment and reoxygenation, are beneficial in a fractionated regimen. The fifth, intrinsic radiosensitivity, is the baseline on which the other modifications work.

1.2.4.1 Reassortment

Cells alter their sensitivity to radiation as they progress through the cell cycle. Cells in mitosis, for example, can be twice as sensitive as those at the end of the DNA synthesis phase. A single dose of radiation therefore preferentially kills cells at particular stages of the cell cycle. Although cells do not progress normally through the cell cycle after exposure to radiation due to checkpoint activation, there is usually some progression between fractions, such that cells in a resistant phase may be reassorted into a more sensitive phase of the cycle.

1.2.4.2 Reoxygenation

Oxygen is critical to the effects of radiation. Cells that are irradiated in hypoxic environments are much more resistant to radiation compared with their well oxygenated counterparts. This is largely because oxygen reacts with free radicals to produce chemically unrepairable peroxy radicals ($R\cdot + O_2 \rightarrow RO_2$), hence oxic cells sustain more DNA damage. The degree of sensitisation by oxygen is often quoted as the oxygen enhancement ratio (OER), which is the ratio of doses needed to produce a given biological effect in the presence or absence of oxygen. For most cells and tissues the OER has a value of 2.5 – 3 (Chapman, Dugle et al. 1974; Hall and Chapman 1974). The decrease in sensitivity depends on the partial pressure of oxygen, which means that most well vascularised normal tissues are not affected by the oxygen effect. However, the vascular supply in tumours is often not adequate to maintain sufficient partial pressure of oxygen for all cells. This is due to insufficient vessel production which results in

chronic hypoxia, and is compounded by the abnormal, inefficient nature of these new vessels which can constrict causing acute hypoxia. The proportion of the tumour that is deficient in oxygen can have an important influence on the success of radiotherapy. As it is not unusual to find hypoxic fractions of 10-20% in solid tumours, this can pose a series threat to treatment outcome.

A number of approaches have been considered in an attempt to overcome this problem in clinical practice. One simple measure for certain tumour types is to correct anaemia with a blood transfusion prior to treatment. More technical advances such as the use of hyperbaric oxygen during radiotherapy, although of proven benefit, are difficult to implement. The prospect of introducing drugs that can radiosensitise hypoxic cells was very popular in the 1980s. The most widely studied group consisted of the nitroimidazoles which in principle mimic the effect of oxygen and have no sensitising effect on normal tissue. However, the validity of this approach was called into question due to concerns over dose limiting neurotoxicity.

There is renewed interest in tackling hypoxia following the recognition that cells react to hypoxia by switching on certain genes. This may allow not only the identification of hypoxic cells, but may lead to specific targeting of hypoxic cells. Targeting the vasculature itself as means of combating tumour cell hypoxia has come under scrutiny recently and will be discussed in more detail in Section 1.4.2.

1.2.4.3 Repopulation

During a course of radiotherapy, cells that survive irradiation may proliferate. Even if this occurs at the tumour's normal doubling rate, this can lead to an increase in the number of cells that must be killed. However, there is evidence that repopulation can actually be stimulated to a significant extent in certain tumour types, particularly in head and neck cancer. Accelerated radiotherapy protocols, in which the overall treatment time is reduced by giving multiple fractions on a daily basis, have been developed to address this issue, and in some cases this is proving effective (Hatton and Martin 2010; Saunders, Rojas et al. 2010).

1.2.4.4 Recovery / repair

Most of the damage induced in cells by radiation is satisfactorily repaired. Evidence for this comes from studies of strand breaks in DNA, the vast majority of which disappear within hours after irradiation. Therefore, between radiotherapy fractions, the cell may have sufficent time to reduce the extent of radiation damage, hence the term recovery. This can promote tumour cell survival, which is unfavourable, but this repair period is obviously crucial for normal cells as well as cancer cells; otherwise early and late radiotherapy related toxicity may be a major problem. Accelerated radiotherapy regimens mentioned above can impact on recovery time as well as repopulation. This is because the multiple fractions given each day have only 6 or 8 hours in between as opposed to the standard 24 hour gaps in treatment. While this approach can increase tumour cell kill, it does not lead to a significant exacerbation in normal tissue toxicity as the dose of radiation per fraction is typically much smaller.

1.2.4.5 Intrinsic radiosensitivity

Intrinsic radiosensitivity is the relative susceptibility of cells, tissues, or organs to the harmful effects of radiation. It is well recognised that the inherent sensitivity of tumour cells varies between tumours of different pathological type and between tumours of the same type taken from different patients. In addition, there are discrepancies in radiosensitivity in cells within a single tumour. There are also differences in the susceptibility of normal cells between individuals. While there are some genetic syndromes, such as ataxia telangectasia, that can explain the significant tissue toxicity in certain individuals exposed to ionising radiation, these syndromes are rare. The reasons for marked variation in the non-syndromic population are largely unknown, but it is likely that DNA repair capabilities play a significant role with each individual possessing a pre-determined ability to control the rate, extent, and fidelity of DNA repair. The mechanisms underlying the variation in tumour cell radiosensitivity are probably even more diverse. On top of the intrinsic radiosensitivity acquired from the tissue of origin, cancer cells will have amassed a range of molecular aberrations such as gene amplifications and/or mutations that are likely to influence DNA repair, cell cycle control and cell survival pathways.

1.3 Advances in radiotherapy delivery and dosimetry

The greatest challenge for radiotherapy (or any cancer therapy for that matter) is to increase the therapeutic ratio, that is, to attain the highest probability of cure with the least morbidity. The simplest way in theory to achieve this with radiation is to encompass all cancer cells with sufficient doses of radiation during each fraction, while simultaneously sparing surrounding normal tissues. In practice, however, it is not always straightforward to identify the cancer cells or target them adequately with radiation. Over the past decade, enormous progress has been made on both fronts. Technical improvements in the application of x-rays, computed tomography scans, magnetic resonance imaging with and without spectroscopy, ultrasound, positron emission tomography scans, and electronic portal imaging — and a clearer understanding of their limitations — have greatly improved the ability to identify the tumour volume.

1.3.1 Conformal radiotherapy

For almost a century, radiotherapy planning of deep seated tumours was based on the use of co-planar, regularly shaped symmetrical fields, with the result that the treated volume approximated a simple geometric box, a distinct disadvantage being the inclusion of much adjacent healthy tissue. Nowadays the x-ray beams are shaped with lead to conform to the size and shape of the target volume, hence the term conformal radiotherapy. The advent of three-dimensional (3D) CT based planning has greatly assisted conformal treatment. Because it takes into account axial anatomy and complex tissue contours, as well as irregular beam shape, it can provide a reasonably accurate assessment of dose distribution across the target volume.

1.3.2 Intensity modulated radiotherapy

While CT based planning has revolutionised the field of clinical oncology, it does have limitations. As its name implies, intensity modulated radiotherapy (IMRT) permits modulation of the intensity of each radiation beam, so each field may have one or many areas of high intensity radiation and any number of lower intensity areas within the same field, thus allowing for greater control of the

dose distribution within the target. By modulating both the number of fields and the intensity of radiation within each field, it is possible to sculpt radiation dose. Advanced treatment planning software has furthered the ability to modulate radiation dose. Instead of the clinician or physicist choosing every beam angle and weighting, computer optimisation techniques can now help determine the distribution of beam intensities across a treatment volume. This form of radiotherapy can be very useful in situations where the tumour is lying very close to an important structure such as the spinal cord, but is not widely available as yet.

1.3.3 Image guided radiotherapy

Image guided radiotherapy (IGRT) is a novel array of techniques designed to minimise the discrepancies which occur due to variations in patient and/or tumour position. Strictly speaking, IGRT is the use of imaging to monitor or modify the delivery of radiotherapy treatment. However, the term IGRT is often used to encompass three broad categories of image-based innovations: (1) the integration of functional and biological imaging into the treatment planning process to improve tumour contouring (or target delineation), (2) the use of various imaging modalities to adjust for tumour motion and positional uncertainty, and finally (3) the adaptation of treatment planning based on tumour response and changes in normal tissue anatomy (Nath, Simpson et al. 2009).

A key innovation in the first category of IGRT is the use of 18-F-Flurodeoxyglucose (FDG)-PET, a radio labelled analogue of glucose that is selectively absorbed in tumour cells more than normal tissues. Hence, it can be effective in highlighting the presence of neoplastic growth in tissues that appear radiologically normal. As such, FDG-PET now has an established role in oncology and is a useful tool in the staging process of several common cancers, including lung (Lardinois, Weder et al. 2003; Halpern, Schiepers et al. 2005) and colorectal cancer (Tutt, Plunkett et al. 2004; Rosenbaum, Stergar et al. 2006; Israel and Kuten 2007). However, there is now increased focus on the value of FDG-PET in target delineation for radiotherapy. Sophisticated software can

perform an accurate overlay of PET and CT images and these fused images can guide the contouring of tumour margins and extended fields.

The second type of IGRT involves the use of modern imaging modalities to assist in daily patient positioning. Most radiotherapy protocols involve several weeks of sequential daily treatment, and each day the patient needs to be repositioned into the exact position obtained during the initial planning CT. Despite the use of immobilisation devices, there is considerable potential for setup variability. A common way to verify patient positioning is through the use of two-dimensional (2D) portal film imaging. This is done using devices attached to the treatment machine that are capable of taking radiographs. However, the image quality is usually quite poor. Improved means of imaging are now being introduced, but there are concerns that the increased radiation dose may correspond with a higher risk of second malignancy (Kan, Leung et al. 2008), although this has yet to be established.

The final form of IGRT is known as adaptive radiotherapy. This is a new, and still evolving, concept with the potential to greatly improve the delivery of radiotherapy. As there can be significant changes in patient anatomy during a course of treatment due to tumour shrinkage or weight loss, the premise of adaptive radiotherapy is to alter the treatment plan in response to these changes. Research in this field is quite preliminary so it is currently unclear when and how often re-planning should be done and how this will impact on the available resources.

1.3.4 Intra-operative radiotherapy

A standard course of traditional external beam radiotherapy lasts for several weeks. This may have financial implications for the patient and can significantly impact on healthcare resources. In the post-operative or adjuvant setting, the possibility of applying a single fraction of radiation immediately after surgery has been suggested. This practice is already performed at a number of cancer centres in the US, but only in selected tumour types. In general, the numbers of patients accrued for each tumour type are insufficient to draw definitive conclusions (Beddar, Biggs et al. 2006; Skandarajah, Lynch et al. 2009). Perhaps the one major exception is breast cancer, where a large international study is

directly comparing a standard course of post-operative radiotherapy with a single fraction of radiotherapy given intra-operatively. If this is equivalent in terms of local disease recurrence, the obvious question is whether a single fraction will lead to increased late toxicity. So far, this does not seem to pose a particular threat (Holmes, Baum et al. 2007; Baum and Vaidya 2008; Kimple, Klauber-DeMore et al. 2010) but long term follow up will be required.

1.3.5 4D radiotherapy planning

Recently, a new technology has been developed; four-dimensional (4D) radiotherapy planning. This can add the dimension of time to conventional spatial (3D) planning. A technique such as this may prove to be particularly useful in the treatment of lung cancers where tumours may move up to 4cm during inspiration and expiration. If organ motion can be recorded, this information may be used to better define the radiotherapy treatment volume. For instance, the target could be defined as the volume that encompasses the tumour throughout the breathing cycle, so that the tumour always stays within the radiation field. In addition, separate treatment plans may be created for different phases of motion and the best one selected for “gated” delivery. To achieve this, it would be necessary to synchronise the delivery of radiation with the target motion so that the dose is only delivered when the tumour is in a certain position. While this form of radiotherapy is an exciting step forward, it does require significantly more input and expertise from radiation physics departments, and both treatment planning and treatment delivery times are longer.

1.3.6 Particle therapy

As already outlined, most solid tumours are treated with traditional external beam radiotherapy from megavoltage linear accelerators which produce high energy x-rays. However, there is interest in exploiting forms of radiation other than x-rays for therapeutic use. Particle therapy is a form of external beam radiation that takes advantage of the biological properties of protons, fast neutrons or heavy ions.

1.3.6.1 Protons

Although protons have a similar biological effect to x-rays, they have superior dose distribution properties, which allow the production of a highly focused, high energy peak in otherwise inaccessible sites. The major limitation in the routine use of proton beams is the expensive and complex cyclotron technology required to produce a beam for medical use; at present only a few centres worldwide offer this form of treatment.

1.3.6.2 Neutrons

Unlike protons, neutrons do not have dose distribution advantages over x-rays. However, they do offer a biological advantage. Both x-rays and protons are forms of low energy transfer radiation as they are sparsely ionising, whereas neutrons are more densely ionising and are therefore considered to be high energy transfer radiation. The latter are biologically more effective per unit dose and have less dependence on oxygen for cell kill. Neutrons have been more extensively studied than protons but the clinical results obtained are controversial due to unacceptably high levels of normal tissue toxicity. To some extent these findings probably reflect the poor dose distributions and beam delivery systems of early experimental neutron facilities. The precise role of neutron beams in radiotherapy continues to be investigated, but this form of treatment is certainly not standard practice at the moment.

1.3.6.3 Heavy ions

Heavy ion therapy is the use of particles more massive than protons or neutrons, such as carbon ions. Similar to neutrons, heavy ions have a higher density of ionisation compared with x-rays. The experience in this type of therapy is restricted to less than a handful of centres worldwide (reviewed by (Kitagawa, Fujita et al. 2010)), but a number of new facilities are under construction. Work is also underway to improve the stability and reproducibility of heavy ion beams. This form of treatment may be of value in selected patients but is unlikely to be routinely employed in clinical oncology practice.

1.4 Novel targets in radiation research

As outlined in the previous section, the planning and delivery of radiotherapy is becoming ever more complex, with the ultimate aim of achieving increased tumour cell kill with minimal normal tissue toxicity. However, it is also possible to augment tumour cell kill by biological means. Cytotoxic chemotherapy, for example, has long been used in combination with radiotherapy. Theoretically, there is an advantage to combined modality treatment as the total cell kill should be greater than that achieved with either agent alone (independent cell kill). In addition to this, chemotherapy can enhance the activity of radiotherapy (synergistic cell kill). Certain drugs can inhibit the repair of radiation damage such as cisplatin, doxorubicin and bleomycin. In addition, cisplatin can increase the number of radiation induced strand breaks. Some drugs, such as taxanes, can also influence radiation response by increasing the proportion of cells in a more radiosensitive phase of the cell cycle. For these mechanisms to be exploited, the two modalites should have non-overlapping toxicities and the chemotherapy should not enhance normal tissue damage. Even so, this is likely to have a wider range of toxicity which must be taken into account.

The combination of chemotherapy and radiotherapy has led to improved treatment results in patients with locally advanced solid tumours. In particular, the concurrent application of both modalities has proven efficacy and resulted in lower recurrence rates and improved survival in several tumour types, such as head and neck cancer (Calais, Alfonsi et al. 1999) and cervical cancer (Rose, Bundy et al. 1999). More recently, it has been shown that the addition of chemotherapy in notoriously radioresistant tumours such as glioblastoma, can improve outcome (Stupp, Hegi et al. 2009).

An exciting development in cancer therapy in the last 20 years, however, is the increased understanding of oncogenes, cellular growth factors and signal transduction pathways that control cell proliferation, differentiation, invasion, and angiogenesis. This has led to the development of targeted agents to use alone or in combination with cytotoxic chemotherapy. Similarly, radiation biology has advanced over this time. It has already been highlighted that radiotherapy exerts its effect by inducing DNA damage, and recent years have

seen much progress in dissecting out the various mechanisms responsible for controlling DNA repair. Furthermore, it is becoming clear that radiation does not always induce cell death or a proliferative block. Conversely, ionising radiation can inhibit cell death and /or stimulate cell survival pathways. Many of these effects have been linked to the induction of growth factor receptors, although there are likely to be other factors involved.

This section will focus on combination strategies with proven clinical efficacy and on approaches that are highly promising for combined use with radiotherapy based on their underlying mode of action.

1.4.1 EGFR signalling pathway

To date, much of the preclinical and clinical experience combining molecularly targeted agents with radiation has involved inhibiting signalling through the epidermal growth factor receptor (EGFR) pathway. EGFR is a member of the receptor tyrosine kinase family of growth factor receptors that are activated under normal circumstances by ligand binding to transmit mitogenic signals to the nucleus. On binding to its ligand, EGF, or transforming growth factor-α (TGFα), EGFR dimerizes and the intracellular domain becomes phosphorylated on tyrosine residues, thereby initiating a cascade of signals from the cytoplasm to the nucleus, predominantly through the activation of the Ras/MAPK and PI3K/ Akt/mTOR pathways, that control a variety of cellular responses modifying cell growth and survival (Ullrich and Schlessinger 1990). An overview of the EGFR signalling cascade is provided in **Figure 4**.

EGFR is often expressed at high levels in human cancer; typically mediated by gene amplification or mutation. Upregulation of this protein has been associated with more aggressive tumours and poor prognosis in a range of tumour types (reviewed by (Hale, Buckley et al. 1993)). The first indication that EGFR was involved in radioresistance was documented over 15 years ago when the addition of exogenous EGF rendered MCF-7 breast cancer cells radioresistant (Wollman, Yahalom et al. 1994). This effect was abrogated by exposure to EGFR antibody. It has since been shown that EGFR tyrosine phosphorylation is stimulated by radiation (Dent, Reardon et al. 1999) and the subsequent activation of the signal

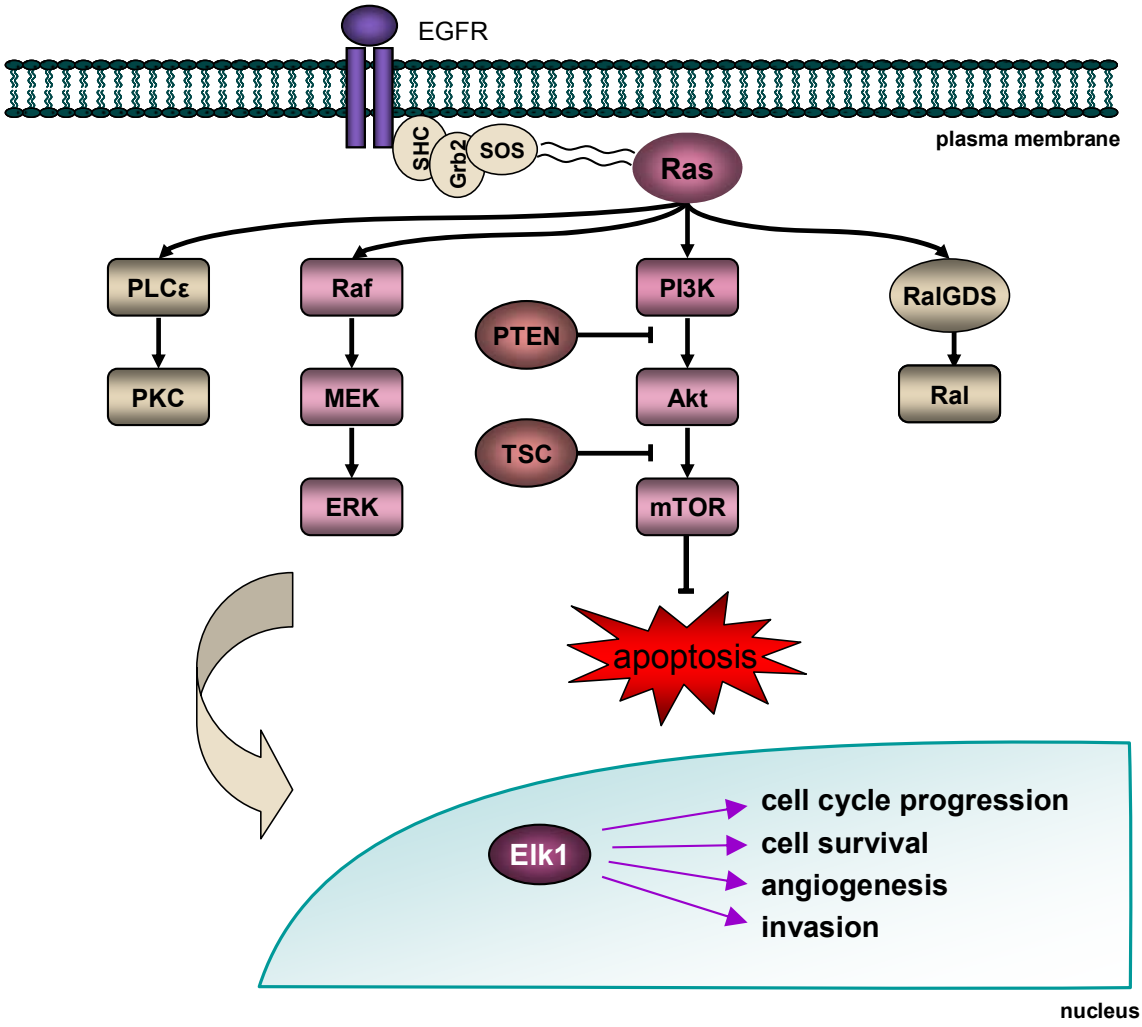


Figure 4 - Simplified model of signalling through the EGFR pathway
The stimulation of the epidermal growth factor receptor (EGFR) results in the assembly of multi-molecular complexes (e.g. SHC, Grb2 and SOS) that interact with Ras at the plasma membrane. Activated Ras then participates in a cascade of signalling events, including Raf/MEK/ERK. The end product of ERK activation is the phosphorylation of cytoplasmic and nuclear targets, such as the Elk1 transcription factor, which regulates transcription of genes involved in crucial cellular processes including cell cycle progression, survival, invasion, and angiogenesis. Activation of PI3K leads to activation of Akt and mTOR, which play critical roles in the control of apoptosis and protein synthesis. Downstream of PI3K, the tumour suppressor genes PTEN and TSC also contribute to the regulation of Akt and mTOR. RalGDS is a guanine nucleotide exchange factor (GEF) for Ral, which has roles in regulating gene transcription and vesicle transport. Phospholipase C (PLC) mediates cross talk between small GTPase signalling pathways and activates protein kinase C (PKC), which has numerous effects on tumour cells including stimulation of proliferation.

transduction pathways outlined in **Figure 4** mimics ligand binding. Consequently, this results in a proliferative response after radiation and counteracts radiation induced growth arrest and cell kill. In addition, it has been reported that radiation induces nuclear translocation of EGFR. This allows DNA-dependent protein kinase (DNA-PK) complex formation which is an important factor in DNA DSB repair (Dittmann, Mayer et al. 2005).

These processes can be inhibited by small molecule inhibitors of EGFR autophosphorylation, such as gefitinib (iressa) and erlotinib (tarceva), or by anti-EGFR antibodies, such as cetuximab. Both modalities have been shown to increase radiosensitivity in a wide range of cancer cell lines (Bianco, Tortora et al. 2002; Shintani, Kiyota et al. 2003; Stea, Falsey et al. 2003; Eller, Longo et al. 2005; Raben, Helfrich et al. 2005; Taira, Doihara et al. 2006; Colquhoun, McHugh et al. 2007). While some of the effects are due to DNA repair, certainly cytosolic sequestration of DNA-PK by cetuximab is thought to impair DSB repair (Dittmann, Mayer et al. 2005; Dittmann, Mayer et al. 2008), changes in cell cycle have also been reported, typically a reduction in the proportion of cells in S phase, which is generally regarded as the most radioresistant phase of the cell cycle.

The use of tumour growth delay experiments in human tumour xenografts treated with either EGFR antibodies or tyrosine kinase inhibitors (TKIs) in combination with radiation have also shown positive results, usually an additive effect although some instances of synergistic cell kill have been reported (Saleh, Raisch et al. 1999; Milas, Mason et al. 2000; Williams, Telfer et al. 2002; She, Lee et al. 2003; Milas, Fan et al. 2004; Nyati, Maheshwari et al. 2004; Krause, Schutze et al. 2005; Colquhoun, McHugh et al. 2007; Feng, Lopez et al. 2007; Geoerger, Gaspar et al. 2008). Effects on cell proliferation seem to be important *in vivo*, at least in single fraction studies. In fractionated studies, decreased repopulation and increased reoxygenation contribute to the outcome. The latter is likely due to an effect on tumour vasculature as EGFR blocking agents have been shown to enhance flow perfusion and reduce hypoxia, ultimately improving tumour response (Qayum, Muschel et al. 2009).

In the clinical setting, the benefit of anti-EGFR treatment in combination with radiotherapy has been demonstrated in locally advanced head and neck cancer. The addition of cetuximab led to increased local disease control and, more

importantly, overall survival (Bonner, Harari et al. 2006; Bonner, Harari et al. 2009). However, this study was initiated before chemoradiotherapy became standard of care in head and neck cancer, so it remains to be seen whether cetuximab can replace chemotherapy in combination schedules or whether cetuximab should be given alongside chemoradiotherapy. While there is some *in vitro* and *in vivo* data (Feng, Lopez et al. 2007; Zhang, Erjala et al. 2009) to support the latter, this may induce considerable toxicity (Pfister, Su et al. 2006). Another interesting question that has yet to be answered is whether the level of EGFR overexpression in tumours is critical in terms of predicting response to combination therapy. While this does seem to be significant *in vitro*, and certainly EGFR is often overexpressed in head and neck cancer (Kalyankrishna and Grandis 2006; Temam, Kawaguchi et al. 2007), the Bonner study did not differentiate between low and high expressors. It is important to point out, however, that based on the evidence from single agent or chemotherapy combination studies in other tumour types, the response to EGFR inhibition is not necessarily dependent on EGFR overexpression, but is influenced by the presence of activating EGFR mutations (Lynch, Bell et al. 2004) or K-Ras status (Ibrahim, Zekri et al. 2010). It has yet to be established whether this type of mutation analysis can be used to select patients who will be most likely to benefit from a combination approach involving radiotherapy. The role of cetuximab (and newer second generation monoclonal antibodies) and various EGFR TKIs in combination with radiotherapy / chemoradiotherapy in other cancers, particularly rectal, pancreas and glioblastoma is currently under investigation and it is likely that future Phase III trials will attempt to address the issue of patient selection / prediction.

Although most of the experience to date has involved targeting EGFR itself in combination with radiation, the constituent components of the downstream signalling pathways are also potential targets. In fact, one of the first attempts at combining targeted agents with radiation concentrated on Ras. This was based on work in the 1990s which demonstrated that constitutive Ras activation promoted radioresistance (Ling and Endlich 1989; McKenna, Weiss et al. 1990; McKenna, Weiss et al. 1990; Samid, Miller et al. 1991; Bernhard, Stanbridge et al. 2000), although in retrospect some of this data is difficult to interpret and confounded by the presence of other oncogenes, such as myc, which can also

influence radiosensitivity. The development of farnesyl transferase inhibitors (FTIs), which prevent membranous localisation of Ras and hence its full biological activity, led to much excitement as Ras mutations are very common in human cancers (Bos 1989). However, these agents failed to meet expectations in the clinical setting, probably because much of the dramatic effects of FTIs observed in early experimental systems were predominantly based on H-Ras induced cancer model systems (Kohl, Mosser et al. 1993); H-Ras mutations being far less frequent than either K-Ras or N-Ras mutations in human cancers. In addition, it is now clear that K-Ras and N-Ras proteins undergo alternative means of modification in the context of farnesyl transferase inhibition (Whyte, Kirschmeier et al. 1997). What is more, the exact mechanism of these agents has been called into question as many intracellular proteins are farnesylated and it has been proposed that alternative targets may be responsible for the anti-tumour effects of FTIs (Clark, Kinch et al. 1997; Lebowitz, Casey et al. 1997; Ashar, James et al. 2000; Zeng, Si et al. 2000). In keeping with this, some wild-type (wt) Ras cell lines exhibited increased radiosensitivity when treated with FTIs (Cohen-Jonathan, Toulas et al. 1999; Delmas, Heliez et al. 2002), although it should be pointed out that these findings have not been completely replicated (Bernhard, McKenna et al. 1998). While there is still some interest in combining these agents with radiotherapy, there is no conclusive evidence as yet from clinical studies to support this.

There are agents available which target Raf and MAPK (MEK) in various stages of clinical development and some pre-clinical data to suggest a possible benefit in combination with radiation. Therapeutic targeting of PI3K and Akt has proved more difficult and the progress of a number of compounds has been limited by specificity and / or toxicity. This is disappointing as Akt is often activated in response to radiation in the *in vitro* setting and inhibiting this pathway with the laboratory based agents wortmannin and/or LY294002 has been shown to have a beneficial effect in combination with radiation in many cell systems (reviewed by (Graham and Olson 2007)). mTOR, however, which is downstream of Akt, and controls the translation of proteins for cell cycle progression and cell proliferation, is perhaps a more promising target. mTOR inhibitors are currently under evaluation, particularly in endometrial cancer and glioblastoma, both of which commonly possess mutations in PTEN (Sansal and Sellers 2004). There is

certainly *in vitro* and *in vivo* data in support of a combination approach with the latter (Eshleman, Carlson et al. 2002) so it will be of interest to see if the proposed clinical studies corroborate this.

1.4.2 Angiogenic factors

Blood vessels are essential for tumour growth and metastasis. Hence, this has prompted interest in therapeutic targeting of the tumour vasculature. The major strategy to date in this endeavour has involved inhibition of vascular endothelial growth factor (VEGF) signalling. VEGF, the key pro-angiogenic growth factor, is secreted by almost all solid tumours (Leung, Cachianes et al. 1989), and acts through VEGFR1, VEGFR2, or VEGFR3 receptors on endothelial cells (Millauer, Wizigmann-Voos et al. 1993), and disruption of this signalling pathway can lead to interference with the formation of new blood vessels.

While preclinical data has suggested that VEGF is upregulated in tumours in response to radiotherapy and that this might be a cause of radioresistance (Gorski, Beckett et al. 1999), VEGF inhibition did not have a radiosensitising effect on cancer cells grown in culture (Gorski, Beckett et al. 1999), although testing this hypothesis was not particularly straightforward as VEGFR is often not expressed on cell lines *in vitro*. However, VEGF inhibition did sensitise endothelial cells, such as HUVECs (which do express VEGFR), to radiation (Gorski, Beckett et al. 1999; Geng, Donnelly et al. 2001). Whether this *in vitro* effect would translate into tumour growth delay *in vivo* was an important question, since an effect on the tumour vasculature could potentially render cells more radioresistant as a result of hypoxia. It has already been highlighted that the generation of highly reactive oxygen intermediates is critical to radiation induced DNA damage and that reduced oxygen levels limit the damaging effect of radiation. Another important consideration that must be taken into account is that the late toxicity from radiotherapy treatment is often attributed to small vessel ischaemia and/or necrosis.

Based on these aspects of tumour microenvironment, the combinaton of anti-angiogenic agents and radiation would seem to be counter-intuitive. However, the concept of a “normalisation window” has been raised as a potential sensitising mechanism (Jain 2005). Essentially, in normal cells there is a balance

between pro- and anti- angiogenic factors resulting in an organised and efficient vascular network and blood supply. In tumours this balance is shifted towards pro-angiogenic factors that promote the formation of an abnormal vasculature with an inefficient supply of oxygen and nutrients. Then, after administration of anti-angiogenic agents, the balance is restored allowing the normalisation of the vascular network with improved perfusion and oxygen delivery. An outline of the connection between radiation and the vasculature is shown in **Figure 5**.

Accordingly, the use of anti-angiogenic agents increased radiosensitivity in a variety of tumour xenograft models such as colon, lung, prostate, breast and ovary (Lee, Heijn et al. 2000; Cao, Albert et al. 2006; Williams, Telfer et al. 2007) (Nieder, Wiedenmann et al. 2007). Most of these studies used fractionated radiotherapy combined with a VEGFR antibody and radiosensitivity was assessed by tumour growth delay experiments. In some cases, the researchers specifically examined the vasculature and found that hypoxia levels decreased after VEGFR inhibition due to vessel regression and reorganisation, primarily as a result of reduced vascular permeability and interstitial fluid pressure (Lee, Heijn et al. 2000). It has been proposed that the "normalisation window" may only be temporary and therefore the timing or sequencing of VEGFR inhibition and radiation may be critical. While some xenograft studies have demonstrated that sequencing can influence the outcome of combined treatment (Williams, Telfer et al. 2004), this does not always appear to be the case, although it is important to remember that the vasculature and tumour environment in a simplified xenograft model is likely to be very different from that of an established more complex human malignancy.

Several Phase I and II trials have been reported detailing the combination of radiotherapy with bevacizumab (avastin), a recombinant humanised monoclonal antibody that binds to and inhibits the biological activity of VEGFR, with some promising results. Cediranib (recentin), a potent orally active tyrosine kinase inhibitor which targets all three VEGF receptors, is also under investigation. As yet, there is no Phase III data on either drug available, so the role of anti-angiogenesis agents in combination with ionising radiation has still to be established. If there is a proven benefit, it will be some time before there is mature data available on the potential long term toxicity.

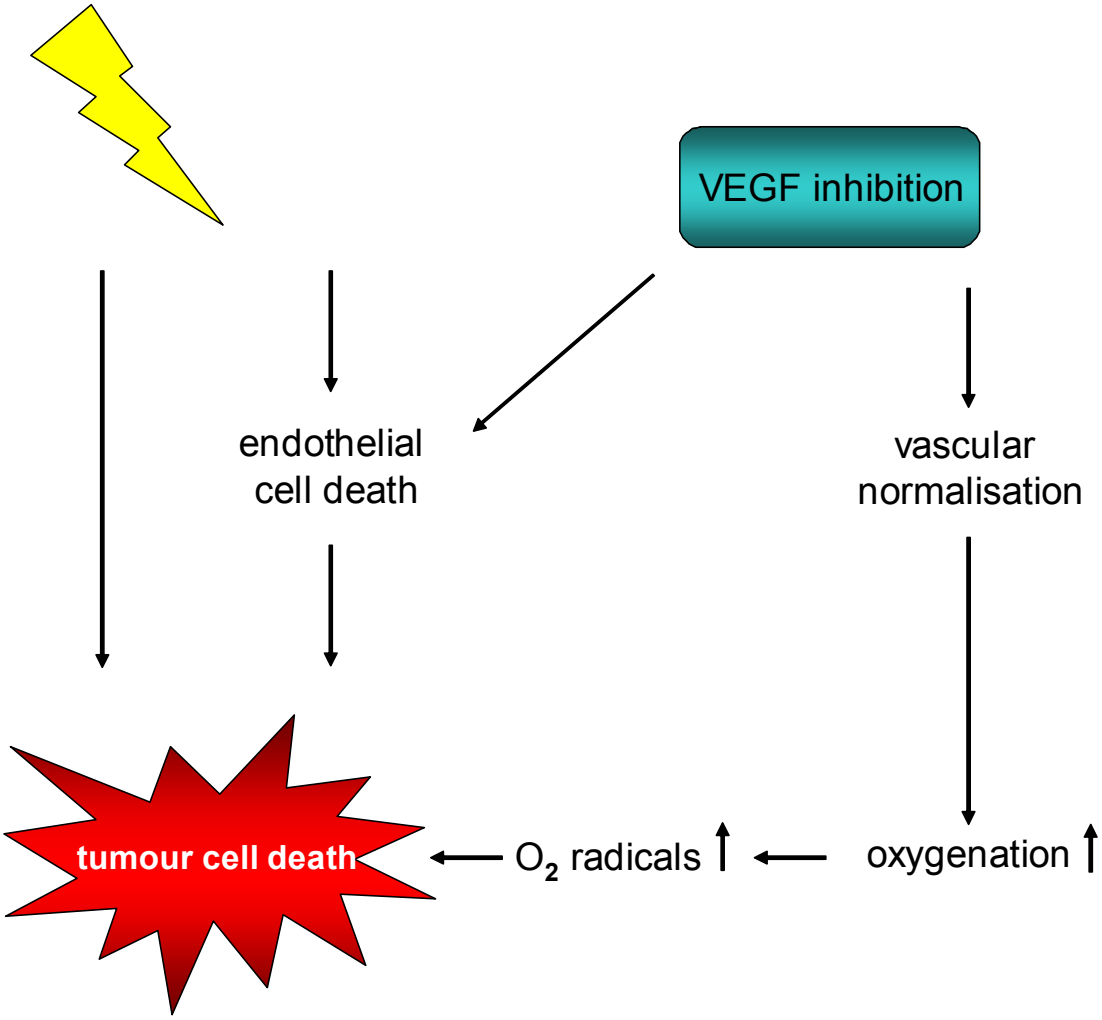


Figure 5 - Mechanistic interaction of radiotherapy and VEGF inhibition
VEGF inhibition enhances radiothearpy induced endothelial cell death. It can also induce “vascular normalisation”, leading to improved tumour oxygenation and sensitisation to radiation.

1.4.3 DNA damage response proteins

Radiotherapy and many anti-cancer chemotherapeutic agents cause cell death by inducing DNA damage and it is recognised that functioning DNA repair pathways can act as mechanisms of radioresistance or drug resistance (Gatti and Zunino 2005; Borst, Rottenberg et al. 2008). In addition, over-expression of these repair pathways has been reported in tumours (Wharton, McNelis et al. 2000; Staibano, Pepe et al. 2005). Thus the potential to inhibit DNA repair enzymes as a means of improving existing cancer treatments has been the focus of drug development programmes. Recent attention has been directed at the poly(ADP-ribose)polymerases (PARPs), a family of highly conserved enzymes found in plants and animals, that were first described over 40 years ago (Chambon, Weill et al. 1963). PARP-1 is the most abundant form of the enzyme and is found in the nucleus, acting as a "molecular nick sensor" to signal DNA single strand breaks (SSBs) and assist in their repair (de Murcia and de Murcia 1994). Commonly used chemotherapeutic agents, such as alkylating agents and camptothecins damage DNA by causing SSBs and for this reason PARP inhibitors were first developed as chemopotentiating agents.

The publication of paired Nature papers in 2005 has revolutionised the clinical research approach to PARP inhibitors (Bryant, Schultz et al. 2005; Farmer, McCabe et al. 2005). These preclinical cell line and xenograft experiments demonstrated that cells with loss of the homologous recombination DSB repair pathway were hypersensitive to blockade of SSB repair with a PARP inhibitor. The proposed mechanism of cytotoxicity is that blocking SSB repair leads to the formation of DSBs at the replication fork. In normal or heterozygote cells the DSB mechanisms can repair the lesion and DNA replication and cell division continues. However, in cells where DSB repair is not functional, such as those with a homozygous mutation in BRCA1 or BRCA2, the loss of two DNA repair pathways causes synthetic lethality and cell death (Evers, Drost et al. 2008). This observation was then tested clinically in Phase II studies in BRCA1 or BRCA2 mutant carriers with breast and ovarian cancer, respectively, with promising results (Audeh, Penson et al. 2009; Tutt, Robson et al. 2009). Although these findings from small non-randomised studies require confirmation in a phase III trial, they do suggest that PARP inhibitors may be useful not only as

chemopotentiators, but they may have an additional exciting role as single agents in appropriately molecularly profiled patients.

Over 20 years ago, tests with early experimental drugs capable of affecting PARP activity showed that addition of PARP inhibitors resulted in enhancement of radiation cell kill *in vitro* (Thraves, Mossman et al. 1985; Kelland, Burgess et al. 1988) and that this was based on DNA repair inhibition (Bowman, White et al. 1998; Veuger, Curtin et al. 2004). Further development of these agents was limited by concerns over excessive toxicity but interest in combining radiation with PARP inhibitors has resurfaced again based on both the general excitement currently surrounding these agents and the introduction of more specific and well tolerated compounds. In keeping with earlier observations, an increase in radiosensitivity has again been demonstrated with these newer agents in both the *in vitro* and *in vivo* settings (Veuger, Curtin et al. 2003; Brock, Milas et al. 2004; Calabrese, Almassy et al. 2004; Albert, Cao et al. 2007; Donawho, Luo et al. 2007; Thomas, Calabrese et al. 2007; Clarke, Mulligan et al. 2009). Clinical trials are now underway investigating the use of PARP inhibitors in combination with radiotherapy so it will be of interest to see if this approach is of clinical benefit. It will also be important to ascertain whether normal tissue toxicity varies depending on the site treated. As the effects of PARP inhibition are more profound in proliferating cells (underlining the model that secondarily induced DSBs resulting from conversion from SSBs are responsible for much of the cell kill as shown in **Figure 6**), organs where there is continuous cell turnover for normal function such as the bowel may be more susceptible than, for example, the brain with its much slower proliferative index. Hence, it may be necessary to select target sites carefully. A further cautionary note is that it has been suggested that blocking a DNA repair pathway may lead to the accumulation of DNA damage. This stems largely from pharmacodynamic data in a Phase I study where the authors found an increase in γH2AX foci in sampled surrogate tissue from patients (Fong, Boss et al. 2009). It is likely that any excess damage could be repaired when pharmacological inhibition is removed but it must be borne in mind that long-term use of a PARP inhibitor could lead to secondary harmful DNA lesions, and possibly further malignancy. Although this is unlikely to significantly impact on a course of radiotherapy lasting a number of weeks, the precise risk has yet to be quantified.

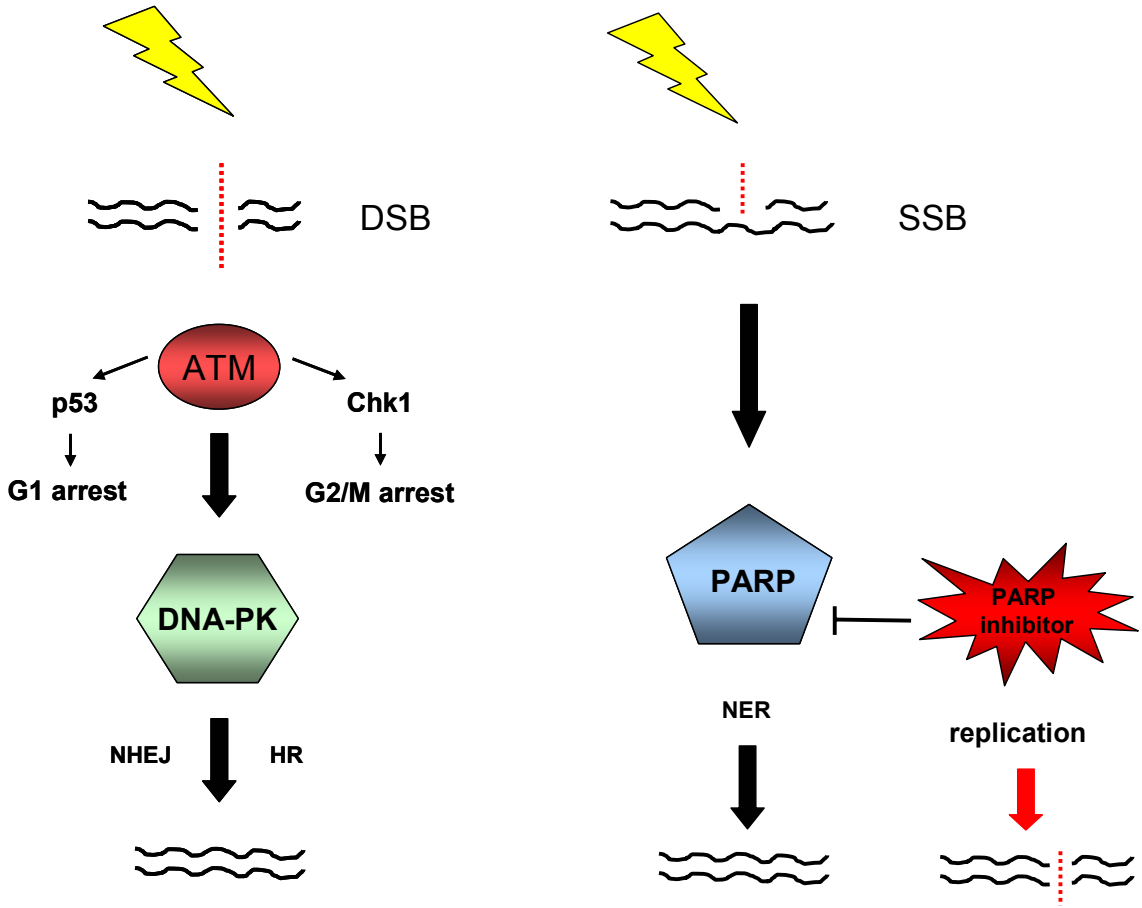


Figure 6 - DNA repair mechanisms in response to ionising radiation
Ionising radiation induces DNA damage, including double strand breaks (DSBs) and single strand break (SSBs) which need to be repaired to maintain genomic integrity and prevent cell death. DSBs are detected by the ataxia telangectasia mutated (ATM) kinase (in conjunction with other molecules), which phosphorylates a series of downstream effectors in a signalling cascade that coordinates cell cycle arrest and DNA repair. In cells with functional p53 tumour suppressor protein, G1 cell cycle arrest may occur. In cells where p53 is dysfunctional, ATM acts through checkpoint kinase-1 (Chk1) to induce a G2/M arrest. The repair of DSBs occurs predominantly through the process of non-homologous end-joining (NHEJ) mediated by the catalytic subunit of the DNA-dependent protein kinase (DNA-PK). Alternatively, DSBs may be repaired by homologous recombination (HR). The repair of SSBs occurs principally through the process of nucleotide excision repair (NER). The poly(ADP-ribose)polymerase (PARP) family of enzymes regulate this process. However, if PARP is inhibited, then SSBs that persist at the time of cell replication will be converted to DSBs, increasing the total number of DSBs that must be repaired.

1.4.4 Apoptosis modulating proteins

The inherent or acquired capacity of tumour cells to resist apoptosis is one of the hallmarks of cancer, and is considered to be an important factor in determining treatment resistance. Even though radiation can induce apoptosis in cancer cells, the significance of apoptosis in terms of tumour response and clonogenicity has yet to be fully elucidated, as already mentioned in Section 1.2.2. Nevertheless, it is clear that in some cases the active modulation of apoptosis can influence radiation reponses to a substantial degree.

Apoptosis can be triggered by either intrinsic or extrinsic pathways as shown in **Figure 7**. Most apoptotic stimuli, including ionising radiation, depend on the intrinsic mitochondrial pathway. DNA damage sensors, such as p53, induce permeability of the mitochondrial outer membrane permitting the release of pro-apoptotic factors into the cytosol (reviewed by (Wang, 2001)). The Bcl-2 protein family mediates mitochondrial permeability and is therefore the key regulator of most apoptotic pathways. Anti-apoptotic Bcl-2 and its family members are frequently overexpressed in a variey of cancers and increased levels often correlate with radio and chemoresistance (Belka and Budach 2002; Wang, Yang et al. 2003; Hara, Omura-Minamisawa et al. 2005). Despite this, and the availability of anti-Bcl-2 agents, the number of clinical trials exploring knockdown of Bcl-2 in combination with radiation has been limited so far.

In contrast to Bcl-2 family members which control the intrinsic pathway, death receptors such as TRAIL activate the extrinsic apoptosis pathway. This receptor is of particular interest because of its proven capacity to induce apoptosis in cancer cells, but relative lack of normal tissue toxicity in preclinical animal models (Ashkenazi, Pai et al. 1999; Walczak, Miller et al. 1999). While of potential value as a therapeutic modality in itself, TRAIL is an excellent candidate for combination therapy, since TRAIL and radiation are known to activate distinct apoptosis signalling pathways. Intriguingly, however, it has been proposed that DNA damage can lead to upregulation or sensitisation of the TRAIL complex, which may explain the synergistic effects seen in experimental systems when TRAIL activation is combined with radiation (Belka, Schmid et al. 2001). The two main approaches involve recombinant human TRAIL ligand or

agonistic TRAIL receptor antibodies. Phase I/II trials with the former are now ongoing. It will be fascinating to determine whether the promising *in vitro* and *in vivo* results of TRAIL modulating agents combined with radiation are reproduced in the more complicated setting of human malignancies.

1.4.5 Other potential radiosensitising targets

In recent years, an increasing number of targeted agents have become available for preclinical evaluation as radiosensitisers. Histone deacetylase inhibitors, for example, which regulate chromatin structure and gene expression, appear be useful in combination with radiotherapy (Camphausen and Tofilon 2007). This may be mediated by effects on DNA repair as well as gene transcription. DNA repair may also be modulated by targeting checkpoint kinases (Chk) such as Chk1 and Chk2. These kinases are activated in response to ionising radiation and result in cell cycle block, which allows time for effective DNA repair. Inhibiting Chk1 and/or Chk2 can promote premature progression through the cell cycle, minimising the time available for DNA repair. The loss of adequate checkpoint control may also lead to increased cell death by mitotic catastrophe (Riesterer, Matsumoto et al. 2009). Full discussion of all the remaining potential radiosensitising targets, including growth factor receptors other than EGFR, is beyond the scope of this review.

One final point on this subject, however, is that the cellular response to radiation is highly complex. As yet, it is unclear which targeted approach is likely to be most successful in combination with radiation in the clinical setting and which patients are most likely to respond to specific targeted agents. Clinical trials must be rationally designed and ideally incorporate the use of biomarkers or predictive assays in order to select out those individuals most likely to benefit. In addition, although there are numerous options for developing novel therapeutics, the potential input from cross talk and alternate signalling mechanisms, many of which are not fully recognised or fully understood, may lead to substantially reduced efficacy. One way of combating this particular problem might be to employ therapeutic agents with a wide range of targets. Heat shock proteins, which act as molecular chaperones for a variety of essential proteins, may be a useful target in this regard.

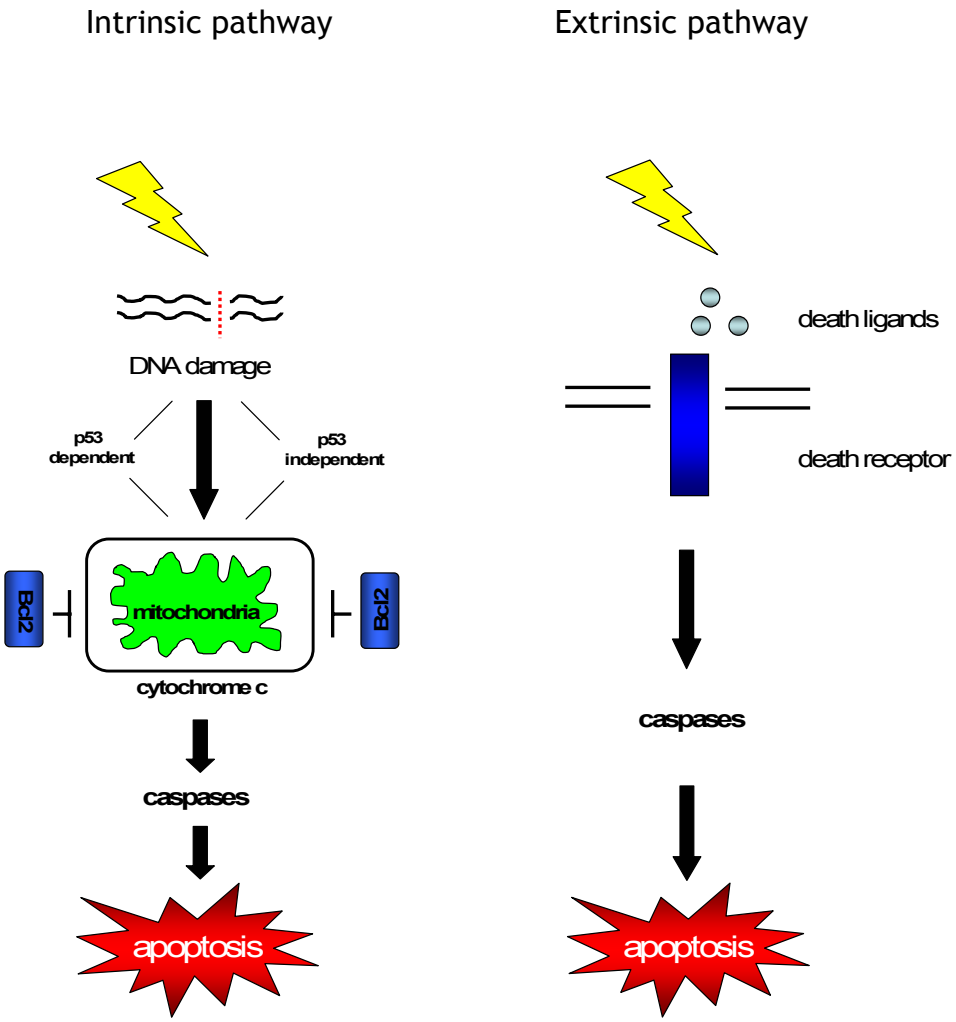


Figure 7 - Ionising radiation induces apoptosis via intrinsic and extrinsic pathways
Induction of apoptosis by ionising radiation may proceed through the intrinsic or extrinsic pathway. In the intrinsic pathway DNA damage is sensed by either p53-dependent or p53-independent pathways. This leads to altered mitochondrial permeabilisation and the release of cytochrome C, which induces caspase activity. Alternatively, ionising radiation may trigger death ligand binding to death receptors, such as the TRAIL family, which sets in motion a caspase cascade. Interestingly, DNA damage may lead to upregulation of death receptors which may sensitise cells to radiation.

1.5 Focal adhesion kinase (FAK)

Focal adhesions are sites where cells contact the extracellular matrix (ECM). These sites are enriched in integrins and in various cytoskeletal and signalling proteins, including talin, vinculin, and paxillin (**Figure 8**). As such, focal adhesions are thought to function as connections between the actin cytoskeleton and the ECM thereby conferring structural integrity as well as transmitting critical signals from the external environment to the nucleus. Following its discovery as a focal adhesion protein that was phosphorylated in response to Src transformation (Hanks, Calalb et al. 1992; Schaller, Borgman et al. 1992), focal adhesion kinase (FAK) has emerged as a key signalling component at these complex dynamic sites. Since then, extensive research has revealed that FAK plays an integral role in normal development and FAK has been implicated in cancer.

1.5.1 FAK structure and function

FAK is a 125 kDa non-receptor protein tyrosine kinase that does not span or associate directly with the plasma membrane. It is structurally distinct from most other non-receptor tyrosine kinases, which generally consist of a tyrosine kinase domain, protein-protein, protein-lipid and other interaction domains; the most common protein-protein interaction domains being SH2 and SH3 domains (reviewed by (Hubbard and Till 2000)). FAK contains a central catalytic domain flanked by an amino-terminal functional ezrin radixin myosin (FERM) domain and a carboxy-terminal Focal Adhesion Targeting domain (FAT) (**Figure 9**), but unlike other non-receptor tyrosine kinases, FAK does not contain SH2 or SH3 domains and only the catalytic domain shows significant homology to any other protein tyrosine kinase.

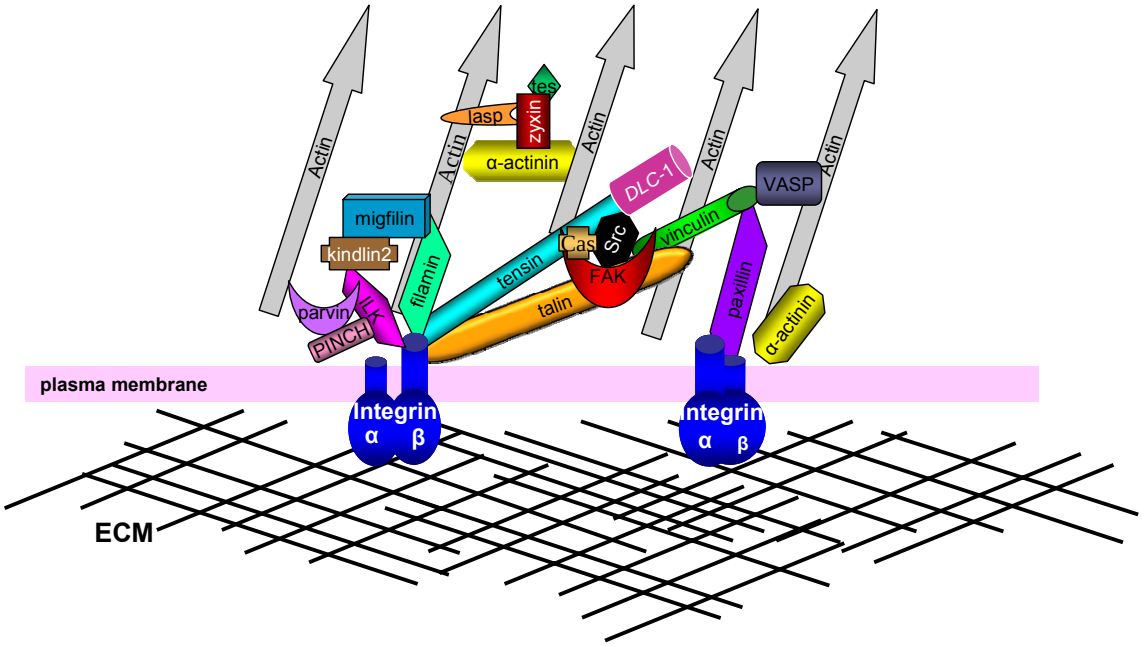


Figure 8 - Schematic diagram of focal adhesion components
Transmembrane integrin receptors transduce signals from the extracellular matirx leading to recruitment of large multi-protein complexes. These complexes contain key signalling proteins such as FAK (shown in red), Src, paxillin and talin and provide a link between integrin receptor signalling and the actin cytoskeleton.

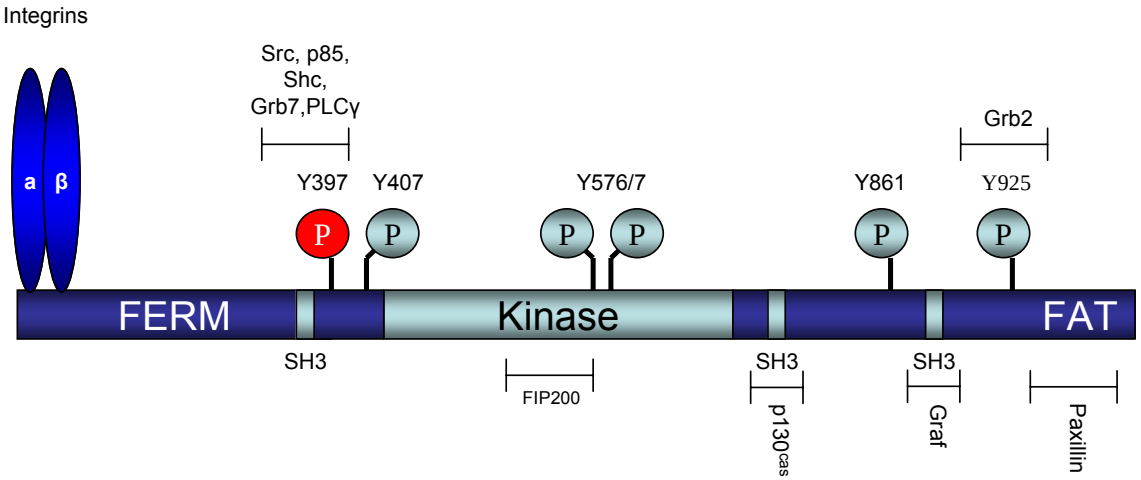


Figure 9 - Schematic representation of FAK structure
FAK contains a central catalytic domain flanked by a large amino-terminal domain and a carboxy-terminal portion. These contain multiple binding sites for proteins such as Src, paxillin and talin. FAK contains six sites of tyrosine phosphorylation with the major site of autophosphorylation at FAK-Y397 highlighted in red. The focal adhesion targeting (FAT) domain is located in the carboxy-terminus and is common to both FAK and FRNK.

1.5.1.1 FAK phosphorylation

Six sites of tyrosine (Y) phosphorylation have been identified on FAK, namely Y397, Y407, Y576, Y577, Y861 and Y925 (**Figure 9**) (Schaller, Hildebrand et al. 1994; Calalb, Polte et al. 1995; Schlaepfer and Hunter 1996). Upon adhesion to the ECM, these sites are phosphorylated in cells, leading to the recruitment of various adaptor molecules and protein kinases, which form complexes with FAK.

FAK-Y397 and FAK-Y407 lie within the amino-terminal half of FAK, and by the use of recombinant FAK protein, FAK-Y397 has been identified as the major site of autophosphorylation (Calalb, Polte et al. 1995). After integrin engagement, phosphorylated FAK-Y397 forms an SH2 binding site for several proteins such as Src family kinases, the regulatory p85 subunit of phosphoinositide (PI) 3-Kinase, the Src homologous and collagen-like protein (Shc), and phospholipase C gamma (PLCγ) (Schaller, Hildebrand et al. 1994; Xing, Chen et al. 1994; Chen, Appeddu et al. 1996; Schlaepfer, Jones et al. 1998; Schaller 2001). Recruitment of Src to phosphorylated FAK-Y397 promotes Src dependent phosphorylation of the remaining tyrosine residues of FAK (reviewed in (Schlaepfer, Hauck et al. 1999)). The resultant increase in phosphorylation further enhances FAK activity, placing FAK at the central hub of multiple signalling pathways.

FAK-Y576/7 lies within the kinase activation loop and phosphorylation of both of these residues is required for maximal FAK kinase activity. However, mutagenesis studies in which FAK-Y397 has been replaced by a phenylalanine (FAK-Y397F) demonstrated that this site is also essential for maximum FAK kinase activity and downstream signalling (Calalb, Polte et al. 1995). To further complicate matters, *in vitro* kinase assays have demonstrated a role for FAK-Y576/7 in the regulation of transphosphorylation of FAK on Y397, with mutation of these residues impairing FAK-Y397 autophosphorylation (Leu and Maa 2002). The same study also highlighted that FAK-Y861 contributes to the activation of FAK-Y397 by regulating its cis-phosphorylation (Leu and Maa 2002). Collectively, this data suggests that FAK-Y397 phosphorylation may be differentially regulated by phosphorylation of distinct tyrosine sites within FAK. FAK-Y861 is heavily phosphorylated in cells by Src family kinases and *in vitro* by c-Src (Brunton, Avizienyte et al. 2005) and is thought to be important in mediating the anti-apoptotic activity of FAK (Abu-Ghazaleh, Kabir et al. 2001). FAK-Y925 is also

phosphorylated by Src upon integrin stimulation. This creates an SH2 binding site for the small adaptor protein Grb2, which links FAK signalling to the MAPK pathway (Schlaepfer, Hanks et al. 1994; Schlaepfer, Hauck et al. 1999).

In addition, FAK can be phosphorylated on multiple serine residues located within the carboxy-terminal domain (S732, S840, S843 and S910), in response to various stimuli (Ma, Richardson et al. 2001). Despite being less well studied than tyrosine phosphorylation, there is evidence which implicates FAK serine phosphorylation in both FAK regulation and the control of biological processes such as microtubule organisation (Xie, Sanada et al. 2003).

1.5.1.2 FAK amino-terminal (FERM) domain

The functional ezrin radixin myosin (FERM) domains serve as a link between the actin cytoskeleton and the plasma membrane and they are primarily involved in mediating both intra-molecular and inter-molecular protein-protein interactions (reviewed by (Sun, Robb et al. 2002)). The FAK FERM domain, for example, links FAK to growth factor receptors at the plasma membrane. However, the FERM domain also regulates FAK activity and phosphorylation. Early studies found that FERM domain truncations of FAK yield proteins with increased tyrosine phosphorylation and associated activity (Chan, Kanner et al. 1994; Schlaepfer and Hunter 1996), thus supporting a negative regulatory role in FAK activation. As the FAK FERM domain can bind the FAK kinase domain and inhibit FAK activity in-trans, a direct auto-inhibitory mechanism for FAK regulation was proposed. Structural data was recently published confirming this (Lietha, Cai et al. 2007). However, the precise mechanisms which drive FAK-FERM-protein interactions have yet to be elucidated. Intriguingly, it seems that the FAK FERM domain has yet another function. Under conditions of cellular stress, the FERM domain can facilitate nuclear translocation of FAK and promote binding to p53 and MDM2 (Lim, Chen et al. 2008). As p53 can inhibit transcription of FAK (Golubovskaya, Finch et al. 2008), this newly described property of the FAK FERM domain may have profound consequences on the level of FAK within the cell. The subject of the FAK–FERM–p53 interaction will be revisited in Section 1.5.3.

1.5.1.3 FAK kinase domain

The activation of FAK requires autophosphorylation of FAK-Y397, recruitment and binding of Src, and subsequent Src induced phosphorylation of FAK-Y576/7 (Calalb, Polte et al. 1995; Schlaepfer, Hauck et al. 1999). Autophosphorylation has been shown to be temporally related to the clustering of integrin receptors in the cell membrane that occurs when these receptors bind their ligand. The integrin receptor clustering triggers a conformational change in FAK that alters the interaction of the FERM domain with the kinase domain. FAK also undergoes autophosphorylation in response to activation by certain growth factor receptors in adherent cells. A number of studies have demonstrated that point mutations within the kinase domain may lead to FAK activation, for example, mutation of residues FAK-K578 and FAK-K581 to a glutamic acid (E) result in a "super-FAK" protein which displays adhesion independent kinase activity (Gabarra-Niecko, Keely et al. 2002). Although mutagenesis experiments are of interest in the laboratory setting, it is notable that spontaneous mutations in FAK have not been reported in any species. While there is evidence that FAK may be upregulated by gene amplification, often the precise mechanism underlying elevated FAK expression and/or activity is unknown. What is known is that the kinase activity of FAK can be regulated in many different ways. These include direct binding of the negative regulator FAK-interacting protein of 200kDa (FIP200) to the kinase domain (Abbi, Ueda et al. 2002) and an intra-molecular interaction involving the FAK-FERM domain and the kinase domain (Lietha, Cai et al. 2007), as outlined above in Section 1.5.1.2. FAK activity can also be regulated by protein tyrosine phosphatases (PTPs) which act upstream to regulate FAK-Y397 phosphorylation in response to integrin stimulation (Zheng, Xia et al. 2009).

1.5.1.4 FAK carboxy-terminal portion

The carboxy-terminal portion of FAK contains two proline rich regions, two paxillin binding sequences, a talin binding sequence, the focal adhesion targeting domain (FAT) and FAK-Y861 and FAK-Y925 (**Figure 9**). The first proline rich region was identified as a binding site for the adaptor protein p130 v-Crk associated tyrosine kinase substrate ($p130^{cas}$) (Polte and Hanks 1995) and c-Cbl-associated protein (CAP) (Ribon, Herrera et al. 1998). The second was identified

as a binding site for the GTPase-activating protein for rho associated with focal adhesion kinase (GRAF) (Hildebrand, Taylor et al. 1996). These proteins are thought to play important roles in the formation of actin stress fibres and cytoskeletal organisation (Hildebrand, Taylor et al. 1996; Honda, Oda et al. 1998; Ribon, Herrera et al. 1998). Binding of this domain to paxillin and talin (Chen, Appeddu et al. 1995; Tachibana, Sato et al. 1995), on the other hand, aids in the recruitment of FAK to adhesion complexes (Tachibana, Sato et al. 1995; Hagel, George et al. 2002).

1.5.1.5 FAK isoforms

An amino-terminally truncated form of FAK, known as the FAK related non-kinase domain (FRNK), can be expressed independently of the entire FAK protein through alternative splicing of the *fak* gene (Schaller, Borgman et al. 1993). FRNK has been shown to function as a physiological dominant negative inhibitor of FAK (Richardson and Parsons 1996). Other isoforms of FAK created by alternative splicing have also been identified, mainly in rat brain tissue (Burgaya, Toutant et al. 1997). Some of these proteins have increased autophosphorylation activity, suggesting that FAK may have specific properties in neurones.

1.5.1.6 FAK family members

Pyk 2, also referred to as cell adhesion kinase-beta (CAK-ß), related adhesion focal tyrosine kinase (RAFTK), or calcium dependent protein tyrosine kinase (CADTK), is the only other FAK family member to be identified (Lev, Moreno et al. 1995). FAK and Pyk2 share significant sequence homology, the majority of which comprises the kinase domain, the conserved tyrosine residues in the surrounding region responsible for binding SH2 containing proteins, the proline rich regions responsible for binding SH3 containing proteins and the paxillin binding sequences (reviewed by (Lipinski and Loftus 2010)). Pyk2 has four tyrosine phosphorylation sites (Y402, Y579/80 and Y881) at analogous positions to that of FAK (Y397, Y576/7 and Y925), and upon phosphorylation recruits Src family kinases. Although Pyk2 is stimulated by cell adhesion, it can also be activated in response to ultra violet (UV) light, hyper-osmotic shock, and changes in intracellular calcium levels (reviewed by (Lipinski and Loftus 2010)).

Interestingly, while FAK is ubiquitously expressed, Pyk2 is often highly expressed in cells of the haemopoetic and nervous system. What is more, Pyk 2 is not typically associated with focal adhesions, and instead is often localised in punctate spots in the peri-nuclear region. The altered distribution and localisation of FAK and Pyk2 at both tissue and cellular levels would suggest that they perhaps have alternate properties. However, in mouse embryo fibroblasts genetically deficient for FAK, Pyk2 can compensate for certain aspects of FAK signalling.

1.5.2 FAK is involved in multiple intracellular processes

By virtue of its scaffolding and kinase domains, FAK interacts with an array of cellular proteins and is involved in a myriad of crucial cell signalling pathways. Work from the FAK knockout mouse model, for example, has demonstrated that FAK is necessary for vascular development and gastrulation in early embryonic life (Ilic, Furuta et al. 1995). In addition, FAK has been implicated in cancer. This is not surprising as an accumulating body of data links FAK with processes that are perturbed in malignancy, such as proliferation, migration, invasion, and angiogenesis. In this section the evidence associating FAK with these processes will be discussed, with emphasis on the relevance to human cancer.

1.5.2.1 Proliferation and cell cycle

FAK has been implicated in the control of cell proliferation where it is thought to mediate signals from growth factor receptors and integrins to regulate cell cycle progression. In fibroblasts it has been shown that FAK controls the G1-S transition and this is linked to its ability to regulate transcription of cyclin D1 (Zhao, Pestell et al. 2001; Zhao, Bian et al. 2003). There is also evidence that FAK can regulate proliferation in cancer cells. In glioblastoma, for example, FAK controls exit of cells from G1 phase of the cell cycle via a mechanism involving cyclin D1 and p27 (Ding, Grammer et al. 2005). This property of FAK appears to be cell line dependent as FAK is often dispensable for proliferation, at least on tissue culture plastic. This does not mean that FAK will be dispensable for anchorage – independent proliferation in the same cell line. In fact, in this thesis we will show that FAK is a prerequisite for 3D, but not 2D, proliferation in a

murine squamous cell carcinoma cell line, and that the kinase activity of FAK is integral to this phenomenon.

1.5.2.2 Migration

Cell migration is central to many processes such as embryonic development, wound healing and cancer invasion and metastasis. Initial studies in FAK deficient mouse embryo fibroblasts (MEFs) illustrated that loss of FAK led to impaired focal adhesion turnover and cell migration (Ilic, Furuta et al. 1995). Subsequently, specific loss of FAK from mouse keratinocytes was also shown to result in impaired migration (McLean, Komiyama et al. 2004). Re-introduction of kinase dead FAK (FAK-KD) into FAK deficient MEFs was not able to restore migration on fibronectin (Sieg, Hauck et al. 2000), indicating that the kinase activity of FAK is required. In contrast, growth factor stimulated migration was not dependent on the kinase activity of FAK, but was instead dependent on interactions within the N-terminal domain of FAK (Sieg, Hauck et al. 2000). Further work suggested that the ability of FAK to control cell migration was related to regulation of cell polarity (Tilghman, Slack-Davis et al. 2005), although this did not specify whether the effect was kinase-dependent or independent. Recent data produced in this laboratory indicates that the ability of FAK to control directional cancer cell migration is indeed mediated via cell polarity. However, this is a complex process that involves both FAK kinase activity (personal communication – Valerie Brunton) and FAK adaptor functions (Serrels, Sandilands et al, submitted). Interestingly, these processes can be overcome by inhibition of FAK kinase activity (personal communication – Valerie Brunton). This finding is significant as it may support the potential use of FAK kinase inhibitors to prevent tumour spread.

1.5.2.3 Invasion

Cell invasion through matrix and tissue barriers requires increased cell motility and usually also regulated proteolytic degradation of the matrix. FAK has been shown to mediate the invasive activity of both normal and v-Src transformed fibroblasts through reconstituted 3D matrix (Hauck, Hsia et al. 2002; Hsia, Mitra et al. 2003). While v-Src can promote transformation in FAK -/- cells and overcome the motility defects associated with FAK deficiency (Hsia, Mitra et al.

2003; Moissoglu and Gelman 2003), these cells are defective in invading 3D matrices *in vitro* compared with v-Src transformed FAK containing cells (Hsia, Mitra et al. 2003). Moreover, dominant-negative inhibition of FAK activity blocked v-Src stimulated cell invasion and experimental metastases in nude mice without effects on *in vitro* motility (Hauck, Hsia et al. 2002). This was associated with decreased levels of matrix metalloproteinase (MMP) expression and activity. Further studies using either dominant negative or anti-sense inhibition of FAK have also shown reduced expression of MMPs in a range of carcinoma cell lines (Shibata, Kikkawa et al. 1998; Hauck, Hsia et al. 2002; Zhang, Thant et al. 2002), implying a general requirement for FAK for the production and activity of MMPs that have cancer-associated matrix-degrading activities.

Taken together, it appears to be the case that FAK regulates cell motility and invasion by distinct pathways; that is, by promoting the dynamic regulation of focal adhesions and peripheral actin structures during migration, as well as by MMP-mediated matrix degradation (reviewed by (Tomar and Schlaepfer 2009)). Interestingly, it is now quite well recognised that cancer cells can invade a 3D matrix by both MMP-dependent and MMP-independent mechanisms – referred to as mesenchymal-like and amoeboid-like invasion, respectively (reviewed by (Tomar and Schlaepfer 2009)). The precise function(s) of FAK during these specific mechanisms of invasion have yet to be elucidated. Since FAK has been implicated in MMP expression, and in the transition from an epithelial to a mesenchymal phenotype in cancer cells (Avizienyte, Wyke et al. 2002), it is likely that FAK is required for mesenchymal-like invasion. By contrast, the amoeboid form of invasion, which does not seem to be dependent on integrin-mediated adhesions within the matrix, may not critically depend on the presence of FAK. However, it has recently been suggested that there is a significant degree of overlap and that aspects of both forms of invasion may be required for sufficient execution of the metastatic process (Sanz-Moreno, Gadea et al. 2008). Exactly how FAK would fit into this model has yet to be determined.

1.5.2.4 Angiogenesis

The importance of angiogenesis in tumour growth and metastasis has already been outlined in Section 1.4.2. Development of blood vessels requires FAK

expression, especially in endothelial cells. This is clear from work in the FAK knockout mouse model, where endothelial cell dysfunction contributed to the embryonic lethal phenotype (Ilic, Furuta et al. 1995). Similarly, conditional knockout of FAK in mouse endothelial cells led to impaired angiogenesis (Shen, Park et al. 2005). It has been shown that FAK expression in endothelial cells is not only necessary for the process of formation of new vessels, but is also required for the stability of vascular networks and survival of endothelial cells (Shen, Park et al. 2005; Braren, Hu et al. 2006). In contrast, overexpression of FAK in vascular endothelial cells directly promotes angiogenesis in transgenic mice (Peng, Ueda et al. 2004). In support of a role for FAK in promoting angiogenesis in human cancer, a positive correlation between microvascular density in tumour specimens and level of FAK expression was found in a clinical study of breast cancer patients (Huang, Khoe et al. 2007). Interestingly, it has been shown that FAK regulates VEGF expression and induces an angiogenic switch in cancers (Mitra, Mikolon et al. 2006). This property of FAK was mediated via FAK-Y925 signalling through the MAPK pathway. This suggests that FAK kinase activity is important in mediating tumour angiogenesis. In keeping with this data, there is some evidence from *in vivo* studies that small molecule inhibitors of FAK activation cause a reduction in the microvascular network (Halder, Lin et al. 2007; Roberts, Ung et al. 2008), suggesting that FAK kinase inhibitors may be useful anti-angiogenesis agents.

1.5.2.5 Cell survival

FAK was first linked to cell survival by Frisch et al., who reported that FAK was able to suppress suspension-induced cell death, known as anoikis, in kidney epithelial cells (Frisch, Vuori et al. 1996). This ability of FAK to protect cells from anoikis depended on both phosphorylation of the Y397 site and FAK kinase activity. Overexpression of FAK also protected the same epithelial cells from UV light induced cell death (Chan, Lai et al. 1999). Further work showed that increased FAK expression in a leukaemia cell line suppressed cell death, specifically apoptosis. Again, FAK phosphorylation and kinase activity were required for the observed effects on cell survival (Sonoda, Matsumoto et al. 2000). Since then, induction of apoptosis has been demonstrated in a range of cell lines by attenuating FAK activity either by antibody injection (Hungerford, Compton et al. 1996; Liu, Yang et al. 2002), antisense oligonucleotides (Xu,

Owens et al. 1996) or expression of the isolated focal adhesion targeting domain (Ilic, Almeida et al. 1998; Jones, Machado et al. 2001; van de Water, Houtepen et al. 2001).

FAK mediated pro-survival functions are thought to play an important role in cancer cell survival. Certainly, adhesion-dependent survival, which is often lost in tumour cells, is regulated by FAK (Frisch, Vuori et al. 1996) (Xu, Owens et al. 1996). Hence, increased FAK expression may encourage matrix-independent survival of cancer cells, which then promotes or contributes to invasion and metastasis. Preliminary evidence in support of this in cancer cells stemmed from work in pancreatic and oral cancer cell lines in which FAK knockdown with siRNA resulted in anoikis (Duxbury, Ito et al. 2004; Zhang, Lu et al. 2004). Since then, modulation of FAK protein levels has been shown to influence the sensitivity of tumour cells to various chemotherapeutic agents (Smith, Golubovskaya et al.; Duxbury, Ito et al. 2003; Halder, Landen et al. 2005; Halder, Kamat et al. 2006; Wu, Yuan et al. 2006; Halder, Lin et al. 2007; Hochwald, Nyberg et al. 2009; Yu-Ying, Zhan-Xiang et al. 2009). Is this simply linked to the ability of FAK to protect against anoikis? In support of this, signalling from integrins is involved in protection from drug induced apoptosis in lung cancer cells (Sethi, Rintoul et al. 1999). However, in leukaemia cells, the increased sensitisation of cells to an anthracycline following reduced FAK expression is not dependent on the ECM (Recher, Ysebaert et al. 2004).

One way in which FAK may contribute to survival (which may be ECM-dependent or independent) is by binding to receptor-interacting protein (RIP) (Kurenova, Xu et al. 2004), a major component of the death receptor complex. It seems that FAK may be able to sequester RIP from the death receptor complex, thereby suppressing apoptosis (Kurenova, Xu et al. 2004). Whether this is a common mechanism for promoting cancer cell survival has yet to be determined. In addition to suppressing apoptosis, there is growing evidence that FAK may contribute to other cytoprotective mechanisms such as autophagy (Sandilands, Serrels et al., submitted) and inhibiting senescence (Pylayeva, Gillen et al. 2009). Even more intriguingly is the potential for FAK to influence cell survival by regulating p53. This will be discussed in more detail in Section 1.5.3.3.

1.5.3 FAK interacts with the tumour suppressor protein p53

1.5.3.1 The role of p53 in cancer

The discovery of p53 in 1979 marked the beginning of a dynamic era in cancer research. Since then, p53 has been established as a key tumour suppressor; with several lines of evidence demonstrating the importance of p53 in the prevention of tumour development. It is well recognised that the p53 gene is mutated in approximately 50% of all human cancers and that this is typically a late event. In the majority of the remaining cancers which retain the ability to express a normal p53 protein, there are defects in the ability to respond to p53. Moreover, germline mutations in p53 give rise to the Li-Fraumeni cancer susceptibility syndrome (Varley 2003). In the laboratory setting, mice deficient in p53 develop cancer, typically lymphoma, within the first six months of life (Donehower, Harvey et al. 1992; Jacks, Remington et al. 1994; Purdie, Harrison et al. 1994). Additionally, in a number of experimental animal models, loss of p53 has been shown to cooperate with a wide variety of other oncogenic events to promote and accelerate cancer development.

p53 is particularly interesting, not only in terms of the frequency in which it is mutated in malignancy, but also in that it is quite distinct from other tumour suppressors whereby a mutation typically causes loss of a specific suppressor function. On the contrary, aberrations in p53 often lead to acquired oncogenic properties, so-called “gain of function” mutations. There is abundant *in vitro* and *in vivo* data in support of this, indicating that mutant p53 proteins can mediate tumour growth, invasion, and metastasis (Dittmer, Pati et al. 1993; Lang, Iwakuma et al. 2004; Olive, Tuveson et al. 2004; Hingorani, Wang et al. 2005; Caulin, Nguyen et al. 2007). In spite of the convincing laboratory evidence linking mutant p53 proteins with more advanced malignancy, coupled with the prominence of p53 mutations in human cancers, it has proved difficult to definitively pinpoint p53 as a prognostic factor. However, this may be explained by poorly designed studies and/or inefficient means of p53 detection, particularly in the older reports.

On balance it appears that while functional normally regulated p53 is not essential for normal growth and development, as evinced by knockout mouse

models (which is perhaps surprising in view of its role as a key transcriptional regulator of cell cycle and apoptosis), it does appear to be crucial in preventing malignancy. Even more intriguingly, some mutant forms of p53 seem to actively promote a more aggressive cancer phenotype. Unsurprisingly, the prospect of targeting p53 in human cancer is an ever increasing field of research.

1.5.3.2 Targeting p53 in cancer

Therapeutic manipulation of p53 in cancer falls largely into two categories; (1) restoring p53 activation and function in tumours containing wt p53 and (2) elimination of mutant p53 or conversion of mutant p53 to wt p53. The latter can be achieved in a number of ways including viral mediated gene therapy (Bischoff, Kirn et al. 1996; Heise, Sampson-Johannes et al. 1997; Rogulski, Freytag et al. 2000) or the application of small molecules such as PRIMA-1 (and its analogues) which can restore wt p53 conformation and promote sequence-specific DNA binding, leading to transactivation of target genes (Bykov, Issaeva et al. 2002; Bykov, Issaeva et al. 2005; Bykov, Zache et al. 2005; Magrini, Russo et al. 2008; Lambert, Gorzov et al. 2009). These approaches do appear to be effective *in vitro* and *in vivo* and are now under investigation in the clinical setting.

Re-enabling the full functional capacity of p53 in cancers with deregulated wt p53 is far more challenging, not least because regulation of the p53 protein is highly complex (reviewed by (Boehme and Blattner 2009)). This occurs predominantly at post-translational level and involves a number of mechanisms, including phosphorylation, acetylation, and ubiquitination. Interestingly, p53 can influence its own regulation by transcriptional activation of the target gene MDM2, which ubiquitinates p53 and targets it for proteasome-mediated degradation (Momand, Zambetti et al. 1992). In this way, p53 and MDM2 form a negative regulatory loop that downregulates p53 expression. Of note, this feedback loop was shown to involve FAK (Lim, Chen et al. 2008), following speculation that FAK and p53 somehow interacted on a functional level. The evidence to date outlining the fascinating relationship between FAK and p53 will shortly be presented.

MDM2 is overexpressed in 5-10% of human cancers (Momand, Jung et al. 1998). Both *in vitro* and *in vivo* studies have indicated that the oncogenic activity of MDM2 is mainly attributable to its binding and degrading p53 (Montes de Oca Luna, Wagner et al. 1995; de Rozieres, Maya et al. 2000). Thus, it has been proposed that inhibiting MDM2 in a subset of human cancers should reactivate wt p53. To this end, two classes of small molecules have been identified which either disrupt MDM2-p53 binding (the most notable being the Nutlins) or inhibit MDM2 ubiquitin ligase activity. While encouraging results are being reported at the pre-clinical level (reviewed by (Wang and Sun 2010)), there are several concerns that must be borne in mind in the clinical development of MDM2 inhibitors for anti-cancer therapy. Firstly, these compounds are likely to induce p53 in normal cells and this may cause adverse effects. Secondly, MDM2 itself is likely to accumulate as a result of p53 activation and this may have deleterious effects (Gu, Zhu et al. 2009).

The first concern is not specific to MDM2 and the activation of wt p53 in normal cells must be a consideration when developing any drug that is designed to restore p53 levels in cancer cells. Indeed, it is quite well recognised that normal tissue toxicity secondary to chemotherapy and/or radiotherapy is at least in part due to p53 activation and induction of apoptosis (Komarova, Chernov et al. 1997; Jiang and Dong 2008). This knowledge has in turn prompted the development of chemical inhibitors of p53 that can be used temporarily to protect against the side effects of cancer treatments (Komarov, Komarova et al. 1999).

In order to target p53 in cancer effectively, it will be crucial to select the correct approach for each individual patient based on their tumour characteristics. This is likely to involve molecular profiling, a technique which is not routinely performed in current clinical practice. While some subsets of patients could be relatively easily identified, such as those with mutant p53 or overexpression of MDM2, some molecular profiles may be more difficult to interpret. In particular, it may not always to be appropriate to elevate tumour levels of wt p53. There is evidence that in some situations possessing wt p53 may be detrimental. This is illustrated not only by its connection with genotoxic induced normal tissue toxicity as outlined above; wt p53 can sometimes promote, rather than inhibit, tumour growth (reviewed by (Kim, Giese et al.

2009)). The exact reasons for this are unclear but merit intensive study. The paradoxical roles of p53 in cancer will be discussed further in Chapters 5 and 6.

While there is no doubt that p53 plays a critical role in protecting against malignant development, understanding exactly how p53 functions will be key to the development of therapies based on activating or mimicking p53 functions.

1.5.3.3 Understanding the FAK – p53 axis and its significance in cancer

The FAK –/- MEFs described by Ilic et al. (Ilic, Furuta et al. 1995) suggested an important functional interaction between FAK and p53, as these cells could only proliferate *in vitro* in the absence of p53. Following this, several studies in normal and/or cancer cells reported an indirect link between FAK and p53, that seemed to be important in mediating cell survival (Ilic, Almeida et al. 1998; Lewis, Truong et al. 2002; Zhang, Lu et al. 2004). Meanwhile, there were a number of reports documenting the presence of a p53 binding site on FAK suggesting a direct interaction (Golubovskaya, Kaur et al. 2004; Golubovskaya, Finch et al. 2005; Golubovskaya and Cance 2007). In keeping with this, definitive evidence of a direct FAK–p53 functional interaction emerged in 2008. The work by Lim et al. demonstrated a scaffolding role for FAK in facilitating cell survival through enhanced p53 degradation under conditions of stress (Lim, Chen et al. 2008). Intriguingly, this process requires translocation of FAK to the nucleus and is dependent on the FERM domain. In fact, it is the FERM domain that binds to p53 and stabilises the p53-MDM2 complex.

Although the authors were able to show that the FAK-p53 axis exists in human fibroblasts and A549 lung cancer cells as well as murine derived tissue, the precise relevance of this axis in terms of human malignancy is unclear. Notably, an additional stress in the form of cisplatin was required in combination with FAK knockdown in order to induce significant levels of apoptosis in fibroblasts. However, cell death was not specifically examined in any cancer cell line.

Further work has also illustrated the presence of a FAK-p53 relationship in cancer cells. Essentially, wt p53 was able to bind to and inhibit FAK promoter activity in a colorectal cancer cell line (Golubovskaya, Finch et al. 2008). Again, the functional significance of this is unclear. Interestingly, this study showed

that FAK expression was increased in human colorectal cancer tumour specimens containing p53 mutations compared to tumours with wt p53. Also, insertion of various p53 mutations into p53 -/- cancer cells failed to inhibit FAK promoter activity unlike wt p53 (Golubovskaya, Finch et al. 2008). However, a similar correlation was not seen in tissue samples from head and neck cancer (Rodrigo, Cabanillas et al. 2007).

Taken together, current evidence suggests that FAK and p53 can exert important regulatory influences over the other, but this may be tissue specific. Exactly how this relationship is related to carcinogenesis or how it affects the response to anti-cancer therapies has yet to be fully elucidated. Interestingly, it hints that lowering FAK levels may be useful in both tumours with wt 53 and mutant p53. In the case of wt p53 tumours, a reduction in FAK may promote p53 mediated apoptosis, although it is likely that another stressor is required. On the other hand, if mutant p53 allows uncontrolled transcription of FAK which then contributes to tumour cell survival, inhibiting FAK under these circumstances may also be advantageous.

However, we believe that the FAK-p53 axis is likely to be cell line and context dependent and in this thesis we will present data that challenges the view that FAK knockdown increases susceptibility to cell death in a wt p53 cancer cell line.

1.5.4 FAK as a potential target in human cancer

1.5.4.1 FAK and carcinogenesis

FAK has been implicated in a wide range of cellular processes that are deregulated in malignancy as just outlined in Section 1.5.2. In addition, elevated levels of FAK have been reported in a variety of human cancers. Confirmation of a causative role for FAK in tumour formation and progression was initially lacking, until work performed in this laboratory demonstrated that FAK was required for the initiation and development of skin cancer in a mouse model (McLean, Komiyama et al. 2004). It had already been shown using *fak* heterozygous (+/-) mice that FAK was involved in the formation of benign papillomas in response to DMBA/TPA induced topical carcinogenesis, but no effects were observed on malignant conversion, probably due to the fact that FAK protein levels were expressed in papillomas of both *fak* +/+ and *fak*+/- mice to equal levels (McLean, Brown et al. 2001). By conditionally deleting *fak* in murine keratinocytes, however, and subjecting the animals to the same topical carcinogenesis protocol, it was clear that FAK was important for both papilloma formation and conversion to squamous cell carcinoma (McLean, Komiyama et al. 2004).

Parallel *in vitro* studies of keratinocytes from this model revealed that loss of FAK expression led to aberrant cell cycle profiles and cell death (McLean, Komiyama et al. 2004). Furthermore, FAK was essential for promoting cell survival via the MAPK signalling pathway. Taken together, this data was consistent with FAK-dependent survival signalling being required for cancer development and progression, at least in this model. More recently, FAK has also been directly implicated in mouse mammary tumour development (Provenzano, Inman et al. 2008; Luo, Fan et al. 2009).

1.5.4.2 FAK and human cancer

Increased expression of FAK was first noticed in high grade and metastatic sarcomas (Weiner, Liu et al. 1994). Since then elevated FAK expression and/or activity has been reported in a variety of epithelial malignancies, including cancers of the thyroid, pancreas, prostate, cervix, rectum, head and neck, and

ovary (Owens, Xu et al. 1996; Tremblay, Hauck et al. 1996; McCormack, Brazinski et al. 1997; Lori 1998; Patricia, Xiaping et al. 1999; Cance, Harris et al. 2000; Lark, Livasy et al. 2003; Furuyama, Doi et al. 2006). Most of these studies are based on immunohistochemical or immunoblotting analysis. Hence, the precise mechanism of upregulation is not always clear. Amplification of the *fak* gene has been reported in a few cancer cell lines, and increased gene copy number are found in cells derived from head and neck cancer (Agochiya, Brunton et al. 1999). It is noteworthy that mutations in FAK have not been reported in human cancer. Elevated FAK protein expression and activity need not be regulated at the genetic level, however, as FAK is regulated by a complex range of mechanisms. These include tyrosine phosphorylation, serine/threonine phosphorylation and many protein binding interactions (reviewed by (Li, Hua et al. 2008)). In light of recent observations, the possibility of p53 mediated FAK regulation must also be considered.

Typically, the lowest levels of FAK expression are found in normal tissues with higher levels present in pre-neoplastic tissue and malignant tissue. In some situations, elevated FAK expression and/or activity has correlated with a poorer outcome (Recher, Ysebaert et al. 2004; Sood, Coffin et al. 2004; Cai, Han et al. 2009). This suggests that FAK may be a useful prognostic indicator. However, a negative correlation between FAK expression levels and overall survival is not always seen, although several studies have failed to reach statistical significance due to insufficient patient numbers. Although FAK appears to be required at slightly different stages in carcinogenesis in the conditional mouse models, it is interesting that the highest levels of FAK recorded in human tumours are often detected in metastatic disease (Cance, Harris et al. 2000; Lark, Livasy et al. 2003; Sood, Coffin et al. 2004). Based on the *in vitro* work outlined in Section 1.5.2.3 and *in vivo* orthotopic studies (van Nimwegen, Verkoeijen et al. 2005; Mitra, Lim et al. 2006), FAK can contribute towards metastasis by promoting a pro-survival invasive phenotype that would permit passage through the ECM and entry to the lymphatic supply or vasculature. Does this mean that FAK is necessary for the maintenance and growth of metastatic deposits? Most of the evidence to date would suggest that this is the case but it is worth noting that in certain cases FAK levels appear to be lower in metastatic tumours compared with the primary (Ayaki, Komatsu et al. 2001).

Further clarification is still required regarding the precise functions of FAK in human cancer. However, in view of increasing circumstantial evidence linking elevated FAK protein levels with a range of human tumour types, coupled with the incriminating role for FAK in promoting carcinogenesis in conditional mouse models, FAK has been selected as a potential target at which to direct novel anti-cancer therapies. The next section will focus on the available anti-FAK agents and their progress in the clinical setting.

1.5.4.3 Anti-FAK targeted agents

Although FAK appears to have important scaffolding functions that are distinct from its catalytic activity, much of the work to date has focussed on inhibiting the latter. This is probably because kinases are more readily druggable targets. Two FAK kinase inhibitors have dominated the recent preclinical literature: PF-562,271 (Pfizer) and TAE226 (Novartis).

PF-562,271 is an ATP-competitive and reversible inhibitor, with selective activity against FAK and the related family member Pyk2. While this drug does not significantly inhibit proliferation or apoptosis *in vitro*, it does significantly inhibit migration *in vitro* and growth *in vivo* (Slack-Davis, Martin et al. 2007; Bagi, Roberts et al. 2008; Roberts, Ung et al. 2008; Bagi, Christensen et al. 2009) Interestingly, some of the effects *in vivo* are thought to be due to anti-angiogenic effects. It has been suggested that compensatory signalling through Pyk2 may circumvent the anti-angiogenic features of selective FAK inhibition (Weis, Lim et al. 2008). As a dual inhibitor of both FAK and Pyk2, this particular function should not be compromised and may explain the promising actions of this drug. In the clinical setting, Phase I testing has now been undertaken in advanced solid malignancies. The drug was well tolerated with mild fatigue and gastrointestinal toxicity as the most common side effects. Although no partial responses were noted, disease stability was reported in several patients (Schwock, Dhani et al. 2010). This is encouraging in a heavily pre-treated population.

TAE226 is a dual-specificity inhibitor targeting both growth promoting and adhesion pathways through its activity against the insulin-like growth factor receptor (IGF-1R) and FAK, respectively. While some very promising results have

been observed in the laboratory setting (Halder, Lin et al. 2007; Liu, LaFortune et al. 2007; Shi, Hjelmeland et al. 2007; Beierle, Trujillo et al. 2008; Golubovskaya, Virnig et al. 2008; Roberts, Ung et al. 2008; Wang, Fukazawa et al. 2008; Watanabe, Takaoka et al. 2008; Sakurama, Noma et al. 2009), the overlapping activities of this agent make it difficult to decipher the precise mechanisms underlying any therapeutic efficacy. This drug has profound effects on glucose metabolism, most likely due to inhibition of IGF-1R, and for that reason did not enter clinical development. A number of second generation compounds with enhanced selectivity for FAK have been developed and are due to undergo Phase I trials.

Although siRNA and anti-sense oligonucleotides can be useful tools with which to inhibit FAK in the laboratory, this type of approach is unlikely to prove useful in the clinic. Instead, it may be appropriate to develop protein-protein interaction inhibitors designed to interrupt FAK regulatory connections. These agents are typically much more complicated to develop than kinase inhibitors. Based on evidence to date, possibilities include FAK-RIP and FAK-p53 specific inhibitors. While there is more evidence in support of the latter, we will present data that suggests an indirect, but kinase-dependent relationship between FAK and p53. It may be that the catalytic versus adaptor functions of FAK vary in a cell line dependent or tissue dependent manner. More information is necessary regarding the specific kinase or scaffolding roles of FAK and how their respective attenuation will contribute to the effectiveness of anti-FAK therapies.

Regardless of the exact underlying mechanism, be it reduced FAK levels, reduced FAK activity, or FAK-p53 deregulation, there is no convincing evidence to date that FAK inhibition alone in cancer cells will be sufficiently efficacious. For that reason, it is likely that modulation of FAK levels and/or activity will need to be combined with traditional anti-cancer modalities, or possibly other targeted agents. There is *in vitro* and *in vivo* evidence demonstrating that FAK knockdown can sensitise cells to cytotoxic chemotherapy (Smith, Golubovskaya et al.; Duxbury, Ito et al. 2003; Halder, Landen et al. 2005; Halder, Kamat et al. 2006; Wu, Yuan et al. 2006; Halder, Lin et al. 2007; Hochwald, Nyberg et al. 2009; Yu-Ying, Zhan-Xiang et al. 2009).

However, several interesting theories exist which may support the use of FAK inhibitors as a means of single agent therapy. Firstly, inhibiting FAK may prevent the progression of pre-invasive conditions to frank malignancy. Secondly, anti-FAK targeted agents may be useful in the adjuvant setting as an anti-metastatic tool. Testing both of these hypotheses would require carefully designed clinical trials with extended periods of follow up.

1.6 Exploring the role of cell adhesion signalling molecules in radiation survival

1.6.1 FAK and radiation

At the time of initiating this study, there was no published data available on FAK and cellular radiation survival. It had been documented that phosphorylation of FAK was induced following exposure to ionising radiation *in vitro* (Beinke, Van. Beuningen et al. 2003), although this report featured only a single cell line and it was unclear whether this phenomenon was simply a transient stress response as no clonogenicity studies were performed. We suspected that FAK may play a role in radiosensitivity in view of its multiple properties within the cell, particularly the accumulating evidence linking FAK to cell survival. In addition, a small body of work was emerging, predominantly from a laboratory in Germany, on the concept of ECM mediated radioresistance, whereby radiation can promote cell survival through cell adhesion signalling pathways. Initially this work did not focus specifically on FAK, but as FAK is an important central point in cell adhesion signalling, it was only a matter of time before FAK was investigated in this context. In fact, data has now been published which demonstrates that FAK knockdown increases radiosensitivity in human pancreatic cancer cell lines (Cordes, Frick et al. 2007). While this was the first time FAK had been definitively linked with radiation survival, the authors were unable to provide a clear explanation of the underlying mechanism(s), prompting a number of questions about how exactly FAK exerted this effect. However, Src, which interacts closely with FAK as previously outlined, has been subject to more rigorous investigation as a potential radiomodulator (discussed below).

1.6.2 Src and radiation

Increased Src activity has also been reported in response to ionising radiation (Li, Hosoi et al. 2006; Park, Park et al. 2006; Dittmann, Mayer et al. 2008; Kim, Byun et al. 2008). The first documented investigation of the role of Src in radiosensitivity was performed in endothelial cells using the Src inhibitor SU6656 (Cuneo, Geng et al. 2006). A significant reduction in radiation survival was observed on clonogenicity assays but the dose employed was far in excess of that required to inhibit Src and the authors acknowledged that this effect was probably due to the off target anti-angiogenic effects of this drug. However, a number of recent studies have consistently shown inhibition of Src activity increases radiosensitivity (Contessa, Abell et al. 2006; Dittmann, Mayer et al. 2008; Dittmann, Mayer et al. 2009; Purnell, Mack et al. 2009). What is more, one of these studies incorporated Src siRNA, indicating that the effects seen with a Src kinase inhibitor are indeed due to inhibition of Src, and not mediated by inhibition of kinases other than Src (Dittmann, Mayer et al. 2009). In terms of mechanism, it was recently shown that Src activation in response to ionising radiation promotes internalisation of EGFR, leading to nuclear translocation of EGFR and stimulation of DNA repair processes (Dittmann, Mayer et al. 2008; Dittmann, Mayer et al. 2009)

1.6.3 ECM mediated radioresistance

It has been recognised for some time that irradiating cells on tissue culture plastic coated with ECM components such as fibronectin can increase radioresistance (reviewed by (Sandfort, Koch et al. 2007)). This phenomenon occurs in both fibroblasts and tumour cells and can often be inhibited by PI3K inhibitors, suggesting reliance on adhesion mediated Akt signalling. Although interesting, the precise relevance of these findings are unclear, other than perhaps hinting at the employment of PI3K inhibitors in combination with radiotherapy. However, it has already been pointed out that these agents have proved troublesome to adapt in the clinical setting. More recently, work has focussed on integrin-linked kinase (ILK), a widely expressed serine/threonine protein kinase bound to the cytoplasmic tail of ß-integrins (Hannigan, Leung-Hagesteijn et al. 1996). Intriguingly, although ILK typically promotes cell survival

in various biological contexts, ILK -/- fibroblasts are more radioresistant than their ILK +/+ counterparts on 2D (Cordes 2004; Eke, Sandfort et al. 2007; Hess, Estrugo et al. 2007). However, this effect is lost when the fibroblasts are grown in a 3D matrix (Hehlgans, Eke et al. 2008). This indicates that ECM mediated signalling may be crucial to radiation survival in fibroblasts and suggests that adhesion linked kinases may have different roles in different cellular environments. While there is some evidence that ILK can also affect radiosensitivity in cancer cells (Eke, Sandfort et al. 2007), and that this might be related to EGFR signalling, whether this will translate into an effect *in vivo* has yet to be examined.

1.7 Summary and Thesis Aims

Radiotherapy is an essential component of cancer therapy but treatment is not always curative. Much effort is currently directed at improving the delivery of radiotherapy by increasingly sophisticated spatial and dosimetric methods in order to improve patient outcome. However, treatment failure may not necessarily result from inadequate dose or coverage of the tumour bed. Ionising radiation can promote activation of receptor and non-receptor tyrosine kinases leading to profound cytoprotective responses such as increased DNA repair, proliferation and reduced apoptosis (Cuneo, Geng et al. 2006; Li, Hosoi et al. 2006; Park, Park et al. 2006; Cosaceanu, Budiu et al. 2007; Dittmann, Mayer et al. 2008; Dittmann, Mayer et al. 2009; Meyn, Munshi et al. 2009). Since these responses contribute to cellular radioresistance, which can limit the effectiveness of radiotherapy in cancer treatment, understanding these phenomena may provide new molecular targets for radiosensitisation and ultimately improve cancer outcomes. The epidermal growth factor receptor is the most extensively studied tyrosine kinase in this field. Strong preclinical evidence relating to the capacity of EGFR inhibition to enhance the antitumour activity of ionizing radiation has translated into the clinical setting based on the results of a pivotal Phase III trial in head and neck cancer (Bonner, Harari et al. 2006; Bonner, Harari et al. 2009).

The role of other tyrosine kinases, especially non-receptor tyrosine kinases, however, is less clear. FAK is a highly complex signalling molecule that has been

implicated in a wide range of crucial cellular processes that are perturbed in malignancy including proliferation, cell cycle, adhesion and invasion (reviewed by (Brunton and Frame 2008; Zhao and Guan 2009)). While FAK has been linked with resistance to certain cytotoxic chemotherapeutics (Smith, Golubovskaya et al.; Duxbury, Ito et al. 2003; Halder, Landen et al. 2005; Halder, Kamat et al. 2006; Wu, Yuan et al. 2006; Halder, Lin et al. 2007; Hochwald, Nyberg et al. 2009; Yu-Ying, Zhan-Xiang et al. 2009) a link between FAK and radiation is more tenuous. Upregulation of FAK activity following exposure to radiation has been reported (Beinke, Van. Beuningen et al. 2003), but whether this observation represented a transient stress response or signified that FAK may be critical in mediating radiation survival was largely unexplored. During the course of this work, other groups did attempt to answer this question. Recently, for example, it was shown that transient FAK knockdown with siRNA sensitised pancreatic cancer cells to radiation (Cordes, Frick et al. 2007). However, there was no clear explanation of a possible underlying mechanism. Hence there was much scope at the start of (and indeed during) this project to investigate whether FAK is important in radiosensitivity and, if so, to elicit the underlying mechanism.

FAK regulates an array of important cellular functions that are often deregulated in cancer. In view of the roles for FAK in mediating adhesion signalling, cell cycle, cell survival, and angiogenesis, we wished to address whether FAK was involved in radiosensitivity. A schematic diagram outlining the potential ways in which FAK may influence radiosensitivity is shown in **Figure 10**.

In this thesis, we set out to:

1. Generate novel squamous cell carcinoma cells with inducible *fak* deletion in order to study the role of FAK in cellular radiation response.

2. Evaluate whether the presence or absence of FAK influences radiosensitivity *in vitro* and *in vivo*.

3. Dissect out the underlying mechanisms linking FAK to cellular radiation response.

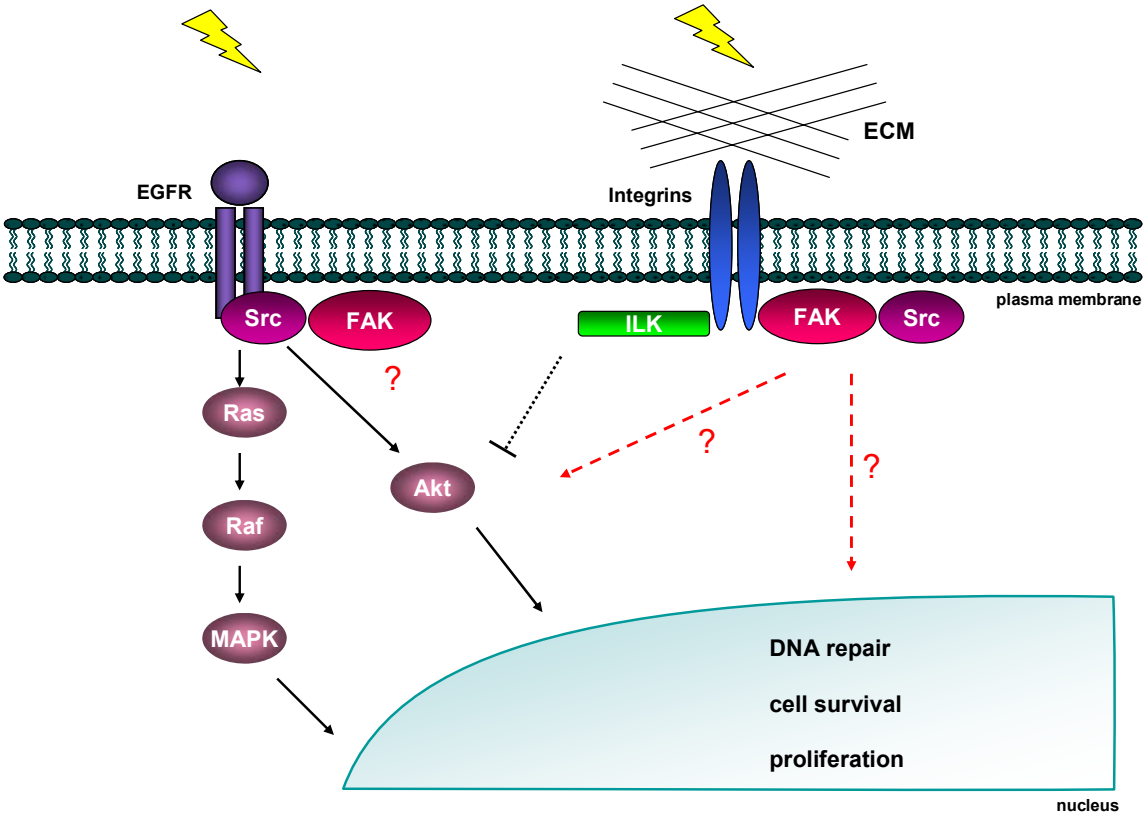


Figure 10 - FAK may be an important mediator of radiation survival
At the time of initiating this study, it was known that EGFR could influence radiation survival by activation of the Ras/PI3K pathways. Also, there was data that indicated involvement of integrin signalling pathways in the cellular response to radiation. ILK, for example, was shown to inhibit signalling through the PI3K pathway and promote radiation induced cell death. Following on from work demonstrating a role for Src in mediating radiosensitivity in cancer cells, we hypothesised that FAK, an important mediator of both growth factor receptor signalling and integrin signalling, could also influence radiosensitivity.

Chapter 2

Materials and Methods

2 Materials and Methods

2.1 Materials

2.1.1 Animal experiments

Supplier: Sigma Chemical Co, Poole, UK
Dimethylbenzanthracene (DMBA)
4-hydroxy-tamoxifen (4-OHT)
Sunflower oil
12-o-tetradecanoyl-13-phorbol acetate (TPA)

2.1.2 Cell culture reagents

Supplier: Amaxa, Lonza, Cologne, Germany
Nucleofector solution V

Supplier: Autogen Bioclear, Wiltshire, UK
Foetal bovine serum

Supplier: BD Biosciences, Oxford, UK
Fibronectin

Supplier: Beatson Institute Central Services
Sterile PBS
Sterile PBS/1mM EDTA

Supplier: Clonetics, Slough, UK
Keratinocyte basal medium
Keratinocyte growth medium singlequotes

Supplier: Dharmacon, Abgene Ltd., Epsom, UK
Dharmafect transfection reagent
Smartpool siRNA pools (p53, p21, scrambled)

Supplier: Invitrogen, Paisley, UK

Dulbecco's modified Eagle's medium (DMEM)

Eagle's Minimal Essential Medium with Earles salts (MEM)

HBSS

200mM L-glutamine

MEM vitamins (100x)

MEM non-essential amino acids (NEAA) (100x)

2.5% trypsin solution

Dispase II

Supplier: Merck Biosciences, Sussex, UK

Hygromycin B

Supplier: R&D systems, Abingdon, UK

Methylcellulose

Supplier: Sigma Chemical Co, Poole, UK

Dimethyl sulphoxide (DMSO)

Sodium pyruvate

2.1.3 Cell culture plasticware

Supplier: BD Biosciences, Oxford, UK

Cell strainers (40 and 70 μm)

Collagen I coated 60mm dishes

FACS polypropylene tubes

Falcon tissue culture dishes (60mm, 90mm and 120mm)

Falcon tissue culture plates (6, 12, 24 and 96 well)

Supplier: Corning, Amsterdam, The Netherlands

Transwell inserts

Supplier: TCS biologicals, Botolph Clayton, UK

Nunc tissue culture flasks

Nunc cryotubes

2.1.4 Cell based assays and inhibitors

2.1.4.1 Cell cycle analysis

Supplier: Qiagen, Crawley, UK
RNase A

Supplier: Sigma Chemical Co, Poole, UK
Propidium iodide (PI)

2.1.4.2 Cell proliferation assay

Supplier: Sigma Chemical Co, Poole, UK
Dimethyl sulphoxide (DMSO)
Thiazolyl blue tetrazolium bromide (MTT)

2.1.4.3 Clonogenic assay

Supplier: Sigma Chemical Co, Poole, UK
Crystal violet solution

2.1.4.4 Inhibitors

Supplier: Bristol Myers Squibb, **Middlesex, UK**
Dasatinib

Supplier: Pfizer, *Groton, CT, USA*
PF-562,271

Supplier: Sigma Chemical Co, Poole, UK
Actinomycin-D
Cisplatin
Doxorubicin
4-hydroxy-tamoxifen
Staurosporine
Taxol
Topotecan

2.1.5 Protein detection

2.1.5.1 Immunofluorescence

Supplier: Invitrogen, Paisley, UK
Anti-mouse/rabbit AlexaFluor conjugated secondary antibodies

Supplier: Sigma Chemical Co, Poole, UK
TRITC phalloidin
Paraformaldehyde

Supplier: Vector Laboratories Ltd, Peterborough, UK
Vectashield mounting medium
Vectashield mounting medium with DAPI

Supplier: Olympus UK Ltd, Hertfordshire, UK
Olympus FV1000 Confocal microscope

Primary antibodies

Supplier: New England Biolabs, Hertfordshire, UK
Anti-FAK rabbit Ab
Anti-LC3 rabbit Ab
Anti-paxillin mouse Ab
Anti- p53 (IC12) mouse Ab
Anti-phospho-Src-Y416 rabbit Ab

Supplier: Upstate (Millipore), Hampshire, UK
Anti-phospho-γH2AX mouse Ab

2.1.5.2 Immunohistochemistry

Supplier: Dako UK Ltd, Ely, UK
DAKO Envision kit ™ (mouse and rabbit)

Supplier: Olympus UK Ltd, Hertfordshire, UK
Olympus BX51 microscope

Supplier: Sigma Chemical Co, Poole, UK
Sodium citrate

Primary antibodies

Supplier: Abcam, Cambridge, UK
Anti-K14 mouse Ab

Supplier: Autogen Bioclear, Wiltshire, UK
Anti-p21 (M-19) mouse Ab

Supplier: New England Biolabs, Hertfordshire, UK
Anti-FAK rabbit Ab

Supplier: R&D systems, Abingdon, UK
Anti-activated-Caspase 3 rabbit Ab

Supplier: Thermo Fisher Scientific, Loughborough, UK
Anti-Ki67 rabbit Ab

Supplier: Vector Laboratories Ltd, Peterborough, UK
Anti-p53 (CM-5) mouse Ab

2.1.5.3 Western Blot and Immunoprecipitation

Supplier: Beckman Coulter UK Ltd, Buckinghamshire, UK
Beckman DU® 650 spectrophotometer

Supplier: Bertin Technologies, Provence, France
Precellys 24 homogeniser
Precellys CK14 homogeniser tubes

Supplier: Chemicon International, Harrow, UK
Re-blot Plus Strong antibody stripping solution

Supplier: GE Healthcare, Little Chalfont, UK

ECL reagent

Full range molecular weight rainbow ™ marker

Supplier: Genetic Research Instrumentation, Dunmow, UK

Atto protein electrophoresis apparatus

Supplier: Invitrogen, Paisley, UK

NuPAGE® Bis-Tris gels

NuPAGE® Electrophoresis Apparatus

NUPAGE® LDS Sample Buffer (4x)

NuPAGE® MOPS Buffer

Supplier: Jencons, Leighton Buzzard, UK

Wet blotting apparatus

Supplier: New England Biolabs, Hertfordshire, UK

Anti-mouse/horseradish peroxidase conjugate

Anti-rabbit/horseradish peroxidase conjugate

Supplier: PERBIO, Glasgow, UK

Micro BCA ™ protein assay kit

Supplier: Schleicher and Schuell, London, UK

Nitrocellulose membrane

Supplier: Sigma Chemical Co, Poole, UK

Ammonium persulphate (APS)

0.1% (v/v) aprotinin

Bovine serum albumin (BSA)

NP-40

2 mM phenylmethylsulponyl fluoride

Protein G sepharose beads

Sodium deoxycholate

Sodium fluoride

Sodium orthovanadate

TEMED
T-PER reagent
Triton X-100
Tween 20

Supplier: Whatman, Maidstone, UK
3MM filter paper

Primary antibodies

Supplier: Abcam, Cambridge, UK
Anti-K14 mouse Ab

Supplier: Autogen bioclear, Wiltshire, UK
Anti-p21 (SC-421) rabbit Ab

Supplier: Chemicon International, Harrow, UK
Anti-FAK (clone 4.47) agarose conjugated mouse Ab
Anti-MDM2 rabbit Ab

Supplier: Invitrogen, Paisley, UK
Anti-phospho-FAK-Y861 rabbit Ab

Supplier: New England Biolabs, Hertfordshire, UK
Anti- Akt rabbit Ab
Anti-ERK rabbit Ab
Anti-FAK rabbit Ab
Anti-LC3 rabbit Ab
Anti-myc tag 9B11 mouse Ab
Anti-Src rabbit Ab
Anti-paxillin rabbit Ab
Anti-PARP(cleaved) mouse Ab
Anti-p53 (IC12) mouse Ab
Anti-phospho-Akt-S473 rabbit Ab
Anti-phospho-ERK-S42/S44 rabbit Ab
Anti-phospho-FAK-Y576 rabbit Ab

Anti-phospho-FAK-Y925 rabbit Ab
Anti-phospho-Src-Y416 rabbit Ab
Anti-phospho-Pyk2-Y402 rabbit Ab
Anti-PUMA(α/ß) rabbit Ab
Anti-Pyk2 rabbit Ab

Supplier: Sigma Chemical Co, Poole, UK
Anti-ß-actin mouse Ab
Anti-γ-tubulin mouse Ab

Supplier: Transduction Laboratories, BD Biosciences, Oxford, UK
Anti-phospho-FAK-Y397 mouse Ab

2.1.6 Molecular biology techniques

Supplier: Appleton Woods, Birmingham, UK
96 well plates

Supplier: *Bio-Rad, Hertfordshire, UK*
DNA Engine ® thermal cycler
DNA Engine ® thermal gradient cycler

*Supplier***:** *New England Biolabs, Hertfordshire, UK*
DyNAmo SybrGreen qRT-PCR kit
Hyperladder IV molecular weight ladder

Supplier: PERBIO, Glasgow, UK
Mastermix PCR mix (22.5µl aliquots)

Supplier: Sigma Chemical Co, Poole, UK
Agarose
Ethidium bromide
Magnesium chloride
NP-40
Potassium chloride

Proteinase K
Tween 20

Supplier: Qiagen, Crawley, UK
miRNeasy mini RNA extraction
Superscript First-Strand cDNA synthesis kit
QIAquick Gel purification Kit
QIAquick PCR purification Kit
Quantitect qRT-PCR validated primer pair assays

Supplier: VH Bio, Gateshead, UK
PCR / Sequencing primers

2.1.7 Stock solutions and buffers

2.1.7.1 Cell culture solutions

Cell culture medium – SCC 1.1 and SCC 7.1 cells
1x MEM supplemented with:
10% FBS
2mM L-glutamine
1% Non Essential Amino Acids
1% MEM vitamins
1% Sodium Pyruvate

Cell culture medium – 83320 cells, FAK +/+ and FAK -/- MEFs
1x DMEM supplemented with:
2mM L-glutamine
10% FBS

Trypsin solution
0.25% trypsin in sterile PBS/1mM EDTA

2.1.7.2 DNA and protein extraction / elution buffers

DNA extraction buffer

50mM KCl

10mM Tris (pH 7.4)

2.5mM $MgCl_2$

0.45% NP-40

0.45% Tween 20

1mg/ml Proteinase K

RIPA buffer

50mM Tris/HCl, pH 7.4

150mM NaCl

Triton X-100

1% Sodium deoxycholate

1% NP40

5mM EGTA

100µM sodium orthovanadate

1mM PMSF

10µg/ml aprotinin

100mM Sodium fluoride

10µg/ml leupeptin

T-PER protein extraction buffer

T-PER supplemented with:

100µM sodium orthovanadate

1mM PMSF

10µg/ml aprotinin

100mM Sodium fluoride

10µg/ml leupeptin

2.1.7.3 Western blotting – gels and electrophoresis buffers

Acrylamide gel – 7%

10ml 30% acrylamide

15ml Tris pH 8.8

15ml H_2O
400µl 10% SDS
375µl 10% APS
20µl TEMED

Stacker gel
3.2ml 30% acrylamide
2.5ml Tris pH 6.8
14ml H2O
200µl 10% SDS
200µl 10% APS
20µl TEMED

Sample buffer – 2x

800µl 2-mercaptoethanol
1.3ml Tris pH 6.8
2ml glycerol
5ml 10% SDS
1.3ml H_2O
bromophenol blue to colour

Tank buffer - 10x
0.05M Tris
0.05M glycine
0.1% SDS

Transfer Buffer
50mM Tris
40mM glycine
0.04% SDS
20% methanol

Wash buffer
0.2% Tween 20 in Tris Base Solution

2.2 Methods

2.2.1 General animal husbandry

Immune compromised CD-1 mice (Charles River, Edinburgh, UK) were housed in the animal unit at the Beatson Institute for Cancer Research. They were maintained in filter cages and all procedures performed according to the Home Office project licence conditions, rules and regulations. For drug experiments, all agents were prepared in a sterile environment and autoclaved where appropriate. For radiation experiments, the animals were irradiated in a specialised mouse holding chamber which allowed a large degree of immobilisation whilst keeping the animals safe with minimal distress. 5Gy γ-irradiation was applied from a cobalt ($_{60}$Co) source (Alcyon II teletherapy unit, General Electric, France) at a distance of 80cm. The typical dose rate was 1 – 1.20 Gy/minute. Breeding and maintenance of K14*Cre*ER FAK$^{flox/flox}$ transgenic FVB mice is described by McLean et al. (McLean, Komiyama et al. 2004).

2.2.2 Routine cell culture and generation of cell lines

2.2.2.1 Routine cell culture

The cell lines were grown in appropriate growth media and maintained in a dry 5% CO_2 incubator at 37^{o}C. To subculture adherent cells the medium was removed by aspiration, the monolayer rinsed with PBS, followed with 10% trypsin/PE solution. Upon detachment the cells were resuspended in media, counted, and transferred into tissue culture flasks or flasks. Cells were typically treated one day after plating unless stated otherwise.

2.2.2.2 Generation of primary murine keratinocyte cultures

Primary murine keratinocytes were isolated from the tail skin of adult mice. The skin from the tail was removed and placed in dispase (8mg/ml in PBS) overnight at 4°C. The dermis and epidermis were separated and the dermis incubated with trypsin at 37°C for one hour. Tryspin was inactivated using DMEM + 20% FBS and the resulting cell suspension filtered through a 75μM cell strainer (Becton Dickinson, Oxford, UK). Cells were pelleted by centrifugation

at 1000 rpm for five minutes, washed in PBS, pelleted again, and resuspended in keratinocyte growth media (KGM, Clonetics). Cells were plated onto collagen-I coated 60mm tissue culture dishes (Becton Dickinson, Oxford, UK), and cultured in a humidified incubator at 37°C / 3% CO_2.

2.2.2.3 Isolation of squamous carcinoma cells from mouse tumours

Squamous carcinoma cells (SCC) were isolated from chemically induced skin tumours removed from K14*Cre*ER FAK$^{flox/flox}$ transgenic FVB mice. Skin carcinomas were induced using a two-stage chemical carcinogenesis protocol as previously described (McLean, Komiyama et al. 2004). Briefly, six week old mice were subjected to one topical application of 7,12-dimethylbenz(a)anthracene (DMBA, Sigma Chemical Co., Poole, UK), followed by twice weekly topical applications of 12-o-tetradecanoyl-phorbol-13-acetate (TPA, Sigma Chemical Co., Poole, UK) for a period of 20 weeks. This treatment schedule gave rise to benign papillomas approximately 6-8 weeks after the first treatment, with subsequent progression of a small proportion of papillomas to invasive squamous cell carcinomas from 15 weeks onwards. Following surgical excision of carcinomas, small tissue pieces were adhered to plastic tissue culture plates and cells allowed to grow out onto the plastic surface. Tumour pieces and cells were maintained in Minimum Essential Medium (MEM) with Earle's Salts and without L-Glutamine, supplemented with 2 mM L-glutamine, MEM vitamins, MEM aminoacids (Invitrogen, Paisley, UK), 1 mM Sodium Pyruvate (Sigma Chemical Co, Poole, UK) and 100 ml/L Foetal Bovine Serum (FBS, Autogen Bioclear, Wiltshire, UK). Outgrowth of cells was observed within one week under normal culture conditions at 37°C / 5% CO_2.

2.2.2.4 Re-expression of FAK constructs

FAK-/- SCC 7.1 cells expressing the various FAK constructs were generated by Amaxa nucleofection kit V (Amaxa, Lonza, Cologne, Germany), program P20, using pWZL FAK wt, FAK KD, FAK-Y397F, or FAK-Y4F-Y9F constructs previously generated in this laboratory. Cells were selected in hygromycin B (Merck Biosciences, Sussex, UK) and pooled populations maintained in 1mg ml^{-1}.

2.2.2.5 Subcutaneous tumour growth

Cells were trypsinised, washed in HBSS (Invitrogen, Paisley, UK), and re-suspended in sterile HBSS at a concentration of $2.5x10^5$ to $1x10^6$ cells / 100µl. 100 µl of cell suspension was injected subcutaneously into the flank of immune-compromised nude mice and tumour growth measured every two to three days using callipers. Measurements were taken from at least 8 mice for each cell line. Tumour volume was calculated in Excel by the formula $l \times s^2 / 2$ where "l" represents the maximal diameter and "s" represents the diameter perpendicular to "l".

2.2.2.6 Transient transfection with siRNA

The Dharmacon Smartpool method (Dharmacon, Abgene Ltd., Epsom, UK) of mammalian cell transfection was used for the transfection of SCC 7.1 FAK -/- and FAK wt cells. Flasks of confluent cells were passaged the day prior to transfection and seeded at an appropriate concentration in 6 well plates to ensure 50% confluence on the day of transfection. These plates were incubated overnight at 37°C in an atmosphere of 95% air and 5% CO_2. The siRNA for transfection was diluted in sterile PBS and added to serum free MEM containing 5µl of Dharmafect Transfection Reagent 1 (Dharmacon, Abgene Ltd., Epsom, UK) to give a total volume of 400µl. The complexes were incubated at room temperature for 20 minutes then added directly to the appropriate well of the transfection plate containing 1600µl of complete medium (final concentration of siRNA, 100nM). The plates were incubated for approximately 24 hours at 37°C in an atmosphere of 95% air and 5% CO_2 prior to any cell treatments and harvesting. In each experiment, a scrambled pool of siRNA was utilised as a control.

2.2.3 Cell based assays and addition of inhibitors

2.2.3.1 Cell cycle analysis

Cells were treated as indicated, trypsinised, and washed twice in ice cold PBS prior to fixation in 70% ethanol/PBS overnight at 4°C. For DNA content analysis (including sub-2n DNA), cells were pelleted and resuspended in PBS containing 1µg/ml RNase A (Qiagen, Crawley, UK) and 10µg/ml propidium iodode (PI)

(Sigma Chemical Co, Poole, UK), incubated at room temperature for 30 minutes, then passed through a FACSCalibur flow cytometer with CELLQuest software (Becton Dickinson, Oxford, UK). The data was analysed on FloJo (Treestar, Stanford, CA, USA) and a graphical representation obtained. Experiments were performed at least 3 times and the data presented represents the combined mean ± SEM, unless stated otherwise.

2.2.3.2 Cell proliferation assay

Cell proliferation was assayed indirectly by a modified MTT assay which provides a measure of cell viability. This assay is based on the enzymatic reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (Sigma Chemical Co, Poole, UK) to formazan crystal by mitochondria and cellular dehydrogenase enzymes (Mosmann 1983). Briefly, 100µl of cell suspension containing 250 - 2500 cells from each cell line of interest was dispensed in quadruplicate into 96-well flat bottomed microplates. Plates were then incubated in a 5% CO_2 humidified incubator at 37^{o}C. At the time points indicated, 50µl of MTT solution (5mg/ml in sterile PBS) was added and the plate(s) incubated for a further 4 hours. The formazan crystals were than dissolved in 100µl DMSO (Sigma Chemical Co, Poole, UK) and the optical density determined at 590nm using a plate reader (SpectraMax Plus 384, Molecular Devices, Wokingham, UK). Experiments were performed at least 3 times. The data presented (mean ± SD) is representative of a single experiment.

2.2.3.3 Clonogenic assay

Cells were irradiated in tissue culture flasks with increasing doses of radiation at 70% confluence and immediately trypsinised, counted, and diluted in complete growth medium to a concentration that would yield approximately 50 – 150 colonies after the required period of incubation. The cell suspension was directly added to 60mm tissue culture dishes and the plates were incubated for sufficient time to allow 6 doubling times. This was previously determined by performing a proliferation assay in each individual cell line under investigation. At the appropriate time point, the plates were washed in PBS, fixed in methanol for 15 minutes and stained with crystal violet (Sigma Chemical Co, Poole, UK). The numbers of colonies present per plate were counted on low power bright field

microscopy. Plating efficiency was determined by dividing the number of colonies present by the number of cells seeded. Surviving fraction was then calculated by dividing the plating efficiency at each radiation dose by the plating efficiency of unirradiated cells. Each experiment was performed in triplicate and on at least 3 separate occasions. The data was combined and displayed graphically as mean ± SEM.

2.2.3.4 Clonogenic assay (limiting dilution method)

Cells were passaged at 70% confluence, counted, and diluted in complete growth medium to yield a final concentration that would permit single colony growth after the required period of incubation. 100µl of this cell suspension was added to each well of a flat bottomed 96 well microplate. The plates were incubated for 6 hours to allow cell adhesion then irradiated at increasing doses of irradiation. After 5 – 8 days (to allow 6 cell doubling times), the plates were washed in PBS, fixed in methanol, and stained with crystal violet (Sigma Chemical Co, Poole, UK). The numbers of colonies present per plate were counted on low power bright field microscopy. Plating efficiency was determined by dividing the number of colonies present by the total number of cells seeded per plate. Surviving fraction was then calculated by dividing the plating efficiency at each radiation dose by the plating efficiency of unirradiated cells. Each experiment was performed in triplicate and on at least 3 separate occasions. The data was combined and displayed graphically as mean ± SEM.

2.2.3.5 Irradiation of cells

Cells were grown to 70% confluence in plates or tissue culture flasks then exposed to γ-irradiation from a cobalt ($_{60}$Co) source (Alcyon II teletherapy unit, General Electric, France). The receptacles were set at a distance of 80cm from the source and a Perspex layer added to the surface of the receptacles in order to achieve build-up. The average dose rate was 1 – 1.20 Gy/minute and doses of 1 – 30Gy were applied.

2.2.3.6 Methylcellulose growth assay

In order to study cell growth and proliferation in a 3-D environment, 2.5 x 10^5 cells were suspended in a solution of 1.4% methylcellulose in growth media, plated over a layer of 0.9% agarose, and grown at 37°C in 5% CO_2. Cells were photographed after 10 days (x10 magnification). When appropriate, the numbers of colonies were counted from 10 random fields; colony area was calculated using Image J.

2.2.3.7 Soft agar assay

2-4x10^4 cells were suspended in a solution of 0.45% low melting point agarose in growth media and plated over a layer of 0.9% agarose in growth media in each well of a 6-well dish. Cells were grown under normal conditions at 37°C / 5% CO_2 and colonies were photographed after the appropriate time periods.

2.2.3.8 Treatment with inhibitors

The various inhibitors were reconstituted in tissue culture grade DMSO (Sigma Chemical Co, Poole, UK) to provide a stock solution of 20mM. This was aliquoted to prevent loss of activity from repeat freeze / thaw and stored at -20°C. At the appropriate time, the inhibitors were diluted to the required concentration in growth medium and added to the cells for the indicated time period.

2.2.4 Protein detection

2.2.4.1 Immunofluoresence

Cells were grown on sterile coverslips for 24 hours then washed in cold PBS and fixed in 4% paraformaldehyde for fifteen minutes. Permeabilisation was then achieved with exposure to 0.2% Triton X-100 in PBS for 5 minutes. This was repeated 3 times and excess Triton solution removed by blotting. The cells were blocked in PBS containing 10% FBS and 1% BSA for one hour then incubated overnight at 4°C with the primary antibody of interest. The appropriate dilution for each primary antibody is outlined below in **Table 2**. The coverslips were washed 3 times with blocking solution and incubated with secondary antibody

conjugated to Alexa® 488 or 594 fluorescent dyes (Invitrogen, Paisley, UK) at a 1/200 dilution for one hour. Finally, the coverslips were mounted using Vectashield (Vector Laboratories Ltd, Peterborough, UK) and cells visualised on an Olympus FV1000 confocal microscope (Olympus UK Ltd, Hertfordshire, UK) under an oil immersion lens. The experiments were performed 3 times and the images shown representative of the localisation observed in the majority of cells. In the case of phosphorylated-γH2AX, 100 cells under each condition and for each separate experiment were counted. Data shown is a mean ± SD of 3 independent experiments.

Table 2 - Antibody dilutions for immunofluorescence

Antibody	Dilution
FAK	1/100
LC3	1/200
Paxillin	1/200
p53	1/200
phospho-γH2AX	1/250
phospho-Src-Y416	1/200

2.2.4.2 Immunohistochemistry

Fixed paraffin embedded (FFPE) tissue sections mounted on slides were dewaxed in xylene solution followed by stepped rehydration via a series of graded alcohols to water. Antigen retrieval was then performed by boiling the slides in sodium citrate solution (pH 6) for 20 minutes. The slides were incubated with peroxidase block for 5 minutes to quench endogenous peroxidase activity and blocked in 10% FBS in 0.01M Tris buffered saline (pH 7.5) for one hour at room temperature. The primary antibody of choice was diluted in blocking solution and added to the slides which were then incubated overnight at 4°C. A negative control was compared in parallel to the investigated sections by omitting the

primary antibody step. Visualisation was carried out with a DAKO EnVision kit ™ (Dako UK Ltd, Ely, UK) as per manufacturers' instructions. The resulting sections were analysed and images captured digitally using an Olympus BX51 microscope and cell^D software (Olympus UK Ltd, Hertfordshire, UK). Primary antibody dilutions are outlined below.

Table 3 - Antibody dilutions for immunohistochemistry

Antibody	Dilution
Activated-Caspase 3	1/400
FAK	1/250
K14	1/500
Ki67	1/250
p21 (M-19)	1/800
p53 (CM-5)	1/150

2.2.4.3 Western blotting and immunoprecipitation

Preparation of whole cell lysates / tissue extracts

Confluent cells were harvested at temperatures less than 4°C using buffers that had been previously chilled to minimise protein degradation in the whole cell extract. The cell culture media was aspirated and the cells were washed twice with ice cold sterile PBS. The cell plates were drained thoroughly and the appropriate volume of RIPA lysis buffer added. For a 6 well plate, 50 - 100µl of cell lysis buffer was added whereas a 90mm plate received 200 - 250µl of cell lysis buffer. The cells were incubated for 5 minutes with buffer on ice and then scraped into a 1.5ml Eppendorf® tube. The cell lysates were centrifuged in a bench-top, refrigerated centrifuge at 13000rpm at 4°C and the supernatant retained. The cell lysates were then snap frozen on dry ice and stored at –80°C.

Animal tissue was removed post mortem and immediately frozen in liquid nitrogen for storage at –80°C. At the required time the frozen tissue was added to a Precellys tube (Bertin Technologies, Provence, France) with 100µl of ice cold T-PER buffer. The samples were homogenised (Precellys 24 device – Bertin Technologies, Provence, France), transferred to a 1.5ml Eppendorf® tube and centrifuged as outlined above.

Determination of protein concentration

Protein content was determined using the MicroBCA™ Protein Assay Kit (PERBIO, Glasgow, UK) as per manufacturers' instructions and quantified by measuring light absorbance with a DU® 650 spectrophotometer at a wavelength of 562nm (Beckman Coulter UK Ltd, Buckinghamshire, UK).

Immunoprecipitation

500µg of whole cell lysate was pre-cleared by adding 20µl protein-G sepharose slurry (Sigma Chemical Co, Poole, UK) and rotating at 4°C for one hour. Following this, the lysate was transferred to a clean tube and incubated with 10µl anti-FAK agarose conjugated primary antibody (Chemicon International, Harrow, UK) overnight at 4°C. As a negative control, lysate was immunoprecipitated with an anti-histidine agarose conjugated antibody (Sigma Chemical Co, Poole, UK). The antibody-protein complexes were then washed in RIPA buffer and resuspended in sample buffer prior to western blot analysis.

SDS-PAGE

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) is a method that is routinely employed to separate proteins by virtue of their molecular weight. Protein samples of 5 - 40µg were denatured and reduced by addition of NuPAGE® 4x LDS sample buffer (Invitrogen, Paisley, UK). The samples were boiled for 5min and then loaded directly to an appropriate well of a NuPAGE® Bis-Tris polyacrylamide gel immersed in Invitrogen™ NuPAGE® MOPS SDS running buffer. 10%, 12% or 4-12% gradient gels were used depending on the molecular weight of the protein of interest. 5µl of pre-stained molecular weight rainbow ™ protein marker (GE Healthcare, Little Chalfont, UK) was also loaded

to the first well of the gel to allow the molecular weight of the proteins within the sample to be determined. The gels were run at 200V for 1h.

Western blotting

Proteins separated by SDS-PAGE can be visualised by a variety of methods. Western blotting allows the detection of individual proteins with specific anti-sera. Hence, the proteins separated by SDS-PAGE were transferred to a nitrocellulose membrane using wet blotting apparatus (Jencons, Leighton Buzzard, UK) with an applied voltage of 30V for 90 minutes. Following transfer of the sample proteins, as indicated by successful transfer of the pre-stained molecular weight markers, the nitrocellulose membrane was blocked in 5% bovine serum albumin (BSA), re-constituted in 20mM Tris-Cl; pH 7.6, 150mM NaCl and 0.1% Tween20 (TBST), for one hour at room temperature with gentle agitation. The primary antibody was added at the appropriate dilution to a 5% BSA - TBST solution. Details of the primary antibodies used in experiments described in this thesis are shown in **Table 4**. The blocked nitrocellulose membrane was then sealed in an airtight plastic carrier containing the primary antibody solution and this was incubated overnight at 4^{o}C, with vigorous agitation. The membrane was then washed several times with TBST before the application of the appropriate horseradish peroxidase (HRP) conjugated anti-immunoglobulin G (IgG) secondary antibody diluted 1:5000 in 5% BSA - TBST solution. This was incubated for 45 minutes at room temperature with vigorous agitation. The membrane was again washed several times with TBST before employing the Amersham Biosciences (Little Chalfont, UK) enhanced chemiluminescence (ECL) Western immuno-blotting kit as the visualisation protocol. Briefly, the bound antibodies were detected by exposure of the membrane, following washing in ECL solution, to blue-light sensitive autoradiography film and developed using the Kodak® X-Omat Model 2000 processor. When necessary, blots were stripped using the Re-Blot Plus Strong antibody stripping solution (Chemicon International, Harrow, UK) according to the manufacturer's instructions. The blots shown are representative of experiments repeated at least twice. Densitometry analysis was carried out using Image J. The relative number of arbitary units was measured and normalised to ß-actin.

Table 4 - Antibody dilutions for western blotting

Antibody	Dilution	Antibody	Dilution
Akt	1/500	p53 (IC12)	1/1000
β-actin	1/5000	phospho-Akt-S473	1/1000
ERK	1/1000	phospho-ERK-S42/S44	1/1000
FAK	1/1000	phospho-FAK-Y397	1/1000
K14	1/2000	phospho-FAK-Y576	1/1000
LC3	1/1000	phospho-FAK-Y861	1/1000
MDM2	1/500	phospho-FAK-Y925	1/1000
myc	1/2000	phospho-Src-Y416	1/1000
Src	1/1000	phospho-Pyk2-Y402	1/1000
paxillin	1/1000	PUMA(α/β)	1/1000
PARP(cleaved)	1/500	Pyk2	1/1000
p21	1/1000	α-tubulin	1/2000

2.2.5 Molecular biology techniques

2.2.5.1 Extraction of genomic DNA and genotyping

Genomic DNA was isolated from cells in culture by lysing in extraction buffer (50mM KCl, 10mM Tris (pH 7.4), 2.5mM MgCl2, 0.45% NP-40, 0.45% Tween 20, 1mg/ml proteinase K) for 10 minutes on ice, followed by heating at 95°C for 5 minutes. The DNA was quantified using a GeneQuant RNA/DNA calculator with a spectrophotometer cell and stored at -20°C. Cells containing the K14*Cre*ER transgene were identified by PCR analysis on a DNA thermal cycler (Bio-Rad, Hertfordshire, UK) using the following primers: 5'-ATT TGC CTG CAT TAC CGG TC-3' and 5'-ATC AAC GTT TTC TTT TCG G-3 '. A *Cre* specific PCR product was

amplified using 25ng of DNA and the following protocol: 94°C for 1 minute; 55°C for 30 seconds; 72°C for 30 seconds (39 cycles). Following amplification PCR products were analysed by agarose gel electrophoresis using a 1.5% agarose gel. Cells positive for the K14*Cre*ER transgene exhibited a single PCR product of 350 bp.

To determine floxed *fak* gene status, the same genomic DNA was subjected to PCR analysis using the following primers: LP2s; 5'-ATT GTG CTA TAC TCA CAT TTG GA-3', LP2as; 5'-TTA ATA AGA CCA GAG GAC TCA GC-3', and LP2h; 5'-GGA AGA AGC TTG TAT ACT GTA TG-3'. A FAK-specific PCR product was amplified using 25ng of DNA and the following protocol: 95°C for 5 minutes (1 cycle); 95°C for 45 seconds + 62°C for 45 seconds + 72°C for 1 minute (35 cycles); 72°C for 7 min (1 cycle). Following amplification, PCR products were analysed by agarose gel electrophoresis. Cells homozygous for the floxed *fak* mutation ($FAK^{flox/flox}$) exhibited a single major band of 580 bp, cells heterozygous for the floxed FAK ($FAK^{wt/flox}$) contained three major bands at 650 bp, 580 bp, and 400 bp, and wild type (wt) cells ($FAK^{wt/wt}$) contained a double band at 650 bp and 400 bp.

2.2.5.2 Extraction of RNA and qRT-PCR analysis

RNA was isolated from $1x10^6$ cells using miRNeasy mini RNA extraction kit (Qiagen, Crawley, UK) as per the manufacturer's instructions. 1µg of RNA was then converted to cDNA using Superscript First-Strand cDNA synthesis kit (Qiagen, Crawley, UK). The cDNA was diluted 1 in 5 then prepared for qRT-PCR analysis by adding 5µl to 45µl SybrGreen master mix (Invitrogen, Paisley, UK) containing 1µM of paired validated primers directed against the target gene of choice (Qiagen, Crawley, UK). All primer pairs were assessed for linearity prior to use and produced a PCR single product of the correct size as outlined by the manufacturer. Real time PCR was performed on a gradient cycler (Bio-Rad, Hertfordshire, UK) with the following programme: 95°C for 15 minutes (1 cycle); 95°C for 15 seconds + 55°C for 30 seconds + 72°C for 30 seconds (39 cycles); 72°C for 5 minutes (1 cycle); melting curve 70 – 95°C, hold every 0.1 seconds; 72°C for 10 minutes (1 cycle); 15°C for 10 minutes (1 cycle). Data was analysed using Opticon software V3.1 (Bio-Rad, Hertfordshire, UK). ß-actin controls were included with each reaction to act as a housekeeping gene and fold change in mRNA levels calculated by the ddC(t) method (Livak and Schmittgen 2001). The

samples were loaded in triplicate and the mean ± SEM from three combined experiments displayed graphically.

2.2.5.3 Routine PCR analysis and sequencing

RNA was isolated from cells and converted to cDNA as outlined above. 2µl of cDNA was added to 22.5µl pre-aliquoted Mastermix PCR mix (PERBIO, Glasgow, UK) containing 1µM of forward and reverse primers. All primers were obtained from VH Bio (Gateshead, UK) and details are listed in **Table 5**. PCR amplification was performed on a DNA thermal cycler (Bio-Rad, Hertfordshire, UK) using the programme: 95°C for 10 minutes (1 cycle); 95°C for 30 seconds; 60°C for 30 seconds; 72°C for 45 seconds (35 cycles); 72°C for 5 minutes (1 cycle). Following amplification, PCR products were separated by agarose gel electrophoresis using a 1.5% agarose gel with an applied voltage of 30V and imaged using SYNGENE UV gel visualisation system.

Table 5 - Primer sequences for RT-PCR

Gene	Sense primer 5'-3'	Anti-sense primer 5'-3'	Fragment size (bp)
H-Ras	CAG CCG CTG TAG AAG CTA TGA	TCA GTT TCC GCA ATT TAT GCT	531
GAPDH	GTG GAT ATT GTG CCC AAT GAC ATC	GGA CTC CAC GAC GTA CTC AGC GCC AGC	214
K14	CCT GGA GAT GCA GAT TGA GAG	ATT CTC CAG GGA TGC TTT CAT	370
p21	AGC CTG ACA GAT TTC CAC	CTT TAA GTT TGG AGA CTG GGA	222
p53	CCT GTC ATC TTT TGT CCC TTC	TGC GGA AAT TTT CTT CTT CTG	494

For sequencing analysis, ten RT-PCR amplification reactions were carried out simultaneously for each line of interest using the method outlined above. The primer pair for H-Ras was as described in **Table 5**. p53 primer pairs were as follows: 5'- AGA TAT CCT GCC ATC ACC TCA -3' and 5'- CCA GAC TCC TCT GTA

GCA TGG -3' which yielded a single product size of 975 bp. The PCR products were then pooled, purified with QIAquick PCR purification Kit (Qiagen, Crawley, UK) as per the manufacturer's instructions, and the concentration of DNA quantified using a GeneQuant RNA/DNA calculator with a spectrophotometer cell as previously mentioned. The DNA was diluted to 50 – 100ng/µl and direct sequencing analysis performed using the Big-Dye method. 20ng/µl of primer was included in the final reaction mix. The primers were designed to sequence codon 61 of H-Ras and the DNA binding domain of p53 (further information is provided in Chapter 4) and are listed below in **Table 6**.

Table 6 - Sequencing primers

Gene	Sense primer 5'-3'	Anti-sense primer 5'-3'
H-Ras	GCA GCC GCT GTA GAA GCT AT	TCA GTT TCC GCA ATT TAT GCT
p53	CCT GTC ATC TTT TGT CCC TTC CGG GTG GAA GGA AAT TTG TAT	TGC GGA AAT TTT CTT CTT CTG CCA GAC TCC TCT GTA GCA TGG

2.2.6 Statistical analysis

Graphs and bar charts were created in Excel and represent the mean value ± SD or mean value ± SEM from three separate experiments. Statistical tests were performed in Minitab 15, $p<0.05$ was considered significant and is denoted by *. An unpaired t-test was used to compare the means of two populations with approximately equal variance and normal distribution, where n = number of data sets that contributed towards the mean. For the purposes of immunohistochemistry data analysis, the mean percentage of positively stained cells per xenograft was calculated based on examination of twenty high powered fields. The mean values of two test groups were then analysed by either the Kruskal-Wallis test or the Mann Whitney U test (typically three to five xenografts from separate mice were included in each defined experimental group).

Chapter 3

Development of a FAK -/- murine squamous cell carcinoma cell line

3 Development of a FAK -/- murine squamous cell carcinoma cell line

3.1 Aims

We set out to evaluate the role of FAK in cellular radiation survival *in vitro* and *in vivo* and opted to use a genetic deletion model to achieve FAK knockout as we believed that this would be a clean and precise biological method. It is widely accepted that gene deletion studies in cells constitute a useful scientific tool for investigating the roles of specific gene products / proteins in particular biological contexts. FAK deficient mouse embryo fibroblasts (FAK -/- MEFs), for instance, have provided invaluable information about the role of FAK in cell adhesion and motility. As opposed to working solely with FAK +/+ and FAK -/- MEFs, which are readily available, we wanted to utilise a tumour cell line as this would be more relevant to human cancer. Also, the use of a cell line of malignant origin would enable us to investigate the role of FAK *in vivo* as well as *in vitro*. There were no such cell lines available when we decided to embark on this project. Therefore, our first aim was to develop a novel murine squamous cell carcinoma with inducible *fak* deletion, based on a conditional mouse model that was already established in this laboratory.

3.2 Results

3.2.1 Skin specific FAK deletion.

Previous work in this laboratory has demonstrated that FAK is important in mouse skin carcinogenesis. Initially, it was shown using *fak* heterozygous (+/-) mice, that reduced FAK expression had a negative impact on chemically induced papilloma formation (McLean, Brown et al. 2001). However, the benign tumours from *fak* +/- mice had elevated expression of FAK to a level that was indistinguishable from papillomas from wild-type *fak* +/+ mice. The key question of whether FAK expression is causally involved in malignant progression therefore remained unanswered. However, this question was addressed by adopting a conditional gene targeting system. Briefly, *Cre*/loxP technology was

used to generate mice that are homozygous for a *floxed fak* allele and that also express a 4-hydroxy-tamoxifen (4-OHT) regulated *Cre* recombinase (*Cre*-ER^{T2}) expressed under the control of the keratin 14 promoter (K14*Cre*ER^{T2}/FAK$^{flox/flox}$), a highly active promoter in dividing cells of the epidermis from day E14.5 (Vasioukhin, Degenstein et al. 1999). A schematic representation of this model is described in **Figure 11A**. It was then possible to study the role of FAK in cancer development by subjecting these mice to the well established chemical carcinogenesis protocol (DMBA-TPA) which is outlined in **Figure 12**. Rendering keratinocytes FAK -/- by 4-OHT treatment (**Figures 11B** and **11C** depict successful skin specific FAK protein loss by western blotting and immunohistochemistry respectively) resulted in a lower frequency of papilloma development and inhibited progression to malignant carcinoma. This work demonstrated that FAK is crucial in carcinogenesis and tumour progression, at least in this system (McLean, Komiyama et al. 2004).

An advantage of this model is that *fak* deletion is under temporal as well as spatial control. It was shown that FAK could be deleted from the epidermis in an established papilloma (**Figure 13A**). In addition, FAK knockout was also achieved *ex vivo* in cultured keratinocytes with low dose 4-OHT (**Figure 13B**). Thus, we hypothesised that it should be possible to delete FAK in an established tumour cell line by inducing malignancy, isolating a cell line and exposing this cell line to 4-OHT *in vitro*.

3.2.2 Generation of murine squamous cell carcinoma cell lines.

Two squamous cell carcinoma (SCC) cell lines were propagated from K14*Cre*ER^{T2}/FAK$^{flox/flox}$ mice which underwent treatment with DMBA/TPA; these were called SCC 1.1 and SCC 7.1. SCC 1.1 was derived from a newly formed carcinoma that developed at 18 weeks following induction, while SCC 7.1 was derived from an advanced ulcerative tumour at 24 weeks post induction. Interestingly, the cell lines proved to be morphologically distinct with the former exhibiting an epithelial morphology, remarkably similar to cultured normal keratinocytes, while the latter was mesenchymal in appearance, suggestive of spindle cell carcinoma, a variant of squamous cell carcinoma that is associated with a more aggressive phenotype.

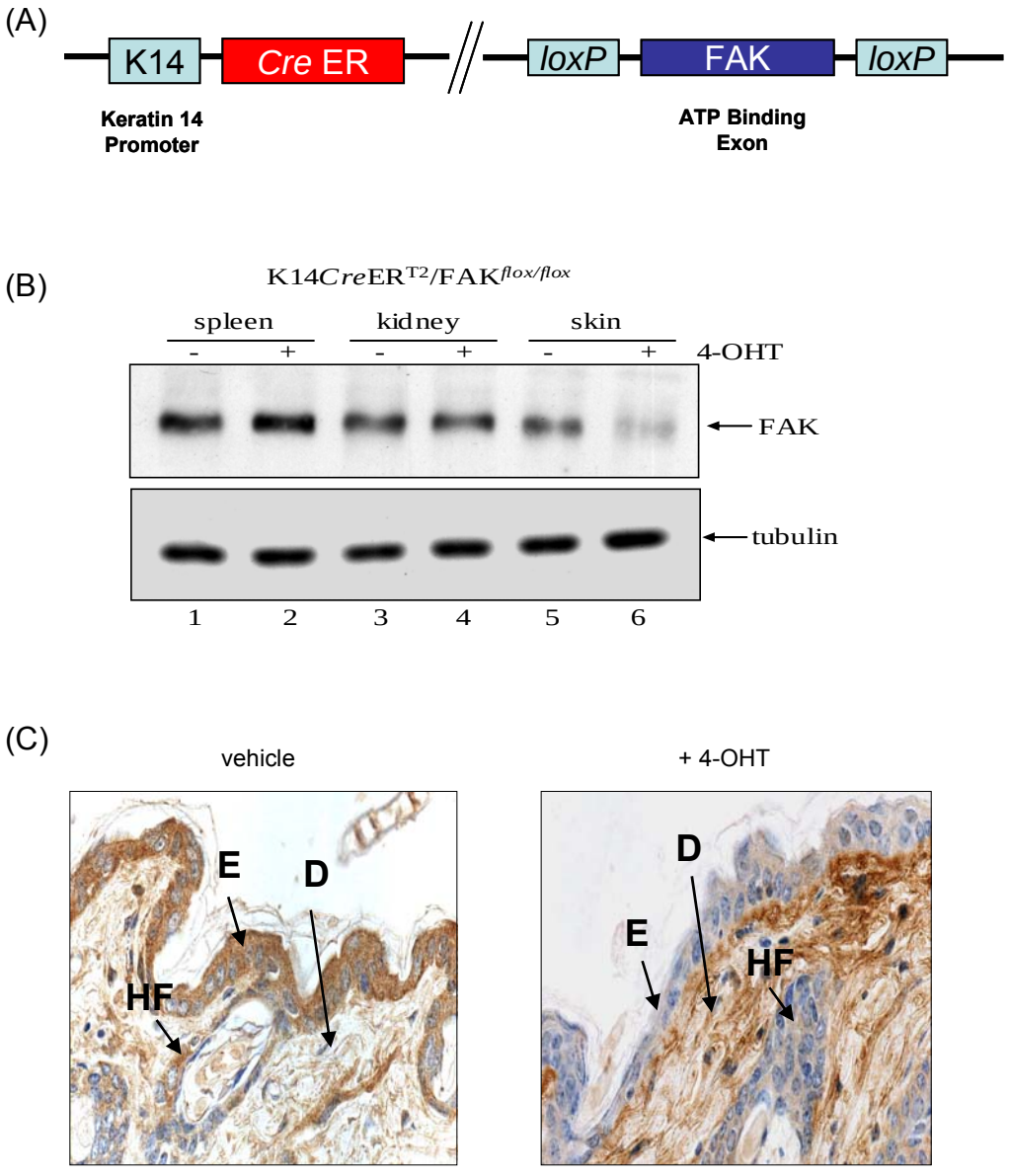


Figure 11 - Deletion of FAK from the epidermis of mouse skin
(A) Schematic diagram representing the K14*Cre*ER^{T2}FAK$^{flox/flox}$ system. *Cre*/LP2 homozygous mice were generated to express a *Cre*ER fusion protein under the Keratin 14 (K14) promoter which is present in developing keratinocytes. After exposure to tamoxifen, *Cre* translocates to the nucleus and excises the loxP sites which flank the FAK kinase domain. A frameshift mutation incorporated into this system ensures that no functional protein is synthesised. (B) Protein extracts were obtained from spleen, kidney and skin of K14*Cre*ER^{T2}FAK$^{flox/flox}$ mice treated with either vehicle or tamoxifen (4-OHT), separated by SDS-PAGE, transferred to nitrocellulose and blotted with either anti-FAK (upper panel) or anti-tubulin (lower panel). (C) K14*Cre*ER^{T2}FAK$^{flox/flox}$ mice were treated with either vehicle (left) or 4-OHT (right). Paraffin embedded dorsal skin sections were stained with an anti-FAK antibody and visualised under bright field microscopy (x20 magnification), E-epidermis, D-dermis, HF-hair follicle. Experiment courtesy of GW McLean.

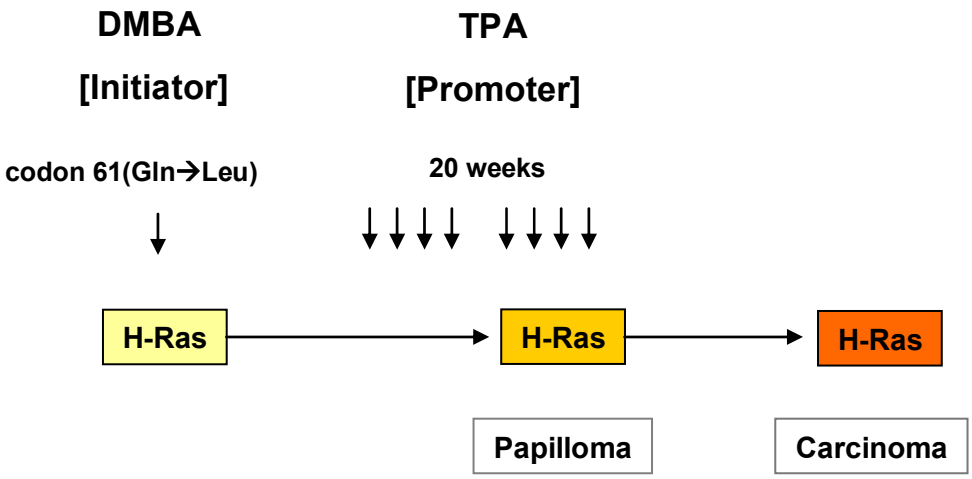


Figure 12 - Topical carcinogenesis
Schematic diagram demonstrating the well described mouse skin carcinogenesis protocol. Briefly, initiation with a single application of DMBA results in an activating missense mutation in codon 61 of the H-Ras gene in which an adenosine is converted to a thymidine. Subsequent twice weekly TPA treatment selects for clonal expansion of initiated cells, giving rise to papillomas after 5-7 weeks and conversion to carcinomas from 16 weeks onwards.

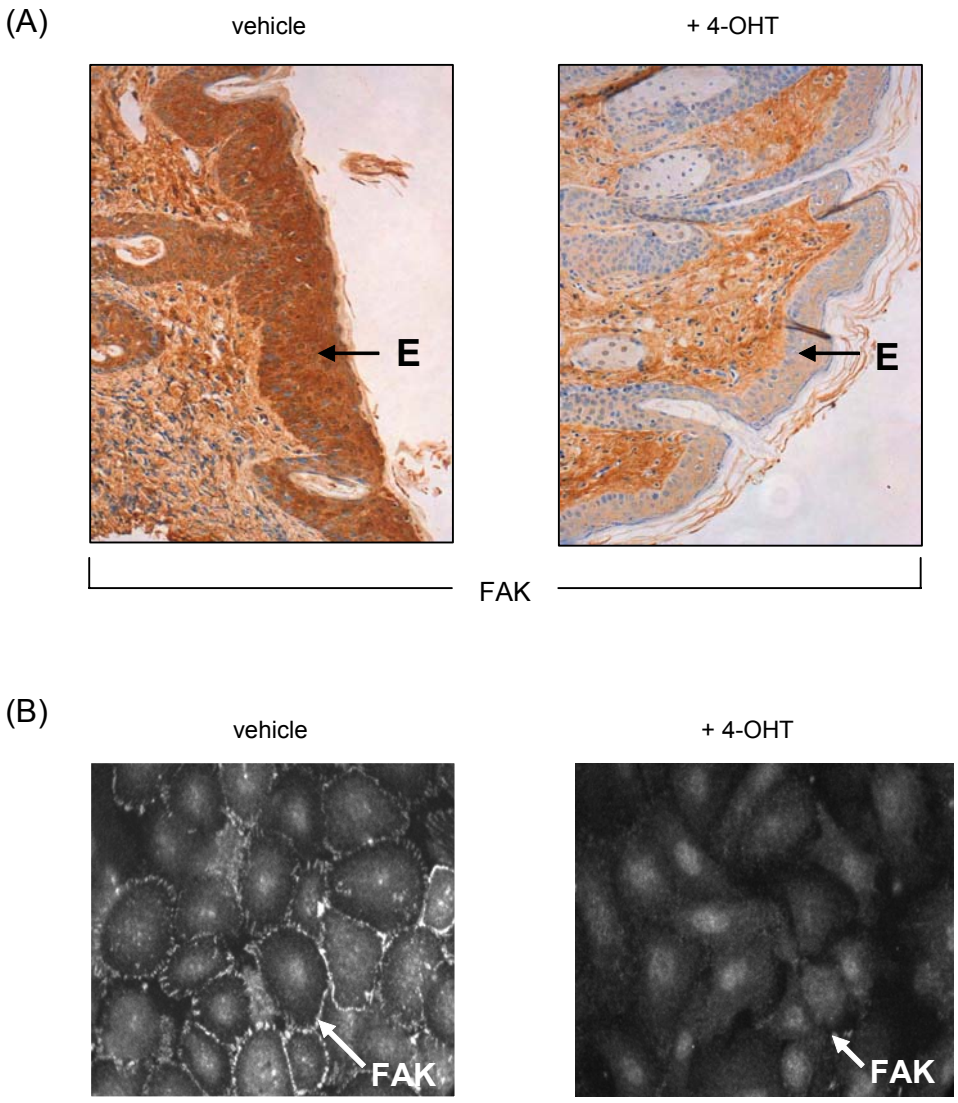


Figure 13 - FAK deletion is temorally and spatially controlled
(A) Immunohistochemistry detailing FAK staining of papilloma sections of K14*Cre*ER^{T2}FAK$^{flox/flox}$ mice treated with either vehicle (left) or 4-OHT (right), E-epidermis. (B) Keratinocytes derived from the skin of 2 day old K14*Cre*ER^{T2}FAK$^{flox/flox}$ pups were plated on glass coverslips, treated with either vehicle (left) or 10nM 4-OHT (right) for 48 hours, fixed, and stained with an anti-FAK antibody prior to visualisation by confocal microscopy. Experiment courtesy of BR Serrels.

Representative phase contrast images of the SCC cell lines are shown in **Figure 14A**. PCR analysis of genomic DNA extracts from both cell lines demonstrated homozygous expression of *Cre* and the loxP sites, confirming the correct genotype (**Figure 14B**). However, prior to 4-OHT treatment we wanted to be certain that the cell lines were in fact malignant, particularly the mesenchymal line, as fibroblast contamination is a common problem in primary cell culture. This was approached in several ways.

Firstly, sequencing analysis of H-Ras was undertaken in DNA prepared from SCC 1.1 and SCC 7.1 cell lines. The reasoning for this is that the DMBA/TPA carcinogenesis protocol promotes an activating missense mutation in codon 61 of H-ras (CAA – CTA), which is typically homozygous in carcinomas (Balmain, Ramsden et al. 1984; Quintanilla, Brown et al. 1986). Examination of codon 61 revealed a single T at the centre base in both cell lines signifying homozygous mutation (**Figure 15**). By contrast, parallel analysis of DNA prepared from K14*Cre*ER^{T2}/FAK$^{flox/flox}$ keratinocytes demonstrated a base sequence of CAA at codon 61, in keeping with wild type (wt) H-Ras. This data indicates that SCC 1.1 and SCC 7.1 cells are tumour cell derived and not fibroblasts.

Secondly, the ability of cell lines to establish xenografts following subcutaneous injection into nude mice is an indicator of malignant origin. Accordingly, both SCC 1.1 and SCC 7.1 cells formed tumours after injection into female nude mice.

Unsurprisingly, the histological appearance was markedly different. SCC 1.1 xenografts demonstrated features of a highly keratinising moderately to well differentiated squamous cell carcinoma with cell nests and keratin pearls. On the other hand, SCC 7.1 xenografts were composed of a homogeneous mass of fusiform cells arranged in fascicles, and to a lesser extent, whorls. Representative H & E images of paraffin embedded sections are shown in **Figure 16A**. Of interest, the SCC 7.1 xenografts grew much more rapidly (data not shown), and examination of the H & E sections under high powered microscopy revealed an abundance of mitotic figures (**Figure 16B**). A high mitotic index is known to be a feature of spindle cell tumours.

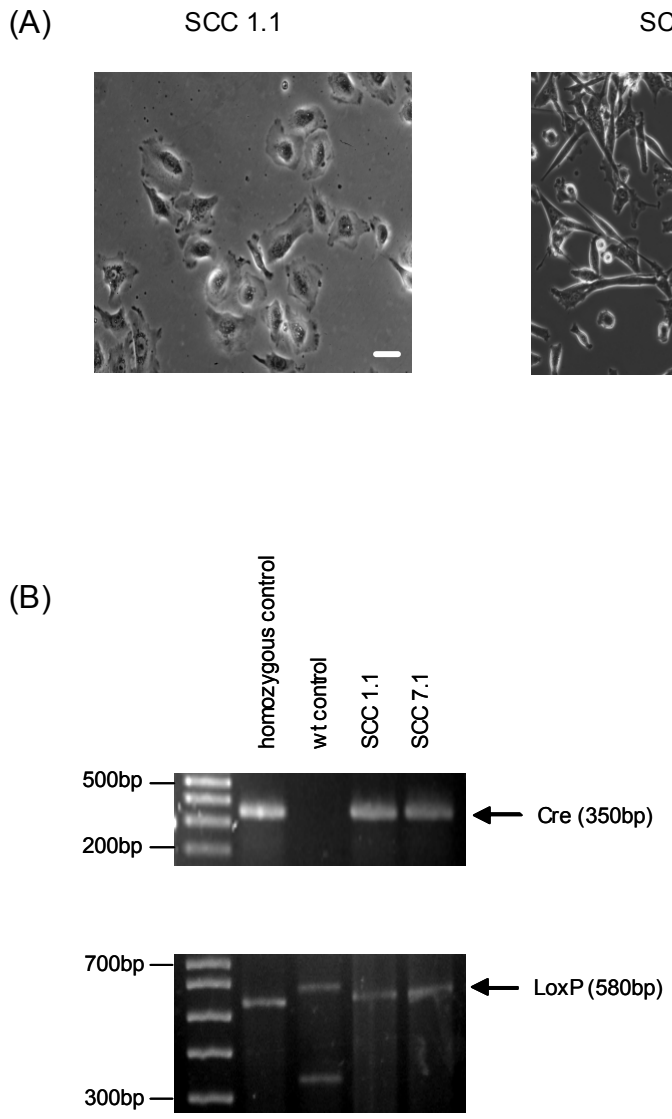


Figure 14 - Isolation of squamous cell carcinoma cell lines
(A) K14*Cre*ER^{T2}FAK$^{flox/flox}$ female mice were subjected to the DMBA/TPA carcinogenesis protocol and monitored regularly for papilloma and carcinoma formation. Tumours were then disaggregated and cell lines established *in vitro* as outlined in Methods section. Phase contrast images of the derived cell lines SCC 1.1 (left) and SCC 7.1 (right) are shown (scale bar, 30μm). (B) Genomic DNA was extracted from cell lines SCC 1.1 and SCC 7.1, subjected to RT-PCR for *Cre* (upper panel) and loxP (lower panel) as outlined in Methods section, and separated on 1.5% and 3% agarose gels respectively.

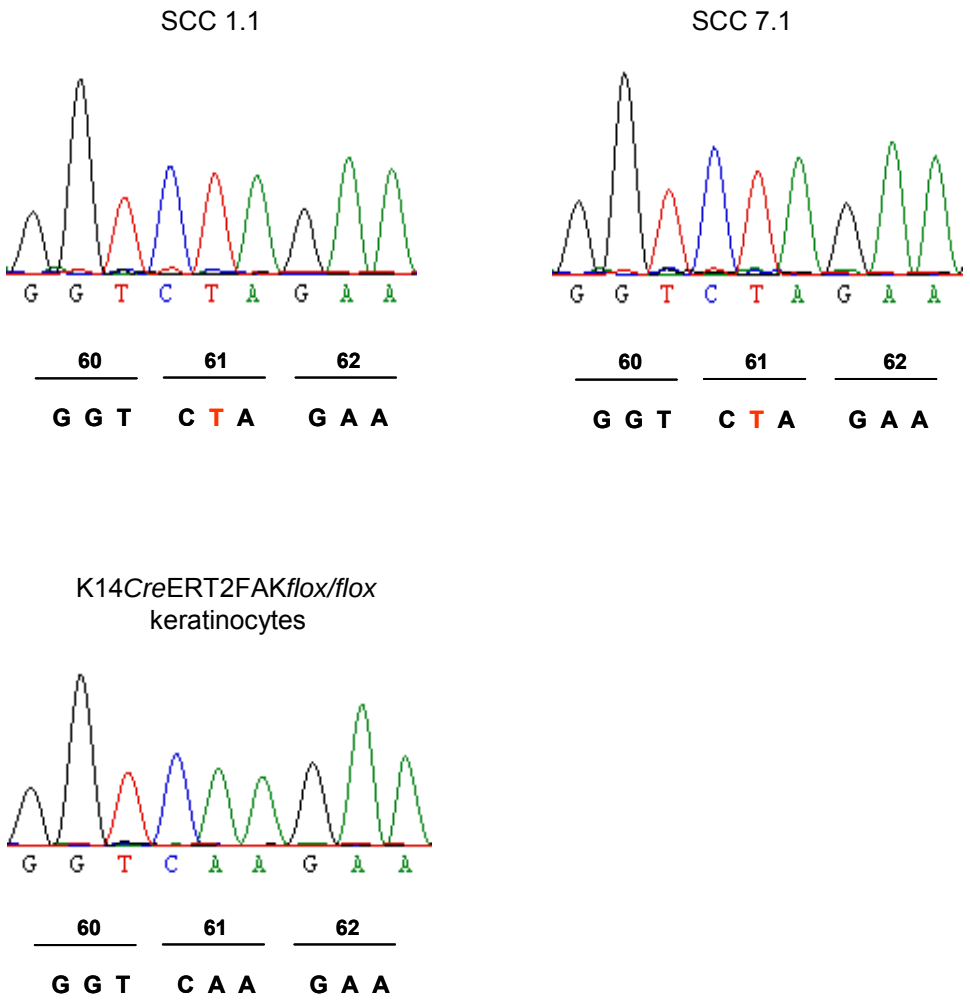


Figure 15 - SCC 1.1 and SCC 7.1 cells possess a mutation in codon 61 of H-Ras
RNA was extracted from SCC 1.1 and SCC 7.1 cell lines, and K14*Cre*ER^{T2}FAK$^{flox/flox}$ keratinocytes, converted to cDNA using Superscript II, amplified by PCR, and purified as outlined in Methods section. The cDNA was then diluted and subjected to direct sequencing analysis. Chromatograms for codons 60-62 of H-Ras are demonstrated for SCC 1.1 cells, SCC 7.1 cells, and K14*Cre*ER^{T2}FAK$^{flox/flox}$ keratinocytes.

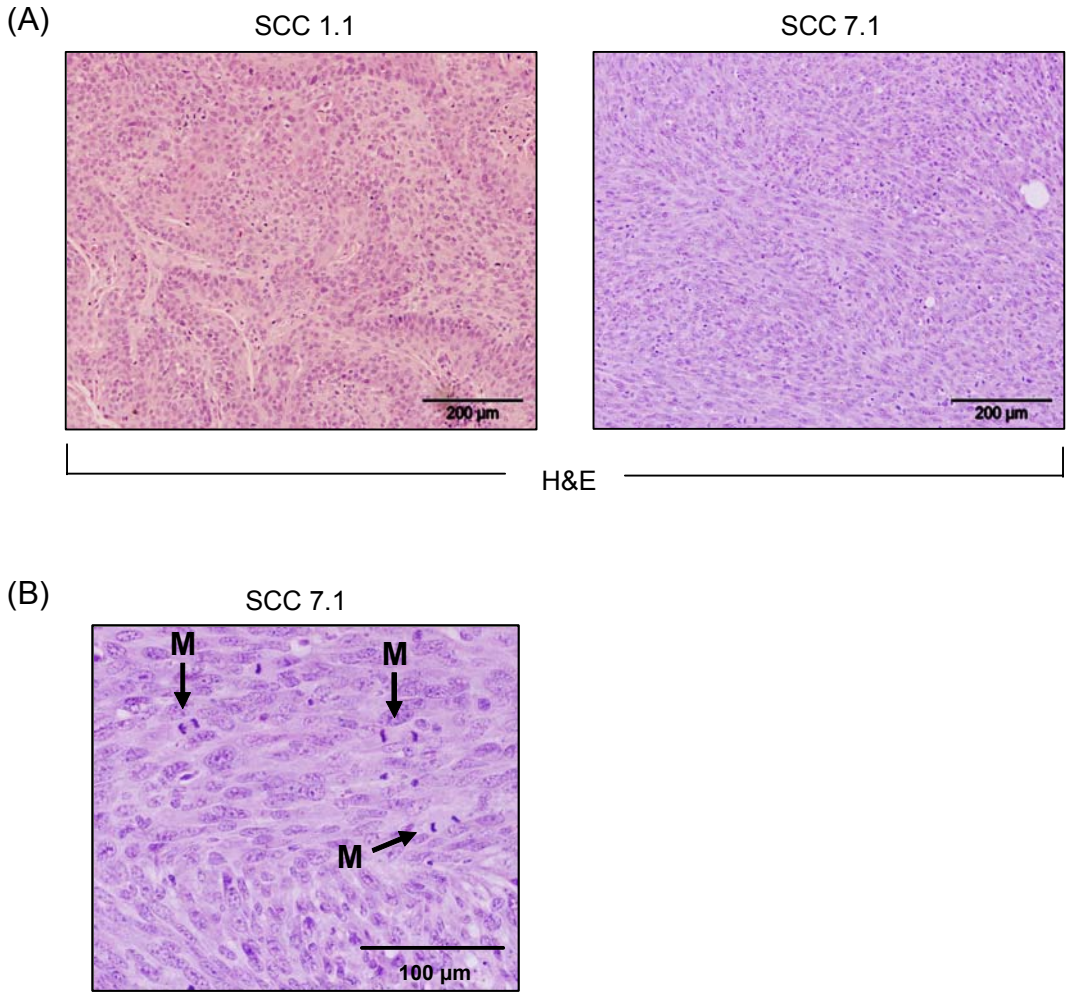


Figure 16 - SCC 1.1 and SCC 7.1 cells produce tumours in nude mice
(A) $1x10^6$ cells were injected subcutaneously into the right flank of 6 week old female CD-1 nude mice. Growth was monitored regularly and the animals sacrificed when the xenograft reached 10mm in maximal diameter. H&E staining of paraffin embedded sections was then performed and representative images of SCC 1.1 (left) and SCC 7.1 (right) are shown (scale bar, 200µm). (B) High powered image of SCC 7.1 xenograft, arrows indicating mitotic figures (M) (scale bar, 100µm).

Taken together, we reasoned that both cell lines were indeed of tumour origin with SCC 1.1 representing a moderately / well differentiated carcinoma and SCC 7.1 a more advanced spindle cell carcinoma.

3.2.3 FAK expression is increased in SCC 1.1 and SCC 7.1 cells.

FAK protein levels have been shown to increase during the process of tumour development in the mouse skin carcinogenesis model, with low levels detected in normal keratinocytes, intermediate levels detected in papillomas, and high levels detected in squamous cell carcinomas, implying that FAK signalling is a prerequisite, at least in certain types of epithelial malignancies (McLean, Brown et al. 2001; McLean, Avizienyte et al. 2003; McLean, Komiyama et al. 2004; McLean, Carragher et al. 2005). In keeping with these findings, FAK protein levels were clearly upregulated in the cancer cell lines we derived compared with cultured K14*Cre*ER^{T2}/FAK$^{flox/flox}$ keratinocytes, as shown in **Figures 17A** and **17B**. Interestingly, FAK (and the related kinase Pyk2) protein levels were equivalent in SCC 1.1 and SCC 7.1 cells (**Figure 18**), despite reports that spindle cell carcinomas may contain more FAK than differentiated squamous cell carcinomas (McLean, Carragher et al. 2005).

FAK phosphorylation levels were also similar in SCC 1.1 and SCC 7.1 cells (**Figure 18**). However, downstream signalling effectors which have been linked to FAK, for example phospho-ERK and phospho-Akt, were present at different levels (**Figure 18**). Obviously, these effectors are subject to control by multiple upstream pathways, and the situation is further complicated by extensive cross-talk and feedback loops between signalling molecules. However, the variance in phospho-protein levels between these cell lines hints that FAK may be involved in different signalling pathways and hence may have different functions in SCC 1.1 cells compared with SCC 7.1 cells.

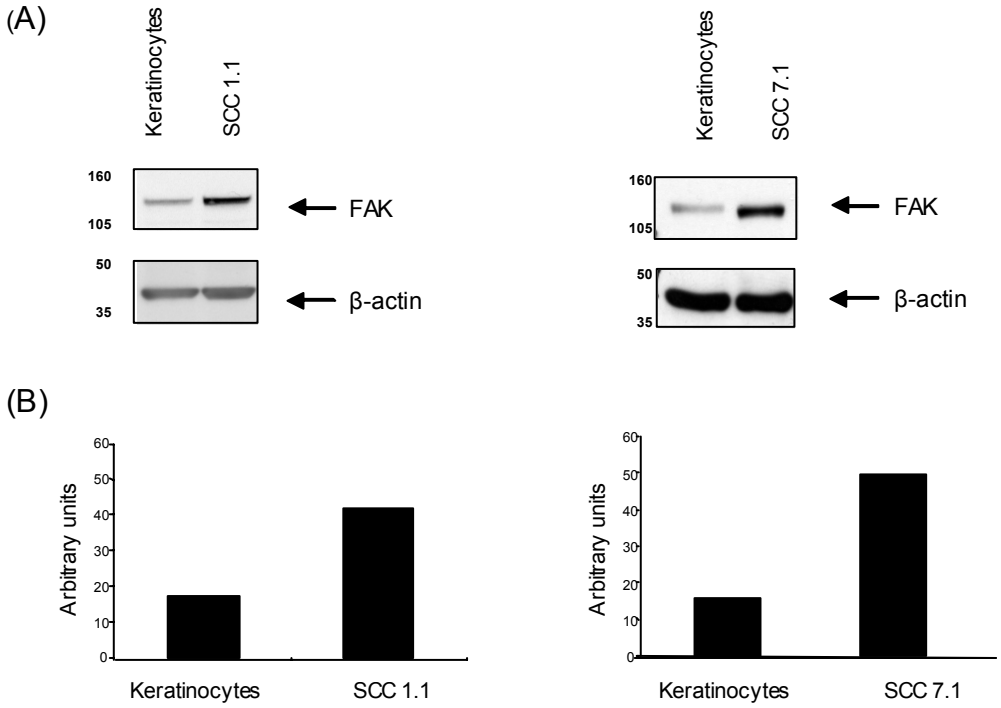


Figure 17 - FAK is upregulated in SCC 1.1 and SCC 7.1 cells
(A) Protein extracts from K14*Cre*ER^{T2}FAK$^{flox/flox}$ keratinocytes, SCC 1.1 cells, and SCC 7.1 cells were separated by SDS-PAGE, transferred to nitrocellulose, and probed with anti-FAK antibody (upper) and anti-β-actin antibody (lower). (B) FAK protein levels in SCC 1.1 and SCC 7.1 cells were compared to keratinocytes by densitometry analysis and normalised to β-actin. The difference is demonstrated by an increase in arbitary units and is representative of one of three separate experiments.

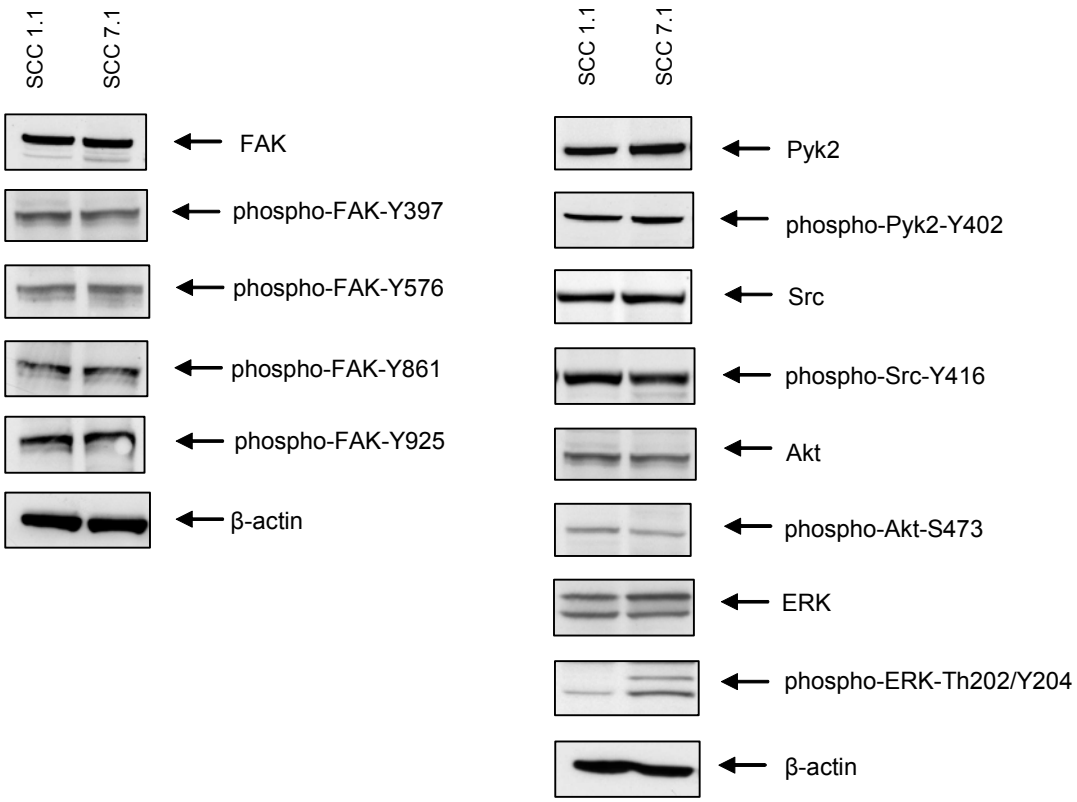


Figure 18 - FAK levels are similar in SCC 1.1 and SCC 7.1 cells
Protein extracts from SCC 1.1 and SCC 7.1 cells were immunoblotted with the various antibodies demonstrated. β-actin loading is also shown.

3.2.4 Tamoxifen treatment does not result in stable FAK knockdown in pooled cell populations.

Increasing doses of 4-OHT were added to pooled populations of SCC 1.1 and SCC 7.1 cells in culture and FAK expression was assessed by western blotting at various time points following drug exposure for a period of 4 weeks. We noted that very high concentrations of drug (10-15μM) were required to induce substantial FAK knockout. Greater knockout was consistently achieved in the SCC 7.1 pools compared with SCC 1.1 pools as judged by both immunoblotting and immunofluorescence. An example of FAK protein levels following 72 hours exposure to either vehicle or 10μM 4-OHT is demonstrated in **Figure 19A**. Within 3 - 4 weeks, however, the FAK expression in 4-OHT treated populations returned to pre-treatment levels as depicted in **Figure 19B**. This observation led us to question whether induced FAK -/- cells have a proliferation, or even survival, disadvantage. We did not address the issue of proliferation at this time due to the lack of a stable knockout cell population, but we did attempt to address the question of survival by evaluating the subG1 fraction on FACS analysis in 4-OHT treated SCC 1.1 and SCC 7.1 cells versus vehicle treated controls (data not shown). However, interpreting this data proved to be problematic as 4-OHT itself is known to cause G1/S phase arrest and the doses of drug used here resulted in a significant degree of cytotoxicity. It was, therefore, very difficult to attribute any differences in subG1 fraction to a FAK -/- mediated survival disadvantage as opposed to the effects of 4-OHT on cell cycle and cell survival alone.

In fact, the concentration of 4-OHT that was required to effect significant FAK knockout in the SCC cell lines surprised us. As the *Cre*-LoxP system is designed for *in vivo* work, and the regimen of 4-OHT which induces optimal recombination *in vivo* well described (Indra, Warot et al. 1999; McLean, Komiyama et al. 2004), we wondered whether the outcome would be different *in vivo* compared with *in vitro*. We therefore treated nude mice bearing SCC 1.1 or SCC 7.1 xenografts with the recommended schedule of intraperitoneal 4-OHT. Interestingly, this did not result in any FAK knockout at all in either cell line (**Figure 20**). This data, coupled with the fact that FAK knockout can be achieved *in vitro* in keratinocytes with only 10nM 4-OHT (Indra, Warot et al. 1999; McLean,

Komiyama et al. 2004) suggests that the high dose of 4-OHT required to produce the desired effect in the SCC cells was probably not related to *in vitro* versus *in vivo* drug activity. Instead, we suspected that underlying cell line factors were more likely to determine the outcome of these experiments.

It is well recognised, for example, that the expression of *Cre* recombinase in conditional mouse models is not 100%. Based on these reports, it seemed reasonable to consider the possibility that the expression of *Cre* was suboptimal in the derived SCC cell lines. Attempts were made to directly quantify the level of *Cre* expression in SCC 1.1 and SCC 7.1 cells by immunofluorescence but the commercially available antibodies were not sufficiently robust and produced non-specific results (data not shown).

As *Cre* recombinase expression is driven by the K14 transgene in this system, we decided to examine the K14 status in more detail. Intriguingly, although both SCC cell lines were positive for mRNA, the mRNA levels were visibly lower in SCC 7.1 cells compared with SCC 1.1 cells; the latter, on the other hand displayed similar mRNA levels to normal keratinocytes (**Figure 21A**). Furthermore, K14 protein could only be detected in SCC 1.1 cells, but not SCC 7.1 cells, as evidenced by immunoblotting of cellular protein extracts (**Figure 21B**) and immunohistochemistry staining of xenografts (**Figure 21C)**. For the latter, skin from a K14*Cre*ER^{T2}/FAK$^{flox/flox}$ mouse which had not been treated with either DMBA/TPA or 4-OHT was included as a positive control (**Figure 21D**); this image demonstrates K14 expression in the basal layer of the epidermis. The appearance of two bands in the SCC 7.1 lane on immunoblotting (**Figure 21B**) is likely to represent the presence of different keratin proteins which are not expressed at detectable levels in either normal keratinocytes or SCC 1.1 cells. We did consider the possibility that these may have simply represented non-specific bands, but they were not present in the other lanes. Review of pathological case series of squamous cell carcinomas reveals that keratin expression is often lost or aberrant in spindle cell carcinomas (Zarbo, Crissman et al. 1986). Hence, the lack of K14 expression in SCC 7.1 cells was not entirely unusual, but we did initially find it a little surprising that we were able to achieve more substantial FAK knockout in this cell line.

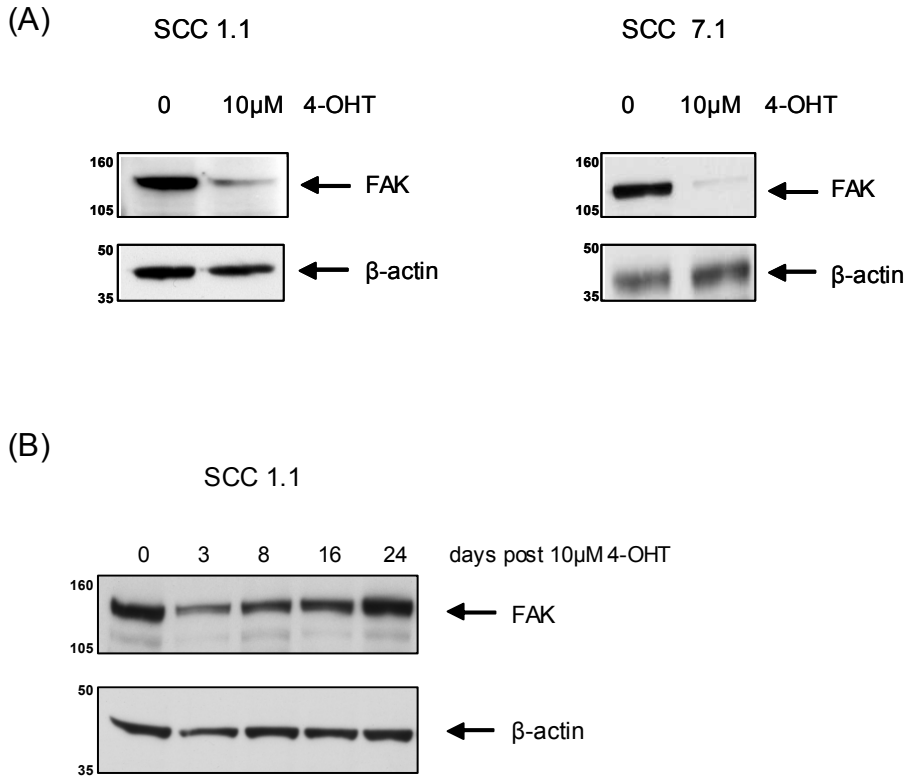


Figure 19 - 4-OHT treatment does not induce stable FAK knockout in pooled cells
(A) Subconfluent populations of SCC 1.1 and SCC 7.1 were treated with either vehicle (DMSO) or 10μM 4-OHT for 72 hours, protein extracts prepared and separated by SDS-PAGE, transferred to nitrocellulose, and probed with anti-FAK antibody (upper) and anti-β-actin antibody (lower). (B) SCC 1.1 cells were treated with 10μM 4-OHT for 72 hours after which time the drug was washed out and lysates were prepared on the indicated days. Immunoblotting was then performed with an anti-FAK antibody (upper) and an anti β-actin antibody (lower).

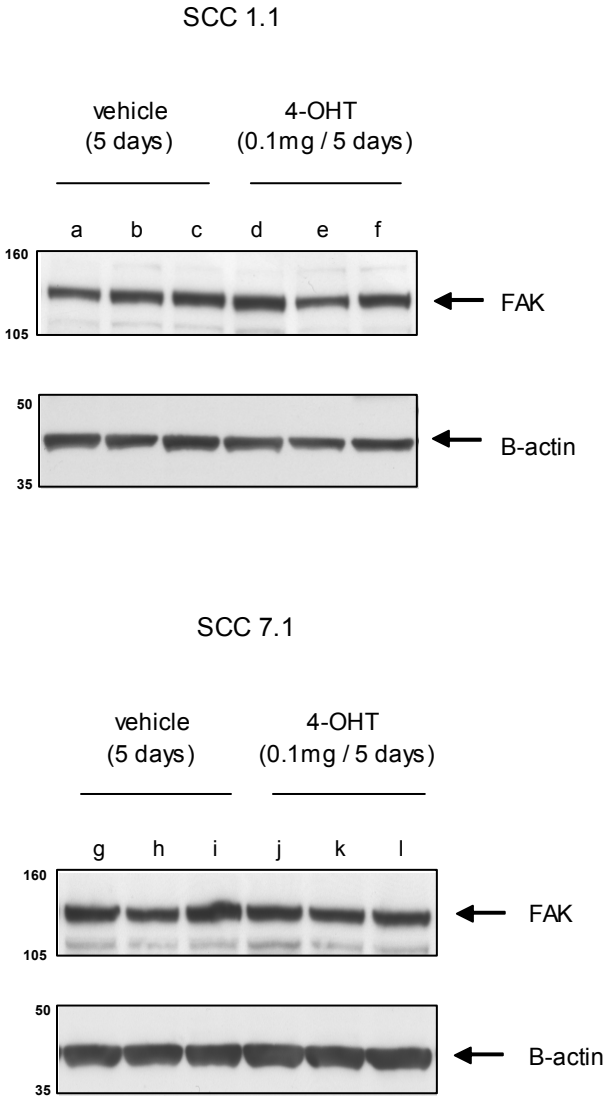


Figure 20 - 4-OHT treatment does not induce FAK knockout *in vivo*
6 week old female nude mice were injected with $5x10^5$ SCC 1.1 or SCC 7.1 cells and monitored regularly. When tumours reached approximately $100mm^3$, the animals then received either vehicle (100µl sunflower oil) or 4-OHT (0.1mg in 100µl sunflower oil) by intraperitoneal injection once daily for 5 days, n=3 per group. Protein extracts were prepared 48 hours after the last injection as detailed in methods, separated by SDS-PAGE, transferred to nitrocellulose, and probed with anti-FAK antibody (upper) and anti-β-actin antibody (lower), a – l represent the individual mice examined.

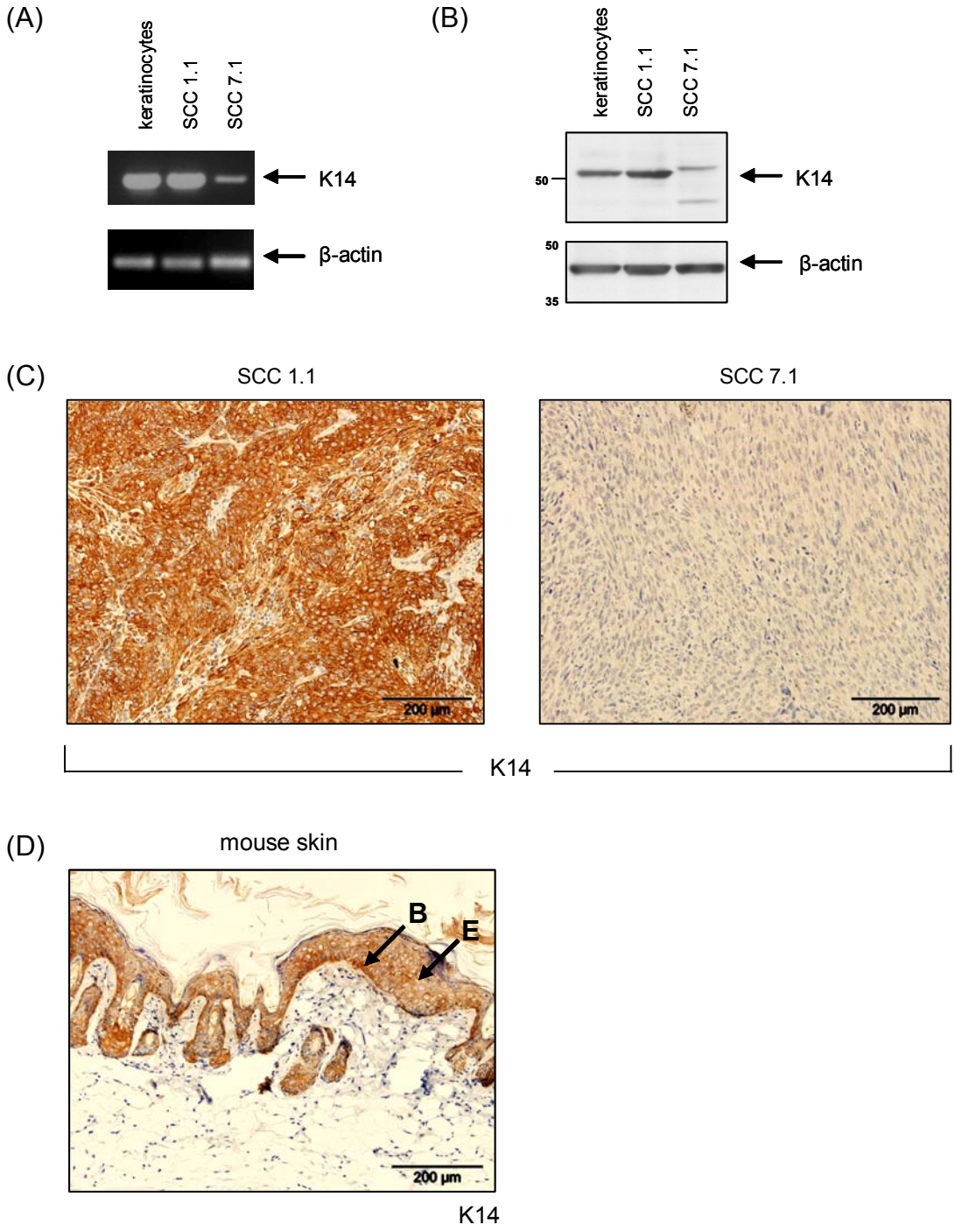


Figure 21 - K14 protein is readily expressed in SCC 1.1 but not SCC 7.1 cells
(A) RNA was extracted from K14*Cre*ER^{T2}FAK$^{flox/flox}$ keratinocytes, SCC 1.1 and SCC 7.1 cell lines, converted to cDNA using Superscript II as described in Methods section, and RT-PCR performed using K14 primers (upper panel). β-actin RT-PCR is shown as a loading control (lower panel). (B) Protein extracts from cultured normal keratinocytes, SCC 1.1, and SCC 7.1 cell lines were separated by SDS-PAGE, transferred to nitrocellulose, and probed with anti-K14 antibody (upper) and anti-β-actin antibody (lower). (C) Paraffin embedded sections from SCC 1.1 and SCC 7.1 xenografts were stained with the same anti-K14 antibody used for immunoblotting following appropriate antigen retrieval as outlined in Methods section. Representative images taken on bright field microscopy are demonstrated (SCC 1.1 – left panel and SCC 7.1 – right panel) (scale bar, 200µm). (D) K14 immunohistochemistry was repeated using skin from a K14*Cre*ER^{T2}FAK$^{flox/flox}$ mouse and a representative image is demonstrated, arrows indicate epidermis (E) and basal layer of the epidermis (B) (scale bar, 200µm).

3.2.5 Isolation of a FAK -/- single cell clone.

In order to overcome the potential problem of suboptimal *Cre*-mediated gene deletion, we adopted a single cell cloning approach and screened up to 40 colonies by western blotting following 72 hours treatment with 10μM 4-OHT. It was not possible to produce a FAK null cell clone from the SCC 1.1 cell line under these conditions (**Figure 22**), and further attempts varying the 4-OHT dose, exposure time and cell density did not yield a knockout cell line.

A FAK-/- cell line was, however, successfully generated from the SCC 7.1 cell line, as demonstrated by the absence of FAK protein in clone 11 on immunoblotting (**Figure 23**). Of note, further immunoblotting analysis of clones 10 and 31, which appeared to display lower levels of FAK, illustrated that this was a protein loading issue rather that a true reduction. There were no obvious morphological differences noted between the parental SCC 7.1 FAK +/+ cells and the derived FAK -/- cell line, both of which were mesenchymal in appearance (**Figure 24A**). The absence of FAK in clone 11 was illustrated by both western blotting (**Figure 24B**) and immunofluorescence (**Figure 24C)**, and the cell line remained stable over several months in culture. RT-PCR analysis of genomic DNA was performed to validate these findings. Both the parental cells and clone 11 demonstrated the presence of *Cre*, as expected, but the loxP sites were excised in clone 11, confirming *fak* deletion (**Figure 24D**).

Interestingly, unlike FAK -/- MEFs which have upregulation of Pyk2 compared with their FAK +/+ counterparts, the levels of Pyk2 recorded here were equivalent regardless of FAK status (**Figure 24B**). This result is significant as it suggests that Pyk2 does not compensate for the loss of FAK in this particular cell line. Also, despite our initial impressions from the 4-OHT FAK knockout experiments that FAK may be important for cell survival in the SCC 1.1 and SCC 7.1 cell lines as it is in keratinocytes (McLean, Komiyama et al. 2004); this was not the case, at least in SCC 7.1 cells. Similar subG1 fractions on propidium iodide based FACS analysis were demonstrated for both parental SCC 7.1 FAK +/+ cells and the derived FAK -/- clone (**Figure 24E**), indicating that FAK was not critical for survival *in vitro* in these cancer cells.

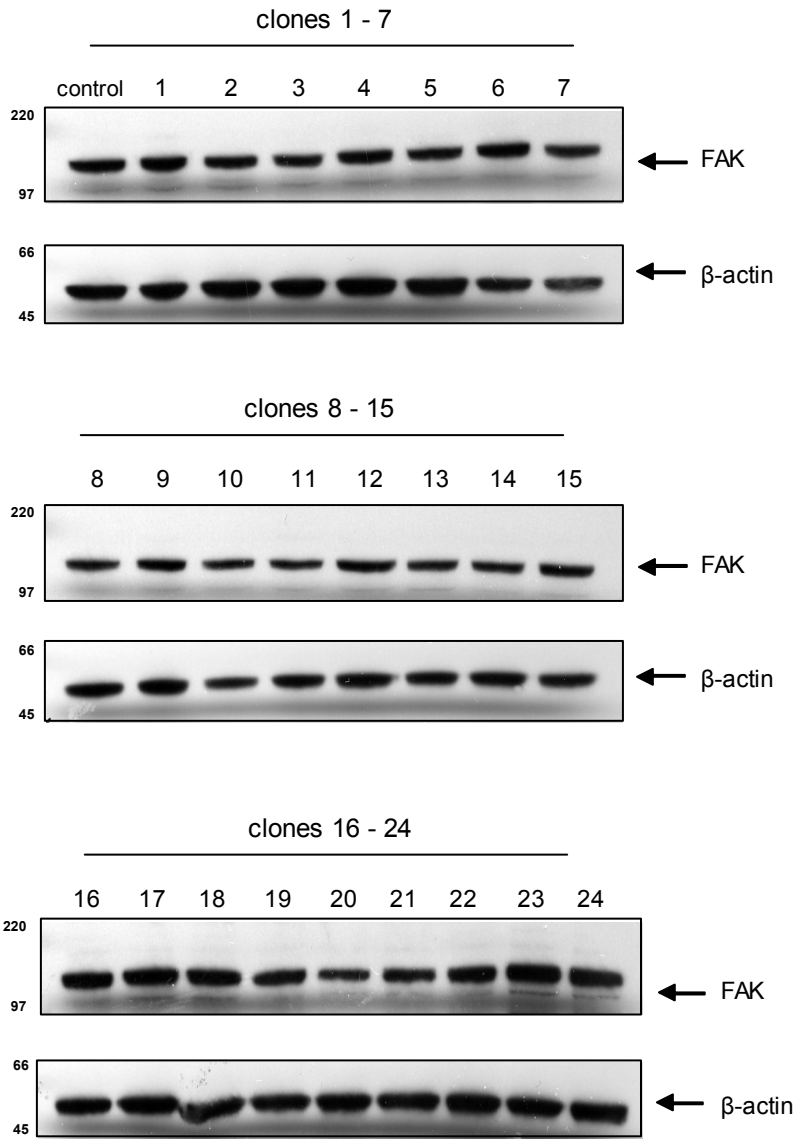


Figure 22 - 4-OHT treatment does not yield a FAK -/- single cell clone in SCC 1.1 cells
A subconfluent population of SCC 1.1 cells was treated with 10μM 4-OHT for 72 hours, trypsinised and replated at low density (100 cells per 90mm dish). After 14 days, 40 colonies were selected with a cloning disc and transferred into 12 well plates. Protein lysates were prepared from surviving clones, separated by SDS-PAGE, transferred to nitrocellulose, and probed with anti-FAK antibody (upper) and anti-γ-tubulin antibody (lower).

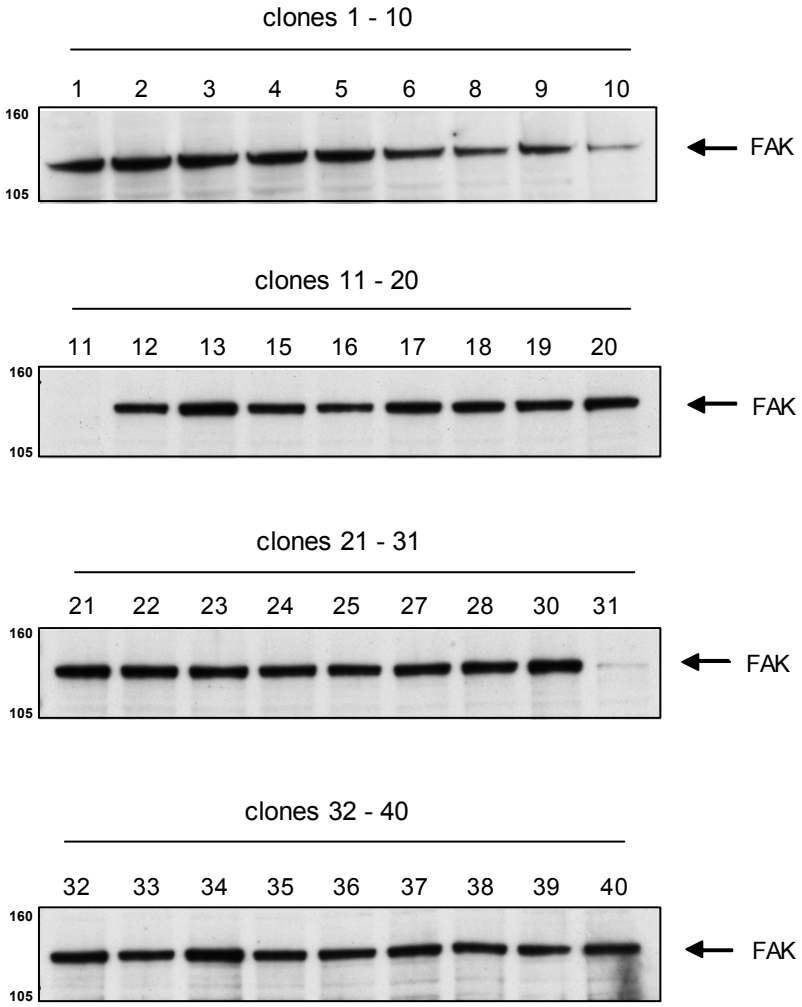


Figure 23 - 4-OHT treatment yields a FAK -/- single cell clone in SCC 7.1 cells
A subconfluent population of SCC 7.1 cells was treated with 10μM 4-OHT for 72 hours, trypsinised and replated in a 96 well plate at a dilution of 1 cell per well. After 10 days, 40 colonies were selected and transferred into 12 well plates. Protein lysates were prepared from surviving clones, separated by SDS-PAGE, transferred to nitrocellulose, and probed with anti-FAK antibody. Experiment courtesy of A Serrels.

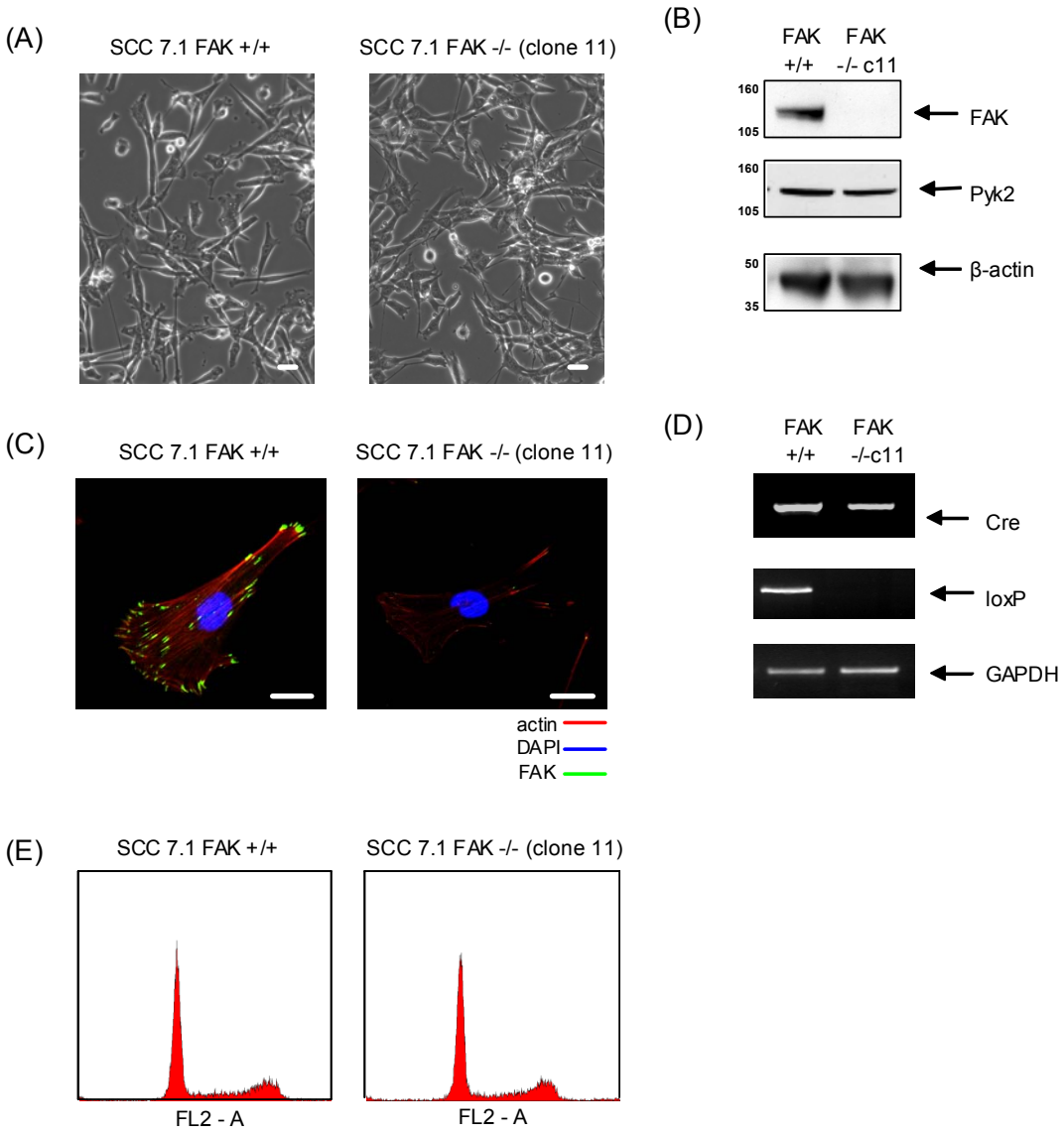


Figure 24 - Isolation of an SCC 7.1 FAK -/- single cell clone
(A) Phase contrast images of SCC 7.1 FAK +/+ parental cells and the derived FAK -/- cell line clone 11 (scale bar, 25μm). (B) SCC 7.1 FAK +/+ and FAK -/- clone 11 cellular protein extracts were separated by SDS-PAGE, transferred to nitrocellulose, and probed with anti-FAK antibody (upper), anti-Pyk2 antibody (middle), and anti-γ-tubulin antibody (lower). (C) Cells were plated on glass coverslips at low density, fixed, and stained with antibodies directed at FAK and actin, prior to visualisation by fluorescent confocal microscopy. Representative images of SCC 7.1 FAK +/+ (left) and FAK -/- clone 11 (right) cells are shown, red – actin, green – FAK, blue – DAPI (scale bar, 20μm). (D) Genomic DNA was extracted from SCC 7.1 FAK +/+ and FAK -/- clone 11 cells, subjected to RT-PCR for *Cre* (left) and loxP (right) as outlined in Methods section, and separated on 1.5% and 3% agarose gels respectively. (E) Subconfluent populations of SCC 7.1 FAK +/+ and FAK -/- clone 11 cells were suspended in ice cold PBS and fixed in 70% ethanol overnight prior to staining with propidium iodide and analysis by FACS as outlined in methods. Representative FACS profiles are demonstrated from one of three separate experiments.

3.2.6 Do underlying biological factors influence cell survival in the absence of FAK?

Thus, we have established that it is possible to derive a FAK -/- cancer cell line. Yet, it is unclear whether this is a cell line specific phenomenon and a number of important questions remain unanswered. For instance, was the lack of production of a FAK -/- single cell clone in the SCC 1.1 cell line a result of technical or biological problems within this system, such as the level of *Cre* recombinase expression? Alternatively, was the deletion of FAK effectively inducing proliferation arrest or cell death in this cell line and, if so, why? If we consider the skin carcinogenesis model outlined earlier in **Figure 12** (**page38**), this protocol typically induces a missense mutation in codon 61 of H-Ras which we were able to demonstrate in both cell lines. However, a number of key genetic mutations and biological alterations are thought to contribute to malignant progression, in addition to the previously described activating mutation in H-Ras, notably loss of important tumour suppressors such as p53, PTEN, and $p16^{INK4a}$ (Frame, Crombie et al. 1998; Suzuki, Itami et al. 2003; Zoumpourlis, Solakidi et al. 2003; Mao, To et al. 2004). With this information in mind, we aimed to identify whether any major genetic differences existed between the two SCC cell lines.

Currently, there is little in the literature to link FAK with these genes, with the exception of p53. A considerable body of evidence is emerging that connects FAK and p53 in cell survival. We, therefore, decided to focus on p53. First we determined that the gene was present in both SCC cell lines by RT-PCR, using 3T3 mouse fibroblasts as a positive control and p53 -/- MEFs as a negative control (**Figure 25A**). Next, we assessed p53 protein levels by immunoblotting. While protein was detected in both cell lines, a discrepancy was noted in the levels of p53 protein (**Figure 25B**). Intriguingly, low basal levels of p53 were present in SCC 1.1 cells. This indicated the presence of an un-mutated gene since wt p53 is normally expressed at very low levels in unstressed cells. On the other hand, greater levels of p53 were detected in SCC 7.1 cells, suggestive of a stabilising mutation. Consequently, the p53 levels in SCC 7.1 cells were found to be comparable with those in a mouse cell line, 83320, with a confirmed accumulating mutation of p53 (R175H +/-) (**Figure 25B**).

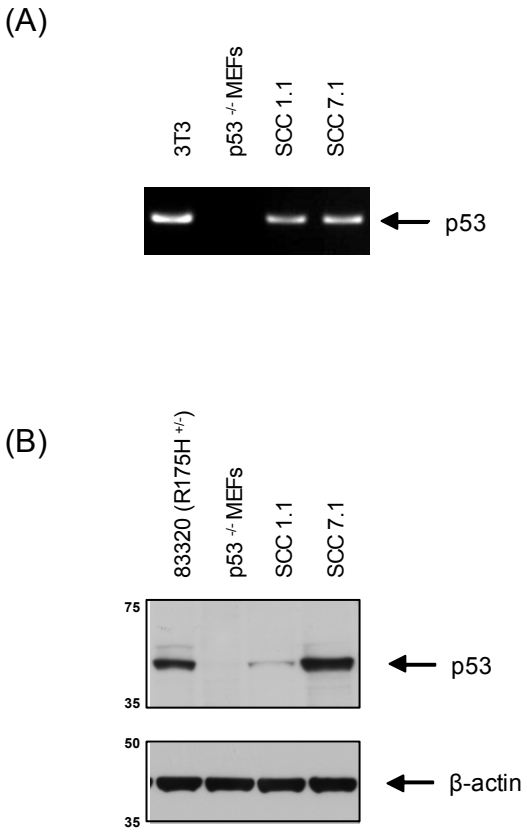


Figure 25 - p53 is overexpressed in SCC 7.1 cells compared with SCC 1.1 cells
(A) RT-PCR for p53 was performed with cDNA from 3T3, p53-/- MEFs, SCC 1.1 and SCC 7.1 cells, and separated on a 1.5% agarose gel. (B) Cellular protein extracts from 83320 cells, p53-/- MEFs, SCC 1.1 and SCC 7.1 cells were separated by SDS-PAGE, transferred to nitrocellulose, and probed with antibodies to p53. For further information on the 83320 cell line see Appendix 1 (page 234).

A full description of the 83320 cell line is provided in **Appendix 1** (page 236). In view of the data linking FAK and p53, these findings may well be biologically relevant and merit investigation in future chapters.

3.3 Discussion

We aimed to evaluate the role of FAK in radiation survival *in vitro* and *in vivo* by performing proof of principle experiments using a genetic deletion model. Rather than restricting our work to FAK +/+ and FAK -/- MEFs, we opted to develop a cancer cell line with inducible *fak* deletion as we believed this would be more relevant to human disease. There is evidence that, compared with tumour cells, fibroblasts respond differently to DNA damaging agents, including ionising radiation. Also, we intended to progress to *in vivo* studies which would not be possible with MEFs. In order to generate such a cell line, we utilised a conditional knock out mouse model that had already been used in this laboratory to investigate the role of FAK in skin carcinogenesis.

The advent of *Cre*/loxP technology has provided researchers with the means to conditionally delete a gene of interest in the mouse (Brault, Besson et al. 2007). This approach is obviously useful in situations where gene deletion in the embryo is lethal, for example in the case of FAK, where profound mesodermal and vascular abnormalities result in death at E8.5 (Ilic, Furuta et al. 1995). However, another major advantage of this system is that it allows temporal regulation in addition to spatial regulation. For the purposes of carcinogenesis, this degree of control is important as the function of the gene of interest can be evaluated at different stages of tumour initiation and progression. Indeed, we have already outlined that this system provided useful insights into the role of FAK in mouse skin carcinogenesis (McLean, Komiyama et al. 2004).

As we wished to develop a FAK -/- cancer cell line with which to investigate radiosensitivity, this model seemed ideal for several reasons, in addition to the obvious advantage of the 4-OHT-inducible system. Firstly, skin carcinogenesis with DMBA/TPA permits the development of squamous cell carcinomas as opposed to adenocarcinomas (or other histological tumour types). The former are more readily treated by radiation and therefore more biologically relevant.

Admittedly H-Ras mutations are not particularly common in human tumours (Bos 1989), at less than 5% (www.sanger.ac.uk/genetics/CGP/cosmic/), but this model has some similarities to human disease (Balmain and Harris 2000). Secondly, tumours form on the skin and the progression to frank carcinoma is often apparent by gross pathological appearance. As these tumours are readily visible and accessible, the likelihood of successfully developing a cancer cell line is high.

Accordingly, the generation of novel cell lines from established tumours was relatively straightforward. Deleting *fak*, however, proved to be more challenging. While 10nM tamoxifen promoted highly efficient *fak* excision in K14*Cre*ER^{T2}/FAK$^{flox/flox}$ keratinocytes (McLean, Komiyama et al. 2004), this dose had no effect on the cancer cell lines we generated. In fact, a 1000-fold dose increase was necessary to induce reasonable knockout. Full knockout was never achievable and we suspected this was due to a combination of suboptimal *Cre* recombinase expression and the dose limiting toxicity of 4-OHT (doses in excess of 15μM resulted in profound cytotoxicity). Despite testing several commercially available *Cre* antibodies in a variety of applications, namely western blotting, immunofluorescence, and immunohistochemistry, we were unable to find a good enough antibody to determine whether *Cre* expression was a problem. For these reasons, we would in the future incorporate a reporter gene such as *lacZ* into the system, to allow visualisation of *Cre* recombinase expression by ß-galactosidase staining.

Although we were unable to confirm our hypothesis regarding *Cre* expression in the SCC lines we generated, another group has since developed a Chk1 knockout cancer cell line using a similar model and have been able to demonstrate approximately 70-80% *Cre* recombinase activity using ß-galactosidase staining as a surrogate marker (personal communication – Lye Mun Tho). The discrepancy in *Cre* activity between normal keratinocytes and cancer cells (and the considerable difference in drug dose required to induce this activity) has led us to question whether a significant change occurs in the *Cre*-ER fusion protein during the process of carcinogenesis. An alteration in the conformation and/or cellular localisation of this fusion protein could perhaps explain why lower doses of 4-OHT were ineffective at inducing FAK loss. This may also explain why 4-OHT was ineffective in the SCC 1.1 and SCC 7.1 xenografts despite using a well

documented regimen. While we accept that this view is largely speculative at present, there is some evidence to suggest that *Cre* undergoes silencing in the form of methylation (Long and Rossi 2009).

One final point on the application of the *Cre*/loxP system is that our results unexpectedly, but consistently, showed lower levels of FAK knockout in the SCC 1.1 cell line. We were able to demonstrate that this was not due to lack of the K14 promoter as this cell line had high levels of both K14 mRNA and protein. Conversely, SCC 7.1 cells, which had very low levels of K14 transcript and did not express K14 at the protein level, displayed more efficient FAK loss. The reasons for this are not entirely clear but our findings would suggest that when K14 is turned on in the embryo it drives the expression of *Cre* which is stably maintained in cells even in the event of aberrant or lost cytokeratins during the process of malignancy. Alternatively, low levels of K14 mRNA may be sufficient to drive *Cre* expression. Either way, this does not necessarily explain why FAK knockout was less efficient in the high K14 expressing cell line, and implies that *Cre* function may be critically important, as we suggested above, and may vary from cell line to cell line.

FAK is elevated genetically, by amplification, and/or at the protein level in a number of epithelial cancers, including breast, colon, thyroid, and ovary (Owens, Xu et al. 1995; Owens, Xu et al. 1996; Agochiya, Brunton et al. 1999; McLean, Avizienyte et al. 2003; Golubovskaya, Kweh et al. 2009). Further, we know from previous work, that FAK protein levels are increased in squamous cell carcinomas of mouse skin compared with normal keratinocytes and papillomas (McLean, Avizienyte et al. 2003; McLean, Komiyama et al. 2004; McLean, Carragher et al. 2005). Similarly, the two isolated cell lines here displayed higher levels of FAK and Pyk2 compared with normal counterpart keratinocytes. This hints that FAK signalling is somehow crucial in the process of carcinogenesis, but does not answer the question of whether FAK is necessary for survival in established cancer cells. Clearly it is not the case in SCC 7.1 cells where a stable FAK -/- clone was isolated and did not demonstrate any increase in subG1 population on FACS analysis, when compared with its FAK +/+ counterpart. However, a number of studies have shown the induction of cell death, primarily by apoptosis, in a variety of cancer cell lines exposed to either FAK siRNA, FAK antisense oligonucleotides, or small molecule tyrosine kinase

inhibitors (Smith, Golubovskaya et al. 2005; Halder, Kamat et al. 2006; Wu, Yuan et al. 2006; Halder, Lin et al. 2007; Liu, LaFortune et al. 2007; Shi, Hjelmeland et al. 2007; Beierle, Trujillo et al. 2008; Golubovskaya, Virnig et al. 2008; Roberts, Ung et al. 2008; Watanabe, Takaoka et al. 2008; Sakurama, Noma et al. 2009). While we struggled to develop an SCC 1.1 FAK -/- single cell clone and strongly suspected that the difficulties were due to *Cre* expression and / or function; in the absence of any definitive data, we had to consider the possibility that SCC 1.1 cells simply could not survive in the absence of FAK, thereby precluding the development of a FAK -/- clone.

We demonstrated that the SCC 1.1 and SCC 7.1 cell lines were morphologically distinct and it seemed likely that they would each possess a different pattern of genetic mutations and biological characteristics. One particular gene product that certainly appeared to differ was p53. The large discrepancy in basal protein level suggested the presence of wt p53 in the SCC 1.1 cell line and mutant p53 in the SCC 7.1 cell line. Interestingly, it has been proposed that the spindle cell carcinoma phenotype arising from the topical carcinogenesis model is dependent on the presence of mutant p53 (Ruggeri, Caamano et al. 1991; Han and Kulesz-Martin 1992), although this is controversial (Buchmann, Ruggeri et al. 1991). More importantly, a link between p53 and FAK and cell survival has already been established (Ilic, Furuta et al. 1995; Lim, Chen et al. 2008). In the pivotal work performed by Ilic et al FAK -/- MEFs could only proliferate in the absence of p53, although it is pertinent to note that the loss of p53 did not rescue embryonic lethality (Ilic, Furuta et al. 1995). In addition, inducing *fak* deletion in K14*Cre*ER^{T2}/FAK$^{flox/flox}$ keratinocytes (which we assume have wt p53) led to increased cell death (McLean, Komiyama et al. 2004).

During the course of this work, further information has come to light indicating a close relationship between FAK and p53. In fact, it was recently documented that FAK and p53 directly interact, and more importantly the FAK – p53 axis is responsible for cell fate decisions (Lim, Chen et al. 2008). It is becoming clear that FAK and p53 maintain a delicate balance of pro-growth/survival and anti-growth/survival signals, at least in normal cells with wt p53. While there is growing indirect and direct evidence demonstrating that this balance also exists in cancer cells (Ilic, Almeida et al. 1998; Zhang, Lu et al. 2004; Lim, Chen et al. 2008), induction of cell death in FAK knockdown cancer cells may require an

additional stress, such as the addition of a cytotoxic drug. A crucial difference between these reported studies and our system is their reliance on FAK siRNA, as opposed to our much cleaner genetic deletion model. It may be the case that even in cancer cells, with their potential for multiple factors to control cell survival, that complete absence of FAK protein is critical in terms of survival. We therefore postulated that p53 status may be a key factor in the survival of cancer cells, as it is in fibroblasts, in the context of a FAK null background (see **Figure 26**).

3.4 Summary

We have generated a FAK -/- squamous cell carcinoma cell line which will now be a useful tool in evaluating the role of FAK in radiation sensitivity. We hypothesised at this point that p53 status may be an important determinant of cancer cell survival in the absence of FAK.

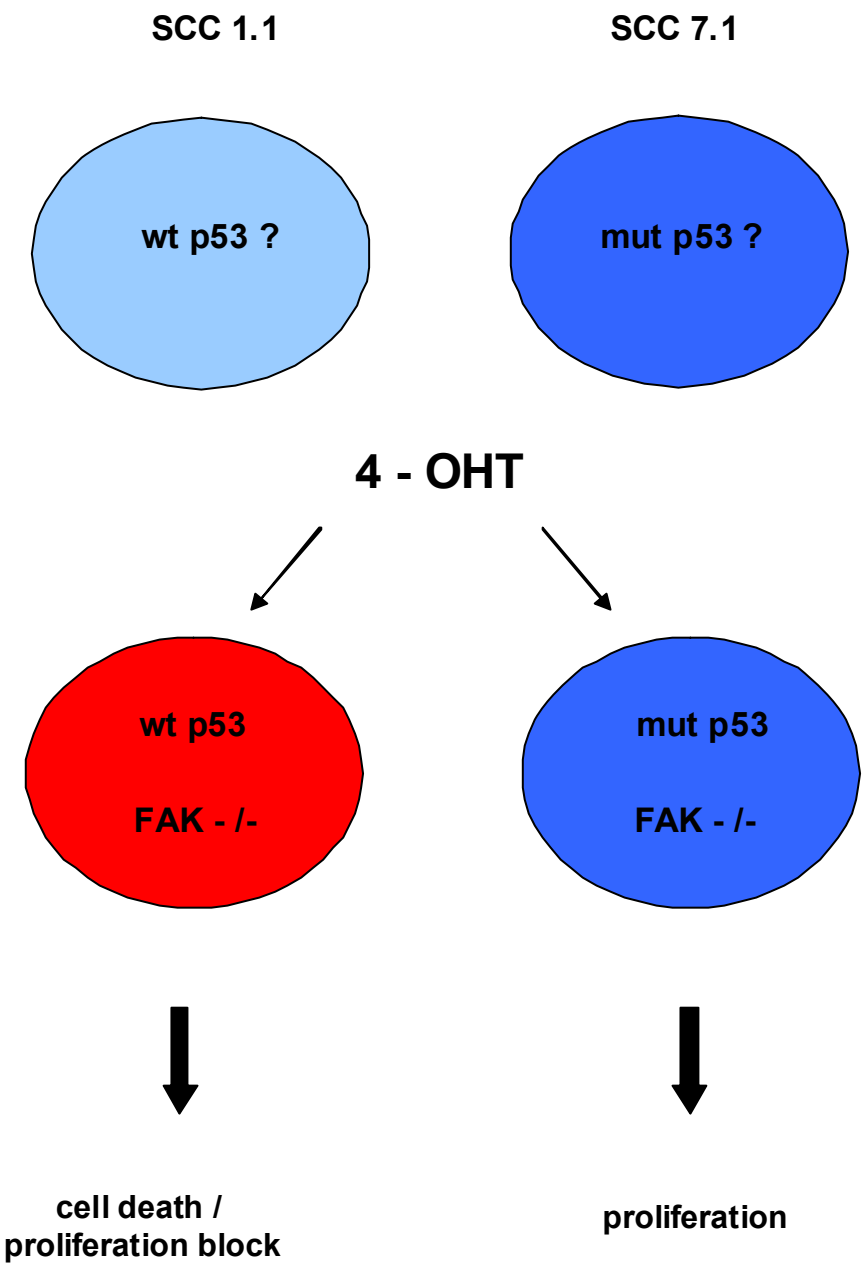


Figure 26 - FAK/p53 hypothesis
We hypothesised that the SCC 1.1 cell line has wt p53 and undergoes cell death or proliferation block following 4-OHT inducible *fak* deletion as opposed to the SCC 7.1 cell line which we suspect has mutant p53 and can propagate in the absence of FAK.

Chapter 4

Evaluating the p53 status of the SCC 1.1 and SCC 7.1 cell lines

4 Evaluating the p53 status of the SCC 1.1 and SCC 7.1 cell lines.

4.1 Aim

In the previous chapter we described the development of two novel squamous cell carcinoma cell lines; SCC 1.1 and SCC 7.1. However, we were only able to successfully excise *fak* from one of these cell lines (SCC 7.1). We suspected that the ability of these cancer cell lines to survive in the absence of FAK was linked to p53. Here, we set out to determine the p53 status of each cell line (and the derived SCC 7.1 FAK -/- single cell clone).

4.2 Results

4.2.1 SCC 1.1 cells have wt functional p53.

The majority of p53 mutations are missense mutations which are readily detectable by direct sequencing. Since more than 90% of these lie within the central DNA binding domain, primers were designed to cover this region (**Figure 27**) and cDNA was analysed following PCR amplification and purification. As expected, no mutations were detected in SCC 1.1 cells.

While a cell can possess wt p53 protein, the p53 pathway may not be fully operational due to dysfunction and / or mutation in either upstream or downstream effectors. Therefore, we attempted to learn more about the function of p53 by studying the effect of ionising radiation on p21 induction. Under normal conditions, p53 is maintained at a low level, largely due to its extremely short half-life. However, in response to stresses such as DNA damage (including ionising radiation), p53 levels rapidly increase. This is predominantly due to stabilisation of the protein, which is in turn triggered by a series of phosphorylation, de-phosphorylation, and acetylation events on the polypeptide. p53 then has an increased ability to bind DNA and mediate transcription by inducing specific target genes. It binds to short genomic sequences, called p53-responsive elements (PREs), and hundreds of potential target genes with PREs

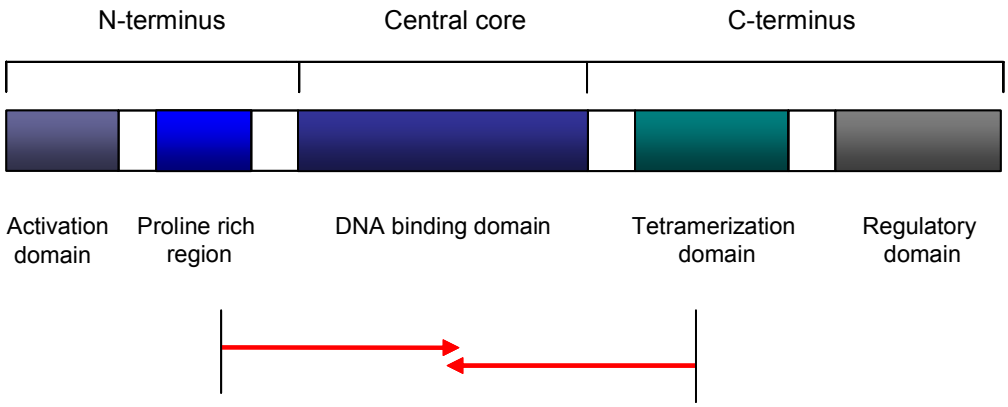


Figure 27 - p53 sequencing region
Primers were designed to amplify the central DNA binding domain of mouse p53 (exons 5 – 8), red arrows denote the specific area under investigation.

have now been identified. One well recognised p53 target gene is CDKN1A, which encodes the protein $p21^{Waf1/Cip1}$ (Kastan, Onyekwere et al. 1991), hereafter referred to simply as p21. This protein belongs to the Cip and Kip family of cyclin-dependent kinase (CDK) inhibitors that includes p21, p27 and p57. p21 inhibits the kinase activity of CDKs and CDK-cyclin complexes. Further, micro-array based studies suggest that p21 expression positively correlates with the suppression of genes that are important for cell cycle progression (Chang, Watanabe et al. 2000). Hence, it is no surprise that p53 mediated induction of p21 in response to DNA damage is often associated with cell cycle arrest. This protein has a number of additional functions that can influence the outcome of DNA damage. For example, it can interfere with PCNA–dependent DNA polymerase activity, thereby inhibiting DNA replication and modulating various PCNA – dependent DNA repair processes. The precise role of p21 in each individual cancer cell line exposed to DNA damaging agents, such as ionising radiation, is likely to be different. For that reason, we opted to investigate whether p21 protein was upregulated in response to stress and use this test as a surrogate for p53 activity. Trying to examine functional downstream effects of p21 upregulation, such as cell cycle arrest, is more problematic in cancer cell lines compared with normal cells like fibroblasts, due to the potential complexity of the DNA damage response.

To assess the effect of ionising radiation on p21 induction and gain insight into the function of p53 in this cell line, subconfluent cell populations were irradiated with 5Gy and lysates prepared for immunoblotting at various time points. Following exposure to radiation, SCC 1.1 cells exhibited an increase in p53 protein levels within 2 hours, quickly followed by induction of p21 at 4 hours (**Figure 28A**). The increase in p21 was maintained until the final 24 hour time point.

A parallel experiment was set up *in vivo* whereby $5x10^5$ cells were injected subcutaneously into nude mice and xenografts grown to 400 – 500 mm^3. The animals were then sacrificed before or 6 hours after 5Gy whole body irradiation. Paraffin embedded sections were then stained for both p53 and p21. Nuclear accumulation of p53 was visible after radiation, but basal levels of the protein were difficult to detect in control animals (**Figure 28B** – upper panels). p21, on the other hand, was readily detected in both groups, allowing an objective

statistical comparison. The proportion of p21 positive cells per field was scored (20 fields per mouse) and the mean values of non-irradiated versus irradiated mice were then analysed. A robust p21 response was visualised (**Figure 28B – lower panels**) suggesting the presence of functional, normally regulated, p53 protein in this cell line although this did not quite reach statistical significance (Mann Whitney, p=0.0809, n=3).

4.2.2 The SCC 7.1 cell line is a mixed population of wt and mutant p53 cells.

Initial sequencing analysis of the SCC 7.1 cell line did not reveal any mutations, but it quickly became apparent that some of the features of this cell line appeared to change over time. We noticed that the basal levels of p53 at early passage were actually very low, and in fact similar to SCC 1.1 cells (**Figure 29A**). However, the levels of p53 increased with passage number, and by passage 25 the levels were comparable with that of the 83320 (R172H +/-) cell line described in the previous chapter (**Figure 29A**). The converse was true for p21; substantial protein levels were detected in early passage cells, but were markedly lower by passage 25 (data not shown). The pattern of p53 immunofluorescence also differed with most of the cells in early cultures illustrating weak diffuse staining of both the cytoplasm and nucleus (although a number of cells did demonstrate stronger predominantly nuclear staining). In contrast, the vast majority of cells in later cultures exhibited robust nuclear staining and minimal cytoplasmic staining (**Figure 29B**).

Further investigation revealed that, in addition to variances in p53 and p21 levels and p53 localisation, the morphology of the cell population seemed to change from predominantly spindle at early passage to a more refractile morphology at later passage (**Figure 30**). Based on these findings, we suspected that the original SCC 7.1 cell line was composed of a mixture of cell populations, some with wt p53 and some with mutant p53. In order to address this, we repeated the sequencing analysis at passage 5 and passage 25. No mutations were detected in low passage cells. Remarkably, examination of the high passage population did reveal a mutation: a T to G substitution was detected at codon 254 (**Figure 31**).

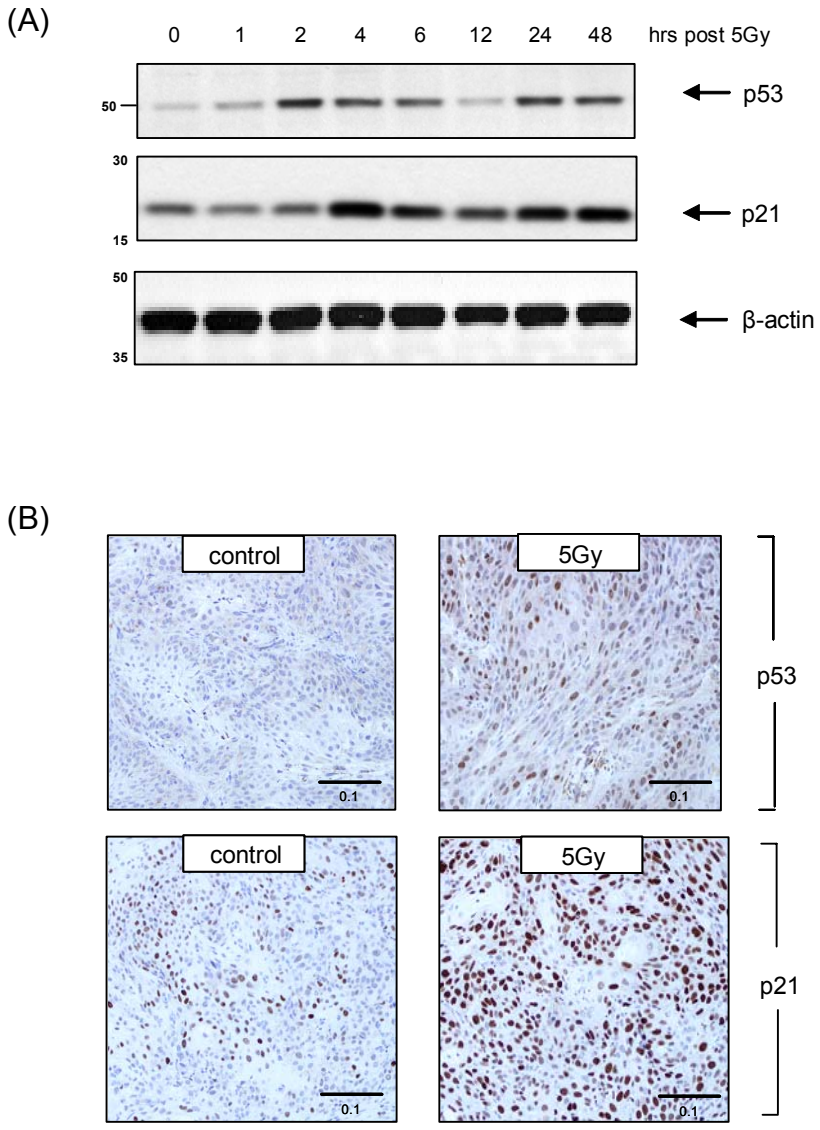


Figure 28 - SCC 1.1 cells have functional wt p53
(A) SCC 1.1 cells were irradiated with 5Gy at 70 – 80% confluence and lysates prepared at various time points. Immunoblotting was then performed for p53, p21, and β-actin. (B) $5x10^5$ SCC 1.1 cells were injected subcutaneously into the right flank of 6 week old female nude mice. Xenograft growth was monitored until tumours reached 400 – 500 mm^3. The animals were then sacrificed either before or 6 hours after 5Gy whole body irradiation. Representative bright field images of paraffin embedded sections stained with p53 (upper) and p21 (lower) are shown (scale bar, 0.1mm).

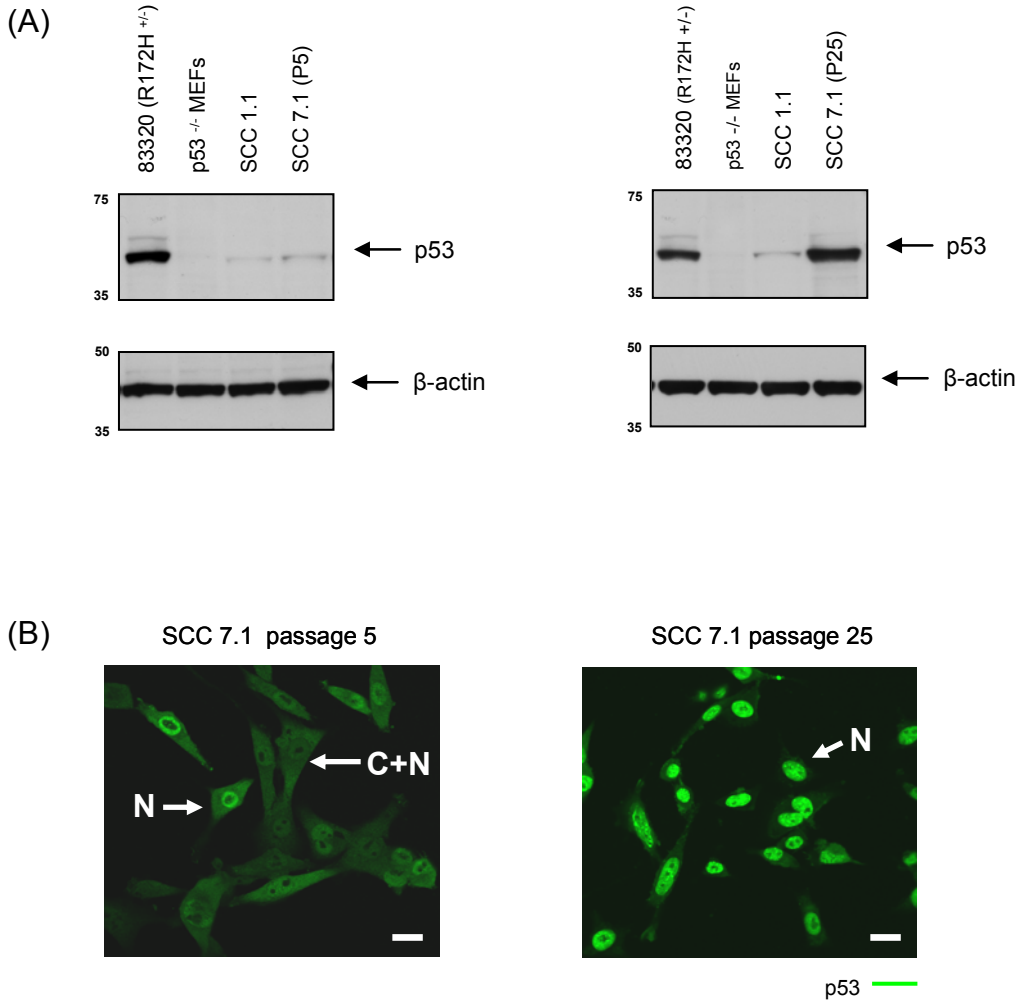


Figure 29 - p53 levels and p53 localisation vary in SCC 7.1 cells depending on passage
(A) Protein extracts were obtained from 83320, p53-/- MEFs, SCC 1.1 cells and either SCC 7.1 passage 5 (left) or passage 25 (right) cells, separated by SDS-PAGE, transferred to nitrocellulose, and blotted with anti-p53 (upper panels), anti-p21 (middle panels) and anti-β-actin (lower panels). (B) SCC 7.1 cells were plated at low density on glass coverslips, fixed, and stained with anti-p53 antibody. Representative images taken on fluorescent confocal microscopy are shown for a passage 5 population (left) and passage 25 population (right), green – p53, arrows indicate cells with predominantly nuclear staining (N) and cells with both cytoplasmic and nuclear staining (C+N) (scale bar, 50μm).

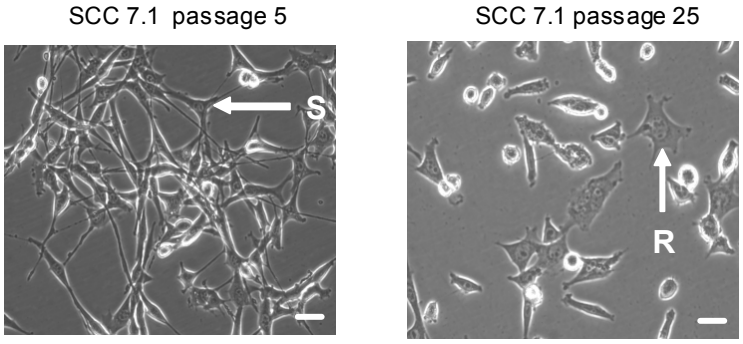


Figure 30 - SCC 7.1 cellular morphology alters with passage number
Phase contrast images of subconfluent populations of SCC 7.1 passage 5 (left) and passage 25 (right), arrows indicate spindle morphology (S) and refractile morphology (R) (scale bar, 25μm).

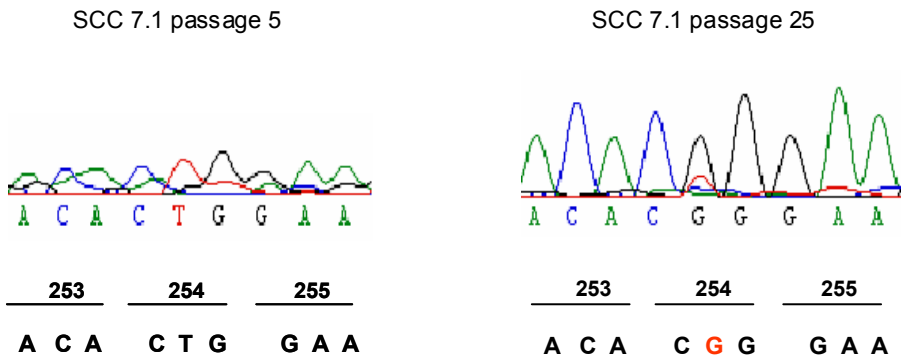


Figure 31 - The SCC 7.1 cell line is a mixed population of cells with different p53 status
RNA was extracted from SCC 7.1 cells at passage 5 and passage 25 and converted to cDNA as previously described. The cDNA was then amplified by PCR, purified, and subjected to direct sequencing analysis. Details of the primers are provided in the Methods section. Chromatograms for codons 253 – 255 of p53 are demonstrated for SCC 7.1 passage 5 (left) and passage 25 (right).

The missense mutation found, L254R, is located in a highly conserved region of the DNA binding domain. The resultant amino acid change (leucine to arginine) is likely to significantly alter the structure and/or function of the p53 protein as leucine is positively charged as opposed to the negatively charged arginine. In addition, leucine is hydrophobic whereas arginine is hydrophilic. However, this is not a well documented mutation, with just a single report in UVA induced mouse squamous cell carcinoma (van Kranen and de Gruijl 1999). The corresponding mutation in humans (L257R) has been documented several times in basal cell carcinoma of the skin (Agar, Halliday et al. 2004) and ovarian adenocarcinoma (Ramus, Bobrow et al. 1999; Rose, Robertson et al. 2003; Wang, Kringen et al. 2004) but, again, has not been studied in much detail.

At this point, we were unsure whether this mutation was present within the original tumour, meaning that the derived SCC 7.1 cell line was a mixed population at the outset, or if the mutation arose in culture. Either possibility was a reasonable conjecture as advanced cancers are likely to consist of populations of cells that have acquired different genetic mutations, plus p53 mutations commonly occur *do novo* in the cell culture environment. We consistently found that different batches of SCC 7.1 cells expressed low levels of p53 protein on immunoblotting at passage 5. The presence of wt 53 was confirmed by sequencing in each case. In contrast, high levels of p53 were always observed in older cells at passage 25. Critically, the same mutation (L254R) was detected in each batch of late passage SCC 7.1 cells. So, we surmised that this mutation had arisen in the original mouse tumour, and that over time the mutant p53 cells overpopulated the culture due to a proliferation advantage. This theory is explored in more detail in the next chapter.

In light of our findings demonstrating a mixed cell population, it seemed appropriate to perform p53 functionality experiments in the SCC 7.1 cell line in early passage versus late passage populations. As shown in **Figure 32A**, p53 levels increased in early passage SCC 7.1 cells in response to radiation, although this was not as marked compared with SCC 1.1 cells. Correspondingly, p21 was induced, albeit transiently (**Figure 32A**). Unlike SCC 1.1 cells, the increase in p21 was not sustained. Low passage SCC 7.1 xenografts also illustrated minimal basal p53 but this was still difficult to detect after radiation (**Figure 32B** – upper left panels). There was a small increase in p21 levels at 6 hours post irradiation

(**Figure 32B** – lower left panels) but this did not reach statistical significance (Mann Whitney, p=0.1914, n=3). However, based on immunoblotting, the transient increase in p21 occurred at 4 hours *in vitro*. Hence, it is possible that the maximal increase *in vivo* was missed, assuming that the cellular response to radiation in these cell lines was similar *in vitro* and *in vivo*.

High passage populations of SCC 7.1 cells, on the other hand, had high basal levels of p53 (in keeping with mutant protein) and no detectable difference could be detected after radiation exposure at all, although there was a late increase in p21 at 24 hours (**Figure 33A**). As expected, SCC 7.1 high passage xenografts displayed abundant levels of p53 in the majority of cells, and this pattern did not change after 5Gy irradiation (**Figure 33B** – upper right panels). p21 levels were generally lower in these specimens and did not alter in response to radiation (**Figure 33B** – lower right panels, Mann Whitney, p=1.0, n=3).

Taken together, this work pointed towards the presence of both wt p53 and mutant 53 cells within the original SCC 7.1 cell line. As a result, compared with the more straightforward data from the SCC 1.1 cell line, the functionality experiments in this section were more difficult to interpret. Nonetheless, early passage SCC 7.1 cells, which demonstrated wt p53 on sequencing analysis, did behave in a manner suggestive of functional p53.

One major unexplained phenomenon was the late increase in p21 protein levels in response to radiation in high passage SCC 7.1 cells (which we believed consisted of predominantly mutant p53 cells). It is likely that there were still wt p53 cells present in the population at passage 25 – 30 which would be capable of transactivating p21 (and indeed a double peak on the chromatogram was highly suggestive of the presence of both wt and mutant p53 containing cells – **Figure 31**). However, this would probably not justify such a substantial delay in p21 induction. For a long time, it was assumed that mutant p53 proteins were incapable of transactivation, but studies have now shown that this is not necessarily the case (Aurelio, Kong et al. 2000; Weisz, Oren et al. 2007). As already outlined, there is minimal information available on the L254R mutant p53 protein. Hence, the specific properties of this protein in murine cancer cells

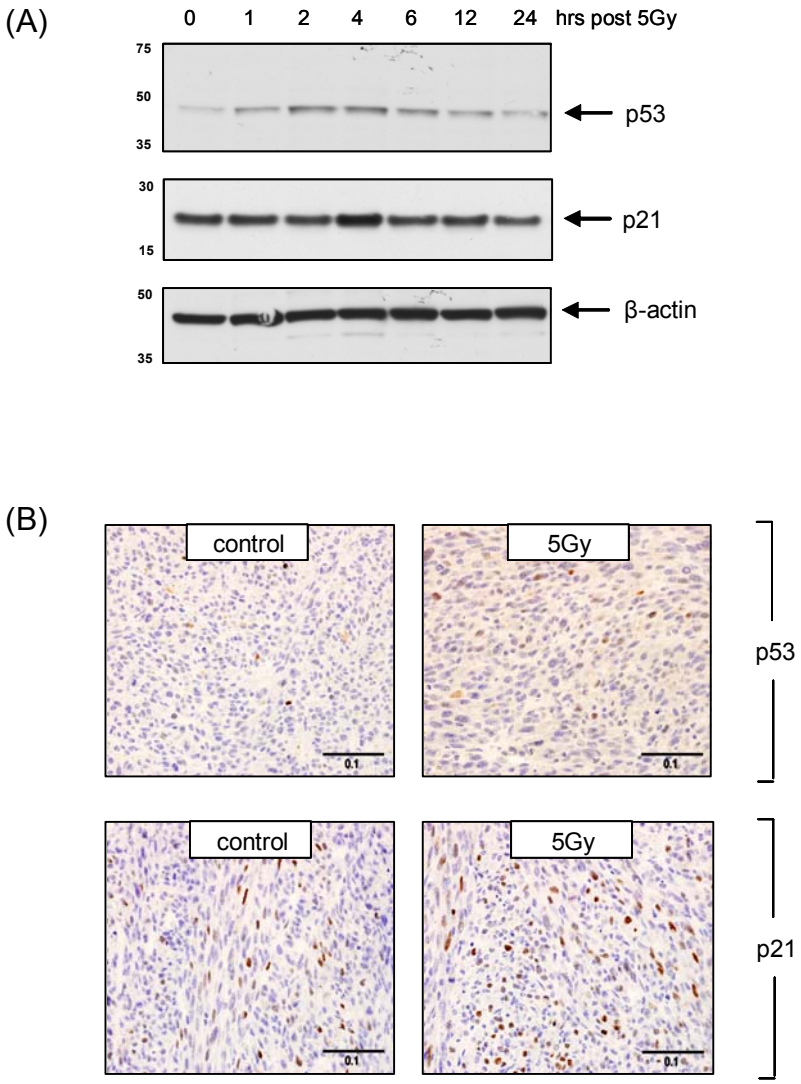


Figure 32 - Investigating p53 function in SCC 7.1 low passage cells
(A) SCC 7.1 cells (passage 5) were irradiated with 5Gy at 70 – 80% confluence and lysates prepared at various time points. Immunoblotting was then performed for p53, p21, and β-actin. (B) $5x10^5$ SCC 7.1 cells (passage 5) were injected subcutaneously into the right flank of 6 week old female nude mice. Xenograft growth was monitored regularly until tumours reached 400 – 500 mm^3. The animals were then sacrificed either before or 6 hours after 5Gy whole body irradiation. Representative bright field images of paraffin embedded sections stained with p53 (upper) and p21 (lower) are shown (scale bar, 0.1mm).

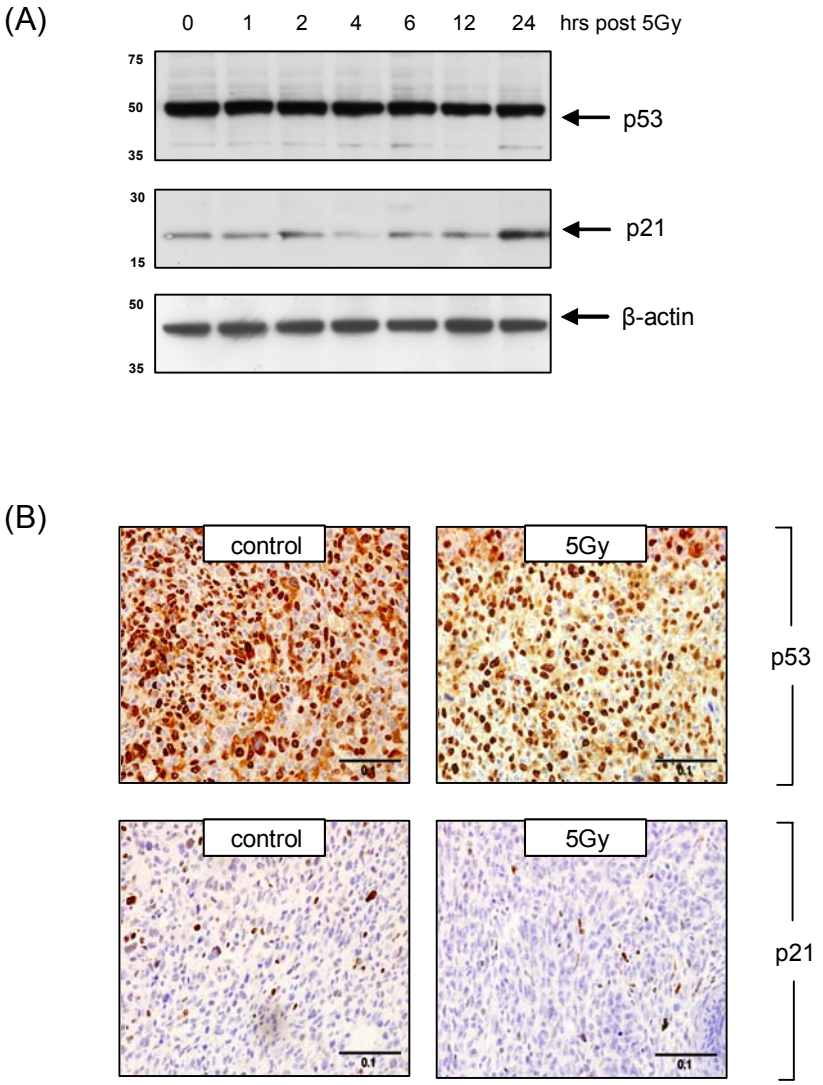


Figure 33 - Investigating p53 function in SCC 7.1 high passage cells
(A) As above, SCC 7.1 cells (passage 25) were irradiated and immunoblotting was performed for p53, p21, and β-actin at the determined time points. (B) $5x10^5$ SCC 7.1 cells (passage 25) were injected subcutaneously into the right flank of 6 week old female nude mice. Xenograft growth was monitored regularly until tumours reached 400 – 500 mm^3. The animals were then sacrificed either before or 6 hours after 5Gy whole body irradiation. Representative bright field images of paraffin embedded sections stained with p53 (upper) and p21 (lower) are shown (scale bar, 0.1mm).

have yet to be elucidated. However, the human equivalent, L257R, has been assessed in a yeast-based functional assay. Kato et al. performed a screen of over 2000 p53 mutations and determined that this particular mutant protein did not lead to transactivation of p53 target genes such as p21 and Bax (Kato, Han et al. 2003). Although these findings have yet to be validated in another cell line, they suggest that the p21 induction seen here is probably not explained by mutant p53 transactivation either. Of course, it must be pointed out that the human and mouse mutant proteins may not have identical properties. On balance, we conclude that the probable mechanism for the pattern of p21 induction in this case is independent of p53. Although p21 is a well known p53 target gene, the regulation of this protein is highly complex and it is subject to control by an array of transcription factors and cellular proteins (reviewed by (Abbas and Dutta 2009)).

4.2.3 The SCC 7.1 FAK -/- single cell clone has wt functional p53.

An important question was posed following the observation that the original SCC 7.1 cell line was a mixed population of wt p53 and mutant p53 cells - did the derived SCC 7.1 FAK -/- single cell clone possess wt or mutant p53? Examination of codon 254 by sequencing analysis did not reveal a T to G substitution and no mutations were detected in the remainder of the DNA binding domain. As it is difficult to ensure that a clonal population is definitely derived from a single cell, we were concerned about possible contamination with a mutant p53 cell. Repeat sequencing analysis was therefore performed at passage 25 – 30. This did not demonstrate any evidence of a mutation (**Figure 34A**). Furthermore, western blotting for p53 showed low basal levels which did not change over time, as did immunofluorescence studies, which also demonstrated weak diffuse p53 staining similar to that of the majority of cells in early passage SCC 7.1 populations (**Figures 34B** and **34C** respectively). The spindle morphology of the clone 11 cell line was also in keeping with the majority of the cells in early passage SCC 7.1 populations and did not vary with increased passage (data not shown). Collectively, this data implied that the FAK -/- single cell clone consisted only of wt p53 cells.

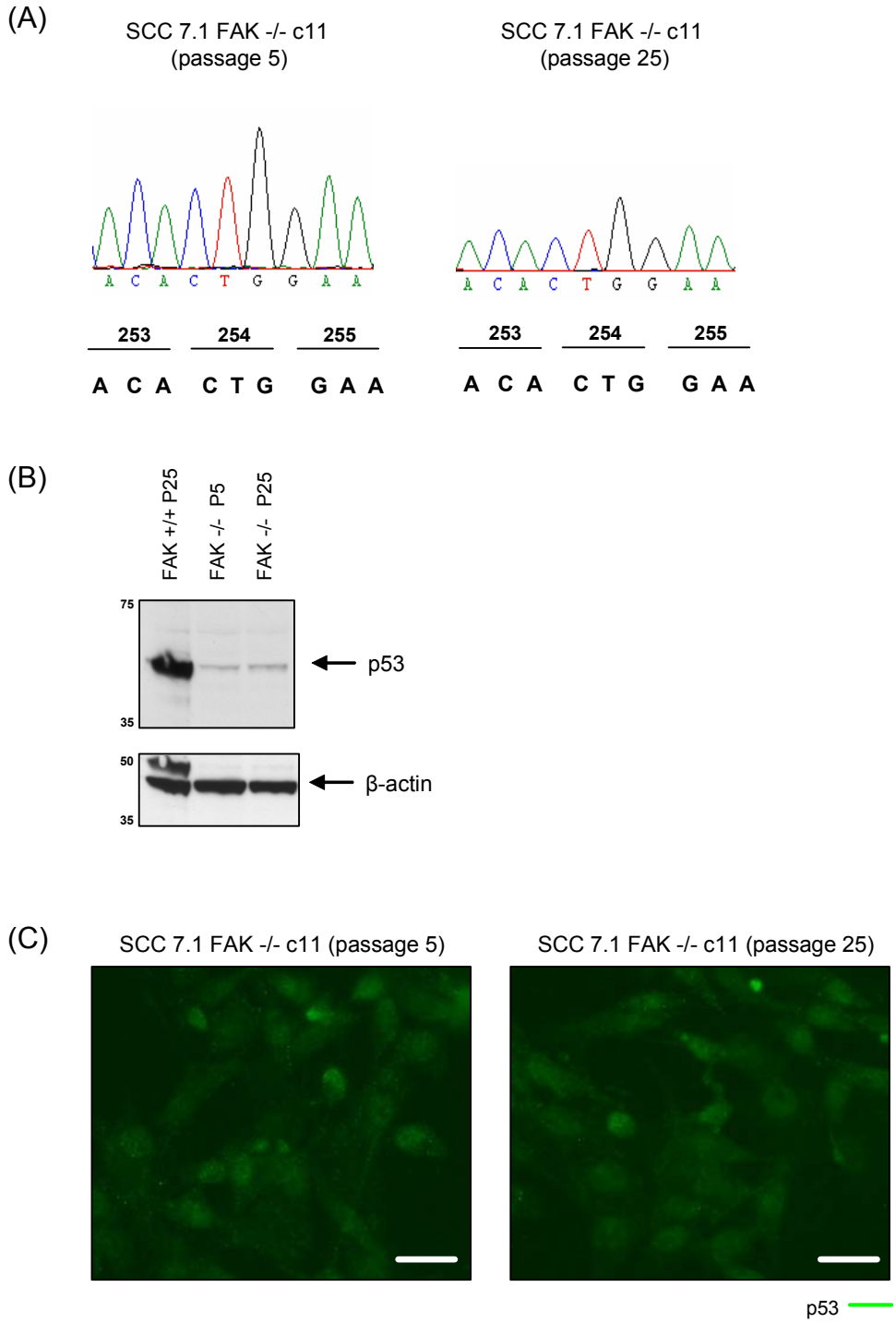


Figure 34 - SCC FAK -/- clone 11 is derived from a wt p53 cell
(A) RNA was extracted from SCC 7.1 FAK -/- cells at passage 5 and passage 25 and converted to cDNA which was then amplified by PCR, purified, and subjected to direct sequencing analysis as previously described. Chromatograms for codons 253 – 255 of p53 are demonstrated for SCC 7.1 FAK -/- clone 11 cells, passage 5 (left) and passage 25 (right). (B) Protein extracts were obtained from SCC 7.1 FAK +/+ passage 25 and SCC 7.1 FAK -/- clone 11 cells at passage 5 and passage 25, separated by SDS-PAGE, transferred to nitrocellulose, and blotted with anti-p53 (upper panels) and anti-β-actin (lower panels). (C) SCC 7.1 FAK -/- clone 11 cells were plated at low density on glass coverslips, fixed, and stained with anti-p53 antibody. Representative images taken on confocal microscopy are shown for a passage 5 population (left) and passage 25 population (right), green – p53 (scale bar, 50μm).

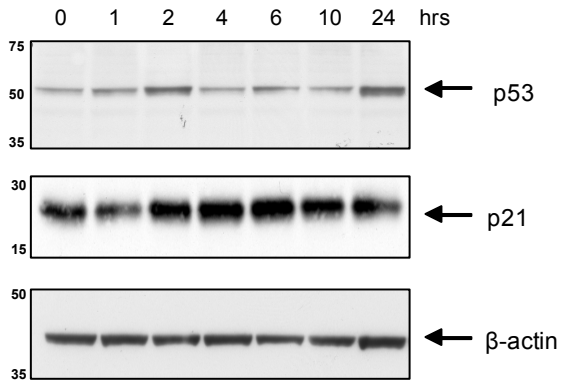


Figure 35 - SCC FAK -/- clone 11 has functional wt p53

SCC 7.1 FAK -/- clone 11 cells (passage 5) were irradiated with 5Gy at 70 – 80% confluence and lysates prepared at various time points. Immunoblotting was then performed for p53, p21, and β-actin.

As previously discussed, the presence of wt p53 protein does not necessarily equate with functional p53 activity. Immunoblotting for p53 and p21 following irradiation demonstrated stabilisation of p53 and a corresponding increase in p21 protein levels, indicating that p53 was functional in this cell line (**Figure 35**). Interestingly, the induction of p53 in this cell line demonstrated a cyclical pattern, with increases in protein levels visible at 2 hours, 6 hours, and 24 hours following irradiation. This finding is not atypical as ionising radiation often produces a biphasic, or even oscillatory, effect on protein induction (Zhang, Liu et al. 2009).

In keeping with our proposal in the previous chapter that the SCC 1.1 cell possessed wt p53 and the SCC 7.1 cell line possessed mutant p53, we have demonstrated the presence of wt functional p53 in the SCC 1.1 cell line. However, we unexpectedly discovered that the SCC 7.1 cell line was composed of (at least) two different cell types, one with wt p53 and one with a p53 mutation, L254R. Even more intriguingly, the derived SCC 7.1 FAK -/- single cell clone was clearly not dependent on mutant p53 for survival. This clone had, in fact, been developed from a cell with wt p53.

4.3 Discussion

The presence of wt p53 was confirmed in the SCC 1.1 cell line and in a subset of SCC 7.1 cells by sequencing the central DNA binding domain. Screening the entire gene was deemed unnecessary given that in excess of 90% of the mutations detected in human cancers occur in this region. What is more, the p53 pathway appeared to be functional based on p21 induction assays, indicating an un-mutated gene. We did consider the fact that there are some crucial differences between human p53 and mouse p53. In general, p53 mutations are not detected as readily in murine solid tumours (Hollstein, Hergenhahn et al. 1999; Zielinski, Liu et al. 2002), possibly due to lower exposure to carcinogens. However, review of the literature regarding p53 mutations in chemically induced mouse skin cancers reveals that the majority of mutations are also missense mutations located in the DNA binding domain (Ruggeri, Caamano et al. 1991; Ruggeri, DiRado et al. 1993; Zhang, Bauer et al. 1995; Wang, Greenhalgh et al.

1998), although gene deletions are probably more common compared with human tumours.

A missense mutation, L254R, was detected in another subset of SCC 7.1 cells. Although this is not a well documented mutation, it has previously been reported in squamous cell carcinoma and basal cell carcinoma of the skin in mice and humans, respectively (van Kranen and de Gruijl 1999; Agar, Halliday et al. 2004), suggesting that it is a physiologically significant mutation in skin cancer. As anticipated, high passage SCC 7.1 populations containing a significant proportion of mutant L254R cells behaved very differently from low passage populations containing mainly wt 53 cells. While the abundant levels of p53 protein present in these late passage populations indicated that L254R was an accumulating mutation, it was difficult to precisely attribute any specific properties to this mutant protein, especially in view of the mixed cell nature of the original SCC 7.1 cell line. Based on our results, we speculated that the mutant p53 cells were not capable of transactivating p53 target genes, such as p21, suggestive of a dominant negative effect. In contrast, we noted that the mutant p53 containing cells appeared to outgrow the wt p53 containing cells, suggestive of a gain of function effect on proliferation. It is important to note, however, that dominant negative and gain of function effects are not mutually exclusive; even if a particular p53 mutant protein does not result in transactivation of target genes, it may exert a gain of function effect on the cell by non-transcriptional means. Hence, these observations regarding p21 induction and proliferation may be significant but we did not attempt to validate our findings at this point; we will return to this point of discussion in the next chapter.

Contrary to our initial impressions, the SCC 7.1 FAK -/- single cell clone originated from a wt p53 cell. This was a fascinating result for two crucial reasons. Firstly, we strongly suspected that mutant p53 offered a proliferative advantage and we were, therefore, extremely surprised that 4-OHT treatment had selected out a wt p53 cell. Secondly, we have obtained conclusive evidence that cancer cells can in fact survive and propagate in the absence of FAK on a wt p53 background (more importantly on a functional wt p53 background). This evidence obviously refutes our earlier hypothesis but does this mean that we can extrapolate these findings to the SCC 1.1 cell line? We have already shown that these cell lines are morphologically and biologically distinct so it cannot be

assumed that FAK is dispensable for survival in both. In fact, if we return to the FAK -/- embryos described by Ilic, the mesoderm was more susceptible to *fak* loss than the ectoderm (Ilic, Furuta et al. 1995). Further studies using conditional *fak* deletion have shown that increased cell death occurs in endothelial cells (Shen, Park et al. 2005), but not necessarily in neuronal cells (Beggs, Schahin-Reed et al. 2003). It is also interesting to note that in the mouse skin model induction of apoptosis following *fak* deletion is seen only within the stem cell bulge region (McLean, Komiyama et al. 2004). Crucially, it has now been demonstrated that p53 is upregulated only in the mesoderm of FAK -/- embryos (Lim, Chen et al. 2008). This indicates that survival in the absence of FAK is not only tissue specific or cell type specific, but suggests that it is likely to hinge on the cross talk and functional interaction between FAK and p53 so it would be of interest to assess whether cell death in the conditional models is p53 dependent. Further work is required to pinpoint the underlying biological reason why a FAK -/- clone was not achievable in the SCC 1.1 population, but we suspect that the ability of cancer cells to survive genetic deletion of FAK will vary from cell line to cell line and depend perhaps not only on the FAK-p53 axis, but also on other as yet undefined mutations arising during cancer progression in each individual tumour.

4.4 Summary

We determined that the SCC 1.1 cell line did, in fact, possess wt p53. Although the SCC 7.1 cell line contained a mixed population of cells with wt p53 and mutant p53, the SCC 7.1 FAK -/- single cell clone was derived from a cell with wt p53. This indicates that some cancer cells can survive in the absence of FAK on a wt p53 background, although this may be a cell line dependent phenomenon.

Chapter 5

Dissecting out the roles of p53 and FAK in the cellular characteristics of the original SCC 7.1 cell line.

5 Dissecting out the roles of p53 and FAK in the cellular characteristics of the original SCC 7.1 cell line.

5.1 Aim

Following the realisation that the original SCC 7.1 FAK +/+ cell line was a mixed population of at least two different cell types, one with wt p53 and one with mutant p53, we were concerned that a direct comparison with the derived SCC 7.1 FAK -/- single cell clone would be unjustifed for a number of reasons. Firstly, the proliferation characteristics of a particular cell line must be established before proceeding to clonogenicity experiments. This is because the doubling time of a cell population determines the length of incubation period for the clonogenic assay. Preliminary observations suggested that there were crucial differences in proliferation rate between SCC 7.1 FAK +/+ cells at various passages, and possibly between SCC 7.1 FAK +/+ and SCC 7.1 FAK -/- cells. However, at this point, it was difficult to pinpoint whether these potential differences were due to p53 (or FAK) and exactly how these differences would influence the outcome of clonogenicity experiments. Secondly, p53 status is thought to be an important determinant of radiosensitivity. The complicated literature on this subject will be discussed in detail later. Suffice to say, the presence of both wt p53 and mutant p53 cells in a cell population could potentially render data interpretation problematic.

Therefore, we set out to determine whether p53 status did affect proliferation rate in the original SCC 7.1 cell line by separating out wt p53 and mutant p53 cells by single cell cloning. In addition, we aimed to establish whether the presence of wt p53 or mutant p53 influenced radiation survival by performing clonogenic assays in these derived single cell clones.

5.2 Results

5.2.1 Early passage and late passage SCC 7.1 populations have different growth characteristics in vitro and in vivo.

It is important to establish certain cellular characteristics prior to performing clonogenicity assays, namely the doubling time of a cell population and the ability to form colonies at low density. We suspected from our previous findings that the SCC 7.1 FAK +/+ mutant p53 subpopulation had a growth advantage *in vitro*. Proliferation curves were therefore obtained for low passage cells (5 – 10) versus high passage cells (25 – 30). Accordingly, high passage populations had a higher proliferation rate as illustrated by MTT assay (**Figure 36**). Both populations yielded colonies when seeded at low density, but perhaps not surprisingly, colonies formed earlier in the late passage populations (at 5 days as opposed to 7 or 8 days in the early passage populations). It was also noteworthy that late passage colonies displayed a regular-shaped, compact appearance as opposed to the very diffuse scattered pattern of early passage colonies (**Figure 37A**). In fact, the latter were so diffuse that the colonies appeared to merge and it was impossible to reliably count the number of colonies present per plate.

Performing a clonogenic assay in soft agar as opposed to standard 2D tissue culture plastic is an acceptable means of assessing radiosensitivity. Therefore, low passage and high passage SCC 7.1 FAK +/+ cells were seeded in soft agar and colony formation was monitored over a period of 2 weeks. Both populations grew readily (**Figure 37B**) but, as the low passage populations contain mutant p53 cells as well as wt p53 cells, we considered the possibility that the mutant p53 containing cells may possess a growth advantage in this environment also and potentially bias the experiment. To answer this question, we plated out low passage cells into soft agar, extracted a number of colonies by fine needle aspiration, and performed p53 sequencing analysis. All of these colonies demonstrated wt p53 (**Figure 38**), indicating that the both the wt p53 containing cells and mutant p53 containing cell types formed viable colonies. It would therefore have been reasonable to assess clonogenicity by this method.

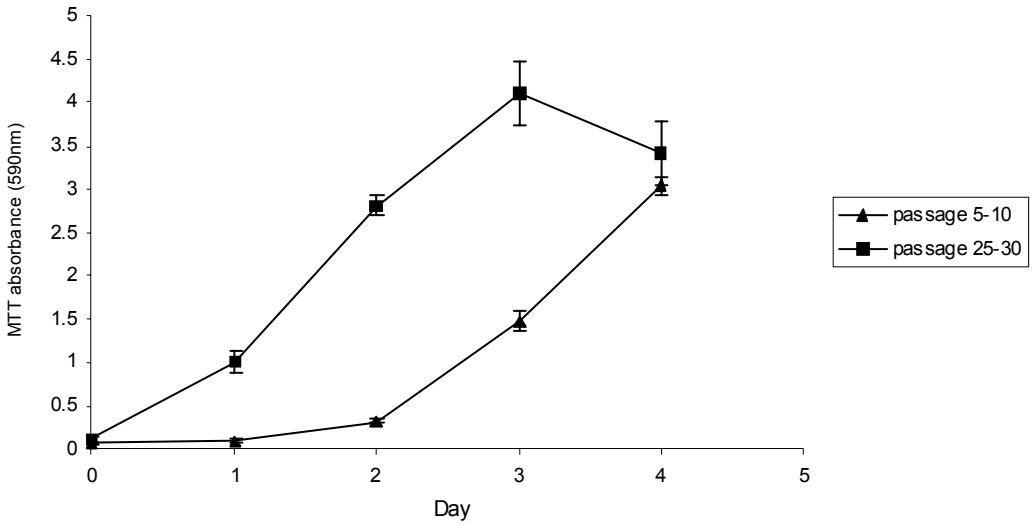


Figure 36 - SCC 7.1 high passage populations have a proliferation advantage *in vitro*
$1x10^3$ SCC 7.1 cells (passage 5-10 and passage 25-30) were seeded into 96 well plates in quadruplicate. After 48 hours incubation, cell viability was evaluated on days 0 – 4 by colorimetric assay (MTT) as described in Methods section. Representative means ± SD from one of three separate experiments are shown.

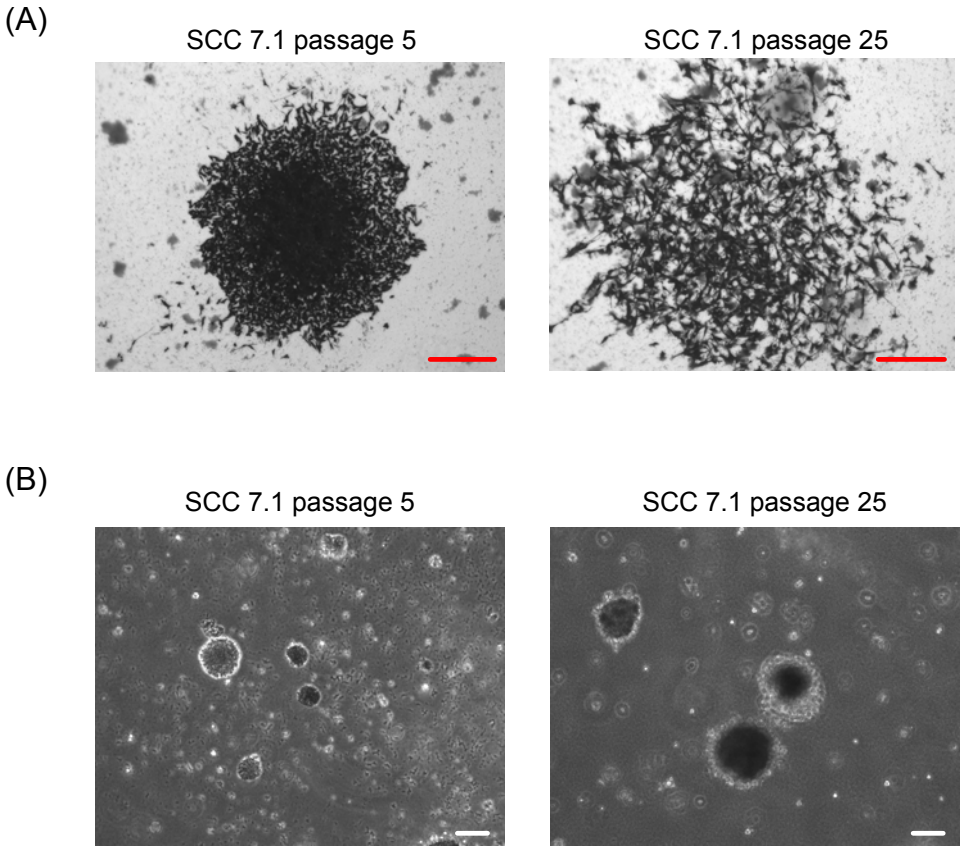


Figure 37 - SCC 7.1 colony formation in 2D and 3D environments
(A) SCC 7.1 cells were seeded into 90mm dishes at low density and observed for colony formation at 5 – 8 days. The colonies were fixed in methanol and stained with crystal violet. Representative phase contrast images are demonstrated for a low passage population (left) and a high passage population (right) (scale bar, 0.5mm). (B) $2x10^4$ SCC 7.1 cells were seeded soft agar as outlined in Methods section and growth monitored over 14 days. Representative phase contrast images are demonstrated for a low passage population (left) and a high passage population (right) (scale bar, 0.5mm).

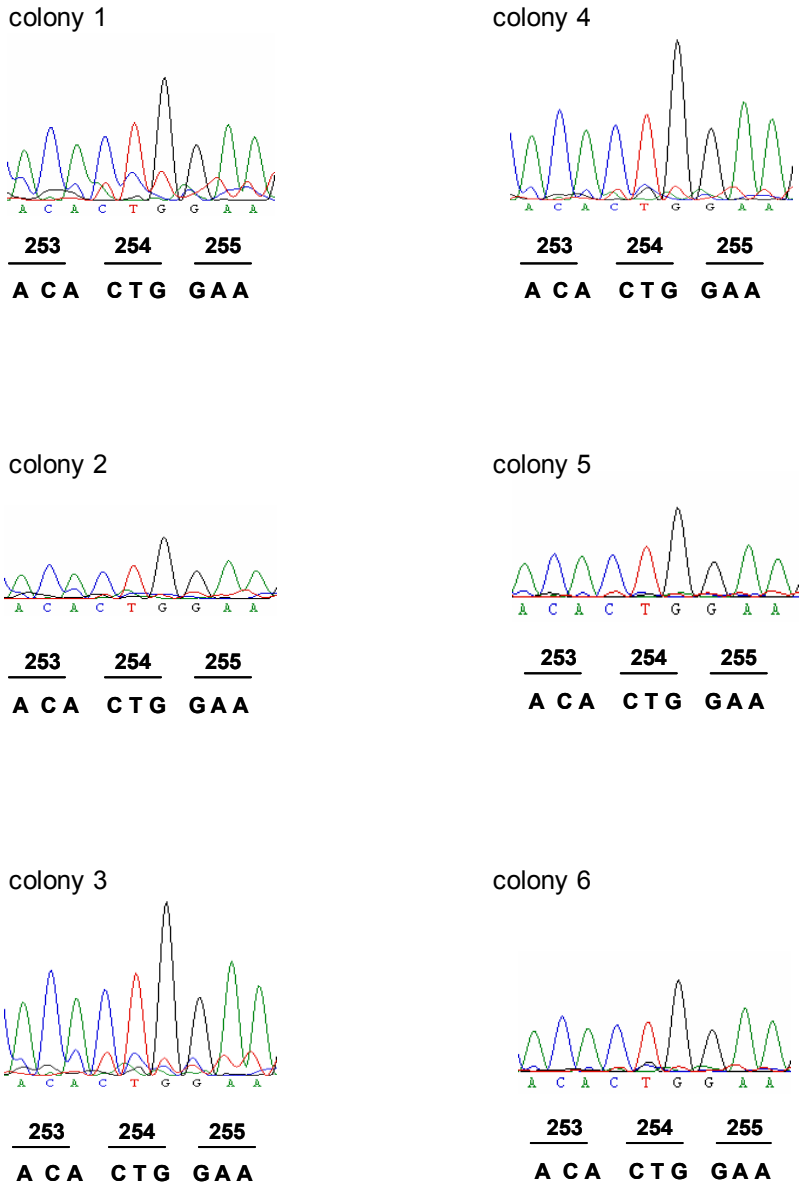


Figure 38 - SCC 7.1 low passage cells containing wt p53 do not have a proliferation disadvantage in soft agar
SCC 7.1 low passage cells were seeded into soft agar and monitored for 14 days. 6 colonies were removed from the agar by fine needle aspiration and RNA was immediately extracted. The RNA was then converted to cDNA which was amplified, purified and subjected to direct sequencing analysis for p53 as previously described. Chromatograms for codons 253 – 255 are demonstrated for each of these 6 colonies.

However, parallel experiments in SCC 7.1 FAK -/- cells demonstrated that these particular cells, which were deficient in FAK, could not survive in an anchorage independent environment. This will be discussed later in Section 5.2.7. In order to maintain uniformity across all cell types and cell populations, we decided to explore another alternative means of assessing clonogenicity. A limiting dilution assay, where cells were seeded in a 96 well plate at a dilution that allowed single colony growth after 5 – 8 days incubation emerged as a viable option. This method proved to be reproducible across all cell populations.

While late passage populations appeared to have a proliferative advantage *in vitro*, this did not translate into a proliferative advantage *in vivo*. In fact, low passage populations appeared to grow much more readily as xenografts as shown in **Figure 39A** and demonstrated a significantly higher proliferation level based on nuclear scoring of Ki67 (**Figure 39B**, Mann Whitney, p=0.0404, n=3). We also observed that while early passage xenografts reached a diameter of well over 1cm before the mice had to be culled, animals bearing late passage xenografts had to be sacrificed by the time the tumours reached 7 or 8 mm due to signs of imminent central necrosis. One of the animals bearing a late passage xenograft developed abdominal swelling which we initially thought may have been due to a B-cell lymphoma, but post mortem examination demonstrated tumour extending from the xenograft into the abdominal cavity. These findings led us to question whether the mutant p53 containing cells have a higher propensity for invasion *in vivo*.

Collectively, this data illustrated that there were indeed crucial differences in proliferative capacity within the cells of the original SCC 7.1 FAK +/+ population in various cellular environments, both *in vitro* and *in vivo*. We were keen to determine if radiosensitivity varied in the low passage and high passage populations but were concerned that the different growth rates would influence the results. Hence, we endeavoured to separate out wt p53 cells from mutant p53 cells before proceeding with clonogenicity assays.

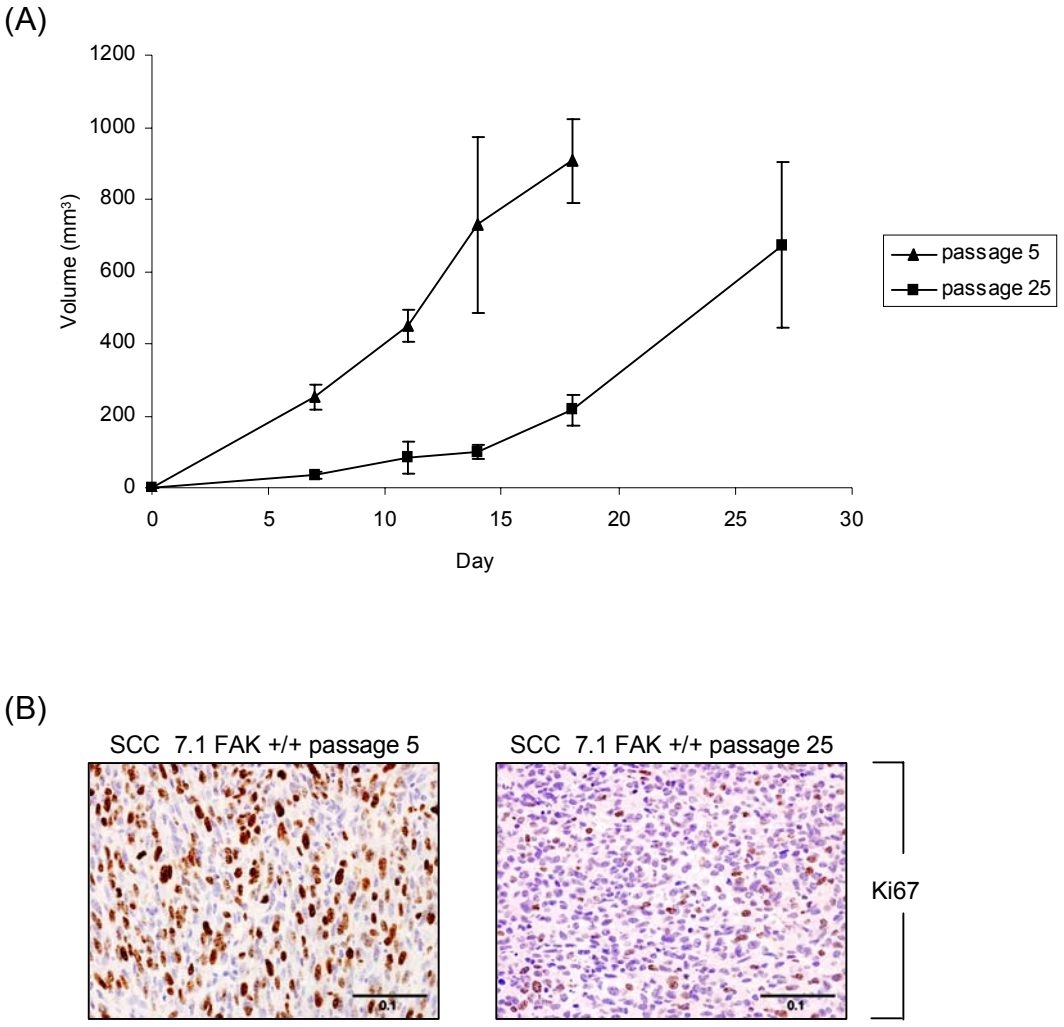


Figure 39 - SCC 7.1 low passage populations have a proliferation advantage *in vivo*
(A) $5x10^5$ SCC 7.1 cells (passage 5 and passage 25) were injected subcutaneously into 6 week old female nude mice and growth monitored regularly. Mice were sacrificed when tumours reached 1.7cm in maximal diameter or developed signs of imminent central necrosis. Tumour volume was calculated as described in Methods section. A graphical representation of mean tumour volume ± SEM is shown. (B) Similar to above, $5x10^5$ SCC 7.1 cells (passage 5 and passage 25) were injected into nude mice. The animals were sacrificed when the tumour volume reached approximately $500mm^3$. Paraffin embedded sections were then stained for Ki67 and representative bright field images are shown (passage 5 – upper panel and passage 5 - lower panel) (scale bar, 0.1mm).

5.2.2 Isolation of wt p53 versus mutant p53 single cell clones.

SCC 7.1 passage 10 cells were trypsinised and diluted such that a single cell was seeded into each well of a 96 well plate. After 10 – 14 days, 10 colonies were selected, of which 7 survived repeated passage. A summary of their morphological appearance, p53 protein levels based on immunoblotting (IB), p53 localisation as judged by immunofluorescence (IF), and sequencing analysis of codon 254 is outlined in **Table 7**. All of these experiments were performed in each cell line between passage 5 and passage 10.

Table 7 - Isolation of wt and mutant p53 single cell clones

	morphology	IB p53	IF p53	Sequencing codon 254
A10	refractile	+++	+++ nuclear > cytoplasmic	CGG
B8	refractile	+++	+++ nuclear > cytoplasmic	CGG
C6	refractile	+++	+++ nuclear > cytoplasmic	CGG
D1	spindle	+	+ cytoplasmic (perinuclear)	CTG
F4	spindle	+	+ cytoplasmic	CTG
H7	refractile	+++	+++ nuclear > cytoplasmic	CGG
H9	spindle	+	+ cytoplasmic	CTG

The actual results for 2 representative wt clones (D1 and H9) and 2 representative mutant clones (A10 and H7) are illustrated in **Figure 40**. Both D1 and H9 cells demonstrated spindle cell morphology, similar to that of early passage SCC 7.1 FAK +/+ cells, whereas A10 and H7 cells demonstrated a more refractile morphology in keeping with late passage SCC 7.1 FAK +/+ cells (**Figure 40A**). As anticipated, low levels of p53 were present in D1 and H9 cells, although there appeared to be less nuclear staining compared with early passage SCC 7.1 FAK +/+ cells and the distribution of staining in the D1 cells was predominantly perinuclear (**Figures 40B** and **40D**). A10 and H7 cells, on the other hand, displayed higher levels of p53, which were predominantly nuclear in distribution (**Figures 40B** and **40D**). Sequencing analysis of p53 revealed wt p53 conformation in D1 and H9 cells and mutant p53 conformation in A10 and H7 cells at codon 254 (**Figure 40C**). Further sequencing of the entire DNA binding domain of p53 in each cell line did not reveal any additional mutations. To be certain that D1 and H9 were not contaminated by mutant cells, all of the above was repeated at passage 25 – 30. There was no evidence of contamination (data not shown). We were interested to learn whether FAK levels varied across these single cell clones, especially in view of recent work which suggests that FAK is upregulated in cells with mutant p53 (Golubovskaya, Finch et al. 2008). However, this did not appear to be the case, and FAK levels were similar in all clones, regardless of p53 status (**Figure 40D**).

5.2.3 Characterisation of wt p53 versus mutant p53 single cell clones.

MTT proliferation assay and xenograft growth curves confirmed that the mutant p53 containing cells had a proliferation advantage *in vitro* (**Figure 41**) but not *in vivo* (**Figures 42A**). Intriguingly, there appeared to be differences between the wt p53 containing clones, for example D1 cells had a much faster proliferation rate than H9 cells *in vivo*. Similarly, there were also differences between the mutant p53 containing clones; A10 cells proliferated more rapidly than H7 cells *in vitro*.

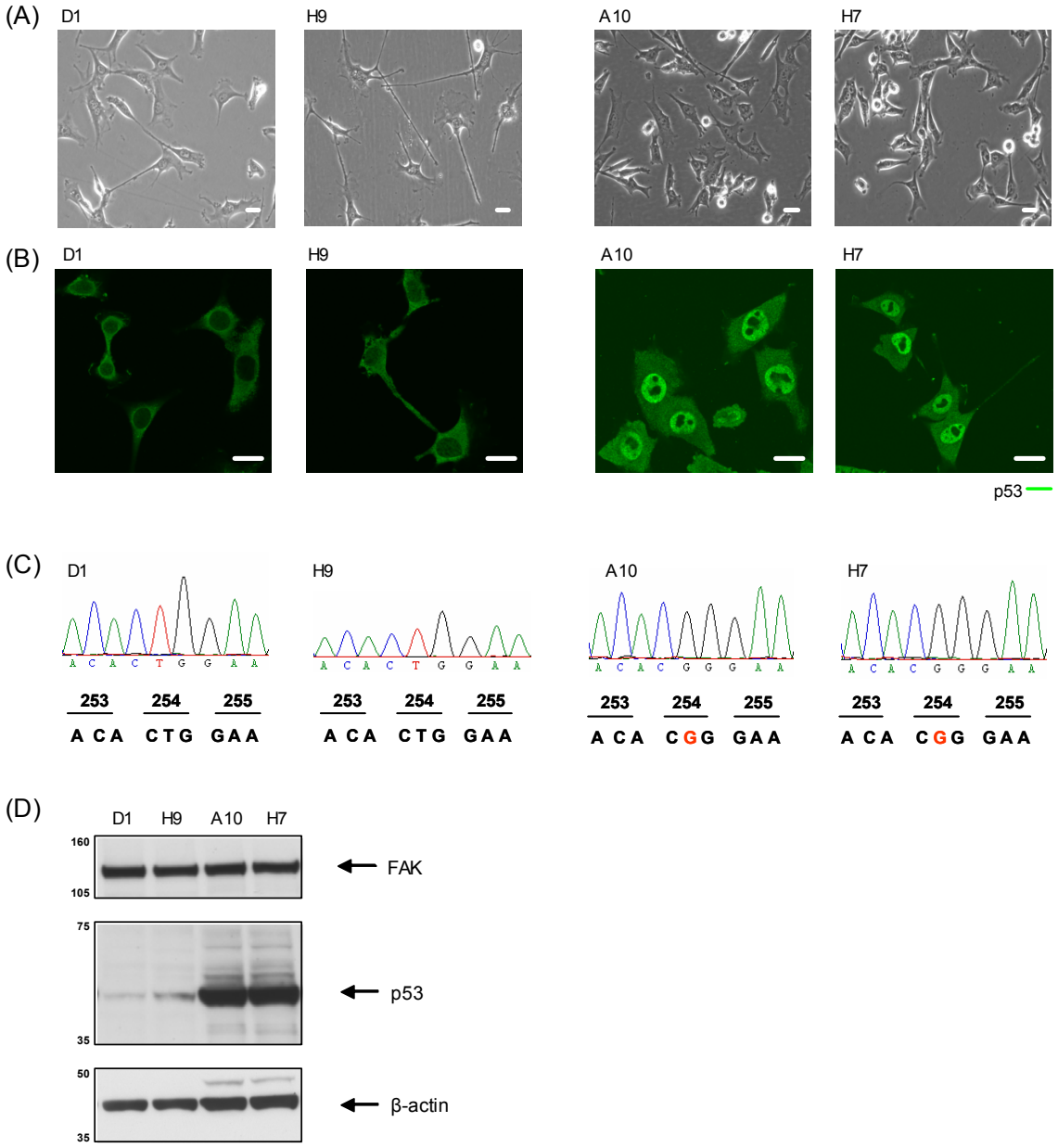


Figure 40 - Isolation of wt p53 and mutant p53 single cell clones
(A) Phase contrast images of two representative wt p53 single cell clones (D1 and H9) and two representative mutant p53 single cell clones (A10 and H7) (scale bar, 25μm). (B) A10, D1, H7 and H9 cells were plated at low density on glass coverslips, fixed and stained with an anti-p53 antibody. Representative images taken on fluorescent confocal microscopy are demonstrated for each of the isolated single cell clones, green – p53 (scale bar, 20μm). (C) RNA was extracted from each of the isolated single cell clones at passage 5 and converted to cDNA which was then amplified by PCR, purified, and subjected to direct sequencing analysis. Chromatograms for codons 253 – 255 of p53 are illustrated. (D) Protein extracts from each of the isolated clones were separated by SDS-PAGE, transferred to nitrocellulose and probed with anti-FAK, anti-p53 and anti-β-actin antibodies.

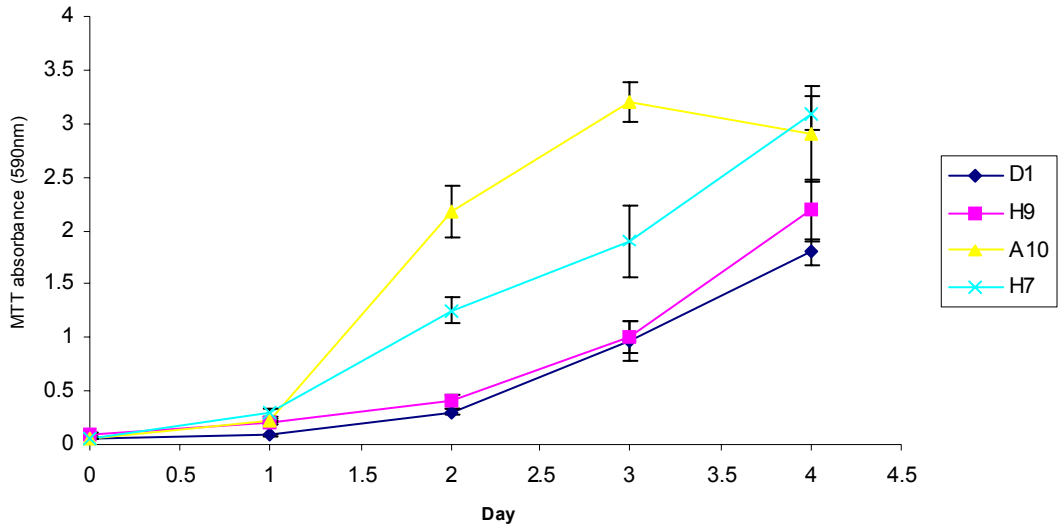


Figure 41 - Mutant p53 single cell clones have a proliferation advantage *in vitro*
Each of the cell lines indicated were seeded at a density of $1x10^3$ cells into wells of a 96 well plate in quadruplicate. After 48 hours incubation, cell viability was evaluated on days 0 – 4 by colorimetric MTT assay. Representative means ± SD from one of three separate experiments are shown.

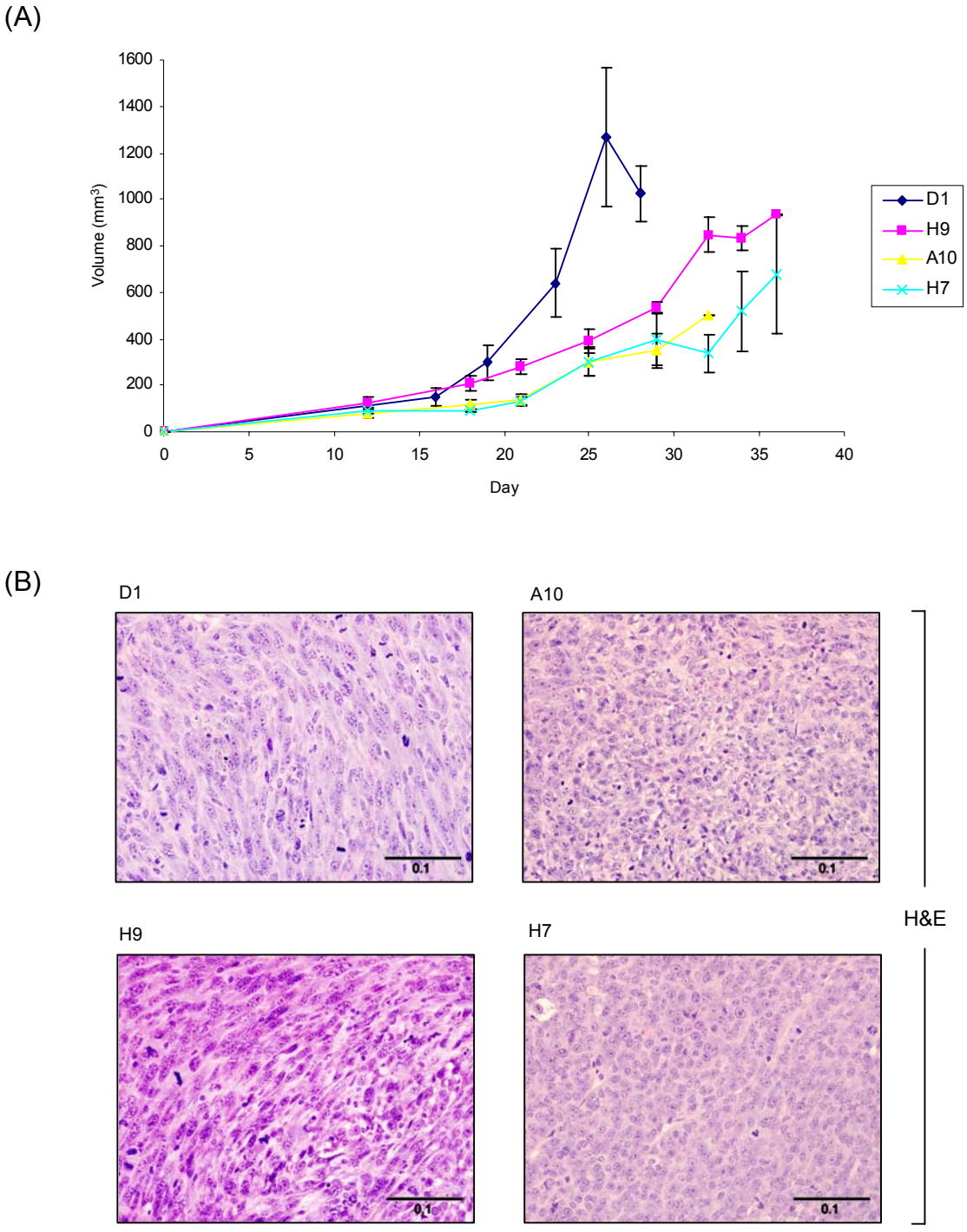


Figure 42 - Mutant p53 single cell clones do not have a proliferation advantage *in vivo*
(A) $5x10^5$ cells from each of the cell lines indicated were injected subcutaneously into 6 week old female nude mice. Xenograft growth was monitored regularly and the animals were sacrificed when tumours reached 1.7cm in maximal diameter or developed signs of imminent central necrosis. Tumour volume was calculated as described in Methods and a graphical representation of mean volume ± SEM is shown (n=8). (B) H&E stained paraffin embedded sections from xenografts generated by the cell lines indicated (scale bar 0.1mm).

In keeping with their distinct cellular morphology patterns, both wt p53 clones formed xenografts consisting of sheets of pleomorphic spindle cells as opposed to the rounder aggregates of cells produced by mutant p53 clones (**Figure 42B**). While D1 xenografts were firm and well circumscribed, generally reaching >1cm in diameter prior to termination, all of the other cell lines, including H9, formed tumours with a more spongy consistency that were difficult to dissect out post mortem (partly due to consistency and partly due to infiltration into host tissue), and which developed signs of necrosis at < 1cm. A number of these infiltrative xenografts extended into the abdominal cavity. **Table 8** outlines the number of invading tumours per total number of xenografts. Only A10 and H7 cells displayed this particular phenotype of frank invasion, and although this data is by no means definitive, again it suggests that the mutant p53 containing cells may have a propensity for invasion *in vivo*. An example of an H7 xenograft invading into underlying tissues is outlined in **Figure 43**.

Table 8 - Number of xenografts invading into the abdominal cavity

Clone	p53 status	Invading xenografts	Total number of xenografts
D1	wt	0	8
H9	wt	0	8
A10	mutant	2	8
H7	mutant	3	8

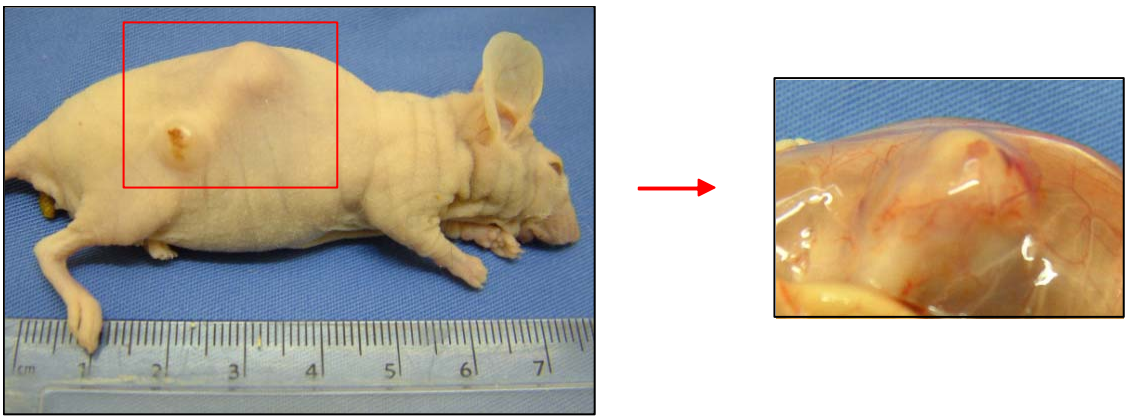


Figure 43 - Mutant p53 single cell clones have a higher propensity for invasion *in vivo*
Photograph of a female nude mouse bearing a xenograft generated by the H7 cell line with a close up image of the area of interest (inset).

5.2.4 Radiosensitivity is not conditional on the basis of p53 status alone in the isolated wt p53 and mutant p53 clones.

Having successfully separated wt p53 from mutant p53 cells in the form of single cell clones, it was now possible to accurately examine radiosensitivity, and we did this by performing a limiting dilution assay at various doses of radiation (up to a maximum of 10Gy in 2Gy increments). Clonogenicity in response to ionising radiation was not identical across these isolated clones (**Figure 44A**). We found that the presence of wt p53 or mutant p53 did not determine either radiosensitivity or radioresistance. In fact, the most radioresistant cell line was consistently H9, which possesses wt p53, and the least radioresistant was the mutant p53 containing H7.

The extremely radioresistant phenotype of H9 may be partly explained by the findings from propidium iodide based FACS analysis (**Figure 44B**). While this revealed a normal cell cycle profile in D1 cells, the H9 cells demonstrated marked aneuploidy. In fact, it was virtually impossible to delineate the separate phases of the cell cycle in this cell line. This would suggest the presence of chromosomal abnormality and genetic instability, which can result in resistance to DNA damaging agents and interestingly, is often linked to mutant p53 as opposed to wt p53 (Mekeel, Tang et al. 1997; Akyuz, Boehden et al. 2002; Okorokov, Warnock et al. 2002; Linke, Sengupta et al. 2003; Okorokov 2003). A10 and H7 cells also demonstrated a degree of genomic instability in the form of both aneuploidy and polyploidy but the level of aneuploidy was not quite as striking compared with the H9 cells. We can only speculate as to why the H9 cell line possessed this particular phenotype, but overall this data highlights both the complexity of cellular radiation survival and hints at the likelihood of additional mutations and / or molecular alterations within these “isogenic” cell lines.

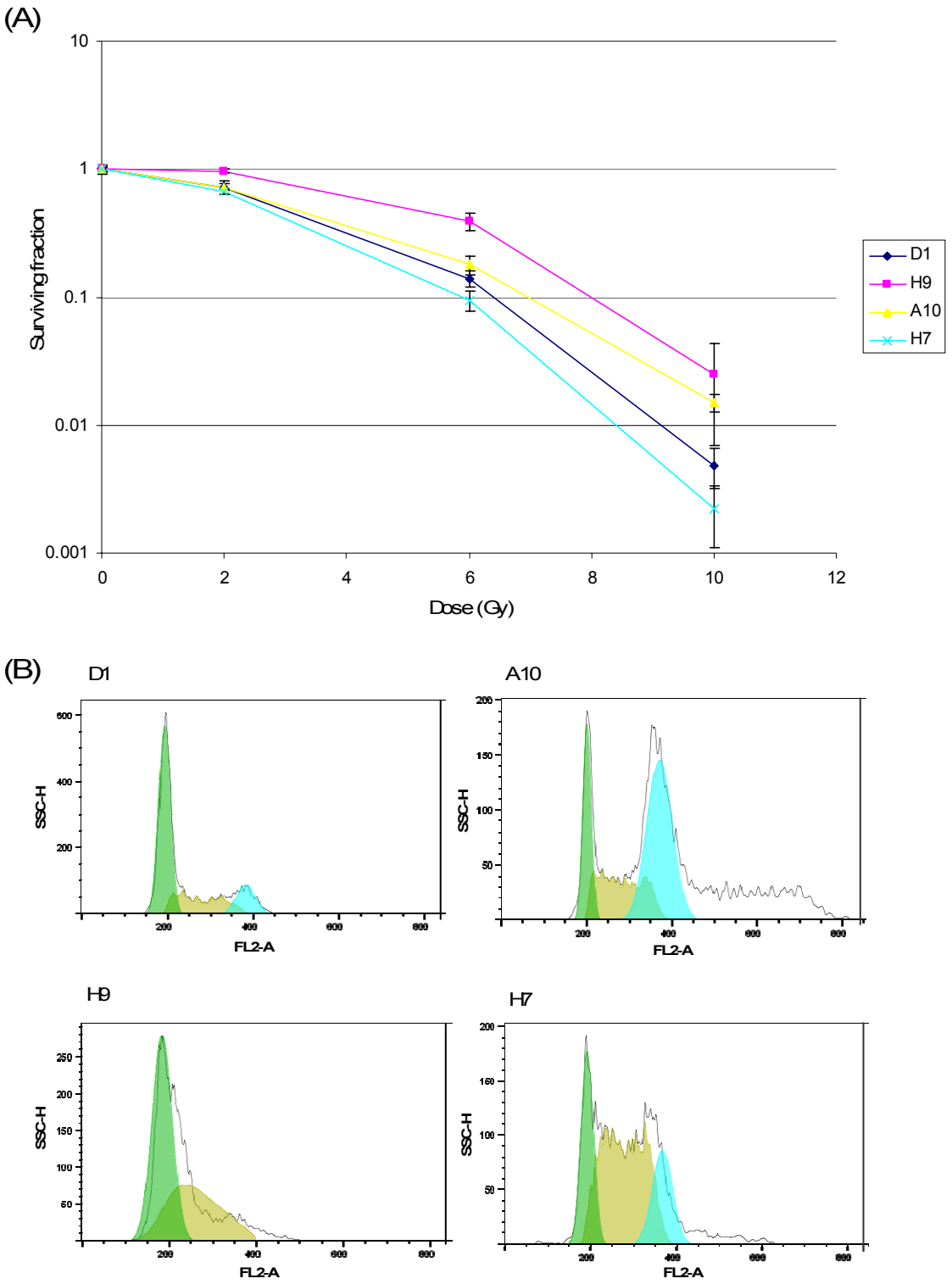


Figure 44 - Radiosensitivity of the isolated wt p53 and mutant p53 clones
(A) The cell lines indicated were diluted and seeded into each well of a 96 well plate at appropriate density to allow single colony growth at 5 – 8 days. After 6 hours the plates were irradiated with various doses of radiation as indicated. Following incubation for 5 – 8 days, the colonies were counted and the surviving fraction calculated at each dose of radiation. Each experiment was performed in triplicate and the combined results (mean ± SEM) from at least three separate experiments are shown. (B) Subconfluent populations of the cell lines indicated were suspended in ice cold PBS and fixed in 70% ethanol overnight prior to staining with propidium iodide and analysis by FACS. Representative profiles from one of three separate investigations are demonstrated.

5.2.5 Development of wt p53 versus mutant p53 FAK -/- cell lines.

Our suspicions that the original SCC 7.1 FAK +/+ cell line contained a mixture of cells with different p53 status and different properties were confirmed. This precluded a direct comparison of radiosensitivity with the strictly wt p53 containing SCC 7.1 FAK -/- cell line. In view of the variation between the D1 and H9 wt p53 single cell clones, which we suspected was due to inconsistencies in their genetic background, we had to acknowledge that the original SCC 7.1 FAK +/+ cell line may have consisted of a vast number of cancer cells containing wt p53 but different additional mutations, any one of which could have given rise to the FAK -/- single cell clone. As we had no evidence that the SCC 7.1 FAK -/- cell line was derived from a D1 type cell or an H9 type cell, it was also inappropriate to compare the SCC 7.1 FAK -/- cell line with either of these wt p53 clones. With both of these means of comparison, it would have proved very difficult to attribute any potential differences in radiation response to FAK alone.

In order to reliably compare FAK -/- cells with their FAK +/+ counterpart, we decided to develop novel FAK -/- cell lines from the isolated clones (A10, D1, H7 and H9), which would have the advantage of a paired parental cell line from an identical genetic background. This work is still ongoing and has not yet yielded a FAK knockout cell line from any of the isolated clones.

5.2.6 Reconstituting wt FAK into FAK -/- cells overcomes the problem of a mixed FAK +/+ parental cell line.

As paired FAK +/+ and FAK -/- cell lines from the isolated clones were not easily forthcoming, we opted to take the alternative approach of reconstituting wt FAK into the SCC 7.1 FAK -/- cell line using a myc-tagged CMV driven expression vector and subsequent selection in antibiotic containing media. Both FAK knockout and FAK reconstituted cells exhibited the same spindle cell morphology (**Figure 45A**). We ascertained that FAK was correctly localised at focal adhesions in these reconstituted cells (**Figure 45B**) and FAK protein levels were comparable to those of low passage original SCC 7.1 FAK +/+ cells (**Figure 45C**).

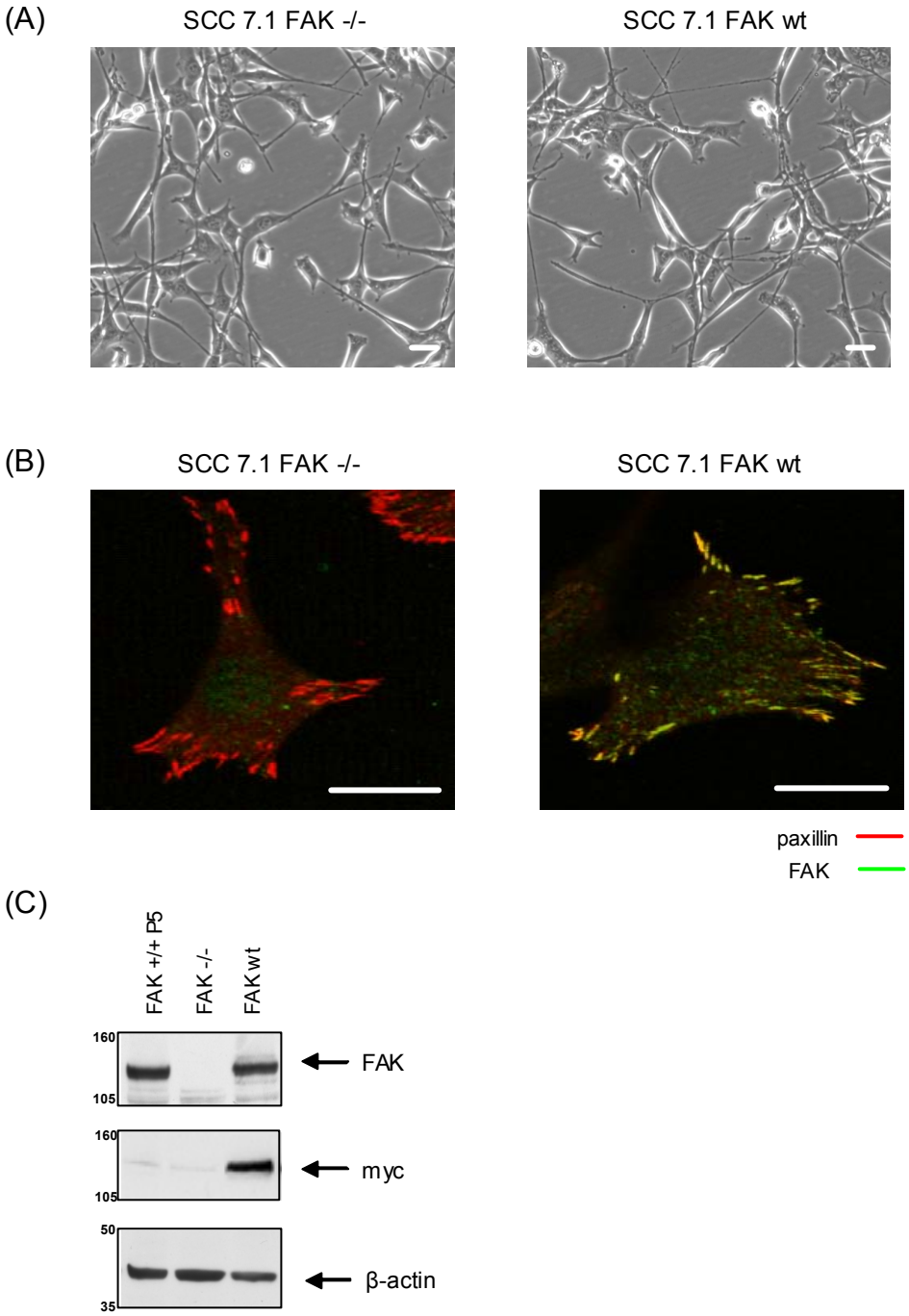


Figure 45 - Reconstituted wt FAK is expressed in focal adhesions
(A) Phase contrast images of SCC 7.1 FAK -/- cell line (left) and SCC 7.1 FAK wt cell line (right), following addition of myc-tagged wt FAK vector and selection in hygromycin (scale bar, 25μm). (B) SCC 7.1 FAK -/- and SCC 7.1 FAK wt cells were plated at low density on glass coverslips, fixed and stained with mouse anti-paxillin antibody and rabbit anti-FAK antibody. Representative images taken on confocal microscopy are shown, red – paxillin and green – FAK (scale bar, 20μm). (C) Lysates from SCC 7.1 FAK +/+ (passage 5), SCC 7.1 FAK -/-, and SCC 7.1 FAK wt cells were immunoblotted for FAK, myc and β-actin.

5.2.7 FAK is dispensable for growth in vitro but is essential for anchorage-independent growth.

FAK has previously been noted to promote proliferation in certain cell lines *in vitro*. Comparison of the proliferation rate in SCC 7.1 FAK -/- versus SCC 7.1 FAK wt cells by MTT assay, however, did not reveal any difference indicating that FAK was dispensable for growth on 2D in this system (**Figure 46A**). Similar to the predominantly wt p53 containing cells of early passage SCC 7.1 populations, both SCC 7.1 FAK -/- and SCC 7.1 FAK wt cells formed dispersed colonies at low density (**Figure 46B**). Colony formation was assessable by limiting dilution assay and there was no significant difference in plating efficiency (**Figure 46C**, student's unpaired t-test, p=0.4290, n=9).

We noted in previous experiments that SCC 7.1 FAK -/- cells failed to establish colonies in soft agar. We wanted to confirm that FAK was essential for anchorage-independent survival and therefore directly compared SCC 7.1 FAK -/- with SCC 7.1 FAK wt cells. At this point, however, we switched soft agar for methylcellulose as it proved easier to extract cells or colonies for further analysis from this medium. As shown in **Figure 47A** FAK was a prerequisite for growth in this medium. Quantification of the number of colonies present in ten random fields revealed an average of 15 - 20 colonies in the FAK wt population, but virtually no colonies in the FAK -/- population (**Figure 47B**).

We speculated whether the kinase function of FAK was responsible for this phenomenon. This was based on previous evidence suggesting that FAK signalling is an important survival mechanism for cells in suspension (Frisch, Vuori et al. 1996; Ilic, Almeida et al. 1998; Xu, Yang et al. 2000) coupled with our own observations. We found that phospho-FAK-Y397 levels were constitutively active in SCC 7.1 FAK wt cells even in suspension as opposed to keratinocytes which switched on phosphorylation following adhesion to fibronectin (**Figure 47C**). To ensure that these results were not related to the FAK plasmid, the experiment was repeated in an early passage parental SCC 7.1 FAK +/+ population. The same outcome was obtained (**Figure 47C** – far right panels).

Further evidence that this phenomenon is kinase dependent was provided by the incorporation of a small molecule FAK kinase inhibitor into the methylcellulose assays. The addition of PF-562,271 to FAK wt cells resulted in a dose-dependent inhibition of colony formation with concentrations of 0.25μM and above resulting in around a 45% reduction in the number of colonies (**Figure 47D**). Notably, substantial FAK inhibition could be demonstrated by western blotting at doses of 0.25μM and above (**Figure 47E**), although complete inhibition of Y397 phosphorylation was never achieved when cells were grown on tissue culture plastic. We did consider the possibility that the drug may have affected proliferation through off-target effects which could restrict colony growth. However, further work demonstrated that the IC_{50} for this particular drug in FAK wt cells was in excess of 3μM (data not shown). Thus, the effects of 0.25μM concentration of drug on colony formation were clearly not due to proliferation.

As there are always some concerns over the specificity of kinase inhibitors, we have generated a FAK kinase dead (FAK KD) cell line using the same system which we used to develop the FAK wt cell line. These cells, which have lost the catalytic function of FAK, behave in a very similar manner to the FAK -/- cells in methylcellulose (personal communication – Kenneth McLeod), indicating that it is indeed the kinase activity of FAK that drives colony growth in a 3D environment.

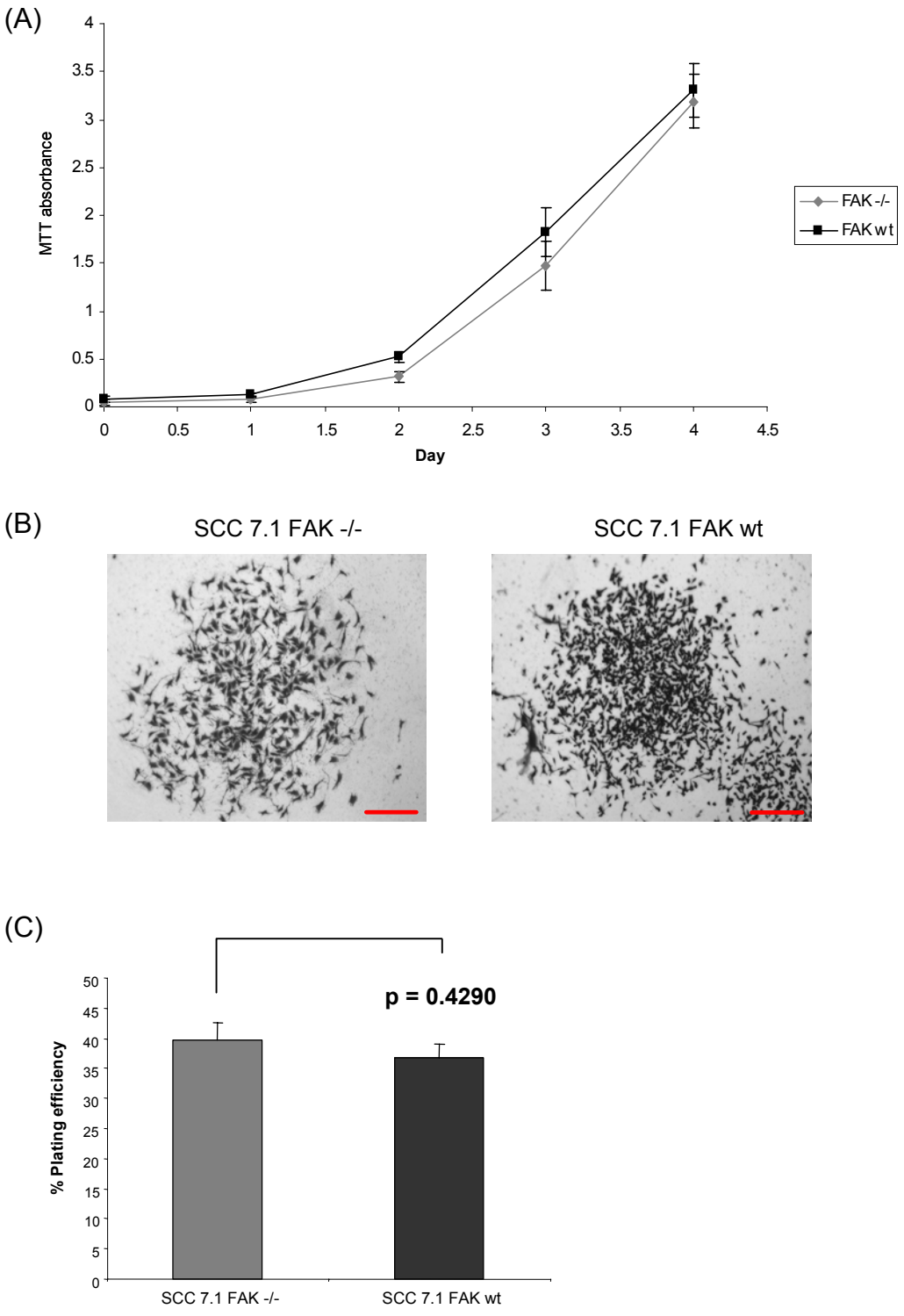


Figure 46 - FAK is dispensable for proliferation *in vitro*
(A) 1x10^3 SCC 7.1 FAK -/- and SCC 7.1 FAK wt cells were seeded into 96 well plates in quadruplicate. After 48 hours incubation, cell viability was evaluated on days 0 – 4 by colorimetric assay (MTT). Representative means ± SD from one of three separate experiments are shown. (B) SCC 7.1 FAK -/- and SCC 7.1 FAK wt cells were seeded at 100 cells per 90 mm dish and growth monitored over 7 days. The colonies were then fixed in methanol and stained with crystal violet. Representative phase contrast images of colony morphology are illustrated (scale bar, 0.5mm). (C) A single cell was seeded into each well of a 96 well plate and the percentage of wells with a viable colony (>50 cells) calculated at 7 days. The experiment was set up in triplicate and the bar chart demonstrates the combined mean ± SEM from three separate experiments based on SCC 7.1 FAK -/- cells (left) and SCC 7.1 FAK wt cells (right). Statistical significance was evaluated by student's unpaired t-test, n=9.

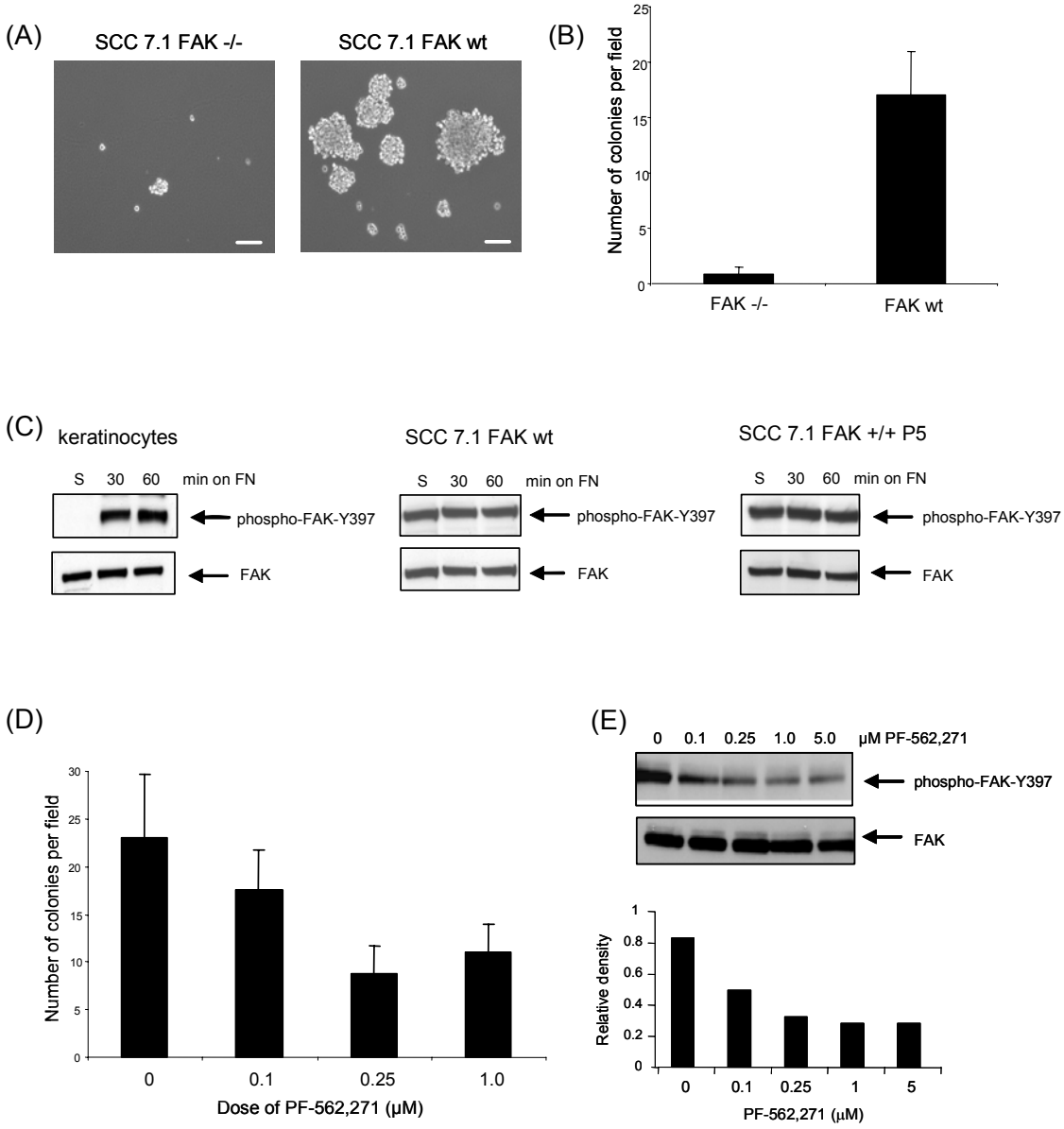


Figure 47 - FAK kinase activity is required for anchorage-independent growth
(A) $2.5x10^5$ cells were resuspended in a solution of 1.4% in growth media and plated over a layer of 0.9% agarose. Cells were photographed after 9 days. Representative phase contrast images at 10 days are demonstrated for FAK -/- cells (left) and FAK wt cells (right) (scale bar, 0.5mm). (B) The numbers of colonies were counted in ten random fields (x10 magnification) and colony area was calculated in Image J. Results are shown from a representative experiment in a series of three, mean ± SEM. (C) $3x10^6$ cells were suspended (S) in ice cold PBS and rotated at 4^oC. After 1 hour, the cells were centrifuged and either collected for protein extraction or resuspended in growth media and seeded on to fibronectin (FN) coated plates. Further lysates were then prepared after 30 minutes and 60 minutes. Protein extracts were separated by SDS-PAGE, transferred to nitrocellulose, probed with anti-phospho-FAK-Y397 antibody then stripped and re-probed with anti-FAK antibody. (D) FAK wt cells were added to methylcellulose as described above in the presence of increasing doses of PF-562,271 and colonies counted at 9 days, results shown are representative mean ± SEM from one experiment in a series of three. (E) FAK wt cells were treated overnight with a range of doses of PF-562,271 prior to western blot analysis for phospho-FAK-Y397 and total FAK. The relative density of the Y397 signal was quantitated by densitometry (lower panel).

5.2.8 FAK is not required for tumour cell growth in vivo.

As we intended to proceed to evaluating the role of FAK in radiation survival *in vivo* as well as *in vitro*, it was essential to elucidate whether loss of FAK prevented or impaired the development of xenografts. In fact, SCC FAK -/- cells formed tumours readily with a success rate in excess of 90%. However, we noted that the FAK -/- xenografts took longer to establish compared with their FAK wt counterparts (**Figure 48A**). Of note, there was no substantial difference in the gross or microscopic appearance of xenografts from either cell line (H & E stained histological specimens from an SCC 7.1 FAK -/- and an SCC 7.1 FAK wt xenograft are shown in **Figure 48B**), although the vasculature did appear to be more tortuous and leaky in the FAK -/- tumours, but this has not yet been formally assessed.

The presence or absence of FAK could be demonstrated by western blotting of tumour lysates (**Figure 48D**). The weak levels of FAK protein in the FAK -/- extracts most likely represents the presence of host infiltrate, such as tumour associated fibroblasts, from the host animal. Interestingly, proliferation was not markedly affected. Ki67 staining of paraffin embedded tissue from SCC 7.1 FAK -/- and SCC 7.1 FAK wt xenografts (**Figure 48C**) measuring approximately 500mm^3 demonstrated median nuclear positivity of 16.0% versus 17.05% respectively which was not statistically significant (Mann Whitney, p=0.5309, n=5)**.** So, it would appear that loss of FAK impedes the initial phase of xenograft development, but does not dramatically impact on growth and development when tumours have managed to establish.

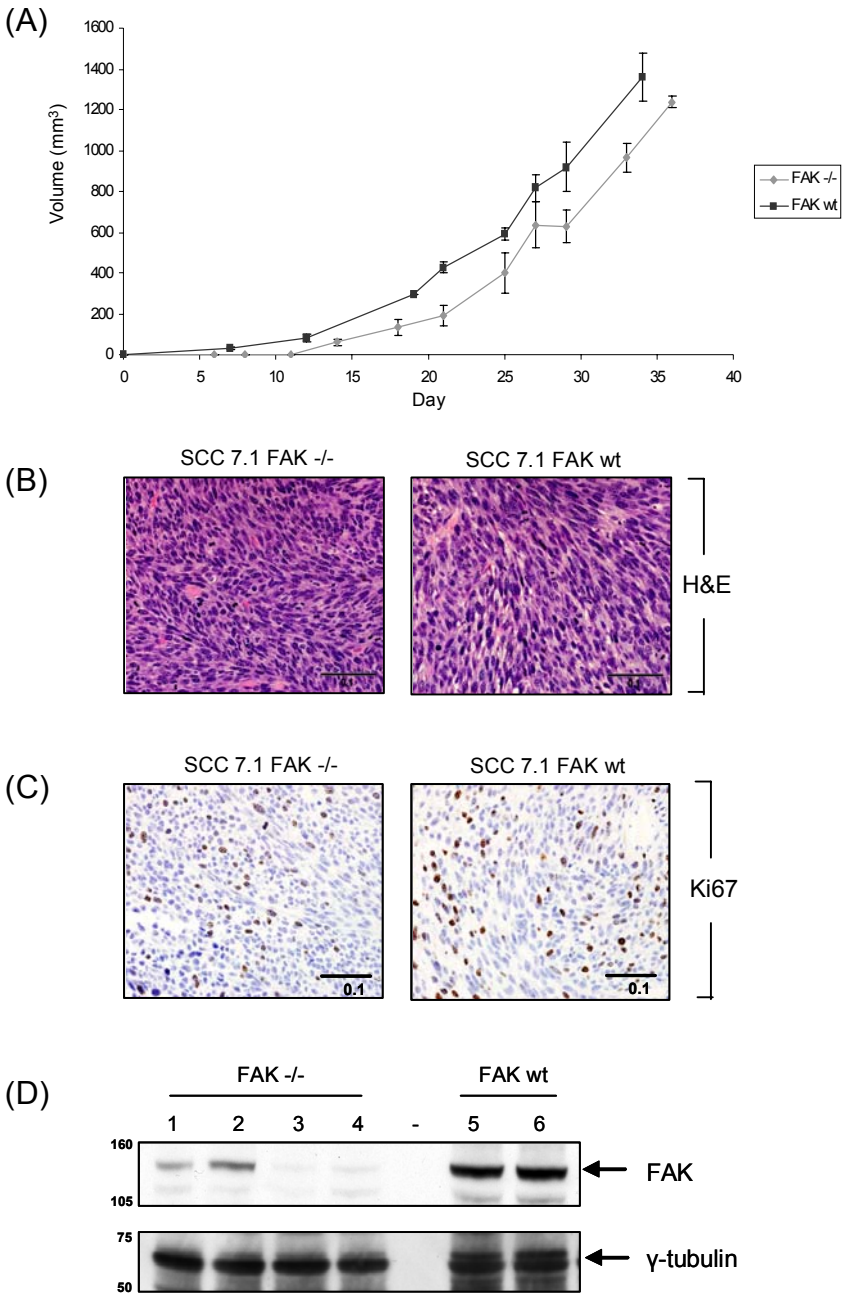


Figure 48 - Loss of FAK delays the establishment of xenografts but does not significantly impede proliferation *in vivo*
(A) 2.5x10^5 SCC 7.1 FAK -/- and SCC 7.1 FAK wt cells were injected subcutaneously into the right flank of 6 week old nude mice. Xenograft growth was monitored regularly and animals were sacrificed when tumours reached 1.7cm in maximal diameter or developed signs of imminent central necrosis. Tumour volume was calculated as previously described and a graphical representation of mean volume ± SEM is shown (n=8). (B) H&E stained paraffin embedded sections from an SCC 7.1 FAK -/- xenograft (upper panel) and an SCC 7.1 FAK wt xenograft (lower panel) (scale bar, 0.1mm). (C) 2.5x10^5 SCC 7.1 FAK -/- and SCC 7.1 FAK wt cells were injected into nude mice and the animals were sacrificed when the tumour volume reached approximately 500mm^3. Paraffin embedded sections were then stained for Ki67 and representative bright field images are shown (FAK -/- in upper panel and FAK wt in lower panel) (scale bar, 0.1mm). (D) Protein extracts from FAK -/- xenografts and FAK wt xenografts were immunoblotted for FAK and γ-tubulin.

5.3 Discussion

The realisation that the SCC 7.1 parental cell line was composed of a mixed population of cells with different properties highlighted the difficulties that can arise working with a novel cell line. Despite the fact that this precluded a direct comparison between the original SCC 7.1 FAK +/+ cell line and the derived FAK -/- single cell clone, it did present some interesting observations. For example, it was fascinating to note that while the mutant p53 containing cells had a proliferation advantage *in vitro* over the wt p53 containing cells, this was not reproduced in the *in vivo* setting, indicating the importance of the cellular environment, a topic that we will return to shortly. The lack of a proliferation advantage *in vivo* could be attributed to a number of factors, but we suspect that this may be at least partly explained by the propensity of these cells to invade locally. In fact, the potential relationship between p53 and invasion is an exciting area of research at the moment (Dong, Tada et al. 2007; Gadea, de Toledo et al. 2007; Dong, Xu et al. 2009; Muller, Caswell et al. 2009; Wang, Wang et al. 2009; Morton, Timpson et al. 2010).

One particular topic of interest that may be relevant to our findings is the work by Gadea et al. which links p53 to proteins involved in actin cytoskeleton dynamics (Gadea, de Toledo et al. 2007). They showed that p53 deficiency in MEFs cultured in 3D matrices induced a switch from elongated spindle morphology to a markedly spherical and flexible one. This morphological transition required the RhoA-ROCK pathway and these rounded, motile cells exhibited amoeboid–like movement and had considerably increased invasive properties. These observations were also extended to cancer cells. The addition of a dominant negative p53 mutation (R273H) to wt p53 A375P melanoma cells induced similar morphological changes and invasive characteristics which were associated with upregulated levels of activated RhoA.

Hence, the presence of the L254R mutation may dictate the more refractile morphology of the mutant p53 cells compared with the fibroblastoid appearance of the wt p53 cells in the SCC 7.1 population. It may also explain the apparent propensity for these mutant cells to invade *in vivo*. However, it is important to point out that it is very likely these cells possess other mutations and / or

molecular aberrations and it cannot be assumed that any of the disparities we see are due to p53 alone, especially as the L254R mutation has yet to be studied in much detail. For this reason, it would be prudent to clarify the effects of the L254R p53 mutant protein on cell morphology and invasion in this cell system by siRNA studies, or alternatively by inserting the mutation into wt p53 and / or p53 -/- cells.

While the role of p53 in cancer cell invasion constitutes a relatively recent field of investigation, the role of p53 in radiosensitivity has been explored for many years. Much of the work in this field is difficult to interpret as often only a small number of cell lines were analysed in each individual study, usually across different histological subtypes. While it is often assumed that the presence of wt p53 denotes a radiosensitive phenotype *in vitro*, the situation is not clear cut and wt p53 has been associated with equivalence or even increased resistance to radiation (Bristow, Benchimol et al. 1996; Servomaa, Kiuru et al. 1996; Chiarugi, Cinelli et al. 1998; Dahm-Daphi 2000; Matsui, Tsuchida et al. 2001; Weber and Wenz 2002; Williams, Zhang et al. 2008; Williams, Zhang et al. 2008). Indeed, some of the most radioresistant cell lines recorded, for example melanoma and glioblastoma, typically possess wt p53, although it is important to point out that p53 may not be functional in some of these cell lines due to defects elsewhere in the p53 pathway. A more rational and scientifically accurate overview of this topic is that wt p53 can increase radiosensitivity and mutant p53 can reduce radiosensitivity when inserted into certain p53 -/- cells (Bristow, Peacock et al. 2003), although this does seem to depend on the specific mutation (Okaichi, Ide-Kanematsu et al. 2008). However, this statement is in itself not without criticism as the addition of wt p53 to p53 -/- cells can result in massive apoptosis (Diller, Kassel et al. 1990; Michalovitz, Halevy et al. 1990; Martinez, Georgoff et al. 1991; Kaeser, Pebernard et al. 2004; Mazzatti, Lee et al. 2005) and attempts to circumvent this using reversible short term systems such as temperature sensitive constructs may be skewed as the conformation of p53 can be altered at low temperature (Cuddihy, Jalali et al. 2008). Similarly, clinical data supporting a role for p53 genotype as in independent predictive factor for radiotherapy outcome in the treatment of various solid tumours continues to be controversial due to variable methodology in detecting p53 function and variable endpoints in clinical trial design.

Thus, it was not surprising that p53 status alone did not determine radiosensitivity here. What was more intriguing was the variation between the two wt p53 clones and the two mutant p53 clones. A small difference in clonal populations (clonal variation) is to be expected and can be explained by discrepancies in gene expression. However, the contrast between the D1 and H9 wt p53 clones was quite marked, not only in terms of radiation survival response, but also in terms of proliferative capacity and gross tumour morphology when cells were injected into nude mice. One of the most significant observations we made while studying these clones was the disparity in their FACS profiles, for two main reasons.

Firstly, the apparent genomic instability which was inherent in the H9 clone probably explained the radioresistant phenotype and may also have explained the slower proliferation rate *in vivo*, as the degree of chromosomal abnormality could prevent effective chromosome segregation and mitosis. If so, why proliferation *in vitro* was not impaired is open to question. Secondly, we suspected that the differences in cellular characteristics between the isolated clones were not simply due to clonal variation alone. The striking difference in the level of aneuploidy between the D1 and H9 clones was a strong indicator of the presence of additional mutations in the H9 cell line. We did confirm that all of the isolated clones possessed the codon 61 mutation in H-Ras (data not shown), but we have not yet screened for the presence or absence of other significant mutations. At this point, it was clear that the SCC 7.1 FAK -/- cell line could not be reliably compared with either the D1 or H9 wt p53 single cell clones, or an early passage SCC 7.1 FAK +/+ population with predominantly wt p53 containing cells, due to significant concerns over the genetic background of these cells.

We then endeavoured to develop FAK -/- cell lines from the isolated clones. This strategy would have the obvious advantage of a paired parental FAK +/+ cell line for comparison. In addition, it would be possible to compare and contrast FAK knockdown as opposed to *fak* deletion. Furthermore, it would provide an opportunity to study *fak* deletion on both a wt p53 and a mutant p53 background. This work is ongoing but has not been straightforward. A potential problem that we have considered is expression of the *Cre* recombinase, especially in view of the single cell lineage. Hence, it may be necessary to

generate new clones from an early passage SCC 7.1 FAK +/+ population and repeat the process.

FAK has been shown to influence proliferation in several cell lines through an effect on cell cycle proteins, but this is not a universal finding, so it is unremarkable that FAK is dispensable for growth in culture in this cell system. FAK is, however, *not* dispensable for colony formation in an anchorage-independent environment in these cells and we have definitive evidence that this property is mediated through the kinase function of FAK. In fact, we have demonstrated constitutive activation of FAK in this cancer cell line. This data provides an intriguing insight into the complex biological functions of FAK in different environments. The uncoupling of FAK activity from adhesion signalling appears to be critical for anchorage-independent growth, although the exact mechanism is unclear. We initially thought that FAK kinase activity was protecting cells from death signals induced by loss of adhesion. This was not unreasonable, particularly as these cells contain wt p53 and the current literature depicts an important functional interaction between FAK and wt p53 in cell survival as already discussed (although the evidence to date linking the two proteins is based on the scaffolding function of FAK rather than its kinase function). However, we did not find that either loss or inhibition of FAK activity had any significant effect on anoikis (data not shown). In addition, although the FAK -/- cells and FAK kinase defective cells did not form large colonies in soft agar, they did form clusters consisting of several cells and would proliferate if they were removed from the agar and replated on tissue culture plastic. This would imply that loss of FAK kinase activity induces growth or proliferation arrest, but not necessarily cell death, in an anchorage-independent environment, which can be overcome by integrin adhesion.

The results discussed above may explain why FAK -/- xenografts can establish, albeit with a delayed onset. Preliminary data suggests that the delayed onset of FAK -/- xenografts is also due to loss of kinase activity (personal communication – Alan Serrels). Similarly, there was no evidence of increased apoptosis *in vivo*, as measured by activated Caspase-3 staining, in the FAK -/- xenografts (data not shown) or FAK kinase defective xenografts (personal communication – Alan Serrels). It may actually be easier for cells deficient in FAK kinase activity to overcome growth / proliferation arrest in the *in vivo* setting compared with soft

agar/methylcellulose due to the infrastructure and integrin signalling provided by the host animal.

A number of important questions stem from this section of work, such as; how exactly does FAK signalling differ in 2D *versus* 3D environments and what precise biological functions are mediated by FAK when it is uncoupled from adhesion signalling, are the anchorage–independent findings here dependent on expression or activity of p53 (and if so how does FAK kinase activity influence p53 function), and does the phenotype that we see in FAK kinase deficient cells grown in soft agar reflect tumour cell dormancy? The subject of tumour cell dormancy, a stage in cancer progression in which residual disease is present but undetectable, is poorly understood. There are several mechanisms that can explain tumour cell dormancy, including immunosurveillance and the inability of a tumour cell population to recruit blood vessels (Aguirre-Ghiso 2007). However, more directly relevant to our work, is that the disruption of crosstalk between growth factor and adhesion signalling can also lead to dormancy. Notably, it has previously been shown that interruption of FAK mitogenic signalling by expression of FRNK induced dormancy in human carcinoma cells *in vivo* (Aguirre Ghiso 2002). It could be argued that the FAK kinase deficient cells undergo senescence in an anchorage-independent environment, but as this is typically a response to replicative or oncogenic stress, and often permanent, we thought that a temporary cellular quiescence was a better mechanistic fit for our data. As quiescent cells are characterised by G0-G1 arrest, we are currently trying to establish the cell cycle status of the small clusters of FAK kinase deficient cells that we see in the soft agar growth medium.

It is already clear from the earlier work in this laboratory that FAK is important in skin carcinogenesis (McLean, Komiyama et al. 2004). However, it was unclear whether the effects of FAK in this context were mediated through its scaffolding function or kinase function. The work presented here suggests that FAK kinase activity is important in contributing to tumour initiation and development. These findings are significant, and alongside the orthotopic data provided by Mitra et al., who showed that expression of FRNK inhibited breast cancer progression and lung metastases in a mammary fat pad tumour model (Mitra, Lim et al. 2006), would support the further investigation of FAK kinase inhibitors. But does this mean that FAK kinase inhibitors will necessarily have a profound effect

on established tumours? We have yet to examine the effect of a FAK kinase inhibitor on established SCC 7.1 FAK wt xenografts, but significantly there was no marked difference in proliferation rate between SCC 7.1 FAK -/- and SCC 7.1 FAK wt xenografts. Other groups have shown some effects on proliferation using FAK kinase inhibitors in human xenograft models (Halder, Lin et al. 2007; Roberts, Ung et al. 2008; Watanabe, Takaoka et al. 2008; Hochwald, Nyberg et al. 2009), although it is important to note that these effects may be mediated by inhibition of kinases other than FAK.

Interestingly, some of these studies have also illustrated an effect on tumour vascularity (Halder, Lin et al. 2007; Roberts, Ung et al. 2008). This is of particular significance as FAK is known to influence VEGF expression via signalling through its 925 phosphorylation site (Mitra, Mikolon et al. 2006). We did find that the FAK -/- xenografts displayed a more tortuous vascular pattern and the vasculature tended to be prone to haemorrhage. While conditional loss of FAK in endothelial cells is known to induce leaky vessels (Ilic, Kovacic et al. 2003; Shen, Park et al. 2005), the vascular endothelial cells supporting a xenograft will be provided by the host animal which is not deficient in FAK, but we do wonder whether the absence of FAK in the surrounding tumour cells affects the signalling and architecture of the developing vasculature. It may well be the case that FAK kinase inhibitors have important anti-angiogenic effects in addition to or instead of anti-proliferative effects.

If so, this may have important implications in terms of radiation. Although work in xenograft models has demonstrated that VEGF-R inhibition in combination with radiation is both beneficial and not usually dependent on scheduling, it may be the case that modulating the tumour vasculature in the more complex setting of human malignancy may either increase or decrease radiosensitivity depending on the timing and sequencing of each modality (Duda, Batchelor et al. 2007). Should FAK kinase inhibitors prove to be useful in combination with radiation, it would be important to assess whether the effects on radiosensitivity are mediated by modulation of the vasculature and, if so, to determine whether scheduling of both agents is critical. One final point on the use of FAK kinase inhibitors, which does not specifically apply to radiation, is a note of caution based on the apparent quiescent state of FAK kinase deficient cells in an anchorage–independent environment. The potential for FAK inhibitors to induce

dormancy in cancer cells has significant implications on the future of these agents as a viable means of anti-cancer therapy and requires further investigation.

5.4 Summary

The original SCC 7.1 cell line we derived was composed of a variety of cells with different clonal origins. Hence, these cells illustrated differential morphological and growth characteristics and radiosensitivity patterns. Some of the properties of each constituent cell were likely to have been influenced by the presence of either wt or mutant p53. However, we strongly suspected the presence of additional mutations within these cells. This mixed cell population precluded a direct comparison with the SCC 7.1 FAK -/- cell line which originated from a single wt p53 cell. Reconstituting wt FAK into the FAK -/- cell line, however, was an acceptable and better controlled means of comparison. We have established that it will be possible to evaluate the radiosensitivity of these FAK -/- and FAK wt cell lines *in vitro* and *in vivo*, and in doing so have learned more about the role of FAK, particularly its kinase function, in various cellular environments.

Chapter 6

FAK deletion is associated with increased radioresistance in vitro and in vivo.

6 FAK deletion is associated with increased radioresistance in vitro and in vivo.

6.1 Aim

In the previous chapters, we described the development of novel SCC 7.1 FAK +/+ and SCC 7.1 FAK -/- squamous cell carcinoma cell lines. However, characterisation of these cell lines revealed that the original SCC 7.1 FAK +/+ parental cell line was composed of a mixture of different cell types, which precluded direct comparison between FAK +/+ and FAK -/- cells. This obstacle was overcome by reconstituting wt FAK into the SCC 7.1 FAK -/- cell line, thereby allowing a more accurate assessment of the functions of FAK in this cell line. Hereafter, these cell lines will be referred to as simply FAK -/- and FAK wt cells. In this chapter, we set out to establish whether the presence or absence of FAK influenced radiosensitivity in these cell lines in both the *in vitro* and *in vivo* settings and, if so, to elicit the underlying mechanism(s).

6.2 Results

6.2.1 FAK deletion is associated with increased radioresistance in vitro and in vivo.

In order to assess radiosensitivity, a limiting dilution clonogenic assay was performed comparing FAK -/- with FAK wt cells at increasing doses of radiation up to 10Gy. This assay revealed that the complete absence of FAK in these cells was associated with increased radioresistance *in vitro* (**Figure 49**). A statistically significant difference in surviving fraction was seen at doses of 4Gy, 6Gy, 8Gy and 10Gy (p values of 0.0136, 0.0097, 0.0045, and 0.0036 respectively, analysed by student's unpaired t-test, n=9).

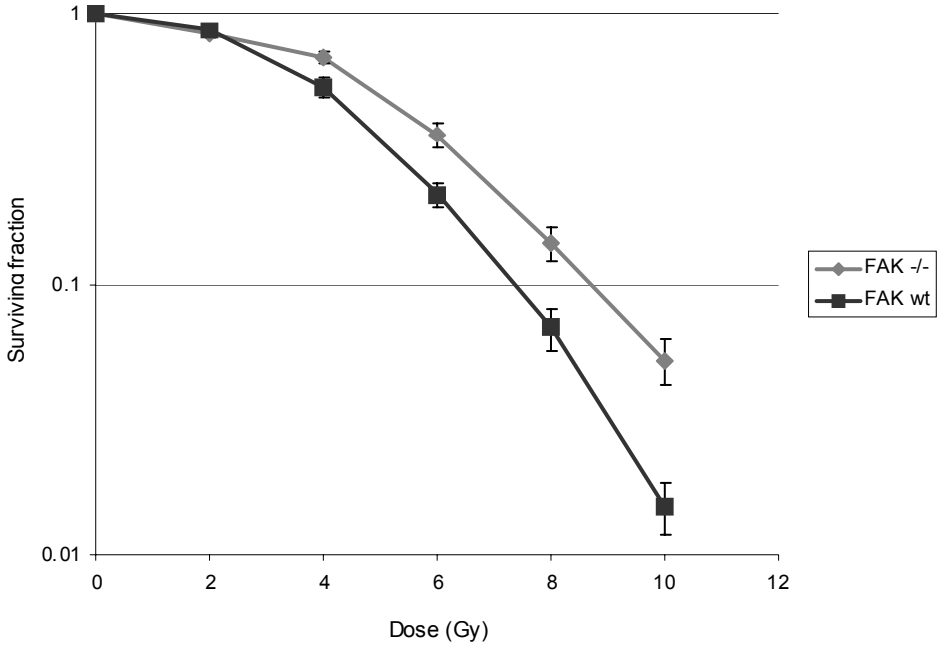


Figure 49 - FAK deletion is associated with increased radioresistance *in vitro*
Subconfluent populations of FAK -/- and FAK wt cells were trypsinised and diluted in growth medium to a final concentration that would permit single colony growth. 100µl of this suspension was added to each well of a 96 well plate. Following incubation for 6 hours to allow cell attachment the plates were irradiated with 0, 2, 4, 6, 8 or 10Gy. The plates were set up in triplicate for each radiation dose. After 7 days the number of colonies per plate was counted and the surviving fraction calculated. The graphical representation shown represents the mean ± SEM from three separate experiments. Surviving fractions at each dose of radiation were compared with unirradiated cells by student's unpaired t-test, n=9.

As one of our initial aims was also to evaluate whether FAK was an important mediator of radiosensitivity *in vivo*, we proceeded to investigate the effect of radiation on FAK -/- versus FAK wt xenografts. $2x10^5$ cells were injected subcutaneously into the right flank of female nude mice and the animals were either irradiated with 5Gy whole body irradiation or mock irradiated when the xenografts reached approximately $150mm^3$. This size was selected as the FAK -/- tumours have become well established by this point and their growth rate does not significantly differ from their FAK wt counterparts. Previous studies demonstrated that this strain of mice could tolerate 5Gy total body dose for 10-14 days. Nonetheless, the mice were carefully observed for haematological and gastrointestinal toxicity afterwards. After 7 days the xenografts were measured and the animals were sacrificed. The tumour volume was calculated before (**Figure 50A**) and after (**Figure 50B**) 5Gy irradiation or mock irradiation and analysed by student's unpaired t-test. A statistically significant reduction in tumour volume was observed in the irradiated FAK wt xenografts compared with the mock irradiated controls ($p=0.0030$, $n=10$), but this was not replicated in the FAK -/- xenografts ($p=0.3300$, $n=10$). Protein extracts were prepared from five mice in each group and subjected to western blotting analysis to confirm appropriate FAK status (**Figure 50C**).

We have shown that FAK is important in mediating radiosensitivity *in vitro* and, for the first time, we have shown that FAK also influences the response to radiation *in vivo*. However, our *in vitro* findings are in contrast to the published work to date (Cordes, Frick et al. 2007), suggesting that FAK may mediate different pro-survival or pro-death mechanisms in response to ionising radiation in different cell lines.

(A)

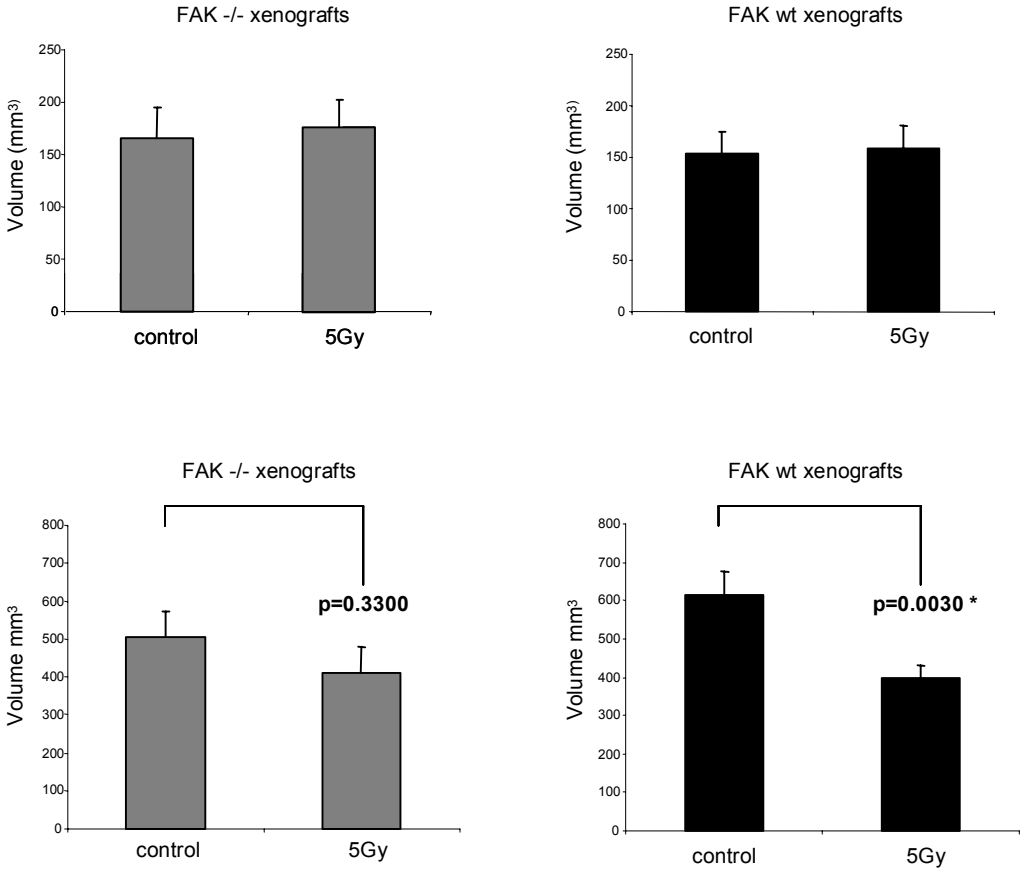


Figure 50 - FAK -/- xenografts are more resistant to radiation

(B)

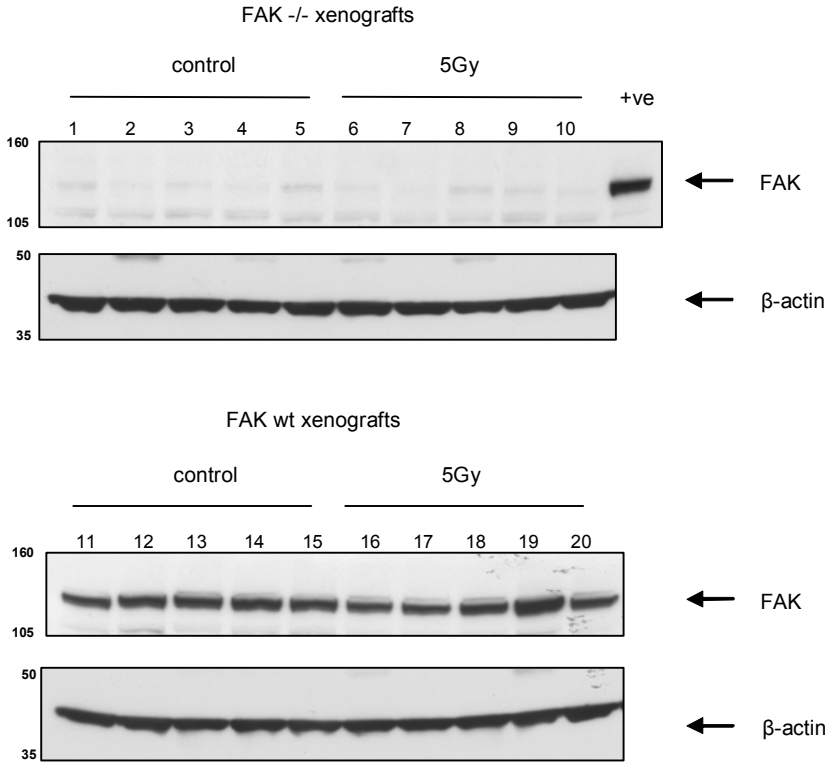


Figure 50 - FAK -/- xenografts are more resistant to radiation (cont)
(A) $2x10^5$ FAK -/- and FAK wt cells were injected subcutaneously into the right flank of female nude mice. Xenografts were allowed to reach approximately 150 mm^3. The animals were then irradiated with 5Gy whole body irradiation or mock irradiated. After 7 days the xenografts were measured and the mice were sacrificed. The mean xenograft volumes ± SEM before radiation (upper panels) and after radiation (lower panels) are shown. Statistical analysis of mock irradiated versus irradiated volumes at 7 days was assessed by student's unpaired t-test, * denotes $p<0.05$, n=10. (B) Protein extracts were prepared from the xenografts, separated by SDS-PAGE, transferred to nitrocellulose, and blotted with anti-FAK (upper) and anti-β-actin (lower) antibodies. A sample of five extracts from each group is shown. A positive control (FAK wt cell extract) was added to the final lane of the FAK -/- xenograft samples.

6.2.2 Ionising radiation results in p21 induction in vitro and in vivo in FAK -/- cells and xenografts respectively, but not FAK wt cells or xenografts.

It has already been shown by sequencing analysis that the cells used here possessed wt p53 but when we attempted experiments to assess p53 functionality, it became clear that there was a difference. Remarkably, exposing both cell lines to 5Gy irradiation and assessing p21 levels, as outlined earlier in Chapter 4, demonstrated p21 induction only in the FAK -/- cells, not FAK wt cells (**Figure 51A**). Interestingly, basal levels of p21 mRNA and protein in subconfluent populations of both cell lines were similar (**Figures 51B** and **51C** respectively). p21 levels were upregulated in both cell lines with increased confluency as expected in view of the widely accepted role of p21 in cell cycle arrest (**Figure 51D**), and this was the same in both cell lines, indicating some similarities in the control of p21 and its function between FAK -/- and FAK wt cells. To ensure that the discrepancy in p21 induction following exposure to ionising radiation was not related to differences in cell density, care was taken to ensure that cells were irradiated at comparable confluency, typically 70%.

This pattern of p21 induction was reproduced *in vivo*. Briefly, nude mice were injected subcutaneously with $2.5x10^5$ FAK -/- or FAK wt cells, xenografts were allowed to establish, and the animals were then irradiated with 5Gy whole body irradiation when tumours reached approximately $500mm^3$ by volume. Mice were sacrificed at 0, 2hrs, 6hrs, and 24hrs post treatment (n=3 per group) and p21 levels assessed by both western blotting of tumour lysates and immunohistochemistry staining of paraffin embedded tissue. The FAK -/- xenografts exhibited a clear increase in p21 protein levels as early as 2 hours post irradiation as shown by western blotting analysis (**Figure 52A**). In fact, the p21 levels were maximal at this point. Admittedly, one of the control samples appeared to be degraded (lane 3) which may have biased the experiment. However, p21 was also visibly increased by immunohistochemistry analysis, again most notably at 2 hours post irradiation (**Figure 52B**). Mean p21 positivity (based on scoring of 20 fields) was then analysed across all time points and this demonstrated a significant difference in the p21 levels in irradiated versus unirradiated animals (Kruskal-Wallis, p=0.038, n=3). Further, individual

comparison of the separate time points illustrated a statistically significant increase in p21 scoring compared with baseline levels (Mann Whitney, p=0.0404, n=3). The FAK wt xenografts, on the other hand, did not demonstrate any consistent increase in p21 levels at any of the time points examined by either means of analysis. Western blotting of FAK wt tumour lysates confirmed the presence of FAK, but there was no appreciable increase in p21 protein levels noted (**Figure 53A**)**.** Representative p21 staining of paraffin embedded tissue at each time point is depicted in **Figure 53B,** again with no appreciable increase in p21 noted. A corresponding boxplot of the proportion of p21 positive cells per group is illustrated in **Figure 53C**. There was no significant increase in p21 expression at 2hrs (p=0.6625), 6hrs (p=0.6625), or 24hrs (p=0.3827) (Mann Whitney, n=3).

This data indicated that the regulation of p21 in response to ionising radiation was different between these cell lines and appeared to be critically dependent on the presence or absence or FAK. It also hinted at a possible mechanism for the difference in radiosensitivity between the FAK -/- and FAK wt cells, as p21 has previously been linked with radioresistance. Most of the evidence in support of p21 as a mediator of radioresistance stems from work in human brain tumour cell lines. Overexpression of p21 was found to increase clonogenic survival in glioma cells (Kokunai and Tamaki 1999) while p21 knockdown using antisense oligonucleotides decreased clonogenic survival in a similar panel of cell lines (Kokunai, Urui et al. 2001). Interestingly, earlier research using the HCT-116 p21 -/- colorectal cancer cell line had suggested that p21 deficient cells were more likely to be killed by apoptosis following ionising radiation than their p21 +/+ counterparts (Waldman, Lengauer et al. 1996; Waldman, Zhang et al. 1997). In fact, this ability of p21 to inhibit apoptosis has been cited as one of the main mechanisms by which p21 can induce radioresistance. However, p21 can also influence DNA repair and cell cycle kinetics, which adds to the complexity of this protein in the DNA damage response. This complexity may explain why p21 has conversely been associated with promoting sensitivity to chemotherapy and radiotherapy in certain situations. Our main priority, therefore, at this point was to determine if the increase in p21 levels in the FAK -/- cell line was indeed responsible for its more radioresistant phenotype.

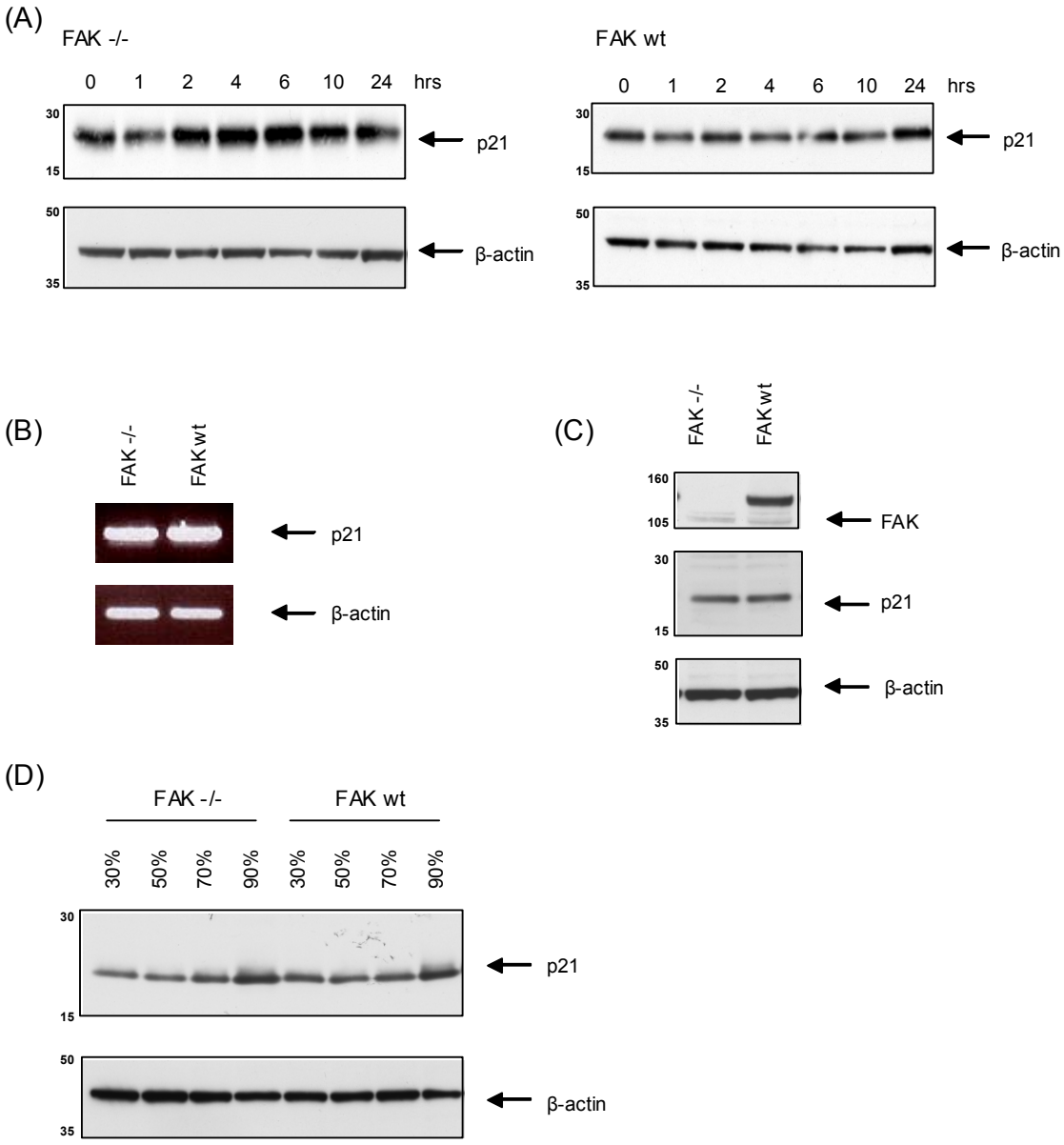


Figure 51 - Ionising radiation results in p21 induction in FAK -/- cells but not FAK wt cells
(A) FAK -/- and FAK wt cells were irradiated with 5Gy at 70% confluence and lysates prepared at the indicated time points. Immunoblotting was then performed with anti-p21 (upper), and anti-β-actin (lower) antibodies. (B) RNA was extracted from subconfluent FAK -/- and FAK wt cell populations and converted to cDNA as previously described. A PCR reaction was then performed and 5µl of PCR product analysed on a 1.5% agarose gel (upper panel). β-actin loading is also shown (lower panel). (C) Protein extracts were prepared from subconfluent FAK -/- and FAK wt cell populations, separated by SDS-PAGE, transferred to nitrocellulose, and blotted with anti-p21 (upper panel) and anti-β-actin (lower panel) antibodies. (D) Protein extracts were prepared from FAK -/- and FAK wt cell populations at the level of confluency indicated. Immunoblotting was then performed with anti-p21 (upper panel) and anti-β-actin (lower panel) antibodies.

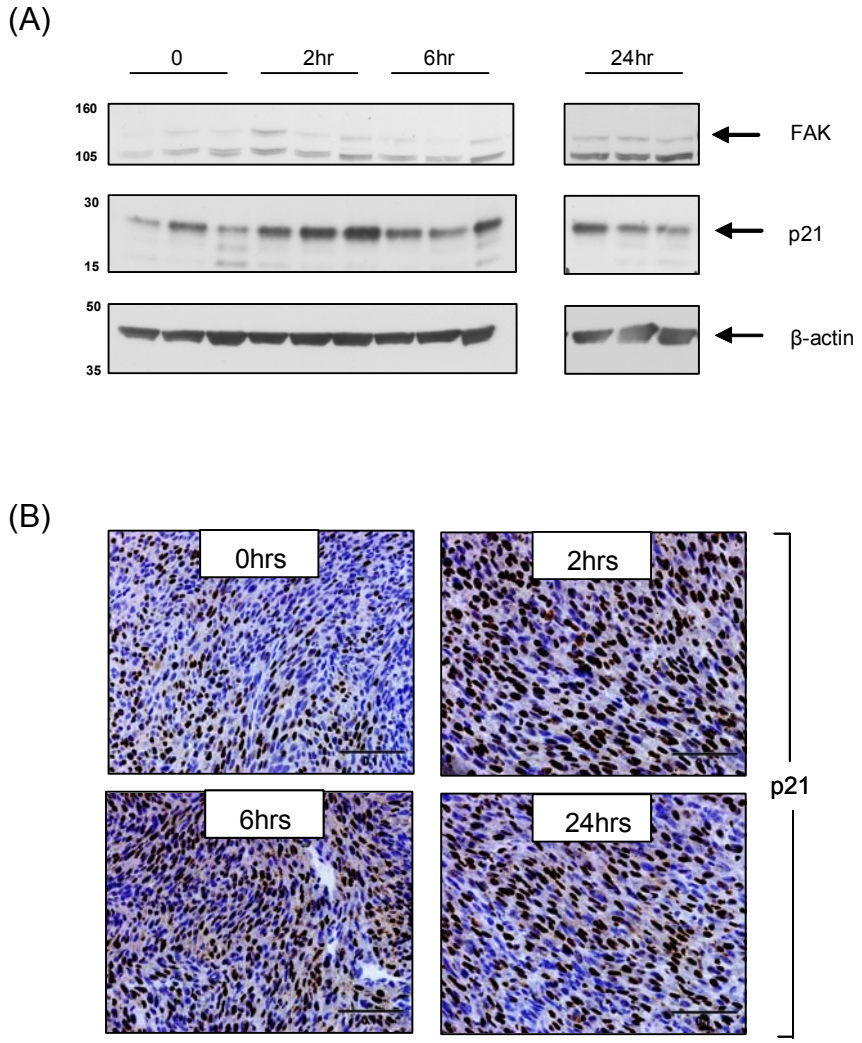


Figure 52 - Ionising radiation results in p21 induction in FAK -/- xenografts
(A) $2.5x10^5$ FAK -/- cells were injected subcutaneously into the right flank of female nude mice. When xenografts reached approximately $500mm^3$, mice were irradiated with 5Gy and sacrificed at 0, 2hrs, 6hrs, and 24hrs (n=3 per group). Half of the xenograft was fixed in formalin then embedded in paraffin and the other half was snap frozen in liquid nitrogen. Protein extracts were prepared from the frozen sections, separated by SDS-PAGE, transferred to nitrocellulose, and blotted with anti-FAK (upper), anti-p21 (middle), and anti-β-actin (lower) antibodies. (B) The paraffin embedded sections were stained with p21 and the proportion of p21 positive cells calculated per high powered field (20 fields per animal). Representative bright field images of p21 stained tissue at 0, 2hrs, 6hrs, and 24hrs post radiation are shown (scale bar, 0.1mm).

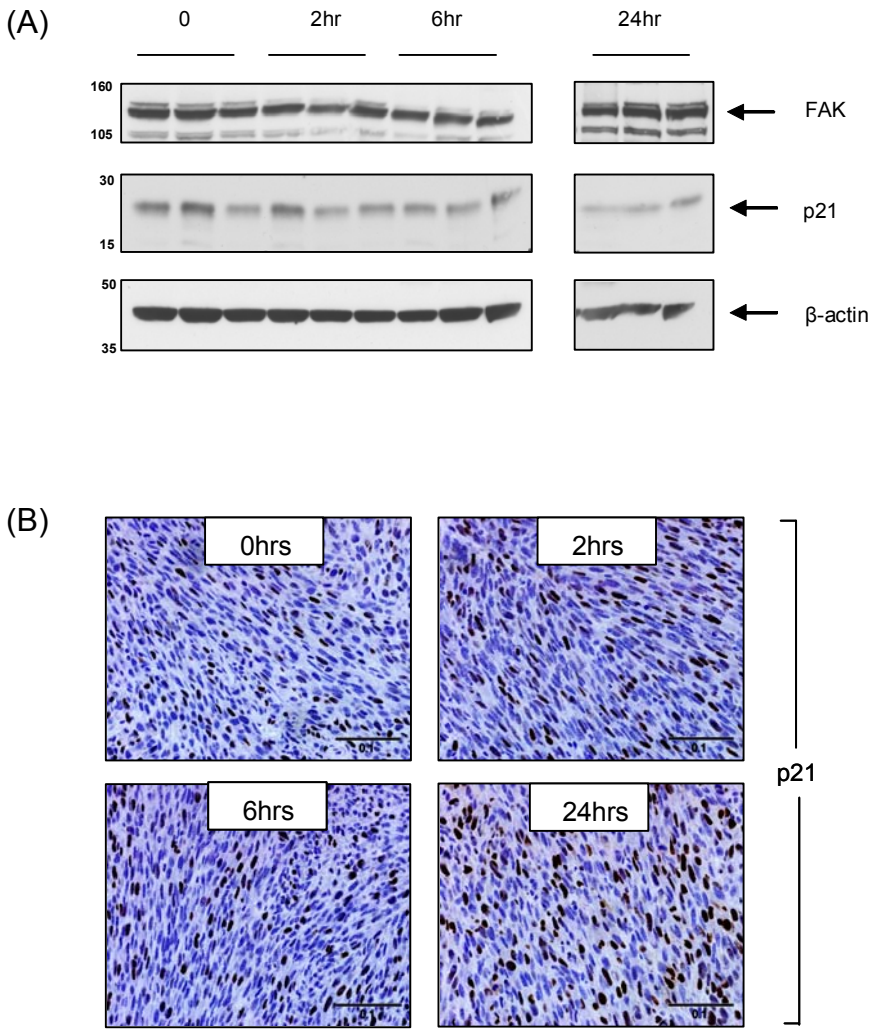


Figure 53 - Ionising radiation does not result in p21 induction in FAK wt xenografts
(A) $2.5x10^5$ FAK wt cells were injected subcutaneously into the right flank of female nude mice. When xenografts reached approximately $500mm^3$, mice were irradiated with 5Gy and sacrificed at 0, 2hrs, 6hrs, and 24hrs (n=3 per group). Half of the xenograft was fixed in formalin then embedded in paraffin and the other half was snap frozen in liquid nitrogen. Protein extracts were prepared from the frozen sections, separated by SDS-PAGE, transferred to nitrocellulose, and blotted with anti-FAK (upper), anti-p21 (middle), and anti-β-actin (lower) antibodies. (B) The paraffin embedded sections were stained with p21 and the proportion of p21 positive cells calculated per high powered field (20 fields per animal). Representative bright field images of p21 stained tissue at 0, 2hrs, 6hrs, and 24hrs post radiation are shown (scale bar, 0.1mm).

6.2.3 p21 mediates radioresistance in FAK -/- cells.

Since p21 has been associated with both resistance and sensitivity to DNA damaging agents, including ionising radiation, it was crucially important to ascertain whether the increase in p21 levels in the FAK -/- cell line was an incidental finding or a key mediator of radiation survival. Consequently, siRNA technology was utilised to achieve transient knockdown of p21 in a pooled population. **Figure 54A** and **Figure 54B** demonstrate western blotting for p21 in scrambled siRNA and p21 siRNA transfected pools and corresponding densitometry analysis indicating 90% p21 knockdown. Clonogenicity was then assessed at 0, 4, and 8Gy and comparison made between cell populations transfected with scrambled siRNA and p21 siRNA. Control cell populations which had been mock transfected were also assessed. As shown in **Figure 54C**, there was a significant difference in surviving fraction at 8Gy ($p=0.0129$) between the scrambled siRNA treated pools and the p21 siRNA treated pools, indicating that p21 did play a role in radioresistance in this cell line.

p21 is known to initiate G1 arrest in response to ionising radiation in a variety of cell lines with wt p53. It is thought that in certain cell lines this ability of p21 may promote radioresistance by allowing ample time for effective DNA repair. Hence, it was important to establish whether the difference in radiosensitivity was explained by altered cell cycle kinetics. FAK -/- and FAK wt cells were irradiated and samples collected for FACS analysis at the time points indicated in **Figure 55**. Radiation induced a temporary G2/M arrest at 8 – 12 hours followed by an accumulation of cells in G1 phase at 24 – 48 hours in both cell lines, as illustrated in **Figure 55A**, which displays the proportion of gated cells in each phase of the cell cycle (G1, S, and G2/M). The actual FACS plots from each time point are shown in **Figure 55B**. There was no obvious difference in cell cycle kinetics which would support the more radioresistant phenotype of the FAK -/- cell line. Although we demonstrated earlier that p21 levels increased with confluency (**Figure 51D, page 173**), indicating a role for this protein in density related cell cycle arrest, it appeared that p21 was not a major driver of cell cycle dynamics in response to radiation in these cells.

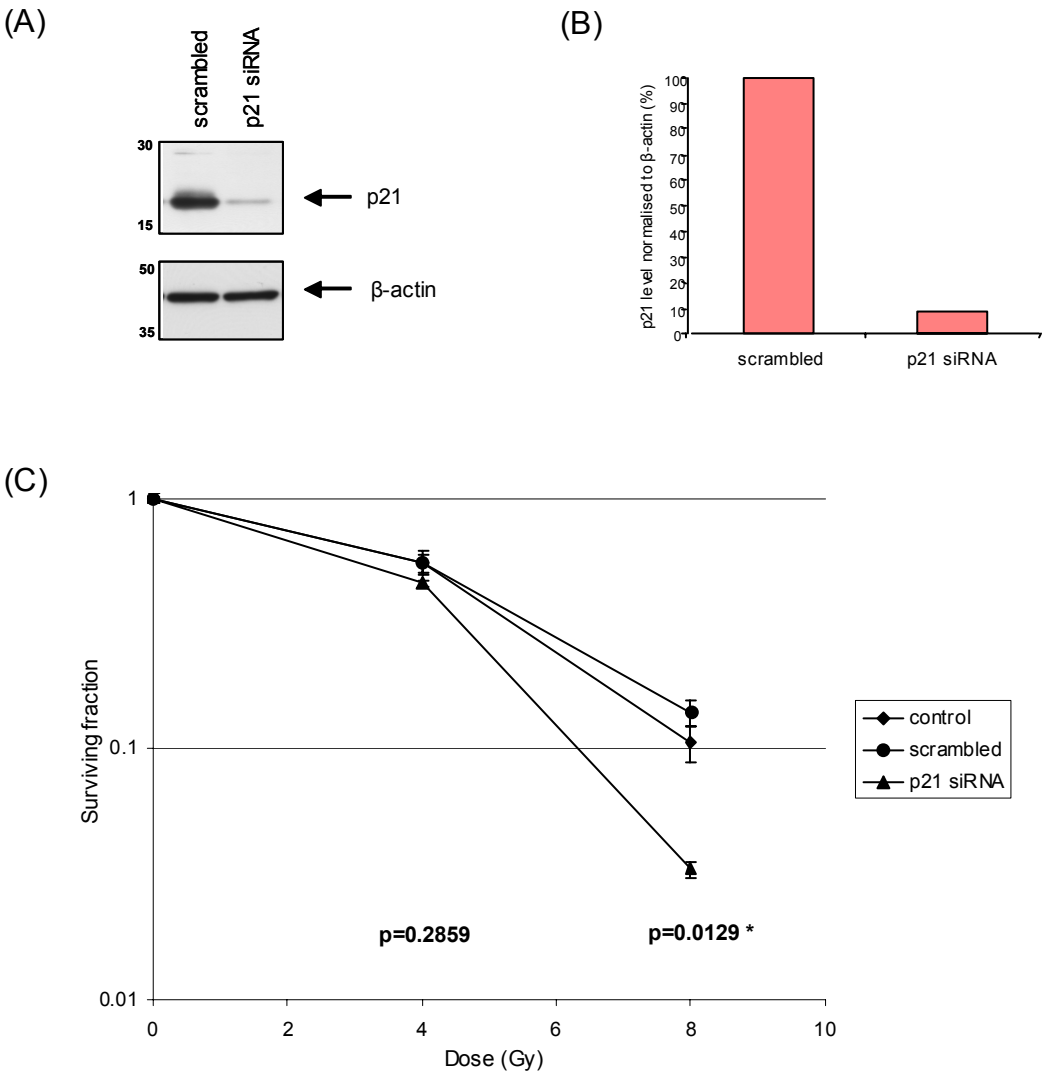


Figure 54 - p21 knockdown reduces radioresistance in FAK -/- cells
(A) FAK -/- cells were transfected with 100nM siRNA (either a scrambled pool or p21 siRNA) at 50% confluency as described in Methods section. Following incubation for 24 hours, protein extracts were prepared and immunoblotted with anti-p21 (upper) and anti-β-actin (lower) antibodies. (B) Densitometry comparing p21 levels in FAK -/- protein extracts treated with either a scrambled pool of siRNA or p21 siRNA was performed. The p21 protein levels were normalised to β-actin level and results shown are representative of one of three separate experiments. (C) FAK -/- cells were mock transfected or transfected with 100nM of either a scrambled siRNA pool or p21 siRNA at 50% confluency. After 24 hours the cell populations were trypsinised and diluted in growth medium to a final concentration that would permit single colony growth. 100 µl of this suspension was then added to each well of a 96 well plate. Following incubation for 6 hours to allow cell attachment the plates were irradiated with 0, 4, or 8Gy. The plates were set up in triplicate for each radiation dose. After 7 days the number of colonies per plate was counted and the surviving fraction calculated. The graphical representation shown represents the mean ± SEM from three separate experiments. Surviving fractions of p21 siRNA treated cells were compared with scrambled siRNA treated cells at each dose of radiation and statistical significance assessed by student's unpaired t-test, * denotes $p<0.05$, n=9.

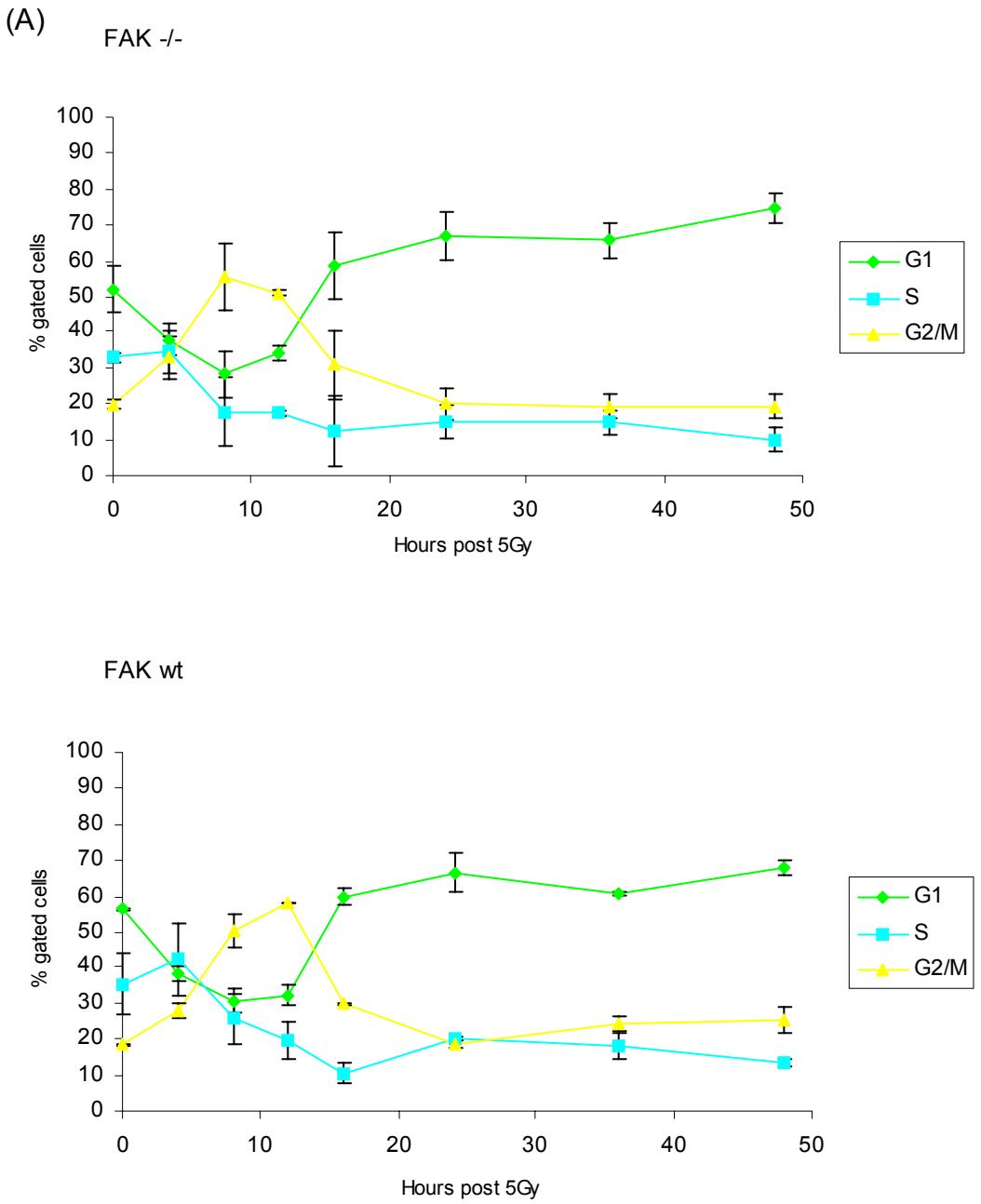


Figure 55 - p21 induction does not promote radioresistance in FAK -/- cells by increasing the length of cell cycle arrest in response to ionising radiation

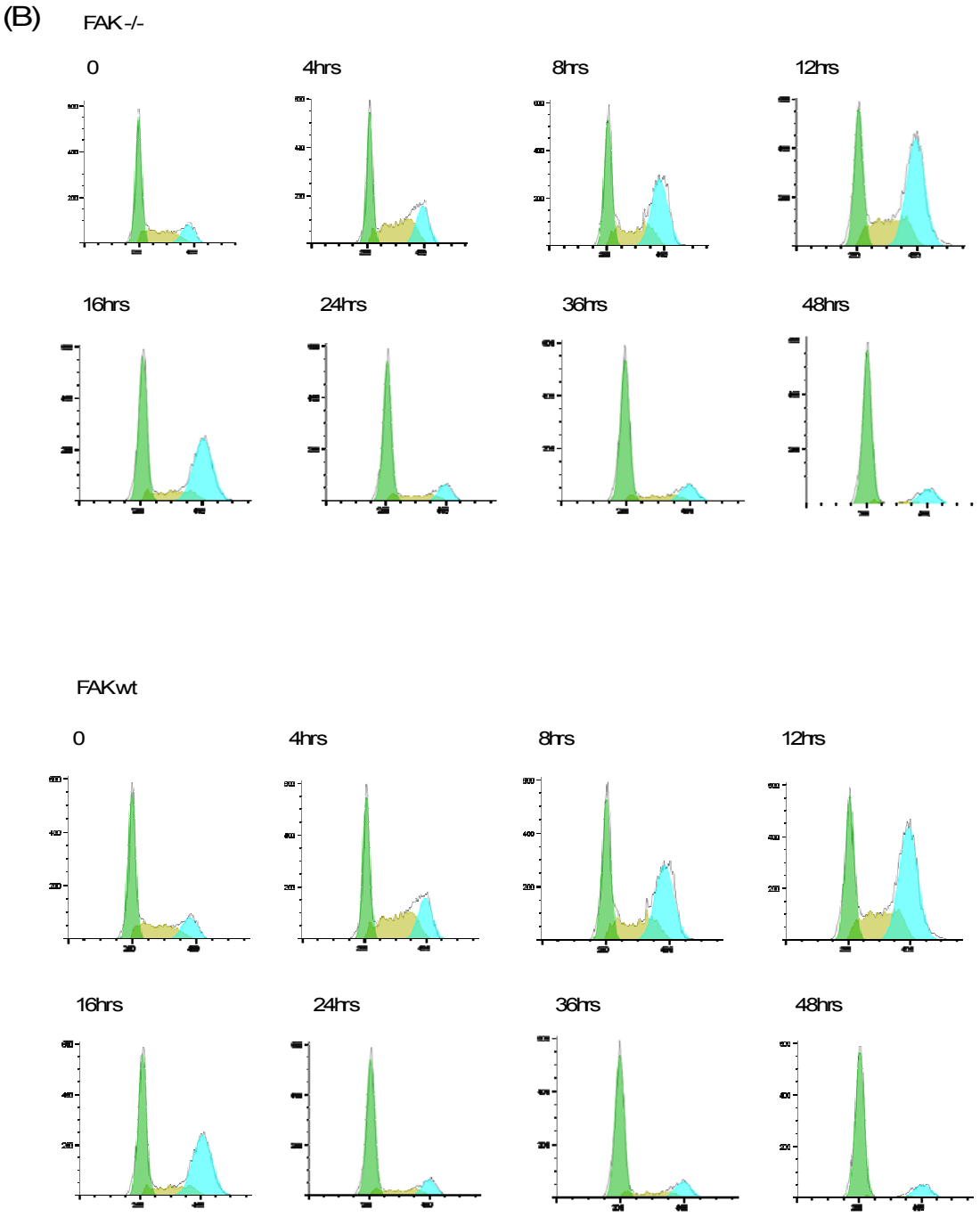


Figure 55 - p21 induction does not promote radioresistance in FAK -/- cells by increasing the length of cell cycle arrest in response to ionising radiation (cont)
(A) FAK -/- and FAK wt cells were irradiated with 5Gy at 70% confluence; at various time points samples were fixed in 70% ethanol, stained with propidium iodide and subjected to cell cycle analysis. The percentage of gated cells in each of the component phases (G1, S, and G2/M) of the cell cycle was evaluated at each time point. The graphs shown represent the mean ± SEM from three experiments. (B) Representative FACS plots from one of the experiments outlined above.

6.2.4 p21 induction following ionising radiation is regulated at transcriptional level in FAK -/- cells.

Ionising radiation can alter the level of various cellular proteins by transcriptional and/or translational mechanisms. Following the observation that p21 levels increased in FAK -/- cells in response to ionising radiation, further studies were undertaken to determine whether this phenomenon was transcription-dependent or independent. RNA was therefore extracted from subconfluent populations of FAK -/- and FAK wt cells at various time points following 5Gy irradiation and qRT-PCR performed using endogenous p21 primers. A biphasic increase in p21 mRNA levels at 2 hours and 6 hours post radiation was seen in the FAK -/- cells whereas p21 mRNA levels remained stable in the FAK wt cell line over the full 24 hour period of investigation (**Figure 56A**).

To determine whether radiation dose was significant, RNA was extracted from both cell lines 2 hours after a range of radiation doses (0, 2, 5, 10, 20, and 30Gy) and again analysed by qRT-PCR. This time point was selected as the maximum increase in p21 mRNA levels in FAK -/- cells in the experiment outlined above occurred at 2hrs. **Figure 56B** shows that p21 mRNA levels increased in the FAK -/- cell line in a dose-dependent fashion from 2Gy to 10Gy with some fall-off at doses in excess of 10Gy. Corresponding p21 protein levels were assessed by western blotting 2½ hours post irradiation (**Figure 56C**). The lowest dose of 2Gy was sufficient to induce a detectable rise in p21 protein levels in FAK -/- cells and even higher levels were achieved with 10Gy. However, there was little difference in protein levels in cells stimulated by either 10Gy or 20Gy irradiation suggesting that p21 induction was dose dependent in FAK -/- cells in the range from 2Gy to 10Gy. Minimal protein response was detected following 30Gy, suggesting that either effective translation was not taking place or p21 was being rapidly degraded.

Small increases in p21 mRNA levels were detected in the FAK wt cell line in response to the highest radiation doses of 20Gy and 30Gy (**Figure 56B** – right panels) but no p21 protein response was elicited in FAK wt cells at any of the examined radiation doses, including 20Gy and 30Gy (**Figure 56C** – right panels). Again, it was unclear on the basis of these results alone to differentiate between

ineffective translation and enhanced protein degradation at doses of or in excess of 20Gy. As it proved difficult to interpret the findings at these higher doses of radiation we opted to focus our studies mainly on doses of 10Gy or less. Taken together, the findings here indicate that increased transcription was responsible, or at least partly responsible, for the increase in p21 protein levels in FAK -/- cells in response to clinically relevant doses of ionising radiation.

6.2.5 p21 is regulated by p53 in FAK -/- cells.

It has already been alluded to that p21 is a transcriptional target of p53. Although p21 is not exclusively regulated by p53, we suspected that p53 was driving p21 in FAK -/- cells as the surges in p21mRNA levels (**Figure 56A**) seemed to correlate with p53 stabilisation (**Figure 35, page 131**). As there is growing evidence linking FAK to p53, we wanted to determine whether p53 was a major regulator of p21 protein levels in these cells, as a FAK mediated effect on p53 functionality or transcriptional activity could potentially explain the discrepancy in p21 induction. To this end, a pooled population of FAK -/- cells were transiently transfected with either scrambled siRNA or p53 siRNA and protein levels of both p53 and p21 measured after 24 hours. It proved difficult to quantify the reduction in p53 levels as the basal levels of this protein were very low, but a reduction in p21 levels of approximately 50% was recorded at 24 hours post transfection suggesting that p53 (at least partially) regulated the basal expression of p21 in these cells (**Figures 57A** and **57B**). More importantly, when FAK -/- cells that had been transfected with p53 siRNA were irradiated with 5Gy, there was no significant increase in p21 levels up to 6 hours post treatment, in contrast with cells that had been transfected with scrambled siRNA which did show an elevation in p21 protein levels within 2 hours (**Figure 57C**).

Here we have shown that p53 exerted a regulatory influence over both basal levels of p21 and the induction of p21 in response to ionising radiation in the FAK -/- cell line. These findings indicated that the role of p53 in these cells warranted further investigation.

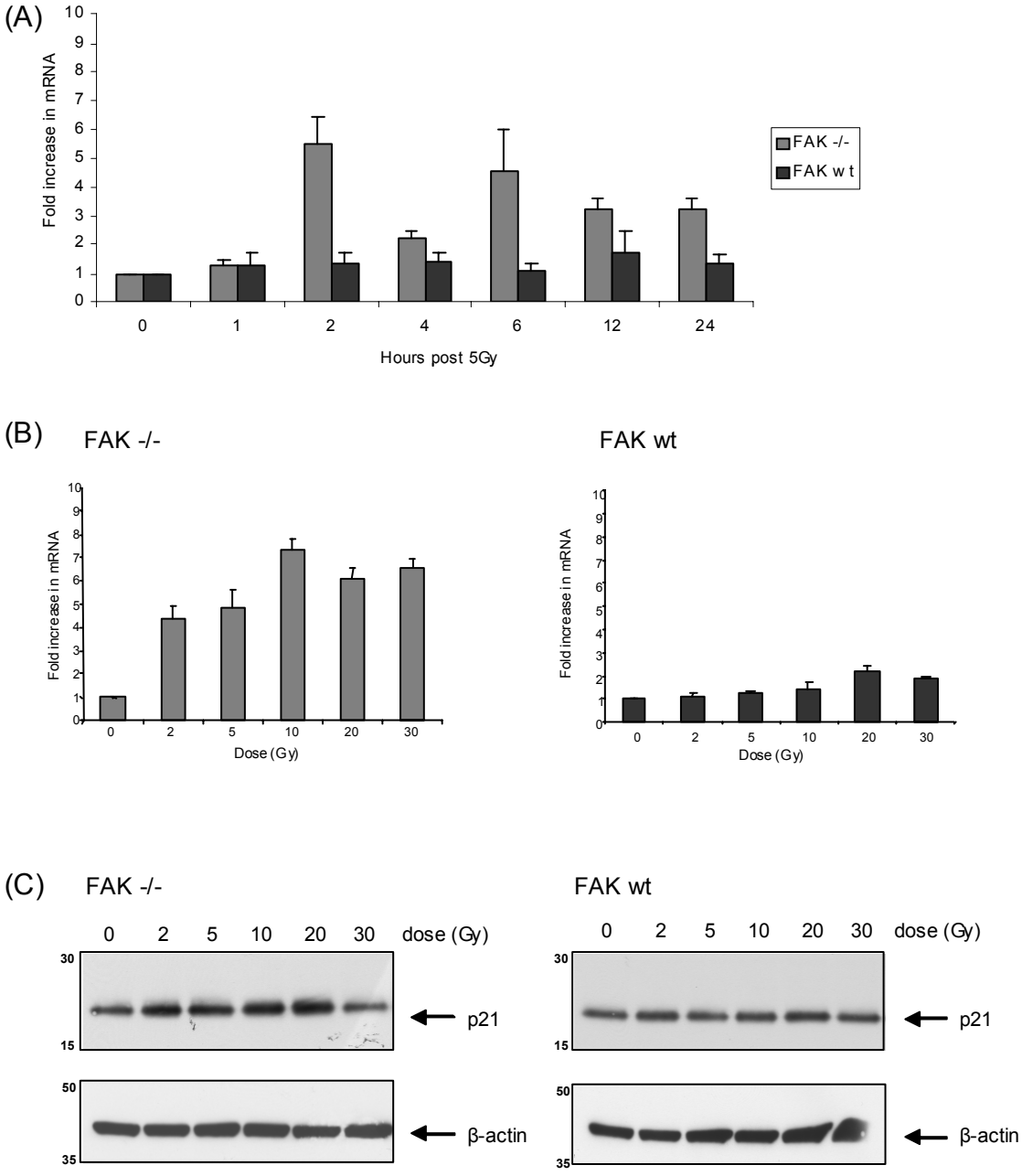


Figure 56 - p21 induction following radiation is regulated at transcriptional level
(A) RNA was extracted from subconfluent FAK -/- and FAK wt cell populations at various time points after 5Gy irradiation and converted to cDNA using Superscript II. The cDNA was diluted 1/5 and 5µl added to 50µl SybrGreen master mix containing 1µM concentration of primer pair. qRT-PCR analysis was then performed in triplicate based on a 96 well plate format. Fold increase in p21 mRNA levels was calculated using the ddC(t) method with β-actin as a loading control. Graphical representation of combined mean ± SEM from three experiments is demonstrated. (B) RNA was extracted from subconfluent FAK -/- and FAK wt cell populations 2 hours after 0, 2, 5, 10, 20, and 30Gy irradiation. cDNA was then generated and qRT-PCR for p21 performed as outlined above. (C) Protein extracts were prepared from FAK -/- and FAK wt cell populations 2½ hours after exposure to various doses of radiation as outlined. The extracts were separated by SDS-PAGE, transferred to nitrocellulose, and blotted with anti-p21 (upper) and anti-β-actin (lower) antibodies.

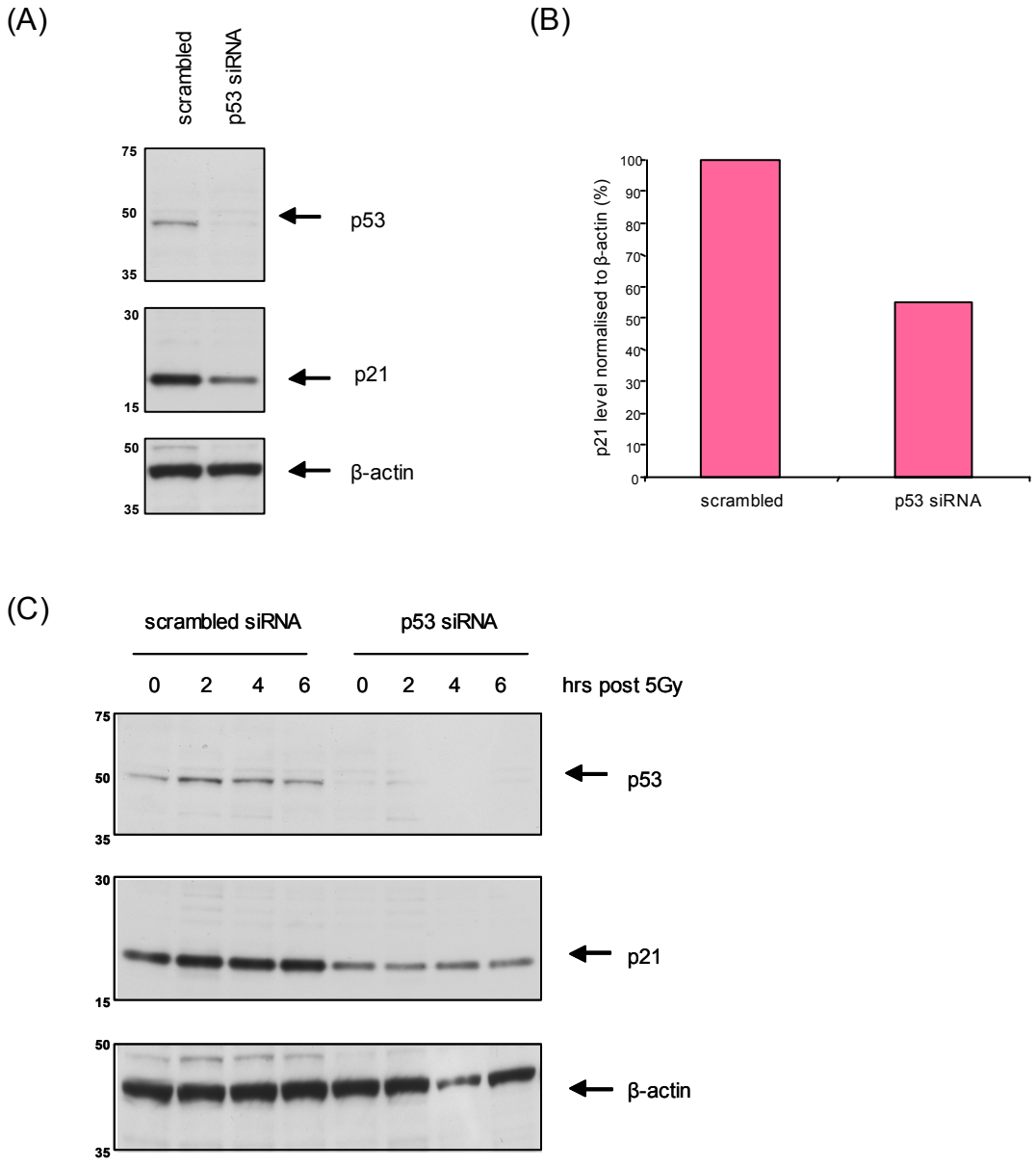


Figure 57 - p53 knockdown reduces basal p21 levels and prevents p21 induction following exposure to ionising radiation in FAK -/- cells
(A) FAK -/- cells were transfected with 100nM siRNA (either scrambled pool or p53 siRNA) at 50% confluency. Following incubation for 24 hours, protein extracts were prepared and immunoblotted with anti-p53 (upper), anti-p21 (middle) and anti-β-actin (lower) antibodies. (B) Densitometry comparing p21 levels in FAK -/- protein extracts treated with either a scrambled pool of siRNA or p53 siRNA was performed. The p21 protein levels were normalised to β-actin and results shown are representative of one of three separate experiments. (C) FAK -/- cells at 50% confluence were transfected with either 100nM scrambled siRNA or 100nM p53 siRNA, incubated for 24 hours, then irradiated with 5Gy. Lysates were collected at the time points indicated and immunoblotted with anti-p53 (upper), anti-p21 (middle) and anti-β-actin (lower) antibodies.

6.2.6 p53 stabilisation in response to ionising radiation is regulated largely at post transcriptional level.

Having established that p21 was regulated by p53 in FAK -/- cells, it was essential to pinpoint why p21 induction in response to radiation was a feature of FAK -/- cells and not FAK wt cells. As the presence or absence of FAK has been shown to influence total protein levels of p53 (Lim, Chen et al. 2008), it seemed pertinent to compare and contrast the ability of both cell lines to stabilise p53 in response to radiation. By measuring p53 protein levels on western blotting at various time points after 5Gy irradiation, it was clear that basal levels of p53 were comparable (**Figure 58A**) and p53 was stabilised in both FAK -/- and FAK wt cell lines (**Figure 58B**). Although a similar pattern was seen in both cell lines initially, i.e. maximal levels of p53 at 2 hours post irradiation followed by a smaller increase at 6 hours, FAK -/- cells exhibited a further increase in p53 protein levels at 24 hours.

While the stabilisation of p53 in response to ionising radiation is principally at the post-translational level, radiation can induce transcription of p53 (Fu and Benchimol 1997; Takagi, Absalon et al. 2005). Interestingly, analysis of p53 mRNA levels by qRT-PCR highlighted an increase in transcription of p53 only at the 24 hour time point in FAK -/- cells (**Figure 58C**). It appeared that the increases in p53 protein levels in response to ionising radiation were largely due to post-translational mechanisms in these cells, which is a typical finding, with the exception of the 24 hour time point in FAK -/- cells, the significance of which was unclear.

6.2.7 p53 stabilisation is stress dependent not FAK dependent.

We noted that p53 levels, although elevated in response to 5Gy irradiation, did not increase as significantly as other cell lines containing wt p53, for example the SCC 1.1 cell line. Exposing the FAK -/- and FAK wt cell lines to higher doses of radiation (up to 30Gy) led to further, albeit small, amplification at 2 hours post treatment (**Figure 59**). In contrast, the addition of cytotoxic agents, such as cisplatin or doxorubicin, effected massive accumulation of p53 levels, although this was dose and drug dependent (**Figure 60**). Topotecan, for

instance, did not induce p53 in either cell line. This was not particularly surprising as topoisomerase I inhibitors do not usually have this effect at the applied doses. The lack of response in either cell line following the addition of taxol was a little unexpected, however, as this drug is known to induce p53 in a range of cancer cell lines, albeit at high concentrations. It may be the case that concentrations in excess of 10μM would be required in order to produce any effect on p53 levels.

With each of the stressors that did induce p53, namely ionising radiation, cisplatin, and doxorubicin, there was no visible difference in the levels of p53 attained between the FAK -/- and FAK wt cells. This indicated that stabilisation of p53 was stress dependent rather than FAK dependent. Intriguingly, p21 protein levels increased in both cell lines following exposure to both cisplatin and doxorubicin (**Figure 60** – left panels). Admittedly, we have yet to investigate whether the drug induced elevation in p21 levels here were regulated at the transcriptional level by p53, but the disparity in p21 induction following radiation as opposed to chemotherapeutic agents led us to question whether this phenomenon was stress dependent as well as FAK dependent.

It is noteworthy that cisplatin and doxorubicin operate via quite different mechanisms compared to ionising radiation. It would therefore be useful to repeat these studies with radiomimetic drugs such as etoposide or bleomycin which induce considerable levels of DSBs.

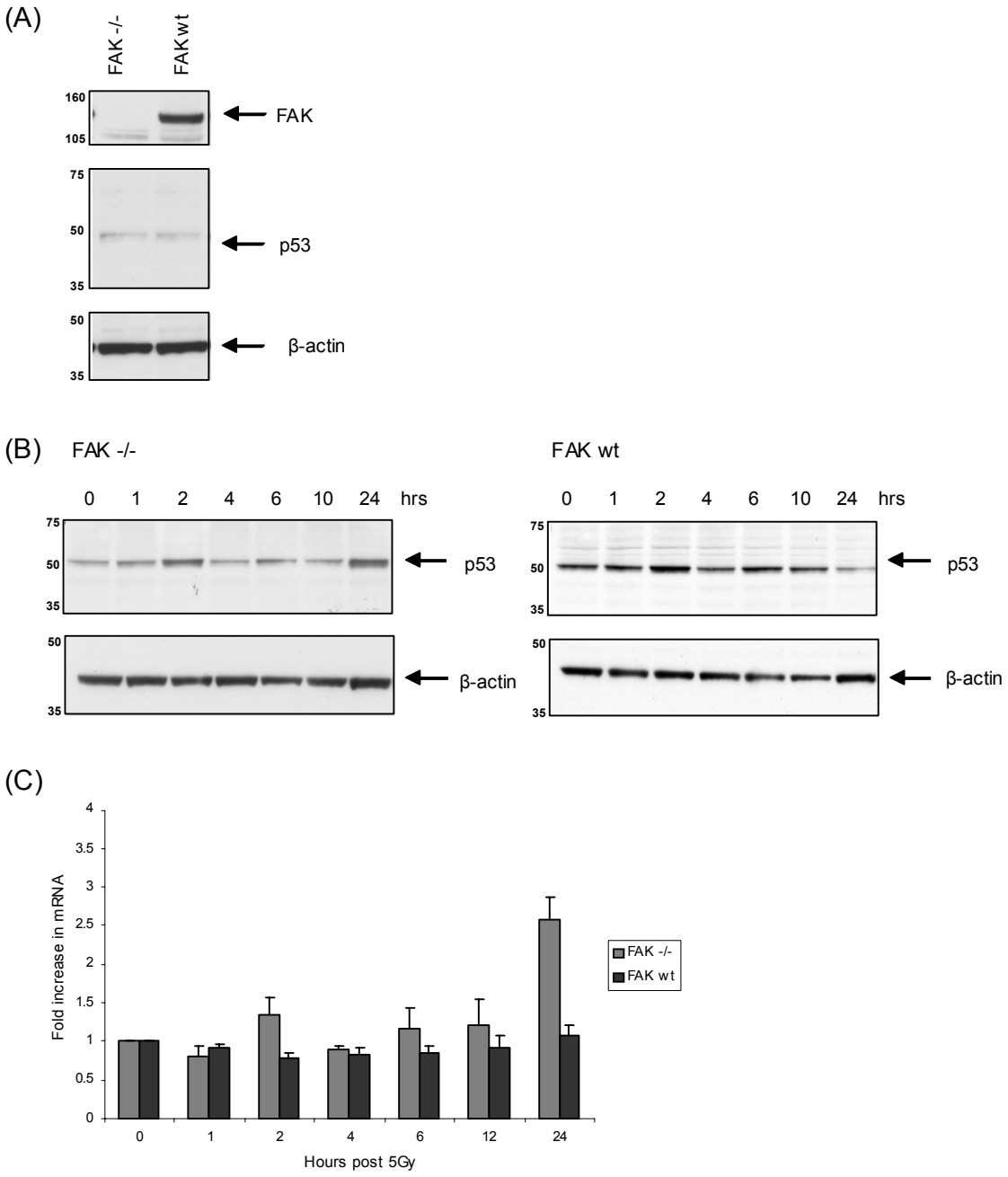


Figure 58 - p53 stabilisation in response to ionising radiation is predominantly regulated at post transcriptional level in both FAK -/- and FAK wt cells

(A) Protein extracts were prepared from subconfluent FAK -/- and FAK wt cell populations, separated by SDS-PAGE, transferred to nitrocellulose, and blotted with anti-FAK, anti-p53 (upper panel) and anti-β-actin (lower panel) antibodies. (B) FAK -/- and FAK wt cells were irradiated with 5Gy at 70% confluence and lysates prepared at the indicated time points. Immunoblotting was then performed with anti-p53 (upper), and anti-β-actin (lower) antibodies. (C) RNA was extracted from subconfluent FAK -/- and FAK wt cell populations at various time points after 5Gy irradiation, converted to cDNA, and qRT-PCR analysis was then performed using 1μM p53 primers as previously described. Fold increase in p53 mRNA levels was calculated using the ddC(t) method with β-actin as a loading control. The combined results from three separate experiments are shown.

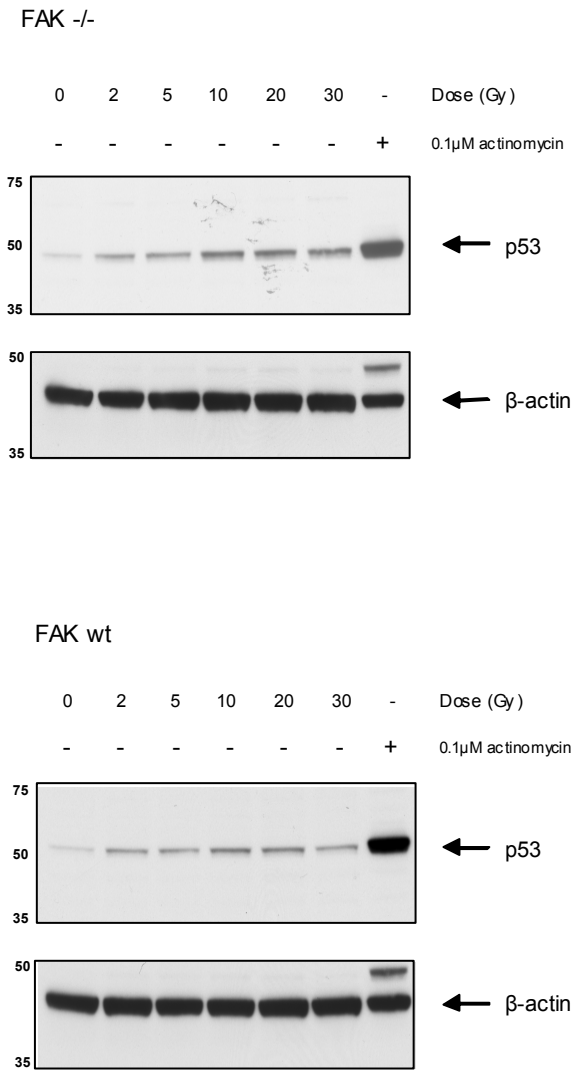


Figure 59 - The level of p53 stabilisation in response to radiation is not FAK dependent
Protein extracts were prepared from FAK -/- and FAK wt cell populations 2 hours after exposure to various doses of radiation (0 – 30Gy). The extracts were separated by SDS-PAGE, transferred to nitrocellulose, and blotted with anti-p53 (upper) and anti-β-actin (lower) antibodies. The last lane in each gel contains protein extracts from FAK -/- or FAK wt cells exposed to overnight treatment with 0.1μM of actinomycin.

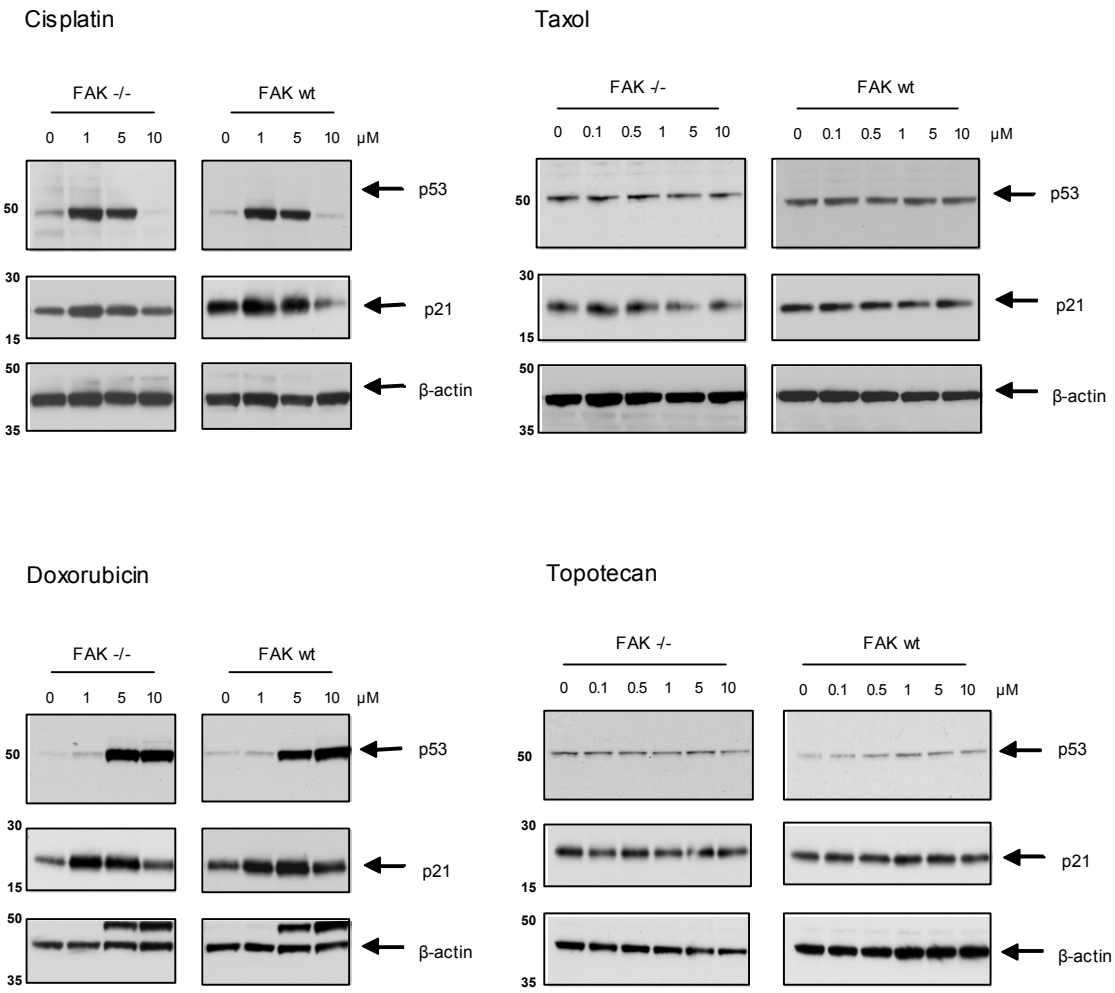


Figure 60 - The level of p53 stabilisation in response to cytotoxic agents is drug and dose dependent rather than FAK dependent

Subconfluent populations of FAK -/- and FAK wt cells were exposed to various concentrations of the cytotoxic drugs indicated for 16 hours, at which point lysates were collected and immunoblotting performed for p53, p21, and β-actin.

6.2.8 ATM/ATR mediated phosphorylation of p53 (serine 15 and serine 20) in response to ionising radiation is not FAK dependent.

The observation that p53 was stabilised in FAK wt cells at both 2 hours and 6 hours post radiation with no corresponding increase in p21 suggested that FAK was somehow suppressing the transcriptional activity of p53 without directly affecting the total protein levels. Before assessing the global transcriptional function of p53, we considered the possibility that FAK may directly or indirectly influence the post translational modification of p53 in response to radiation and hence the conformation of the protein, as opposed to total protein level, which could perhaps have an effect on transcriptional activity. Following ionising radiation, a well described modification of p53 is the phosphorylation of key serine residues. We opted to focus on two of these serine residues, namely serine 15 and serine 20. The ATM and ATR kinases are activated within minutes of radiation and phosphorylate Chk1 and Chk2 kinases respectively, which in turn phosphorylate p53 at serine 20 (Chehab, Malikzay et al. 2000; Shieh, Ahn et al. 2000). In addition, ATM can directly phosphorylate p53 at serine 15 (Canman, Lim et al. 1998).

Accordingly, subconfluent FAK -/- and FAK wt cell populations were irradiated with 5Gy and lysates collected at various time points. Western blotting revealed an increase in phospho-Chk2-S68 at 15 minutes post radiation in addition to an increase in phospho-Chk1-S345 at 30 minutes post radiation in both cell lines. Similarly, increases in phospho-p53-S15 and phospho-p53-S20 were visible at 1 -2 hours post radiation in both cell lines (**Figure 61**). It could therefore be concluded that there was no obvious disparity in ATM/ATR activation of the serine 15 and serine 20 sites which would explain the difference in p53 function.

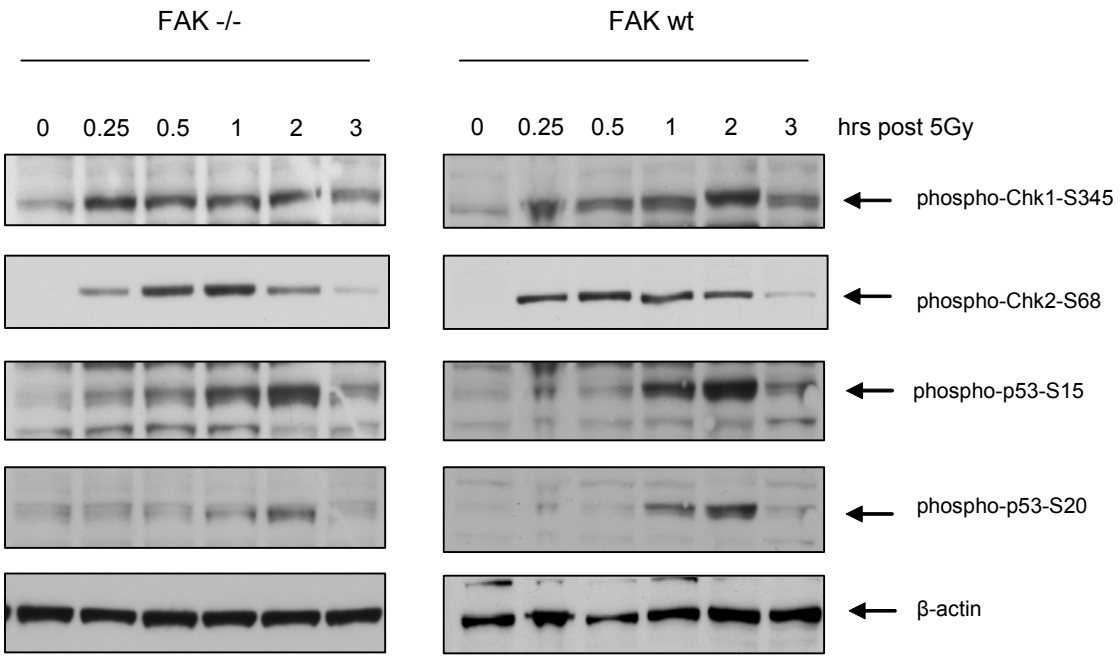


Figure 61 - ATM/ATR mediated phosphorylation of p53 (serine 15 and serine 20) in response to ionising radiation is not FAK dependent
Subconfluent populations of FAK -/- and FAK wt cells were irradiated with 5Gy and protein extracts were prepared at various time points. The extracts were then separated by SDS-PAGE, transferred to nitrocellulose, and blotted with the antibodies indicated.

6.2.9 Induction of the p53 target gene MDM2 is altered in FAK -/- versus FAK wt cells.

The altered p21 transcriptional response pattern following exposure to ionising radiation led us to question whether this difference was restricted to p21 alone or if other p53 target genes were involved. We first examined MDM2 as this protein is known to increase following exposure to radiation. Also, the work by Lim et al. detailed a direct interaction between FAK and MDM2 as well as p53 (Lim, Chen et al. 2008). Since MDM2 can target p53 for ubiquitination, it exerts powerful control over the levels of p53 in the cell. However, in view of the fact that p53 levels did not vary between FAK -/- and FAK wt cells (at least not in the first 12 hours after exposure to ionising radiation), we were not convinced that we would see any significant FAK dependent difference in MDM2 induction.

Interestingly, qRT-PCR analysis showed that mRNA levels increased 4-fold in FAK -/- cells at 2 hours post 5Gy irradiation (**Figure 62A**). This corresponded with elevated protein levels (**Figure 62B**). FAK wt cells also demonstrated an increase in mRNA levels in response to the same dose of radiation, but this increase was less dramatic (2.5-fold as opposed to 4-fold) and occurred later, at 4 hours rather than 2 hours (**Figure 62A**). An elevation in protein level was seen in the FAK wt cells at this later time point (**Figure 62B**). From these results it seemed unlikely that MDM2 was responsible for the discrepancy in p53 mediated p21 induction, but it was interesting to note that the increase in MDM2 at both the mRNA and protein level was lower and delayed in FAK wt cells compared with FAK -/- cells.

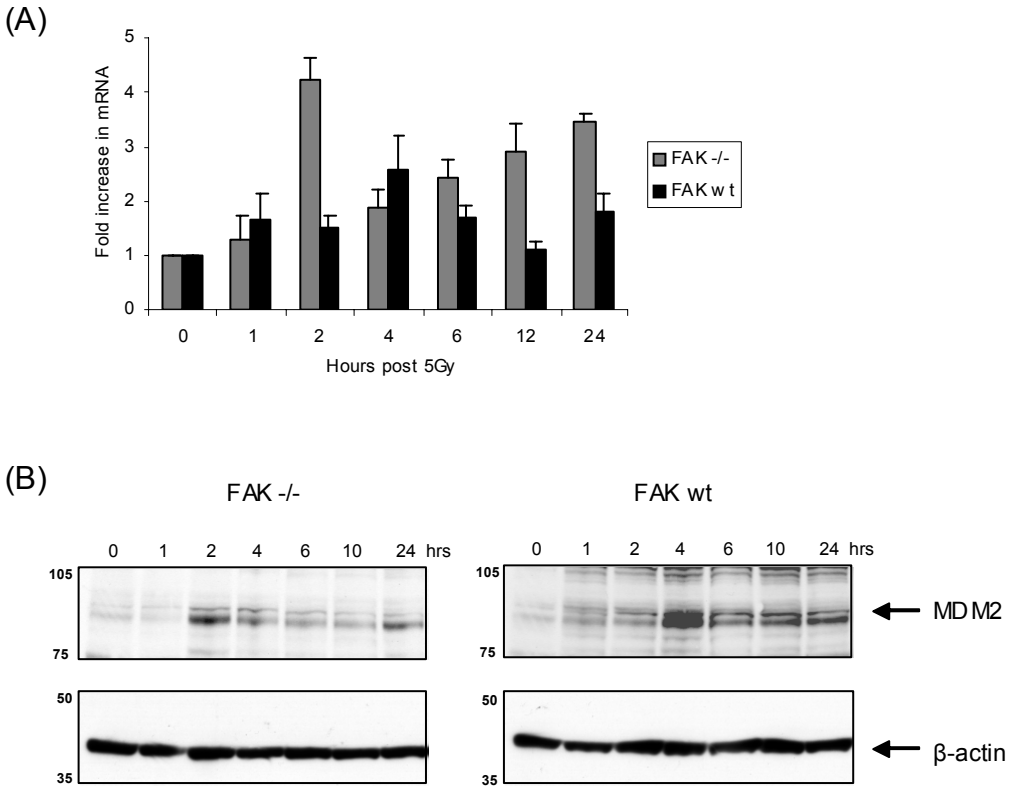


Figure 62 - Induction of MDM2 is altered in FAK -/- versus FAK wt cells
(A) RNA was extracted from subconfluent FAK -/- and FAK wt cell populations at various time points after 5Gy irradiation and converted to cDNA. qRT-PCR analysis was then performed as previously described with 1μM MDM2 primers, using β-actin as a loading control. (B) FAK -/- and FAK wt cells were irradiated with 5Gy at 70 % confluence and lysates prepared at the indicated time points. Immunoblotting was then performed with anti-MDM2 (upper), and anti-β-actin (lower) antibodies.

6.2.10 Induction of p53 target genes involved in DNA repair is altered in FAK -/- versus FAK wt cells.

p53 is traditionally thought to mediate radiosensitivity, but this is paradoxical in view of its role in inducing p21 which is often linked with radioresistance. Therefore, it is likely that in certain situations p53 may provide a cytoprotective role. With this in mind we set out to determine whether p53 induced transcription of genes in FAK -/- cells that may contribute to radioresistance, other than p21. As DNA repair is such an integral part of cellular radiation response, we examined mRNA levels in a number of p53 target genes that are known to be involved in repair following ionising radiation; gadd45, p53R2, Ddb2, and BRCA1. Although the results were not clear cut, a pattern emerged whereby mRNA levels for all but BRCA1 increased to a greater extent in the FAK -/- cells (**Figure 63**). Intriguingly, BRCA1 mRNA levels were moderately but consistently higher in the FAK wt cells.

Despite the fact that the qRT-PCR results appeared to be inconsistent, we were able to show that DNA repair was more efficient in the FAK -/- cells by γH2AX immunofluorescence. Phosphorylation on the serine 139 site of the histone γH2AX occurs in response to ionising radiation (Rogakou, Pilch et al. 1998) and is generally considered to be a reliable surrogate of double strand break repair. **Figure 64A** depicts the percentage of nuclei containing <5 or ≥5 γH2AX foci at 0 – 48 hours after a single dose of 5Gy irradiation. Unsurprising, both FAK -/- and FAK wt populations demonstrated ≥5 γH2AX foci in virtually all cells 1 hour post irradiation. However, the FAK -/- cells had started to clear these foci within 6 hours and reached baseline level within 24 hours, as opposed to the FAK wt cells which appeared to have a slower foci clearance rate. This is suggestive of more efficacious DNA repair abilities in the FAK deficient cells. Representative images of γH2AX immunofluorescence in FAK -/- cells (before and 1 hour after 5Gy irradiation) are illustrated in **Figure 64B**. An interesting observation we made during this experiment is that the FAK -/- cells appeared to have generally higher levels of γH2AX foci under control conditions, with most cells displaying several foci and around 10-15% displaying ≥5 foci. The reasons for this are unclear but hint that these cells are either more genetically unstable than their FAK wt counterparts, or have generally enhanced DNA repair functions.

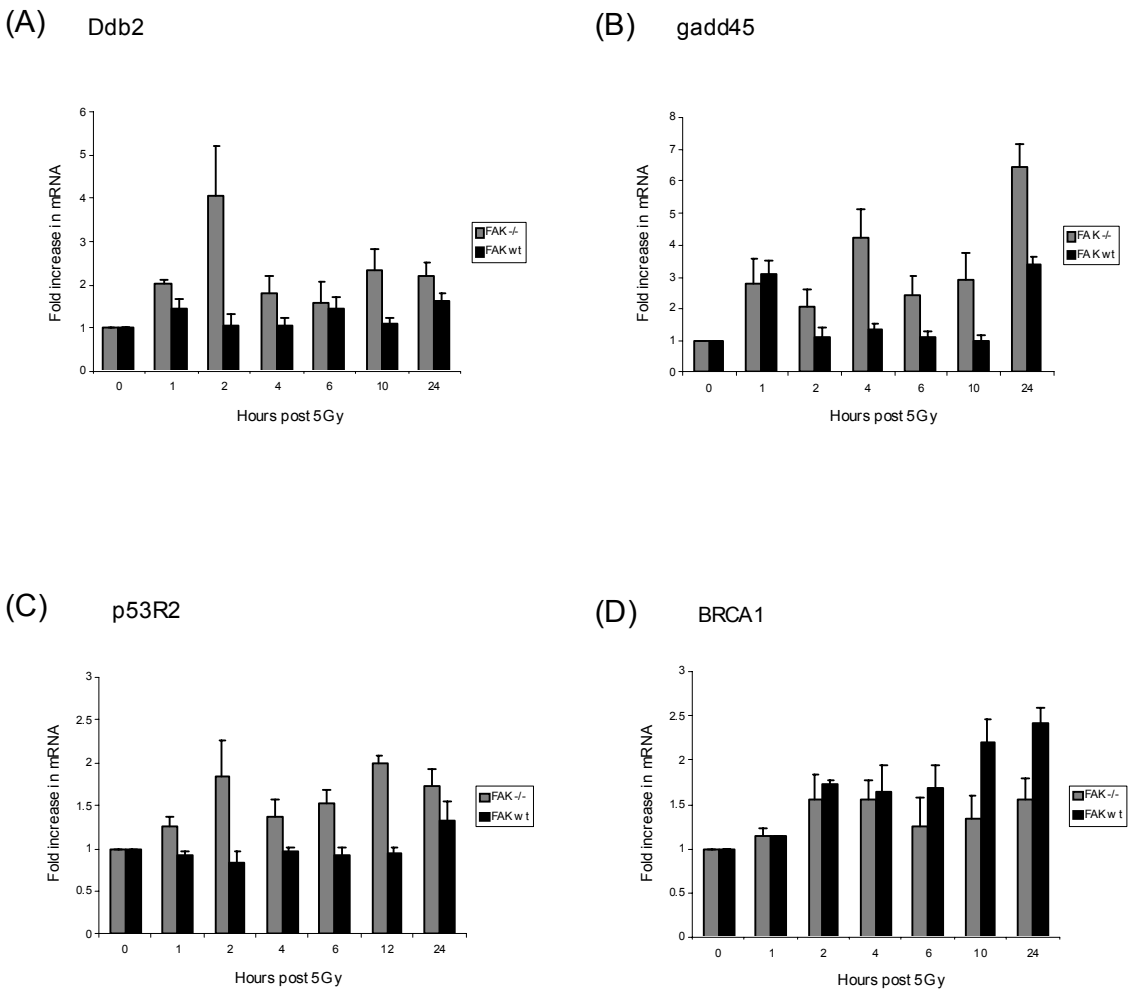


Figure 63 - Induction of p53 target genes involved in DNA repair is altered in FAK -/- versus FAK wt cells

RNA was extracted from subconfluent FAK -/- and FAK wt cell populations at various time points after 5Gy irradiation. qRT-PCR analysis was then performed as previously described using primers directed against Ddb2 (A), gadd45 (B), p53R2 (C), and BRCA1 (D) with β-actin as a loading control.

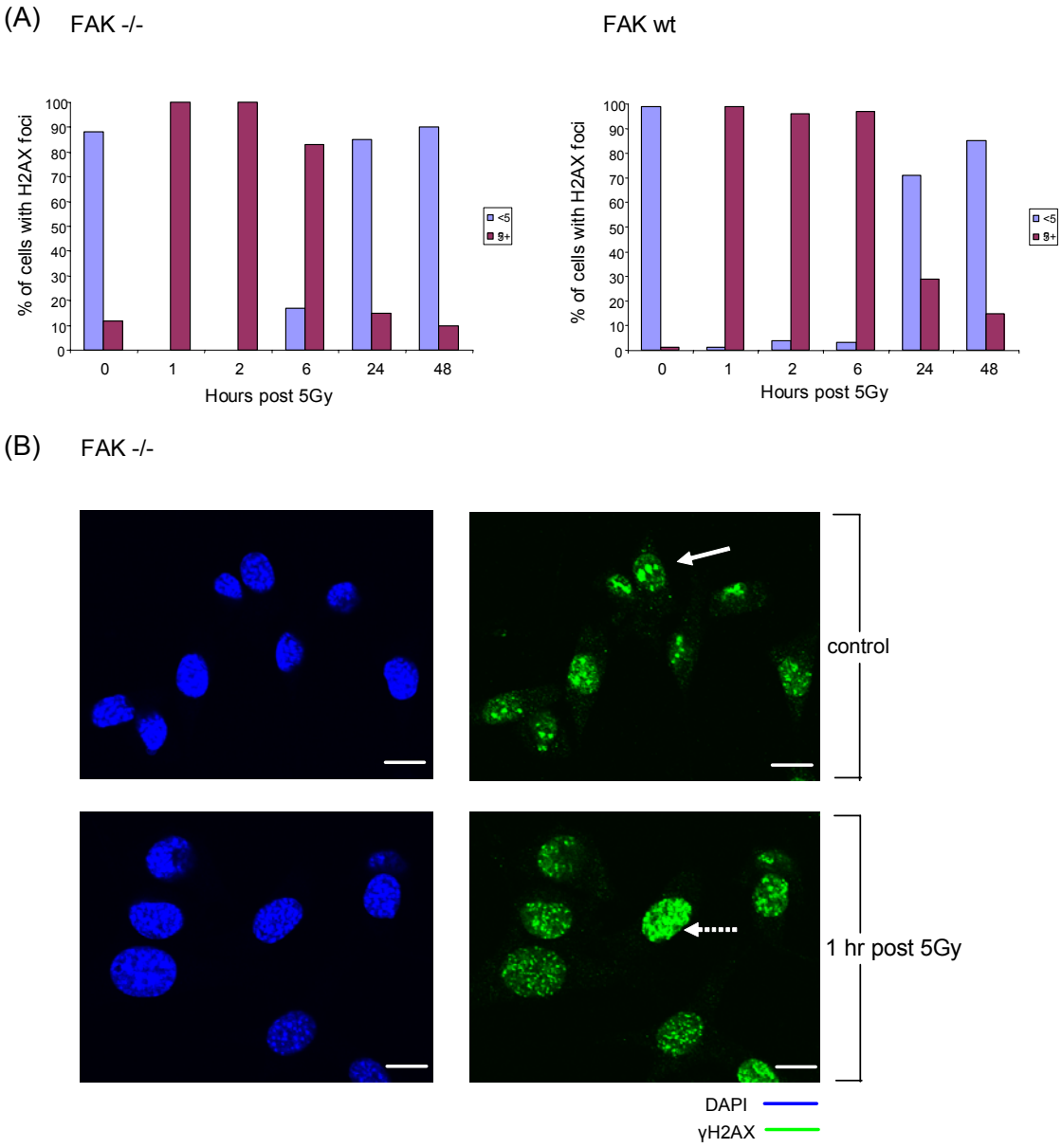


Figure 64 - FAK -/- cells are more efficient at DNA repair
(A) FAK -/- and FAK wt cells were plated at low density on glass coverslips, incubated for 24 hours and irradiated with 5Gy. At various time points, the cells were fixed, permeabilised, stained with anti-phospho-γH2AX (serine 139) antibody and visualised under confocal microscopy. The number of foci per nucleus (<5 foci or ≥5 foci) was documented in at least 100 cells. Results shown are representative of one of two separate experiments. (B) Representative images of unirradiated and irradiated FAK -/- cells at 1 hour post 5Gy are shown, green – phospho-γH2AX and blue – DAPI (scale bar, 20μm), arrow in top right hand box pointing at a nucleus with <5 foci and broken arrow in bottom right hand box pointing at a nucleus with ≥5 foci.

6.2.11 Induction of p53 target genes involved in apoptosis is altered in FAK -/- versus FAK wt cells.

p53 can downregulate as well as promote target gene transcription (Mack, Vartikar et al. 1993). It was therefore of interest to evaluate whether apoptosis was impaired in the FAK -/- cell line, particularly as p53 has long been linked to radiation induced apoptosis. We opted to study Bax and PUMA as both are well known p53 targets and have also been linked to radiation induced apoptosis (Garcia-Barros, Paris et al. 2003; Jeffers, Parganas et al. 2003; Yu, Wang et al. 2003; Rotolo, Maj et al. 2008). As described earlier, RNA was extracted at various time points after 5Gy irradiation and analysed by qRT-PCR. FAK wt cell populations did not demonstrate any increase in mRNA or protein levels for either Bax or PUMA (**Figure 65, A-D**). However, PUMA mRNA levels were dramatically increased in FAK -/- cells at 2 hours, 6 hours, and 24 hours post radiation (**Figure 65C**). Corresponding western blots showed a gradual increase in PUMA-α levels (but not PUMA-ß) over the 24 hours post irradiation (**Figure 65D**). Interestingly, basal PUMA protein levels were higher in FAK -/- cells compared with FAK wt cells. Although Bax mRNA levels did show an approximately 2 fold increase in the FAK -/- cell population (**Figure 65A**), this was not associated with an increase at protein level (**Figure 65B**).

The FAK -/- cell line was more radioresistant so it would appear slightly counter-intuitive that apoptosis would be more marked in this cell line, although it must be noted that radiation induced apoptosis often plays a very small part of radiation induced cell death and does not necessarily reflect cellular radiosensitivity. Intriguingly, when we progressed to formally assess apoptosis by trying to examine the percentage of subG1 cells on FACS analysis at various time points following 5Gy, the levels were very low in both FAK -/- and FAK wt cells. In fact, it was difficult to detect any apoptosis by this method, with typical values of less than 1%. Representative FACS profiles are shown in **Figure 66A**. This data was corroborated by the absence of PARP cleavage on western blotting at various time points after 5Gy irradiation (**Figure 66C**). Increasing the dose of radiation up to a maximum of 30 Gy did not induce any PARP cleavage in either cell line (**Figure 66D**). Of note, 24 hours was selected for analysis as this allowed sufficient time for cells to be released from the temporary G2/M block

which occurs in response to radiation. In addition, PUMA-α protein levels were maximal at this time point in the previous experiment. As a positive control, we induced PARP cleavage with 0.5μM staurosporine (**Figure 66B**), indicating that these cells can undergo marked apoptotic cell death in response to certain triggers. Overall, these findings indicated that ionising radiation did not induce significant cell death by apoptosis in either FAK -/- or FAK wt cells and hinted that PUMA may have an additional or alternative cellular function here.

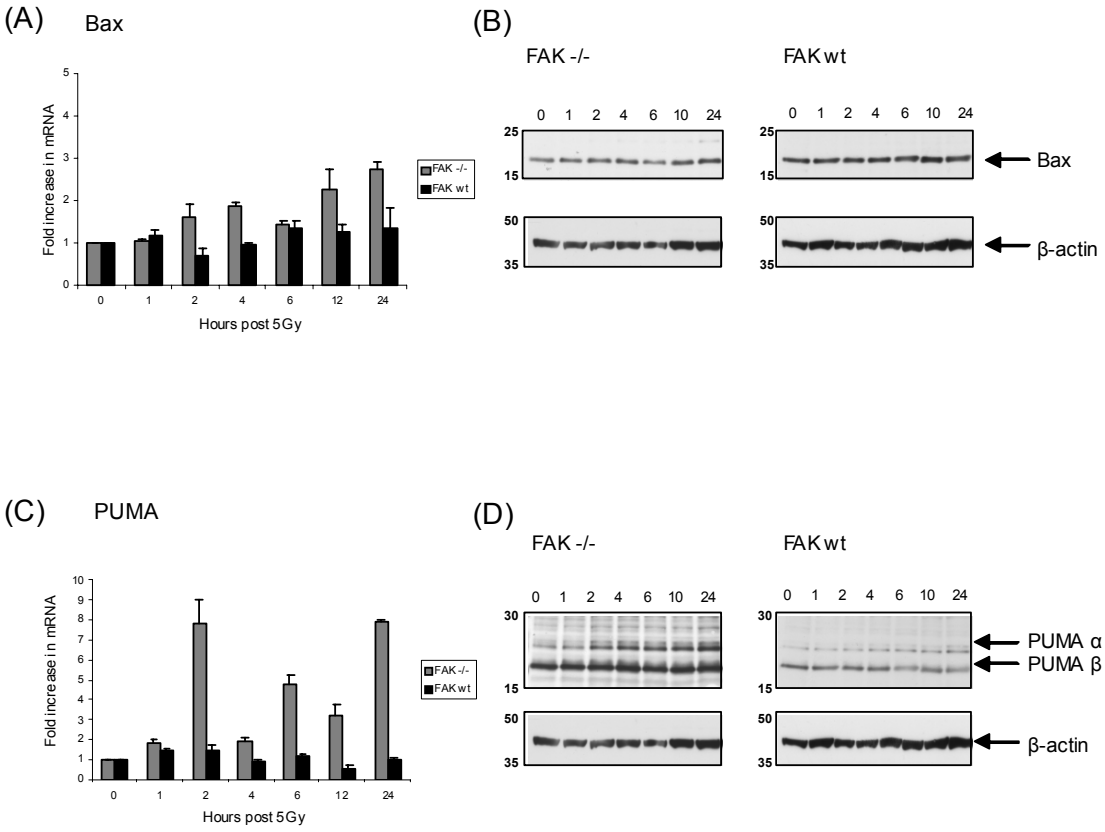


Figure 65 - Induction of p53 target genes involved in apoptosis is altered in FAK -/- versus FAK wt cells

(A) RNA was extracted from subconfluent FAK -/- and FAK wt cell populations at various time points after 5Gy irradiation. qRT-PCR analysis was then performed as previously described using Bax primers with β-actin as a loading control. (B) FAK -/- and FAK wt cells were irradiated with 5Gy at 70% confluence and lysates prepared at the indicated time points. Immunoblotting was then performed with anti-Bax (upper) and anti-β-actin (lower) antibodies. (C) RNA was extracted from subconfluent FAK -/- and FAK wt cell populations at various time points after 5Gy irradiation. qRT-PCR analysis was then performed using PUMA primers with β-actin as a loading control. (D) FAK -/- and FAK wt cells were irradiated with 5Gy at 70% confluence and lysates prepared at the indicated time points. Immunoblotting was then performed with anti-PUMA α/β (upper) and anti-β-actin (lower) antibodies.

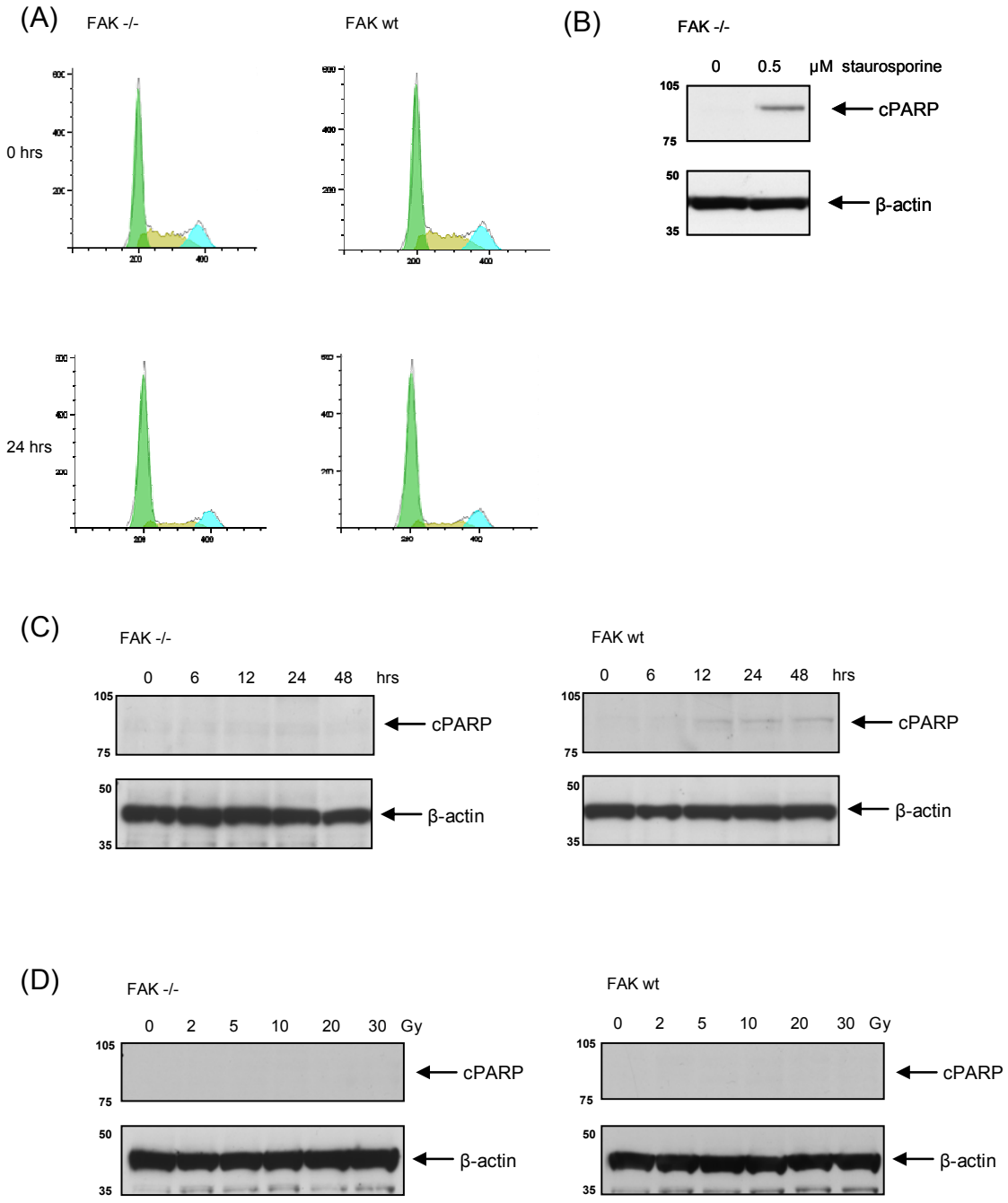


Figure 66 - Ionising radiation does not induce significant apoptosis in FAK -/- or FAK wt cells

(A) $1x10^6$ FAK -/- and FAK wt cells were irradiated with 5Gy at 70% confluence, fixed in 70% ethanol, stained with propidium iodide and cell cycle profiles obtained by FACS analysis. Representative images are shown (FAK -/- on the left and FAK wt on the right) at 0 and 24 hours. (B) Subconfluent populations of FAK -/- and FAK wt cells were irradiated with 5Gy and protein extracts were prepared at 0, 6, 12, 24, and 48 hours. Immunoblotting was then performed with anti –cleaved PARP (upper) and anti-β-actin (lower) antibodies. (C) Subconfluent populations of FAK -/- and FAK wt cells were irradiated with increasing doses of radiation and protein extracts prepared at 24 hours. Immunoblotting was then performed with anti–cleaved PARP (upper) and anti-β-actin (lower) antibodies. (D) FAK -/- and FAK wt cells were treated overnight with 0.5μM staurosporine. Lysates were then prepared and immunoblotting performed with anti–cleaved PARP (upper) and anti-β-actin (lower) antibodies.

6.2.12 FAK does not interact directly with p53 or prevent nuclear localisation and/or accumulation of p53 in response to ionising radiation.

It has been reported that FAK binds to p53 in the cytoplasm before shuttling into the nucleus (Lim, Chen et al. 2008). In certain cancer cell lines with wt p53, particularly breast cancer and neuroblastoma cells, the p53 protein is not fully functional as it is sequestrated in the cytoplasm (Moll, Riou et al. 1992; Moll, LaQuaglia et al. 1995). We therefore wondered whether FAK was binding p53 and preventing its accumulation in the nucleus, which would explain the relative lack of transcriptional activity as nuclear localisation is necessary to achieve full transcriptional functionality. Immunofluorescence studies, however, refuted this. Accumulation of p53 in the nucleus was visible in the majority of cells at 2 hours post 5Gy irradiation in both FAK -/- and FAK populations (**Figure 67**). In addition, we did not find any evidence that FAK translocated to either the cytoplasm or nucleus following irradiation (data not shown).

To confirm that FAK did not directly interact with p53, we proceeded to immunoprecipitation studies. FAK wt lysates were collected 2 hours after 5Gy irradiation or mock irradiation and immunoprecipitated with a FAK antibody. As expected, Src binding was visible on western blotting in the FAK wt mock irradiated and irradiated immunoprecipitates, but there was no interaction with p53 (**Figure 68A**). As a negative control, irradiated FAK wt lysate was immunoprecipitated with a histidine antibody. Paired lysates were probed for FAK, Src and p53 to ensure equal loading (**Figure 68B**).

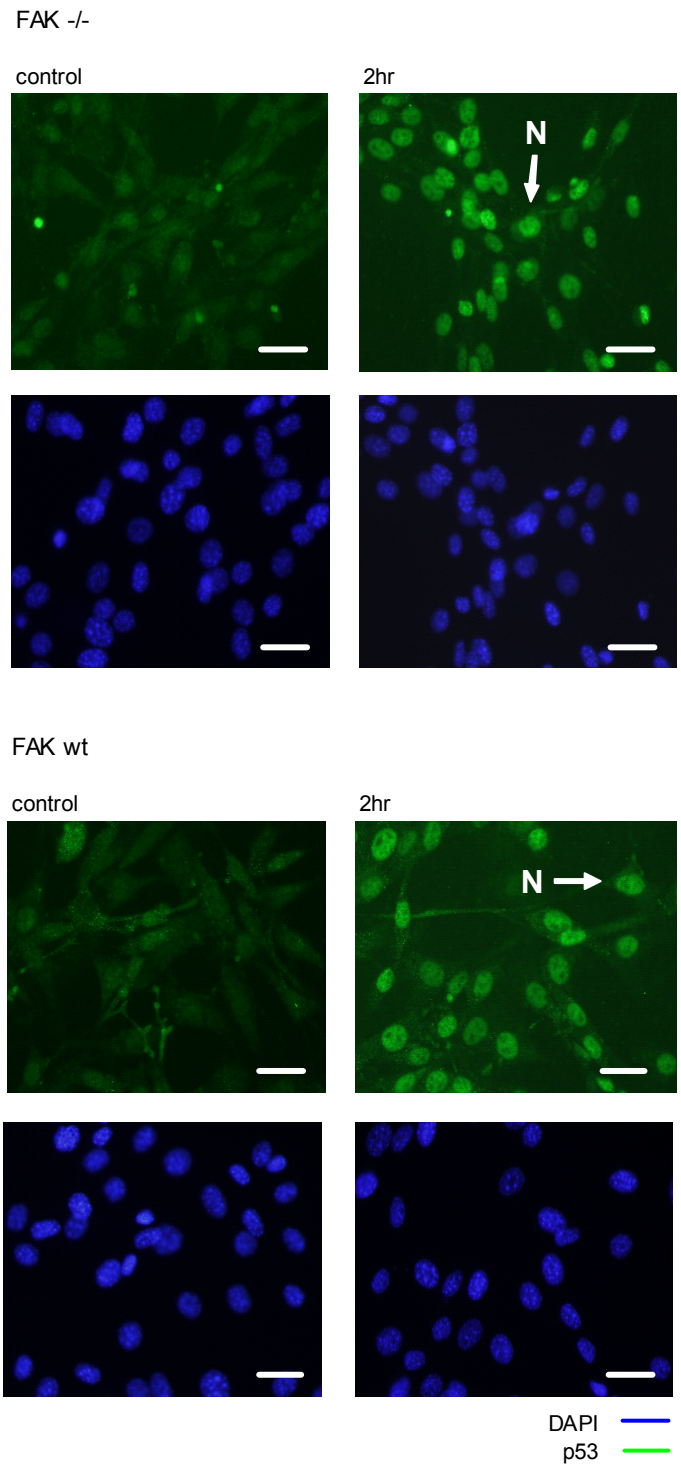


Figure 67 - FAK does not prevent nuclear accumulation of p53 in response to ionising radiation
FAK -/- and FAK wt cells were plated at low density on glass coverslips and subjected to 5Gy irradiation after 24 hour incubation. At 0 and 2 hours the cells were fixed, permeabilised, stained with anti-p53 antibody, and visualised under fluorescent confocal microscopy. Representative images from 1 of 3 experiments are shown, green – p53 and blue – DAPI, arrows represent nuclear accumulation of p53 (N) (scale bar, 50μm).

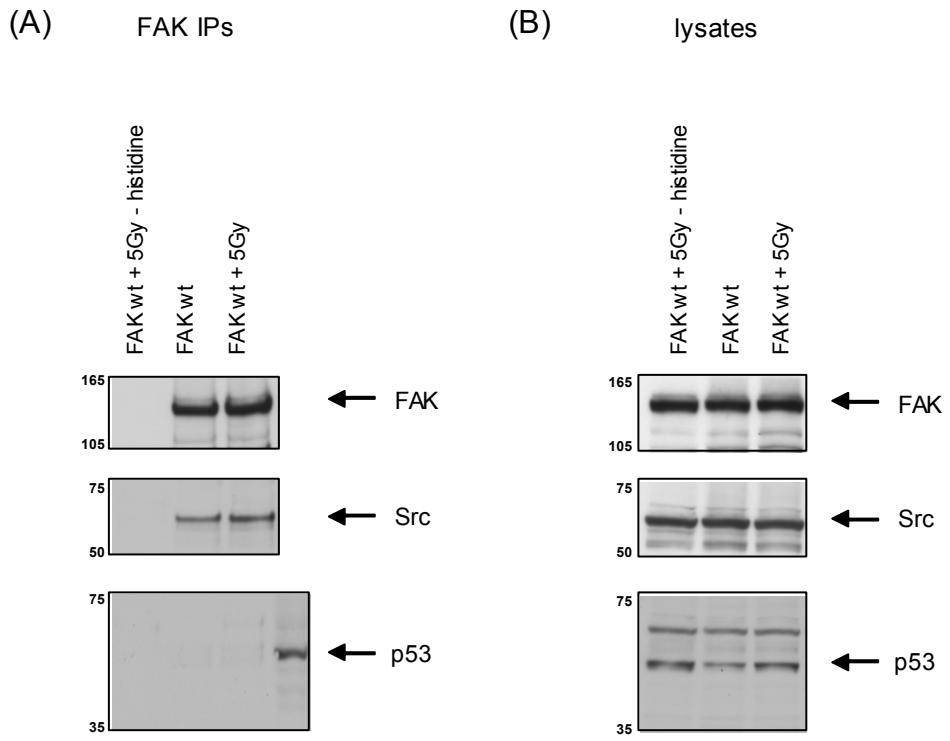


Figure 68 - FAK and p53 do not directly interact in response to ionising radiation
(A) FAK wt cells were irradiated at 70 – 80% confluency and lysates prepared at 0 and 2 hours. 1mg of protein was immunoprecipated with an anti-FAK agarose conjugated antibody at 4^{o}C overnight. The IPs were separated by SDS-PAGE and immunoblotted with anti-FAK, anti-Src, and anti-p53 antibodies. As a negative control, irradiated FAK wt cell lysates were also immunoprecipitated with an anti-histidine agarose conjugated antibody. (B) 20µg of protein lysate was separately immunoblotted with anti-FAK, anti-Src, anti-p53, and anti-β-actin antibodies.

6.2.13 FAK kinase inhibition with a small molecule inhibitor induces p21 protein levels in response to ionising radiation.

We have acquired evidence that the presence or absence of FAK influenced the function of p53 in this cell system. In addition, our data suggested that the FAK/p53 interaction was indirect. It is well recognised that FAK has kinase-dependent as well as kinase-independent functions. To assess whether the kinase domain was integral to this phenomenon, we treated FAK wt cells with a FAK kinase inhibitor (PF-562,271) at a range of doses (0, 0.1μM, 0.2μM, and 0.5μM) for 2 hours prior to irradiation. Lysates were collected for immunoblotting analysis at 0, 2, 4, and 6 hours after 5Gy. There was no difference in the level of p53 induction at any of the applied concentrations but p21 levels were visibly increased 2 hours after radiation in 0.5μM PF-562,271 treated cells. **Figure 69** demonstrates p53 and p21 levels in response to 5Gy irradiation in vehicle treated FAK wt cells (**left**) and 0.5μM PF-562,271 treated FAK wt cells (**right**).

These results were surprising as the current literature indicates that the FAK-p53 functional interaction is mediated by the scaffolding function of FAK and is not kinase–dependent. In order to confirm our findings, we plan to evaluate the ability of FAK kinase defective mutants to affect the function of p53 by using p21 induction in response to radiation as readout of p53 activity. We also plan to evaluate the radiosensitivity of these mutants and, in addition, incorporate the FAK kinase inhibitor into clonogenic assays with FAK wt cells and assess whether this reproduces the radiation survival curve of FAK -/- cells.

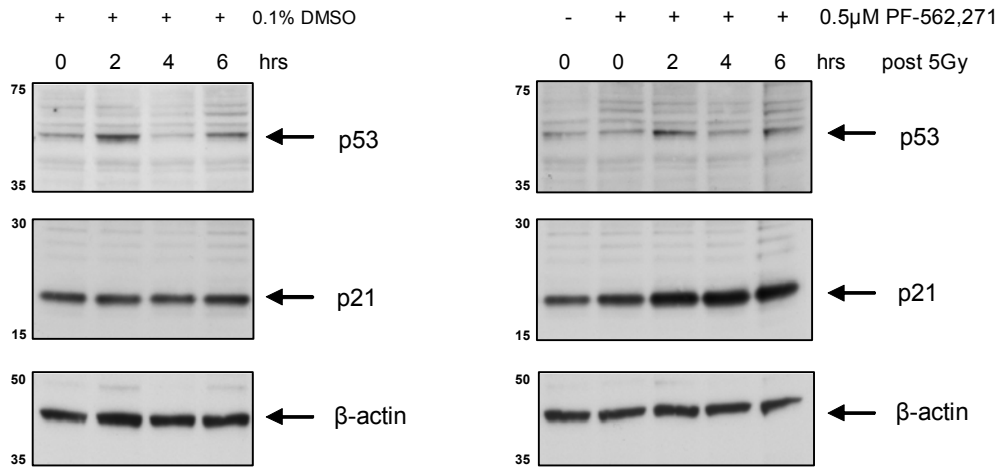


Figure 69 - p21 is induced in FAK wt cells in response to ionising radiation in the presence of a FAK kinase inhibitor

FAK wt cell populations at 70% confluence were incubated for 2 hours with either PF-562,271 (0.1μM, 0.2μM, and 0.5μM) or 0.1% DMSO then irradiated with 5Gy. Protein extracts were prepared at 0, 2, 4, and 6 hours and immunoblotting performed with anti-p53, anti-p21, and anti-β-actin antibodies. Representative immunoblots from 0.1% DMSO (left) and 0.5M drug treated (right) cell populations are shown.

6.3 Discussion

The evidence to date suggests that knockdown of FAK protein levels increases radiosensitivity (Cordes, Frick et al. 2007). Although the authors were unable to pinpoint a clear mechanism, there are several theoretical reasons that could potentially explain this phenomenon. These include the effects of FAK on cell cycle, cell survival, and adhesion signalling. However, results in our cell system clearly show that the absence of FAK has a detrimental effect on radiosensitivity. An explanation for this discrepancy might be that this phenomenon is entirely cell line dependent. The cell lines examined by Cordes et al originated from *human pancreatic adenocarcinomas*, whereas the cell lines of interest in this case were derived from a *murine squamous cell carcinoma* of the skin. Aside from the possibility that there may be crucial disparities in the function of FAK in humans versus mice, it is likely that FAK has different properties in cancer cells depending on both the tissue of origin and cell type of origin. It is also likely that FAK has different properties depending on the underlying molecular aberrations present within the cell. Notably, the pancreatic cancer cell lines outlined above have mutant p53 whereas the cell lines under evaluation here have wt p53. The potential relevance of p53 will be discussed in detail later.

A key issue that needs to be addressed, however, is that the complete absence of FAK may be a very different biological entity compared to reduced levels of FAK. It is possible that genetic deletion generates a particularly resistant phenotype which may not be fully recapitulated by simply lowering levels of FAK in the cell. It would therefore be crucial to repeat the radiosensitivity experiments in FAK +/+ cells using siRNA to knockdown the levels of FAK and compare with a paired FAK -/- population. We are currently developing paired FAK +/+ and FAK -/- cell lines from the isolated single cell clones and aim to explore this avenue of research.

Regardless of whether this is a cell line dependent phenomenon, we have shown that the presence or absence FAK does influence radiation survival in this cell system; specifically FAK deletion promotes increased radioresistance. Furthermore, we determined that radioresistance is mediated, or at least partly

mediated by p21. Interestingly, this could not be explained by a difference in cell cycle kinetics. Since the radiosensitivity pattern of a cell line is governed by many factors, we acknowledged that it was possible other elements were at play here. As p21 is driven by p53 in this system, and p53 is deemed to be a critical mediator of radiation survival, it seemed reasonable to consider the possibility that p53 could be influencing radiation survival via both p21 dependent and p21 independent mechanisms. One of the areas we focussed on was the transcriptional control of p53 target genes involved in DNA repair as the ability of a cell to repair damage can greatly influence radiosensitivity, as evidenced by various genetic syndromes such as ataxia telangectasia and Nimegen breakage syndrome. Although there was a general trend, specifically FAK -/- cells demonstrated higher levels of transcript for 3 out of 4 genes tested, these genes are primarily involved in single strand break repair. While single strand breaks undoubtedly do occur after exposure to ionising radiation, it is generally accepted that the ability to repair double strand breaks is crucial to cell survival after such an insult. Careful review of the literature reveals an abundance of data linking the transcriptional function of p53 to single strand break repair but the evidence connecting the transcriptional activity of p53 to double strand repair is rather limited (reviewed by (Gatz and Wiesmuller 2006)). Interestingly, BRCA1, which is involved in double strand repair (Scully, Chen et al. 1997; Moynahan, Chiu et al. 1999), was transcribed to a slightly higher level in the FAK wt population. However, it would be important to examine whether this corresponds to an increase in protein level. Also, BRCA1 is not necessarily a straightforward p53 target gene and, conversely, BRCA1 has been shown to influence p53 stabilisation (MacLachlan, Takimoto et al. 2002). Nevertheless, this may represent a genuine increase and BRCA1 may be a critical mediator of DNA repair in FAK wt cells.

Although we were unable to definitively link p53 transcriptional activity to double stranded DNA repair in FAK -/- cells, it was clear from observing the rate of γ-H2AX foci that this process is more efficient in FAK -/- cells compared with FAK wt cells. Whether this is mediated by p21 which probably has a role in DNA repair (reviewed by (Abbas and Dutta 2009)), or through p53 target genes that we did not examine, or even unrelated to p53 is unknown. However, there are several possibilities that ought to be considered. Firstly, p53 has recently been

shown to play a direct role in cellular metabolism. Through the induction of target genes such as TIGAR, p53 can alter the way in which a cell utilises glucose (Bensaad, Tsuruta et al. 2006). This is important because if cells are diverted into a pentose phosphate shunt, this may encourage the synthesis of nucleotides and other products that might be important for the repair of DNA lesions. Incidentally, a further by-product of this pathway is an increase in glutathione levels which can promote scavenging of reactive oxygen species (and theoretically lessen the effects of ionising radiation). It may be significant that p53R2, one of the target genes we examined, appears to be involved in regulating nucleotide pools within damaged cells (Tanaka, Arakawa et al. 2000). We have carried out some preliminary qRT-PCR studies with Gpx-1, a p53 target gene that is known to be involved in metabolism, and we found that this was upregulated only in FAK -/- cells (data not shown). While Gpx-1 has not been formally linked with resistance to ionising radiation, overexpression of the protein has been associated with resistance to UV irradiation (Baliga, Wang et al. 2007). Secondly, mounting evidence links non-transcriptional activities of p53 to double strand DNA repair. Essentially, p53 can bind directly to damaged DNA and institute repair mechanisms directly (Ko and Prives 1996; Levine 1997; Albrechtsen, Dornreiter et al. 1999). The role of FAK (if any) in inhibiting this process is open to debate.

Similarly, p53 transcription-dependent and transcription-independent mechanisms have been described in apoptosis (Moll, Wolff et al. 2005). One key difference is that the transcription-independent function of p53 in this case is related to its localisation in the cytoplasm and / or mitochondria as opposed to the nucleus (Mihara and Moll 2003; Chipuk, Kuwana et al. 2004; Leu, Dumont et al. 2004). Radiation can induce significant p53 dependent apoptosis *in vitro*, for example, in cells of haemopoetic origin (Lorimore, Goodhead et al. 1995) and *in vivo* in tissues such as spleen, gut and thymus (Clarke, Purdie et al. 1993; Lowe, Schmitt et al. 1993; Clarke, Gledhill et al. 1994; Komarova, Christov et al. 2000), but this is not necessarily the case in cancer cell lines. Indeed, we found that trying to quantify apoptosis was not particularly valuable in this system as the level of apoptotic cell death induced by ionising radiation was exceedingly low. Studying the induction of Bax and PUMA, however, again highlighted the significant difference in the control of p53 target genes in FAK -/- versus FAK wt

cells. Surprisingly, although PUMA-α protein levels increased in the FAK -/- cell line, this did not result in any detectable increase in apoptosis. While PUMA has been undisputedly linked to p53 dependent apoptosis, including specifically radiation induced apoptosis (Jeffers, Parganas et al. 2003; Yu, Wang et al. 2003), most research studies do not specify which of the four isoforms (α, ß, γ, δ) is under investigation so it is difficult to delineate the specific functions of each protein. There is now some preliminary evidence which suggests that PUMA-α is both p53 dependent (Jiang, Wei et al. 2006; Sole, Hernandez-Guillamon et al. 2008) and required for apoptotic response (Jiang, Wei et al. 2006; Sole, Hernandez-Guillamon et al. 2008), but whether this is cell line dependent has yet to established. Another critical point is that PUMA induced apoptosis requires co-operation between nuclear and cytoplasmic proteins (Chipuk, Bouchier-Hayes et al. 2005) and for some reason this interaction may be defective in FAK -/- cells. Of course, it may simply be the case that PUMA has another function here.

Taken together, the results up to this point highlight several important issues. Firstly, p53 appears to be mediating radioresistance through p21 dependent (and possibly p21 independent) mechanisms. Secondly, we believe that the loss of FAK promotes the pro-survival function of p53 in response to ionising radiation in these cells. Admittedly, a critical experiment would be to perform clonogenicity assays in the context of p53 knockdown but we did not have sufficient time. However, we were able to demonstrate that p21 knockdown reduces radioresistance in FAK -/- cells, and that p21 is in turn driven by p53 transcription, in these same cells. This combined data gives credence to our above proposal that FAK mediated effects on p53 are responsible for the variations in radiosensitivity that we see in FAK -/- compared with FAK wt cells. As discussed in Chapter 5, wt p53 is typically associated with radiosensitivity rather than radioresistance but this is by no means definitive and there is data which challenges this view. One theory which would support a role for functional wt p53 in mediating radioresistance is that low levels of p53 induction tend to activate genes associated with cell cycle whereas high levels of p53 typically lead to apoptosis (reviewed by (Chen, Ko et al. 1996; Weinberg, Veprintsev et al. 2005)). This may explain the relative radioresistance of certain glioblastoma cell lines, some of which show a selective impairment of the apoptotic functions

of wt p53, while retaining the potential to mediate p53 responses relevant for DNA repair and control of cell cycle (Shu, Kim et al. 1998). Obviously, this would depend on how "stressful" ionising radiation is to the cell. Certainly in our cell system radiation did not effect dramatic levels of p53 stabilisation, as opposed to certain chemotherapeutic agents, and perhaps this is not surprising as ionising radiation is a transient insult unlike the sustained high level stress imposed by prolonged exposure to cytotoxics. To counter this, the precise role of cell cycle arrest and radiosensitivity has yet to be clarified and p53 is not solely responsible for checkpoint activation in response to radiation. Indeed, we were unable to illustrate any difference in cell cycle between FAK -/- and FAK wt cells. Further evidence against this hypothesis stems from *in vivo* work where researchers have been able to show that p53 target gene induction is tissue dependent and does not critically hinge on the level of p53 (MacCallum, Hupp et al. 1996; Bouvard, Zaitchouk et al. 2000; Komarova, Christov et al. 2000; Fei, Bernhard et al. 2002).

As increasing information is obtained pertaining to the activation and function of p53 in response to stress, it is becoming clear that this process is highly complex and dependent on many factors including cell type, cell environment, and both the nature and duration of stress (reviewed by (Vousden and Lu 2002; Murray-Zmijewski, Slee et al. 2008; Vousden and Prives 2009)). For that reason, it is difficult to make generalisations on the role of p53 in radiosensitivity but it seems that maintenance, or at least partial maintenance, of the pro-survival functions of wt p53 might be an important factor contributing to the survival of certain cancer cells in response to ionising radiation and /or other cellular stress. Whether this is an acquired property during the process of cancer cell evolution or a manifestation of the predetermined developmental pattern of a specific tissue or distinct cell types within a tissue has yet to be determined. A greater understanding of this phenomenon is vital, especially as there is currently interest in augmenting or restoring the function of wt p53; in certain situations this may prove to be detrimental.

In light of recent publications, it was important to characterise the relationship between FAK and p53 in these cells, and in particular determine whether this relationship was direct or indirect and kinase-dependent or independent. As nuclear localisation of p53 is necessary for full transcriptional activity, we

anticipated that FAK may directly (or indirectly) prevent nuclear accumulation of p53 in response to ionising radiation, but this was not the case. Further to this, we have shown, in contrast to a recent study (Lim, Chen et al. 2008), that FAK does not translocate to either the cytoplasm or nucleus and it does not directly bind to p53. We do not dispute the mounting evidence that a p53 binding site exists on FAK (Golubovskaya, Kaur et al. 2004; Golubovskaya, Finch et al. 2005; Golubovskaya and Cance 2007) or vice versa (Golubovskaya, Finch et al. 2008), and, as there is accumulating data to suggest that p53 exists in various pools within the cell, it would not be inconceivable for binding between p53 and FAK to occur. Nonetheless, in this particular cell line, any FAK/p53 functional interaction in this context is indirect. We accept that this may be stress dependent and it would be interesting to see if FAK does directly cooperate with p53 under different cellular stresses.

Although the presence or absence of FAK does not influence either basal levels of p53 or stabilisation of p53, at the total protein level, in response to radiation in these cells (with the exception of the 24 hour time point), we did wonder whether FAK indirectly influences the phosphorylation status of p53, particularly as various post translational modifications can affect the transcriptional activity of p53. There are many residues on p53 which are phosphorylated in response to DNA damage but it proved difficult to obtain sufficiently robust phospho-specific antibodies directed at mouse p53. We were able to examine the serine 15 and serine 20 sites, however, which constitute two of the most significant phosphorylation sites on p53 following DNA damage, specifically ionising radiation. Significantly, there was no difference in phosphorylation levels between FAK -/- and FAK wt cells at these sites in response to irradiation. p53 is further regulated at the post translational level by multiple additional mechanisms, for example, acetylation, sumolyation, neddylation, methylation, and ubiquitination. It is difficult on the basis of current evidence to link FAK to any of these mechanisms, with the exception of ubiquitination. FAK has been shown to form a scaffold with p53 and Mdm2 and promote Mdm2 mediated degradation of p53 (Lim, Chen et al. 2008). We did not formally assess this but the slower rate of Mdm2 increase after radiation in FAK wt cells, coupled with relatively similar levels of p53 mRNA and protein in both cell lines would perhaps argue against increased degradation.

As FAK does not appear to alter the localisation or level of p53, there must be another explanation for the differential transcription pattern of p53 target genes in response to ionising radiation. We outlined earlier that the selective functioning of p53 in response to stress is dependent on cell line factors as well as the type of stress. However, it is becoming increasingly realised that the function of p53 is also dependent on cofactors, proteins that interact with p53 and influence both its transcription-dependent and transcription-independent activities. While some cooperate directly with p53 to influence target gene activity by binding to the DNA-binding domain, such as the evolutionary conserved ASPP family (reviewed by (Sullivan and Lu 2007)), more recently other modifiers have been identified which indirectly affect p53 function by regulating the proteins that are directly involved in its activation and repression, for example p300/CBP (reviewed by (Lee and Mapp 2010)). Although recent work suggests that there is a direct interaction between FAK and p53 (Lim, Chen et al. 2008), an indirect mechanism has previously been described. FAK is known to associate with the FAK-interacting protein (FIP200) which can bind p53 and enhance its half-life (Abbi, Ueda et al. 2002). We have yet to examine whether FIP200 is significant here but, as this protein tends to alter the levels of p53 as opposed to selective functioning, this may not be a particularly productive line of investigation. The possible link between FAK and p53 cofactors involved in selective functioning, however, opens up a novel area of research. As a signalling molecule, FAK would be well placed to exert an effect over many cellular proteins, some of which may interact with p53. We suspect that the kinase function of FAK is integral to this phenomenon, but accept that we only have limited evidence in support of this at present. Notably, Src signalling has been linked both directly to p53 (Wang, Zheng et al. 2009) and indirectly via the ASPP family (personal communication – Karen Vousden). While this information may strengthen our hypothesis that adhesion signalling can affect the function of p53, we then have to question whether our findings are Src dependent rather than FAK dependent. In fact, we intend to investigate the significance of Src in our system.

While there is no direct evidence in the literature which connects the kinase activity of FAK to p53, this has not yet been examined by other research groups in the context of ionising radiation. It has already been shown that FAK activity

can be upregulated by radiation (Beinke, Van. Beuningen et al. 2003). While this may represent indirect activation (e.g. via EGFR), it is possible that FAK may be directly activated by radiation through the production of highly reactive oxygen species by radiation. Certainly, FAK tyrosine phosphorylation can be induced by reactive oxygen species generated by alternative means such as hydrogen peroxide (Sonoda, Watanabe et al. 1999; Meriem, ValÉrie et al. 2000). As previously discussed, the effect of ionising radiation on the cell is quite different from that of other stresses such as cytotoxic chemotherapy. Thus, the type of damage induced by radiation may preferentially activate certain kinase functions of FAK. Just as the effects of p53 on the cell are stress dependent, it may well be the case that the effects of FAK are also stress dependent. Based on our preliminary data and the published results to date, the precise nature of the FAK – p53 functional interaction may vary not only from cell line to cell line, but may be direct or indirect and kinase–related or unrelated depending on the nature and severity of stress.

Much effort was spent trying to establish exactly how any FAK–p53 functional interaction operates in these cells and further clarification is still required. One probing question that persisted throughout the course of this work, however, was why should the presence of FAK in the cell dampen down the pro-survival function of p53? Although FAK does not strictly function as an oncogene, much of the evidence in the literature points toward upregulation of FAK levels and/or activity in association with the cancer phenotype. Further, FAK has been directly linked to carcinogenesis in the mouse skin cancer model (McLean, Brown et al. 2001; McLean, Komiyama et al. 2004) and, more recently, other carcinogenesis models such as breast cancer (Provenzano, Inman et al. 2008; Luo, Fan et al. 2009). However, it is important to note that high levels of FAK expression and activity need not always be associated with be a more aggressive phenotype. Reduced FAK activity, for example, has also been shown to promote motility, invasion and metastases in the *in vitro* and *in vivo* settings (Lu, Jiang et al. 2001; Yano, Mazaki et al. 2004). This suggests that FAK may have tumour suppressor as well as tumour promoter properties. While the evidence for the former is rather limited at present, intriguing data is emerging from conditional FAK knockout experiments in the mouse hepatobiliary system. Loss of FAK appears to accelerate carcinogenesis in the pancreas and liver on a background of

constitutive K-Ras activation (personal communication – Owen Sansom). It is therefore not unreasonable to speculate that FAK may possess a tumour suppressor type function in our cell system. This function may have evolved in order to keep the pro-survival activity of p53 in check. Why this property of FAK should emerge specifically in response to ionising radiation is rather puzzling and requires further investigation.

One of our initial aims was to evaluate the role of FAK in radiosensitivity *in vivo*. As the effects of radiation can be more or less pronounced *in vivo* compared with *in vitro*, we wanted to establish whether any of the effects attributed to FAK would translate in a more clinically relevant setting. This is especially important as the functions of FAK appear to be very context dependent, as exemplified by the requirement of FAK for growth in an anchorage-independent environment while it is redundant for growth on cell culture plastic. Setting up this experiment was incredibly technically challenging due to various underlying biological factors. The cell lines were very radioresistant and we were limited by the dose of radiation we could use as whole body irradiation is poorly tolerated. Doses in excess of 5Gy in nude mice result in fatal haematological and gastrointestinal toxicity. Also, the xenografts became necrotic at around 1cm in diameter and did not reach the maximum diameter of 1.7cm permitted by the Home Office.

Despite these limitations, we were able to show the absence of FAK was associated with reduced tumour growth inhibition in response to radiation. Furthermore, the pattern of p21 induction suggested that similar mechanisms were invoked *in vitro* and *in vivo*. We intend to do further studies to assess whether any additional mechanisms contribute to radioresistance *in vivo*. One area of interest is the potential difference in vascularity between FAK -/- and FAK wt xenografts. A defective vascular system could create a hypoxic environment which may have important implications on radiation survival.

It could be argued that studying the response to radiation in xenografts is still rather artificial. For this reason, we did consider irradiating K14*Cre*ER^{T2}FAK$^{flox/flox}$ mice bearing tumours but this was precluded by the long latency period necessary for topical carcinogenesis. In retrospect, this may have been problematic as, based on our experience with both the SCC 1.1 and SCC 7.1

xenografts, the application of 4-OHT is unlikely to have induced sufficient FAK deletion in carcinomas. Even if 4-OHT treatment had been successful, the results would have proved difficult to interpret in view of heterogeneity between tumours and even within the different cellular components of the same tumour.

Although we have yet to ascertain whether or not genetic deletion of FAK would result in similar effects in response to radiation in another cancer cell line, we strongly suspect that the underlying heterogeneous nature of cancer cells and the multiple roles of FAK within the cell suggest that the outcome is likely to be tissue and / or cell line specific. The interplay between FAK and p53 and how this axis is programmed to respond to stress is likely to be a critical factor, at least in cells with wt p53. Attempting to predict the effect of FAK on radiosensitivity in the context of a p53 null or mutant p53 background is more difficult, not least because it is unclear exactly how mutant p53 proteins can influence radiation survival. Also, the relationship between FAK and mutant p53 is even less well described. Whether FAK can directly or indirectly impinge on the transcription-dependent or transcription-independent functions of mutant p53 proteins and whether this in turn depends on the specific mutation or conformation of p53 is largely unexplored.

Interestingly, there is some preliminary evidence to suggest that cancer cells with mutant p53 have higher levels of FAK due to uncontrolled transcription (Golubovskaya, Finch et al. 2008). The precise function of FAK in such cells is unclear but if high levels of FAK are contributing to cell survival then FAK knockdown under these circumstances could be beneficial. It is noteworthy that Liu et al. found that treatment with the FAK kinase inhibitor TAE226 induced apoptosis in mutant p53 containing cells and not in wt p53 containing cells (Liu, LaFortune et al. 2007), although it must be pointed out that this drug also inhibits the growth factor receptor IGF-1R and it cannot be assumed that its effects are exclusively FAK mediated. It is also difficult to dissect whether any of the possible FAK mediated effects on survival are related to p53 or not. Unfortunately the Cordes group were unable to pinpoint the exact mechanism responsible for increased radiosensitivity following FAK knockdown in the pancreatic cancer cell lines they evaluated. Notably, the combination of FAK siRNA and radiation induced slightly different effects on cell cycle dynamics and cell signalling / cell survival proteins such as Akt in each cell line, again

demonstrating the range of tumour cell heterogeneity. Indeed, it may well be relevant that each cell line possesses a different p53 mutation.

6.4 Summary

This work has demonstrated that loss of FAK has a detrimental effect on radiation survival in this cell line. We have shown that this effect is mediated by transcriptional regulation of p21, which is driven by p53, and we believe that wt p53 is acting as a radioprotectant in this system. Why FAK should repress the pro-survival function of p53 is unclear, but this data indicates that inhibition of FAK may not always be advantageous in the clinical setting and contributes to a small body of literature highlighting a close interaction between FAK and p53.

Chapter 7

Concluding Remarks and Future Perspectives

7 Concluding Remarks and Future Perspectives

7.1 Concluding Remarks

Radiotherapy remains one of the principal anti-cancer modalities and is the treatment of choice in a variety of solid epithelial tumours. For many years, it was accepted that radiotherapy exerted its effect by indirect DNA damage secondary to free radical formation and the field of radiation biology remained relatively quiescent. Meanwhile, massive technological advances in medical imaging and radiation physics were driving the discipline of clinical oncology forward. The advent of 3D, and now 4D, treatment planning means that radiotherapy is better directed at the target, and safer, than ever before. In recent years, however, it has become increasingly apparent that cancer cells can evade radiation–induced cell death. The reasons for this are complex. At the cellular level, the ability of cancer cells to repair damaged DNA, and the activation of growth factor receptor mediated survival pathways in response to ionising radiation, appear to be critical. However, the tumour microenvironment also has a significant effect on radiosensitivity. This is often related to tumour oxygenation, which is dependent on the efficiency and stability of the supporting vasculature, but it is likely that other factors are also involved, and there is much more to learn. The inherent radioresistance present in certain cancer cells contributes to treatment failure and probably explains why dose escalation studies in radiotherapy practice are not always successful. Hence, there is a great need to gain a better understanding of the complex processes that govern radioresistance. This will help to identify certain cellular or tumour characteristics that could be selectively targeted in order to improve the efficacy of radiation treatment.

To this end, a number of putative novel signal transduction targets have already been identified. Amongst the most advanced is EGFR. There is evidence that inhibiting EGFR signalling can enhance the effectiveness of radiation in head and neck cancer (Bonner, Harari et al. 2009). However, this is not standard practice as this combination has yet to show superiority over chemoradiotherapy in a clinical trial. Nonetheless, the Bonner study indicated that targeted agents could feasibly be combined with a radical course of radiotherapy, paving the way for

further evaluation of EGFR inhibitors (and other targeted agents) in combination with radiation in a wide range of tumour types.

FAK is a non-receptor tyrosine kinase that is located in focal adhesions and therefore plays an integral role in transmitting signals from growth factor receptors and integrins to the nucleus. As ionising radiation can stimulate cell survival through both growth factor receptor and integrin signalling pathways, we hypothesised that FAK may be an important mediator of radioresistance. We aimed to test this hypothesis by developing a novel cancer cell line with inducible *fak* deletion.

Using a conditional mouse model which was already well established in this laboratory (McLean, Komiyama et al. 2004), we were able to generate SCC cell lines from mouse skin (SCC 1.1 and SCC 7.1) and successfully delete *fak* from one of these cell lines (SCC 7.1). The precise reason why we were unable to produce a FAK -/- cell line in SCC 1.1 cells is unknown, but we did consider whether this was related to the presence of wt p53. Although it is likely that *Cre* expression and/or function was suboptimal in this cell line, we suspected that SCC 1.1 cells may have undergone growth arrest or cell death in the complete absence of FAK, due to activation of p53, therefore precluding the proliferation of a stable knockout. This is obviously conjecture as the growth arrest/cell death inducing abilities of high dose 4-OHT made it difficult to interpret the results of FACS analysis. The reasoning behind this conjecture was based, in part, on an emerging body of data outlining a key functional interaction between FAK and p53. In fact, the significance of p53 and the possible interplay between FAK and p53 in these derived cell lines are recurring themes throughout this thesis, although this was not what we had set out to test.

p53 was important in the SCC 7.1 cell line for a number of reasons. Firstly, the FAK -/- cell line contained functional wt p53, demonstrating that, at least in certain cancer cells, the complete absence of FAK does not promote uncontrolled activation of p53 and resultant growth arrest or cell death. This suggests a different "wiring" or "functional inter-dependency" between normal MEFs (Ilic, Furuta et al. 1995) and the cancer cells we derived here. Secondly, the observation that the SCC 7.1 FAK +/+ parental cell line was composed of a mixture of cell populations with either wt p53 or mutant p53 (L254R), and that

these populations had different cellular characteristics, made it clear that a direct comparison could not be performed between SCC 7.1 FAK +/+ cells and SCC 7.1 FAK -/- cells. Thirdly, it is likely that the poorly described L254R p53 mutant protein has gain of function properties, including increased proliferative capacity *in vitro* and the ability to invade *in vivo*. Admittedly, these characteristics may be related to other mutations and we have yet to confirm that mutant p53 alone is the cause of these effects.

Although in the short term it was disappointing that we could not immediately proceed to clonogenic assays in the SCC 7.1 FAK +/+ and FAK -/- cell lines, it was of the utmost importance to create a system that would provide the most accurate representation of any potential FAK mediated effects on clonogenicity. We achieved this by introducing a FAK wt construct into the FAK -/- clone, ensuring that FAK wt was both correctly localised and expressed at a similar level to endogenous FAK. In doing so, we were then able to evaluate the role of FAK in radiation survival in an advanced, invasive squamous cell carcinoma cell line under highly controlled and reproducible conditions.

Much to our surprise, and in contrast to published literature to date based on human pancreatic cancer cells (Cordes, Frick et al. 2007), the absence of FAK in our SCC cell lines was associated with a more radioresistant phenotype. This result raises questions of course about reduction of FAK levels *versu*s deletion of FAK and the possibility of different roles for FAK in humans *versus* mice which have yet to be addressed. It seems more likely, however, that FAK will play different roles in cell survival depending on the particular cell of interest and the various mutations and/or molecular aberrations it has acquired during the process of carcinogenesis. Indeed, we believe that p53 status is likely to be critical. However, we acknowledge that in certain cell lines or tissues, gene products other than FAK or p53 may be more important in determining cell fate in response to ionising radiation or other cellular stresses.

The reason why we believed that p53 status *was* critical in determining radiation survival in the SCC 7.1 FAK -/- versus FAK wt cell lines was based on the ability of p53 to transcriptionally activate p21 following a radiation insult in the absence of FAK. Accordingly, p21 knockdown led to a significant increase in radiosensitivity in FAK -/- cells. As the cellular response to ionising radiation is

complex, factors unrelated to p21 are probably also involved. Nonetheless, this data suggested that wt p53 promoted cell survival in response to radiation. It also indicated that FAK somehow inhibited the pro-survival function of p53 in these cancer cells. While wt p53 is traditionally characterised by its tumour suppressor functions, including a more radiosensitive phenotype, this ability of p53 is very much cell line and context dependent. The fact that our SCC cell line had survived transfer from the original mouse tumour to the cell culture environment, followed by repeated passage and high dose 4-OHT treatment without acquiring a p53 mutation, implies that we had selected out a particularly aggressive wt p53 cell line.

Similarly, while FAK typically has tumour promoting properties, emerging data highlights that in certain cellular systems FAK acts as a tumour suppressor (personal communication – Owen Sansom). Hence, it seems that both FAK and p53 can play very different roles depending on the precise cellular context. It is reasonable to conclude that in our SCC cell line FAK suppresses radiation induced survival mediated by wt p53.

There are several important questions that stem from this. Firstly, what is the major mechanism for cell death in the FAK containing cells exposed to radiation? We did not demonstrate any increase in apoptosis in FAK wt cells compared with FAK -/- cells at any of the time points examined. However, as we have already pointed out apoptosis propensity does not always reflect cellular radiosensitivity, and from our experience radiation induced apoptosis is difficult to detect in many cancer cell lines in the laboratory setting. The possibility of enhanced cell death by alternate events, such as mitotic catastrophe, should be considered. Secondly, why does the radiation enhancing role of FAK appear to be stress dependent? Intriguingly, we have yet to demonstrate a similar effect in response to cytotoxic chemotherapy. This may be specifically related to the property of ionising radiation to induce DSBs. FAK may somehow sense that these are particularly damaging to the cell and prevent p53 from activating survival mechanisms through p21 (and other target genes / pathways). However, FAK has not been directly implicated in the DNA damage/response pathway. While FAK activity can be stimulated directly in response to reactive oxygen species (which are the cause of DSBs), it is unclear whether this alone could explain the phenomenon, especially as cytotoxic agents can also induce free radical

formation. At this point, we can only speculate why FAK has different functions in response to different stresses in these cancer cells.

Despite increasing evidence highlighting a direct functional interaction between FAK and p53 in both normal and cancer cells, we were unable to demonstrate this in the SCC 7.1 cell line. On the contrary, our results were indicative of an indirect FAK/p53 functional interaction. Moreover, preliminary data based on the use of a small molecule inhibitor in the FAK wt cells suggests that the kinase activity of FAK is important. This is also in direct opposition to most of the published work to date, with the most convincing evidence produced by Lim et al. clearly delineating the requirement of the FAK FERM domain in establishing a structural connection between FAK and p53 (Lim, Chen et al. 2008). This discrepancy may be explained by cell line dependent factors. It is certainly reasonable to speculate that the FAK/p53 axis may vary from cell line to cell line. However, we have considered the possibility that the indirect relationship between FAK and p53 in our cells may be specific to ionising radiation (and/or other cellular stresses). It may be the case that FAK does directly influence the function of p53 in SCC 7.1 cells in response to other cellular stresses.

To summarise, in the SCC 7.1 cell line, p53 is stabilised in response to ionising radiation within a few hours. This occurs regardless of FAK status. In the absence of FAK, upregulation of p53 leads to transcriptional activation of p21 which increases the ability of the cell to survive the radiation insult. Increased survival is not secondary to effects on cell cycle, but may instead be related to the DNA repair function of p21. It is likely that additional p53 transcription-dependent or independent pathways also have a part to play. In the presence of FAK, p53 is still stabilised in response to radiation. However, we believe that FAK signalling suppresses these p53 mediated survival mechanisms. Any FAK/p53 functional interaction in this context is indirect and mediated by an unknown pathway, the end result of which may be the recruitment and/or activation of a cellular protein(s) that can selectively control p53 transcriptional function. A schematic diagram of this hypothesis is shown in **Figure 70**. Interestingly, evaluation of the radiomodulating properties of other non-receptor tyrosine kinases such as Src and ILK has revealed that the interaction of these proteins with EGFR appears to be critical. While we did not specifically investigate this possibility, it is worth noting that the SCC 7.1 cell line expresses very low levels of EGFR and we did

not find any obvious differences in the activation of downstream signalling pathways as assessed by immunoblotting for phospho-ERK and phospho-Akt between FAK -/- and FAK wt cell populations in response to ionising radiation (data not shown). However, our SCC 7.1 cells do express high levels of other receptor tyrosine kinases such as the Ret oncoprotein, and Ret interacts with FAK (personal communication – Emma Sandilands). This could be an area for further study. Also, it may be that FAK can influence the translocation of EGFR to the nucleus, and hence its DNA repair abilities, but is possible that we have discovered a protein kinase-dependent effect on radiosensitivity that is completely distinct from EGFR.

In addition, we have shown for the first time that FAK can influence radiation survival *in vivo* as well as *in vitro*. This was a particularly pertinent experiment to do in view of the potentially different roles for FAK depending on the cellular context. Indeed, we demonstrated that FAK was imperative for proliferation in a 3D but not a 2D environment in our SCC cells. In this case, FAK is constitutively active and FAK kinase activity drives growth and proliferation in the absence of adhesion signalling. We believe that loss of FAK (specifically loss of FAK kinase activity) delays the onset of xenograft formation, but this deficit is soon overcome by the supporting infrastructure provided by the host animal. Hence, when xenografts have established and FAK is no longer critical for performing an anchorage-independent type function, it may be able to resume similar roles that it plays *in vitro*. Notably, an increase in p21 was seen in FAK -/- xenografts but not FAK wt xenografts, indicating that similar FAK mediated mechanisms are provoked *in vitro* and *in vivo*. However, there are a number of characteristics specific to the *in vivo* setting, such as tumour oxygenation and angiogenesis, that may have contributed to the overall outcome and which require further investigation.

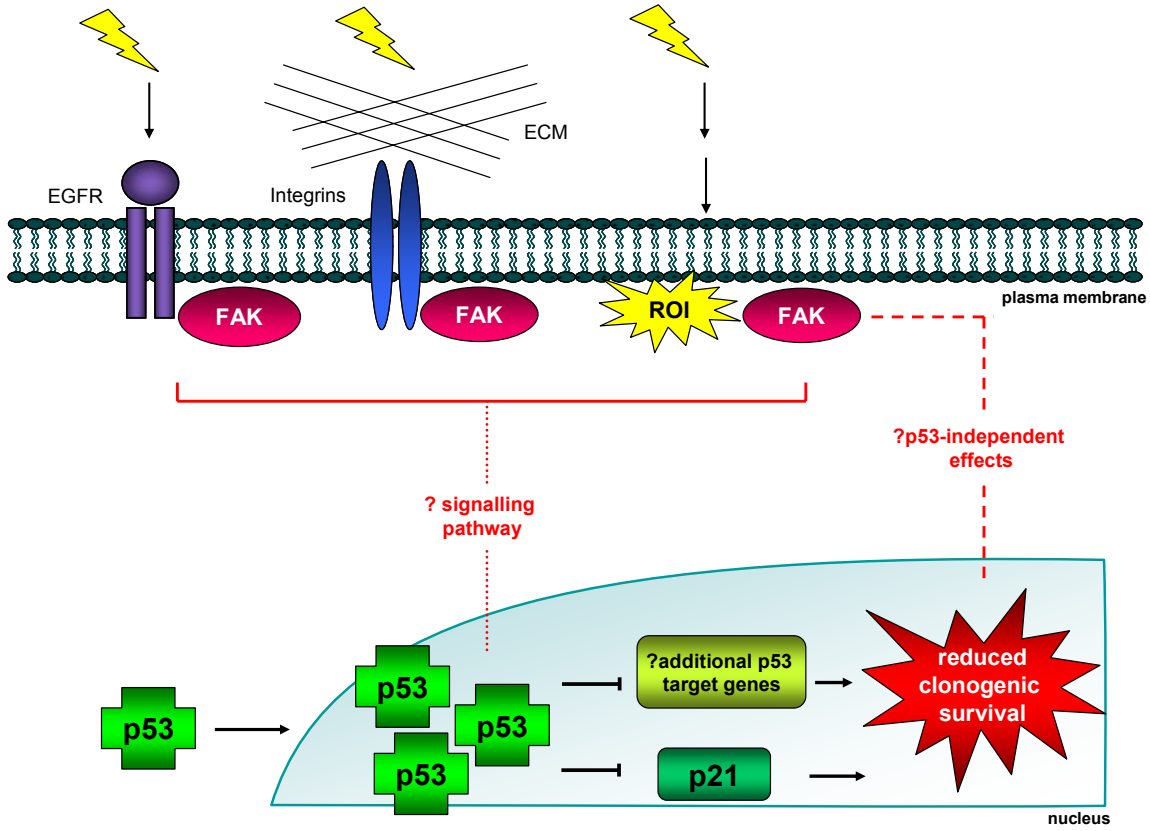


Figure 70 - FAK mediated effects on radiosensitivity
In response to ionising radiation, FAK may be activated indirectly by growth factor receptor or integrin signalling. However, FAK may also be phosphorylated by reactive oxygen species. p53 is able to detect cellular stress induced by radiation and accumulates in the nucleus. FAK does not interact directly with p53, but through an unknown mechanism (which may involve recruitment of a p53 co-factor via a signalling pathway) FAK inhibits transcription of p21 and other p53 target genes that promote cell survival. There may be additional mechanisms that contribute to reduced clonogenic survival; these may be p53 transcription-independent or even p53-independent.

Interestingly, work has just been published assessing the role of the FAK inhibitor TAE226 as a radiosensitising agent in head and neck cancer cell lines (Hehlgans, Lange et al. 2009). The authors found that radiosensitivity was increased in a 3D (laminin-rich ECM) but not a 2D environment. While these results may corroborate our preliminary data suggesting that the kinase activity of FAK has an important role in mediating radiation survival, we do wonder whether TAE226 was applied too early in the experiment, i.e. when FAK kinase activity was necessary to stimulate colony formation. Even more intriguingly, two of the three pancreatic cancer cell lines evaluated by Cordes et al. (Cordes, Frick et al. 2007) were also examined in this study and TAE226 did not radiosensitise either of these cell lines in 2D or 3D, indicating that reduced FAK levels as opposed to reduced FAK activity was the critical factor in promoting radiosensitivity in the Cordes study.

Thus, just as the cellular response to ionising radiation is complex, the relationship between FAK and radiosensitivity is also complex. The effects of FAK on radiation survival appear to vary from cell line to cell line. It is likely that either the scaffolding function of FAK or the kinase activity of FAK may be important depending on the cell line of interest and the various roles that FAK plays in each particular cell line. We believe that the functional interaction between FAK and p53 is also critical, at least in cell lines with wt p53 where p53 strongly influences the outcome of ionising radiation via predetermined pro-survival or pro-death pathways. As we have already outlined, trying to predict the effect of the FAK/p53 axis on radiosensitivity in cells with mutant p53 is much more difficult. Hopefully, future radiation studies in a wide panel of cell lines with varying p53 status will help to dissect out the roles of FAK and p53 in the highly intricate cellular response to ionising radiation. This highlights the need for greater understanding of the underlying critical factors and mechanisms before embarking on trials of FAK inhibitors along with radiotherapy in clinical trials.

7.2 Future Perspectives

The work presented in this thesis represents a thorough investigation into the role of FAK in mediating radiosensitivity *in vitro* and *in vivo* using mouse squamous cell carcinoma cell lines. In addition, it has prompted a wide range of potential research questions. In this final section we will focus on the most pertinent topics for future study:

1. Dissecting out the general complexities of the FAK/p53 interaction and its role in mediating radiosensitivity in a heterogeneous set of cancers with defined molecular properties

2. Evaluating whether the FAK mediated effects on p53 and radiosensitivity in the SCC cells we derived are Src-dependent

3. Investigating the role of autophagy in radiation induced survival in our SCC FAK -/- and FAK wt cells

4. Exploring the role of FAK in normal tissue radiosensitivity

7.2.1 Dissecting out the general complexities of the FAK/p53 interaction and its role in mediating radiosensitivity

One particular topic that requires further clarification is the precise relationship between FAK and p53 in our SCC cells. We have shown that any FAK/p53 functional interaction in response to ionising radiation in these cells is indirect. While we think that the catalytic activity of FAK is a prerequisite, more definitive evidence is needed. Firstly, we plan to incorporate the small molecule inhibitor PF-562,271 into clonogenic assays and evaluate whether FAK kinase inhibition in combination with radiation reproduces the radioresistant phenotype of FAK -/- cells. Secondly, we aim to compare and contrast the radiation survival of cell lines expressing FAK kinase mutants, and also assess the ability of these cells to induce p21 in response to radiation. Indeed, we have already generated FAK KD mutants as described in Chapter 5. Further, we have also generated FAK-Y397F and FAK–YF4-Y9F kinase mutants and expressed these in the FAK deficient SCC 7.1 cells. In the former, Src is unable to bind due to mutation of the Y397

site, and this in turn inhibits full FAK catalytic activity. In the latter, all of the Src–dependent phosphorylation sites on FAK have been mutated from tyrosine to phenylalanine and so FAK cannot be phosphorylated by Src. This also inhibits full FAK catalytic activity. The advantage of this work is that it may also reveal whether or not Src is critical for any FAK mediated effects on p53, and the signalling determinants in FAK that controls its effects.

Clearly, one of the major differences between our work and the Cordes study (Cordes, Frick et al. 2007) is the presence of wt p53 in the mouse SCC cells versus mutant p53 in the pancreatic cancer cell lines. We had hoped to address this by developing FAK -/- cell lines from the isolated mutant p53 single cell clones (A10 and H7) by the addition of high dose 4-OHT and comparing radiosensitivity in FAK +/+ *versus* FAK -/- cells in the context of a mutant p53 background. Despite much effort, this endeavour has not yet proved successful. In fact, it may be necessary to generate new single cell clones from an early passage SCC 7.1 population and repeat the 4-OHT selection process. An alternative approach would be to return to the original mouse model and incorporate various p53 mutations into this system in addition to deleting *fak*. New SCC cell lines with altered p53 genetic backgrounds could then be propagated by the topical carcinogenesis protocol we described earlier. In this way, we could examine a number of common “hotspot” p53 mutations, including R172H and R270H, and their contribution to radiosensitivity in the presence or absence of FAK. Ideally, we would also incorporate a reporter gene such as *lacZ* into this model, as this would allow visualisation of *Cre* in the derived cell lines. One problem with this method is the likelihood of the various SCC cell lines to have amassed different mutations during the process of carcinogenesis and in some of these cell lines it is quite possible that p53 will not significantly affect radiation survival. This makes standardisation and direct comparison between cell lines difficult. One way to circumvent this might be to conditionally delete out p53 as well as FAK and thus create a p53 null SCC cell line. A variety of p53 mutant constructs could then be transfected into the p53 -/- cells and the behaviour of these mutant proteins compared in FAK +/+ and FAK -/- cells. It may even be possible to insert wt p53 into the p53 -/- cells, although the presence of wt p53 in p53 knockout cells is often only tolerated for a short

period of time and therefore this approach may prove difficult due to the prolonged duration of a clonogenic assay.

7.2.2 Evaluating whether the FAK mediated effects on p53 and radiosensitivity are Src-dependent

It can be difficult to separate out the effects of FAK on the cancer phenotype from those of Src. In fact, until the work by McLean et al. demonstrated a causative role for FAK in carcinogenesis (McLean, Komiyama et al. 2004), it was suggested that any apparent cancer related properties of FAK may be due to Src. We aim to evaluate whether Src is driving the radiation enhancing function of FAK in the SCC 7.1 cell line. As outlined above, assessing the FAK kinase mutants which have lost either the Src binding site or the Src-dependent phosphorylation sites on FAK will provide very useful information. In addition, we intend to study the effect of Src knockdown using siRNA and/or a Src kinase inhibitor such as dasatinib on p21 induction and clonogenicity in the SCC 7.1 FAK wt cell line. It would also be reasonable to delete Src in the conditional mouse model as opposed to FAK and investigate the radiosensitivity of Src +/+ and Src -/- cell lines. However, it is important to point out that analysis may be confounded due to functional redundancy between Src family kinases – such that Fyn and Yes will still be present when Src is knocked out.

7.2.3 Investigating the role of autophagy in radiation induced survival in FAK -/- and FAK wt cancer cells

We have reiterated several times that the cellular response to radiation is complex. Although we were able to show that activation of p53 led to transcriptional upregulation of p21 in FAK -/- cells following exposure to radiation, we accepted that this mechanism may only partly explain the radioresistant phenotype of this cell line. One cell protective mechanism that has provoked increasing levels of interest over the past few years is autophagy. This process is not yet fully understood and there is data suggesting that autophagy may either promote radiosensitivity (Paglin, Hollister et al. 2001; Zois and Koukourakis 2009) or radioresistance (Moretti, Cha et al. 2007; Apel, Herr et al. 2008; Lomonaco, Finniss et al. 2009). We are currently exploring whether

autophagy is an important means of cell survival in response to ionising radiation in the SCC 7.1 cell line.

The specific reasoning behind this possibility stems from very recent work in this laboratory demonstrating increased autophagy in the SCC 7.1 FAK -/- cell line (Sandilands, Serrels et al., submitted). Intriguingly, this phenomenon appears to be mediated via Src. It was first noted that although the SCC 7.1 FAK -/- and FAK wt cells displayed similar levels of Src, activated Src was present at a lower level in the FAK -/- cells (**Figure 71A**). Furthermore, localisation studies revealed that the absence of FAK caused a dramatic relocation of Src from focal adhesions to distinct intracellular punctate structures (**Figure 71B**) which were shown to represent autophagosomes (**Figure 71C**). Treatment with an autophagy inhibitor restored normal Src localisation and, more importantly, led to increased cell death through apoptosis in FAK -/- cells (personal communication – Emma Sandilands). Collectively, this data describes a novel form of autophagy by which active Src is selectively targeted from focal adhesions to autophagosomes, and on for degradation, thus explaining the overall loss of active Src from the cell after perturbation of integrin signalling caused by FAK deletion. Physiologically, this is needed for these cancer cells to evade cell death in the absence of FAK.

Preliminary investigations are underway to determine whether protein expression levels and/or localisation of key autophagy regulators, such as LC3, beclin-1 and ATG7, are altered in response to ionising radiation in the SCC 7.1 FAK -/- and FAK wt cell lines. The definitive experiment, of course, would be to inhibit autophagy in combination with radiation in the FAK -/- and FAK wt cell lines and evaluate the effect on clonogenicity. The options include the commonly used autophagy inhibitor 3MA which has the disadvantage of interrupting a large range of intracellular proteins and may not be particularly specific or alternatively using siRNA to knockdown autophagy regulators such as ATG7.

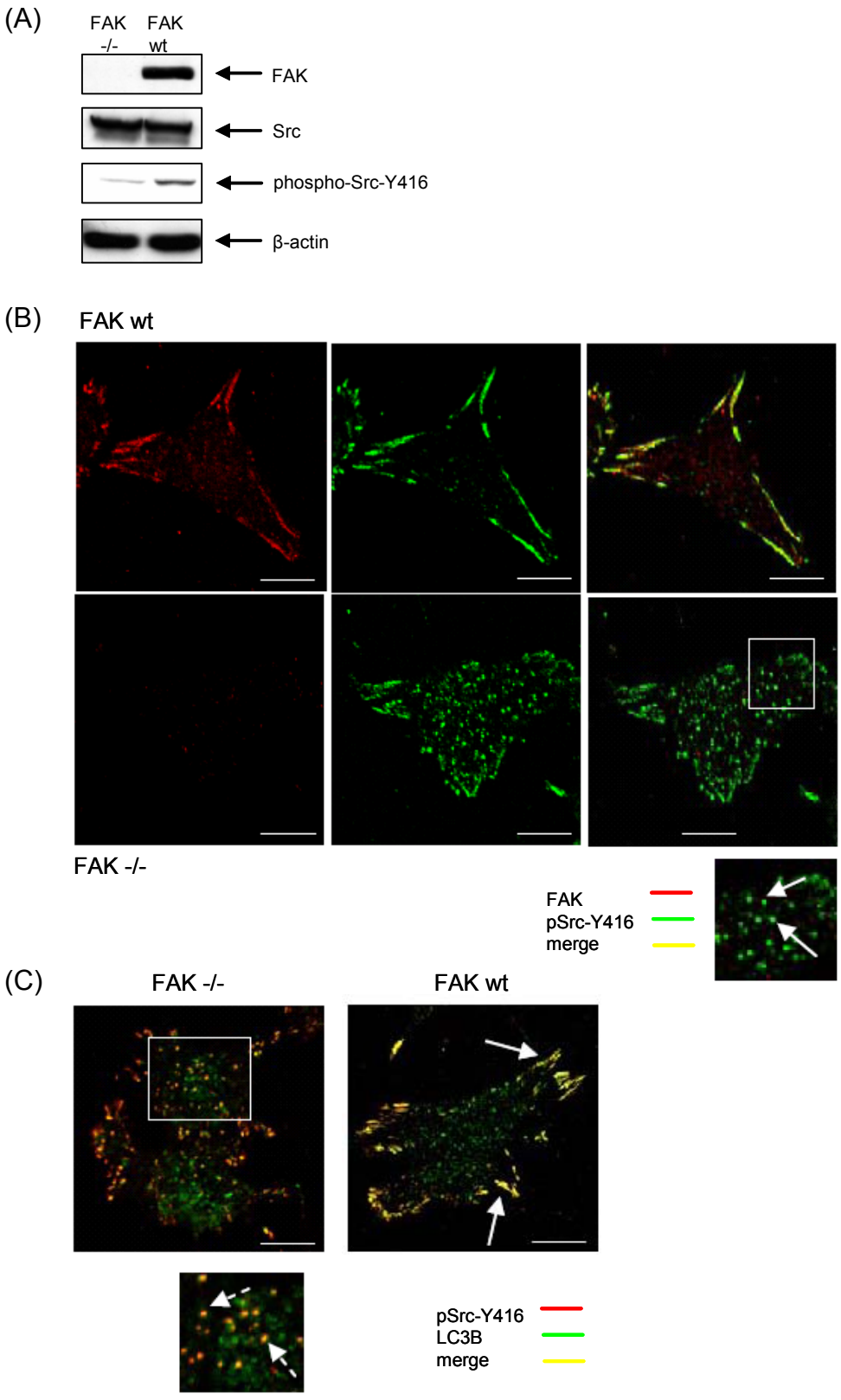


Figure 71 - Active Src is trafficked to autophagosomes in the absence of FAK
(A) Western blot analysis for FAK, Src and activated Src in FAK -/- and FAK wt cells. β-actin loading is also shown. (B) Immunofluorescence analysis demonstrating localisation of FAK and activated Src in FAK -/- and FAK wt cells, FAK – red and pSrc-Y416 – green, arrows demonstrating punctuate distribution of activated Src in FAK -/- cells (scale bar, 20μm). (C) Cells were fixed and stained for pSrc-Y416 (red) and LC3B (green), complete arrows represent co-localisation at focal adhesions while broken arrows in zoomed image show co-localisation in puncta. Experiment courtesy of E Sandilands.

7.2.4 Exploring the role of FAK in normal tissue radiosensitivity

Our results demonstrated a negative effect on radiosensitivity following *fak* deletion in a squamous cell carcinoma cell line. This has provided a new insight into the role of FAK in cellular radiation survival and the relationship between FAK and p53. Should future work confirm that manipulating FAK levels and / or activity in combination with radiation is advantageous in certain cell lines and/or tumour types, there are a number of important questions that would need to be addressed before considering progression into the clinical phase of investigation. For instance, which patients would be most likely to benefit and would this depend on tumour type alone? If not, would patient selection be based at the molecular level and if so, what molecular signature would predict response? Is it likely to be as straightforward as p53 status given the contrasting pro-survival and pro-death functions of the wt p53 protein depending on cell line and context, coupled with the vast number of potential p53 mutations and their diverse (and often largely unknown) properties? There are also issues regarding the best form of pharmacological approach – would this entail the use of a protein-protein interaction inhibitor which is complex to develop or a small molecule tyrosine kinase inhibitor, several of which are already in existence, and would this depend on the treatment context? Also, are FAK inhibitors safe in the long term or will they induce dormancy, allowing late reactivation of tumour cell activity?

Finally, how will altered FAK levels affect normal tissue response to radiation? It is slightly concerning that Ashton et al. have just reported that FAK is necessary for intestinal regeneration following irradiation of mouse gut (Ashton, Morton et al. 2010). However, their findings were based on a background of genetic deletion and perhaps low levels of FAK protein as opposed to FAK deletion would be sufficient to prevent this. Interestingly, it has been proposed that cells with a high turnover may be more susceptible to FAK inhibition due to the role of FAK in focal adhesion turnover. If the gut is definitely intolerant of low FAK levels and/or activity, then it may be necessary to focus combination regimens at other sites. Towards the end of this project, we did some work looking at whether FAK mediates radiation survival in fibroblasts, using FAK +/+ and FAK -/- MEFs. Preliminary data indicates that the presence or absence of FAK has no

effect on radiosensitivity in these cells (**Figure 72**). This suggests that some normal tissues may not be compromised by FAK inhibition or FAK loss in the context of radiation induced DNA damage. However, it is important to point out that the MEFs are deficient in p53. Hence, the possibility of a completely different outcome *in vivo* in the presence of wt p53 must be considered, although this is difficult to test.

Also, the FAK -/- MEFs have upregulation of Pyk2 (this was not evident in our SCC cells) and in the MEFs it is possible that Pyk2 may compensate for the loss of FAK. Currently, there is no data in the literature regarding Pyk2 and cellular radiation response. Intriguingly, it has just recently been reported that Pyk2 can facilitate cell survival in FAK-/- / p21-/- MEFs by translocation to the nucleus and limiting levels of p53 in a FERM domain–dependent manner (Lim, Miller et al. 2010). While this highlights some important similarities in the function of FAK and Pyk2, obviously a Pyk2 mediated effect on p53 would not explain our radiation findings in the MEFs based on their p53 null background. It may be the case that Pyk2 (and FAK) have significant p53-independent influences on cell survival that have yet to be fully explored.

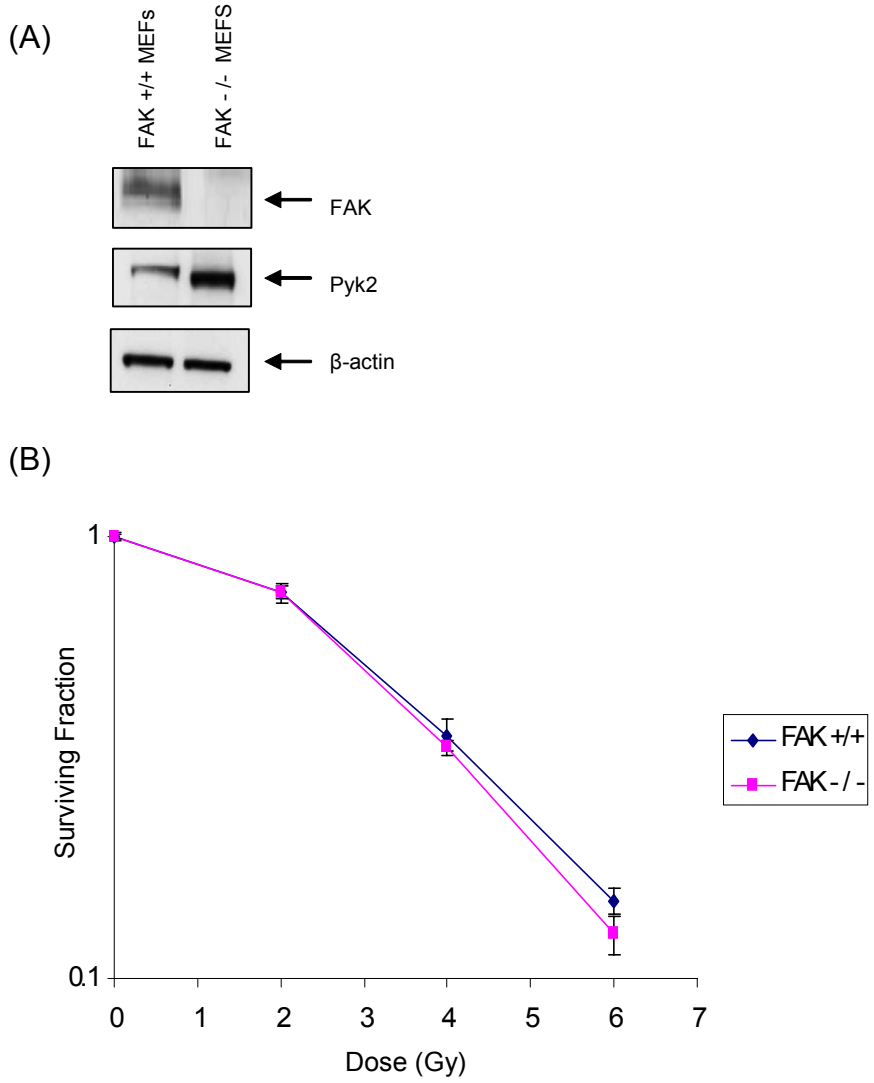
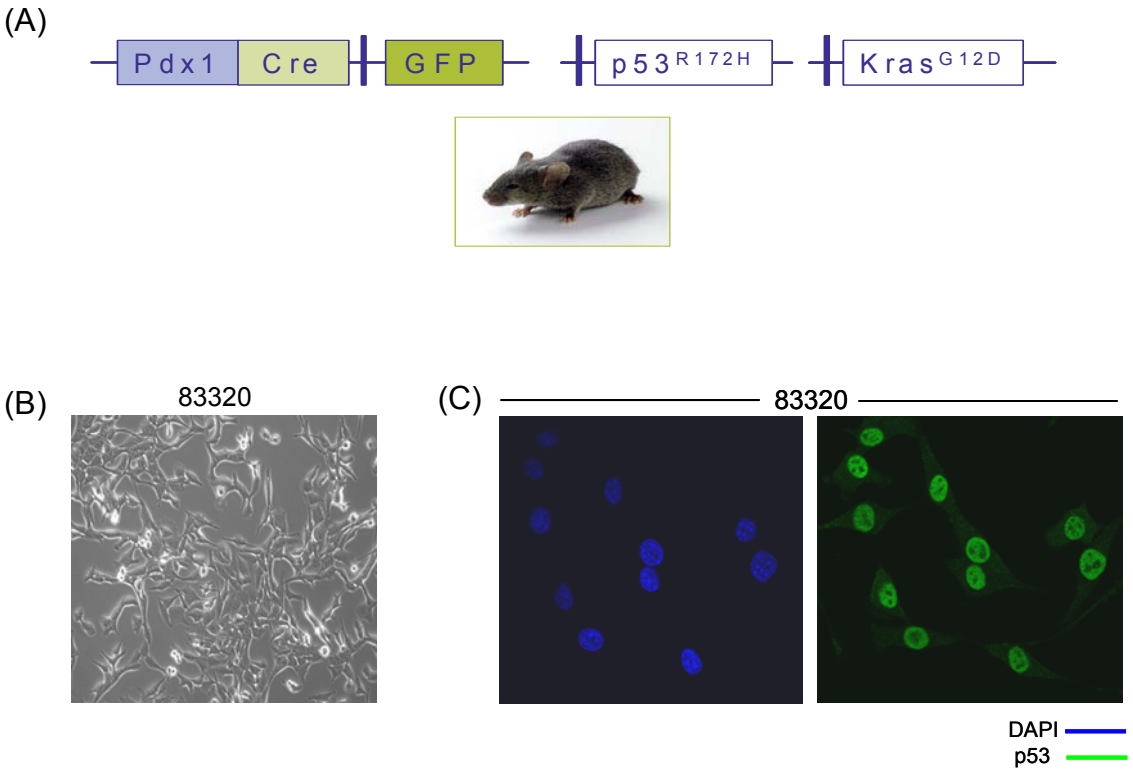


Figure 72 - FAK is not an important mediator of radiosensitivity in MEFs
(A) Western blot analysis of FAK and Pyk2 protein levels in MEFs. (B) FAK +/+ and FAK -/- MEFs were irradiated (0, 2Gy, 4Gy and 6Gy), immediately trypsinised and seeded into 60mm dishes at a density that would permit colony growth of 50–150 colonies per plate. After seven days the plates were fixed in methanol and stained with crystal violet. The numbers of colonies per plate were counted under low power microscopy and surviving fraction calculated at each dose of radiation. Results shown represent mean ± SEM from two combined experiments.

Final Comment

I have now returned to clinical practice and in my future career as a clinical oncologist I look forward to contributing to the development of radiotherapy practice. Whilst there are ever increasing technological advances in radiotherapy planning and delivery (even in the four years I spent out of practice), the research work that I described here has made me much more aware of the complexity that underpins the biological effects of radiation at the cellular level. Understanding this complexity and being cogniscent of the potential context specificity of the various putative novel radiosensitising targets is crucial if radiotherapy is to be successfully combined with targeted therapies. It is likely that the effects of targeted agents in combination with radiation will be tumour type specific, or may even be dependent on patient-specific response factors. This highlights the need for further and extensive basic laboratory research in order to develop informative biomarkers of response and/or predictive assays, such that the optimal combined treatment could be offered to each individual patient.

Appendices



Appendix 1 – Development of 83320 murine pancreatic cancer cell line

(A) Targeting expression of p53 (R172H) and K-Ras (G12D) to the mouse pancreas results in the formation of invasive carcinomas (Hingorani, Wang et al. 2005). (B) A cell line was derived from a mouse pancreatic tumour – 83320; a phase contrast image is shown. (C) This cell line demonstrated high levels of nuclear p53 on immunofluorescence in keeping with an accumulating mutation of p53. (D) Sequencing analysis was carried out on the p53 DNA binding domain in mouse keratinocytes (left) and 83320 cells (right). This revealed a G-A base change at codon 172 in 83320 cells in keeping with the presence of R172H mutation.

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