2009-09-01

Metal-induced Bystander Effects: Mechanism and Implicationis.

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Metal-Induced Bystander Effects: Mechanism and Implications

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Thesis submitted for the award of PhD

Dublin Institute of Technology

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September 2009

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Acknowledgements

For my mum and dad

Thanks to my supervisors Fiona and Patrick for your guidance throughout this thesis. Thanks also to Nino and Kat for sharing your knowledge and practical skills with me.

Thanks to Miguel Rubio for providing the fibroblast cell lines and Duncan Baird for the thyroid carcinoma cell line and telomere analysis. Thanks to Roger Newson for statistical analysis, Kate Heesom for proteomics, Kate Nobes for help with microinjections and Maeve Caldwell and Lucy Crompton for the human embryonic stem cell work.

I must also thank Ryan. I think we made an excellent team in the lab.

Thanks to Vikki, Kate Harvey and Kate George for your friendship and support.

Finally, thanks to Chris for looking after me over the last few months of writing.

"*We can only see a short distance ahead, but we can see plenty there that needs to be done.*" Alan Turing

Abstract

The radiation-induced bystander effect is a phenomenon known to occur post irradiation, characterised by the induction of biological effects in unirradiated cells as a result of receiving signals from irradiated cells or their culture medium. Chemical-induced bystander effects are poorly characterised and there are no reports of a bystander effect induced by metals. Heavy metals and in particular chromium (VI) are known to cause persistent genomic instability. For the first time, this study provides evidence that a short, low-dose exposure of human fibroblasts to chromium (VI) causes a bystander effect in human fibroblasts that persists for at least thirty days post metal treatment. The biological effects induced in these bystander cells include induction of micronuclei, nucleoplasmic bridges, cytogenetic abnormalities and DNA double strand breaks.

Crucially, this effect depends on whether the bystander cells are telomerase positive or negative. Telomerase negative human fibroblasts respond to a medium-transmissible bystander signal from all 'donor' cell types; but telomerase positive cells, whether they express ectopic hTERT or physiological telomerase as is the case for the immortalized k1 thyroid carcinoma cell line and human embryonic stem cells, are resistant to a signal from other telomerase positive cells. Treatment of cells with ascorbic acid revealed that the role of oxidative stress in transmission of bystander responses is more important for telomerase positive cells than telomerase negative cells. The role of TNF-alpha was also explored. In telomerase negative cells, addition of a neutralizing antibody to TNF-alpha to conditioned medium from either cell type caused a significant reduction in bystander γ-H2AX foci. In contrast, there was a significant reduction in γ-H2AX foci in bystander telomerase positive cells exposed to conditioned medium from telomerase negative but not telomerase positive donor

cells. Inhibition of p38MAP Kinase by SB203580 had no effect on bystander signalling.

This study highlights novel considerations for predicting the outcome of metal exposure and the importance of the indirect biological effects of heavy metals in cancer risk assessment, which has implications for cancer therapy.

List of Abbreviations in alphabetical order

53BP1-p53 binding protein-1

A-T -ataxia telangiectasia

ATM -ataxia-telangeictasia mutated

ATR -ataxia-telangiectasia and rad3-related

BER -base excision repair

BRCA1 -breast cancer gene 1

CBMN -cytokinesis block micronucleus assay

CO_2 -carbon dioxide

Cyt-B -cytochalasin-B

DAPI -4',6-diamidino-2-phenylindole

DMEM -Dulbecco's modified eagles medium

DMSO -dimethyl sulfoxide

DN -dominant negative

DNA -deoxyribonucleic acid

DNA-PK -DNA dependent protein kinase

DPBS -Dulbecco's PBS

DPBST -DPBS containing triton x-100

EDTA -diaminoethanetetraacetic acid, edetic acid, edetate, ethylenedinitrilotetraacetic acid, versene, ethylene diamine tetraacetic acid

FBS -foetal bovine serum

FISH -fluorescence in situ hybridization

GJIC -gap junctional intercellular communication

hES cells -human embryonic stem cells

hTERT -human telomerase reverse transcriptase

ICCM -irradiated cell conditioned medium

ICM -inner cell mass

$K_2Cr_2O_7$ -potassium dichromate

KCl -potassium chloride

MAP Kinase -mitogen activated protein kinase

mEF -mouse embryonic fibroblast

MMR -mismatch repair

MN -micronucleus

MRM -minichromosome maintenance complex

MRN -protein complex of Mre11, Rad50 and Nbs1

NaCl -sodium chloride

NPB -nucleoplasmic bridge

p38 MAP kinase -p38 mitogen activated protein kinase

pBABE-PURO-hTERT -5' sequencing primer,

PBS -phosphate buffered saline

PDT -photodynamic therapy

Pfa –paraformaldehyde

PI3-Kinase -phosphoinositide 3-kinase

pLXSN -retroviral vector designed for retroviral gene delivery and expression

PURO-selectable marker, telomerase reverse transcriptase

RPM -revolutions per minute

ROS -reactive oxygen species

SB203580 -p38 MAP kinase inhibitor [4-

(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridinyl) imidazole]

SCE -sister chromatid exchange

STELA -single telomere length analysis

TAM -transcription associated mutation

TAR -transcription associated recombination

TNR –trinucleotide repeat

TRAP -telomere repeat amplification protocol

TRF2 telomere repeat binding factor 2

WT -wild type

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CHAPTER 1 GENERAL INTRODUCTION

1.1 Genomic Instability

1.1.1 Role of Genomic Instability in Cancer

The idea that cancer is a disease of impaired genome stability was initially proposed by Boveri in 1914 (reviewed in (Balmain 2001)). Work carried out over the last century has expanded this knowledge and our understanding of cancer genetics. The molecular mechanisms governing the maintenance and replication of the genome are invariably diminished in cancer cells. However, there is debate surrounding the origin of these genomic defects and their role in tumour progression. According to Tomlinson et al (Tomlinson et al. 1996), 'the process of tumourigenesis is a form of evolution at the somatic level' in which, mutation and selection are the essential components. However, the relative importance of these components remains unknown (Tomlinson et al. 1996). The role of genomic instability in cancer is thought to be in enabling the accumulation of genetic alterations required for malignant transformation.

Persuasive evidence for the genetic basis of human cancer is provided by the observation that numerous genes controlling growth are altered in neoplastic cells (summarised in (Lengauer et al. 1998)). Genomic instability can occur through a variety of mechanisms including defective DNA damage responses, defective DNA replication and defective chromosome segregation. The importance of these mechanisms is demonstrated by their role in human pathological disorders that are characterised by decreased genomic stability, cancer predisposition, premature aging and genetic disease. For example, Cleaver (Cleaver 1968) observed that DNA damage leading to higher rates of *in vitro* mutations and neoplastic transformation in response to UV radiation in cells from patients with Xeroderma Pigmentosum was due to defective DNA repair and

replication. Paterson and Smith (Paterson and Smith 1979) identified the inherent genomic instability in patients with Ataxia Telangiectasia (A-T), an inherited disorder involving hypersensitivity to ionizing radiation and related DNA damaging chemicals as a factor driving the onset and progression of carcinogenesis. The defective gene in A-T patients is Ataxia–telangiectasia-mutated (ATM), a member of the phosphoinositide 3-kinase (P13-kinase) family that also includes the catalytic subunit of DNA-dependent protein kinase (DNA-PKcs) and A–T and Rad3-related protein (ATR) all of which are involved in DNA damage recognition (Abraham 2001).

Preservation of genomic integrity during cell proliferation depends on the strict coordination of numerous processes. DNA replication before chromosome segregation must be efficient and error free. DNA replication is tightly coordinated with detection of DNA damage and repair and cell cycle progression. This ensures genomic integrity during cell divisions preventing mutations and DNA rearrangements. The term genomic instability can refer to a variety of alterations from point mutations to whole chromosome rearrangements. According to Lengauer et al (Lengauer et al. 1998), these alterations can be divided into four main categories: subtle sequence changes, changes in chromosome number, chromosome translocations and gene amplifications. Subtle sequence changes involve base substitutions or insertions or deletions of a small number of nucleotides. Changes in chromosome number involving loss or gain of whole chromosomes are found in nearly all known human tumours as detailed in the catalogue of chromosome aberrations in cancer (Mitelman 1998), which presents information on over thirty thousand cases. Chromosome translocations can lead to fusion of two different genes leading to transcription of a protein with tumourigenic properties exemplified by the Philadelphia chromosome translocation in chromic myeloid leukaemia (CML) (Nowell 1962). At the molecular level, the carboxyl terminus of the

c-*abl* gene on chromosome 9 is joined to the amino terminus of the *BCR* gene on chromosome 22. This 9:22 translocation results in transcription of a 210 kD fusion gene product, conferring selective growth advantage to these cells that differentiate down the myeloid lineage (Nowell 1997). Failures in mitotic chromosome transmission or the mitotic spindle checkpoint can lead to chromosome rearrangements and changes in chromosome number as a result of chromosome loss or gain. Microsatellite instability leads to repetitive DNA expansions and contractions and can occur by replication slippage and impaired mismatch repair (MMR) or homologous recombination (HR). Instability leading to mutations like base substitutions or micro -insertions or –deletions is mainly associated with replication errors or repair. If instability leads to chromosome rearrangements involving changes in the genetic linkage of two DNA fragments, increases in HR-mediated events such as sister chromatid exchange and ectopic HR between non-allelic repeated DNA fragments or end-joining between non-homologous DNA fragments can result in gross chromosomal rearrangements such as translocations, duplications, inversions or deletions. A broad spectrum of proteins and breakpoints are associated with rearrangements and a common feature is their association with replication stress.

According to Gorgoulis et al (Gorgoulis et al. 2005), inactivation of the DNA damage response in human lung hyperplasia leads to malignant transformation. This demonstrates the role of checkpoints as a natural anticancer barrier. Replication failure is a primary cause of cancer and checkpoints detecting DNA damage and replication failures comprise an anticancer mechanism. A common factor linking the instability events leading to these rearrangements is the induction of DNA double strand breaks (DSBs).

1.1.2 DNA Double Strand Breaks

Throughout the work presented in this PhD thesis, the main biological endpoint described to detect DNA damage is the induction of DNA double strand breaks, assayed using an antibody to the phosphorylated form of histone H2AX termed γ-H2AX, which accumulates at sites of DNA DSBs. Therefore, it is important to give an overview of DNA DSB induction under natural conditions in addition to their induction by extraneous factors such as irradiation, exposure to toxic metals or bystander factors.

DNA double strand breaks are particularly dangerous lesions. Both strands of the double helix are damaged, which prevents the use of a complementary DNA strand as a template for repair. In precancerous lesions, aberrant stimulation of cell proliferation leads to DNA replication stress, which either directly, or through the formation of DSBs activates DNA damage checkpoints and induces cell cycle arrest. In those cells that do not undergo permanent cell cycle arrest or apoptosis, there is a likelihood that errors in DSB repair lead to allelic imbalances. These imbalances will preferentially target common fragile sites, which are most sensitive to replication stress and a source of DNA double strand breaks (Gorgoulis et al. 2005). The high incidence of chromosomal translocations and frequent malignancies in A-T lymphoid cells is a demonstration of the biological consequences of failure to enforce DSB-induced cell cycle checkpoints (reviewed in (van Gent et al. 2001)).

1.1.3 DNA Replication as a Source of DNA Double Strand Breaks

Under normal physiological conditions, DNA is most vulnerable during S-phase of the cell cycle. During replication, the replisome must overcome obstacles such as DNA adducts, secondary structures or tightly bound proteins that can cause replication fork stalling, which can compromise genome integrity if not properly processed.

Therefore, S-phase checkpoints are crucial for maintenance of genome integrity. They respond to stalled replication forks and intra-S-phase damage by preventing the collapse of replication forks. Maintenance of replication fork stability helps prevent conversion of primary DNA lesions into double strand breaks and facilitates the subsequent recovery of DNA replication (Kastan and Bartek 2004). The replisome multisubunit complex catalyses replication and contains replication elongation factors. The eukaryotic minichromosome maintenance (MCM) helicase unwinds parental duplex to allow access to the DNA polymerase α primase, which synthesis RNA primers. Processive elongation is catalysed by the replicative polymerases δ and ε. A host of obstacles can be responsible for replication fork stalling. Single stranded DNA gaps and DNA double strand breaks are sensed by S-phase checkpoints activated by ATM and ATR kinases, which phosphorylate the effector kinases CHK1 and CHK2 to trigger the checkpoint response. Phosphorylation of MCM contributes to its association with active replication forks. Restarting of stalled replication forks is mediated by the S-phase checkpoint to prevent unscheduled recombination. If DSBs are generated, H2AX becomes phosphorylated by ATM/ATR forming γ-H2AX, thereby amplifying the initial damage signal. Large megabase lengths of chromatin domains are responsible for the stable accumulation of damage response and cohesion factors that favour repair by sister chromatid exchange (Strom et al. 2004). If replication stress or S-phase checkpoint inactivation disrupt these processes, leading to replication stress and in turn replication fork collapse, breaks accumulate that could trigger genomic instability mediated by replisome disassembly, single stranded (ss) DNA gaps and double strand breaks (Sogo et al. 2002; Cobb et al. 2005). Replication associated DNA DSBs can be generated in several ways. For example, a replication fork that encounters a ss DNA nick or gap leading to discontinued synthesis of the nascent strand can result in formation of a DSB

(Cortes-Ledesma and Aguilera 2006). If the nick is on the leading strand, the DSB is one-ended and the restart of synthesis by break-induced replication is promoted. Blockage of replication fork progression or leading strand synthesis could lead to fork reversal in which Holliday junctions are formed if the replisome is destabilized. Lesions block the synthesis of only one DNA strand without impeding fork progression. Blockage of lagging strand synthesis by single strand nicks or gaps can lead to induction of DSBs if the lesion is a nick between two flanking Okazaki fragments. Lesions blocking leading strand synthesis are bypassed by the replication fork, which restarts downstream of the lesion leaving a single stranded gap.

Many lines of evidence indicate that replication impairment is a main cause of genomic instability. According to Cobb (Cobb et al. 2005), increased chromosome breaks and rearrangements correlate strongly with replication fork collapse. Replication fork collapse entails rapid displacement of DNA polymerases and RNA polymerase due to the loss of ATR kinase activity at sites of replication stalling. The yeast homolog Mec1, when mutated but catalytically active led to partial displacement of polymerases. Nakamura et al (Nakamura et al. 2004) showed that H2A phosphorylation in fission yeast at stalled replication forks regulates accessibility to DNA of enzymes involved in repair and fork restart maintains checkpoint arrest and influences DNA repair. Apart from mis-incorporation of nucleotides, during this stage of the cell cycle, the relatively chemically stable double stranded DNA structure is temporarily suspended at the replication fork, this fragile structure susceptible to collapse resulting in DSB formation (Costanzo et al. 2003). Replication fork stability is maintained by a variety of mechanisms including activation of the ATR-dependent checkpoint pathway. This activation occurs upon recognition of extended stretches of single stranded DNA at stalled replication forks. Replication forks are very robust and precise but have to

overcome obstacles, such as DNA binding proteins and transcription complexes operating on replicating templates and secondary structures in DNA. These are natural impediments as opposed to external or genetic factors. Replication fork restart and checkpoint controls ensure completion and accuracy of replication.

Exogenous factors can also interfere with replication and jeopardize genomic integrity. These factors damage the DNA template or deplete nucleotide pools, resulting in insufficient deoxyribonucleoside triphosphates necessary for DNA synthesis. UV light, gamma-irradiation, DNA modifying agents and topoisomerase poisons block replication at sites of damage because of the inability of the replication fork to pass through a corrupted DNA template.

The function of ATR and its orthologs in maintenance of genome integrity were identified in yeast through the observation of increased chromatid breaks in ATR $^{-/-}$ cells (Brown and Baltimore 2000). Subsequent studies in S. cerevisiae allowed the observation that mutation of Mec1 or the downstream checkpoint kinase Rad53 lead to increased chromatid breaks at regions of the genome that were inherently difficult to replicate and a decreased ability to reinitiate replication fork progression following DNA damage or deoxyribonucleoside depletion (Cha and Kleckner 2002). Rad53 and Mec1 allow slow S-phase progression in response to DNA alkylation as a means of reducing replication fork catastrophe. Replication forks terminate irreversibly in *Rad53* and *Mec1* mutants at a high rate. Replication fork catastrophe is a reason for the very high rate of lethality caused by alkylating agents in checkpoint mutants (Tercero and Diffley 2001). RAD51 and Mec1 mutants are unable to survive with their existing, unviable nucleotide levels. Also, they cannot replicate chromosomes after nucleotide depletion. Mec1 and Rad53 are not just concerned with cell cycle transitions, but DNA damage and DNA replication block stress response pathways. Fragile sites are the most

unstable in the absence of ATR and CHK1 indicating a role in replication fork stability. Depletion of CHK1 using siRNA leads to extensive chromosome fragmentation and instability and breaks at fragile sites (Durkin et al. 2006). An early response occurring in replication stress responses is represented by prevention of replication fork collapse. Chanoux et al (Chanoux et al. 2009) showed that combined deficiency in ATR and H2AX dramatically enhanced DSB induction upon replication fork stalling. Their elimination (either partial or complete) demonstrates that they work cooperatively to suppress DSBs during S-Phase.

1.1.4 Key contributors to genomic instability

Tumour suppressors are proteins that act in *trans* to prevent genomic instability. The ability of different repair mechanisms to process replication-dependent DNA breaks can influence the outcome and rate of genomic instability. Fragile sites are DNA regions frequently found at breakpoints of genomic instability events and show gaps and breaks following partial inhibition of DNA synthesis (Sutherland 1977). There are two types of fragile sites, common and rare. Common fragile sites account for 95 % of all fragile sites and are naturally present in the mammalian genome. Rare fragile sites account for less than 5% and arise as a consequence of DNA repeat expansion. Rare fragile sites are composed of AT rich sequences and are found at the break point of chromosome rearrangements that can be seen in tumours (Yunis and Soreng 1984). Fragile sites often contain trinucleotide repeat sequences (TNRs) that are highly conserved in mammals (Shiraishi et al. 2001). AT-rich TNRs are able to adopt unusual secondary structures such as hairpins and DNA triplexes (Wells 1996). Unusual DNA structures such as cruciforms, H-DNA (triplex), G-quartets, Z-DNA and S (slipped strand) DNA, which can occasionally form from the usual B-form (right handed double helical) structured DNA depends on symmetry, base composition, DNA supercoiling and ambient

conditions. DNA structure has an important role to play in providing direction for the selective association of architectural proteins such as histones. Functional evidence for the importance of unusual DNA structures in genomic instability is provided by the role of expanded triplet repeats associated with the human genetic diseases myotonic dystrophy and fragile X syndrome, suggesting that DNA hair pins explain aberrant DNA replication (Samadashwily et al. 1997). In order to undergo a structural transition, DNA must have some symmetry or structural regularity. The fact that eukaryotic DNA is rich in simple repeats could be responsible for human disorders characterised by expansions of microsatellites (Cox and Mirkin 1997). Unusual DNA structures inhibit DNA replication, DNA polymerisation stops in the middle because newly synthesised DNA reaches a repeat, its remaining single stranded segment folds back, forming a triplex behind and trapping DNA polymerase.

It is possible that hairpins, loops and triplexes at fragile sites could present an impediment to DNA synthesis and enhance replication stress, leading to DNA breaks or gaps (Glover et al. 1984) in an S-phase dependent manner (Casper et al. 2002). Fragile sites appear when replication progression is impaired mainly at a DNA stem loop or a triplex that impedes replication fork progression, presumably leading to replication fork stalling and formation of DNA breaks that are responsible for rearrangements.

1.1.5. Transcription as a Source of Genomic Instability

Highly transcribed DNA regions show high recombination frequencies. Transcription of a DNA sequence increases its frequency of recombination and mutation. These phenomena are referred to as transcription associated recombination (TAR) and transcription associated mutation (TAM) respectively and could be two different outcomes of the same intermediates. The whole basis behind the mechanism for TAM or TAR is the fact that chemically, single stranded DNA is less stable than

double stranded DNA. Evidence for this lies in the observation that the deamination of cytosine to uracil is 140-fold more efficient in single stranded DNA than double stranded DNA *in vivo* (Frederico et al. 1990). DNA strand opening occurs during transcription forming transient single stranded regions. Genotoxic agent-induced recombination can increase up to 100-fold when a gene is transcribed (Aguilera and Gomez-Gonzalez 2008). There is evidence that the non-transcribed strand is more vulnerable to damage than the transcribed strand. The transcribed strand forms an RNA DNA hybrid 8-9 nucleotides in length inside the transcription bubble within the catalytic pocket of RNA polymerase, leaving the non-transcribed strand single stranded. The nascent mRNA may hybridize with the transcribed strand leading to formation of R-loops. R-loops are linked to transcription-associated genomic instability in mutants defective for synthesis and processing of messenger ribonucleoprotein particles (Huertas and Aguilera 2003; Li and Manley 2005).

1.2 Telomeres and Telomerase

1.2.1. Introduction

Normal human BJ fibroblasts and BJ fibroblasts transfected with telomerase (Rubio et al. 2002) were studied throughout this project. Previous work has identified the protective effects of the human catalytic telomerase reverse transcriptase (hTERT) against Cr (VI)-induced genomic instability (Glaviano et al. 2006). Therefore, the role of hTERT was investigated in the response of human fibroblast cells to metal-induced bystander effects.

Telomeres are the structures that cap the ends of eukaryotic chromosomes and are critical for the maintenance of genome integrity. Telomere structure is highly conserved and in vertebrates comprises several kilobase pairs of double stranded DNA

containing the repetitive sequence TTAGGG with a 3′ single stranded extension 130-210 bases in length known as the 3′ overhang (Makarov et al. 1997). This overhang is folded round to form a t-loop, which is embedded in the double stranded part of the telomere (Griffith et al. 1999). Various proteins, collectively known as shelterin, play a key role in shaping the structure of vertebrate telomeres (reviewed in (de Lange 2005)). Six shelterin proteins have been identified in vertebrates: TRF1, TRF2, POT1, TIN2, TPP1 and Rap1.

Telomeres were first discovered in the 1930s when Barbara McClintock (McClintock 1938) and Herman Muller (Muller 1938) observed that broken chromosome ends always fused together but natural chromosome ends did not. More recent work has shown that telomeres are protected from the machinery that repairs DNA breaks. Telomeres thus provide a mechanism for cells to distinguish between the natural ends of chromosomes and chromosome breaks that require repair (Chan and Blackburn 2004). Telomeres serve at least three essential functions: (1) protecting natural chromosomal DNA ends from being inappropriately recognised as double-stranded breaks and therefore preventing the initiation of inappropriate DNA damage response (DDR), (2) protecting chromosomal ends from inappropriate enzymatic degradation and (3) preventing chromosomal end-to-end fusions (Deng and Chang 2007). Telomeres do not merely function to prevent chromosome fusions; they play a major role in the protection of chromosomes against the terminal erosion that occurs with every cell division.

As cells divide, telomeres become reduced to a critical short length because conventional DNA polymerases cannot complete the synthesis of chromosome ends. This is known as the end replication problem (Watson 1972). A gradual shortening of telomere repeats occurs over time, connecting telomeres to cellular senescence. In

culture, all cells will eventually become senescent; that is, they eventually stop dividing usually within 40-60 population doublings (Hayflick 1965). This activates cellular checkpoints similar to those induced by DNA damage. The limit on cell division is known as the Hayflick limit, which is activated when cells become senescent and stop dividing (Hayflick 1985). The telomere dependent senescence checkpoint is mediated by ATM, which activates p53 and p21.

1.2.2 Oxidative stress and telomere loss

It is not just the end replication problem that contributes to telomere shortening. There is considerable heterogeneity in both the number of divisions cells can undergo (Smith and Hayflick 1974) and in the rate of telomere shortening at each cell division (Martin-Ruiz et al. 2004), which indicates that other factors must play a role in telomere attrition. Reactive oxygen species (ROS) are present in the cell as a natural by-product of metabolism. Oxidative stress causes damage to DNA and can result in base deletion. If DNA is damaged in the telomeric region of the chromosome and not repaired before the next cell division, DNA replication will terminate at that locus and any telomere repeats beyond will be lost. Telomeres are more sensitive to oxidative damage than the rest of the genome, due to the presence of many GGG triplets which are a major target for ROS due to π-stacking between adjacent guanine residues (Saito et al. 1995; Oexle and Zwirner 1997). Telomeres also have a much lower rate of damage repair than other parts of the genome (Petersen et al. 1998). Thus, as damage accumulates in the main body of the chromosomes, it will be accumulating at a faster rate in the telomeres. By the time damage in the main body of the chromosomes has reached dangerous levels, the telomeres will already have become short enough to trigger senescence. This could be an adaptive response for protection against tumourigenesis. The longer a cell has been actively dividing and incurring damage, the greater this risk of cancer progression

becomes (Campisi 2003). By using telomeres to set a limit on cellular lifespan, the chances of this occurring are much reduced.

Inactivation of p53 and RB growth inhibitory pathways can breach the replication limit set by telomere shortening and cells can continue to proliferate. Further telomere erosion creates massive genomic instability and telomere dysfunction culminating in a period of cell death termed cellular crisis.

1.2.3 Telomerase

In order to avoid damage induced by the telomere loss that occurs with cell division, telomeric repeat sequences can be synthesised by Telomerase. Telomerase is a ribonucleoprotein complex containing an RNA component, TER, and a catalytic reverse transcriptase domain, TERT, which stabilizes telomere length by adding -TTTAGGG- repeats onto telomere ends using its intrinsic RNA as a template for reverse transcription in the 5′ to 3′ direction (Feng et al. 1995). In humans, TERT expression is down-regulated in most somatic cells (Kim et al. 1994) and limited to some stem cells, embryonic tissues and activated lymphocytes (Maser and DePinho 2002). However, the level of telomerase expression in somatic cells varies among species. For example, telomerase is not downregulated in mice (Prowse and Greider 1995).

Three sequence specific DNA binding proteins are recruited to chromosomal ends: the duplex telomere-binding proteins, TRF1 and TRF2, and the single-stranded TTAGGG repeat binding protein, POT1. These proteins are interconnected by three additional proteins: TIN2, TPP1 and RAP1 to form a functional complex that caps telomeric ends and prevents inappropriate activation of non-homologous end joining (NHEJ) and homologous recombination (HR) pathways at telomeres. Some gross chromosomal rearrangements arise as a result of telomere end-to-end fusions, a de novo

telomere addition, which should normally be prevented by Pif1, a 5′ -3′ DNA helicase. Perez-Rivero et al (Perez-Rivero et al. 2008) identified a direct relationship between telomerase deficiency and increased oxidative stress in mammals. Telomerase deficiency was found to cause a reduction in catalase activity in cultured murine embryonic fibroblasts (mEFs) isolated from telomerase deficient mice, which gave rise to an oxidant-antioxidant imbalance that promoted the overexpression of cytokines. Induction of oxidative stress is a very important factor in both metal-induced genomic instability and the radiation-induced bystander effect, described in detail in sections 1.3 and 1.4 respectively.

1.2.4. Telomere Loss – Role in Cancer

One proposed mechanism for chromosome instability in cancer is through loss of telomeres (Blackburn 2001). Growth arrest induced by short telomeres may be a potent anticancer mechanism. Telomeres are only maintained in specific subsets of human cells, shortening with age in most somatic cells due to lack of telomerase expression. An additional step in oncogenesis is required for ongoing proliferation of cancer cells and the escape from cellular senescence allowing cells to become immortal is the activation of telomerase or an alternative mechanism to maintain telomeres. In fact, telomerase is active in most human cancers (Kim et al. 1994). Maintenance of telomere stability is thought to be required for the long-term proliferation of tumours (Shay and Bacchetti 1997). When telomeres become critically short during crisis, extensive chromosome fusion occurs. Fusions are thought to be responsible for the chromosome rearrangements resulting from telomere loss in cancer (Rudolph et al. 2001). However, telomere loss is not confined to cells in crisis, as many early passage tumour cells demonstrate telomere instability (Fouladi et al. 2000). Sister chromatid fusion and prolonged periods of chromosome instability leading to spontaneous

telomere loss may provide a mechanism for chromosome instability in human cancer. Even when cancer cells actively maintain their telomeres, they continue to show a fundamental defect that promotes telomere loss. For example, an *in vivo* study by Chin et al (Chin et al. 2004) showed that breast cancer cells continue to experience telomere loss and anaphase bridges (an indicator of chromosome fusion) even after telomerase expression is restored.

An early step in carcinogenesis could involve unregulated cell growth due to overexpression of oncogenes, possibly leading to a high rate of DSBs. High DSB rates select for cells with defective cell cycle checkpoints, resulting in continued growth of cells with chromosomal instability. A high rate of loss of regions containing fragile sites indicates that problems with DNA replication maybe responsible for increased DSBs (Sutherland et al. 1998). These are known to have an increased frequency of stalled replication forks in cells under stress. Telomeres are similar to fragile sites in posing problems for DNA replication and may too be prone to stalled replication forks and increased DSBs in cancer cells (Sutherland et al. 1998). In precancerous lesions, aberrant stimulation of cell proliferation leads to DNA replication stress. This stress directly or through formation of DSBs activates DNA damage checkpoints. If a cell does not undergo cell cycle arrest or apoptosis, errors in DNA DSB repair could result. This could lead to allelic imbalances, targeted preferentially to fragile sites, which are most sensitive to replication stress. Telomere attrition could also contribute to checkpoint activation and genomic instability. Targeting tumour suppressor loci, e.g. p53 will eventually release the cells from the suppressive effects of DNA damage checkpoint pathways and facilitate tumour progression. According to Gorgoulis et al (Gorgoulis et al. 2005), cancer development is associated with DNA replication stress,

which leads to DNA DSBs, genomic instability and selective pressure for p53 mutations.

1.2.5. Telomere shortening and senescence

Telomere shortening is thought to lead to replicative senescence as a result of altered telomere state rather than telomere loss. Therefore, replicative senescence is induced by a change in the protected status of shortened telomeres rather than by a complete loss of telomeric DNA (Karlseder et al. 2002). This altered telomere state activates a DNA damage checkpoint response bringing about replicative senescence (d'Adda di Fagagna et al. 2003). The senescence checkpoint activated by telomere shortening is mediated by ATM, p53 and p21 in human cells (Herbig et al. 2004).

Cells that fail to senesce enter a crisis-like state involving extensive chromosome fusion, aneuploidy and cell death. Chromosome fusions occur as a result of telomere loss. Naturally, loss of telomeric DNA with each cell division leads to growth arrest signals, which limit the lifespan of normal cells by activation of two mortality checkpoints: M1 and M2. Cells that fail to senesce bypass M1. This occurs as a result of loss of cell cycle inhibitory signals such as functional RB or p53 proteins. These cells can extend their life spans, eventually reaching a second mortality checkpoint, M2, also known as crisis (Shay et al. 1991). Therefore, cells that overcome the M2 crisis checkpoint have acquired the ability to grow indefinitely via telomerase activation or to maintain telomere length sequences or Alternative lengthening of telomeres (ALT), a recombination mechanism for maintaining telomeres. Telomerase is active in many cancers and ALT has also been observed in tumours (Reddel and Bryan 2003). According to Shay et al (Shay et al. 2001), when telomeres become sufficiently short, cells enter an irreversible growth arrest called cellular senescence. In most cases,

cells become senescent before they can accumulate enough mutations to become cancerous.

1.3. Metal-Induced Genomic Instability

1.3.1 Introduction

Heavy metals can accumulate in human tissue as a result of exposure to chemicals in the environment, corrosion of medical and dental prostheses, agricultural contamination of ground water and cigarette smoke (Coen et al. 2001). A wide variety of metals including arsenic, cadmium, chromium, cobalt, nickel and vanadium are known carcinogenic agents (Leonard et al. 2004). Genomic instability induced by metals is marked by reduced clonogenic survival in the progeny of treated cells several generations post-exposure (Coen et al. 2003). Cells begin to show high levels of non-clonal cytogenetic aberrations, reproductive failure and induction of a persistent level of cytogenetic abnormalities. It has been observed for all instability-inducing agents, that low dose exposure has the most important consequences (Coen et al. 2003). Low dose exposure allows greater survival of cells to express damage. Failure to respond to this damage by death increases the probability that the unstable cell will survive and divide. Cytogenetic analysis of metal-exposed cells reveals the persistent induction of tetraploidy, which is not seen post-irradiation (Coen et al. 2003). Aneuploidy occurs as a result of failed segregation of replicated chromosomes between progeny cells, resulting in the production of daughter cells with an abnormal chromosome number (Aardema et al. 1998).

1.3.2. Chromium-Induced Genomic Instability

The work presented in this thesis focuses on the effects of Chromium (VI) on human fibroblast cells. Chromium is a naturally occurring heavy metal, which mainly

exists in the environment in trivalent (Cr (III)) or hexavalent (Cr (VI)) forms (Bryant et al. 2006). The International Agency for Research on Cancer (IARC) classifies compounds of chromium (VI) as group 1 carcinogens, which means they are carcinogenic to humans. The importance of chromium exposure in human health monitoring was highlighted in a report published by the Department of Health on 9th February 2006. This report, 'Biological Effects of Wear Debris Generated from Metal on Metal Bearing Surfaces: Evidence for Genotoxicity' discusses evidence for the dispersion of metallic material from hip replacements. This demonstrates that the committee on mutagenicity of chemicals in food, consumer products, and the environment has identified a concern regarding the biological effects of particulate debris from metal prostheses (Department of Health (2006)). A study carried out by The Rizzoli Orthopaedic Institute showed a highly significant release of Cr and Co ions, detected in serum of patients with metal-on-metal bearings compared with ceramic on-ceramic bearings (Savarino et al. 2006). This supports earlier findings of a study by the Bristol Implant Research Centre (Doherty et al. 2001). These studies indicate that exposure to metals is a widespread problem and one that is facing more and more people as orthopaedic joint replacements are becoming increasingly common with approximately one million artificial hips implanted annually, worldwide (Malchau et al. 1993).

1.3.3. Intracellular Reduction of Cr (VI)

The toxicity of metal compounds largely depends on their bioavailability; i.e. their ability to enter cells and interact with cellular macromolecules. Plasma membranes are impermeable to Cr (III). Therefore, cellular uptake favours Cr (VI) as chromates are isostructural with physiological sulphate and phosphate ions, enabling Cr (VI) to readily enter cells via anion transport channels (Alexander and Aaseth 1995). Mammalian cells

are capable of massive accumulation of Cr (VI), with cellular levels 10–20 times above those outside the cell within 3 h (Reynolds et al. 2007). A 24-h long incubation can lead to 100-fold or higher accumulations (Messer et al. 2006).

Cr (VI) is a pro-carcinogen that by itself at physiological pH and temperature is completely unreactive towards DNA. In biological systems, however, Cr (VI) undergoes a series of reduction reactions yielding thermodynamically stable Cr (III). This reduction is the activation event that is responsible for the generation of genotoxic damage and other forms of toxicity (Hamilton and Wetterhahn 1986; Levy and Venitt 1986). Cr (VI) is reduced to its lower oxidation states by direct electron transfer in three one-electron steps via Cr (V) and Cr (IV) to the thermodynamically stable form of Cr (III). Unlike the majority of other human pro-carcinogens, Cr (VI) metabolism in mammalian cells does not depend on enzymes and relies on electron transfer by enzymatic and non-enzymatic low molecular weight reductants and non-protein thiols such as glutathione, ascorbate, and cysteine (Shi et al. 1999; Zhitkovich 2005). The intracellular reduction of Cr (VI) produces many reactive intermediates, including Cr (V), Cr (IV), Cr (III), reactive oxygen species (ROS) and other reactive free radicals. Production of these reactive intermediates is an important characteristic of Cr (VI)-induced cellular responses. Cr (V), Cr (IV), and Cr (III) are all able to contribute to •OH- radical formation from H_2O_2 via a Fenton-like reaction, with Cr (V) acting as a catalyst with H_2O_2.

$$\mathbf{Cr(V) + H_2O_2 \rightarrow Cr(VI) + \bullet OH + OH^-}$$ **(Shi et al. 1994)**

In addition, both Cr (VI) and Cr (V) are capable of generating hydroxyl radicals via the Haber-Weiss cycle (Ding and Shi 2002)

$$\mathbf{O_2 + H_2O_2 \xrightarrow{Cr(VI)Cr(V)} O_2 + \bullet OH + OH^-}$$ **(Ding and Shi 2002)**

Excessive quantities of free radicals and ROS generated by these reactions can cause damage to proteins, lipids and DNA, interacting with guanine residues to generate a range of products such as 8-oxo-2'-deoxyguanosine (8-oxo-dG) leading to a state known as oxidative stress (Dizdaroglu 1991; Leonard et al. 2004). Through ROS mediated reactions, Cr (VI) causes apoptosis, growth arrest, DNA damage, activation of nuclear transcription factors, oncogene expression and activation of members of the MAP kinase (mitogen activated protein kinase) family (Leonard et al. 2004). MAP kinases play an important role in signal transduction pathways activated by a range of stimuli and mediate a number of physiological and pathological changes in cell function (Camps et al. 2000). Therefore, massive production of ROS during the reduction of Cr (VI) in the cell is one of the most important damages caused by extraneous Cr (VI). The importance of chromium reduction in genotoxicity was highlighted in a study by Hamilton et al (Hamilton and Wetterhahn 1986), using chick embryos, in which intra-embryonic tissue differences were observed. Chromium (VI) induced transient DNA cross-links in liver, whereas in blood cells chromium (VI) induced DNA double strand breaks, that persisted for 24 hours post treatment. These intra-embryonic tissue differences in chromium (VI)-induced DNA damage may be a result of the differences in glutathione, cytochrome P-450, other pathways of chromium (VI) metabolism or differences in chromatin organisation which exist between liver and blood cells.

1.3.4. Cr-Induced DNA lesions

Metals can affect cellular processes in several ways, through direct binding to cellular components via ionic or coordinating bonds. The generation of numerous reactive species by different mechanisms of *in vivo* reduction of Cr (VI) to Cr (III) may result in various types of DNA lesions. Many types of DNA damage have been observed with Cr (VI), including DNA interstrand cross-links (Bridgewater et al. 1994a;

Zhitkovich et al. 1995; Zhitkovich 2005), DNA-protein cross-links (Xu et al. 1994), DNA strand breaks (Reynolds and Zhitkovich 2007), Cr—DNA adducts (Xu et al. 1994; Zhitkovich et al. 1995; Singh et al. 1998; Zhitkovich 2005) and 8-oxo-2'-deoxyguanosine (8-oxo-dG) (Sugden and Martin 2002).

Chromium-induced DNA adducts and DNA-protein crosslinks are important factors to consider in Cr-induced genomic instability (Xu et al. 1994). Small Cr-DNA adducts are the most abundant form of Cr (VI)-induced genetic lesions in mammalian cells (Zhitkovich 2005). Reductive metabolism of Cr (VI) always yields Cr (III), which is able to form stable complexes with subcellular components. This ability to form stable complexes with macromolecules and the presence of six coordination sites enables Cr (III) to generate cross-links with other molecules (Zhitkovich 2005). Ternary Cr (III) adducts, which include Cr (III) atom bridging DNA and small cellular molecules represent the major form of Cr-DNA adducts and interstrand DNA crosslinks in Cr (VI)-exposed mammalian cells (Zhitkovich et al. 1995). Four major forms of ternary adducts are glutathione-Cr-DNA, cysteine-Cr-DNA, histidine-Cr-DNA, and ascorbate-Cr-DNA complexes. Cr-DNA–protein cross-links have significant genotoxic potential as they represent an impediment to replication and transcription processes culminating in replication fork arrest (Zhitkovich et al. 1998). Correlation between the presence of interstrand DNA cross-links (binary Cr (III)−DNA adducts) and polymerase inhibition led to the conclusion that these lesions may be responsible for the blockage of DNA replication *in vitro* (Bridgewater et al. 1994a). Polymerase arrest sites were preferentially observed at positions one nucleotide upstream of guanine residues, but templates with high numbers of adducts showed more uniform distribution of replication-blocking damage (Bridgewater et al. 1994b).

In Cr-treated cells, increased production of H_2O_2 could occur as a result of mitochondrial damage or elevated activity of NADPH oxidase. Damage to the base component of DNA can involve either loss of bases (i.e., production of abasic sites) or chemical modifications with the retention of altered bases in DNA duplexes. Cr (III) ternary complexes are mutagenic in human fibroblasts, predominantly resulting in single base substitutions (Voitkun et al. 1998).

An interesting feature of Cr (VI)-associated cancers is the presence of microsatellite instability (Hirose et al. 2002), indicating a loss of functional mismatch repair (MMR) (Modrich 2006). The absence of MMR leads to the inability of cells to correct replication errors, and these cells accumulate high levels of mutations, especially in the areas of nucleotide repeats known as microsatellites. Thus, chromate-associated cancer cells express a mutator phenotype caused by the loss of MMR. Once MMR is inactivated, the subsequent acquisition of mutations in the critical growth-controlling genes is greatly accelerated since these cells maintain high rates of random mutagenesis and no longer need continuous exposure to Cr (VI) for additional mutagenic events. This specific form of genomic instability induced by Cr (VI) is uncommon for other lung carcinogens. The reason for this may lie in the active role that MMR plays in the toxic and genotoxic effects of Cr (VI) (Peterson-Roth et al. 2005). Repair of lesions at stalled replication forks by MMR results in formation of a DSB as part of the repair process. The entire MMR complex was required for the processing of Cr-DNA adducts into highly toxic DSBs. The damage-promoting effects of MMR extended to the full range of Cr (VI) concentrations from very low nontoxic (<1 μM) to highly toxic doses (>90% clonogenic lethality) (Reynolds and Zhitkovich 2007). These DNA double strand breaks induce a prolonged G2 arrest and can lead to centrosome amplification and spindle assembly checkpoint bypass, which both contribute to numerical

chromosome instability, possibly leading to neoplastic transformation and cancer. If DNA double strand breaks remain unrepaired, the resulting structural chromosome instability also contributes to cancer progression; summarised by Holmes et al (Holmes et al. 2008). DSBs were preferentially found in G2. This cell cycle stage specificity of DSB production was caused by the requirement for Cr-damaged DNA to pass through S-phase in order for the MMR system to activate aberrant processing (Reynolds et al. 2007). p53 has been shown to play no significant role in the toxicity of Cr (VI) (Reynolds and Zhitkovich 2007). Metal-DNA adducts could be considered as biomarkers of exposure to toxic and carcinogenic metals such as Cr, in cultured cells, animals, and humans (Singh et al. 1998).

A chromium-mediated pathway for DNA damage may result in direct interaction of chromium with DNA, producing Cr-DNA binding, DNA-protein cross-links and DNA interstrand cross-links. However, a free radical-mediated pathway may result indirectly in oxidative damage, producing DNA single-strand breaks and 8-oxo-dG adduct formation.

1.4. Bystander Effects

1.4.1 Introduction

Radiation exposure occurs naturally from terrestrial rocks and the atmosphere. Due to its uses in industry and energy production and its military and medical applications, radiation has become an essential component of everyday life. This widespread use of radiation presents hazards associated with exposure to radiation and research has been carried out to understand and eventually minimize the deleterious effects of exposure to radiation. Ionizing radiation is known to act as a potent carcinogen and DNA damaging agent (Morgan 2003). The radiation-induced bystander

effect is characterised by the occurrence of biological effects in unirradiated cells as a result of receiving signals from irradiated cells or their culture medium (Kadhim et al. 2004). This challenged the paradigm of radiation biology that biological effects were caused by the passage of energy directly through the 'hit' cell. Bystander effects present a major concern to the field of radiation protection as they represent an effective amplification of the deleterious effects of cytotoxic agents. These effects include the introduction of mutations (Zhou et al. 2000), micronuclei (Prise et al. 1998), sister chromatid exchange (Nagasawa and Little 1992), changes in patterns of gene expression (Ghandhi et al. 2008), transformation (Sawant et al. 2001) and cell death (Morgan et al. 2002).

Early evidence for radiation exposure causing biological effects in unirradiated cells emerged in the 1950s when Parsons et al (Parsons et al. 1954) reported changes in sternal bone marrow, characterised by a reduction in cellularity in all cells of the granulocytic series after children received radiation to their spleens for the treatment of leukaemia. The first direct observation of radiation-induced bystander effects was reported in 1992. Nagasawa and Little (Nagasawa and Little 1992) examined the induction of sister chromatid exchanges (SCE) in Chinese hamster ovary cells irradiated with very low doses of alpha-particles (0.31 mGy). An enhanced frequency of SCE was observed in 30% of cells even though fewer than 1% of cell nuclei were actually traversed by an α-particle.

Numerous approaches have been used to study radiation-induced bystander effects. These include exposing donor cells to α-particles (Han et al. 2007), exposure to X-rays (Anzenberg et al. 2008), γ-rays (Baskar et al. 2007) and UV (Dahle et al. 2005) radiation. The study of bystander effects requires some degree of spatial separation

between directly irradiated cells and bystander cells to ensure no direct irradiation occurs in bystander cells. To this end, conditioned medium generated from directly irradiated cultures can be transferred to unirradiated bystander cells (Vines et al. 2009). Directly irradiated cells can also be co-cultured with unirradiated bystander cells after irradiation (Shao et al. 2004; Yang et al. 2005) or simultaneously, using a computerised system that automatically records the positions of irradiated cells targeted with a microbeam (Shao et al. 2004; Sokolov et al. 2005).

1.4.2. Microbeams

It has long been considered that the nucleus is the target for the important biological effects of radiation, and that the consequent genetic alterations arise from radiation-induced DNA damage. However, the development of precision microbeam irradiators has permitted the study of the effects of radiation localized to specific parts of the cell. Direct nuclear irradiation is not required for the induction of important genetic effects of irradiation; and cytoplasmic irradiation results in significant increase in mutation frequency in mammalian cells (Wu et al. 1999). Targeted cytoplasmic irradiation of a single human glioblastoma cell in a population with a single $^{3}He^{2+}$ ion has been shown to significantly increase micronucleus induction in over 36% of the unirradiated bystander population one hour post irradiation, similar to the effects of a single direct nuclear traversal (Shao et al. 2004), indicating that nuclear irradiation in target cells is not necessary for induction of bystander responses in unirradiated cells.

The value of partial and microbeam irradiation of cells has been appreciated for a long time. Classical studies by Zirkle and Bloom (Zirkle and Bloom 1953) used a microbeam of ionizing particles to traverse a selected portion of an individual cell, which he described as being a widely applicable method with which small fractions of average-sized cells could be irradiated. This enabled the analysis of the normal

functions of various cell parts by selectively altering them. In addition to these functionality investigations, irradiation of a highly localized cellular region allows questions regarding cell-to-cell communication to be addressed directly. Crucially, microbeam techniques allow selected cells or subcellular components in an irradiated population to remain *unirradiated* allowing direct investigation of bystander effects. Many modern studies of radiation-induced bystander effects have made use of charged particle microbeams

A number of facilities have been developed worldwide that use microirradiation techniques to study radiation damage. Among these is the Gray Cancer Institute microbeam, one of the first such operational facilities in Europe (Folkard et al. 2009). In addition, the Columbia university microbeam has allowed the delivery of a specific number of alpha particles to the cytoplasm of individual cells without nuclear exposure. The original objective of the Columbia University microbeam was to study 'the ability of single α-particles to produce transformational and mutational events in mammalian cells irradiated through the nucleus' (Randers-Pehrson et al. 2001).

Biological interest in the microbeam stems from the potential to define the ionizing energy absorbed by a cell in terms of space, time and quantity. Many studies have taken advantage of the ability radiation experiments afford to deliver known quantities of radiation to known cells in a population. Included among these was that of Prise et al (Prise et al. 1998), who used alpha particles to irradiate four cells in a population with 15 α-particles and observed that the fraction of micronucleus-containing cells was 0.03 compared to 0.008 in controls. Sokolov et al (Sokolov et al. 2005) delivered 2 or 20 individual α-particles to selected cell nuclei, the locations of which were automatically recorded by an imaging system for computer driven

irradiation. Bystander cells were stained with a vital dye CellTracker CMRA and analysed for DNA double strand break induction using the γ-H2AX focus formation assay. Without irradiation, 7% of CMRA-stained bystander cells contained four or more foci per cell. 18 hours post irradiation in cells targeted with 20 α-particles, over a quarter of the bystander population contained four or more foci per cell, a 3.7-fold increase in DNA double strand break induction.

Two main models have been suggested to explain the mode of transmission of bystander responses, communication via gap junction intercellular communication (GJIC) and via medium-based soluble factors (Kadhim et al. 2004).

1.4.3 Bystander effects and Gap Junctions

Azzam et al (Azzam et al. 2001) explored the role of gap junctional intercellular communication in transmission of bystander responses using exposed cells compromised in their ability to perform GJIC in the presence of gap junction inhibitors. Cells were exposed to low fluences of α-particles where only a small fraction of nuclei were traversed. The endpoints studied were increased Ser-15 phosphorylation of p53 and induction of the stress-inducible $p21^{Waf1}$, which were found to correlate with the induction of DNA damage as measured by micronucleus formation. This study presented evidence for the involvement of connexin43-mediated intercellular communication in the transmission of damage signals to non-irradiated cells. Only gap junction-proficient cells had $p21^{Waf1}$ protein in neighbouring cells far exceeding the fraction of cells whose nucleus has been traversed. Hu et al (Hu et al. 2005) irradiated AGO1522 fibroblasts with low doses of α-particles from a ^{421}Am source and studied γ-H2AX focus formation. At a dose of 1 cGy, 9.2% of nuclei were estimated to have been traversed by at least one charged particle. γ-H2AX focus formation was observed in

over 42% of the cells, which was reduced to 19% if the gap junction inhibitor lindane was added prior to irradiation.

1.4.4 Medium Transmissible Effects

Microbeams are valuable tools in the study of bystander responses as they can irradiate certain subsets of cells within a population, allowing specific cells to remain unirradiated. Alternatively, a medium transfer technique can be used in which, one whole cell population is directly irradiated with a broad beam source, and conditioned medium, termed irradiated cell conditioned medium (ICCM) is harvested and filtered before being transferred to non-irradiated bystander cells. This approach has been widely used and has the advantage of effective separation of target cells and bystander cells. For example, Yang et al (Yang et al. 2005) used a transwell co-culture system allowing irradiated and non-irradiated cells to share the same medium without coming into direct contact. X-irradiation at doses of 0.1 Gy and 1 Gy was found to induce medium-mediated increases in micronuclei, $p21^{waf1}$, reactive oxygen species (ROS), clonogenic survival and γ-H2AX foci in bystander AGO1522 normal human fibroblasts. GJIC was not involved because the irradiated and unirradiated cells never came into direct cell-cell contact with each other. Novel mechanisms for transmission of bystander effects have been proposed by Fakir et al (Fakir et al. 2009), which describes the bystander component as a sequence of two distinct processes: triggering of signal emission from irradiated cells and the response of non-irradiated, recipient cells. Their model suggests signal transmission through the medium rather than gap junctions and that for medium transfer experiments, a moderate increase in medium volume should have about the same effect as a moderate decrease in the fraction of irradiated cells. Baskar et al (Baskar et al. 2007) showed that bystander effects are alleviated by the dilution of conditioned medium obtained from irradiated cell cultures. It is possible that

cell density is important for bystander responses since this factor may influence the relative concentration of soluble factors released from the irradiated cells (Mothersill and Seymour 1997). Low linear energy transfer (LET) microbeam irradiation of the human keratinocyte HPV-G cell line with 3.2MeV protons delivered directly to the nucleus resulted in induction of early apoptotic events including mitochondrial membrane potential depolarisation, increased ROS, bcl-2 expression and release of cytochrome c in bystander HPV-G cells neighbouring those directly traversed through the nucleus with 1 or 10 protons. It is unlikely that gap junctional intercellular communication was involved, as the directly irradiated and bystander cells were effectively two distinct populations separated by around 6 mm (Lyng et al. 2006a). Therefore, t is also possible that in the absence of close cell-to-cell contact, soluble factors may play more of a role in bystander signalling than gap junction communication. However, it is still possible that medium transmissible factors still contribute to any bystander effects observed in gap junction studies as both the directly irradiated and bystander cells grow in the same culture medium.

1.4.5 Linear Energy Transfer (LET)

An important aspect underlying bystander effects is the dependence on radiation quality, or linear energy transfer (LET). LET is defined as the average amount of energy lost per unit of distance travelled. Low LET radiation includes X- and γ- rays. Alpha particles and neutrons are high LET, because they lose their energy within a few microns; and the density of ionisations is high. LET dependency has important consequences for biological dosimetry. High LET irradiation using α-particles, is sufficient to induce a bystander response in fibroblasts (Zhou et al. 2000; Azzam et al. 2001; Belyakov et al. 2001; Shao et al. 2003). Conflicting reports exist as to whether low LET irradiations such as X- or γ-rays are able to elicit a bystander response in

fibroblast cells. The co-culture study by Yang et al (Yang et al. 2005) was among the first to provide direct evidence that low LET, X-irradiation can induce a medium-mediated bystander response in bystander normal human fibroblasts. Mothersill and Seymour (Mothersill and Seymour 1997) showed that cell killing as a bystander effect extends to low-LET irradiation. This study showed that supernatants from immortalized HaCaT keratinocytes that were exposed to ^{60}Co γ-rays induced apoptosis and reduced the cloning efficiency of unirradiated cells. Perhaps exposure to high concentrations of high LET radionuclides inhibits cellular capacity to generate bystander signals.

1.4.6 Cell Type and Endpoint Dependence of Bystander Responses

The expression of non-targeted effects is influenced by cell-type and genetic factors and in some cases by the type of radiation exposure. There are many inducers of bystander effects, which have many possible consequences in different cell systems. The precise consequence of a specific trigger might depend on various factors. For example, medium transfer experiments have shown that signal production by an irradiated cell and response to that signal by a recipient cell can be distinguished as separate processes (Mothersill and Seymour 1997; Mothersill et al. 2001). For example, Vines et al (Vines et al. 2008) used a medium transfer method to expose three individual cell lines: E89, CHO-K1 and HPV-G to conditioned medium from each of the three variants and observed that they responded differently. Medium generated from irradiated HPV-G and CHO-K1 cells reduced the surviving fraction of those cell lines but had no effect on E89 cells. Conditioned medium derived from irradiated E89 cells had no effect on the surviving fraction of any of the three cell lines. Each cell line seemed able to generate its own characteristic bystander signal, their responses reflecting the varying toxicity of bystander signals generated from different sources.

Both signal production and response seem to be p53-independent, although there is evidence that the response in fibroblast cells might require a functional p53 pathway. For example, Azzam showed involvement of the p53 damage-response pathway in cells that were exposed to very low fluences of α-particles (1cGy) (Azzam et al. 2002). However Zhang et al (Zhang et al. 2008) showed a lack of p53 involvement in bystander γ-H2AX focus formation. A possible explanation for these conflicting reports may lie in the fact that different endpoints in bystander cells require different signal transduction mechanisms. Another factor to consider is that the presence of wild-type p53 facilitates the transduction of the signal, but that in its absence, alternative p53-independent pathways could transduce the signal.

Bystander responses that occur following exposure to various forms of radiation have been shown to be very cell-type specific. For example, Mothersill and Seymour found that low LET -irradiation of epithelial cells could elicit a bystander response in unirradiated epithelial cells with no effect in fibroblasts whereas γ-irradiation of fibroblasts could not elicit a response in unirradiated cells of the same type (Mothersill and Seymour 1997). Groesser et al (Groesser et al. 2008) exposed cells to 20 MeV nucleon nitrogen ions, 968MeV .nucleon iron ions and 575MeV nucleon iron ions (both high and low LET radiation). Conditioned medium was transferred to recipient cells that were DNA repair proficient and deficient hamster and human cell lines. Early cytogenetic endpoints were studied including DSBs, γ-H2AX foci, chromatid breaks and micronuclei. No bystander effects were discovered. The investigators attributed this lack of effect to the epigenetic status of the cell lines and concluded that in addition, the precise culture conditions and medium supplements used were critical factors for inducing the bystander response.

1.4.7. Dose

Bystander cells are the population at risk from the delayed effects of radiation exposure including genomic instability and cancer progression but at low doses, below 0.5 Gy, the actual radiation dose seems to be irrelevant (Mothersill and Seymour 2004). Belyakov et al (Belyakov et al. 2001) observed radiation-induced bystander effects in non-hit cells neighbouring those targeted through the nucleus with individual charged particles when only a single cell in a population was targeted with a single helium ion, independent of the dose delivered to the target cell. Sokolov et al (Sokolov et al. 2005) observed only a slight increase in bystander γ-H2AX foci when directly-irradiated target cells had been exposed to 20 rather than 2 α-particles, indicating that radiation dose was not a critical parameter. This lack of linear dose response in bystander cells was first described by Seymour and Mothersill (Seymour and Mothersill 2000) who reported that bystander clonogenic death in a human keratinocyte cell line is predominant at doses below 0.5 Gy when conditioned medium is harvested from donor cells approximately 1 hour post irradiation. Lyng et al (Lyng et al. 2002) used the same human keratinocyte cell line and reported that there was no significant difference in bystander effects induced by medium conditioned in cells one hour after irradiation at 0.5 Gy or 5 Gy for loss of mitochondrial membrane potential and increased reactive oxygen species. Similar observations have been reported following non-nuclear irradiations. Bystander micronucleus induction in human glioblastoma cells irradiated with a $^{3}He^{2+}$ ion through the cytoplasm was independent of the number of cells traversed and increased at the same level whether 1 or 10 cells were targeted (Shao et al. 2004). The influence of radiation dose on bystander effects varies depending on the endpoint under investigation, but on the whole, is thought to predominate following low dose exposure.

1.4.8. Involvement of Oxidative stress

Mitochondrial function in donor cells has been implicated to play a role in bystander effects. Tartier and colleagues (Tartier et al. 2007) treated HeLa cells with ethidium bromide to deplete mitochondrial DNA to less than 5% of the normal level to show that mitochondrial dysfunction impairs bystander signalling. Co-culture of normal HeLa cells with these pseudo ρ^0 cells showed that bystander induction of 53BP1 foci, which parallel DNA DSB levels did not occur in ρ^+ HeLa cells if ρ^0 cells were irradiated through the nucleus or the cytoplasm but did occur in ρ^0 cells if ρ^+ HeLa cells were irradiated. Chen et al (Chen et al. 2009b) treated normal (ρ^+) and mitochondrial DNA depleted (ρ^0) human-hamster hybrid cells with α-particles from a ^{421}Am source at a dose rate of 1.1 cGy/s. ROS production, mutation and delayed cell death were assayed in bystander ρ^0 and ρ^+ cells exposed to conditioned medium from irradiated donor cells for 24 hours. γ-H2AX focus formation was assayed in recipient AG1522 normal human diploid skin fibroblasts exposed to conditioned medium from ρ^0 and ρ^+ irradiated donor cells for 30 minutes. Significantly higher ROS formation, γ-H2AX focus formation and mutation induction were observed following medium transfer from irradiated ρ^+ cells than from ρ^0 donor cells. There was no immediate difference in colony formation between directly treated ρ^0 and ρ^+ donor cells. However, following a seven day incubation of recipient cells with conditioned medium, there was a significant reduction in surviving fraction in the progeny of recipient cells exposed to ρ^+ conditioned medium but not ρ^0 conditioned medium, which had no significant effect on surviving fraction of recipient cells. These results indicate that irradiated cells initiate bystander signalling via a mitochondria-dependent pathway, which stimulates upregulation of ROS in bystander cells. Since mitochondria are the main source of energy production and

generators of free radicals in cells, particularly under stressful conditions, they are the prime target for the source of these radical species. There is recent evidence that increases in mitochondrial mass (Nugent et al. 2007) are induced in directly irradiated human keratinocytes exposed to a 5Gy dose of gamma-rays or in bystander cells exposed to conditioned medium from irradiated cells.

Upregulation of ROS leads to increased induction of bystander DSBs, which if incorrectly repaired, may contribute to chromosomal instability and delayed genomic instability in bystander cells. Therefore, mitochondria-dependent bystander signalling mechanisms are at least partly responsible for the genotoxicity induced by radiation-induced bystander effects. In the transwell system used by Yang et al (Yang et al. 2005), DCFH-DA (2'7'-dichlorodihydrofluorescein diacetate) was used to detect oxidative stress in bystander cells. ROS levels were unchanged 6 hours after irradiation but increased 2-fold 30 hours after radiation. These findings indicate that bystander cells are under oxidative stress after co-culture with irradiated cells. At the same time, the ROS scavengers Cu-Zn superoxide dismutase (SOD) and catalase were found to decrease the induction of micronuclei, $p21^{waf1}$ and γ-H2AX foci. However, ROS scavengers did not have any effect on the loss of cloning efficiency of bystander cells. One possibility for this difference is that, in addition to ROS, irradiated cells might release other soluble factors into the medium that contribute to bystander cytotoxicity. ROS appear to participate in the signalling pathway for some end points, but the existence of other signalling mechanisms is indicated, especially for cell killing. Lyng et al (Lyng et al. 2002) showed that long-term irreversible increases in reactive oxygen species occur in the progeny of irradiated cells and that the effect of medium from progeny was the same as the initial effect and did not diminish with increasing passage number, even up to 35 population doublings. These data suggest that initiating events in

the cascade that leads to apoptosis are induced in unirradiated cells by a signal produced by irradiated cells and that this signal can still be produced by the progeny of irradiated cells for several generations. Many studies have shown the importance of ROS in bystander effects by demonstrating that inhibition of ROS blocks radiation-induced bystander signalling. In the system used by Tartier et al (Tartier et al. 2007), induction of bystander 53BP1 foci was prevented by treating cells with DMSO, a free radical scavenger, which shows that the bystander signal is mediated through the production of ROS that, in turn, induces an increase in 53BP1 focus formation in bystander cells. In the system used by Chen et al (Chen et al. 2009b), pretreatment of donor cells with DMSO decreased bystander γ-H2AX foci to control levels.

Nitric oxide (NO) has also been implicated to play a role in bystander responses (Han et al. 2007). In one study, irradiated cells were shown to secrete certain DSB-inducing factors into the medium as early as 2.5 min post-irradiation, and this resulted in the excessive DSBs in non-irradiated medium recipient cells (Han et al. 2007). By treating the cells with L-NMMA to inhibit the activity of nitric oxide synthase (NOS), induction of bystander DNA double strand breaks was significantly reduced, suggesting NO as a possible signalling molecule. Shao et al (Shao et al. 2004) showed that incubation of microbeam irradiated populations with c-PTIO, a NO scavenger completely abrogated all bystander micronucleus induction. In addition, Tartier et al (Tartier et al. 2007) showed that bystander 53 BP1 focus formation is mediated through the production of NO as well as ROS. Treatment of microbeam irradiated HeLa cell populations with aminoguanidine, a NO synthase inhibitor suppressed 53BP1 induction to near zero.

1.4.9 Cytokines

ROS are known to be released from irradiated cells, possibly into growth medium or transferred between cells in close contact via gap junctional intercellular communication. However, since free radicals of ROS and NO have relatively short half lives, they are unlikely candidates for the longer term signalling factors present in conditioned medium derived from directly irradiated cells in medium transfer studies. Cytokines such as interleukin-8 can be induced by ROS and NO/NOS (Xiong et al. 2001) providing a possible link between different factors potentially involved in bystander signalling. Shao et al (Shao et al. 2008a) detected TGF-β1 in conditioned medium derived from irradiated T89G glioblastoma cells. Inhibition of NO synthase reduced TGF-β1 in conditioned medium to control levels indicating that TGF-β1 is a downstream product of NO generation in irradiated cells. TGF-β1 was found to contribute to the generation of NO and micronuclei in bystander cells. Further evidence that TGF-β1 production is mediated by NO was provided using DEANO, which reliably generates NO in aqueous solution leading to increased levels of TGF-β1(Shao et al. 2008b).

Production of cytokines, ROS and free radicals is a feature of inflammatory responses and has the potential for persistent induction of damage (Lorimore et al. 2001). The cyclooxygenase-2 (COX-2) pathway is involved in mediating the cellular response to inflammation. The role of COX-2 in bystander signalling was studied by Zhou et al (Zhou et al. 2005), who showed that mutagenesis at the *HPRT*⁻ locus in normal human lung fibroblast (NHLF) cells irradiated with a 0.5-Gy dose of α-particles using the track segment beam was reduced by >6-fold after inhibition of COX-2 activity by NS-398. MAP Kinase signalling cascades are activated by cytokines and expression of *COX-2* is dependent on the activation of ERK by phosphorylation. Western blot

analysis demonstrated strong up-regulation of phospho-ERK levels in both α-irradiated and bystander cells 4 h after treatment. Because NHLFs respond to different forms of stress by up-regulation of the production of various cytokines, anti-TNF-α monoclonal antibody was used to determine the probable role of TNF-α in bystander activation. Addition of anti-TNF-α significantly blocked ERK activation in bystander cells leading to suppression of COX-2 expression (Zhou et al. 2005). Addition of PD 98059, a specific inhibitor of MAPK kinase (MEK)-ERK immediately after irradiation for a period of 4 h ERK activation was suppressed in both α-particle-irradiated and bystander cells leading to an increase in the surviving fraction of bystander cells, almost to control levels (Zhou et al. 2005).

NF-κβ is a transcription factor critical for regulation of many signalling genes including *COX-2* and inducible nitric oxide synthase *(iNOS)*. Azzam et al (Azzam et al. 2002) exposed confluent human skin fibroblasts to low fluences of α-particles and observed a rapid upregulation of NF-κβ, c-JUN-N-terminal kinase (JNK) and ERK suggesting a role for these stress-inducible signalling pathways in bystander cells.

In later work, Zhou et al (Zhou et al. 2008) explored the role of NF-κβ in bystander responses. NF-κβ–binding activity was found to be up-regulated in both directly α-particle irradiated and bystander cells. Treatment of cells with Bay 11-7082, a pharmacologic inhibitor of IKK–NF-κβ activation 2 hours before α-particle irradiation led to suppression of NF-κβ activity and down-regulation of COX-2 and iNOS expression levels in both directly irradiated and bystander fibroblasts. Micronucleus formation and induction of mutations at the *HPRT* gene locus, which were readily detected in directly irradiated and bystander cells were significantly reduced when NF-κβ activity was inhibited by Bay 11-7082 (Zhou et al. 2008). Another

mediator of COX-2 expression is TNF-α, which is induced after α-particle irradiation of NHLF. Addition of inhibitory anti-TNF-α to the cell culture medium substantially decreased levels of NF-κβ and COX-2 expression in both irradiated and especially, in bystander cells and also led to a significant increase in bystander clonogenic survival (Zhou et al. 2008).

1.4.10 Transcript Profiles

Another feature of radiation-induced bystander effects is the introduction of changes in patterns of gene expression. Rzeszowska-Wolny et al (Rzeszowska-Wolny et al. 2009) observed that human melanoma cells cultured in the medium of X-irradiated cells had similar transcript profiles to directly irradiated cells. Conditioned medium was harvested 1 hour post irradiation and transferred to recipient cells for 36 hours. Directly irradiated cells were cultured for 36 hours post treatment. Sampling was carried out after 36 hours, corresponding to more than one division cycle to determine any long-term effects that could be relevant in terms of radiotherapy. Genome-wide transcript profiles were investigated for changes that were common to treatment by both ICCM and direct irradiation. Common significant changes included groups of transcripts involved in different functional pathways, namely neuroactive ligand receptor interactions, oxidative phosphorylation, cytokine-cytokine receptor interactions (consistent with observations that IL-8 is upregulated in bystander cells (Facoetti et al. 2006)), proteasomes and ribosomes. An upregulation of the calcium signalling pathway group was also reported, consistent with observations that activation of a calcium influx pathway is responsible for induction of micronuclei in bystander cells (Shao et al. 2006) and that calcium signalling plays an important role in bystander cell death, mediated by ROS in unirradiated cells (Lyng et al. 2006b). Global gene expression analysis in

human lung fibroblast cells 4 hours after bystander exposure to α-particles showed that 305 genes were differentially expressed compared to controls. Pathway analysis of the differently expressed gene sets revealed that the most significant gene groups were inflammation and chemokine-cytokine signalling related genes (Ghandhi et al. 2008). The importance of cytokines and inflammatory signalling molecules was also suggested by Zhou et al (Zhou et al. 2005), who used a signal transduction pathway finder array, which includes 96 genes involved in 18 different signal transduction pathways to compare the differentially expressed genes among the nonirradiated control NHLF and bystander cells. Among the 96 genes represented on the platform, the transcription level of one gene, *COX-2*, was found to be consistently up-regulated by >3-fold.

1.4.11 Cell Signalling Pathways

Several studies have demonstrated that a particularly harmful form of DNA damage, DNA double strand break induction, occurs in bystander cells (Sokolov et al. 2005; Burdak-Rothkamm et al. 2007; Chen et al. 2009b). Rogakou et al (Rogakou et al. 1998) reported that a variant of histone H2A, H2AX becomes rapidly phosphorylated at a conserved serine residue 4 amino acids from the carboxyl-terminus of histone H2AX when DNA double stranded breaks are introduced by ionising radiation. This phosphorylation site corresponds to serine 139 in mammalian cells (Nakamura et al. 2006). The phosphorylated form of H2AX is termed γ-H2AX, which has been used as a reliable selectable marker for identification of DNA DSBs (Rogakou et al. 1998).

Several proteins co-localise with γ-H2AX foci at sites of DNA DSBs including ataxia telangiectasia mutated (ATM), ataxia telangiectasia and Rad3-related (ATR) and DNA dependent protein kinase (DNA-PK), which have all been found to participate in H2AX phosphorylation. Use of ATR mutated fibroblasts revealed that H2AX

phosphorylation in bystander cells is ATR dependent, whereas ATM and DNA-PK functions are not essential for induction of bystander γ-H2AX foci (Burdak-Rothkamm et al. 2007). According to Burdak-Rothkamm et al (Burdak-Rothkamm et al. 2008) ATM acts downstream of ATR in DNA damage response signalling of bystander cells. S-Phase was found to be the only stage when cells were at increased risk of developing bystander γ-H2AX foci (Burdak-Rothkamm et al. 2007). These observations support the hypothesis that accumulation of stalled replication forks occurs in bystander cells to result in ATR-mediated γ-H2AX focus formation. DNA replication fork stalling can be caused by DSBs, single strand breaks and secondary DNA structures, as well as impediments such as tightly bound proteins such as transcription factors or machinery such as RNA polymerase. ATR is involved in recognition of stalled replication forks. Failure to stabilize stalled forks can result in fork collapse and genomic instability. ATM, ATR and DNA-PK are members of the phosphoinositol3-kinase-like kinase family (PIKK) involved in sensing DNA damage and translation of the signal into an appropriate response such as cell cycle arrest and DNA repair (Rouse and Jackson 2002).

According to Stiff et al (Stiff et al. 2006), ATR is recruited in an ATM-MRN complex-dependent manner in response to direct radiation-induced DNA damage, resulting in Chk1 phosphorylation. In contrast, recruitment of ATR in response to UV damage or stalled replication forks occurs in an ATM-independent manner. Under these conditions (UV/stalled forks) ATM activation is ATR-dependent. There is substantial overlap between ATR and ATM signalling, which both constitute an expansive network (Matsuoka et al. 2007). Bystander γ-H2AX foci are thought to be generated through different pathways to those resulting from direct irradiation. Burdak-Rothkamm et al (Burdak-Rothkamm et al. 2008) reported a radiation-induced decrease in clonogenic

survival in ATR/ATM proficient bystander cells, which is abrogated in ATM/ATR deficient cells but not in those cells deficient for DNA-PK. Therefore, ATM and ATR both participate in bystander signalling leading to decreased survival in bystander cells. In contrast, in directly hit cells, survival decreased upon inhibition of ATM, ATR and DNA-PK, suggesting the possibility of differential modulation of targeted and non-targeted effects through ATM and ATR inhibitors. Burdak-Rothkamm et al (Burdak-Rothkamm et al. 2007) also observed co-localization of ATR, 53BP1, ATM, p21 and BRCA1 foci in bystander cells. Induction of 53BP1 foci occurred in an ATR-dependent manner in S-Phase, similar to γ-H2AX focus formation. ATM activation was found to be dependent on ATR function. ATR-dependent induction of γ-H2AX foci occurs over a prolonged time in bystander cells and there was a similar response for 53BP1 induction. Thirty minutes post transfer of conditioned medium from irradiated cells to bystander cells, H2AX foci were found to co-localize with 53BP1 and ATR foci, all DNA repair-associated proteins. Induction of DNA damage may be linked to stalled replication forks in S-phase cells and consecutive activation of proteins involved in DNA damage recognition and repair and cell cycle checkpoint control involving ATM, BRCA1 and p21/CiP1/WAF1. Reduction in bystander clonogenic survival in DNA-PK mutated but not ATR/ATM deficient cells indicated that bystander signalling to result in reduced clonogenic survival is dependent on ATR and ATM but not DNA-PK. A potential downstream effect is cell cycle checkpoint activation and induction of apoptosis. p53 status appears not to be critical in the bystander responses. According to Zhang et al (Zhang et al. 2008), p53 status does not play a role in production or response to bystander signalling leading to mutation induction. For certain endpoints, bystander DNA damage is independent of p53 even though it is a known ATM target. However, p53 may be involved in selected bystander responses. If ATM/ATR function

is impaired, the long term fate of bystander cells is increased mutation rate and genomic instability.

1.4.12 Mechanism

Irradiated cells communicate with non-irradiated cells via secreted factors and GJIC pathways, eliciting biological responses in those non-targeted cells. Bystander effects appear to predominate at low doses of radiation after both low LET X or gamma rays (Seymour and Mothersill 2000) and low doses of high LET alpha particles (Little et al. 2002). Bystander interactions have been reported to involve production of cytokines (Iyer et al. 2000; Xiong et al. 2001; Azzam et al. 2002; Zhou et al. 2005; Shao et al. 2008a; Shao et al. 2008b; Zhou et al. 2008), free radicals including ROS (Lyng et al. 2000; Lyng et al. 2002; Rugo et al. 2002; Yang et al. 2005; Tartier et al. 2007; Chen et al. 2009b) and NO (Shao et al. 2004; Han et al. 2007; Tartier et al. 2007). Lyng et al (Lyng et al. 2000) reported a role for calcium signalling in bystander responses. Treatment of unirradiated human keratinocytes with medium from irradiated cells led to rapid and transient increases in intracellular calcium in bystander cells. This rapid increase in intracellular calcium may be responsible for some of the biological endpoints observed in bystander cells. Blocking of Ca^{2+} channels by calciculidine eliminated bystander micronucleus induction (Shao et al. 2006). In addition, chelation of extracellular calcium by EGTA and addition of verapamil to block Ca^{2+} channels led to a significant reduction in bystander apoptosis (Lyng et al. 2006b). These results suggest that calcium entry into the cell is an important transducer for several biological endpoints in bystander cells. These rapid calcium fluxes may be dependent on reactive oxygen and nitrogen species, as addition of aminoguanidine and DMSO led to inhibition of calcium fluxes and micronucleus induction in bystander cells, suggesting a further role for these radical species in bystander signalling (Shao et al. 2006).

The membrane-bound NAD(P)H-oxidase enzymes have also been implicated as a source of ROS in bystander signalling pathways (Narayanan et al. 1997). Addition of the flavoprotein oxidase inhibitor diphenylenelodonium (DPI), a blocker of NAD(P)H-oxidase activity significantly decreased intracellular H_2O_2 production in α-particle irradiated human fibroblasts (Narayanan et al. 1997). DPI has also been shown to inhibit ROS production by NAD(P)H-oxidase enzymes (Azzam et al. 2002). NAD(P)H-oxidase-mediated ROS production, implicated in the enhanced accumulation of p53 and $p21^{Wafl}$ immunoreactive protein occurring after mean doses of 1–3 cGy was also significantly reduced in the presence of DPI. NAD(P)H-oxidases transfer electrons to oxygen to form superoxide ($O_2\bullet^-$) (Bedard and Krause 2007), which is rapidly dismutated into H_2O_2, a stable, neutral long-lived species that can cross cell membranes and diffuse extracellularly. Further evidence for the role of NAD(P)H oxidases in bystander responses is provided by reports that the E89 cell line, which is null for the glucose-6-phosphate dehydrogenase pathway, an important producer of the substrate for these oxidase enzymes, NADPH, show no bystander responses for induction of reduced clonogenic survival (Vines et al. 2008).

1.4.13. Summary

In general, bystander studies examine either the process of signal production from the irradiated donor cell and/or the bystander response in unirradiated, recipient cells. Fundamental observations reported in the bystander literature:

Bystander effects are cell type dependent – several cell lines do not demonstrate bystander effects.

Detection of any biological effects is dependent on the endpoint used.

Damage attributable to the bystander effects is most significant at low doses, saturates at doses above 0.5 Gy and is insignificant at higher doses where direct effects of radiation dominate.

The bystander effect can be induced by a single irradiated cell.

Transmission of bystander signals occurs through gap junctions, medium transmissible/soluble factors or a combination of both.

1.4.14. Implications

Traditionally, the detrimental effects of radiation were considered to be the result of radiation depositing its energy in the nucleus of the irradiated cell, damaging the critical nuclear target, DNA. The subsequent fate of the cell, tissue, organ or system was thought to reflect the DNA damage response. Interest is now focusing on the non-targeted effects, which seriously challenge the notion that radiation-induced energy deposition in the nucleus of an irradiated cell is responsible for its detrimental effects.

Radiation-induced genomic instability defines a host of potentially detrimental effects observed in the progeny of an irradiated cell, characterised by genetic changes including chromosome rearrangements, induction of micronuclei, transformation, amplifications, mutations and reduced plating efficiency (delayed reproductive cell death or lethal mutations) in cells derived or clonally expanded from an irradiated cell many cell divisions after the initial insult. Delayed death, gene mutations and a variety of chromosomal abnormalities can be demonstrated in cells that are not themselves irradiated but are the progeny of cells exposed to ionizing radiation many cell divisions previously. Tissue culture medium from clones of genomically unstable cells, when transferred to unirradiated, normal cells leads to the Death Inducing Effect (DIE) (Nagar et al. 2003). Factors from unstable cells generate chromosomal changes and cell death

in normal cells. The factors responsible for DIE may be responsible for perpetuating the instability phenotype. According to Nagar et al (Nagar et al. 2003), DIE is a separate endpoint from the bystander effect, and cells exhibiting bystander effects are refractive to DIE. Clastogenic factors manifest when blood plasma isolated from individuals exposed to radiation either through occupation, environment or therapeutically, causing chromosomal aberrations in blood lymphocytes from non-irradiated cells after co-culture. Radiation elicits secretion of blood-borne factors that cause chromosomal rearrangements in peripheral blood lymphocytes when mixed with blood from unirradiated subjects. Blood plasma from patients with cancer prone disorders including ataxia telangiectasia can cause chromosomal aberrations in peripheral blood samples from normal healthy subjects after co-culture (Huang et al. 2003). These dysgenetic effects have been interpreted as manifestations of a radiation-induced genomic instability. All together, DIE, clastogenic factors and bystander effects indicate that secreted factors and direct cell-to-cell communication mediated by GJIC operate to transfer signals from unstable cells to unirradiated normal, healthy cells. The non-targeted effects indicate that these signals are capable of causing genetic damage that can ultimately lead to cell death. Therefore, in practice, radiation protection may need to be restructured to emphasise individual susceptibility, taking environmental and lifestyle factors in to account. Little is known about the bystander factor or process but the fact that communication is observed in a variety of flora and fauna, what we now know as the bystander effect may form part of a complex and ancient control mechanism that facilitates adaptation to new environmental challenges.

Standards and guidelines regarding acceptable radiation doses to the general public and to radiation workers are developed and reviewed by assuming a linear no-threshold (LNT) hypothesis, which relates dose to biological effect. The LNT

hypothesis states that the dose–effect relationship is linear even at very low doses, which assumes that there is no threshold below which radiation ceases to have a biological effect. Bystander effects therefore challenge this hypothesis, and ultimately, prediction of radiation effects cannot depend on the amount of energy deposited but on the cellular response to that energy deposition, the signals generated and the bystander cell response. A key aim of radiation protection guidelines is the prevention of radiation-induced cancer. It is important therefore to consider the bystander effect as there is no direct correlation between the number of cells exposed to radiation and the number of cells exhibiting deleterious biological effects. Ultimately, these effects depend on complex interactions between irradiated and bystander cells.

1.4.15 Bystander Effects Induced by Agents Other Than Ionising Radiation

Ionising radiation is not unique in generating bystander responses. Genomic instability can be induced by a number of agents and bystander effects have been observed following other forms of cell stress. Examples have been published for unirradiated plasma from ataxia telangiectasia patients, or genomically unstable clones (Morgan 2003), suggesting that radiation may not be necessary as the source of genomic instability in bystander effects.

Photodynamic therapy (PDT) is a cancer therapy under investigation for the treatment of solid malignancies and some non-malignant diseases. PDT consists of photosensitisation by uptake of porphyrin followed by light irradiation, typically from a laser source. This photoactivation is followed by oxidative damage and subsequent cell death (Dabrowska et al. 2005). Dabrowska et al (Dabrowska et al. 2005) reported bystander reduction in plating efficiency in human ovarian carcinoma OVP10 cells co-cultured with PDT-treated cells. The role of ROS in PDT-induced bystander signalling in EMT6 murine breast cancer cells was explored by Rubio et al (Rubio et al. 2009).

Induction of ROS was observed in bystander cells, which was decreased in the presence of catalase, suggesting a role for H_2O_2 in increased bystander ROS levels. Inhibition of NAD(P)H oxidases by DPI resulted in a complete blockage of ROS induction in bystander cells. This indicates that bystander ROS induction depends on NAD(P)H oxidase to generate H_2O_2 that can readily cross cell membranes. However, DPI treatment did not completely block ROS generation in target cells, suggesting NAD(P)H oxidase as just one of the sources of ROS in damaged cells.

Dabrowska et al (Dabrowska et al. 2005) also observed a heat-damage-induced bystander effect characterised by slower growth in OVP10 cells co-cultured with cells that had been previously heated to 75°C for 10 minutes. This heat-damage-induced bystander effect was reversible upon removal of damaged cells.

The phenomena of genomic instability and bystander effects have also been observed in the cellular responses to UV irradiation(Whiteside and McMillan 2009) (McMillan et al. 2008). McMillan et al (McMillan et al. 2008) observed a significant reduction in clonogenic survival of unirradiated HaCaT bystander cells co-cultured with UVA treated ($1x10^5$ J m^{-2}) cells. This bystander effect was reduced upon incubation of bystander cells with diphenyleneiodoium (DPI) to inhibit NAD(P)H oxidase, implicating ROS as a signalling factor in the response to UVA exposure.

Bystander effects have also been observed in response to treatment of target cells with chemical agents. A recent study by Asur et al (Asur et al. 2009), reported induction of bystander micronuclei in response to donor cell mitomycin c or phleomycin exposure using a medium transfer approach. Alexandre et al (Alexandre et al. 2007) studied bystander signalling in response to the chemotherapeutic agent

paclitaxel using a co-culture method. A H_2O_2-dependent inhibition of proliferation was observed in bystander cells.

The phenomenon of chemical-induced bystander effects is poorly characterised and no evidence is available to support the existence of a metal-induced bystander effect.

1.5. Aims

The aim of this thesis is to explore the possibility that metal ions can induce a bystander effect similar to the phenomenon observed post irradiation. Potassium dichromate was selected for direct metal treatment because chromium exposure through industry, orthopaedic wear debris and agricultural means poses a relevant health risk. Also the use of chromium builds on previously reported studies by this group, which outlined some of the DNA damage endpoints induced by hexavalent chromium (Glaviano et al. 2006). In order to determine whether or not there is a metal-induced bystander effect, a medium transfer approach is described, in which culture medium harvested from Cr-exposed human fibroblasts was transferred to non-exposed cells. The medium transfer recipients were assayed for DNA damage endpoints including micronucleus induction, γ-H2AX focus formation and induction of cytogenetic aberrations. Experiments will also be presented, exploring the role of close cell-to-cell contact in addition to medium-borne factors in transmission of bystander signalling. The cells used throughout this project were BJ normal human fibroblasts and BJ fibroblasts infected with the human catalytic subunit of telomerase reverse transcriptase (hTERT). hTERT cells have long telomeres and are telomerase positive. In order to gain mechanistic insights into metal-induced bystander signalling, the roles of some key modulators of the radiation-induced bystander effect including MAP Kinase signalling

pathways, cytokines and oxidative stress were explored. To put the results obtained for bystander interactions in telomerase positive fibroblast cells into context, experiments will also be described using cells that are naturally telomerase positive, including human embryonic stem cells and a thyroid carcinoma cell line.

A schematic summary diagram of the experimental work described in the thesis is shown in Figure 1.1.

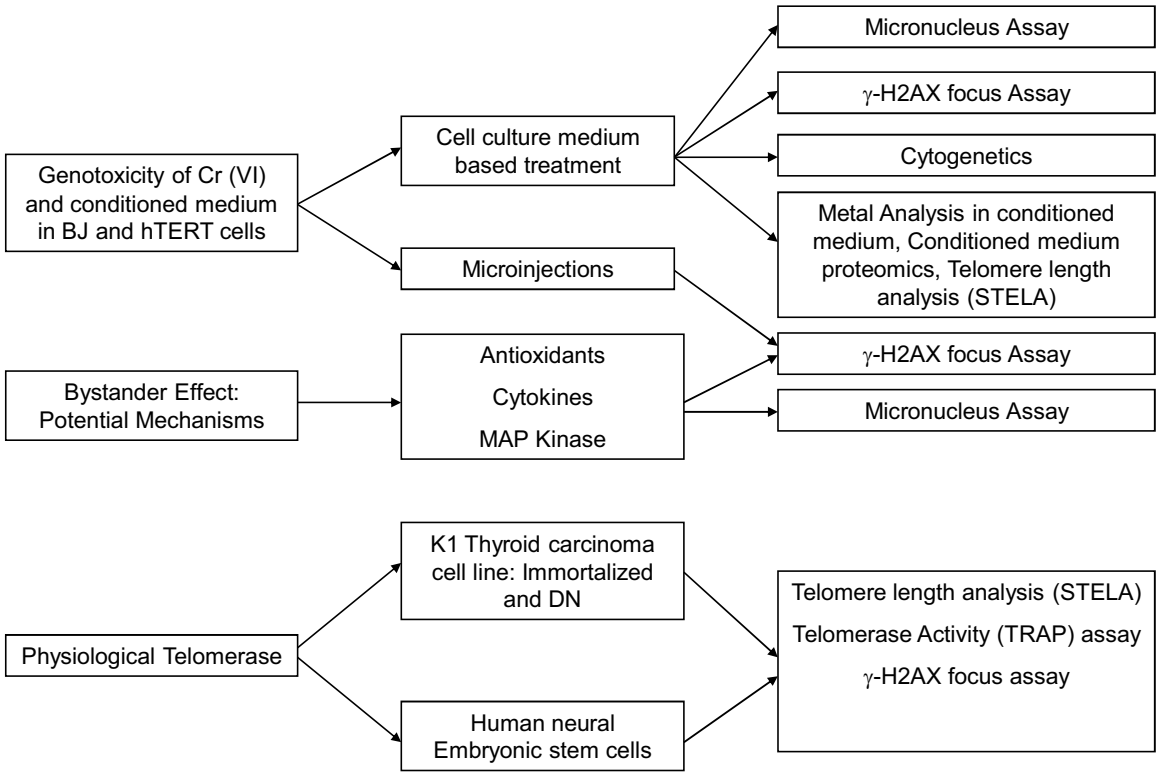

Figure 1.1 Schematic summary diagram outlining the experimental work described in the thesis

CHAPTER 2 GENERAL MATERIALS AND METHODS

2.1 Cell Culture

2.1.1 Materials and Solutions

- Dulbecco's modified Eagles medium (DMEM) (Sigma) was supplemented with: 20% medium 199 (Sigma), 10% Foetal bovine serum (FBS) (Gibco), 5% 1M Hepes buffer (Sigma), 5% 200mM L-glutamine (Gibco), 5% 100mM sodium pyruvate (Sigma) and 2.5% 100 X antibiotics antimycotic (Sigma).

This medium was stored at 4^{o}C for up to one month, after which L-Glutamine was replenished. This cell culture medium will be hereafter referred to as 'complete DMEM' for simplicity.

- Tissue Culture Flask 75cm^2 (T-75) or 150 cm^2 (T-150) gamma sterilized flasks for tissue culture (Triple Red)
- Trypsin-EDTA 1-X, stored at -20^{o}C (Gibco)
- Improved neubauer haemocytometer
- Inverted light microscope (Wild Heerbrugg)
- Virkon **(**10g) (Antec™ International supplied by Anachem) in 1 litre tap water, used to decontaminate cell culture implements
- 70% analytical reagent grade ethanol (Fisher Scientific) (v/v) in distilled water
- Cell freezing medium-DMSO 1X (Sigma)
- Nunc™ 1.8 ml Cryovials
- Phase contrast light microscope

2.1.2 Cell Lines

The majority of this study was carried out using two types of BJ human foreskin fibroblasts obtained from Miguel A. Rubio (Netherlands Cancer Institute). These were BJ normal human skin fibroblasts (hTERT-), which normally senesce around population doubling (PD) 80 and BJ fibroblasts immortalized at PD 22 with hTERT retrovirus (hTERT +) as according to Rubio (Rubio et al. 2002). BJ normal human foreskin fibroblasts were infected at PD 22 with pLXSN-hTERT retroviruses. The plasmid was created using hTERT from pBABE-PURO-hTERT in the pLXSN retroviral vector (Clontech). Cells were selected with G418 and the population expanded prior to being frozen.

2.1.3 Culture Conditions

Both human fibroblast cell types were grown in complete DMEM at 37^{o}C with no ambient CO_2. Cells were cultured in a class II cabinet under aseptic sterile conditions, achieved by regular spraying of all items used with 70% ethanol. Cells were generally not allowed to exceed 80% confluence. Experiments were carried out on cells at passage 7.

2.1.4 Subculturing and Passaging Cells

To passage cultures, the following procedure was used for trypsinizing cells in a T-75 flask:

Enzymatic disruption of the cellular monolayer was achieved using trypsin/EDTA 1X. Trypsin cleaves peptide bonds towards the carboxyl terminus of the positively charged amino acid residues arginine and lysine if the next residue is not proline.

Growth medium was aspirated and approximately 0.5 ml trypsin at 37^{o}C was added to the flask to wash surface. The trypsin was then aspirated and 1.5 ml trypsin added to the

flask, distributed evenly over the monolayer and incubated for 2 minutes at 37^{o}C to allow cells to detach. Cells were then viewed under an inverted light microscope using the 20x objective. If cells were not separated from each other or from the flask surface, a further minute of trypsin incubation was allowed and flasks were agitated by tapping. When satisfactory detachment was achieved, an equal volume of complete DMEM (37^{o}C) was added to neutralize the trypsin, and cells were dispersed by pipetting. The concentration of cells in the suspension was estimated each time the population was passaged using a haemocytometer as described in 2.1.7. Cells were expanded or split as required. The volume of fluid in each flask was made up to 20 ml using fresh, complete DMEM (37^{o}C). The passage frequency was generally every 3-4 days. Cells were incubated overnight, allowing them to adhere to the flask surface before medium was replaced, which removed any residual traces of trypsin or unattached cells.

2.1.5 Generation of Cell Banks and Cryopreservation

'Working' and 'master' cell banks were established at the start of the study. In order to create a master cell bank, cells were initially subcultured for 2-3 passages and the majority harvested via trypsinization. Some cells remained in culture for the production of a working cell bank. The population density was determined using a haemocytometer. The cell suspension was then centrifuged at 1200 RPM for 8 minutes; the cell pellet was retained and resuspended in cell freezing medium-DMSO (1X) at a concentration of 10^{6} cells/ml. One ml of this cell suspension was aliquoted into cryovials. Cryovials were placed in a methanol-filled cell freezing chamber at -70°C overnight. This chamber allowed the cell to freeze gradually at a rate of 1°C/ minute. Cryovials were then transferred to two separate liquid nitrogen tanks for long term storage.

In order to create a working cell bank, cells which were not harvested for cryopreservation at 2-3 passages for the master cell bank were expanded for a further 4 passages. They were then harvested, centrifuged, resuspended in cell freezing medium-DMSO (1X), frozen in the same way as the master cell bank and the cryovials transferred to the two separate liquid nitrogen tanks for long term storage.

2.1.6 Resuscitation of Cryopreserved Cells by Defrosting

Cryovials were defrosted quickly in a water bath at 37°C. Cell freezing medium was diluted in complete DMEM and the resulting cell suspension was transferred to a 15ml centrifuge tube and further complete DMEM was added. These tubes were centrifuged at 1200 RPM for 8 minutes, the DMSO-containing supernatant was discarded and the cell pellet retained and resuspended in complete DMEM. DMSO must be decanted immediately as it is especially toxic to cells at higher temperatures. The cell suspension was seeded in to a T-150 sterile tissue culture flask for incubation at 37°C overnight, which allowed cells to attach to the flask. Complete DMEM was then replaced to remove any residual traces of cryoprotectant and unattached cells.

2.1.7 Determination of Cell Concentration in a Single Cell Suspension

A drop of well mixed single cell suspension was transferred to the counting chamber of an improved neubauer haemocytometer allowing the chamber to fill completely by capillary action. Viable cells observed in 10 triple lined squares were counted under phase contrast light microscopy using the 10x objective. The number of cells in 10 of these squares represented cells/μl cell suspension. Numerous observations were made ensuring that at least 100 cells were counted for accuracy.

2.2 Metal Exposures and bystander treatments

2.2.1 Materials and Solutions

- PBS Solution: 3 PBS tablets (Sigma) were dissolved in 600ml distilled water to produce - 0.01 M Phosphate buffer, 0.0027 M KCl, 0.137 M NaCl at pH 7.4
- Potassium Dichromate 200 μM stock solution: 29 mg $K_2Cr_2O_7$ (Sigma) dissolved in 500 ml PBS
- Potassium Dichromate 20 μM stock solution: 1 ml of the above 200 μM solution is diluted 1/10 (v/v) with PBS
- Potassium Dichromate 0.4 μM treatment: 400 μl of the above 20 μM stock solution was added to each T-75 cell culture flask. The total liquid volume in the flask was made up to 20 ml with complete DMEM at 37^{o}C.

This treatment will be referred to hereafter as Cr (VI)

- 0.2 μm sterile syringe filter (Corning)
- Sterile Dulbecco's PBS (DPBS) solution (Sigma)

2.2.2 Methods

2.2.2.1 Direct Metal Exposures

Cells were seeded into sterile tissue culture flasks at a frequency of $3x10^5$ cells in 12 x T-75 flasks for each cell type and incubated overnight. The following day, cells were exposed to a treatment of 0.4 μM $K_2Cr_2O_7$ or 400 μl filtered PBS for 24 hours at 37°C. Six flasks of each cell type were treated with Cr (VI) and 6 of each type with PBS. To remove any contaminants from the treatments, they were filtered using a sterile 0.2μm syringe filter. After the 24 hour treatment period, cells were washed 5 times with sterile DPBS (37°C) to remove any trace of metal ions. After washing, 3 Cr (VI)-exposed flasks of each cell type and 3 PBS-treated flasks of each cell type were

harvested via trypsinization in order to carry out assays to determine the genotoxicity caused by direct metal treatment. These assays will be described in this chapter. Cells exposed to Cr (VI) or PBS for 24 hours were cultured for a further 30 days, passaging every 3-4 days. Genotoxicity assays were carried out immediately after Cr (VI) exposure on day 0. Treated cells were also assayed 5 and 30 days post treatment, as outlined in table 2.1.

2.2.2.2 Bystander Effects: Medium Transfer

To study a metal-induced bystander effect, a medium transfer technique was used. This was based on the idea that Cr (VI)-treated 'donor' cells secrete a bystander factor or factors into their growth medium to induce biological effects in any 'recipient' cell that subsequently comes into contact with the culture medium and that has not been directly exposed to metal. Two sets of cells were required; donors and recipients. The donor cells were plated out in the same manner as directly treated cells (as described in 2.2.2.1): $3x10^5$ cells of each cell type were seeded into 12 x T-75 flasks (6 for each cell type). Donor cells were treated the following day with either 0.4μM $K_2Cr_2O_7$ or PBS (negative control) for 24 hours as described in (2.2.2.1). Three flasks of each cell type were treated with Cr (VI) and 3 flasks of each type were treated with PBS. On the same day, $3x10^5$ recipient cells of each type were seeded into 12 x T-75 flasks (6 for each cell type). The next day (after 24 hours treatment), donor cells were washed 5 times with DPBS at 37^oC to remove any trace of metal ions, and 20 ml fresh growth medium was added to each of the flasks for 1 hour. The medium was then harvested from donor cells and filtered using a sterile 0.2μm syringe filter to exclude any directly Cr (VI)-exposed cells. The filtered medium harvested from donor cells will be referred to hereafter as 'conditioned medium'. Conditioned medium was transferred to recipient cells replacing their growth medium for incubation at 37°C for 24 hours. Medium from 3 Cr-exposed

donor flasks of one cell type was transferred to 3 separate recipient cell flasks of the same type and medium from 3 PBS-exposed donor flasks of one cell type was transferred to 3 separate recipient cell flasks of the same type for both BJ and hTERT cells. Figure 2.1 shows an illustration of the medium transfer process. Following 24 hours treatment with conditioned medium, the level of DNA damage was determined using the micronucleus assay, γ-H2AX focus formation assay and cytogenetic analysis.

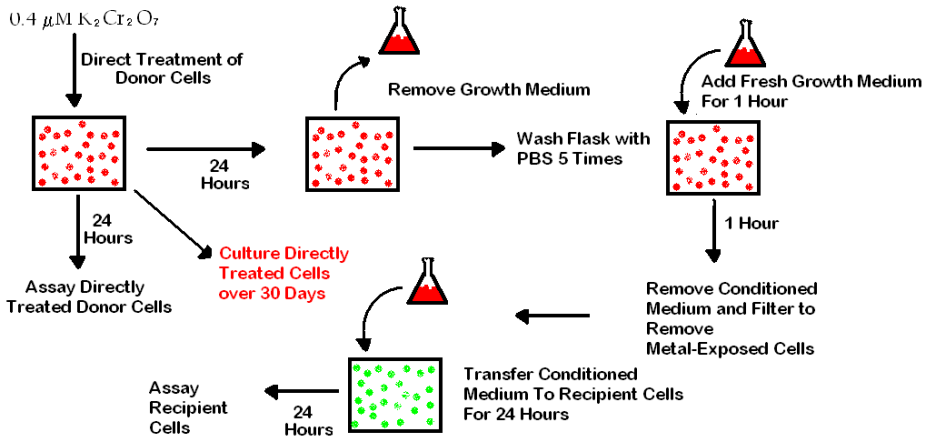

Figure 2.1 Schematic showing medium transfer procedure

2.2.2.3 Sampling over 30 days

Directly treated cells were passaged over a 30 day period; and analysis carried out on day 0 (immediately following 24 hours Cr (VI)/PBS exposure) and on day 5 and day 30 after treatment. To assay recipient cells on day 5 and day 30, medium was conditioned in Cr/PBS-exposed donor cells for 1 hour on day 5 and day 30 after direct treatment. Conditioned medium was filtered and transferred to recipient cells for a 24 hour period. The recipient cells for medium transfer treatment were passaged from day 0 to day 5. For medium transfer on day 30, a vial of cryopreserved cells of each type was defrosted around days 22-25.

2.2.2.4 Medium Transfer between Cell types

Initially, as described above, conditioned medium was harvested from one cell type and transferred to cells of the same type. It became necessary to explore the difference in signalling between the two cell types. In order to do so, conditioned medium was transferred from Cr (VI)/PBS-exposed BJ cells to hTERT cells and vice versa at all three time points (day 0, day 5 and day 30).

Table 2.1: Timetable of Treatments

Day	Directly treated cells	Donor Cells	Recipient Cells
-1	Plate out $3x10^5$ cells in T-75 flasks (Duplicate)	Plate out $3x10^5$ cells in T-75 flasks	
0	Treat with Cr (VI) or PBS for 24 hours	Treat with Cr (VI) or PBS for 24 hours	Plate out $3x10^5$ cells in T-75 flasks
1	Wash both sets of cells 5 times with DPBS. Harvest 1 set for assay, Culture the second set for assay 5 days and 30 days post metal treatment.	Wash cells 5 times with DPBS. Add 20 ml fresh medium, incubate for 1 hour, aspirate medium, filter and transfer to recipients	Treat with 'conditioned medium' from donor cells for 24 hours
2			Harvest for assay
4	Plate out $3x10^5$ metal-treated cells in T-75 flasks	Plate out $3x10^5$ metal treated cells in T-75 flasks	
5	Harvest for assay	Add 20 ml fresh medium, incubate for 1 hour, aspirate medium, filter and transfer to recipients	Treat with 'conditioned medium' from donor cells for 24 hours
6			Harvest for assay
7-28	Maintain metal and PBS-treated cell cultures		Defrost new vial of cells for day 30 recipients (day 22-25 approx)
29	Plate out $3x10^5$ metal-treated cells in T-75 flasks	Plate out $3x10^5$ metal treated cells in T-75 flasks	
30	Harvest for assay	Add 20 ml fresh medium, incubate for 1 hour, aspirate medium, filter and transfer to recipients	Treat with 'conditioned medium' from donor cells for 24 hours
31			Harvest for assay

2.3 Metal analysis in conditioned medium and complete DMEM

2.3.1 Materials and solutions

- Cr (VI) exposed cells
- Warm (37°C), sterile DPBS (Sigma)
- Sterile metal-free containers

2.3.2 Methods

When preparing conditioned medium, 5 DPBS washing steps were carried out in metal-exposed donor cells after 24 hours metal treatment. The washings from each of these 5 steps were collected in separate, sterile, metal-free containers and stored at 4°C. After the washings, fresh medium was added to the metal exposed donor cells in order to obtain chromium-exposed cell conditioned medium. This medium was harvested from the donor cells after one hour incubation at 37°C and filtered using a 0.2μm syringe filter to exclude any directly metal-exposed cells. A sample of this conditioned medium was also collected in a sterile metal-free container and stored at 4°C. The washing solutions labelled (wash 1-5) wash 1 for the first PBS wash 2 for the second etc, the conditioned medium (labelled con med 1 hour) and a sample of complete DMEM (labelled medium control) were sent to the ALS laboratory group, Analytica in Sweden for total chromium analysis. ICP-AES (inductively coupled plasma atomic emission spectrometry) and ICP SFMS (inductively coupled plasma sector field mass spectrometry) were carried out according to EPA methods 200.7 and 200.8 respectively (U.S. Environmental Protection Agency)

2.4 Proteomics

2.4.1 Methods

To determine the nature of the complement of proteins present in metal-conditioned medium compared to control medium, a sample of conditioned medium was harvested from both BJ and hTERT donor cell types 5 days post their initial 24 hour exposure to either Cr (VI) or PBS. 300,000 cells were plated into four T-75 flasks for BJ and four flasks for hTERT cells. Two flasks of each cell type were treated with 0.4 μM potassium dichromate and two with PBS for 24 hours as described in section 2.2 after which, cells were washed 5 times with PBS. Fresh complete DMEM was added to flasks and incubated at 37°C for one hour to obtain conditioned medium. This conditioned medium was stored at -20°C until the samples were sent to the University of Bristol Department of Biochemistry for protein analysis by two dimensional gel electrophoresis, which was kindly carried out by Kate Heesom.

2.5 Telomeres and Telomerase

2.5.1 Single telomere length analysis (STELA)

Cells were detached from culture flasks by trypsinization and washed into a centrifuge tube. The cell suspension was centrifuged and the cell pellet was retained and resuspended in PBS. This cell suspension was sent on ice to Duncan Baird (Cardiff University) for single telomere length analysis (STELA). DNA extractions and STELA reactions were performed as described by Baird et al (Baird et al. 2003) with the following adaptations: The Taq/Pwo ratio was adjusted from 25:1 to 10:1 and reactions were cycled with an MJ PTC-225 thermocycler (MJ Research) under the following conditions: 22 cycles of 94°C for 15 s, 65°C for 30s and 68°C for 10 minutes. To ensure specificity of telomeric bands, only those that hybridized to both the telomere-adjacent

and telomere-repeat containing probe were analysed. STELA is a single-molecule PCR based telomere length analysis technology that determines the full spectrum of telomere lengths from specific chromosome ends (Baird 2005).

2.6 Micronucleus Assay

2.6.1 Materials and Solutions

- Cytochalasin-B 6 μg/ml (Sigma). In a total volume of 20 ml, 120 μl cytochalasin B (stock solution 1mg/ml) was added to each flask
- Sterile DPBS (Sigma)
- Centrifuge (Harrier 15/80 Sanyo) Samples were centrifuged at 1200 rpm for 8 minutes unless otherwise stated
- Cytospin 3 (Shandon)
- Methanol Analytical reagent grade methanol (Fisher Scientific) stored at -20°C
- Coplin jar
- 5% (v/v) Giemsa (twice filtered) in deionized water (Gurr ® BDH Laboratory supplies)

2.6.2 Background

The cytokinesis-block micronucleus assay (CBMN) is used for the assessment of chromosome breakage, chromosome loss and to determine the frequency of cell division. Cytochalasin-B (Cyt-B) is a fungal toxin that inhibits cytokinesis by interfering with the formation of contractile microfilaments. It inhibits cell movement, glucose transport and induces nuclear extrusion. Inhibition of cytokinesis by Cyt-B results in the accumulation of binucleate cells at the first cell division.

2.6.3 Methods

This assay was performed according to the methods and criteria outlined by Fenech (Fenech and Morley 1985). 3 x 10^5 cells were seeded into T-75 cell culture flasks and incubated overnight at 37°C. Around 24 hours post seeding, medium was replaced with 20 ml treatment-containing medium at 37°C. Cells were either treated: 1) directly with Cr/PBS or 2) via medium transfer of conditioned medium. Simultaneously with this treatment, 120 µl Cyt-B (1mg/ml) was added to the flask to achieve a final concentration of 6 µg/ml. This concentration has been described as optimal for induction of binucleate cells in whole human blood (Ellard and Parry 1993). As Cyt-B is light sensitive, care was taken to use it in reduced ambient light and store it in light-resistant containers. After 24 hours treatment, medium was carefully discarded into a sealable container, as Cyt-B is toxic. Cells were harvested by trypsinization separately for each dose and washed into 15 ml centrifuge tubes. The detached cells were centrifuged at 1200 rpm for 8 minutes and the cell pellet was retained and resuspended in approximately 1-1.5 ml DPBS depending on the concentration of cells in the suspension. 200 µl of the cell suspension was dropped onto slides in a cytospin chamber. Slides were spun at 500 rpm for 6 minutes. Slides were allowed to air dry in around 10 minutes and were then fixed by immersing in prechilled methanol (-20 ^{o}C) for 10 minutes. The slides were later stained using 5% Giemsa (v/v in distilled water) for 8 minutes.

2.6.4 Criteria for Scoring the Micronucleus Assay

The micronucleus assay was scored according to the criteria defined by Fenech et al (Fenech et al. 2003). In order to be scored, cells must be binucleated. The two nuclei in a binucleate cell should have intact nuclear membranes and be situated within the same

cytoplasmic boundary. The micronucleus is morphologically identical to but smaller than the main nuclei with the following characteristics:

1. Micronuclei are round or oval in shape

2. Micronuclei are non-refractile and they can therefore be readily distinguished from artefacts such as staining particles

3. Micronuclei are not connected to the main nuclei

4. Micronuclei may touch but not overlap the main nuclei and the micronuclear boundary should be distinguishable from the nuclear boundary

5. Micronuclei usually have the same staining intensity as the main nuclei

Nucleoplasmic bridges (NPBs) may be found in binucleated cells and are thought to originate from rearranged chromosomes with more than one centromere such as dicentric chromosomes. NPBs are considered to be biomarkers of dicentric chromosomes resulting from telomere end fusions or DNA misrepair. They are thought to serve as a measure of chromosome rearrangement that occurs when centromeres of dicentric chromosomes are pulled to opposite poles of the cell at anaphase. The CBMN assay allows NPBs to accumulate and be observed because cytokinesis is inhibited and the nuclear membrane forms around chromosomes (Umegaki and Fenech 2000; Fenech 2006). NPBs have the following characteristics:

1. NPBs are a continuous nucleoplasmic link between the nuclei in a binucleate cell

2. The width of a nucleoplasmic bridge may vary but usually does not exceed one-quarter of the diameter of the main nuclei

3. NPB should have the same staining characteristics as the main nuclei

4. A binucleated cell with a nucleoplasmic bridge may contain one or more micronuclei.

Slides were coded to allow blind scoring. 1000 binucleate cells were scored for each sample and the numbers of micronuclei and nucleoplasmic bridges were observed.

2.6.5 Statistical Methods: Micronucleus Assay

Statistical analysis for the micronucleus assay was kindly carried out by Roger Newson (Imperial College London) as follows. The experiment involved measurements of 2 binary outcomes, namely micronuclei and nucleoplasmic bridges on cells using varying sample sizes per assay. Factors varied in the design were cell type (BJ or hTERT cells), treatment (PBS or 0.04μM potassium dichromate), treatment type (direct or medium transfer), and number of days post treatment at which the cells were sampled and counted (0 days, 5 days or 30 days). Each of the 2x2x2x3=24 combinations of the 4 factors were replicated in 3 replicate assays. In each assay, the outcome was the number of abnormal cells out of the total number assessed.

A generalized linear model with binomial error, log link and a common overdispersion parameter was fitted to the samples for each outcome. A binomial total equal to the number of cells sampled in the assay was used to estimate 24 binomial proportions, one for each combination of the 4 factors. Classical standard errors were used in conjunction with the assumption that the level of overdispersion of binomial rates was the same for all factor combinations. 95% confidence intervals were calculated for the 24 binomial proportions for each outcome, and also for two sets of rate ratios, namely the hTERT/BJ rate ratio for each of the 2x2x2x3=24 combinations of outcome, treatment, treatment type and day and the Cr/PBS ratio for each of the 2x2x2x3=24 combinations of outcome, cell type, recipient type and day. Confidence intervals were also calculated for

the hTERT/BJ ratio of the Cr/PBS ratios for the 2x2x3=12 combinations of outcome, treatment type and day.

For each set of rate ratios, the set of corresponding *P*-values were entered into the Simes procedure (Benjamini et al. 2001), controlling the false discovery rate at 0.05, to define a corrected critical *P*-value, below which a rate ratio might be considered "significant" even considering that it is one of the more significant ratios of a set of 24 or 12 rate ratios measured. (If you measure 20 *sample* rate ratios, then, on average, one of these will be significant at the 5% level, even if all *population* rate ratios are one.) The Simes procedure is less conservative than the Bonferroni procedure, and defines a subset of rate ratios, with the feature that we are 95% confident that *some* of them are different from one in the population at large, or 90% confident that *most* of them are different from one in the population at large (Team 2003).

2.7 γ-H2AX Focus Formation Assay

The protocol for immunofluorescence using anti-γ–H2AX was adapted from a protocol published by Nakamura et al (Nakamura et al. 2006).

2.7.1 Materials and Solutions

- Glass Coverslips round, 12mm diameter, (VWR International Ltd)
- 1 Litre Conical flask
- Nitric Acid 69% ($HN0_3$) (AnalaR BDH)
- 2 Litre glass Beaker
- Methanol **(**Fisher Scientific Ltd)
- Round glass Petri Dish
- Autoclave

- 4- Well Plate Nunclon flat-bottom tissue culture dish, (Nunc) supplied by Fisher Scientific Ltd
- Complete DMEM
- Fixative 4% paraformaldehyde (Pfa) (v/v) in distilled water (AnalaR BDH)
- DPBS (Sigma)
- DPBST 0.1% Triton x-100 (Sigma) in DPBS (Sigma)
- Blocking Solution 1% BSA (Sigma) (1g/100ml) (w/v) in DPBST
- γH2AX Primary antibody: Polyclonal antibody to histone γH2AX, human, phosphorylated ($pSer^{139}$) from rabbit ((Alexis) supplied by Axxora UK Ltd), diluted in blocking solution 1/500 (v/v)
- Parafilm cut into circles, 12mm in diameter (Pechiney Plastic Packaging)
- Secondary Antibody AP 182 C, Donkey anti-Rabbit IgG, Cy3-Conjugated, Species Adsorbed: Hu, Ms, Rt, Bov, Gt, Sh, Chk, GP, Eq (Chemicon International), diluted in blocking solution 1/500 (v/v)
- Hoechst-33342 (2'-[4-hydroxyphenyl]-5-[4-methyl-1-piperazinyl2,5'-bisbenzimidazole]) (Sigma) diluted 1/10,000 in DPBS (v/v)
- Microscope slides **(**VWR International Ltd)
- Mounting Medium antifade, without DAPI VectaShield (Vector Laboratories Inc)
- Sealant Clear nail polish

2.7.2 Preparation of glass coverslips

Glass coverslips were acid washed and sterilized: Glass coverslips were placed in a 1 litre conical flask. Enough 69% nitric acid was poured into the flask to cover the bottom surface. The opening of the conical flask was covered with foil and the coverslips were left there for at least 5 minutes, swirling occasionally. A 2 litre glass beaker was filled

with water and left in the sink. Nitric acid from the conical flask was poured into the water and discarded down the sink. Coverslips in the conical flask were rinsed with tap water for around 10 minutes. They were then rinsed twice with single distilled water. Excess water was poured away and methanol was added to cover the bottom of the conical flask. The liquid was swirled to aid the transfer of the coverslips into a glass Petri dish. Excess methanol was discarded. The lid was placed on the Petri dish and it was autoclaved.

2.7.3 Cell Culture on Glass Coverslips

Cells were treated for 24 hours in a T-75 flask either directly with Cr (VI) or with conditioned medium and then harvested via trypsinization. 25,000 cells were seeded onto a sterile acid-washed coverslip placed in each well of a 4-well tissue culture dish. 1 ml of warm, complete DMEM was added to each well. Cells were incubated overnight at 37°C in 5% ambient CO_2 to allow attachment to coverslips. After which, culture medium was replaced to prevent FBS depletion. The cells were then incubated for a further 2 hours at 37°C in a 5% CO_2 incubator.

2.7.4 Immunostaining

Titration controls were carried out to ensure optimal antibody dilution for both the primary and secondary antibodies. Several dilutions of antibody stock solutions were tested (1:100, 1:250, 1:500 and 1:1000). 1:500 was the concentration that yielded optimal staining in both cases.

All immunostaining steps were carried out at room temperature unless otherwise stated. After 2 hours incubation with fresh medium, coverslips were washed with warm DPBS at 37°C to maintain the cells in a stress-free state. During washing steps, the 4-well plate was tilted to an approximately 45° angle to the work bench. Wash solution was poured

over each coverslip at the uppermost edge and aspirated from the bottom of the well. The coverslips were not allowed to dry out. The DPBS was aspirated and the cells were fixed for 5 minutes using 4% Pfa (v/v) in distilled water. Cells were permeabilized by washing twice in DPBST for 5 minutes each time at room temperature. All antigens were blocked using 1% BSA (w/v) in DPBST for 30 minutes at room temperature.

Primary antibody to histone γ-H2AX polyclonal antibody from rabbit was diluted 1/500 (v/v) in the BSA blocking solution and mixed by vortexing in an eppendorf tube. 50µl of the diluted antibody solution was dropped on to the centre of each coverslip. This drop was covered with a circle of parafilm, which creates a surface tension and prevents the coverslip from drying out. A polystyrene box was moistened using wet paper towels. The plates were placed inside this box and incubated overnight (or for approximately 18 hours) at 4°C.

After primary antibody incubation, coverslips were washed 3 times with DPBST for 5 minutes each time. Following the primary antibody incubation step, coverslips were protected from light at all times.

Secondary antibody (donkey-anti-rabbit Cy3 labelled antibody) was diluted 1/500 (v/v) in blocking solution. 50 µl was dropped on to the centre of each coverslip. Cells were incubated at 37°C for 25 minutes (light protected). After secondary antibody incubation, coverslips were washed 3 times with DPBST for 5 minutes each time. Cells were counterstained with Hoechst-33342, a stain used to visualise DNA (Loken 1980) diluted 1/10,000 (v/v) in DPBS. The surface of the coverslip was immersed in diluted Hoechst solution for 5 minutes at room temperature. After Hoechst-33342 incubation, coverslips were washed with DPBS for 5 minutes at room temperature.

A drop of mounting medium (Vectashield) was placed on a microscope slide. Coverslips were removed from the well using forceps, inverted and placed on this drop. During the transfer of the coverslip to the microscope slide, care was taken not to scratch the surface of the coverslip that holds the stained cells. Excess medium was removed by pressing over the mounted coverslip with a paper towel. Coverslips were sealed using clear nail polish around the circumference of the coverslip. Nail polish was allowed to dry slowly at room temperature before the slides were transferred to 4° C for storage.

2.7.5 Criteria for Scoring γ-H2AX

Following staining of cells on glass coverslips, the cells were stored at 4 °C in the dark as secondary antibody and Hoechst staining is light-sensitive. Cells were viewed by fluorescence microscopy. Cells were viewed under the blue filter (excitation at 355 nm and emission at 465 nm) to locate nuclei stained blue by Hoechst -33342. Cells were also viewed under the red filter with excitation wavelength 550 nm and emission 570 nm to570 nm to identify the Cy3 labelled anti γ-H2AX. Cells were scored according to the number of punctate red foci per cell. A record was made of all the cells containing no foci, 1-3 foci and more than 4 foci per cell. According to Sokolov et al (Sokolov et al. 2005), using a threshold of ≥4 foci per cell is optimal for determining the fraction of affected cells. Fewer than 0.3% of cells should contain ≥4 foci by random Poisson distribution because in early passage normal fibroblast cultures there is an average of <1 focus per cell (Sedelnikova et al. 2004). A minimum of 300 nuclei were counted for each sample.

2.7.6 Statistical Methods: γ-H2AX focus formation assay

Statistical analysis was kindly carried out by Roger Newson (Imperial College London) as follows for all γ-H2AX data unless otherwise stated. The experimental sampling units were samples of cells (3 samples per treatment combination) and cells within samples. The outcome variable, measured in each cell in each sample, was number of foci, coded on an ordinal scale with 3 levels, namely "No foci", "1-3 foci", and "4+ foci". Treatment variables were day of exposure at which foci were measured (0, 5 or 30), cell type (BJ or hTERT), treatment mode (direct or medium transfer), and treatment (PBS or Cr). The variable whose effect was of primary interest was treatment (i.e. Cr or PBS), and the outcome was on an ordinal scale. We therefore defined the "effect" of Cr, for each combination of day, cell type and transfer mode, as being Somers' *D* of number of foci with respect to Cr treatment, in cells with the appropriate combination of day, cell type and transfer mode. Somers' *D* is the parameter behind the Wilcoxon rank sum test, and is here defined as the difference between 2 probabilities, namely the probability that a random Cr-treated cell has more foci than a random PBS-treated cell and the probability that a random PBS-treated cell has more foci than a random Cr-treated cell. Methods for calculating confidence intervals for Somers' *D* are discussed in (Newson 2002) and (Newson 2006).

2.7.7 Statistical Methods for γ-H2AX focus formation assay following medium transfer between cell types

The experimental sampling units were samples of cells (3 samples per treatment combination) and cells within samples. The outcome variable, measured in each cell in each sample, was number of foci, coded on an ordinal scale with 3 levels, namely "No foci", "1-3 foci", and "4+ foci". Treatment variables were day of exposure at which foci were measured (0, 5 or 30), subject cell type (BJ or hTERT), medium donor cell type

(BJ or hTERT), and treatment of donor cell (PBS or Cr). The treatment variable whose effect was of primary interest was treatment type of donor cell, and the outcome was on an ordinal scale. We therefore defined the "effect" of Cr, for each combination of day, subject cell type and donor cell type, as being Somers' *D* of number of foci with respect to Cr treatment, in cells with the appropriate combination of day, subject cell type and donor cell type. Somers' *D* is the parameter behind the Wilcoxon ranksum test, and is here defined as the difference between 2 probabilities, namely the probability that a random Cr-treated cell has more foci than a random PBS-treated cell and the probability that a random PBS-treated cell has more foci than a random Cr-treated cell.

2.8 Microinjections

2.8.1 Background

Microinjections were carried out to study transmission of bystander factors between cells in close contact possibly via gap junctional intercellular communication. This was designed to mimic experiments carried out by radiation biologists in the study of radiation-induced bystander effects in which a targeted microbeam can allow selected cells or subcellular components in an irradiated population to remain unirradiated (Randers-Pehrson et al. 2001). Access to a microinjector was kindly allowed by Dr Kate Nobes (University of Bristol Department of Physiology). An image of the microinjection apparatus is shown in Figure.2.2.

Figure.2.2 Microinjection Equipment

2.8.2 Materials and solutions

- FITC dextran Excitation 490 nm and emission wavelength 425 nm
- Injection Buffer:

  -Filtered 0.4 μM $K_2Cr_2O_7$ with 10% (v/v) FITC dextran or

  -Sterile DPBS with 10% (v/v) FITC dextran

- Zeiss microscope injector
- Materials required for γ-H2AX focus formation assay (as described in 2.7)

2.8.3 Methods

Cells were cultured on acid washed glass coverslips as described in section 2.4.3. During injections, coverslips were placed in a Petri dish containing 5 ml complete DMEM at 37°C and 5% ambient CO_2. Cells were injected with either 0.4 μM or DPBS. Green FITC dextran in the injection buffer enabled the injected cell to be visualized and identified. Cells were injected with indefinable amounts of injection buffer. The duration of injection depends on the amount of pressure exerted on the injection release and the amount of time the release button is depressed for. Injections were carried out

approximately 10 cell widths apart. Following injection, coverslips were replaced in the 4-well plates with 1ml complete growth medium and incubated at 37°C with 5% ambient CO_2 overnight (or for 18 hours). The coverslips were then washed and stained (as described in 2.7).

2.9 Cytogenetics

2.9.1 Background

This assay is performed as described by Coen et al (Coen et al. 2001). The cells were treated with colchicine, an alkaloid extract from meadow saffron that arrests cells in metaphase.

2.9.2 Materials and solutions

- Microscope Slides washed in liquid detergent and then rinsed in deionized water, stored in 70% ethanol at 4°C
- Colchicine 200 μg/ml (Sigma) Colchicine stock solution is at 10 mg/ml
- Hypotonic Solution 0.5% (w/v) potassium chloride (KCl) (Sigma) in distilled water
- Fixative: methanol and acetic acid in a 3:1 ratio (v/v) stored at -20°C
- 5% Giemsa (v/v) (twice filtered) in deionized water (Gurr BDH Laboratory supplies)

2.9.3 Methods

5 x 10^5 cells were plated out in a T-75 flask and treated for 24 hours the following day with either Cr (VI) or PBS (as described in 2.2.2). For the final 6 hours of the treatment the treatment, cells were treated with 200 μg/ml colchicine. Cells were harvested and centrifuged at 1200 RPM for 8 minutes. The cell pellet was retained and resuspended drop-wise in a hypotonic solution consisting of 0.5% (w/v) KCl in distilled water. The

cell suspension was incubated for 12 minutes at 37°C. This treatment breaks open the cells to release chromosomes and is followed by centrifugation at 1000 RPM for 8 minutes. The cell pellet was retained and resuspended in approximately 1 ml of the remaining hypotonic solution. The cells were then fixed in cold (-20°C) methanol-acetic acid (3:1 v/v) by adding the fixative slowly drop-wise, with constant agitation using a vortex mixer. The cell suspension was centrifuged again and the fixation step was repeated. Samples could at this stage then be stored at -20°C for future use. Fixed cells were dropped onto clean, pre-chilled slides and allowed to air-dry before solid-staining in 5% Giemsa for 10minutes. 100 metaphase spreads were examined under oil immersion (x 100 objective) with light microscopy. The number of abnormalities per metaphase were counted, including chromatid breaks, gaps, fragments, dicentric chromosomes, tetraploid cells (those with 92 chromosomes) and aneuploidy (chromosome loss and gain). 100 metaphases were scored for each sample and the experiments were repeated in triplicate.

2.9.4 Statistical Methods: Cytogenetics

A 2-tailed student's t-test assuming equal variance was carried out to determine *p* values.

2.10 γ-H2AX focus formation on metaphase spreads

2.10.1 Materials and Solutions

- Colchicine 200 μg/ml (Sigma)
- 0.5% KCl (Sigma)
- Immunopen liquid blocker (Vector Laboratories Inc)
- DPBS (Sigma)
- DPBST: 0.05% triton x-100 (v/v) (Sigma) in DPBS

- Blocking solution 1% BSA (Sigma)(1g/100ml) (w/v) in DPBST
- Rabbit polyclonal antibody to human histone γ-H2AX (Alexis) supplied by Axxora UK Ltd diluted 1/500 in blocking solution (v/v).
- Secondary antibody: Donkey IgG, Cy3 conjugated diluted 1/500 in blocking solution (Chemicon International)
- Hoechst-33342 (Sigma) 1/10,000 (v/v) in PBS
- Microscope slides (VWR International Ltd)
- Rectangular glass coverslips 22x50 mm (VWR International Ltd)
- Mounting medium (Vectashield)
- Sealant: Clear nail polish

2.10.2 Methods

The method for γ-H2AX detection on metaphase spreads was adapted from a protocol published by Nakamura (Nakamura et al. 2006). $3x10^5$ cells were plated out into T-75 flasks and treated the following day with either Cr (VI) or conditioned medium for 24 hours (as described 2.2.2) at 37°C. The cells were treated with colchicine for 4 hours prior to harvesting to enrich the population of mitotic cells, after which medium was aspirated leaving mitotic cells undisturbed. The cells were incubated in hypotonic solution (0.5% KCl) at 37°C for 15 minutes and subsequently detached by agitating flasks. Cells were cytospun onto slides by adding 200 μl of cell suspension to each cytospin chamber spinning for 8 minutes at 1800 RPM. Slides were allowed to air dry and were subsequently fixed using pre-chilled methanol (-20°C) for 30 minutes. In order to stain for γ-H2AX, a hydrophobic ring was drawn around the cytospin spots using an immunopen. Once this ring had dried, slides were rehydrated using DPBST, dropped into the circle for 5 minutes. Blocking of all antigens was achieved using 1 %

BSA in DPBST (w/v) in a humid chamber for 1 hour. Rabbit polyclonal antibody to human histone γ-H2AX (primary antibody) was diluted 1/500 in blocking solution and incubated in a humid chamber for 2 hours. Cells were then washed twice with DPBST for 5 minutes each time to remove any excess primary antibody. Donkey IgG, Cy3 conjugated secondary antibody was diluted 1/500 in blocking solution and incubated in a humid chamber for 1 hour. Slides were then washed twice with DPBST for 5 minutes each time to remove any secondary antibody. Nuclei were counterstained with Hoechst -33342 (1/10,000 in PBS) for 5 minutes at room temperature. Antifade mounting medium was used to mount coverslips and slides were sealed using clear nail polish. This process was repeated on day 5 and day 30 for both direct metal treatment and conditioned medium transfer.

2.11 Low Dose Cr (VI) Treatment: Residual Chromium in Conditioned Medium after Washings

2.11.1 Background

In order to confirm that the metal-induced bystander effects observed during this project were due to secretion of bystander factor/s, analysis of the Cr (VI) content in conditioned medium was carried out by the Analytica laboratory (Sweden) (as described in section 2.3). The residual concentration of chromium found in conditioned media after the 5 washing steps was 1.08 μg/L. The concentration of Cr in fresh complete DMEM was found to be 0.983 μg/L. BJ and hTERT cells were directly exposed to the low concentration of Cr (VI) for 24 hours – fresh complete DMEM supplemented with 1.08μg/L $K_2Cr_2O_7$. The cells were then subsequently assayed for hallmarks of genotoxicity by the micronucleus assay and the γ-H2AX focus formation assay.

2.11.2 Materials and Solutions

- 12 T-75 cell culture flasks of hTERT cells
- 12 T-75 cell culture flasks of BJ cells
- PBS tablet solution: 3 Tablets in 600 ml distilled water
- $K_2Cr_2O_7$ stock solution 54 μg/L in PBS tablet solution
- 0.2 μm syringe filter
- Materials required for Cyt-B block micronucleus assay (as described in 2.6)
- Materials required for γ-H2AX focus formation assay (as described in 2.7)

2.11.3 Methods

BJ and hTERT cells were seeded in to 12 x T-75 tissue culture flasks for each cell type at a density of $3x10^5$ cells per flask. The following day, 6 flasks for each cell type were treated with 400 μL PBS (control) and 6 flasks for each cell type were exposed to 400 μL of the Cr (VI) stock solution (54 μg/L) to give a final concentration of 1.08 μg/L in a total flask volume of 20 ml. Prior to treatment with either PBS or Cr (VI), solutions were sterilized using a 0.2 μm syringe filter. Six BJ flasks (3 PBS-treated and 3 Cr-treated) and 6 hTERT flasks (3 PBS-treated and 3 Cr-treated) were investigated for chromosome breakage and loss using the micronucleus assay (as described in section 2.6). In these flasks, Cyt-B (6μg/ml) was administered to the cells simultaneously with the Cr (VI) or PBS treatment solutions as Cyt-B treatment took place over 24 hours. After 24 hours incubation at 37°C, the cells were harvested by trypsinization and the micronucleus assay was carried out in triplicate (as described in section 2.6). The remainder of the flasks (3 BJ exposed to Cr, 3 BJ exposed to PBS, 3 hTERT exposed to Cr and 3 hTERT exposed to PBS) were treated for 24 hours before they were harvested

by trypsinization and plated out onto coverslips in triplicate at a density of 30,000 cells per coverslip for the γ-H2AX focus formation assay (as described in section 2.7).

CHAPTER 3 MATERIALS AND METHODS: MECHANISTIC INSIGHTS

3.1 Antioxidants

3.1.1 Background

An antioxidant is defined as 'any substance that, when present at a low concentration compared to that of an oxidizable substrate, significantly delays or prevents oxidation of that substrate'(Halliwell 1990). L-ascorbic acid (vitamin C) is an antioxidant that is able to act rapidly due to its water solubility (Wu et al. 1991), and when combined with a reactive free radical, readily undergoes oxidation to produce a less reactive ascorbate radical (Bielski et al. 1975). Some proposed mediators of the bystander effect are known to increase the intracellular reactive oxygen species (ROS) (Narayanan et al. 1997). The antioxidant properties of ascorbic acid were summarized by Halliwell (Halliwell 1996). Ascorbic acid was used in this study to eliminate the DNA damaging actions of molecules present in the medium of Cr (VI) treated fibroblasts and also by direct treatment of Cr (VI). Its effects on the frequency of γ-H2AX focus formation were investigated in both recipient cells and Cr (VI) exposed cells.

3.1.2 Materials and Solutions

- PBS Tablet Solution: 3 PBS tablets (Sigma) were dissolved in 600ml distilled water to produce - 0.01 M Phosphate buffer, 0.0027 M KCl, 0.137 M NaCl at pH 7.4
- 40 mM Stock solution l-ascorbic acid (Sigma) 1.76 g in 250 ml PBS

(– use 200μl stock per 20 ml total flask volume to give 0.4 mM treatment)

- 0.4 μM $K_2Cr_2O_7$
- Materials required for γ-H2AX focus formation assay (as described in 2.7)

3.1.3 Methods

Two sets of cells were directly Cr exposed during this experiment; 1) Cells to be assayed for the effects of ascorbic acid on directly Cr (VI)-exposed cells and 2) Donor cells, in which medium was conditioned prior to its transfer to recipient cells.

$3x10^5$ cells were plated out in 24xT-75 flasks for each cell type and incubated overnight before being directly treated with Cr (VI) (12 flasks for each cell type) or PBS (12 flasks for each cell type) (as described in 2.2.2). After a 24 hour Cr (VI) or PBS treatment, cells were washed to remove Cr and were then exposed to either the antioxidant l-ascorbic acid (0.4mM); or no additive, as a control, also for 24 hours after metal treatment to avoid potentially harmful reduction of Cr by ascorbate. This concentration was selected because the antioxidant activity of ascorbic acid becomes attenuated above 2 mM possibly adopting pro-oxidant activity (Park and Lee 2008). On the same day as treatments were administered, $3x10^5$ recipient cells were plated out into 24 flasks for each cell type. These would receive conditioned medium from either the same cell type (12 flasks) or the other cell type (12 flasks) from donor cells that had been exposed to either Cr (VI) or PBS and 0.4mM ascorbic acid, or no additive. A summary of these treatment combinations is shown in Figure 3.1.

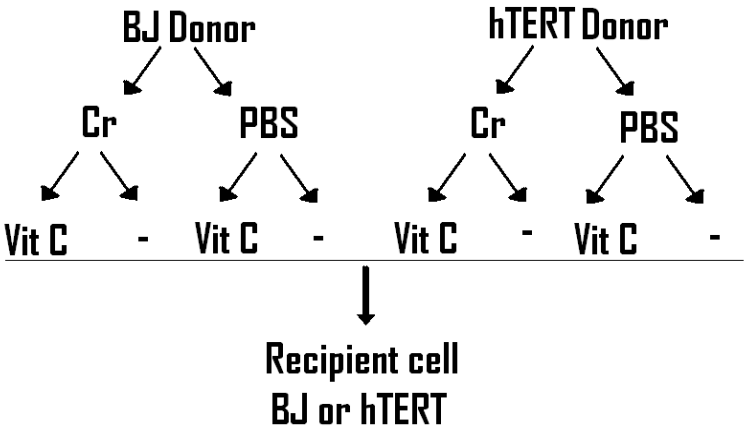

Figure 3.1Summary of Treatments with Vitamin C

After 24 hours treatment, cells were washed with sterile DPBS (37°C). Directly treated cells were seeded onto coverslips and stained for the γ-H2AX focus formation assay (as described in section 2.7.4). Fresh complete DMEM was added to donor flasks for one hour in order to condition medium. This medium was filtered and transferred to recipient cells as described in section 2.2.2.2. The recipient cells were exposed to conditioned medium for 24 hours before being seeded onto coverslips and stained for the γ-H2AX focus formation assay (as described in section 2.7). Cells were scored according the criteria outlined in section 2.7.5. A minimum of one hundred randomly selected nuclei were scored from at least three independent experiments and ranked as containing 0, 1-3 or four or more foci per cell.

3.2 p38 MAP Kinase and gap junctions

3.2.1 Background

Members of the MAP Kinase family have been implicated as mediators of bystander responses (Azzam et al. 2002; Zhou et al. 2005; Lyng et al. 2006b) and MAPK is known to become activated by hexavalent chromium (Tessier and Pascal 2006). MAP Kinase cascades regulate critical cellular signalling processes involved in proliferation,

differentiation and apoptosis. The p38 MAP Kinase signalling module can be activated by environmental stresses such as oxidative stress, UV irradiation, hypoxia, ischaemia, and inflammatory cytokines and is important for survival after radiation exposure (Dent et al. 2003). Activated MAPK translocates from the cytoplasm to the nucleus where they regulate target transcription factor activity through phosphorylation (Su and Karin 1996).

p38 MAP Kinase signalling was manipulated in order to investigate whether the differences in bystander signalling between the two cell types can be attributed to differences in the nature of their gap junctional intercellular communication (GJIC) as gap junctions are proposed to mediate the bystander effect (Azzam et al. 1998; Azzam et al. 2001). Perhaps BJ cells have a more permissive 'open' gap junction structure than hTERT cells. With this hypothesis in mind, anisomycin, an antibiotic produced by streptomyces griseolus was used to reduce GJIC in BJ cells. Anisomycin activates p38 MAP Kinase leading to enhanced Connexin 43 phosphorylation and reduced GJIC (Ogawa et al. 2004). Treatment of BJ donor cells with anisomycin may induce a less permissive more 'closed' gap junction structure enabling the secretion of fewer bystander factors from BJ donor cells. The Pyridinyl imidazole inhibitor SB203580 inhibits p38 MAP Kinase and is able to restore GJIC (Ogawa et al. 2004). Donor hTERT cells were incubated with SB203580 at 10μM for 3 hours to alter their gap junction signalling (Ogawa et al. 2004; Lyng et al. 2006b). The concentration of anisomycin was chosen according to that selected by Ogawa (Ogawa et al. 2004). Treatment of hTERT donor cells may induce more open gap junction signalling, enabling the cells to secrete additional bystander factors, possibly enabling so far unobserved biological effects in recipient hTERT cells, investigated via the micronucleus and γ-H2AX focus formation assays.

3.2.2 Materials and solutions

- SB203580 (Alexis) 10μM use 77μl of stock (1mg/ml in DMSO) in 20 ml total T-75 flask volume.
- Anisomycin (Calbiochem) 10μg/ml use 20μl of stock (10mg/ml in distilled H_2O) in 20 ml total T-75 flask volume.
- Cyt-B 6μg/ml for MN assay (as described in 2.6)
- DMSO (Sigma)
- Materials required for γ-H2AX focus formation assay (as described in 2.7)

3.2.3 Methods

Donor cells were treated with 0.4 μM potassium dichromate for 24 hours as described in section 2.2, after which, cells were washed 5 times with sterile DPBS at 37°C. On day 5 post Cr-exposure, BJ donor cells were incubated with anisomycin or no additive (control and Cr-exposed hTERT cells were incubated with SB203580 or DMSO (control) at 37°C for 3 hours. After 3 hours incubation with the chemical additive, the cells were washed with DPBS, and fresh complete DMEM was added for conditioning for one hour. Conditioned medium was harvested from both donor cell types and transferred to hTERT recipients for 24 hours to be assayed for γ-H2AX focus formation. After 24 hours exposure to conditioned medium, hTERT cells were harvested and plated out onto coverslips (as described in section 2.7). Conditioned medium from Cr-exposed BJ cells treated with anisomycin (or no additive) was supplemented with 6μg/ml Cytochalasin-B in order to carry out the micronucleus assay as described in section 2.6. Cyt-B-containing conditioned medium from BJ donor cells was transferred to BJ recipient cells for 24 hours incubation at 37°C prior to harvesting for the micronucleus assay.

3.2.4 Statistical Analysis of Micronucleus and γ-H2AX Focus Formation Following p38 Map Kinase Manipulation in Donor Cells

The experimental sampling units were samples of cells (3 samples per treatment combination) and cells within samples. The outcome variable, measured in each cell in each sample, was number of foci, coded on an ordinal scale with 3 levels, namely "No foci", "1-3 foci", and "4+ foci". All measurements in this experiment were made on exposure day 5, and all samples of cells were hTERT cells, treated with Cr medium from donor cells. Treatment variables were donor cell type (BJ or hTERT) and additive additional to Cr (None, Anisomycin, DMSO or SB203580). There were 2 sub-experiments. In the first sub-experiment, the donor cells were BJ throughout, and the additive was either none (control) or Anisomycin. In the second sub-experiment, the donor cells were hTERT throughout, and the additive was either DMSO (control) or SB203580. The treatment variable whose effect was of primary interest was the additive (control or non-control), and the outcome was on an ordinal scale. The "effect" of the non-control additive, for each sub-experiment, was therefore defined as being Somers' *D* of number of foci with respect to non-control additive. Somers' *D* is the parameter behind the Wilcoxon rank sum test, and is here defined as the difference between 2 probabilities, namely the probability that a random non-control cell has more foci than a random control cell and the probability that a random control cell has more foci than a random non-control cell.

3.2.5 Scrape loading dye transfer of Lucifer yellow

3.2.5.1 Materials and Solutions

- 35 mm diameter round sterile plastic cell culture dish (Nunc)
- DPBS (Sigma)

- Lucifer yellow CH 0.05% in PBS (Sigma)
- Scalpel
- 4% formaldehyde in distilled water

3.2.5.2 Methods

Gap junctional intercellular communication (GJIC) has been proposed as a possible mechanism for the transmission of bystander signals (Azzam et al. 2001). In order to investigate whether the fibroblasts have gap junctions, scrape loading dye transfer of Lucifer yellow was carried out. Cells were cultured in sterile round plastic Petri dishes 35 mm in diameter. When cells had reached 80-90% confluence, they were washed with DPBS (37°C). Lucifer yellow CH 0.05% in PBS was washed over the cell-containing surface. 3 scrapes were made in the cell monolayer using a scalpel. This allowed the dye to enter the cells damaged by the scrape. Transfer of the dye to cells distant from the scrape may occur only via gap junctional intercellular communication. After 3 minutes incubation with the dye at room temperature, the cell monolayer was washed several times with PBS and cells were fixed using 4% formaldehyde. The extent of dye transfer from primary cells to adjoining cells gives a measure of GJIC and was determined by examination under a Zeiss epifluorescence microscope. Lucifer yellow is excited at 425 nm and emits at 528 nm according to Olympus Microscopy Centre fluorochrome data tables.

3.3 TNF–alpha neutralization in conditioned medium

3.3.1 Background

TNF-alpha is a cytokine involved in systemic inflammation and is known to induce apoptotic cell death and inflammation and to inhibit tumourigenesis (Tracey and Cerami 1993). TNF-alpha has been implicated to play a role in bystander responses (Shareef et

al. 2007). To investigate whether the metal-induced bystander effect is influenced by TNF-alpha signalling, a neutralizing antibody, anti-TNF-alpha was used to sequester functional TNF-alpha from the conditioned medium.

3.3.2 Materials and Solutions

- Rabbit polyclonal Antibody to TNF-alpha (Abcam) 0.1 μg/ml
- Materials required for γ-H2AX focus formation assay (as described in 2.7)

3.3.3 Methods

Conditioned medium was harvested from donor cells on day 5 post Cr (VI) treatment. A neutralizing antibody, anti-TNF-alpha (0.1 μg/ml) (optimal for neutralization according to manufacturer) was added to the filtered conditioned medium prior to its transfer to recipients. Following 24 hours treatment with conditioned medium, cells were harvested and seeded onto coverslips for the γ-H2AX focus formation assay (as described in 2.7).

3.4 K1 Thyroid Carcinoma Cell Line-Telomerase Control

3.4.1 Background

An immortalized cell line was used in which a dominant negative hTERT is expressed to abolish telomerase activity. These cells could be compared to the BJ – hTERT cells used in this project to abolish the confounding effects of using a retrovirally created hTERT positive cell line. The K1 papillary thyroid cancer cell line is well characterized and is known to be well differentiated. The cells are telomerase positive and immortal (Jones et al. 1998). A retrovirus expressing a dominant negative (DN) version of the catalytic subunit of telomerase was created by Hahn et al (Hahn et al. 1999) using pBABE DNhTERT puro. K1 cells were infected with either DNhTERT puro or pBABE puro (control) retroviruses. This DN expression in K1 cells is sufficient

to abolish telomerase activity (Preto et al. 2004). The K1 thyroid cancer cell line was kindly provided by Duncan Baird (Cardiff University).

3.4.2 Materials and Solutions

- PBS or 0.4 μM $K_2Cr_2O_7$ (As described in 2.2)
- Complete DMEM
- K1 thyroid carcinoma cells DNhTERT puro (Dominant Negative) – donor cells
- K1 thyroid carcinoma cells pBABE puro (control) – telomerase positive donor cells
- BJ and hTERT recipient cells
- Materials required for γ-H2AX staining protocol (as described in 2.7)

3.4.3 Methods

K1 donor cells (DN or immortal control) were treated with Cr (VI) or PBS (as described in 2.2) for 24 hours, after which they were washed to remove any trace of metal ions. On day 5 post metal treatment, treated cells were incubated in complete DMEM. This conditioned medium was harvested after one hour incubation, filtered to remove any directly metal-exposed cells and transferred to BJ or hTERT recipient cells. After 24 hours incubation of BJ and hTERT recipient cells in Cr-conditioned medium from either DN or control K1 cells, recipient cells were plated onto coverslips for the γ-H2AX focus formation assay (as described in section 2.7) and scored using the criteria outlined in 2.7.5.

3.5 Human Embryonic Stem Cells

3.5.1 Background

To compare the effects of physiological telomerase to the hTERT cells used in this project, a study was carried out using human embryonic stem (hES) cells. hES cells

form colonies which are routinely cultured with mouse embryonic fibroblasts (mEFs). The mEFs serve as a feeder cell layer to maintain the stem cells in an undifferentiated state and provide a sort of connective tissue forming the matrix upon which the stem cells grow. hES cells were co-cultured with mEFs under a combination of different treatment conditions. Stem cells were kindly supplied and cultured by Maeve Caldwell and Lucy Crompton (University of Bristol).

3.5.2 Materials and Solutions

- PBS or 0.4 μM $K_2Cr_2O_7$ (as described in 2.2)
- Human embryonic stem cells (hES) Cell line - Shef3 (UK Stem Cell Bank)
- Mouse embryonic fibroblasts (mEFs) – feeder cells, Mitomycin-C inactivated
- Growth media was as follows; knockout DMEM supplemented with 20% serum replacement, 1% non-essential amino acids, 1x glutamax, 0.1mM β-mercaptoethanol, penicillin/streptomycin/fungizone (recommended concentrations) [all from Invitrogen], and 4 ng/ml basic fibroblast growth factor [Peprotech]
- Stem cells were propagated on mitotically inactivated mouse embryonic fibroblasts (MEF's) and subcultured manually using a glass needle
- Materials required for γ-H2AX staining protocol (as described in 2.7)

3.5.3 Methods

hES cell colonies were cultured on a mitotically inactivated feeder cell layer of mEFs on glass coverslips in a humidified 37°C incubator, with 5% CO_2. mEFs were inactivated using Mitomycin-C and seeded into four well plates at a density of 50,000 per well (approximately 15,000/cm^2). hES cells and mEFs were exposed to 0.4 μM potassium dichromate or PBS only in the normal growth media for a 24 hour period before the cells came into contact with each other. After this the cells were washed in PBS to

remove all traces of Cr, and the normal growth media was replaced. The cells were cultured for 5 days, with media changes occurring every second day. Five days post treatment with either Cr (VI) or PBS control, mEFs and hES cells were co-cultured for 24 hours to give the following combinations:

Table 3.1 Stem cell and mouse feeder cell treatment combinations

hESCs	mEFs
hESC exposed control PBS only	mEFs exposed to control PBS only (h-/m-)
hESC exposed control PBS only	mEFs exposed to Cr (h-/m+)
hESC exposed to Cr	mEFs exposed control PBS only (h+/m-)
hESC exposed to Cr	mEFs exposed to Cr (h+/m+)

After this 24 hour period, the cells were fixed in 4% Pfa and the coverslips were processed for analysis using the γ-H2AX focus formation assay (as described in 2.7.4), using the same scoring criteria as described for BJ and hTERT human fibroblasts (2.7.5).

3.6 Human Embryonic Stem Cells - Human Fibroblast Feeder Cells

3.6.1 Background

The identification of hES cells using anti-Oct-4, a specific stem cell marker was considered to be important as embryonic stem cells spontaneously differentiate into embryoid bodies, which no longer possess telomerase activity.

3.6.2 Materials and Solutions

- PBS or 0.4 μM $K_2Cr_2O_7$ (as described in 2.2)
- Human embryonic stem cells (hES) Shef3 line (UK Stem Cell Bank)

- BJ and hTERT human fibroblast feeder cells (Mitomycin C-inactivated)
- BJ and hTERT human fibroblast feeder cells - Active (NOT Mitomycin C-inactivated)
- Chamber Slides (LAB-TEK)
- Fixative 4% paraformaldehyde (Pfa) (v/v) in distilled water (AnalaR BDH)
- DPBS (Sigma)
- DPBST 0.1% triton x-100 (Sigma) in DPBS (Sigma)
- Blocking Solution 1: 10% and 2% Goat Serum (Sigma) (v/v) in DPBST
- Blocking Solution 2: 10% and 2% Donkey Serum (Sigma) (v/v) in DPBST
- Blocking Solution 3: 1% BSA (Sigma) (1g/100ml) (w/v) in DPBST
- Anti Oct- 4 Stem Cell Marker (Primary antibody 1): Monoclonal IgI_{2b} antibody to Oct 4 from mouse (clone C-10) diluted in 2% goat serum 1/25
- anti-γ-H2AX (Primary antibody 2): Polyclonal antibody to histone γ-H2AX, human, phosphorylated ($pSer^{139}$) from rabbit ((Alexis) supplied by Axxora UK Ltd), diluted in 2% donkey serum: 1/250 (v/v)
- Secondary Antibody 1: Goat anti-Mouse Alexa fluor 488 fluorescein-conjugated IgG_{2b} (Invitrogen), diluted in 2% blocking solution 1: 1/125 (v/v)
- Secondary Antibody 2: AP 182 C, Donkey anti-Rabbit IgG, Cy3-Conjugated, Species Adsorbed: Hu, Ms, Rt, Bov, Gt, Sh, Chk, GP, Eq (Chemicon International), diluted in 2% blocking solution 2: (1/250) (v/v)
- Hoechst-33342 (2'-[4-hydroxyphenyl]-5-[4-methyl-1-piperazinyl2,5'-bisbenzimidazole]) (Sigma) diluted 1/10,000 in DPBS (v/v)
- Glass coverslips rectangular, 22 mm x 55 mm **(**VWR International Ltd)
- Mounting medium antifade, without DAPI VectaShield (Vector Laboratories Inc)
- Sealant Clear nail polish

3.6.3 Culture Methods

Human embryonic stem cells were cultured in chamber slides using either BJ or hTERT human fibroblasts as feeder cells in a humidified 37°C incubator, with 5% CO_2. Feeder cells were plated out at a density of approximately 15,000/cm^2 and were either normal or Mitomycin-C inactivated. Chromium (VI) or PBS control exposure was carried out in either the human fibroblasts or the hES cells for 24 hours before the stem cells and feeder cells came into contact. The cells were cultured for 5 days, with media changes occurring every second day. Five days post exposure to Cr (VI) or PBS control, the human fibroblast feeder cells and the hES cells were co-cultured for 24 hours to give the following combinations:

Table 3.2 Stem cell and human feeder cell treatment combinations

hESCs	BJ or hTERT feeders (Bh)
hESC exposed control PBS only	Inactive BJ or hTERT exposed to control PBS only (h-/Bh-)
hESC exposed control PBS only	Inactive BJ or hTERT exposed to Cr (h-/ Bh +)
hESC exposed to Cr	Inactive BJ or hTERT exposed control PBS only (h+/Bh-)
hESC exposed to Cr	Inactive BJ or hTERT exposed to Cr (h+/ Bh +)
No hESC	Non-inactivated BJ or hTERT cells exposed to Cr
No hESC	Non-inactivated BJ or hTERT cells exposed to control PBS only

After this 24 hour period, the cells were fixed in 4% Pfa at 4°C for 20 minutes. Chamber slide gaskets were washed in PBS and the slides were stored in PBS at 4°C

until use. The slides were processed for analysis using the γ-H2AX focus formation assay (as described in 2.7.4), using the same scoring criteria as described for BJ and hTERT human fibroblasts (2.7.5).

3.6.4 Immunostaining

All Immunostaining steps were carried out at room temperature unless otherwise stated. Cells were permeabilized by washing three times in DPBST. Blocking of antigens was achieved using the serum of the species that secondary antibodies were raised in. The secondary antibodies used in this procedure were raised in goat and donkey so equal volumes of 10% goat and 10% donkey serum were mixed together so both sera were at 5%. The mixed blocking solution was added to each well of the chamber slide to block for 2 hours. Two primary antibodies were used in this experiment and these were added in 2% serum at concentrations of 1/25 for anti-Oct-4 and 1/250 for anti-γ-H2AX. Both primary antibodies were added at the same time in equal volumes producing final concentrations 1/50 and 1/500 for anti-Oct-4 and anti-γ-H2AX respectively. Primary antibody incubations were carried out overnight at 4°C on a rocker.

Cells were washed three times in DPBST. Goat anti-mouse Oct 4-secondary IgG_{2b} 1/125 in 2% goat serum was mixed with an equal volume of donkey anti rabbit H2AX secondary IgG 1/250 in 2% donkey serum to give final concentrations of 1/250 and 1/500 for goat-anti-mouse and donkey-anti-rabbit respectively each in 1% serum. From this point on, all work was carried out in the dark as the secondary antibody conjugated fluorophores were light sensitive. Cells were incubated with secondary antibodies for two hours. Cells were then washed three times in DPBST. Cells were counterstained with Hoechst-33342, 1/10,000 (v/v) in DPBS for 5 minutes. After Hoechst-33342 incubation, chamber slides were washed with DPBST before gaskets were removed. A

drop of mounting medium with antifade was placed on the slides and a coverslip was placed on top. Excess mounting medium/buffer was blotted away using even pressure and a paper towel. Coverslips were sealed using clear nail polish around the edge of the coverslip. Nail polish was allowed to dry slowly at room temperature before the slides were transferred to 4° C for storage. Slides were scored according to the criteria described in 2.7.5.

3.6.5 Statistical Methods

A 2-tailed student's t-test assuming equal variance was carried out to determine *p* values in experiments involving embryonic stem cells cultured on human fibroblast feeder cells.

CHAPTER 4 RESULTS : CELL CULTURE MEDIUM BASED TREATMENT

4.1 Micronucleus Assay

4.1.1 Background

Heddle proposed the measurement of micronuclei in dividing cell populations as a means of indicating levels of chromosomal damage(Heddle 1973). Micronuclei enclose acentric fragments or whole chromosomes that are not incorporated into the main nuclei during cell division. According to Heddle (Heddle et al. 1991), there are four mechanisms by which micronuclei are recognized to arise:

1. Mitotic loss of acentric fragments
2. Chromosome breakage and exchange
3. Mitotic loss of whole chromosomes
4. Apoptosis

Micronuclei are small extranuclear bodies, formed during mitosis from acentric chromosomal fragments as the result of a clastogenic event. They may also arise from chromosomes that lag behind during anaphase; that is, they fail to segregate to the cellular poles as the result of an aneugenic event. During telophase, a nuclear membrane forms around the acentric fragment or lagging chromosome leading to formation of nuclei much smaller than the main nuclei residing in the cytoplasm. Induction of micronuclei only occurs in cells that have undergone at least one cell division. Therefore, the level of micronuclei depends on the proportion of dividing cells (Fenech and Morley 1985). Accurate estimation of micronuclei can be acquired in cells that have divided only once; achieved by addition of Cytochalasin-B (Cyt-B) to cultures before mitosis and after a chromosome damaging event. Cyt-B inhibits cytoplasmic cleavage

(cytokinesis) without interfering with nuclear division (karyokinesis) (Carter 1967). The concentration of Cyt-B selected for use in this study was based on preliminary work carried out by this group, which established that 6μg/ml raised the nuclear division index(Glaviano et al. 2006) and produced an optimal yield of binucleated cells. Cells that have undergone one division accumulate as binucleated cells, easily differentiable from the mononucleated cells that have not divided and all the products of mitosis are in one cell. This increases the sensitivity of the assay.

The micronucleus assay was carried out in this experiment to provide a sensitive measure of the chromosome damage induced by either direct Cr (VI) exposure or by treatment of cells with conditioned medium transferred from a Cr (VI)-exposed cell, by identifying cells containing one or more micronuclei and/or a nucleoplasmic bridge (NPB), which provide a means of quantifying chromosome rearrangements. A micronucleus and nucleoplasmic bridge in a binucleate cell are shown in Figure 4.1.

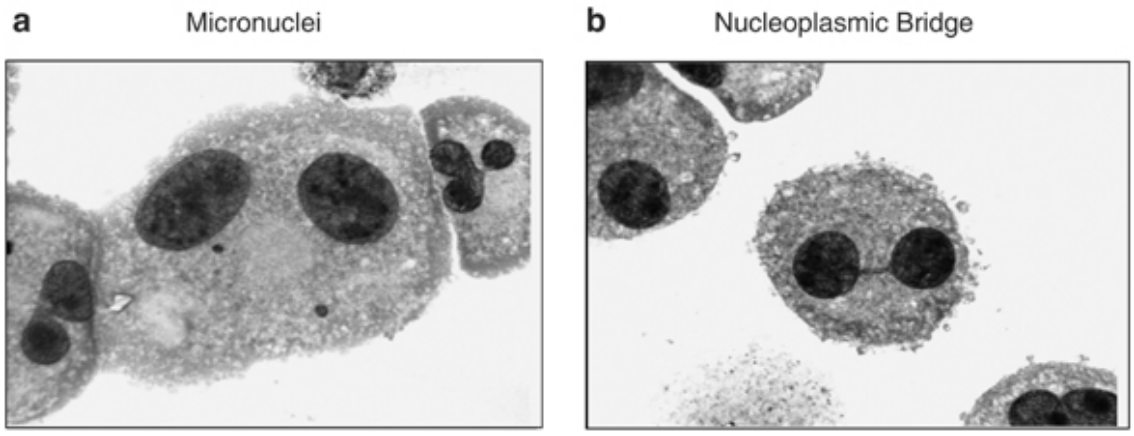

Figure 4.1 Micronuclei in a binucleate cell (a) and a nucleoplasmic bridge in a binucleate cell (b) at 100x magnification. Reproduced with permission from (Glaviano et al. 2006)

BJ and hTERT cells were exposed to either 0.4μM potassium dichromate or PBS (control) for 24 hours. Directly treated cells were then immediately harvested, cytospun and Giemsa stained for the micronucleus assay; or donor cells were incubated

for one hour with fresh complete DMEM in order to obtain conditioned medium. Recipient cells were incubated in conditioned medium for 24 hours, after which, they were harvested, cytospun and Giemsa stained for the micronucleus assay. Directly treated cells were cultured for a further 30 days after initial metal exposure and a proportion of these cells were harvested at day 5 and day 30 time points in order to harvest, cytospin and Giemsa stain them for the micronucleus assay. The remainder of the directly metal-exposed cells were used as donors from which to condition medium. Conditioned medium was harvested from these donor cells on days 5 and 30 post initial exposure and medium was transferred to a fresh population of recipient cells of the same type. In this medium transfer experiment, conditioned medium was only transferred between cells of the same type i.e. BJ to BJ; and hTERT to hTERT.

4.1.2 Micronuclei: Results

Mean numbers of micronuclei and nucleoplasmic bridges in binucleates (and the standard deviations) were calculated from three independent experiments and are expressed here as a percentage (%).

Figure 4.2a shows the percentage of binucleate BJ cells containing one or more micronucleus 0, 5 and 30 days post direct Cr (VI) exposure; and micronuclei in recipient BJ cells exposed to conditioned medium from Cr-treated donor cells 0, 5 and 30 days post Cr (VI) treatment in donor cells. Cr (VI) caused an increase in micronuclei observed in BJ cells compared to control immediately (on day 0) and 5 days post metal exposure, showing similar levels of micronuclei at both of these time points (at around 2% compared to 1.2% in controls). 30 days post direct Cr exposure, the percentage of binucleate BJ cells containing micronuclei was higher than that observed on day 0 and day 5 at over 3%, which was more than two-fold higher than the percentage of micronuclei observed in control cells, which was 1.43%. A similar pattern was observed

in the BJ cells that had been exposed to conditioned medium from a Cr-treated cell. The conditioned medium recipient had a slightly lower level of micronuclei than cells directly exposed to Chromium. On day 0, there was a significant increase in percentage micronuclei in recipient cells that had received medium from Cr treated donor cells (at 1.57%) compared to PBS control treated donor cells (at 1.18%). The percentage of micronuclei on day 5 was similar to that observed on day 0. This value increased by day 30 so that there were around 50 % more micronuclei in cells that had received medium from a Cr treated donor cell (at around 3.2%) compared to those that had received conditioned medium from a PBS treated control donor cell (at around 1.5%).

Figure 4.2b shows the percentage of hTERT cells containing micronuclei after direct Cr (VI) treatment and transfer of conditioned medium 0, 5 and 30 days post initial Cr (VI) treatment in donor or directly treated cells. Direct exposure to Cr (VI) caused an increase in micronuclei in hTERT cells (Figure 4.2b) but not to such an extent as that observed in BJ cells. The control level of micronuclei was also lower in hTERT cells, ranging from 0.35% after 24 hours exposure to PBS and increasing to 0.54% on day 5. No further increase occurred on day 30. There was a 2-fold increase in micronuclei observed in hTERT cells at all 3 time points following direct exposure to Cr (VI) compared to control values. There was a particular peak on day 5 where the percentage micronuclei observed was greater than at the other time points and it was at its most pronounced magnitude above the control value at around 1.1% compared to 0.54% in the control. Although the direct chromium exposure induced micronuclei in hTERT cells, there was no significant difference in micronuclei caused by exposure to conditioned medium from hTERT cells that had been exposed to Cr at any of the three time points (around 0.56% on average) compared to control values (around 0.51% on average).

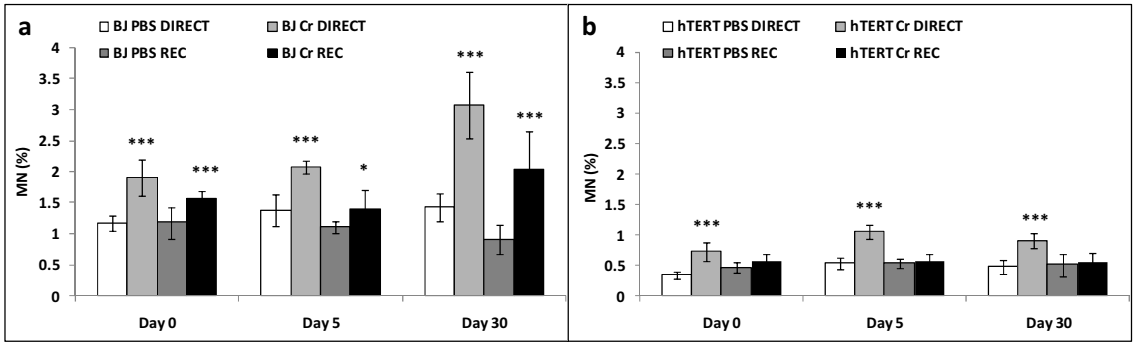

Figure 4.2 Micronuclei in BJ (a) and hTERT (b) cells treated with Cr (DIRECT) or Conditioned Medium from the same cell type (REC)

4.1.3 Nucleoplasmic Bridges: Results

Figure 4.3a shows the percentage of binucleate BJ cells containing nucleoplasmic bridges (NPBs) 0, 5 and 30 days post direct Cr (VI) exposure; and NPBs in recipient BJ cells exposed to conditioned medium from Cr-treated donor cells 0, 5 and 30 days post Cr (VI) treatment in donor cells. Direct treatment with Cr (VI) caused an approximate two-fold increase in nucleoplasmic bridges (NPBs) in BJ cells at all three time points post exposure compared to controls (Figure 4.3a). Immediately after treatment (on day 0), the percentage of NPBs increased from 0.61% in controls to 1.53% in Cr exposed cells and this pattern persisted over the 30 day sampling period. The general level of NPBs was lower than that observed for micronuclei, with control levels of around 0.7%. Treatment of cells via transfer of conditioned medium also induced an increase in NPBs at all three time points, which was slightly lower than the effect observed for direct Cr (VI) treatment. For recipient cells sampled immediately (on day 0), the level of NPBs increased from 0.57% in controls to 1.17% in cells exposed to Cr-conditioned medium, this pattern persisting over the 30 day sampling period.

Figure 4.3b shows the percentage of binucleate hTERT cells containing nucleoplasmic bridges (NPBs) 0, 5 and 30 days post direct Cr (VI) exposure; and NPBs in recipient hTERT cells exposed to conditioned medium from Cr-treated donor cells 0, 5 and 30 days post Cr (VI) treatment in donor cells. hTERT cells showed a lower level of NPBs than BJ cells and neither direct Cr (VI) treatment nor transfer of conditioned medium caused any induction of NPBs at any of the three time points (Figure 4.3b). For all treatment conditions (Cr, PBS and conditioned medium) the percentage of NPBs remained around 0.3-0.4% with no significant alterations occurring at any time point.

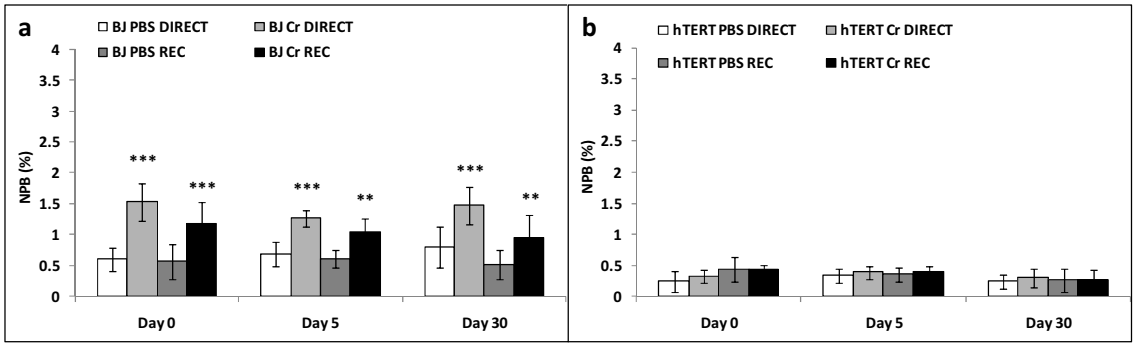

Figure 4.3 Nucleoplasmic Bridges in BJ (a) and hTERT (b) cells treated with Cr (DIRECT) or Conditioned Medium from the same cell type (REC)

4.1.4 Micronucleus Assay: Summary

The analysis shown in Figure 4.4 indicates that there was a highly significant ($p.\leq 0.001$) increase in induction of micronuclei and NPBs in BJ cells treated directly with potassium dichromate on day 0, day 5 and day 30. Induction of micronuclei by direct Cr treatment increased over time and percentages for nucleoplasmic bridges were lower than those for micronuclei. There was also a significant increase in the mean number of micronuclei observed following transfer of conditioned medium in BJ cells on day 0 ($p.\leq 0.001$), day 5 ($p.\leq 0.05$) and day 30 ($p.\leq 0.001$) compared to controls. Induction of nucleoplasmic bridges by medium transfer was significantly higher following treatment with conditioned medium compared to control on day 0 ($p.\leq 0.001$),

day 5 ($p.\leq 0.01$) and day 30 ($p.\leq 0.01$). Induction of micronuclei and nucleoplasmic bridges in BJ cells was greater when the cells were treated directly with Cr than with medium transfer. The statistical analysis shown in Figure 4.4 supports the view that there was no significant difference in the levels of micronuclei in hTERT cells treated with conditioned medium compared to control. There was, however, a significant increase in the number of micronuclei induced in hTERT cells by direct treatment with potassium dichromate ($p.\leq 0.001$). There was no significant increase in induction of nucleoplasmic bridges in hTERT cells under any circumstances i.e. treated either directly with potassium dichromate or via transfer of conditioned medium.

These results suggest that 0.4 μM potassium dichromate can cause a bystander effect to induce micronuclei and nucleoplasmic bridges in normal human BJ cells via transfer of conditioned medium. In addition, production of the bystander factor in Cr (VI)-treated BJ cells persisted for at least 30 days. The results also suggest that hTERT cells were resistant to this bystander effect.

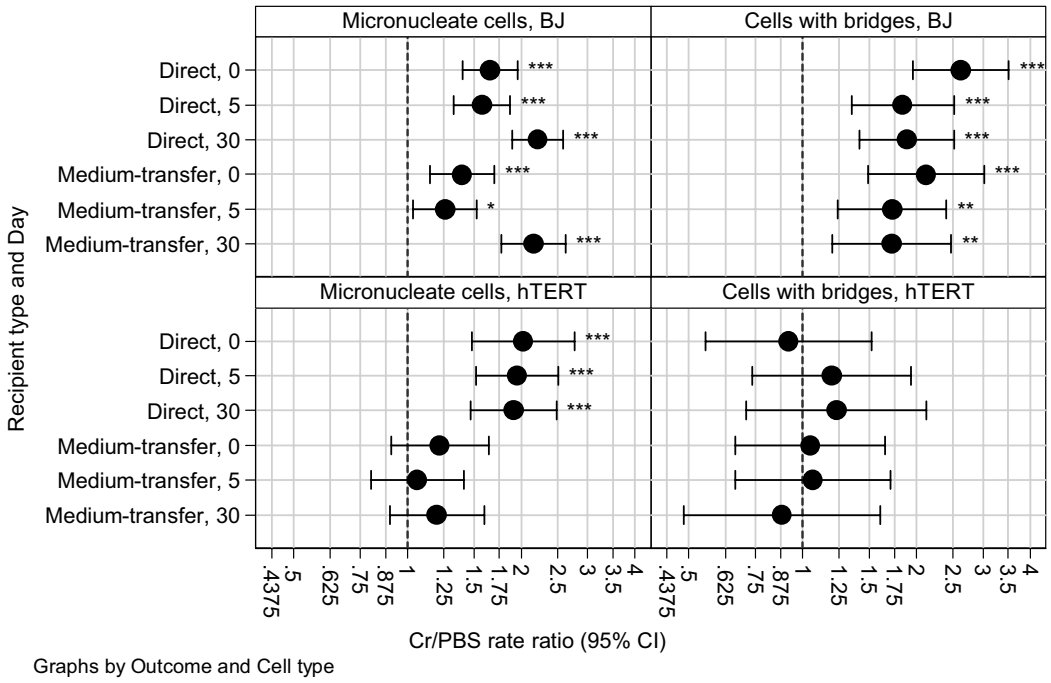

Figure 4.4 Cr/PBS ratios of aberration rates with 95% confidence limits

4.2. γ-H2AX Focus Formation Assay

4.2.1 Background

As centromere labelling was not carried out during the micronucleus assay, it could not be determined whether the micronuclei observed occurred as the result of a genetic event causing chromosome breakage (clastogenic) or chromosome loss (aneugenic). In order to discriminate between these types of damage, the γ-H2AX focus formation assay was carried out. This assay has been widely used to evaluate genotoxicity (Ha et al. 2004).

Histone H2AX is a core histone H2A variant that becomes incorporated into nucleosomes during DNA replication, and accounts for 5-10% of the total H2A in chromatin (Ikura et al. 2007). H2AX contains a unique *SQE* motif at its carboxyl

terminus, which becomes rapidly phosphorylated at a specific serine residue (Ser^{139}) in response to a DNS DSB. Members of the phosphatidylinositol-3-kinase related (PI3KK) family of protein kinases including ATM, ATR and DNA-PK are implicated in this phosphorylation step (Furuta et al. 2003; Friesner et al. 2005). This phosphorylated form of H2AX is known as γ-H2AX and appears over a megabase region of chromatin adjacent to DSB sites. The γ-H2AX foci are sites for recruitment of many DNA repair and chromatin remodelling factors (Rogakou et al. 1999) including the MRN complex, 53BP1 and BRCA1(Bassing and Alt 2004; Stucki and Jackson 2006). Practically, DNA DSB foci can be visualized and quantified using antibodies to γ-H2AX as each DSB has been demonstrated to give rise to one focus (Rogakou et al. 1999). A commonly used commercially available antibody for γ-H2AX detection is the rabbit polyclonal raised by Nakamura et al (Nakamura et al. 2006) against the peptide '*CKATQAS(PO$_4$)QEY'* derived from the human sequence.

BJ and hTERT cells were exposed to either 0.4μM potassium dichromate or PBS (control) for 24 hours. Directly treated cells were then immediately seeded onto coverslips and stained for γ-H2AX foci or donor cells were incubated for one hour with fresh complete DMEM in order to obtain conditioned medium. Recipient cells were incubated in conditioned medium for 24 hours after which, they were seeded onto coverslips and stained for the γ-H2AX assay. Directly treated cells were cultured for a further 30 days after initial metal exposure and a proportion of these cells were harvested at day 5 and day 30 time points in order to seed them onto coverslips and stain them for γ-H2AX foci. The remainder of the directly metal-exposed cells were used as donors from which to condition medium. Conditioned medium was harvested from these donor cells on days 5 and 30 post initial exposure and medium was

transferred to a fresh population of recipient cells of the same type. In this medium transfer experiment, conditioned medium was only transferred between cells of the same type i.e. BJ to BJ; and hTERT to hTERT. Cells were scored according to the number of H2AX foci they contained. These were grouped into three categories, those containing 0 foci, those containing 1-3 foci and those containing four or more foci per cell (4+). The ≥4 foci per cell threshold is thought to be optimal for determining the fraction of affected cells and rules out the possibility that the foci occurred at random (Sokolov et al. 2005).

4.2.2 γ-H2AX Focus Results

Figure 4.5a shows percentages of BJ cells containing 0, 1-3 and 4+ γ-H2AX foci per cell immediately (i.e. on day 0) after a 24 hour direct Cr (VI) treatment or following transfer of conditioned medium 24 hours post Cr (VI) exposure in donor cells. The percentage of BJ cells containing four or more foci immediately after treatment increased from 1% in controls to 4.7% in cells that had been directly treated with potassium dichromate. The percentage of recipient BJ cells containing 4+ foci per cell increased from 2% in controls to over 7% in cells that had received conditioned medium immediately after potassium dichromate treatment in BJ donor cells. Both direct treatment and transfer of conditioned medium induced a significant increase in γ-H2AX foci in BJ cells on day 0. Figure 4.6a shows the percentages of BJ cells containing 0, 1-3 and 4+ γ-H2AX foci per cell 5 days post direct Cr treatment or following exposure of BJ recipient cells to conditioned medium 5 days post Cr treatment in donor cells. The percentage of BJ cells containing 4+ foci per cell on day 5 was higher than that observed on day 0. The background level of instability increased over time. The percentage of directly exposed BJ cells containing 4+ foci per cell increased to 6.8%, which was over two-fold higher than the control value. For conditioned medium

recipients 5 days post donor cell Cr treatment, there was no increase in damage compared to day 0, however, there was a significant increase in induction of γ-H2AX foci compared to control. This is illustrated by the decrease in the number of cells containing no γ-H2AX foci from 82.6% in the control to 57.3% in recipients of conditioned medium from a Cr treated donor cell. Figure 4.7a shows the percentage of BJ cells containing 0, 1-3 and 4+ γ-H2AX foci 30 days post direct Cr treatment or following transfer of conditioned medium 30 days post Cr treatment in donor cells. On day 30, the percentage of cells containing 4+ foci per cell following direct treatment was higher than that observed on day 0 and day 5 for both control and Cr-treated cells. The control cells may have become more unstable and prone to DNA damage including DSBs after 30 days of culturing as almost 5% contained four or more foci per cell, which is higher than the 1% observed for control cells on day 0. The increase in γ-H2AX foci induced 30 days post direct Cr treatment was significantly higher than that observed in controls at 36% cells containing 4+ foci. For medium transfer on day 30, there was a significant increase in the percentage of recipient BJ cells containing 4+ γ-H2AX foci following exposure to chromium conditioned medium at 7.8% compared to 0.9% in controls. Neither the percentage of damaged control cells nor the percentage of damaged Cr-conditioned medium recipient cells appeared to increase over time.

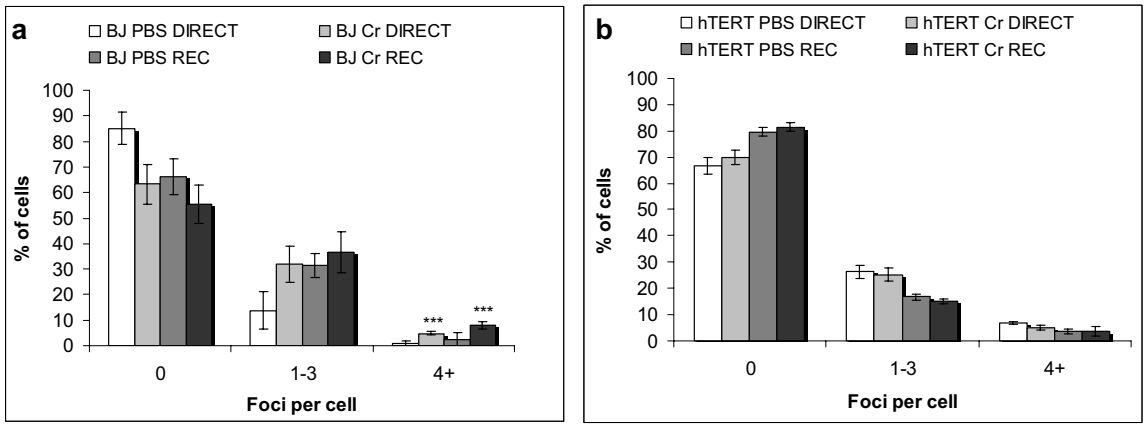

Figure 4.5 γ-H2AX foci in BJ (a) and hTERT (b) cells 24 hours post Cr exposure (DIRECT) or in recipient cells following transfer of conditioned medium 24 hours post Cr-exposure in donor cells of the same type (REC)

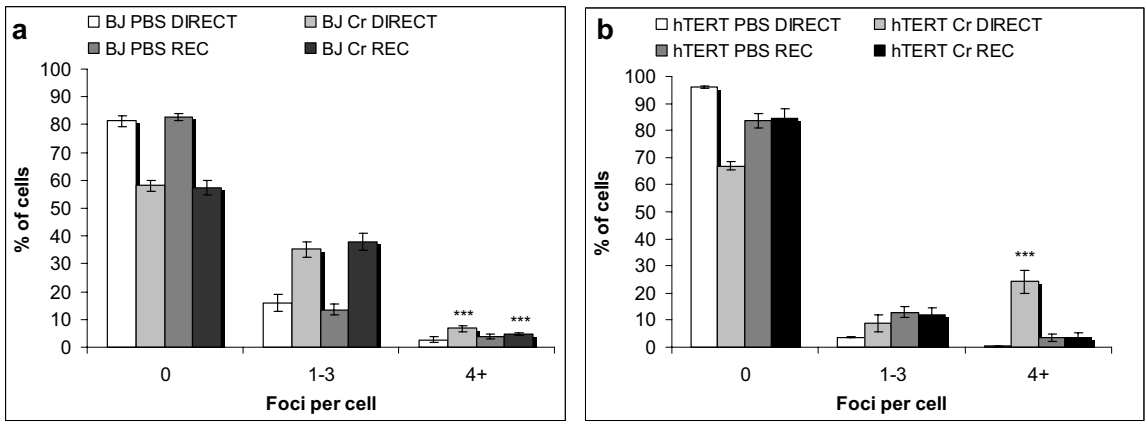

Figure 4.6 γ-H2AX foci in BJ (a) and hTERT (b) cells 5 days post Cr exposure (DIRECT) or in recipient cells following transfer of conditioned medium 5 days post Cr-exposure in donor cells of the same type (REC)

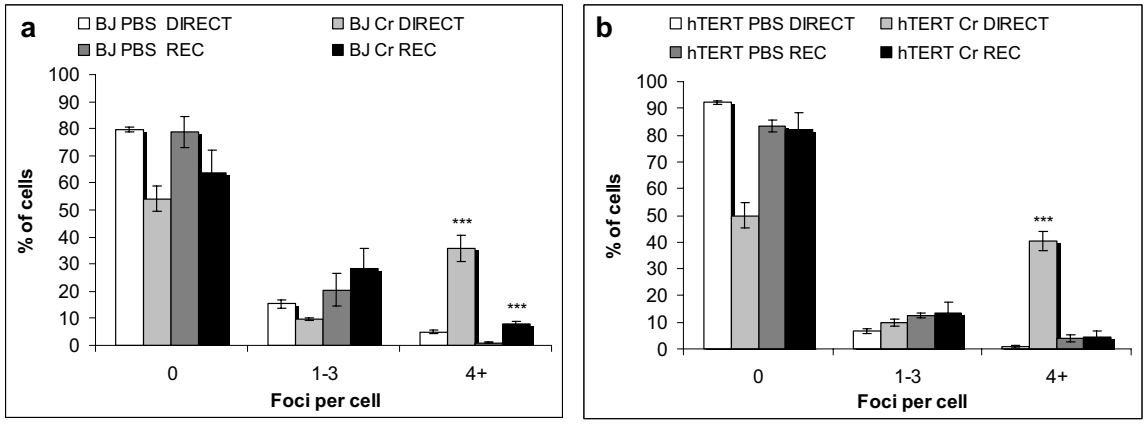

Figure 4.7 γ-H2AX foci in BJ (a) and hTERT (b) cells 30 days post Cr exposure (DIRECT) or in recipient cells following transfer of conditioned medium 30 days post Cr-exposure in donor cells of the same type (REC)

Figure 4.5b shows percentages of hTERT cells containing 0, 1-3 and 4+ γ-H2AX foci per cell immediately (i.e. on day 0) after a 24 hour direct Cr (VI) treatment or following transfer of conditioned medium 24 hours post Cr (VI) exposure in donor cells. On day 0, there was no significant difference in the number of γ-H2AX foci in hTERT cells regardless of whether the cells had been treated with Cr, PBS or conditioned medium. There was neither a direct effect of potassium dichromate to induce foci nor was there a medium transferred bystander effect. Figure 4.6b shows percentages of hTERT cells containing 0, 1-3 and 4+ γ-H2AX foci per cell 5 days post direct Cr treatment or following exposure of hTERT recipient cells to conditioned medium 5 days post Cr treatment in donor cells. This figure shows that the transfer of conditioned medium had no effect on induction of γ-H2AX foci in hTERT cells on day 5, which mimics the result observed on day 0. However, direct treatment induced a large increase in the number of cells containing 4+ foci from 5% on day 0 to over 24% on day 5. This value was also significantly higher than the control value, which was less than1%. Figure 4.7b shows percentages of hTERT cells containing 0, 1-3 and 4+ γ-H2AX foci per cell 30 days post direct Cr treatment or following exposure of hTERT recipient cells to conditioned medium 30 days post Cr treatment in donor cells. On day

30, there was another large increase in the percentage of hTERT cells containing four 4+ γ-H2AX foci following direct Cr treatment. This value increased to over 40%, which was higher than that observed for direct treatment on day 0 or day 5. Again, the control value was less than 1%. In hTERT cells there did not appear to be an increase in instability in control cells as they were cultured over time as there did in BJ cells. However, the effect of direct Cr treatment persisted and increased over time as the percentage of cells containing high levels of γ-H2AX foci increased from 5% on day 0 to 24% on day 5 and reached over 40% on day 30. Again, there was no induction of γ-H2AX foci by medium transfer. The percentage of hTERT recipient cells containing 4+ foci per cell remained between 3 and 4% regardless of what day the medium was conditioned and whether the donor cells had been treated with Cr or PBS (control). It is notable that even in control cells at day 0, small numbers of γ-H2AX foci are present even without the deliberate introduction of DSBs (Figure 4.5). These so called 'cryptogenic' foci have been observed during senescence in humans and during ageing in mice and are thought to signify irreparable DNA lesions (Sedelnikova et al. 2004). This supports the observation of this study that control cells contain slightly more foci on days 5 and 30 than on day 0 (Figure 4.6 and Figure 4.7).

4.2.3 γ-H2AX Results Summary

Both direct and conditioned medium treatment in BJ cells was able to induce a significant increase in γ-H2AX foci 0, 5 and 30 days post direct Cr exposure or donor cell treatment ($p<0.001$). A particularly high peak of γ-H2AX foci occurred in directly treated cells 30 days post Cr exposure. This indicates that DNA double strand breaks persist and accumulate in the progeny of the Cr exposed cells. In recipient BJ cells, the

highest level of γ-H2AX foci appeared to be induced on day 5. This could be an optimum time for production of the bystander signal in donor cells.

hTERT cells, however, seemed more resistant to the induction of DNA double strand breaks at least on day 0, when there was no significant difference in the induction of γ-H2AX foci whether the cells were treated with Cr or PBS. A large peak appeared on day 5, which persisted at least until day 30 after the initial exposure to Cr. On both days 5 and 30, there was a significant increase in the induction of γ-H2AX foci in directly treated hTERT cells compared to control ($p<0.001$). There was no response of hTERT cells to medium conditioned in hTERT donor cells. This indicates that either the hTERT donor cells did not produce a signal to induce γ-H2AX foci in the recipients or that the recipient cells did not respond to any DNA double strand break-inducing signal.

Somers' D is the difference between the probability that a random Cr-treated cell has more foci than a random PBS-treated cell and the probability that a random PBS-treated cell has more foci than a random Cr-treated cell. Estimates of Somers' D are given in Figure 4.8. Under most conditions, most cells contain no foci. For direct treatment of BJ cells on day 0 i.e. 24 hours post Cr exposure, (Figure 4.5a) the Cr-treated cell is 22.3% more likely to have more foci than the PBS-treated cell than vice versa. In the population of such cells at large, we can be 95% confident that the true population difference is between 18.3 percent more likely and 26.4 percent more likely. BJ cells treated with Cr typically have more foci than comparable cells treated with PBS, for all combinations of treatment type (direct or medium transfer) and exposure day.

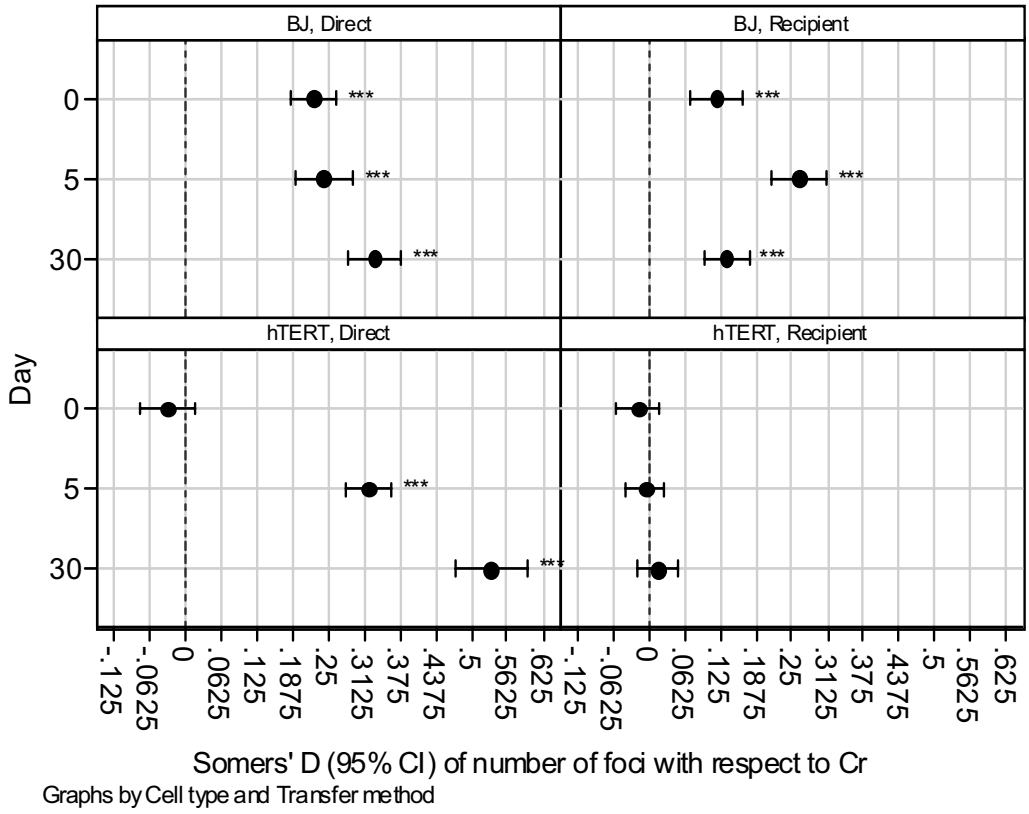

Figure 4.8 Somers' D of numbers of foci with respect to Cr treatment

Examples of nuclei containing 0 foci and 4+ foci per cell are shown in Figure 4.9.

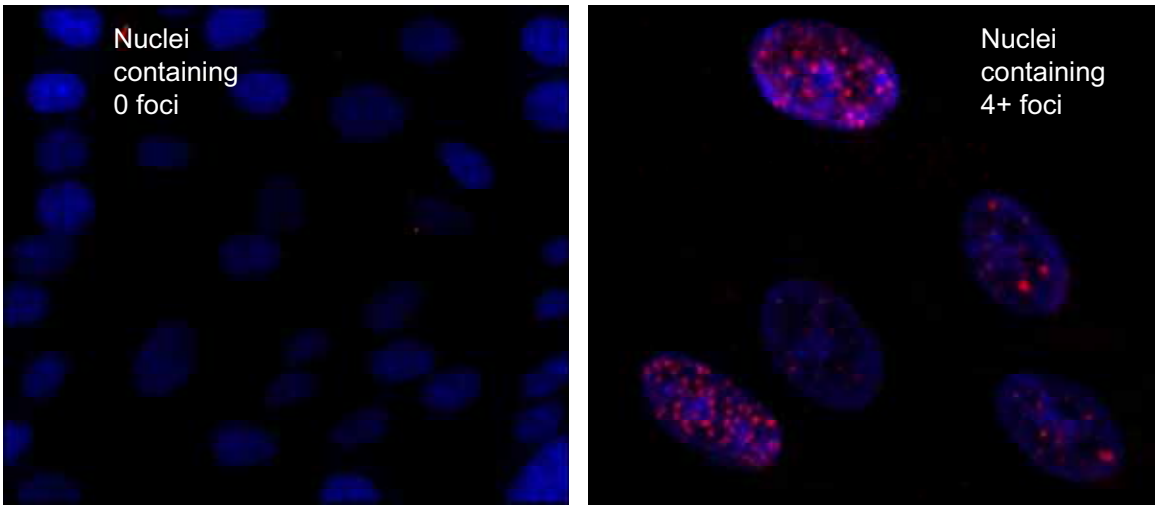

Figure 4.9 Nuclei containing no γ-H2AX staining at 10x magnification (left) and nuclei containing 4+ foci at 40x magnification (right)

4.3 Cytogenetics

4.3.1 Background

In order to determine whether any cytogenetic aberrations were induced by potassium dichromate or conditioned medium, metaphase spreads were obtained for both directly treated and recipient cells. Initially, BJ and hTERT cells were exposed to potassium dichromate or PBS (control) for 24 hours and either harvested immediately to obtain metaphase spreads for direct treatment or they were used as donors from which to condition medium for transfer to recipient cells. Directly treated cells were cultured over 30 days and a proportion of these were harvested on day 5 and day 30 to obtain metaphase spreads for these time points following direct treatment. The remainder were used as donors in which to condition medium to transfer to recipient cells on day 5 and day 30. The cells were arrested in metaphase by colchicine, fixed in methanol: acetic acid and dropped on clean, refrigerated microscope slides. The metaphase spreads were scored via light microscopy using the 100 x objective lens. The results shown in Figure 4.10 are an account of the cytogenetic aberrations observed through solid Giemsa staining of metaphase spreads obtained from at least three independent experiments in which at least one hundred metaphases were scored for each. For medium transfer in this experiment, medium was only transferred between cells of the same type i.e. BJ to BJ and hTERT to hTERT.

4.3.2 Cytogenetics Results

Figure 4.10 shows the chromosome breaks observed in BJ (left) and hTERT (right) cells 24 hours (0 days), 5 days and 30 days post direct exposure or exposure of donor cells to potassium dichromate. There was a very large increase in the number of chromosome breaks observed in BJ cells on day 0, from 0.14% in the control to 6.9% in

cells exposed to Cr. However, this data may not be very reliable as there was a lot of variation in the values observed in different slide preparations, hence the very large error bars. For medium transfer in BJ cells 24 hours after metal treatment in donor cells, there was a slight increase in the level of chromosome breaks, rising from 3.6% in the control to 4.9% in cells exposed to conditioned medium. For hTERT cells 24 hours post direct metal treatment, there was a large increase in chromosome breaks from 0% in controls to 3.3% following direct Cr treatment. For medium transfer however, no effect was observed as the mean number of chromosome breaks actually decreased in those cells receiving conditioned medium and the error bars overlap between control and treatment. It does appear that 24 hours after direct metal treatment, both cell types showed an increase in chromosome breaks compared to control values. However, at this time point, no chromosome breaks were induced by medium transfer in either BJ or hTERT cells. On day 5 after direct metal exposure in BJ cells, there was a 1.7-fold increase in the mean number of chromosome breaks observed, from 4.6% in the control to 8.1% in cells treated with 0.4 μM potassium dichromate. In the BJ cells treated via medium transfer, a large increase in the number of chromosome breaks was observed. There was a 2.7-fold increase, rising from 2.7% in the control to 7.4% in the cells exposed to conditioned medium. On day 5, the medium transfer effect appears greater than direct metal treatment and was also larger than that observed at the 24 hour stage. For hTERT cells on day 5, there was a 4.8-fold increase in the number of chromosome breaks observed following direct treatment, rising from 0.8% in the control to 3.8% post direct exposure to chromium ions. There did not appear to be any change in the level of chromosome breaks observed in hTERT cells following medium transfer on day 5, as the error bars overlap for control and treatment. On day 30 in directly treated BJ cells, there was a 6-fold increase in the level of chromosome breaks, as they rise to the highest

level observed, at 17.6%. For medium transfer in BJ cells, chromosome breaks increased from 2.5% to 8.7%. This is approximately half of the level seen with direct treatment. This is the opposite of what was observed on day 5, where the mean number of chromosome breaks in cells exposed to conditioned medium was 1.6 times higher than in the directly treated cells. On day 30, all levels of chromosome breaks observed in both cell types were higher than observed on either day 0 or day 5. This may be due to the stresses of being cultured for 30 days. For hTERT cells on day 30, the mean number of chromosome breaks in the control metaphases was 2.3%. This value is very high, perhaps due to the 30 day culture duration. Following direct Chromium treatment, the value drops to zero. This could be an anomalous outlier. For medium transfer in hTERT cells on day 30, values for control and treatment are very similar at 1.5% and 1.4% respectively.

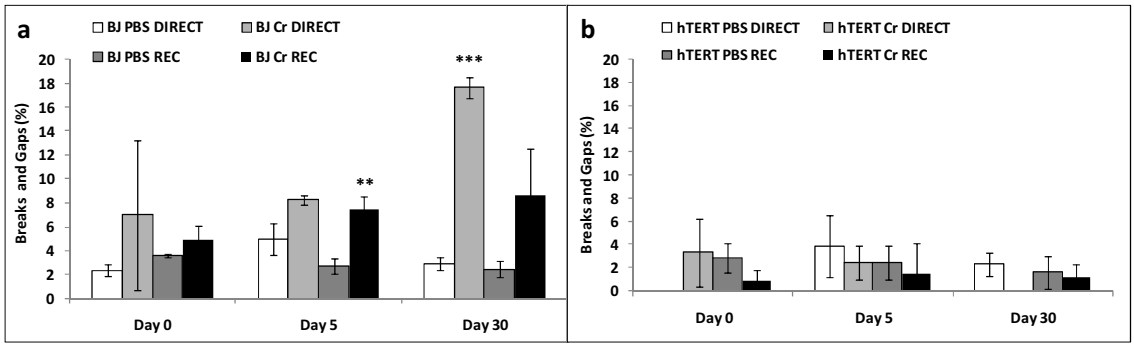

Figure 4.10 Percentage of metaphases containing chromosome breaks in BJ (left) and hTERT (right) cells 0, 5 and 30 days post Cr exposure (DIRECT) and in recipient cells following transfer of conditioned medium 0, 5 and 30 days post Cr-exposure in donor cells of the same type (REC)

4.3.3 Cytogenetics Summary

In general, the data for chromosome breaks agree with those for γ-H2AX foci. The direct effect of potassium dichromate was to cause an increase in the percentage of chromosome breaks in BJ cells over 30 days. Medium transfer also caused an increase in chromosome breaks but to a lesser extent than direct treatment. It is difficult to be certain if there was an immediate effect of medium transfer on chromosome breaks due

to the large overlap of error bars. The level of chromosome breaks observed in hTERT cells was lower than that observed in BJ cells; hTERT cells may have been protected against this type of instability. In general, direct exposure to potassium dichromate caused an increase in chromosome breaks in hTERT cells especially on day 5. There did not appear to be any effect of medium transfer on hTERT cells in the induction of chromosome breaks, which correlates with the data from the γ-H2AX focus formation assay.

4.3.4 γ-H2AX focus formation on metaphase spreads

In addition to the problems identified while trying to score metaphases. Some similar problems were identified in metaphases that had been stained for γ-H2AX foci. Figure 4.11 shows metaphase spreads stained for γ-H2AX foci 5 days post control treatment in BJ (left) and hTERT (right) cells. These images are typical of those obtained for γ-H2AX staining of metaphase spreads. There did appear to be some DNA double strand break foci. However, it was difficult to distinguish between individual chromosomes, rendering it difficult to determine whether any numerical chromosomal aberrations had occurred.

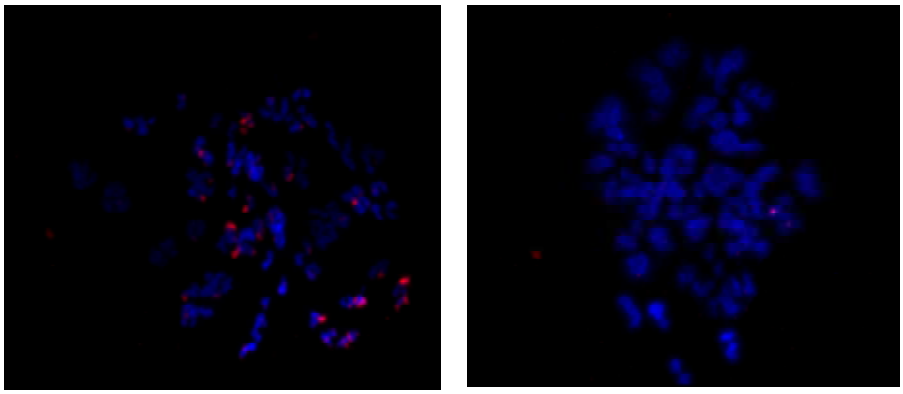

Figure 4.11 γ-h2ax foci on metaphase spreads of BJ (left) and hTERT (right) cells 5 days post exposure to PBS (control) at 100x magnification.

Figure 4.12 shows γ-H2AX foci in metaphase spreads of BJ (left) and hTERT (right) 5 days post direct treatment with 0.4 μM $K_2Cr_2O_7$. It can be observed that the image of BJ cells (left) appears to contain a tetraploid metaphase containing many DNA double strand break foci. Any structural or numerical aberrations were difficult to determine as the chromosomes were not a regular shape. The hTERT metaphase (Figure 4.12, right) also appears to contain many DNA double strand break foci. Genotoxicity was not quantified in these samples due to the nature of the reparations. No reliable data could be obtained.

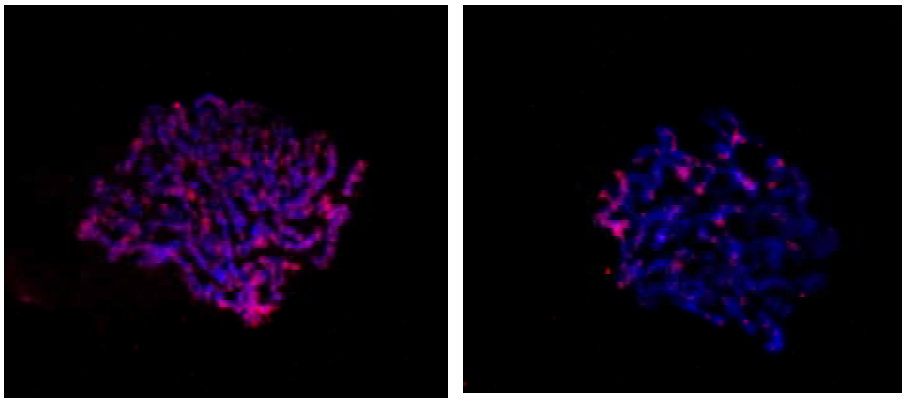

Figure 4.12 γ-H2AX foci on metaphase spreads of BJ (left) and hTERT (right) cells 5 days post direct exposure to 0.4 μM $K_2Cr_2O_7$ at 100x magnification

4.4 Transfer of Conditioned Medium between Cell Types

4.4.1 Background

As there seemed to be a difference in the nature of the bystander signal transmitted into the medium between BJ and hTERT cells, an experiment was devised to transfer medium from one cell type to the other and vice versa i.e. BJ to hTERT and hTERT to BJ. The lack of bystander response in hTERT cells may have been because these cells could not produce a bystander factor or because they did not respond to a signal. The possibility that they could quickly repair any damage induced by the bystander signal was also considered. Clearly, BJ cells could both produce a signal and respond to the signal from their own cell type; so transferring conditioned medium from BJ cells to hTERT cells ensured that hTERT cells were receiving medium that had been shown to produce damage in BJ cells.

Medium was conditioned in either BJ or hTERT cells that had been chromium treated for 24 hours and then transferred to either BJ or hTERT cells at 0, 5 and 30 days post exposure to potassium dichromate in donor cells. The assays used to determine whether any differences lay in the conditioned medium signal were the γ-H2AX focus formation assay and the micronucleus assay.

4.4.2 γ-H2AX focus formation assay: Results

Figure 4.13a shows the percentage of BJ cells containing four or more foci following exposure to medium conditioned in 1) PBS-treated BJ donor cells, 2) Cr-treated BJ donor cells, 3) PBS-treated hTERT donor cells and 4) Cr-treated hTERT donor cells 0, 5 and 30 days post donor cell treatment. As expected from previous experiments (Section 4.2. γ-H2AX Focus Formation Assay), exposure of BJ cells to conditioned medium from a Cr-treated BJ donor cell induced a significant increase in γ-H2AX foci ($p<0.001$) compared to control at all three time points. For BJ recipient cells exposed to medium conditioned in hTERT donor cells, there was also a significant increase in induction of γ-H2AX foci compared to control at all three time points especially on day 0 (Figure 4.14a) and day 30 (Figure 4.16a) ($p<0.001$). There seemed to be a slight decrease in the potency of the signal on day 5 (Figure 4.15a) but there was still a significant increase in induction of γ-H2AX foci ($p<0.05$) compared to control. Figure 4.13b shows the percentage of hTERT cells containing four or more foci following exposure to medium conditioned in 1) PBS-treated BJ donor cells, 2) Cr-treated BJ donor cells, 3) PBS-treated hTERT donor cells and 4) Cr-treated hTERT donor cells 0, 5 and 30 days post donor cell treatment. As expected, based on the results of previous experiments (Section 4.2. γ-H2AX Focus Formation Assay), exposure of hTERT cells to conditioned medium from a Cr-treated hTERT donor cell did not induce

any significant change in the percentage of cells containing γ-H2AX foci when compared to control at any of the three sampling times. However, at all three time points, there was a highly significant induction of γ-H2AX foci and therefore DNA DSBs in hTERT recipient cells that had been exposed to medium from Cr-exposed BJ donor cells compare to control ($p<0.001$).

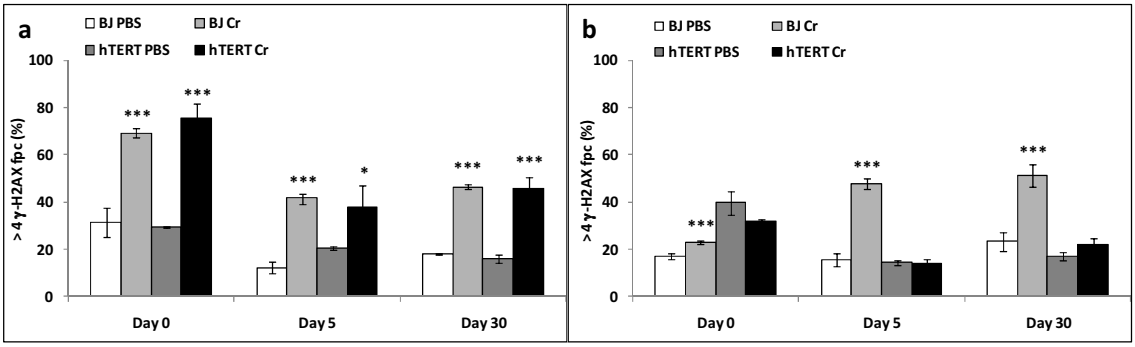

Figure 4.13 Percentage of BJ (a) and hTERT (b) recipient cells containing 4+ γ-H2AX foci following exposure to conditioned medium 0, 5 or 30 days post treatment in donor cells of either type

Figure 4.14a shows γ-H2AX foci in BJ recipient cells following exposure to conditioned medium 24 hours post donor cell treatment from BJ or hTERT donor cells exposed to Cr or PBS (control). Figure 4.14b shows γ-H2AX foci in hTERT recipient cells following exposure to conditioned medium 24 hours post donor cell treatment from BJ or hTERT donor cells exposed to Cr or PBS (control).

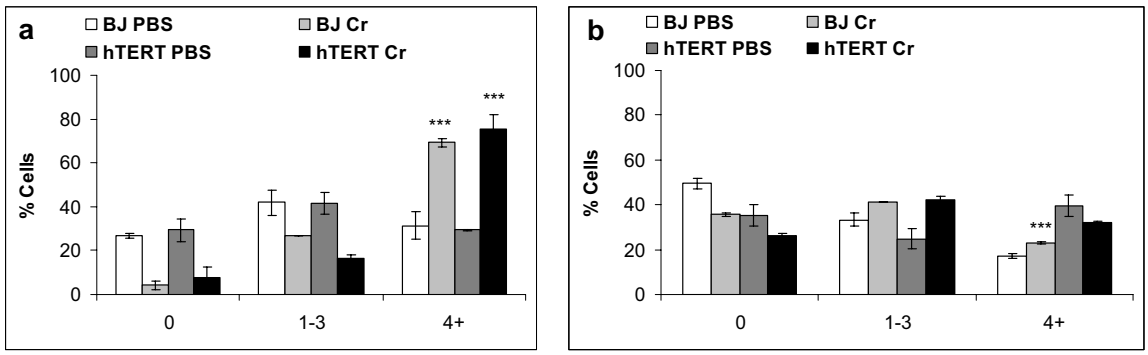

Figure 4.14 γ-H2AX foci in BJ (a) and hTERT (b) recipient cells following exposure to conditioned medium from either BJ or hTERT donor cells 24 hours post treatment in donor cells of either type

Figure 4.15a shows γ-H2AX foci in BJ recipient cells following exposure to conditioned medium 5 days post donor cell treatment from BJ or hTERT donor cells exposed to Cr or PBS (control). Figure 4.15b shows γ-H2AX foci in hTERT recipient cells following exposure to conditioned medium 5 days post donor cell treatment from BJ or hTERT donor cells exposed to Cr or PBS (control).

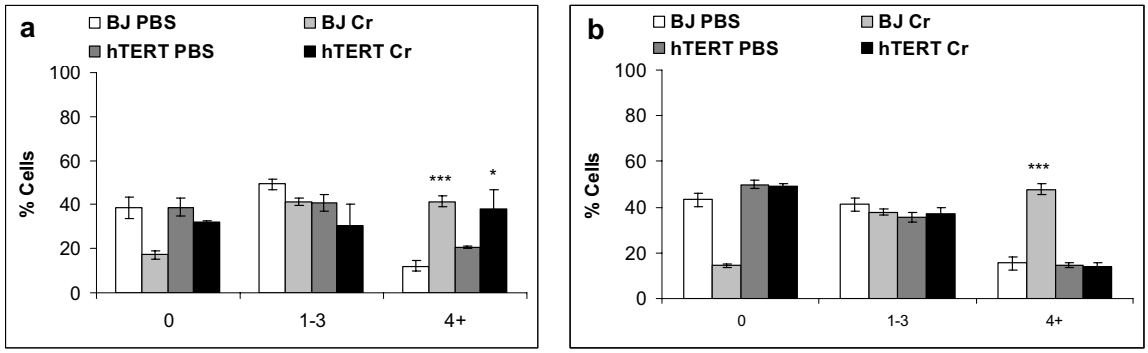

Figure 4.15 γ-H2AX foci in BJ (a) and hTERT (b) recipient cells following exposure to conditioned medium from either BJ or hTERT donor cells 5 days post treatment in donor cells of either type

Figure 4.16a shows γ-H2AX foci in BJ recipient cells following exposure to conditioned medium 30 days post donor cell treatment from BJ or hTERT donor cells exposed to Cr or PBS (control). Figure 4.16b shows γ-H2AX foci in hTERT recipient cells following exposure to conditioned medium 30 days post donor cell treatment from BJ or hTERT donor cells exposed to Cr or PBS (control).

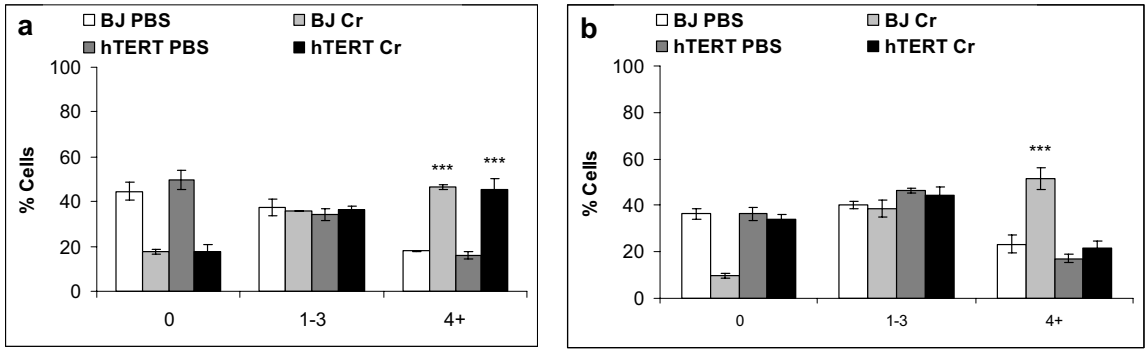

Figure 4.16 γ-H2AX foci in BJ (a) and hTERT (b) recipient cells following exposure to conditioned medium from either BJ or hTERT donor cells 30 days post treatment in donor cells of either type

4.4.3 γ-H2AX focus formation assay: Summary

Both BJ and hTERT Cr-exposed donor cells produced a signal to induce γ-H2AX foci and therefore DNA DSBs in the other cell type at all three time points that was significantly higher than in controls. hTERT cells did not produce a signal to induce γ-H2AX foci in hTERT cells but could both produce a signal to affect BJ cells and respond to the BJ cell signal.

Figure 4.17 shows estimates of Somers' *D* with respect to Cr treatment. Somers' *D* is the difference between the probability that a random Cr-treated cell has more foci than a random PBS-treated cell and the probability that a random PBS-treated cell has more foci than a random Cr-treated cell, assuming that the two cells are selected randomly from cells with the same combination of recipient cell type, donor cell type and exposure day. For instance, for two cells, one Cr-treated and the other PBS-treated, sampled from the BJ recipient cells and the BJ donor type on exposure day zero, the Cr-treated cell is 43.0% more likely to have more foci than the PBS-treated cell than vice versa. In the population of such cells at large, we can be 95% confident that the true population difference is between 35.6 percent more likely and 49.9 percent more likely. Cells treated with Cr typically have more foci than comparable cells treated with PBS, for all combinations of subject cell type, donor cell type, and exposure day, except for

where both the recipient cells and the donor cells are hTERT. This seems to be true for all exposure days.

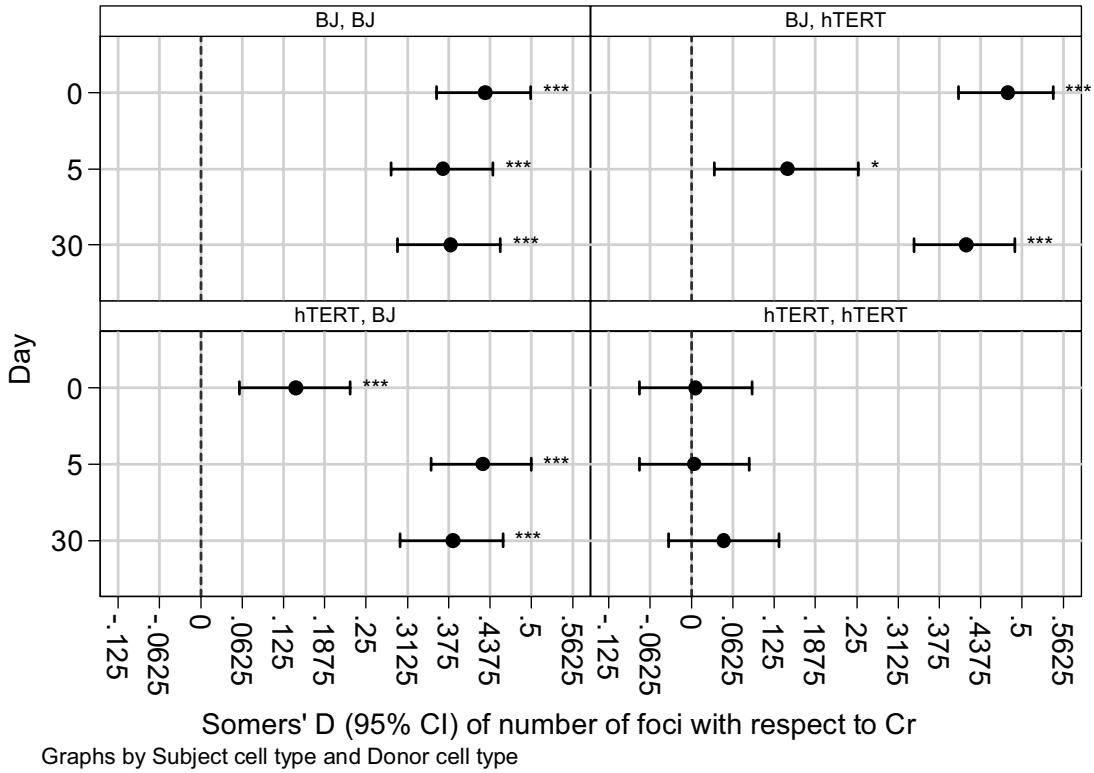

Figure 4.17 Somers' *D* of numbers of foci with respect to Cr treatment

4.4.4 Micronucleus Assay: Results

The micronucleus assay was carried out to indicate the levels of chromosomal damage induced in BJ and hTERT recipient cells by treatment with conditioned medium from either BJ or hTERT donor cell type. These experiments were scored by an additional observer. Inter-observer error was considered minimal as test training slides were used and both observers scored the same slides, using the same criteria and obtained consistent, comparable results. Figure 4.18a shows the percentage of binucleated BJ recipient cells containing micronuclei following exposure to conditioned medium from: 1) BJ PBS-treated donor cells, 2) BJ Cr-treated donor cells, 3) hTERT PBS-treated donor cells and 4) hTERT Cr-treated donor cells 0, 5 or 30 days post donor

cell treatment. As expected, based on the results of previous investigation, (Section 4.1 Micronucleus Assay) conditioned medium from Cr-treated BJ donor cells induced a highly significant increase in micronuclei compared to control medium on day 0 ($p<0.001$). On day 5, as previously observed, there was a significant increase in induction of micronuclei by Cr-conditioned medium compared to control medium ($p<0.05$). However, there was an unexpected result on day 30. There was no significant difference in the induction of micronuclei in cells exposed to Cr-conditioned medium compared to control medium. Figure 4.18b shows the percentage of binucleated hTERT recipient cells containing micronuclei following exposure to conditioned medium from: 1) BJ PBS-treated donor cells, 2) BJ Cr-treated donor cells, 3) hTERT PBS-treated donor cells and 4) hTERT Cr-treated donor cells 0, 5 or 30 days post donor cell treatment. Treatment of hTERT cells with BJ Cr conditioned medium induced a significant ($p<0.001$) increase in micronuclei compared to control on day 0 and day 30. Although there does appear to be a slight increase in the induction of micronuclei in hTERT cells on day 5, Somers' *D* analysis indicated that it was not significant (Figure 4.20).

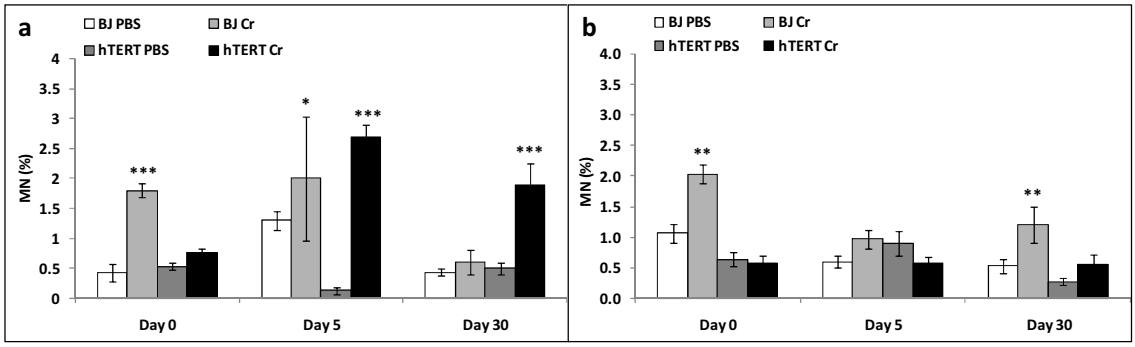

Figure 4.18 Percentage of BJ (a) and hTERT (b) recipient cells containing micronuclei following exposure to conditioned medium from either BJ or hTERT donor cells 0, 5 or 30 days post treatment in donor cells of either type

Figure 4.19a shows the percentage of binucleated BJ recipient cells containing NPBs following exposure to conditioned medium from BJ or hTERT donor cells treated with either PBS (control) or Cr (VI) 0, 5 and 30 days post donor cell treatment. In general, the level of NPBs was much lower than the level of micronuclei. On day 0, there did not appear to be a significant induction of NPBs by any treatment. On day 5, there was a significant increase ($p<0.05$) in NPBs by both hTERT and BJ Cr-conditioned medium compared to control medium in BJ recipient cells. On day 30, there was no longer a significant increase in NPBs caused by BJ medium, however, hTERT Cr-conditioned medium induced a significant increase in NPBs ($p<0.001$) in BJ recipient cells compared to control medium. Figure 4.19b shows the percentage of binucleated hTERT recipient cells containing NPBs following exposure to conditioned medium from BJ or hTERT donor cells treated with either PBS (control) or Cr (VI) 0, 5 and 30 days post donor cell treatment. In hTERT recipient cells, there did not appear to be any significant induction of NPBs at any time point by conditioned medium from either donor cell type, except on day 5 by hTERT conditioned medium. This result was unexpected as in the previous study (Section 4.1 Micronucleus Assay) no induction of NPBs was observed in hTERT cells following either direct Cr treatment or exposure to conditioned medium. This unexpected result on day 5 was due to the very low response observed in hTERT cells exposed to control medium. The percentage of hTERT cells containing NPBs following treatment with BJ control or conditioned medium was around 4%, the same as that observed following treatment with hTERT conditioned medium. On day 30, there was no significant difference in the percentage of hTERT recipient cells containing NPBs following treatment with conditioned medium compared to control medium from either donor cell type.

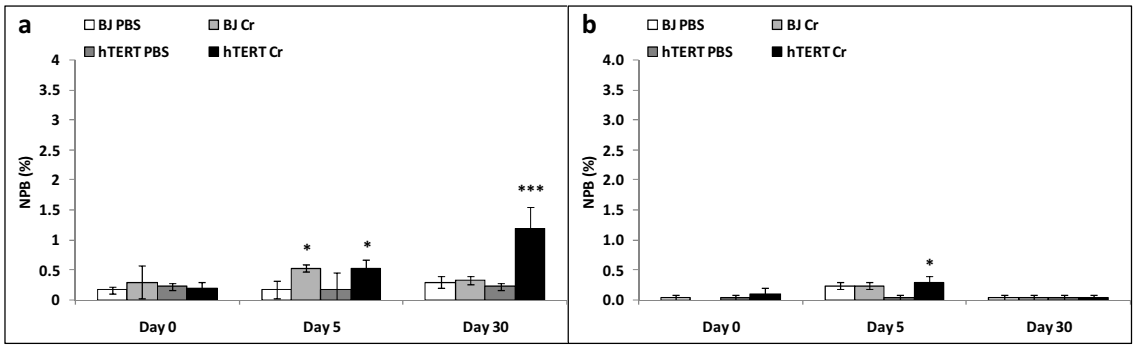

Figure 4.19 Percentage of BJ (a) and hTERT (b) recipient cells containing nucleoplasmic bridges following exposure to conditioned medium from either BJ or hTERT donor cells 0, 5 or 30 days post treatment in donor cells of either type

4.4.5 Micronucleus Assay: Summary

In general the results of this micronucleus assay were in agreement with those obtained during previous investigations (Section 4.1 Micronucleus Assay), suggesting that these results were reproducible even when different observers were employed.

Compared to control, conditioned medium from BJ donor cells induced a significant increase in micronuclei in recipient cells of either cell type at most time points except on day 5 in hTERT recipient cells and day 30 in BJ recipient cells. Compared to control, conditioned medium from hTERT donor cells had no effect in hTERT recipient cells, as expected; and induced a significant increase in micronuclei in BJ recipient cells on day 5 and day 30. On day 0, there was no significant difference in the numbers of nucleoplasmic bridges in either cell type following exposure to either conditioned medium or control medium from any donor cell type. 5 days post donor cell treatment, compared to control, there was a significant induction of NPBs in BJ recipient cells exposed to conditioned medium from either BJ or hTERT donor cells. The day 5 response in hTERT recipient cells exposed to conditioned medium from hTERT donors was possibly an anomaly due to a particularly low control value. 30 days post treatment in donor cells, the only value where treatment was significantly higher than control was in BJ recipient cells exposed to conditioned medium from Cr-treated

hTERT donor cells. Conditioned medium from Cr exposed BJ donor cells did not induce NPBs in hTERT recipient cells at any time.

Both BJ and hTERT Cr-treated donor cells were able to secrete a signal into their conditioned medium to induce micronuclei and, to a lesser extent, nucleoplasmic bridges in BJ recipient cells. In addition, conditioned medium from BJ donor cells was able to cause response to increase micronuclei but not NPBs in hTERT recipient cells. hTERT conditioned medium did not cause a response in hTERT recipient cells except the anomaly observed on day 5. In general, this conditioned medium response persisted for 30 days post donor cell exposure to Cr.

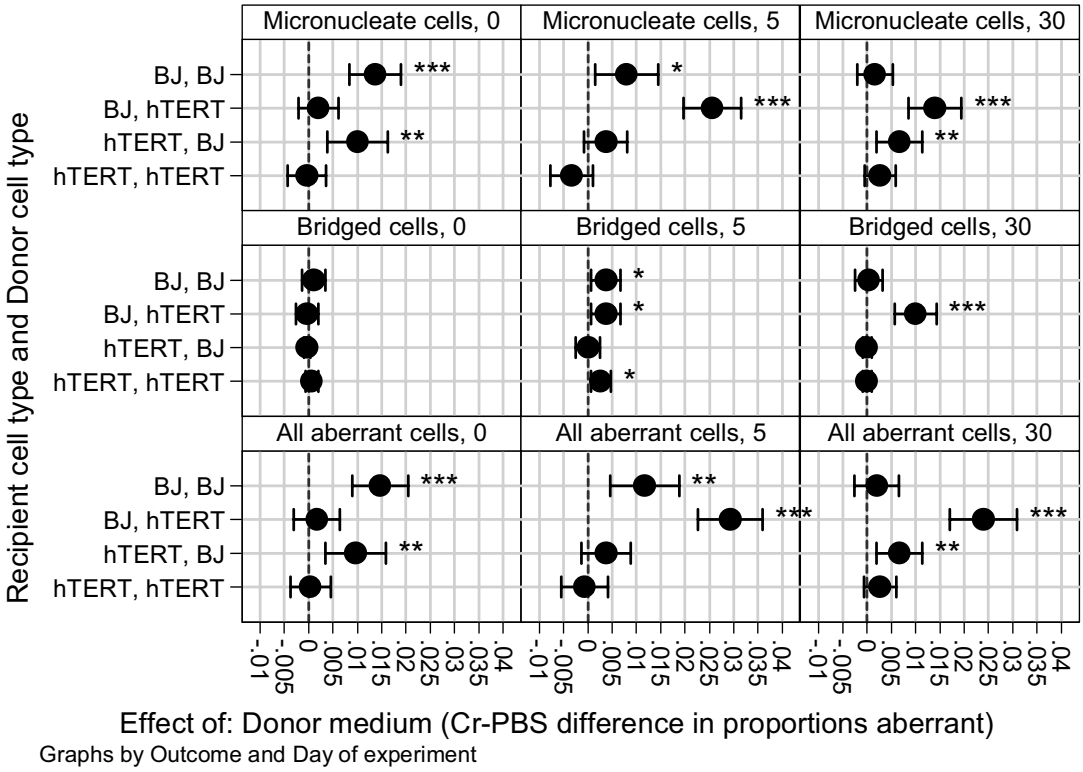

Figure 4.20 Donor medium effects (Cr-PBS differences)

4.4.6 Medium Transfer between Cell Types: Summary

Conditioned medium from BJ and hTERT donor cells was able to induce a significant increase in DNA damage in recipient cells illustrated by the difference in numbers of micronuclei, DNA DSBs (γ-H2AX foci) and to a lesser extent nucleoplasmic bridges compared to controls. In general, this conditioned medium was able to induce DNA damage for at least 30 days after donor cell Cr treatment. Therefore, hTERT cells were able to both secrete the bystander signal into the conditioned medium and respond to a bystander signal when it had been secreted by a BJ donor cells but not an hTERT donor cell. This suggests that there was an inherent difference in the nature of the bystander signal secreted by BJ cells compared to that secreted by hTERT cells.

4.5 Conditioned Medium

Various experiments were carried out to determine the nature of the conditioned medium harvested from a Cr-exposed donor cell. The first of these determined the total chromium content in the washings from all 5 of the washing steps involved in harvesting conditioned medium. Chromium analysis was also carried out in the final conditioned medium that would be transferred to recipient cells. This analysis was carried out to ensure that the biological effects observed in recipient cells were due to bystander signalling rather than the direct action of any contaminating Cr (VI).

The protein content of conditioned medium was analysed by MALDI mass spectrometry for medium harvested from both BJ and hTERT cells treated with either Cr or PBS control. This experiment was carried out to explore whether the differences in bystander signalling between BJ and hTERT could be attributed to secretion of different proteins into the conditioned medium by the two different cell types.

4.5.1 Metal analysis and residual Cr concentration

Analytica (Sweden) analysed total Cr content by ICP-SFMS and K^+ ion concentration by ICP-AES in the washings after the 5 washing steps, medium after Cr-conditioning for 1 hour and in fresh complete DMEM. The values are shown in Table 4.1 below.

Table 4.1 Chromium and K+ ion concentration in washings, conditioned medium and fresh complete DMEM

Sample	*Total Cr analysis by ICP-SFMS (μg/L)*	**K+ ion analysis by ICP-AES (mg/L)**
Wash 1	30.5	16.1
Wash 2	8.12	14.4
Wash 3	2.65	14.1
Wash 4	1.83	14.2
Wash 5	1.67	14.4
Cr-Conditioned (bystander) medium	1.08	19.5
Fresh complete DMEM (control)	0.983	

It is notable that the washing steps were effective, reducing the concentration of Cr in the washes from 30.5 μg/L to less than 1 μg/L in conditioned medium. It is also notable that with each wash, the concentration of K^+ released by the Cr-exposed cells increased. This may have been a response to the metal treatment. The concentration of chromium found in fresh complete DMEM was 1.08 μg/L. This concentration would be used to determine whether any bystander effects observed in this experiment were due to the secretion of a bystander factor into the medium by Cr-exposed cells or whether any DNA damage response was due to the residual Cr that remained in the medium.

However, as the same fresh DMEM was used to culture both control and Cr-treated cells, it is unlikely that this would have been responsible for any differences.

4.5.2 Proteomics

4.5.2.1 Background

To determine the nature of the complement of proteins present in metal-conditioned medium compared to control medium, a sample of conditioned medium was harvested from both BJ and hTERT donor cell types 5 days post their initial 24 hour exposure to either Cr (VI) or PBS. The conditioned medium samples were stored at -20°C before being sent to the University of Bristol, Department of Biochemistry for analysis by two dimensional gel electrophoresis, which was kindly carried out by Kate Heesom. Two samples were analysed for each of the following treatment combinations: BJ PBS; BJ Cr; hTERT PBS; hTERT Cr

4.5.2.2 Proteomics Results

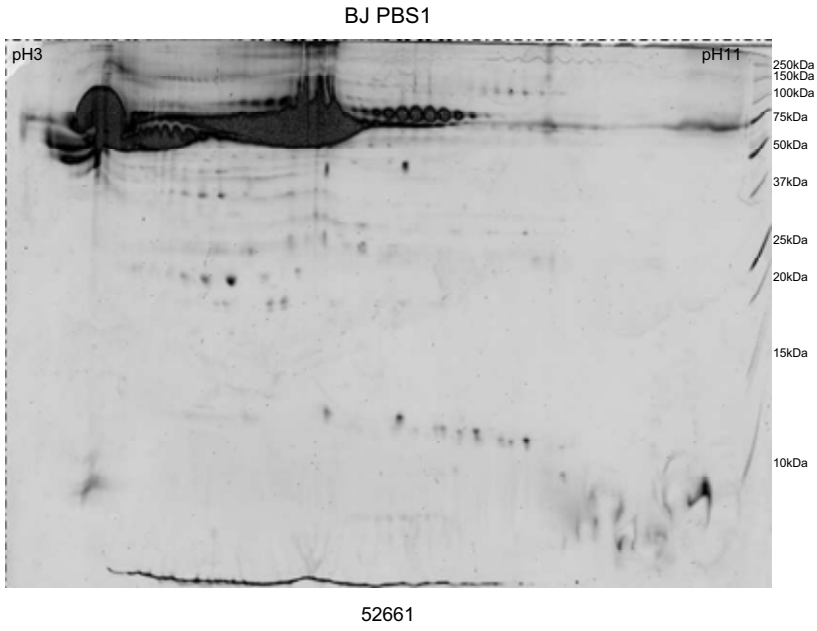

Figure 4.21 Proteomics gel of conditioned medium from a PBS-treated BJ cell

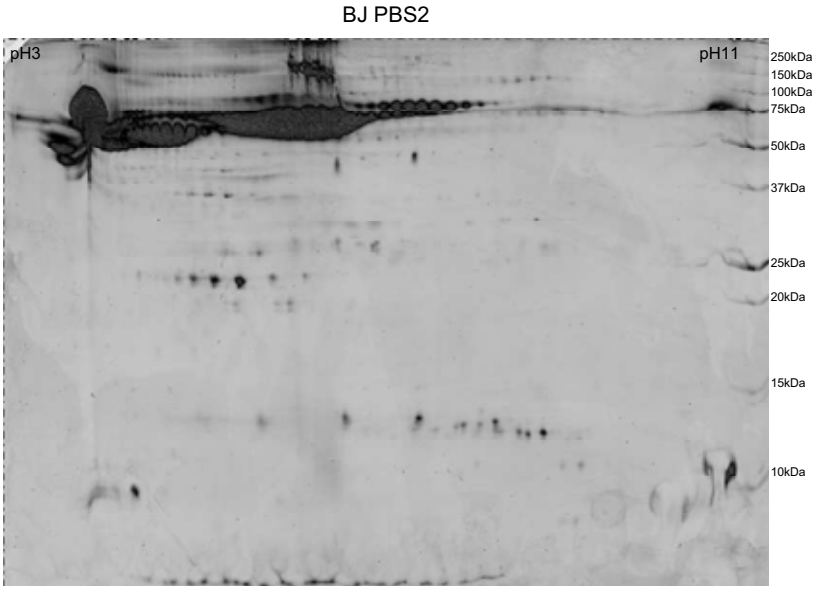

Figure 4.22 Proteomics gel of a PBS treated BJ cell

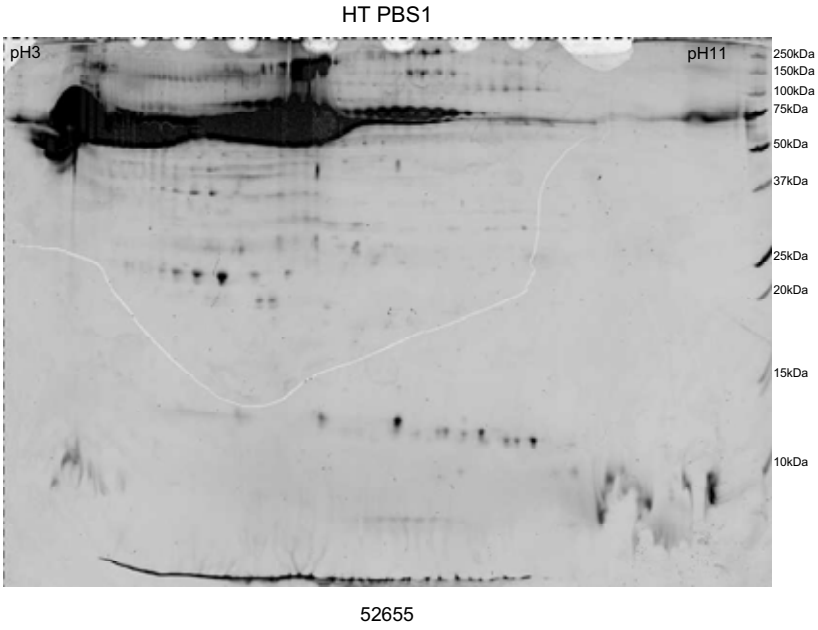

Figure 4.23 Proteomics gel of a PBS treated hTERT cell

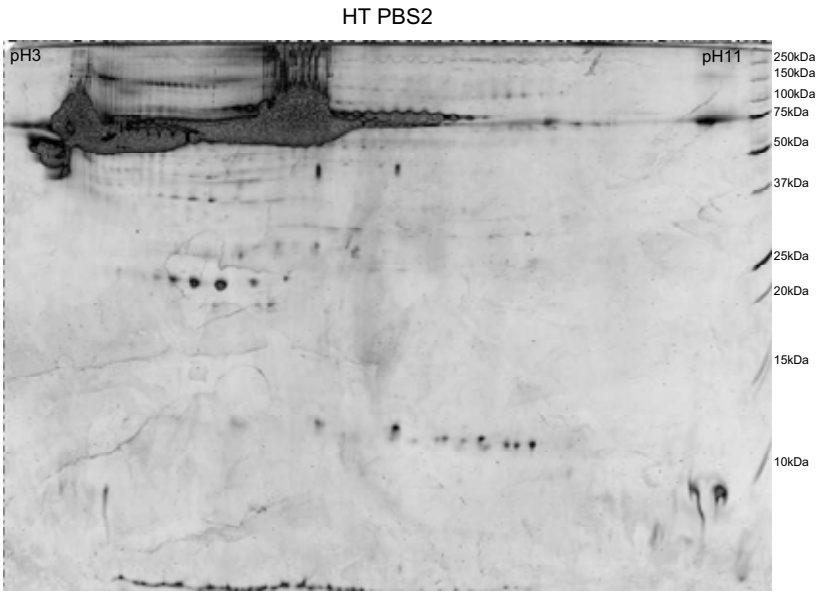

Figure 4.24 Proteomics gel of a PBS treated hTERT cell

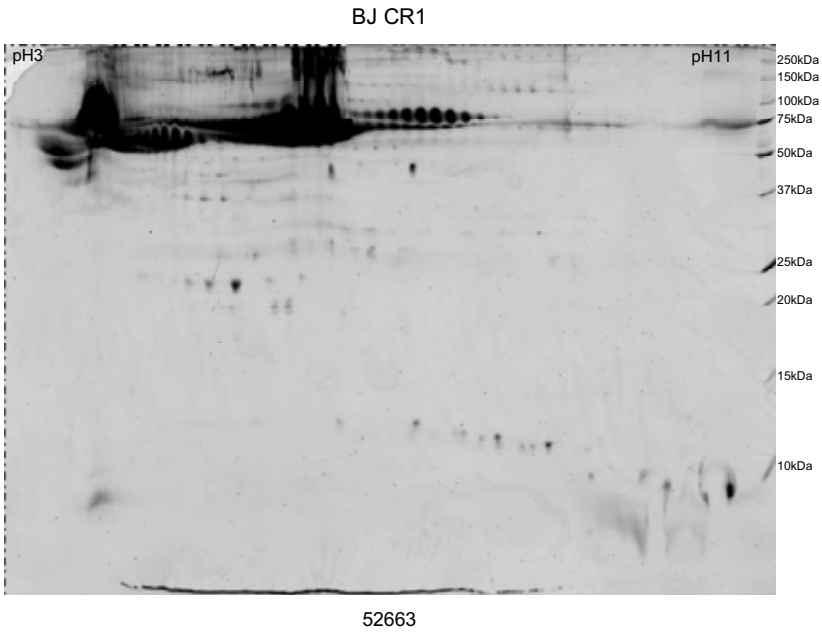

Figure 4.25 Proteomics gel of a Cr-treated BJ cell

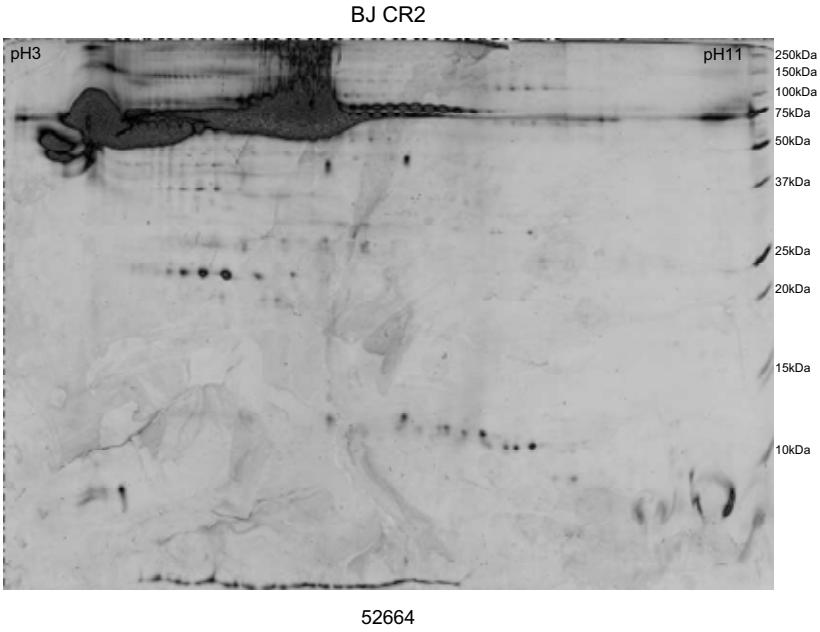

Figure 4.26 Proteomics gel of a Cr-treated BJ cell

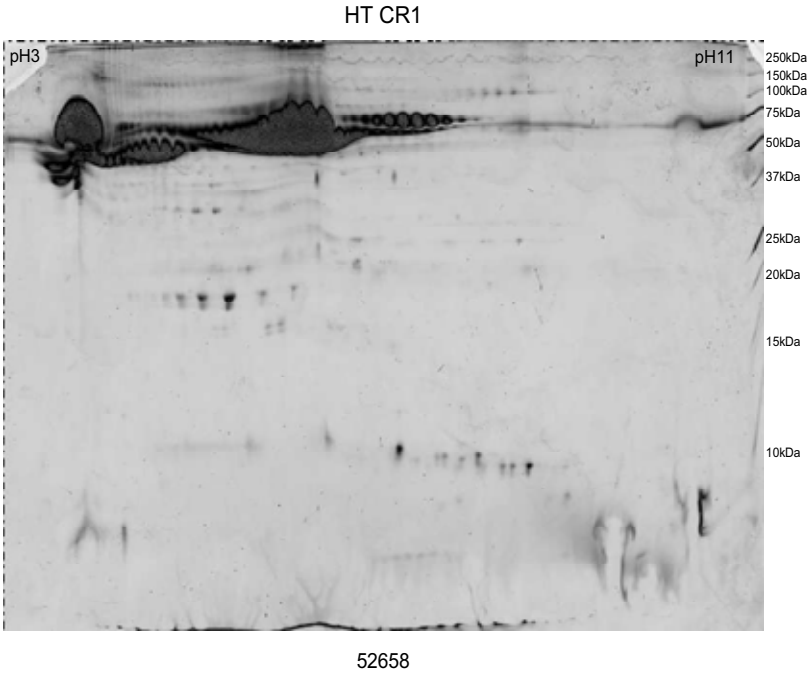

Figure 4.27 Proteomics gel of a Cr-treated hTERT cell

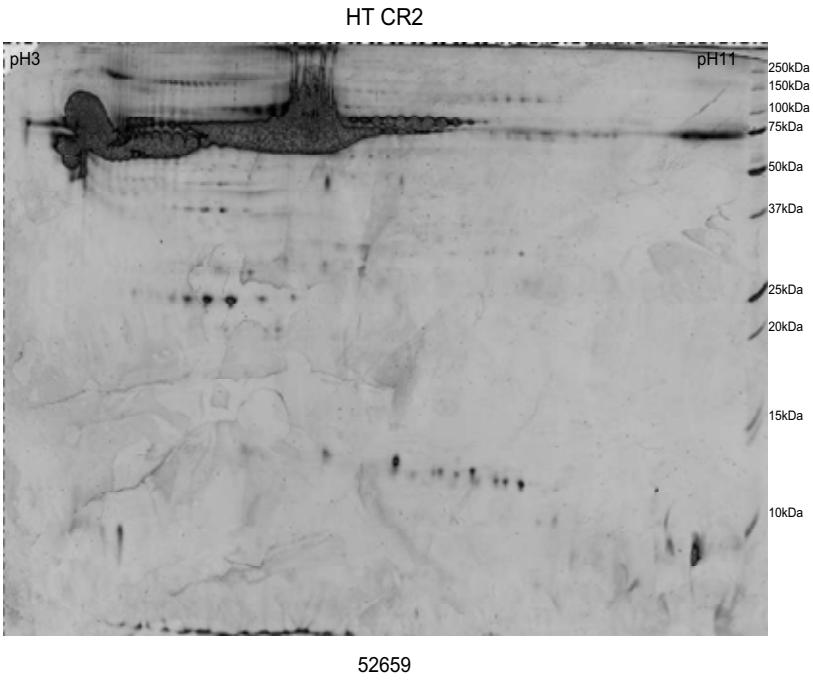

Figure 4.28 Proteomics gel of a Cr-treated hTERT cell

4.5.2.3 Proteomics Summary

There were no bands that appeared in any of the conditioned medium gels that were exclusive to a particular cell type or treatment. All the same proteins appear to have been secreted into the medium, whether the cells were PBS or Cr-treated and whether they were BJ or hTERT. This indicates that the bystander factor was perhaps too small to be detected by 2D gel analysis or that no 'new' factor was secreted – perhaps there was just a change in the balance of what is normally secreted.

4.5.3 Telomeres and Telomerase

4.5.3.1 Background

Single telomere length analysis (STELA) was carried out as described by Baird et al (Baird et al. 2003). Figure 4.29 shows gels obtained from STELA analysis of the XpYp telomere (left panel) in hTERT and passage four BJ cells that had not been Cr-exposed This analysis confirms that hTERT positive cells had longer telomeres than BJ cells.

However, this was not due to an overall increase in all telomere lengths. Rather, it was because BJ cells presented with a bimodal allelic-like distribution of lengths 0-3 kb and 3-11 kb that was lacking in hTERT cells, having only the larger fraction. According to Glaviano et al (Glaviano et al. 2006), there is no evidence that metal exposure in BJ or hTERT cells had any effect on telomere length or distribution The level of telomerase activity was measured in BJ and hTERT cells and as expected, the level of telomerase in hTERT cells was much higher than that in BJ cells at 2.04±0.25 total product generated (TPG) units and 0.01±0.008 TPG units in hTERT and BJ cells respectively (Glaviano et al. 2006) No significant change was noted after Cr treatment. Therefore, it was confirmed that genomic instability induced by metal treatment was not due to metals altering overall telomere lengths or telomerase activity (Glaviano et al. 2006) and that hTERT positive cells did indeed have significantly longer telomeres than BJ fibroblasts.

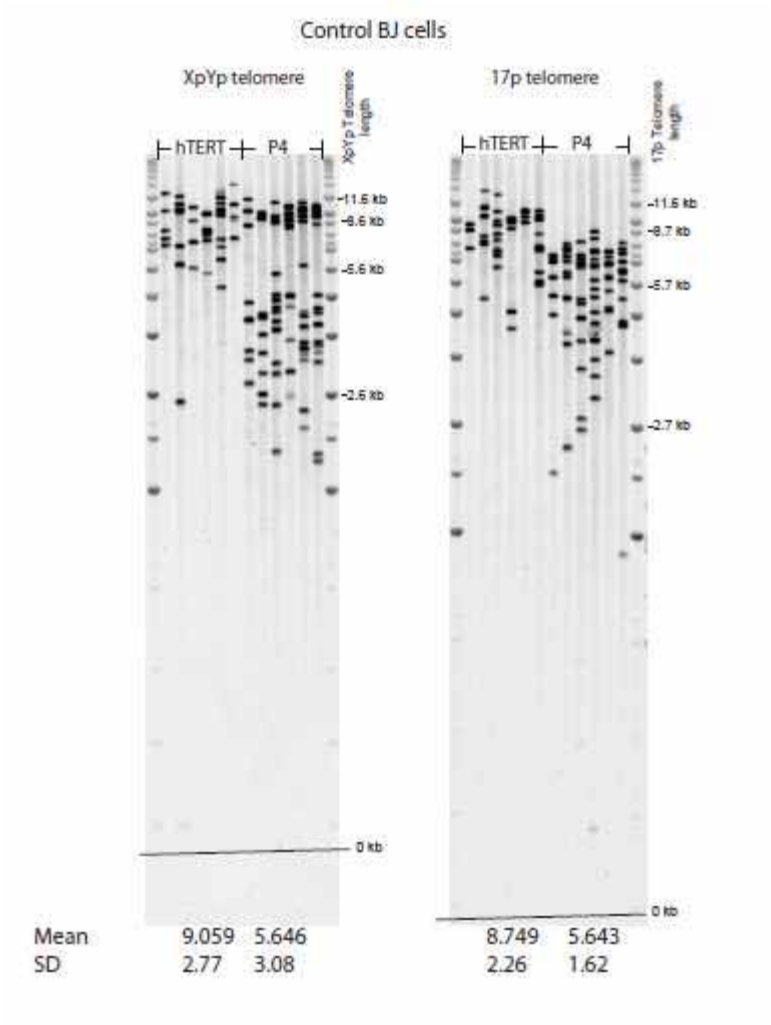

Figure 4.29 Telomere lengths for BJ and hTERT cells

4.6 Direct treatment of BJ and hTERT cells with residual Cr concentration

4.6.1 Background

Chromium analysis was carried out in conditioned medium, fresh complete DMEM and at the 5 washing stages by the Analytica lab in Sweden (as described in section 4.5.1). The concentration of Cr present in conditioned medium was found to be 1.08μg/L (0.003 μM) and was used to directly treat both hTERT and BJ cells for 24 hours. This low dose treatment was over 133-fold less concentrated than the 'normal' Cr treatment of 0.4 μM $K_2Cr_2O_7$. After the 24 hours incubation with this low dose of Cr (VI), the

micronucleus assay and γ-H2AX focus formation assays were carried out on both BJ and hTERT cells. This was in order to determine whether the residual Cr concentration that persisted after the washings steps could induce any significant levels of DNA damage and therefore confirm whether the bystander effect that had been observed was due to this residual metal or a bystander factor released from the metal-exposed cells.

4.6.2. Micronucleus Assay: Results

BJ and hTERT cells were plated out into T-75 cell culture flasks at a density of $3x10^5$ cells per flask as described for direct metal exposures in 2.2.2.1. The following day, cells were incubated at 37°C with the residual Cr concentration identified in Table 4.1 (1.08μg/L or 0.003μM) or PBS (control) for 24 hours. Simultaneously with this treatment, cytochalasin B was added to the cultures at a concentration of 6μg/ml in order to obtain binucleate cells. After 24 hours incubation with metal and Cyt-B, cells were harvested from flasks via trypsinization for the micronucleus assay (as described in 2.6). Figure 4.30a shows the percentage of BJ and hTERT cells containing micronuclei after residual Cr treatment. There was no significant difference in induction of micronuclei between the residual Cr treatment and the PBS control treatment, as illustrated by the statistical analysis in Figure 4.31. Figure 4.30b shows the percentage of BJ and hTERT cells containing NPBs following residual Cr treatment. Again, there was no significant difference in NPB induction between Cr-treated and control cells (Figure 4.31).

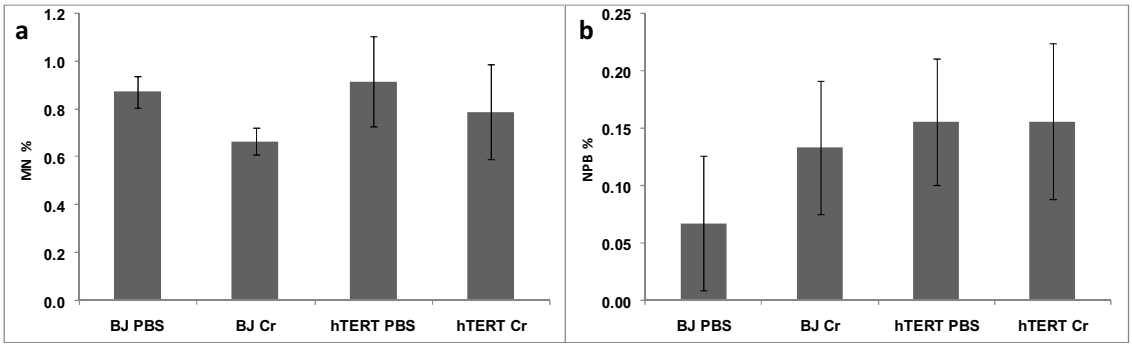

Figure 4.30 (a) Micronuclei in BJ and hTERT cells following treatment with PBS and 1.08 μg/L $K_2Cr_2O_7$ (residual Cr concentration in conditioned medium after washings) (b) Nucleoplasmic Bridges in BJ and hTERT cells following treatment with PBS and 1.08 μg/L $K_2Cr_2O_7$ (residual Cr concentration in conditioned medium after washings)

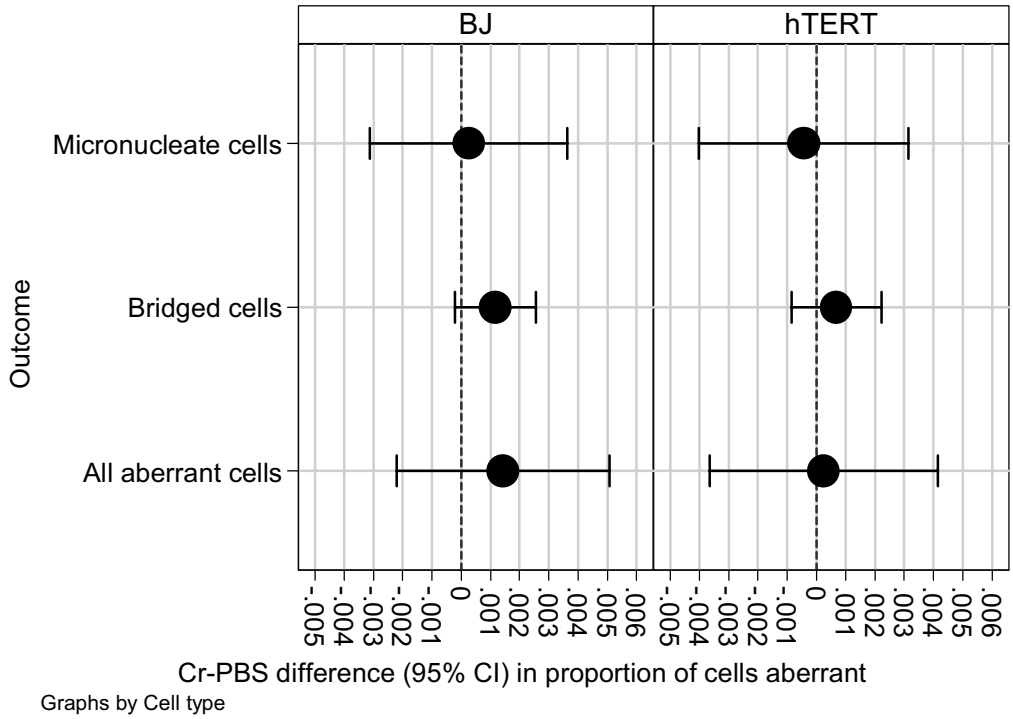

Figure 4.31 Cr-PBS differences in proportions of cells with micronuclei or nucleoplasmic bridges

4.6.3 Micronucleus Assay Low Dose: Summary

There was no significant induction of micronuclei or nucleoplasmic bridges by the residual Cr dose used in this experiment. This suggests that the micronuclei and NPBs previously observed in recipient cells exposed to conditioned medium from metal

exposed donor cells (Sections 4.1 Micronucleus Assay and 4.2. γ-H2AX Focus Formation Assay) was caused by a significant bystander effect and not just a confounding effect of direct metal treatment.

4.6.4 γ-H2AX Focus Formation Assay: Results

BJ and hTERT cells were plated out into T-75 cell culture flasks at a density of $3x10^5$ cells per flask as described for direct metal exposures in section 2.2.2.1. The following day, cells were incubated at 37°C with the residual Cr concentration (1.08μg/L) or PBS (control) for 24 hours. Cells were then harvested and plated onto coverslips at a density of 30,000 cells per coverslip. When attached to the coverslips, cells were stained for γ-H2AX focus formation as a means of detecting the induction of any DNA double strand breaks as described in section 2.7. Figure 4.32 shows the percentage of BJ and hTERT cells with 4+ foci per cell. Again, as stated for the micronucleus assay, there was no significant difference between Cr- treatment and control for either cell type as illustrated by the statistical analysis in Figure 4.33.

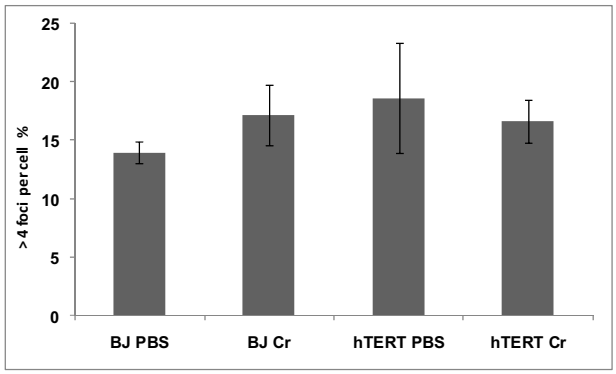

Figure 4.32 Percentage of BJ and hTERT cells containing 4+ γ-H2AX foci after direct exposure to 1.08μg/L potassium dichromate

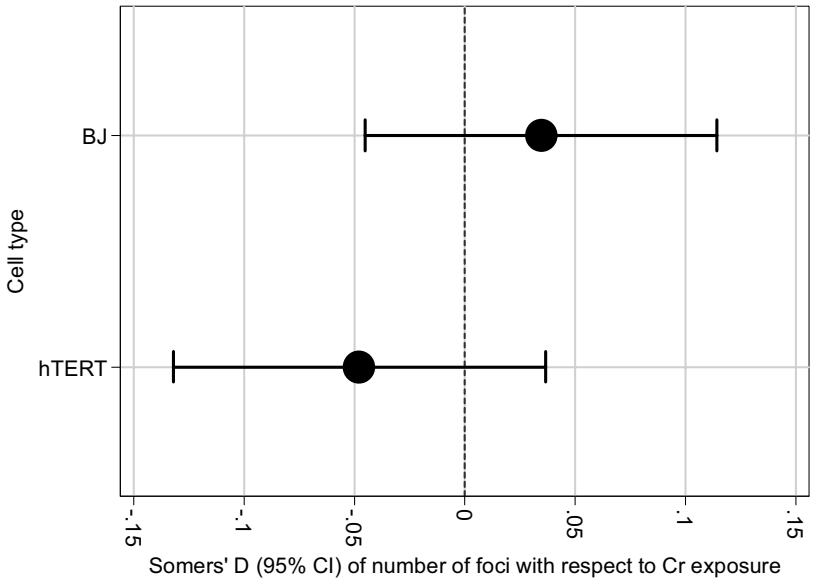

Figure 4.33 Somers' *D* estimates of numbers of foci with respect to Cr exposure

4.6.5 γ-H2AX Focus Formation Assay Low Dose: Summary

There was no significant induction of γ-H2AX foci in either BJ or hTERT cells, which suggests that the low dose of Cr was not able to induce the levels of DNA damage that were observed for transfer of conditioned medium to recipient cells during previous experiments (Sections 4.1 Micronucleus Assay, 4.2. γ-H2AX Focus Formation Assay and 4.3 Cytogenetics). Therefore, the biological effects observed in response to conditioned medium can be attributed to bystander signalling rather than chromium contamination.

CHAPTER 5 : MICROINJECTIONS

5.1 Background

Here, in order to study the metal-induced bystander effect, microinjection with chromium ions in solution was carried out to target individual cells to mimic microbeam irradiation experiments. Cells were microinjected with either PBS (control) or 0.4 μM potassium dichromate. Injected cells were labelled green with FITC dextran. Cells were fixed 6 hours post injection and stained for the γ-H2AX focus formation assay as described in 2.7.

5.2 Microinjection: Results

Figure 5.1 below, shows hTERT cells microinjected with chromium. Injected cells are labelled green with a large FIT-C dextran molecule. They were then fixed and stained with anti-γ-H2AX and counterstained with a Cy3-conjugated secondary antibody. Therefore, anti-γ-H2AX appears as red foci.

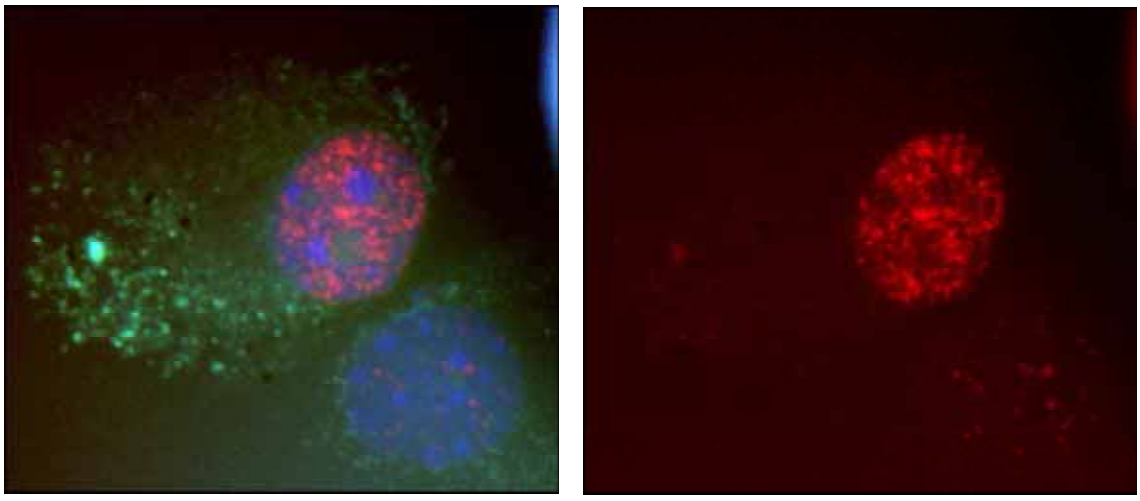

Figure 5.1 Microinjected cell labelled green (left) and under the red filter (right) at 100x magnification

Only the cellular nuclei are visible in Figure 5.2 and Figure 5.3 below. Although some of the nuclei may look far apart, the cell membranes were actually in close contact.

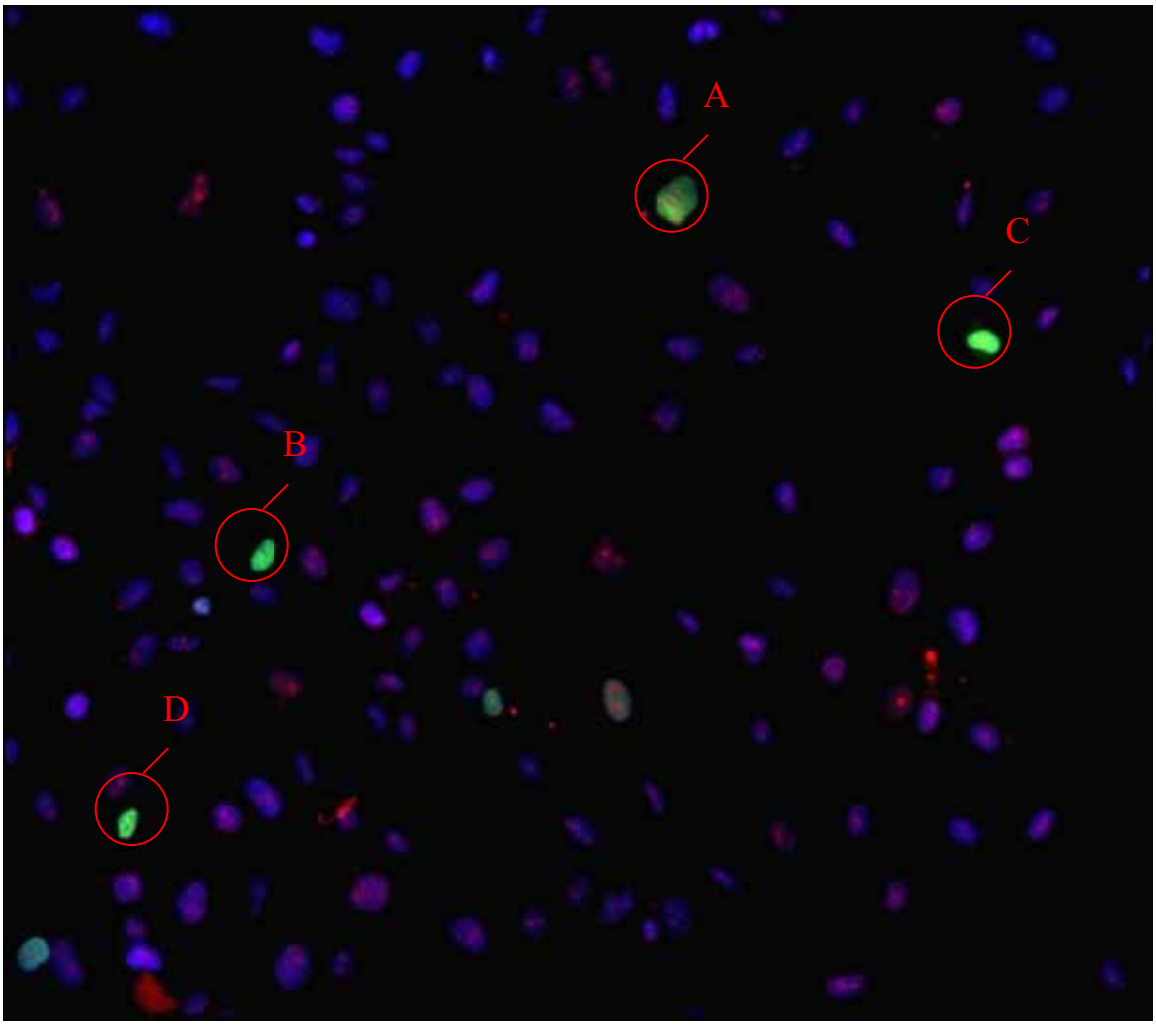

Figure 5.2 hTERT cells microinjected with 0.4 µM $K_2Cr_2O_7$. Injected cells are labelled green. γ-H2AX foci are observed in cells neighbouring the injected cells at 10x magnification

The injected cell labelled 'A' was the only injected cell in Figure 5.2 containing γ-H2AX foci. Five of its nearest neighbouring cells also contained foci. The cells labelled B, C and D do not contain any DNA double strand break foci but are in close proximity to cells that do contain γ-H2AX foci. For example, there are two cells close to the cell labelled C, which contain foci; there are also three cells in contact with both cells B and D, which contain foci. This indicates that there may be a bystander effect

generated in hTERT cells in response to microinjection of 0.4 μM potassium dichromate that induces γ-H2AX foci in neighbouring cells.

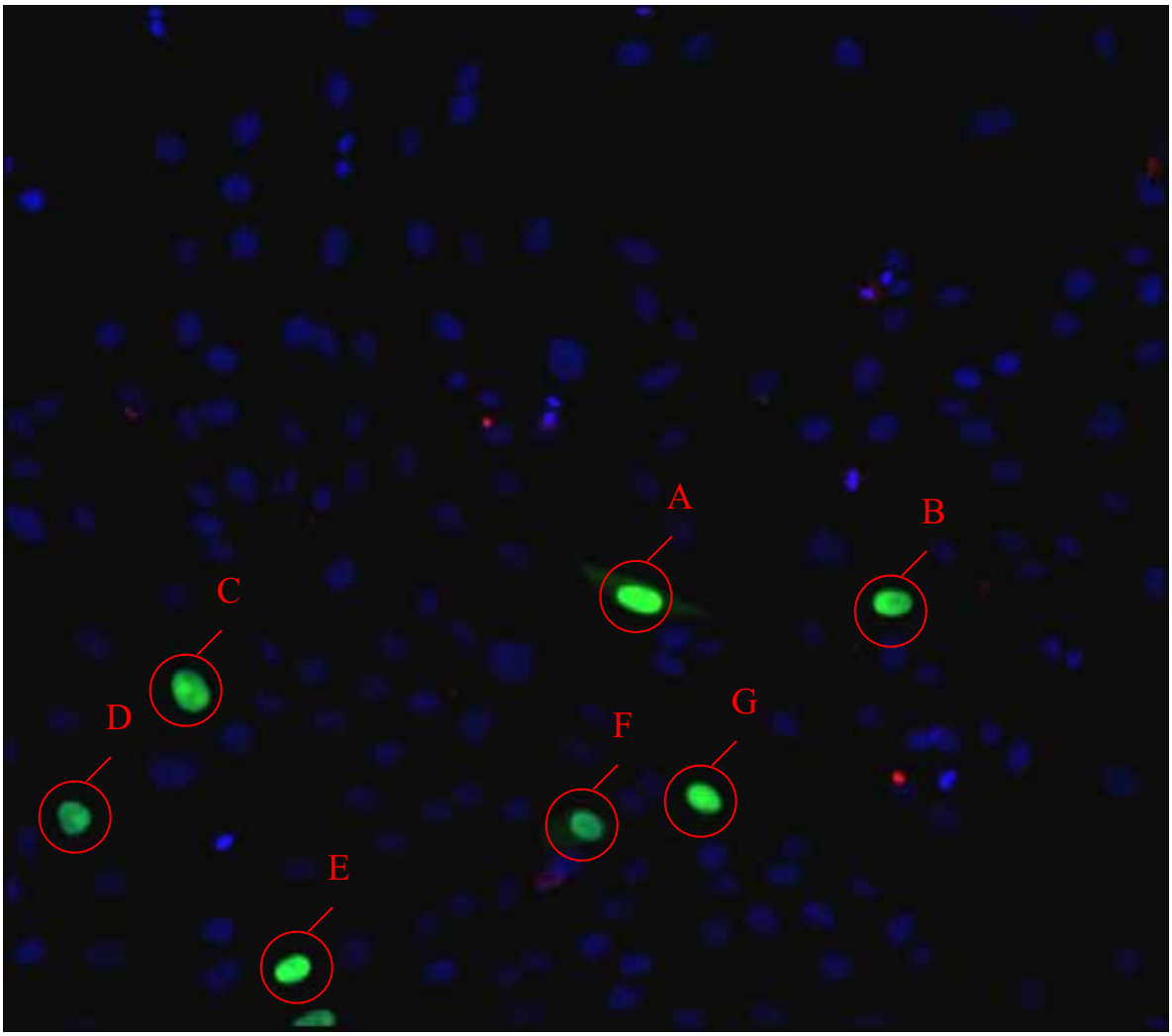

Figure 5.3 hTERT cells microinjected with PBS. Injected cells labelled green at 10x magnification

In Figure 5.3, fewer γ-H2AX foci are present than can be observed in Figure 5.2. Figure 5.3 shows control cells that have been injected with PBS and no Chromium. There do not appear to be any foci in the nuclei of the injected cells as there was in Figure 5.2. This indicates that it was the chromium that induced the foci and not the act of microinjection. None of the nuclei surrounding the injected cells seem to contain γ-H2AX foci in the control, Figure 5.2. Therefore, there appears to be a bystander effect induced by microinjection of chromium that was not observed when PBS was injected.

This indicates that it is not merely the stress of microinjection that causes cells to express γ-H2AX foci. Also to support this idea, there are fewer injected cells in the Cr injection image (Figure 5.2) than there are in control injections (Figure 5.3) which suggests that the injection process was not responsible for inducing the DNA double stranded breaks observed in Figure 5.2. Only the hTERT cells were successfully injected as BJ cells were incapable of surviving the process, the majority becoming detached from the surface of the coverslip.

5.3 Microinjection: Summary

The results that are indicated above in Figure 5.2 and Figure 5.3 are not completely in agreement with the results obtained so far. Up until now, no bystander responses have been observed in hTERT cells exposed to bystander signals from hTERT cells using both the micronucleus assay and γ-H2AX staining via medium transfer. In the case of microinjections, however, there does appear to be a bystander effect to induce DNA double-stranded breaks in hTERT cells.

CHAPTER 6 : MECHANISTIC INSIGHTS

A common theme in the radiation induced bystander effect is the important role of mitochondria to produce NO and calcium dependent NOS (Chen et al. 2008) and secondary more long lasting radicals possibly involving the activation of NADPH (Azzam et al. 2002; Azzam et al. 2003). Indeed increased numbers of mitochondria have been observed in bystander cells indicative of a stress response to mitochondrial dysfunction (Nugent et al. 2007). It is therefore of interest with respect to the results of this study that hTERT can shuttle from the nucleus to the mitochondrion, facilitated by a leader sequence at its N-terminus (Santos et al. 2006; Passos et al. 2007b) and may confer stress resistance to cells with improved antioxidant defense and mitochondrial protection (Ahmed et al. 2008).

6.1 Antioxidants

6.1.1 Background

Some proposed mediators of the bystander effect are known to increase the intracellular reactive oxygen species (ROS) (Narayanan et al. 1997). Therefore, l-ascorbic acid (0.4 mM) was used in this study to eliminate any oxidative damage that may have been caused by molecules present in the medium of Cr (VI)-treated BJ and hTERT fibroblasts and also by direct treatment of Cr (VI). Its effects on the frequency of γ-H2AX focus formation were investigated in both conditioned medium recipient cells and directly Cr (VI)-exposed cells. Cells were incubated in 0.4 mM l-ascorbic acid for 24 hours, after a 24 hour Cr or PBS (control) treatment in directly metal-exposed or donor cells. A minimum of one hundred nuclei were scored from at least three independent experiments to obtain a mean estimate for DNA double strand break induction.

6.1.2 Antioxidants: Results

Figure 6.1a shows the percentage of BJ recipient cells containing four or more γ-H2AX foci per cell following exposure to conditioned medium from BJ or hTERT cells exposed to Cr (VI) or PBS, in the presence of ascorbic acid or not. Figure 6.1a shows that significantly higher levels ($p<0.001$) of DNA double strand breaks were induced in BJ recipient cells that had received conditioned medium from Cr-exposed cells of either type (hTERT or BJ). Incubation of donor cells with ascorbic acid resulted in a considerable reduction in DNA double strand break induction caused by Cr-conditioned medium from either cell type in BJ recipient cells. Figure 6.1b shows that antioxidant treatment in hTERT donor cells did not affect the level of γ-H2AX foci induced in hTERT recipient cells. However, in response to conditioned medium from BJ donor cells that had been exposed to both Cr (VI) and ascorbic acid, there was a significant increase in γ-H2AX foci. Notably, based on the results of previous study (Chapter 4, (4.5)), it would be expected that significantly more DNA double strand breaks would be induced in hTERT recipient cells that had been exposed to conditioned medium from Cr-exposed BJ cells compared to control than were seen here (Figure 6.1b). However, in this study, the percentage of hTERT cells containing a high level of γ-H2AX foci (4+ foci per cell) following exposure to control medium from BJ donor cells exposed to PBS (rather than Cr (VI)) was unusually high at around 33%.

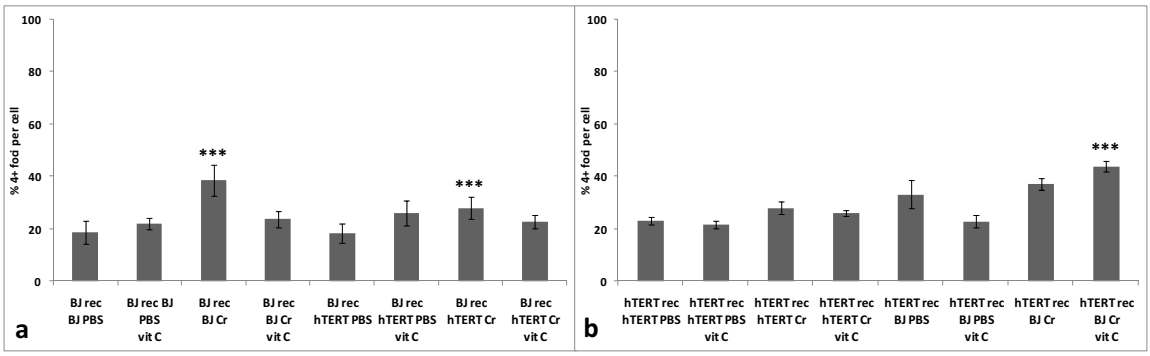

Figure 6.1 BJ (a) and hTERT (b) recipient cells containing four or more γ-H2AX foci following medium transfer 5 days post Cr treatment in donor cells in the presence of 0.4 mM ascorbic acid

Figure 6.2a shows the effect of ascorbic acid on induction of γ-H2AX foci by direct Cr (VI) exposure. There was a significant ($p<0.01$) difference between the percentage of BJ cells containing high levels of DNA double strand breaks following direct treatment with Cr and those cells that were also exposed to ascorbic acid. In this case, the antioxidant completely abrogated the DNA damaging effects of direct Cr exposure in BJ cells. This may explain why the percentage of BJ recipient cells containing 4+ DNA double strand break foci per cell was much lower when Cr treatment in donor BJ fibroblasts was combined with antioxidant treatment. However, this does not explain the result observed in hTERT recipient cells that had been exposed to conditioned medium from Cr treated fibroblasts with or without ascorbic acid (hTERT rec BJ Cr ±vit C). In that case, antioxidant did not eradicate any DNA damaging factors present in conditioned medium from Cr-exposed BJ donor cells. Figure 6.2b shows that direct Cr exposure in hTERT cells induced a high level of DNA double strand breaks and that this effect was completely diminished when ascorbic acid was also present ($p<0.001$), reducing γ-H2AX focus induction to similar levels to that observed in control cells. There even appeared to be a decrease in γ-H2AX focus formation caused by ascorbic acid in control cells.

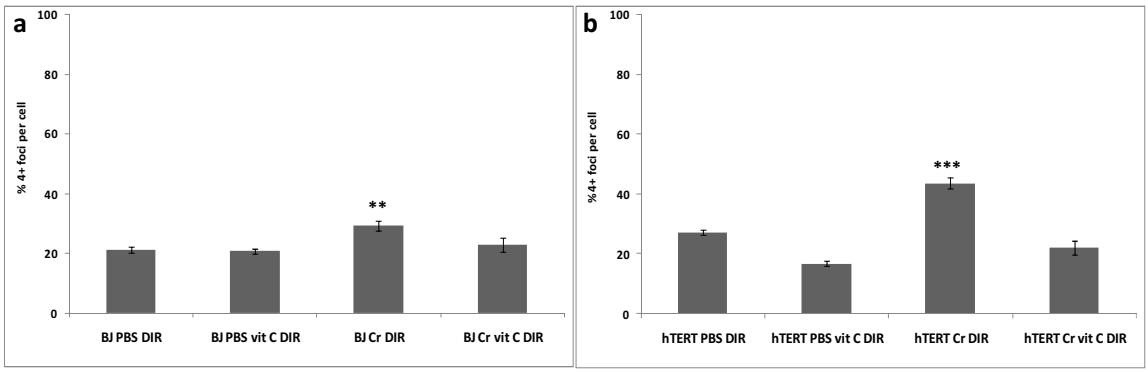

Figure 6.2 BJ (a) and hTERT (b) cells containing four or more γ-H2AX foci 5 days post direct exposure to Cr in the presence of 0.4 mM ascorbic acid

Figure 6.3 shows Somers' D estimates for numbers of foci with respect to Cr treatment. It illustrates that in BJ cells in the presence of. Vitamin C (top right panel) following either medium transfer from BJ donors or hTERT donors or direct Cr (VI) exposure, there was no significant difference in numbers of γ-H2AX foci compared to control. Conversely, all three of these treatments caused a significant induction of DNA double strand breaks when no antioxidant was present (top left panel). This means that antioxidant treatment significantly reduced DNA double strand break induction following direct Cr treatment in BJ cells and transfer of conditioned medium from Cr exposed BJ donor cells or Cr exposed hTERT donor cells to BJ recipient cells.

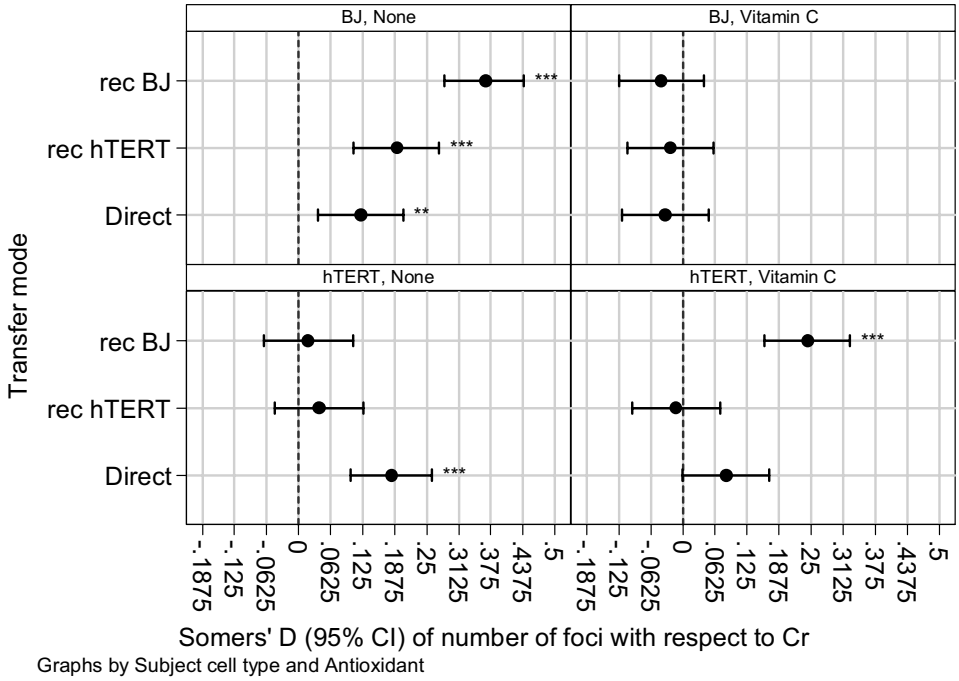

Figure 6.3 Somers' D estimates of numbers of foci with respect to Cr exposure in the presence of 0.4 mM ascorbic acid

6.1.3 Antioxidants: Summary

Direct exposure of human fibroblasts to potassium dichromate ions caused a substantial increase in DNA double strand break induction compared to non-Cr-exposed control cells, analysed by means of the γ-H2AX focus formation assay. Combination of Cr-exposure with ascorbic acid treatment caused a reduction in DNA double strand breaks compared to Cr-exposure alone. This difference was significant in both BJ ($p<0.01$) and hTERT ($p<0.001$) cells. Therefore, it can be assumed that L-ascorbic acid caused a decrease in the percentage of conditioned medium donor cells containing a significantly high level (4+ foci per cell) of DNA double strand breaks. Conditioned medium from hTERT donor cells caused an increase in DNA double strand breaks in BJ recipient cells and this damage was completely abrogated when donor cells had been incubated in vitamin C simultaneously with chromium exposure. hTERT conditioned medium did not have a DNA damaging effect on hTERT recipient cells and this was not

altered in the presence of ascorbic acid. Although, DNA double strand breaks in directly Cr-exposed BJ donor cells were diminished in the presence of vitamin C, this had no effect on γ-H2AX focus formation in hTERT recipient cells exposed to conditioned medium from Cr-exposed BJ donor cells that had been incubated with the antioxidant. Cr-conditioned medium and in particular, Cr-conditioned medium from BJ donor cells incubated with ascorbic acid caused a large increase in DNA double strand breaks in hTERT recipient cells, which was significantly higher than in controls ($p<0.001$). Also the combination of antioxidant with Cr treatment in BJ donor cells produced conditioned medium that caused a significant reduction in the percentage of BJ recipient cells containing a high level of γ-H2AX foci compared to Cr-exposure with no vitamin C.

6.2 p38 MAP Kinase

6.2.1 Background

Members of the MAP Kinase family have been implicated as mediators of bystander responses (Azzam et al. 2002; Zhou et al. 2005; Lyng et al. 2006b) and MAPK is known to become activated by hexavalent chromium (Tessier and Pascal 2006). MAP Kinase cascades regulate critical cellular signalling processes involved in proliferation, differentiation and apoptosis. The p38 MAP Kinase signalling module can be activated by environmental stresses such as oxidative stress, UV irradiation, hypoxia, ischaemia, and inflammatory cytokines and is important for survival after radiation exposure (Dent et al. 2003). Activated MAPK translocates from the cytoplasm to the nucleus where they regulate target transcription factor activity through phosphorylation (Su and Karin 1996).

p38 MAP Kinase signalling was manipulated throughout this study to investigate whether the differences in bystander signalling between the two cell types can be attributed to differences in the nature of their gap junctional intercellular communication (GJIC) as gap junctions are proposed to mediate the bystander effect (Azzam et al. 1998; Azzam et al. 2001). Perhaps BJ cells have a more permissive 'open' gap junction structure than hTERT cells, enabling them to secrete more bystander factors or perhaps larger molecules. With this hypothesis in mind, anisomycin, an antibiotic produced by *Streptomyces griseolus* was used to reduce GJIC in BJ cells. Anisomycin activates p38 MAP Kinase leading to enhanced Connexin 43 phosphorylation and reducing GJIC (Ogawa et al. 2004). Treatment of BJ donor cells with anisomycin may induce a less permissive more 'closed' gap junction structure enabling the secretion of fewer bystander factors from BJ donor cells. The Pyridinyl imidazole inhibitor SB203580 inhibits p38 MAP Kinase and is able to restore GJIC (Ogawa et al. 2004). Donor hTERT cells were incubated with SB203580 at 10μM for 3 hours to alter their gap junction signalling (Ogawa et al. 2004; Lyng et al. 2006b). The concentration of anisomycin was chosen according to that selected by Ogawa (Ogawa et al. 2004). Treatment of hTERT donor cells may induce more open gap junction signalling, enabling the cells to secrete additional bystander factors, possibly enabling so far unobserved biological effects in recipient hTERT cells, investigated via the micronucleus and γ-H2AX focus formation assays. A minimum of one hundred nuclei for the γ-H2AX assay and 1000 binucleate cells for the micronucleus assay were scored from at least three independent experiments. Figure 6.4 shows a model diagram of the

proposed effects of anisomycin on BJ cells and SB203580 on hTERT cells.

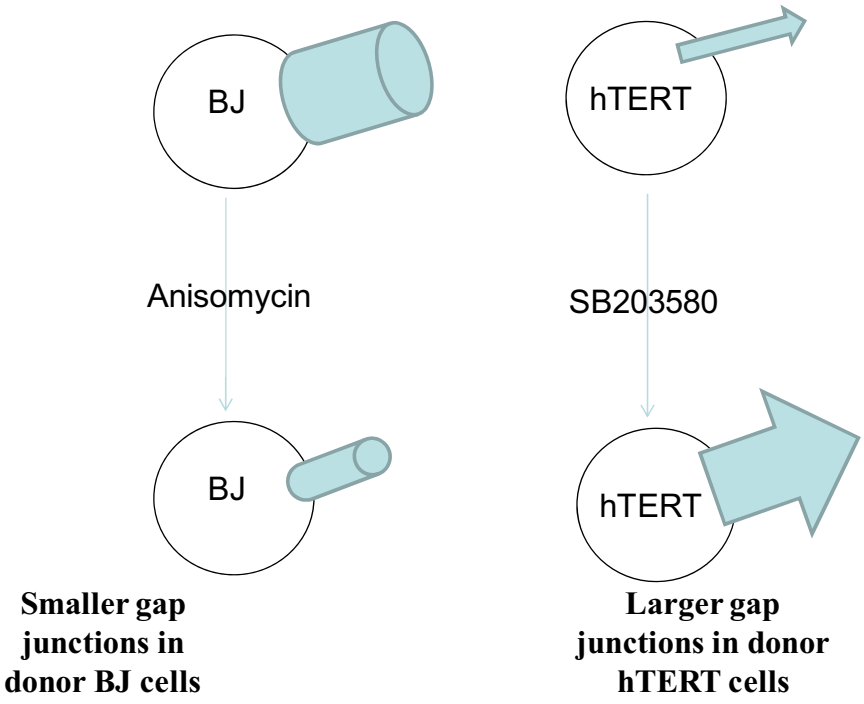

Figure 6.4 Model diagram of effects of anisomycin on BJ gap junction structure to produce smaller channels and less effective conditioned medium and proposed effects of SB203580 on hTERT gap junction structure to promote larger gap junction channels and secretion of more effective conditioned medium

6.2.2 p38 MAP Kinase: Results

Figure 6.9 shows that the fibroblasts used in this study do indeed possess the potential to use gap junctional intercellular communication, as demonstrated by the scrape loading dye transfer technique. The cells were scraped using a scalpel and the Lucifer yellow dye was able to enter the cells that were scraped due to disruption of the plasma membrane. The dye was able to pass to cells that were not scraped via intercellular communication channels, connexins, which make up gap junctions. Gap junctions may be a mechanism for the transmission of bystander signals and the difference between hTERT and BJ fibroblast signalling may be related to differences in their gap junction structure. For this reason, anisomycin and SB203580 were used to interfere with gap junctions.

6.2.2.1 hTERT donor Cells

Figure 6.5 shows the percentage of BJ recipient cells containing micronuclei or NPBs following exposure to conditioned medium from hTERT donor cells that had been exposed to Cr (VI) and SB203580. It shows that although there appeared to be a slight increase in the percentage of binucleated BJ recipient cells containing micronuclei in response to treatment with conditioned medium from SB203580-treated hTERT donor cells compared to the DMSO control-treated hTERT donor cells, this change was not significant. However, it does indicate that there was a slight inclination towards a more open gap junction structure in hTERT donor cells when SB203580 was used. Figure 6.6 shows the differences in proportions of micronuclei and NPBs in BJ recipient cells damage and illustrates that treatment of Cr-exposed hTERT cells with SB203580 had no significant effect on induction of MN and NPBs compared to the DMSO control.

In order to determine whether the gap junctions of hTERT cells could be altered using SB203580, medium transfer was carried out from Cr-exposed hTERT donors to hTERT recipients in the presence of the additive. Figure 6.7 and Figure 6.8 illustrate the levels of γ-H2AX foci in hTERT recipient cells when medium was transferred from hTERT donor cells. There was no difference in the percentage of hTERT recipients containing DNA double strand breaks between Cr exposed donors with or without SB203580. This suggests that either the gap junctions did not change with the use of this additive or that the metal-induced bystander effect is not dependent on gap junctional intercellular communication in telomerase positive fibroblasts.

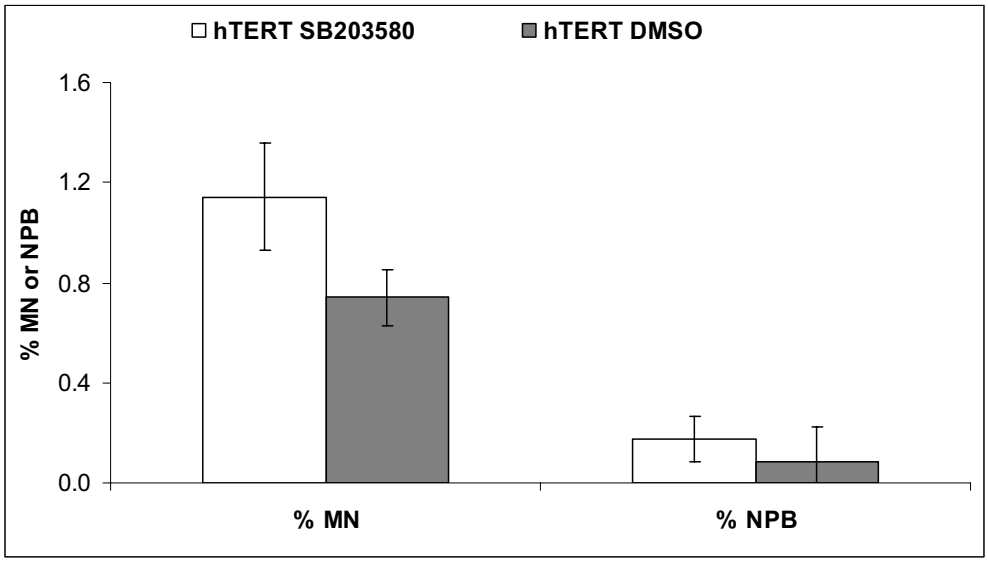

Figure 6.5 Micronuclei and Nucleoplasmic Bridges in BJ recipient cells following exposure to conditioned medium from hTERT donor cells 5 days post Cr exposure in donor cells in the presence of either SB203580 or DMSO (control)

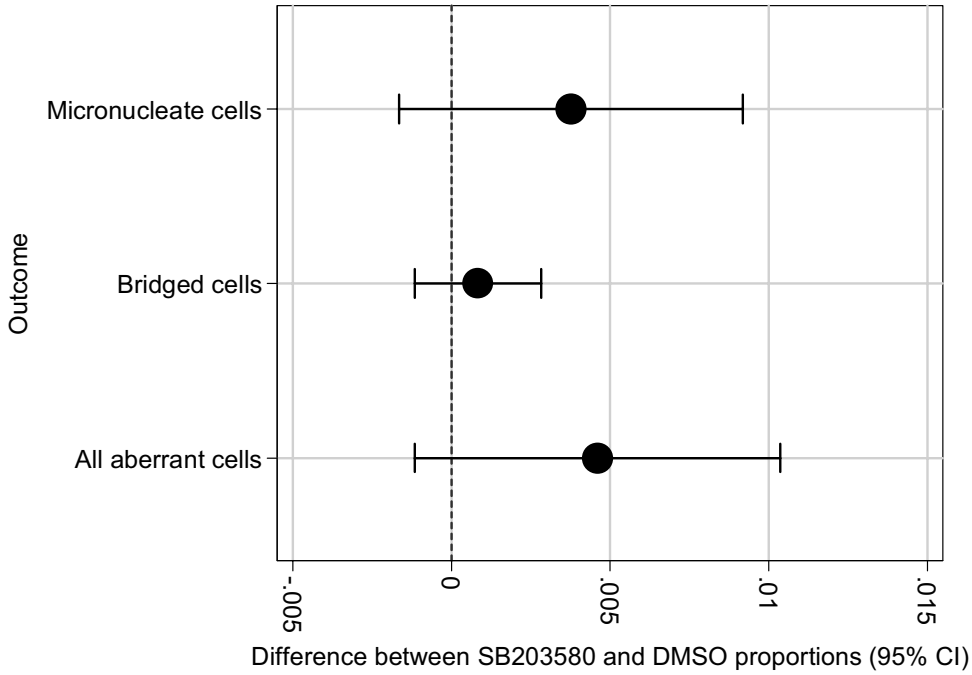

Figure 6.6 Differences in proportions of micronuclei and nucleoplasmic bridges in BJ recipient cells treated with conditioned medium from SB203580 and DMSO exposed hTERT donors

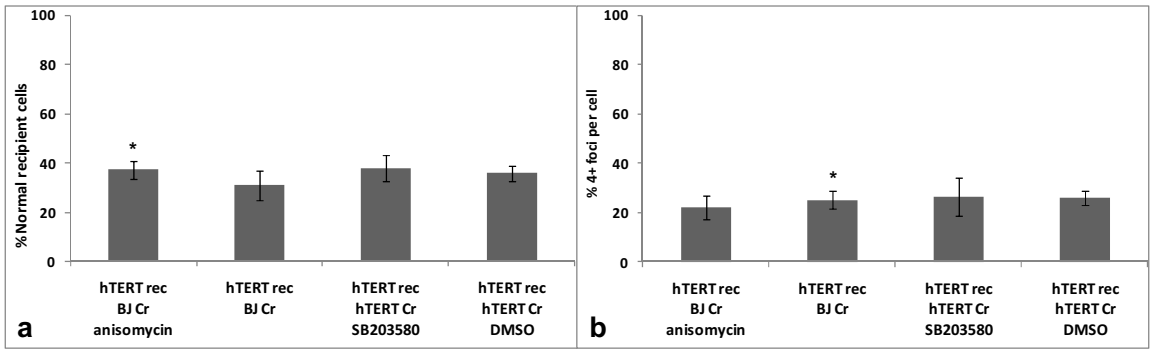

Figure 6.7 no γ-H2AX foci (a) or more than four foci per cell (b) in hTERT recipient cells treated with conditioned medium from BJ donor cells exposed to anisomycin or no additive and hTERT donor cells exposed to SB203580 or DMSO (control)

6.2.2.2 BJ Donor Cells

Figure 6.7a shows the percentage of hTERT recipient cells containing four or more DNA double strand break foci per cell and illustrates that when hTERT recipient cells were treated with conditioned medium from BJ donor cells, anisomycin significantly ($p<0.05$) reduces the percentage of hTERT recipient cells containing a significantly high (four or more foci per cell) level of DNA double strand breaks. Figure 6.7b shows the percentage of hTERT recipient cells containing no DNA double strand breaks following exposure to conditioned medium from Cr-exposed BJ cells in the presence of anisomycin and illustrates that on average in the presence of anisomycin over 37% of the cells were normal i.e. containing no DNA DSBs compared to an average of less than 31% of normal cells in hTERT recipient cells exposed to conditioned medium from Cr exposed BJ cells that lacked anisomycin treatment. This suggests that BJ cells were affected by anisomycin treatment and its effect on their gap junctions may have caused a less open structure and perhaps secretion of fewer bystander factors. Figure 6.8 shows Somers' D estimates of numbers of foci with respect to anisomycin or SB203580 treatment in donor cells and shows that the anisomycin significantly reduces the number of DNA double strand break foci induced in hTERT recipient cells by conditioned medium from Cr-exposed BJ donor cells.

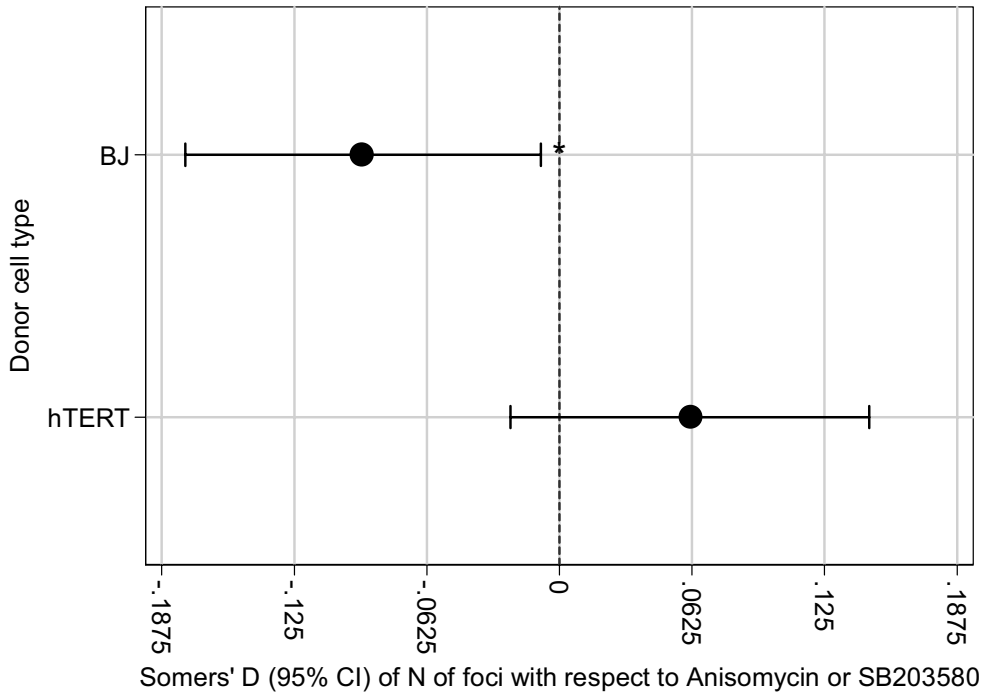

Figure 6.8 Somers' D estimates of number of foci with respect to non control additive

6.2.3 Scrape Loading Dye Transfer Technique

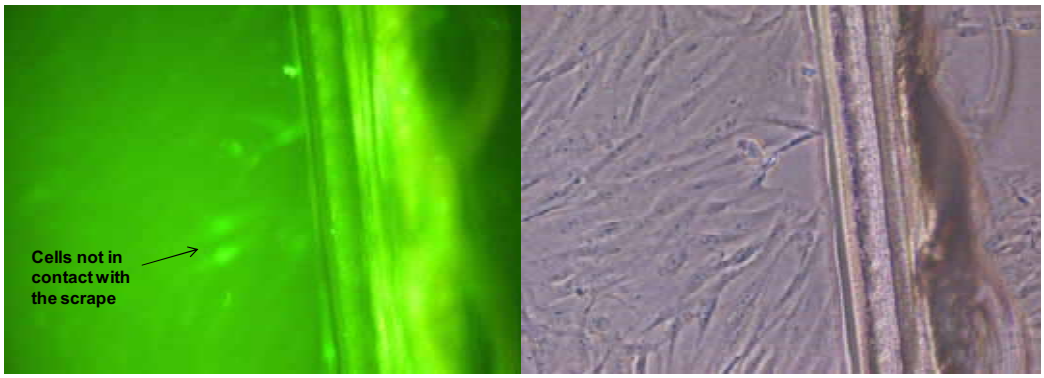

Figure 6.9 Lucifer yellow dye enters cells distant from the scrape via gap junctional intercellular communication at 40x magnification

6.2.4 p38 MAP Kinase: Summary

Figure 6.7 shows that anisomycin treatment in BJ donor cells caused a significant ($p<0.05$) reduction in DNA double strand breaks in hTERT recipient cells compared to conditioned medium from BJ donor cells Cr exposed in the absence of

anisomycin. This suggests that anisomycin may have caused a reduction in gap junction permeability, reducing the bystander signal secreted from BJ donor cells resulting in the decrease in DNA DSBs observed in recipient hTERT cells. SB203580 had no significant effect on micronuclei or NPB induction in recipient cells when additive was present in Cr-exposed hTERT donor cells. Perhaps the release of a bystander signal from hTERT donor cells is not dependent on gap junctional communication, whereas, one of the factors released by BJ cells may have been dependent on gap junction signalling.

6.3 TNF-alpha Neutralization in Conditioned Medium

6.3.1 Background

It was considered that there may be a range of bystander signals produced by Cr-treated fibroblasts that differ between BJ and hTERT cells. It was determined that the conditioned medium obtained from either cell type in response to Cr treatment could contain a factor capable of causing oxidative damage as the antioxidant l-ascorbic acid was able to influence bystander signaling (Chapter 6.1). It was hypothesized that hTERT cells were protected against this oxidative damage but that BJ cells produced an additional factor that hTERT cells were vulnerable to when exposed via medium transfer to conditioned medium from Cr-treated BJ donor cells. This second factor may be a cytokine or any molecule that is not associated with oxidative stress. TNF-alpha is a cytokine involved in systemic inflammation and is known to induce apoptotic cell death and inflammation and to inhibit tumourigenesis (Tracey and Cerami 1993). TNF-alpha has been implicated to play a role in bystander responses (Shareef et al. 2007). Although 2D gel electrophoresis could not detect TNF-alpha in conditioned medium from either cell type following treatment with either Cr or PBS (chapter 4), to

investigate whether the metal-induced bystander effect could be influenced by TNF-alpha signalling, a neutralizing antibody, anti-TNF-alpha was used to sequester functional TNF-alpha from the conditioned medium of both BJ and hTERT cells. This TNF-alpha null medium was transferred to recipient cells of either type; and its effect on DNA double strand break induction in recipient cells investigated using the γ-H2AX focus formation assay. A minimum of one hundred nuclei were scored from at least three independent experiments for each treatment type considered.

6.3.2 TNF-alpha Neutralization: Results

Figure 6.10a shows the percentage of BJ recipient cells containing four or more γ-H2AX foci per cell following exposure to conditioned medium from BJ or hTERT donor cells treated with either Cr (VI) or PBS (control) in the presence of neutralizing anti-TNF-alpha.

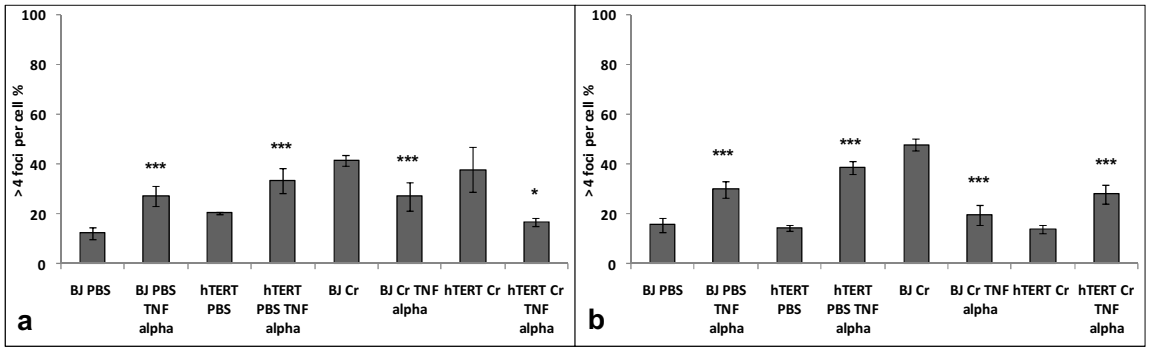

Figure 6.10 Percentage of BJ (a) and hTERT (b) recipient cells containing four or more γ-H2AX foci per cell following exposure to conditioned medium from either cell type exposed to Cr or PBS (control) in the presence of neutralizing anti-TNF-alpha

Figure 6.10a illustrates that the presence of neutralizing anti-TNF-alpha in conditioned medium caused a significant ($p<0.001$) increase in induction of DNA double strand breaks in BJ recipient cells that were treated with control medium from either cell type compared to conditioned medium from PBS-treated donor cells lacking anti-TNF-alpha. Therefore removing TNF-alpha functionality from control conditioned medium actually

caused an increase in DNA damage in BJ recipient cells to over 27% from BJ control donors and over 33% for hTERT control donors. These values are lower than those observed for chromium-conditioned medium but are still over 1.6 fold higher than in controls where no neutralizing anti-TNF-alpha was present. However, when neutralizing anti-TNF-alpha was added to conditioned medium from Cr-exposed BJ donor cells, there was a 1.5-fold decrease in the percentage of BJ recipient cells containing four or more DNA double strand break foci per cell compared to that caused by conditioned medium from Cr-exposed BJ donor cells lacking anti-TNF-alpha. When neutralizing anti-TNF-alpha was added to conditioned medium from Cr exposed hTERT donor cells, the resulting percentage of BJ recipient cells containing four or more γ-H2AX foci per cell decreased more than two-fold compared to the DNA double strand breaks induced by Cr-conditioned medium from hTERT donor cells that lacked anti-TNF-alpha neutralization. Puzzlingly, the antibody increased DNA damage in controls but significantly decreased DNA damage in BJ recipients caused by conditioned medium from both Cr-treated BJ donor cells ($p<0.001$) and Cr-treated hTERT donor cells ($p<0.05$).

Figure 6.10b shows the percentage of hTERT recipient cells containing four or more γ-H2AX foci per cell following exposure to conditioned medium from BJ or hTERT donor cells treated with either Cr (VI) or PBS (control) in the presence of neutralizing anti-TNF-alpha. Figure 6.10b illustrates that the presence of neutralizing anti-TNF-alpha in conditioned medium caused a significant ($p<0.001$) increase in induction of DNA double strand breaks in hTERT recipient cells that were treated with control medium from either cell type compared to conditioned medium from PBS-treated donor cells lacking anti-TNF-alpha. Therefore removing TNF-alpha functionality from control conditioned medium actually caused an increase in DNA

damage in hTERT recipient cells to over 29% from BJ control donors and over 38% for hTERT control donors. These values are lower than those observed for chromium-conditioned medium but are still over 1.9-fold higher than in controls where no neutralizing anti-TNF-alpha was present. However, when neutralizing anti-TNF-alpha was added to conditioned medium from Cr-exposed BJ donor cells, there was a 2.4-fold decrease in the percentage of hTERT recipient cells containing four or more DNA double strand break foci per cell compared to that caused by conditioned medium from Cr-exposed BJ donor cells lacking anti-TNF-alpha. As expected based on the results of previous experiments, conditioned medium from chromium-treated hTERT donor cells did not induce DNA double strand breaks in hTERT recipient cells, the percentage of which containing four or more foci per cell was low, at under 14%. When neutralizing anti-TNF-alpha was added to conditioned medium from Cr exposed hTERT donor cells, the resulting percentage of hTERT recipient cells containing four or more γ-H2AX foci per cell increased two fold compared to the DNA double strand breaks induced by Cr-conditioned medium from hTERT donor cells that lacked anti-TNF-alpha neutralization. Again, as for BJ recipient cells, hTERT recipient cells underwent more DNA damage when neutralizing anti-TNF-alpha was added to the conditioned medium of PBS-treated control donor cells than when control conditioned medium was lacking neutralizing anti-TNF-alpha. When chromium conditioned medium was transferred from hTERT donors to hTERT recipients, there was a significant ($p<0.001$) increase in γ-H2AX foci in recipient hTERT cells when neutralizing anti-TNF-alpha was present.

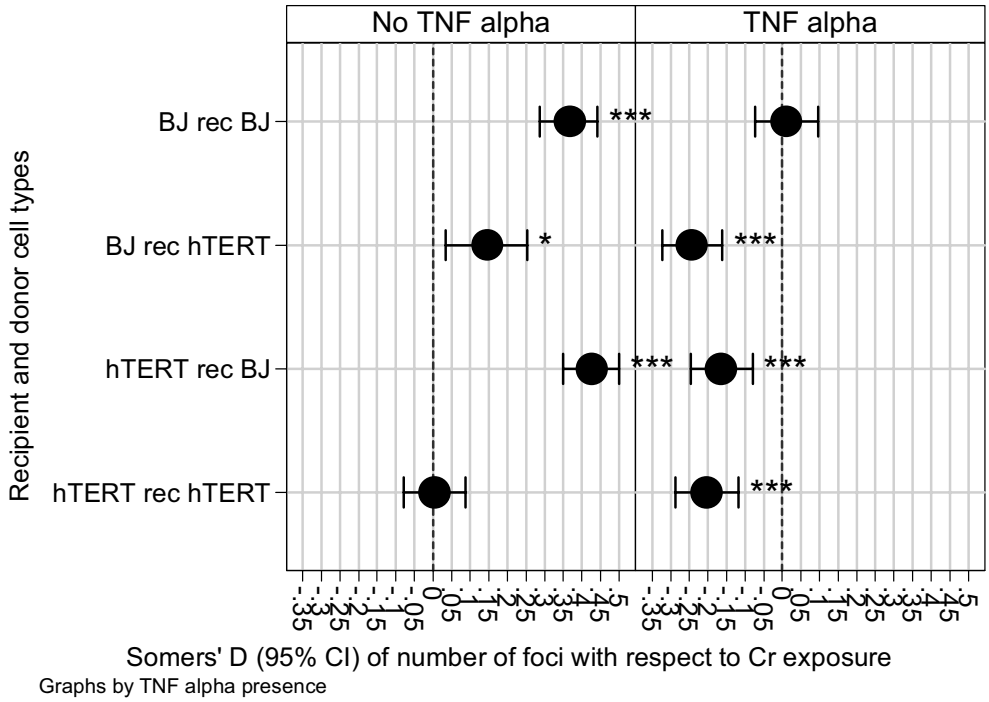

Figure 6.11 Somers' D estimates of numbers of foci with respect to Cr exposure

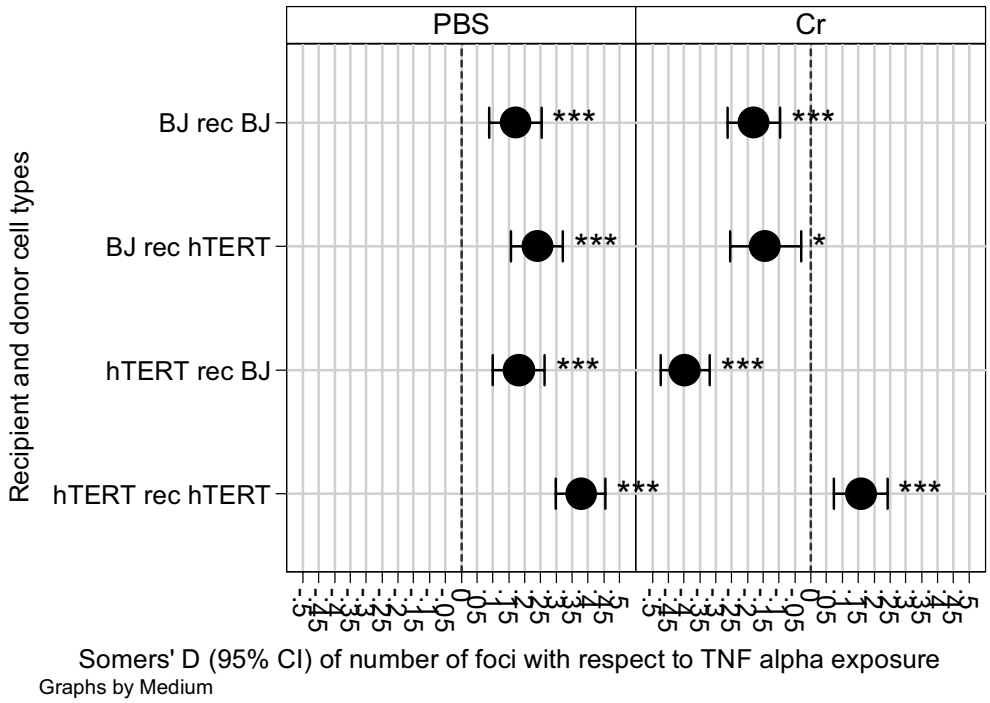

Figure 6.12 Somers' D estimates of numbers of foci with respect to anti-TNF-alpha exposure

6.3.3 TNF-alpha Neutralization: Summary

The statistical analysis shown in Figure 6.11 shows Somers' D estimates of numbers of foci with respect to Cr exposure and illustrates that these results are consistent with the results of previous experiments (chapter 4 (4.2), (4.5)) as the numbers of foci observed in BJ recipient cells were significantly higher than controls for Cr conditioned medium from either donor cell type and numbers of foci in hTERT recipient cells were only significantly higher when cells were treated with Cr-conditioned medium from BJ donor cells and not hTERT donor cells. The statistical analysis shown in Figure 6.12 shows Somers' D estimates of numbers of foci with respect to anti-TNF-alpha exposure and illustrates that when the percentage of cells containing γ-H2AX foci was low (BJ rec BJ PBS, BJ rec hTERT PBS, hTERT rec BJ PBS, hTERT rec hTERT PBS and hTERT rec hTERT Cr), addition of anti-TNF-alpha to conditioned medium caused a transformation, which significantly increased the percentage of cells containing γ-H2AX foci. Figure 6.12 also illustrates that when the percentage of cells containing γ-H2AX foci was high (BJ rec BJ Cr, BJ rec hTERT Cr, hTERT rec BJ Cr), addition of anti-TNF-alpha to conditioned medium significantly reduced the percentage of recipient cells containing DNA double strand break foci.

6.4 Overall Summary: Mechanistic Insights

hTERT cells may have more stable mitochondria (with telomerase within them) which might restrict their response and signalling after metal damage (Ahmed et al. 2008). The hTERT cells might still be able to secrete non mitochondrially mediated bystander signals for example cytokines such as TNF alpha, which induces chromosome abnormalities independent of ROS through IKK,KNK p38 and Caspase pathways. In summary, neither anisomycin nor SB203580 had any consistent effect (Figure 6.6, Figure 6.8). Vitamin C abolished the increase of γ-H2AX foci in hTERT

and BJ cells after direct exposure to Cr and the increase in BJ cells after receiving conditioned medium from Cr exposed BJ cells (Figure 6.3). However, it did not alter γ-H2AX foci in hTERT cells after being exposed to conditioned medium from Cr exposed BJ cells. In contrast, the antibody to TNF alpha had unexpected actions. It increased the number of γ-H2AX foci in either hTERT or BJ cells after receiving conditioned medium from PBS exposed cells (perhaps due to toxicity) and in hTERT cells exposed to conditioned medium from Cr (VI)-exposed hTERT cells (in which there was no effective bystander signalling). However, it reduced the number of γ-H2AX foci in BJ cells after receiving conditioned medium from either cell type and in hTERT cells receiving conditioned medium from BJ cells (Figure 6.10). These data are suggestive of differences in oxidative and non oxidative signalling from hTERT and BJ cells.

CHAPTER 7 : k1 THYROID CARCINOMA CELL LINE

7.1 Background

Chapter 4 outlined the nature of the bystander response of hTERT cells, which was fundamentally different to that of BJ cells. Although hTERT cells were susceptible to acquiring micronuclei and DNA double strand breaks when exposed to conditioned medium from Cr-exposed BJ cells, they did not respond to any bystander signal produced by their own cell type, as there was no significant induction of γ-H2AX foci, micronuclei or NPBs when hTERT cells received conditioned medium from Cr-exposed hTERT cells. However, hTERT conditioned medium was able to induce micronuclei and DNA double strand breaks in BJ cells (Chapter 4.4). In order to test whether this difference in signalling between the two cell lines might be physiologically relevant, analogous experiments were performed using cells which are naturally telomerase positive, thyroid carcinoma cells, with and without a dominant negative down regulation of telomerase. To explore whether this difference could be attributed to the telomerase activity of hTERT cells, a dominant negative hTERT was expressed in the immortalized thyroid carcinoma cell line to abolish telomerase activity. These cells could also be compared to the hTERT cells used in this project to abolish the confounding effects of using a retrovirally created hTERT positive cell line.

The K1 papillary thyroid cancer cell line is telomerase positive and immortal (Jones et al. 1998). A retrovirus expressing a dominant negative (DN) version of the catalytic subunit of telomerase was created by Hahn et al (Hahn et al. 1999) using pBABE DNhTERT puro. K1 cells were infected with either DNhTERT puro or pBABE puro (control) retroviruses. This DN expression in K1 cells was found to be sufficient to

abolish telomerase activity (Preto et al. 2004). The K1 thyroid cancer cell line was kindly provided by Duncan Baird (Cardiff University).

K1 cells either infected with control retrovirus or with DN retrovirus were treated with 0.4 μM potassium dichromate or PBS (control) for 24 hours. After this, cells were washed 5 times with PBS to remove all trace of residual chromium ions. Five days post exposure to Cr or control, fresh medium was added to K1 cells for one hour. This 'conditioned medium' was filtered to remove any directly metal-exposed cells and transferred to BJ or hTERT cells for 24 hours incubation at 37°C. After medium transfer, recipient BJ and hTERT cells were seeded onto glass coverslips for the γ-H2AX focus formation assay as described in chapter 2.7. γ-H2AX foci were scored in a minimum of one hundred nuclei from at least three independent experiments and cells were graded as containing 0, 1-3 or four or more foci.

7.2 Results

Conditioned medium from the K1 thyroid carcinoma cell line donors was transferred to BJ and hTERT recipient cells. Figure 7.1a shows γ-H2AX foci in BJ recipient cells and illustrates that a significant ($p<0.01$) induction of γ-H2AX foci occurred in BJ recipient cells exposed to conditioned medium from the Cr-exposed pBabe control K1 cells. This means that telomerase positive K1 cells were capable of inducing a bystander response in BJ cells. This supports earlier work carried out (Chapter 4.4) in which conditioned medium was transferred between the two fibroblast cell types i.e. from BJ to hTERT and hTERT to BJ. These experiments revealed that hTERT conditioned medium was capable of inducing micronuclei and DNA double strand breaks in BJ cells. Conditioned medium from the DN K1 cell line was not capable of inducing γ-H2AX foci in BJ recipient cells, which was surprising, as

telomerase negative BJ cells in this study have been less selective about their bystander responses than hTERT positive cells.

Figure 7.1b shows that in hTERT recipient cells, there was no significant difference in γ-H2AX focus formation under any circumstance, whether donor cells were telomerase positive or telomerase negative and whether or not they had been exposed to Cr. This supports previous observations that telomerase positive hTERT cells could not induce damage in hTERT cells (Chapters 4.1 and 4.2); however, BJ cells were able to produce a response in hTERT cells. Therefore, we may have expected the DN K1 cells to cause damage in hTERT cells; however, this was not the case. Somers' D estimates of numbers of foci with respect to Cr treatment are shown in Figure 7.2, which illustrates that the only significant induction of γ-H2AX foci occurred in BJ recipients of conditioned medium from K1 cells expressing pBabe control retrovirus. Somers' D estimates of numbers of foci with respect to hTERT recipient cell type are shown in Figure 7.5, which indicates that no significant changes in induction of DNA double strand breaks occurred in hTERT recipient cells.

Figure 7.3a shows that BJ cells contained significantly more γ-H2AX foci following medium transfer from Cr-exposed telomerase positive K1 pBabe control cells than Cr-exposed K1 DN cells ($p<0.05$). In experiments where medium was transferred between the two different fibroblast cell types, (Figure 4.13, Chapter 4.4) BJ and hTERT donor cells induced a similar level of γ-H2AX foci in BJ recipient cells when medium was transferred day 5 post Cr-exposure. Therefore, we might have expected both cell lines to induce a similar level of γ-H2AX foci in BJ cells; however, the telomerase positive line induced the most DNA double strand breaks. Figure 7.4 shows Somers' D estimates of numbers of foci with respect to donor cell type and illustrates

that conditioned medium transferred from the telomerase positive K1 pBabe control cell line induced significantly more γ-H2AX foci in BJ cells than the DN K1 cell line. In this K1 thyroid carcinoma cell line experiment, the general damage level is much lower than that observed for medium transfer between the cell types in which four or more foci per cell 5 days post Cr-treatment in donor cells occurred in around 40% of recipient cells. This may be because the donor cells were different, or could because recipient cells were plated out later and therefore had not been in culture for as long. In this experiment, recipient cells were seeded into flasks 24 hours before conditioned medium treatment. This factor may be important as γ-H2AX foci are known to increase in number during senescence in culture (Nakamura et al. 2006). The importance of this difference of 5 days in culture can be illustrated by referring to the data on γ-H2AX foci in BJ cells cultured over 30 days which were sampled at day 0, day 5 and day 30 post their initial plating out and assayed for DNA double strand break foci (Chapter 4.2). This data shows that the percentage of control BJ cells containing four or more foci increased from 1% on day 0 to 2.7% on day 5 and then to 4.9% on day 30. However, this small increase in DNA double strand breaks in cells cultured over time cannot account for such a difference in levels of γ-H2AX foci between this K1 experiment and the medium transfer experiment detailed in chapter 4.4

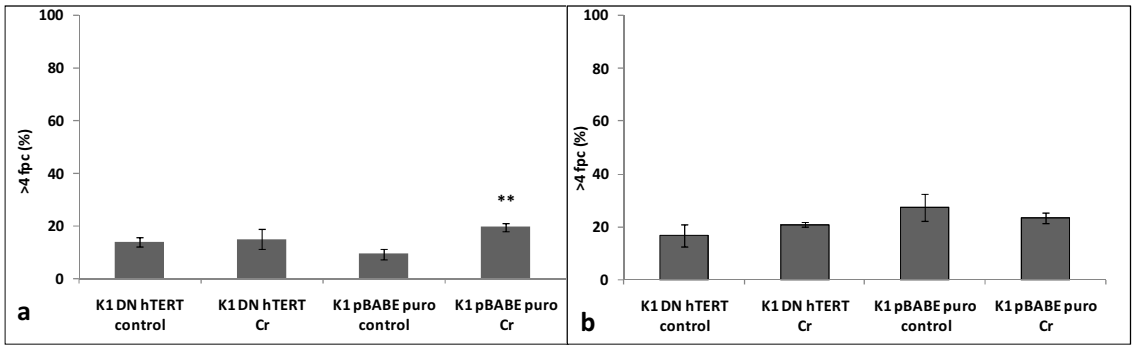

Figure 7.1 γ-H2AX foci in BJ (a) and hTERT (b) recipient cells following exposure to conditioned medium from a Cr- or control treated- K1 thyroid carcinoma cell line expressing a dominant negative hTERT (K1 DN hTERT) or immortalized thyroid carcinoma cell line (K1 pBabe puro)

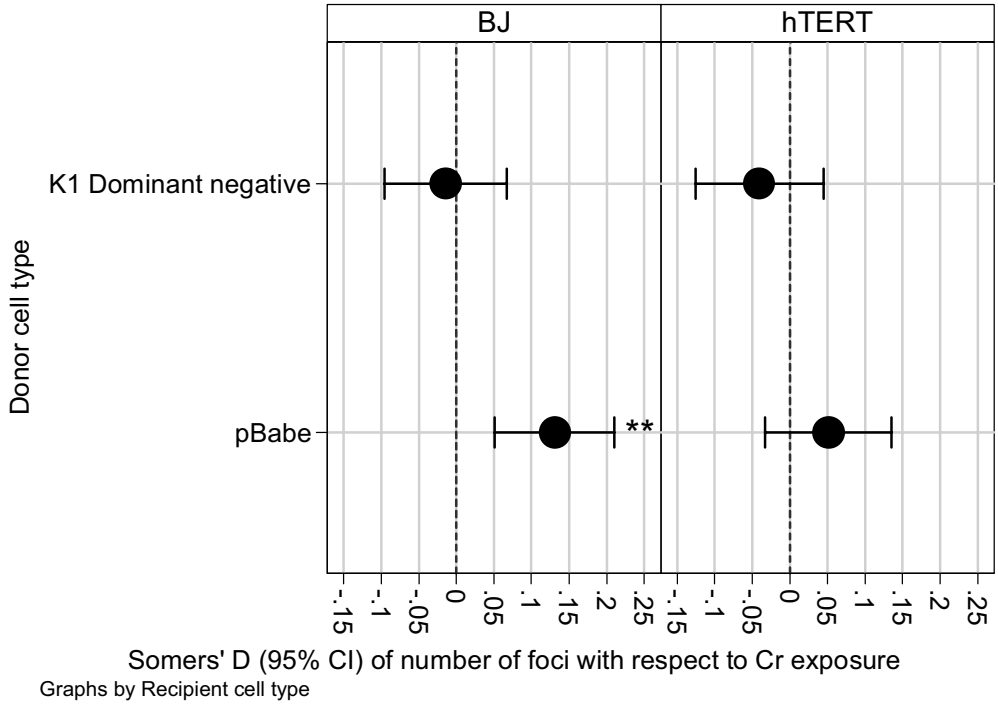

Figure 7.2 Somers' D estimates of number of foci with respect to Cr exposure

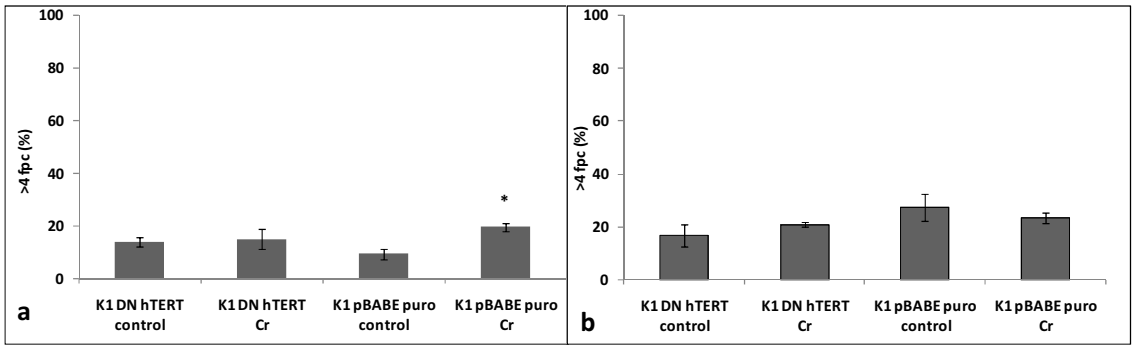

Figure 7.3 γ-H2AX foci in BJ (a) and hTERT (b) recipient cells following exposure to conditioned medium from a Cr- or control treated- K1 thyroid carcinoma cell line expressing a dominant negative hTERT (K1 DN hTERT) or immortalized thyroid carcinoma cell line (K1 pBabe puro)

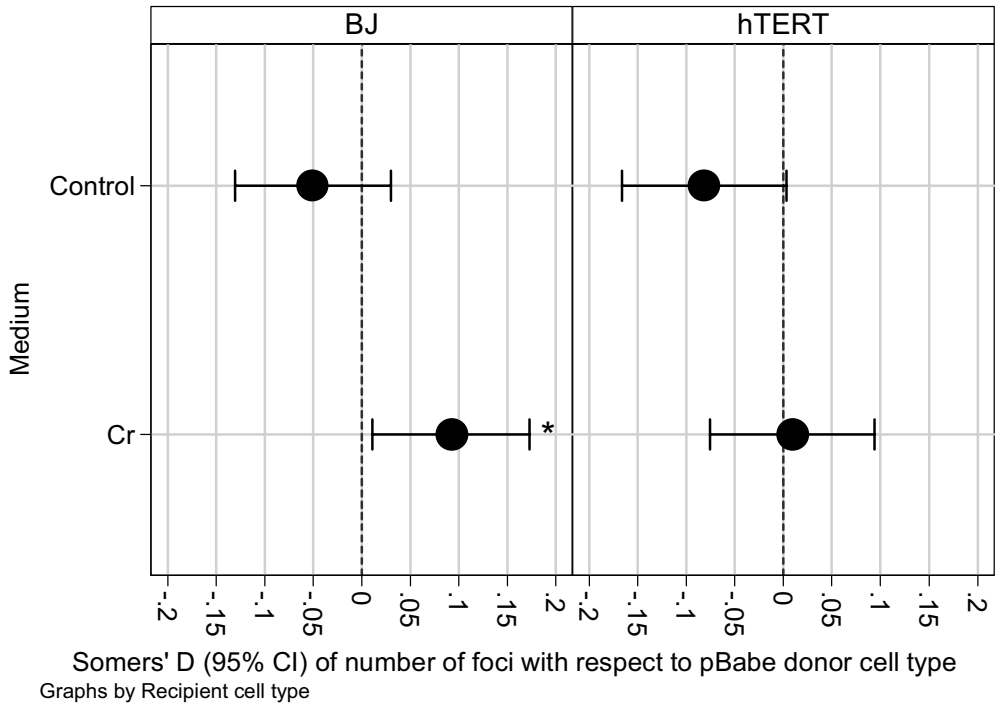

Figure 7.4 Somers' D estimates of number of foci with respect to donor cell type

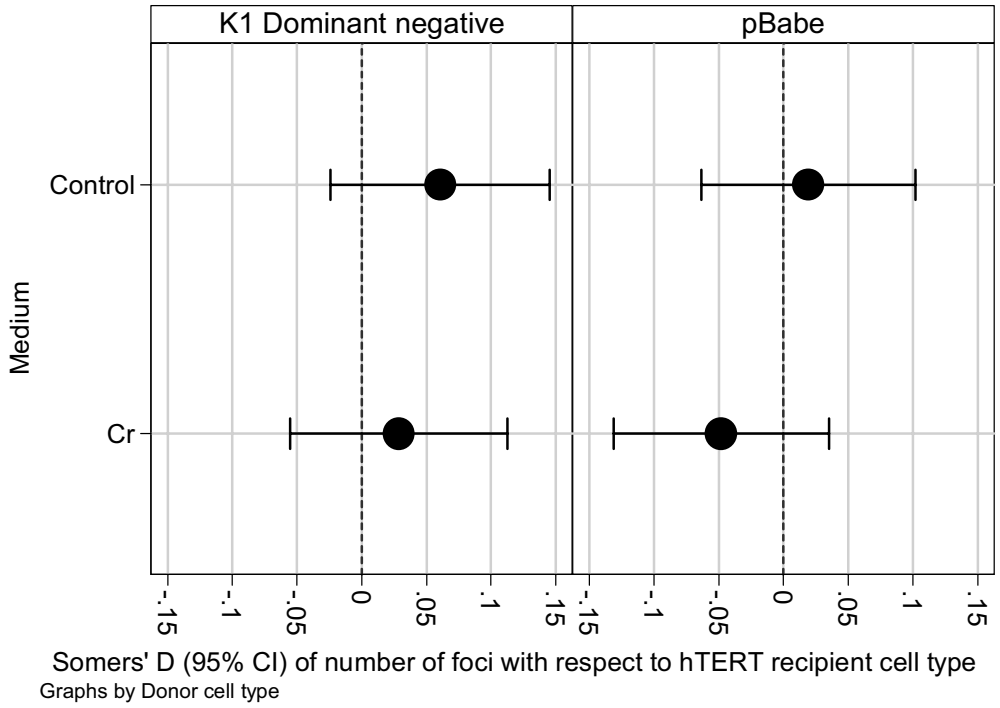

Figure 7.5 Somers' D estimates of number of foci with respect to recipient cell type

Single telomere length analysis (STELA) was carried out on K1 Thyroid cells, the gels for which are shown in Figure 7.6 with means and standard deviations below. Analysis of the XpYp telomere and the 17p telomere confirmed that the dominant negative–expressing K1 cells had considerably shorter telomeres than the K1 cells expressing pBabe puro control retrovirus. The addition of chromium did not influence telomere length in either cell type.

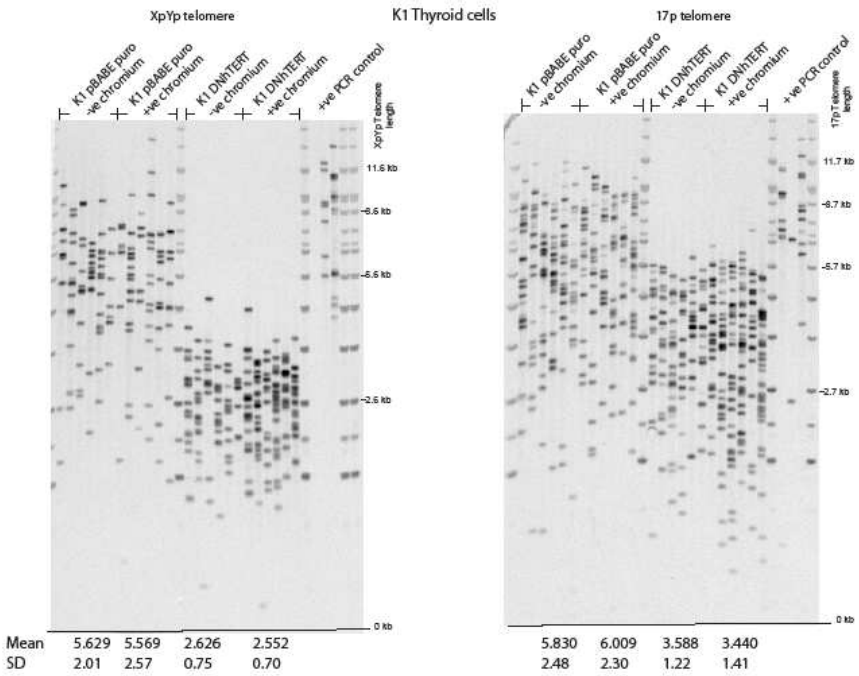

Figure 7.6 STELA analysis of K1 cell line

7.3 K1 Thyroid Carcinoma Cell line: Summary

More γ-H2AX foci were induced in BJ recipient cells when exposed to conditioned medium from Cr exposed pBabe K1 cells than K1 dominant negative cells. Therefore, the telomerase positive immortalized thyroid cell line was able to produce a bystander factor 5 days post exposure to 0.4 μ M potassium dichromate. No significant induction of DNA double strand breaks occurred in hTERT recipient cells even when the origin of the conditioned medium was the telomerase negative DN K1 cell line. The statistical analysis in Figure 7.5 shows that there was no significant induction of γ-H2AX foci in hTERT recipient cells. In summary, the medium from Cr-exposed KI thyroid carcinoma cells (empty vector-pBabe control) induced γ-H2AX foci in BJ but not hTERT fibroblasts. However there was no corresponding induction by the medium of the KI thyroid carcinoma cells whose telomerase was suppressed by over expression of

dominant negative hTERT. The dominant negative hTERT is mutated in the reverse transcriptase motif (Hahn et al. 1999) and not the N-terminal leader sequence, which aids mitochondrial entry (Santos et al. 2006). These dominant negative K1 cells have short telomeres (Figure 7.6) with extremely high telomerase expression, which could potentially play a role in the non-telomere extending functions of telomerase but are specifically ineffective in maintaining telomere length. The failure of bystander signalling in this experiment between two cells with high telomerase and long telomeres (i.e hTERT fibroblasts) suggests again that a critical factor in bystander signalling is not telomere length but the non telomeric actions of telomerase for example, on mitochondrial protection. Cr had no effect on the telomere lengths of these cells (Figure 7.6).

CHAPTER 8 RESULTS: HUMAN EMBRYONIC STEM CELLS

8.1 Background

Previous chapters have found that hTERT fibroblast cells appear to display a reduced sensitivity to the chromium-induced bystander effect compared to normal BJ human fibroblasts. In order to determine whether this trait could be attributed to the increased telomerase activity of hTERT cells compared to BJ cells, a bystander experiment was designed, which utilised cells that naturally express telomerase. In addition to the medium transfer experiments carried out using a telomerase positive and dominant negative thyroid carcinoma cell line (chapter 7), further work was carried out using human embryonic stem (hES) cells. The naturally occurring 'physiological' telomerase in these hES cells would eliminate any confounding effects resulting from the use of a viral transfection system, as was the case for hTERT fibroblasts.

Human embryonic stem cells were first derived from the pluiripotent inner cell mass (ICM) cells of the pre-implantation, blastocyst stage embryo in 1998 by Thomson et al (Thomson et al. 1998). One of the classical markers of hES cells is the POU-domain transcription factor Oct4, which plays a crucial role in maintaining self-renewal and pluripotency of ES cells in a concentration-dependent manner (Zhang et al. 2007). Differentiation of pluripotent cells is associated with downregulation of Oct4 levels and downregulation of the *Oct4* gene in ES cells. These cells are naturally telomerase positive and have the long telomeres associated with potency. Stem cells are routinely cultured on a layer of feeder cells, which provide an extracellular matrix and growth factors required to prevent stem cell differentiation, maintaining their pluripotent status. This system is ideal for studying interactions between cells in close contact as the different cell types are cultured alongside each other and are therefore vulnerable to any

factors secreted by the other cell type. The first set of experiments carried out used mitomycin-C inactivated mouse embryonic fibroblasts (mEFs) as feeder cells. The experimental design involved a combination of different treatments.

1) ES cells Cr exposed, mEFs not exposed
2) ES cells not exposed, mEFs Cr exposed
3) ES cells Cr-exposed, mEFs Cr-exposed
4) ES cells not exposed, mEFs not exposed.

For metal exposure, ES cells or mEFs were treated with 0.4 μM potassium dichromate for 24 hours, after which, they were washed 5 times, which removes all trace of residual chromium ions (Table 4.1, section 4.5). Five days post exposure to Cr or no treatment (control) stem cells were seeded onto mEF feeder cells that had been exposed to chromium or no treatment (control) and they were co-cultured for 24 hours before being fixed in 4% paraformaldehyde and then stained for the γ-H2AX focus formation assay (as described in section 2.7). The bystander experiment in this case was co-culturing of mEFs with Cr-treated ES cells and co-culturing of ES cells with Cr-treated mEFs. In one case both the mEFs and the ES cells had been Cr-exposed and as a control, unexposed ES cells were co-cultured with unexposed mEFs. This experimental design is more able to closely mimic the experiments of radiation biologists that use microbeam irradiation of small numbers of cells in a single population and it removes the need to carry out medium transfer. Another interesting factor emerging from this experiment is the observation of cell-cell interactions between two telomerase positive cell lines belonging to two different species.

It was easy to determine which cell type was which and therefore know which cells were Cr exposed, using morphological features to distinguish between ES cells and

mEFs. ES cells have a more uniform rounded shape than fibroblasts, which have a more elongated, stringy morphology. In addition, the ES cells grow in colonies where the cells are compact and close together, whereas, fibroblasts appear more far apart from one another. Therefore, even though the cells are co-cultured, there is no risk of mistaking a directly-metal-exposed cell for a bystander cell, which increases the validity of the results.

Chapter four outlines the nature of the bystander response presented by telomerase positive hTERT fibroblasts. It was evident from the results of the micronucleus assay and the γ-H2AX focus formation assay that hTERT cells were more protected against the bystander response than normal BJ fibroblasts, as fewer DNA double strand breaks and chromosomal abnormalities occurred in hTERT cells exposed to conditioned medium from Cr-exposed hTERT donor cells. So it was considered interesting to compare other telomerase positive cell lines with hTERT fibroblasts in terms of their ability to produce or respond to bystander factors. The second set of experiments carried out with hES cells involved using human fibroblast feeder cells in place of the mEFs. Both BJ and hTERT cells were used in separate experiments for co-culturing with hES cells. The human fibroblasts had to be inactivated by mitomycin-C. Although attempts were made to culture the stem cells with 'active' feeder fibroblasts, this accelerated the differentiation of the ES cells and it could no longer be assumed that the cells retained the high level of telomerase activity or the long telomeres associated with stem cell pluripotency. In fact, the ES cells were more prone to differentiation when co-cultured with human fibroblast feeder cells than with mEFs. This could be because the human fibroblasts were at a higher passage number than the mEFs as a low passage number is an essential property of feeder cells for ES cell maintenance. For this reason, it was decided to positively identify ES cells using an antibody to the

transcription factor Oct4. The *Oct4* gene has been suggested to play a role in totipotency(Pesce et al. 1998) and is expressed specifically in pluripotent stem cells but not their differentiated daughters (Solter 2000; Tai et al. 2005). Alongside γ-H2AX staining, an antibody to Oct4 was used and stained with a fluorescein-conjugated secondary antibody to distinguish it from the red signal of the Cy3-conjugated secondary antibody that signals γ-H2AX. Oct4 staining is shown in Figure 8.6. Again, the same combinations of chromium exposures were carried out with human fibroblasts as for mEFs. γ-H2AX foci were scored in a minimum of one hundred cells obtained from at least three independent experiments for each of the three types of feeder cells used; mEFs, BJ and hTERT.

Stem cell culture was kindly carried out by Lucy Crompton (University of Bristol) with the support of Maeve Caldwell (University of Bristol).

8.2.1 hES cell Results: mEF Feeders

Figure 8.1a shows the percentage of cells containing more than four foci per cell in mEFs with respect to hESC Cr exposure and illustrates that there was a significant induction of γ-H2AX foci in untreated mEFs that had been co-cultured with Cr exposed stem cells with a p value of <0.001(mEFs (hESC Cr)). There was no significant difference in numbers of foci in mEFs that were directly Cr treated compared to directly treated mEFs when companion hES cells were also treated (Figure 8.1a). Figure 8.1b shows that unexposed hES cells received a bystander signal from Cr exposed mEFs with a p value of <0.001. Directly Cr-exposed stem cells contained a higher number of γ-H2AX foci than directly exposed stem cells co-cultured with Cr-treated companion mEFs with a p value of <0.001. This is also illustrated in Figure 8.3b, which shows percentages of mEFs and hES cells containing no DNA double strand breaks where

there were fewer damaged ES cells when Cr treatment occurred in both ES cells and feeder cells than just from direct treatment.

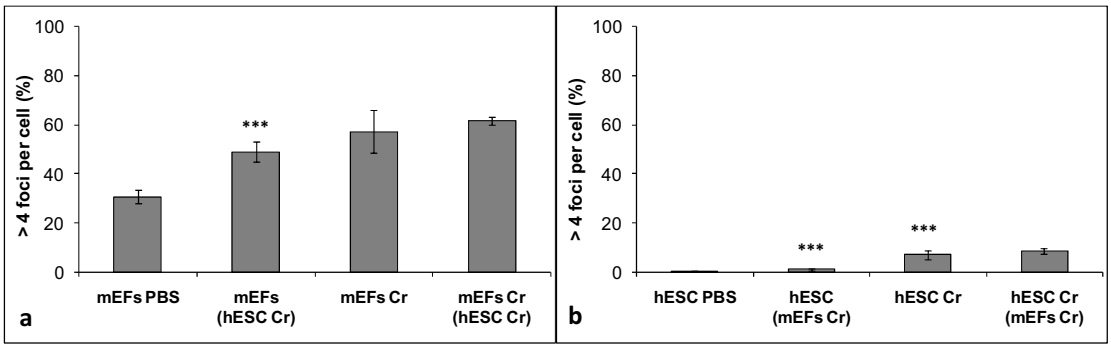

Figure 8.1 γ-H2AX foci 5 days post Cr exposure in a combination of cell types prior to cell mixing in (a) mEFs with respect to hES cell Cr exposure and (b) in hES cells with respect to mEF Cr exposure

Direct Cr exposure in mEFs caused a significant induction of γ-H2AX foci, an approximate two-fold increase compared to control (where neither mEFs nor ES cells were Cr-exposed), with a *p* value of <0.001 (Figure 8.2a). Figure 8.2a shows that mEFs exposed to both direct Cr treatment and a bystander signal from hES cells (mEFs Cr (hESc Cr)) contained more γ-H2AX foci than when only exposed to the hESC bystander signal (mEFs (hESc Cr)) with a *p* value of <0.01.

Direct Cr treatment in hES cells induced a significant increase in γ-H2AX foci compared to control (where neither ES cells nor their companion mEFs were Cr-exposed) with a *p* value of <0.001 (Figure 8.2b). Figure 8.2b shows that there was no significant difference in numbers of γ-H2AX foci in hES cells that were co-cultured with Cr-exposed mEFs (hESc (mEFs Cr)) compared to numbers of foci in hESCs that were directly treated and were also co-cultured with Cr-exposed mEFs (hESc Cr(mEFs Cr)).

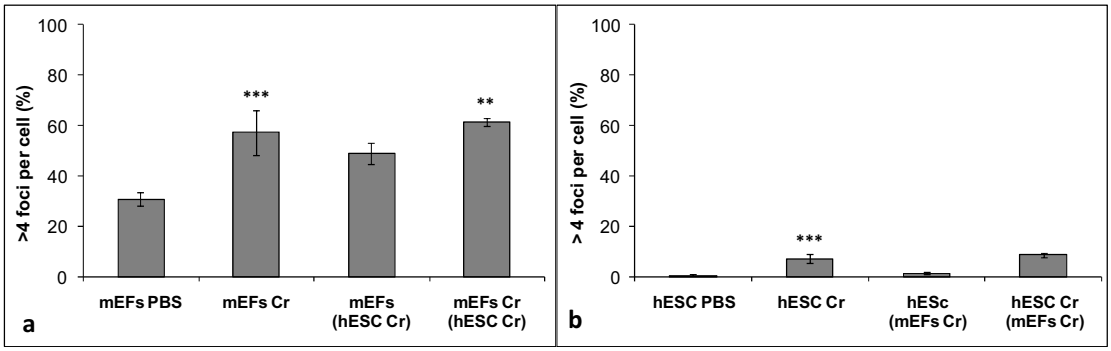

Figure 8.2 γ-H2AX foci 5 days post Cr exposure in a combination of cell types prior to cell mixing in (a) mEFs with respect to mEF Cr exposure and (b) hES cells with respect to hES cell Cr exposure

Figure 8.3 illustrates the difference in numbers of cells containing no γ-H2AX foci and therefore no DNA double strand breaks. Figure 8.3 shows that stem cells contained fewer γ-H2AX foci when both stem cells and their companion feeder mEFs were Cr-exposed than when just the stem cells were Cr-exposed. Whereas this comparison in MEF feeder cells indicates that there was no significant difference in numbers of foci between cells in which just mEFs were Cr-exposed and when mEFs and their companion hES cells were Cr-exposed.

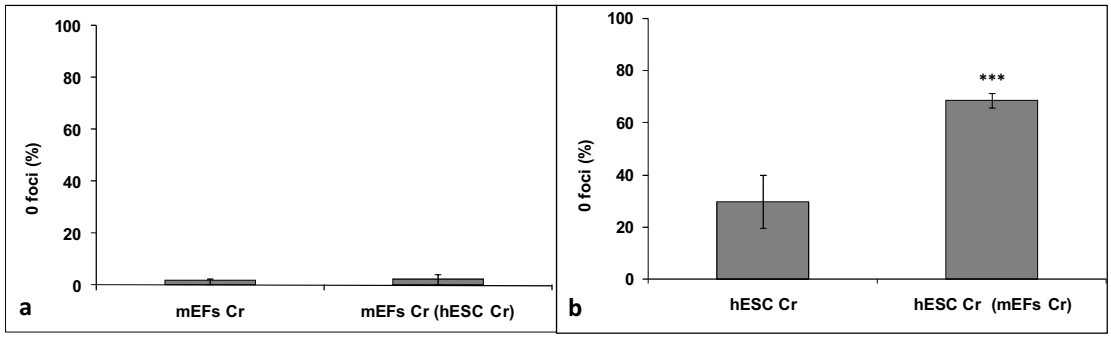

Figure 8.3 Cells containing no γ-H2AX foci 5 days post Cr exposure in combinations of cell types prior to cell mixing in (a) mEFs with respect to hES cell Cr exposure and (b) hES cells with respect to MEF Cr exposure

Figure 8.3 shows that stem cells contained fewer γ-H2AX foci when both cell types were Cr-exposed than when just hES cells were Cr-exposed.

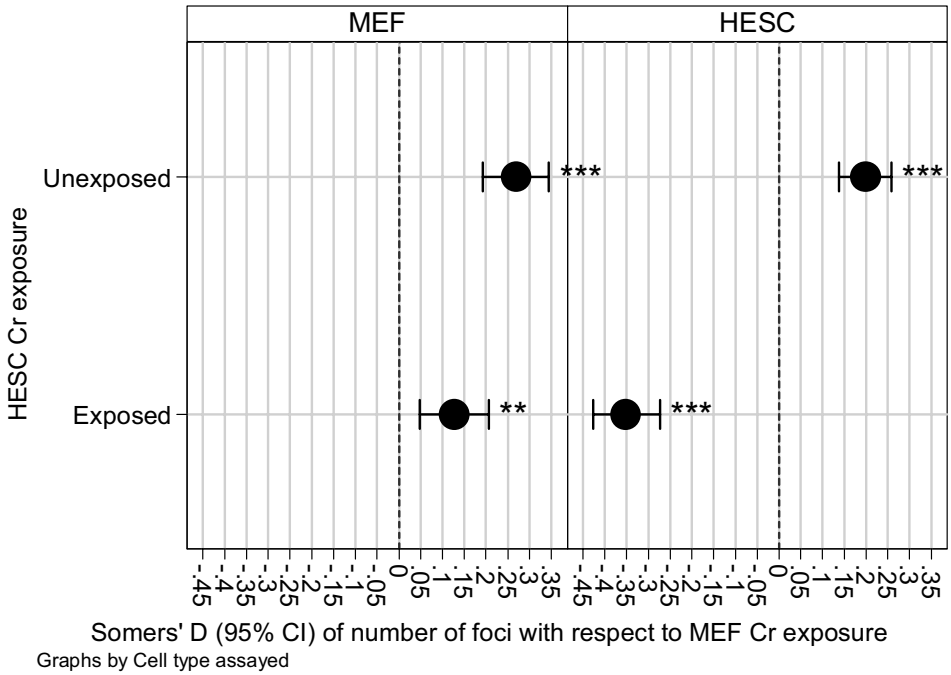

Figure 8.4 Somers' D estimates of number of foci with respect to mEF Cr exposure

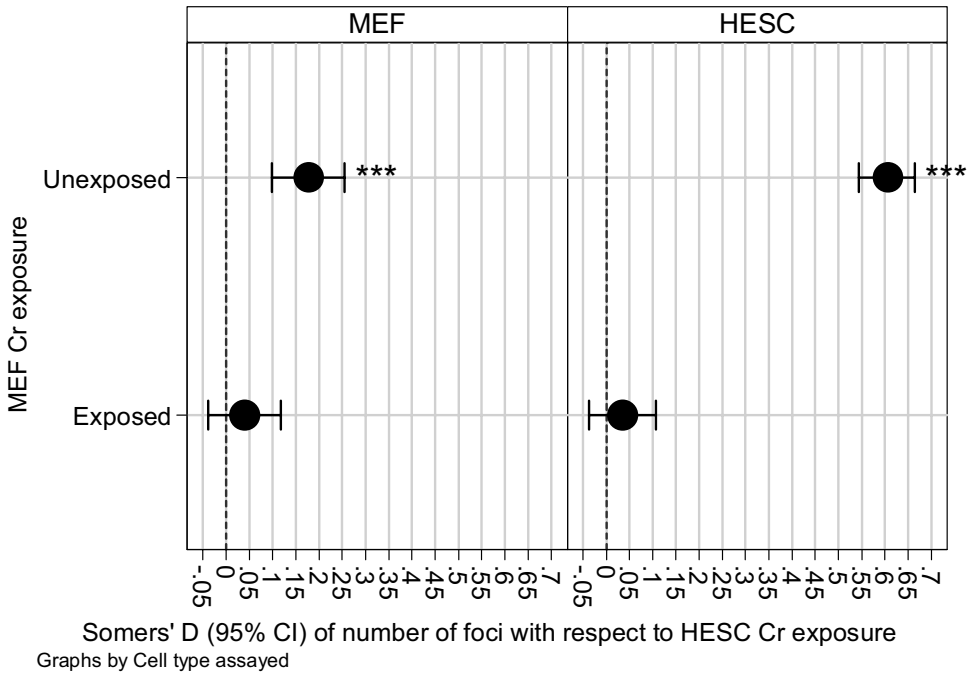

Figure 8.5 Somers' D estimates of number of foci with respect to hES cell Cr exposure

8.2.2 hES cell Summary

Both hES cells and mEFs were sensitive to direct Cr treatment to cause a significant increase in γ-H2AX focus formation (Figure 8.2). Both cell types were also capable of sending and receiving bystander signals as when only one cell type was Cr-exposed, the other cell also developed γ-H2AX foci (Figure 8.1a). For both cell types, direct treatment when supplemented with bystander treatment does not induce more foci than just direct treatment on its own without bystander treatment (Figure 8.1).

8.3.1 hES cell Results: Human Feeders

Human feeder cells were more sensitive to mitomycin C-inactivation than mEFs. Figure 8.7b and Figure 8.9a show that mitomycin C inactivation alone, without Cr exposure caused a significant ($p \leq 0.001$) increase in the percentage of BJ cells containing 4+ γ-H2AX foci of around 30% (based on the observation of 624 nuclei from six independent experiments). Figure 8.8b and Figure 8.9b show that mitomycin C inactivation without Cr treatment caused a significant ($p \leq 0.001$) increase in the percentage of hTERT cells containing 4+ γ-H2AX of around 25%. Mitomycin-C-induced DNA damage was not so prominent in the mouse feeders, in which the background level of DNA double strand breaks was around 30%.

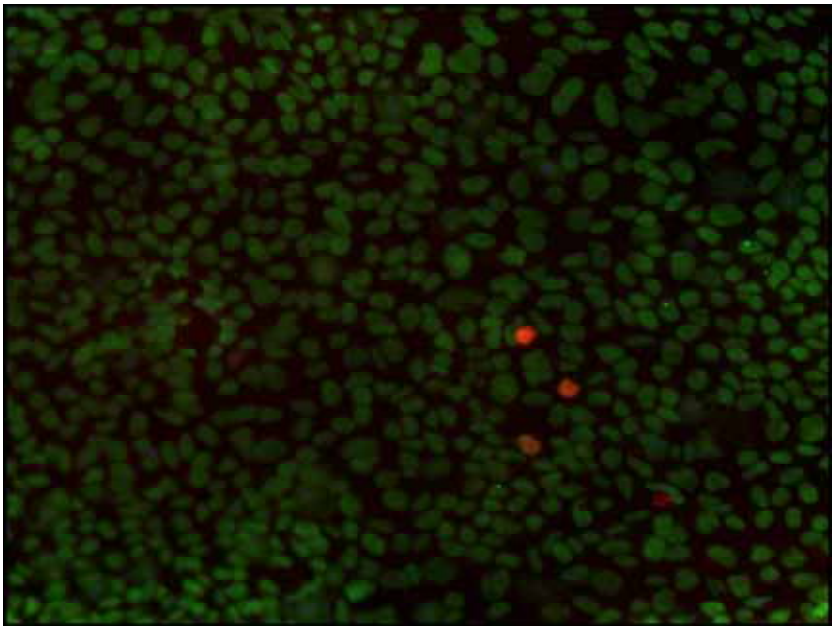

Figure 8.6 Oct4 staining of embryonic stem cells at 10x magnification.

The addition of direct Cr treatment to mitomycin-C inactivation induced significantly high ($p \leq 0.001$) levels of γ-H2AX foci in 55% of BJ cells (shown in Figure 8.7b). Figure 8.8b shows that 4+ γ-H2AX foci were induced in around 42% of hTERT cells when mitomycin-C inactivation was combined with direct exposure to potassium dichromate ($p \leq 0.001$). The DNA double strand break level in hTERT cells was therefore slightly lower than in BJ cells.

Figure 8.10 shows that there was no significant induction of bystander signalling to induce γ-H2AX foci in ES cells after co-culture with Cr-exposed BJ or hTERT fibroblasts.

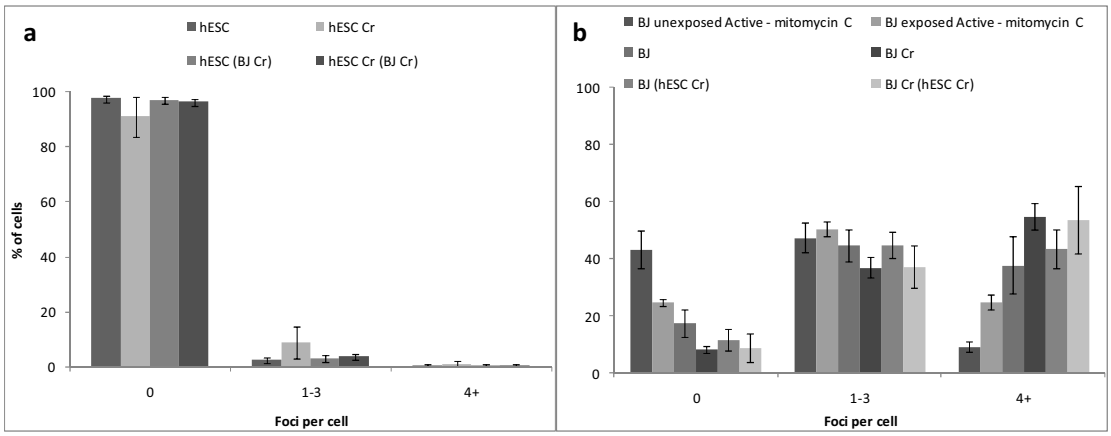

Figure 8.7 γ-H2AX foci in hES cells (a) and BJ cells (b) following 24 hours co-culture on day 5 post Cr treatment in donor cells.

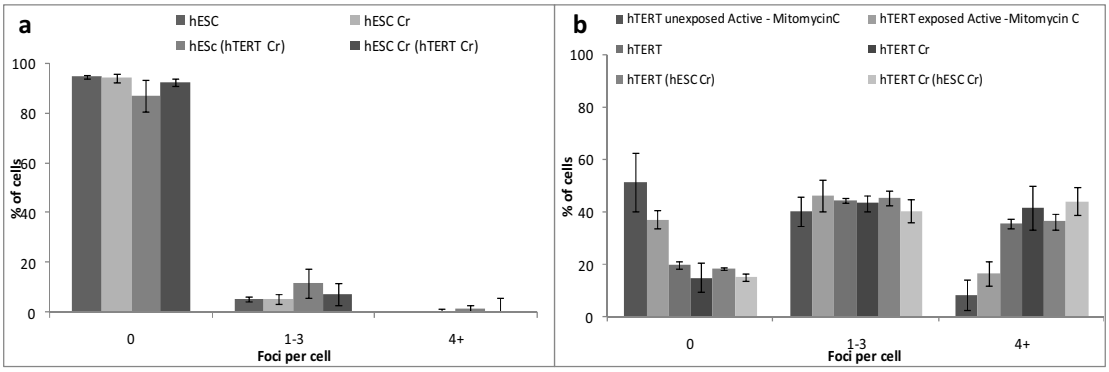

Figure 8.8 γ-H2AX foci in hES cells (a) and hTERT cells (b) following 24 hours co-culture on day 5 post Cr treatment in donor cells

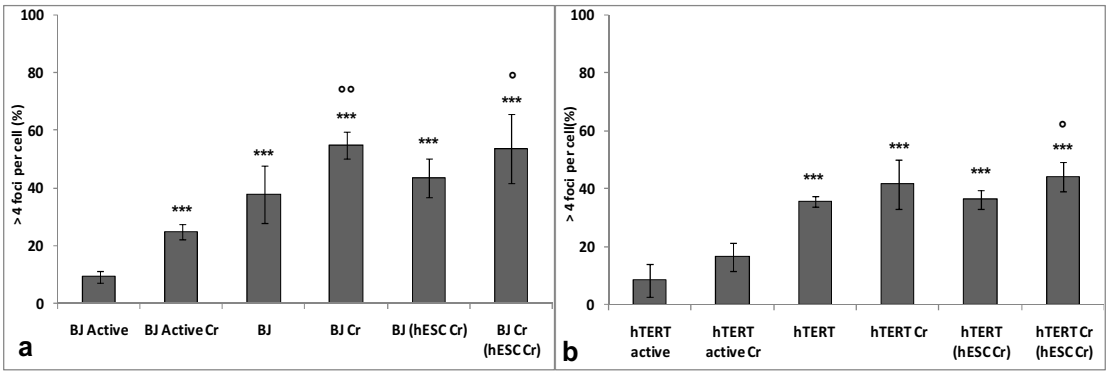

Figure 8.9 BJ (a) and hTERT (b) cells containing 4+ γ-H2AX foci following 24 hours co-culture with hES cells 5 days post Cr treatment. Symbols: * refers to t-test *p* values comparing unexposed

active (not mitomycin-C inactivated) cells to other conditions. ° refers to t-test *p* values comparing inactive unexposed cells to inactive exposed cells

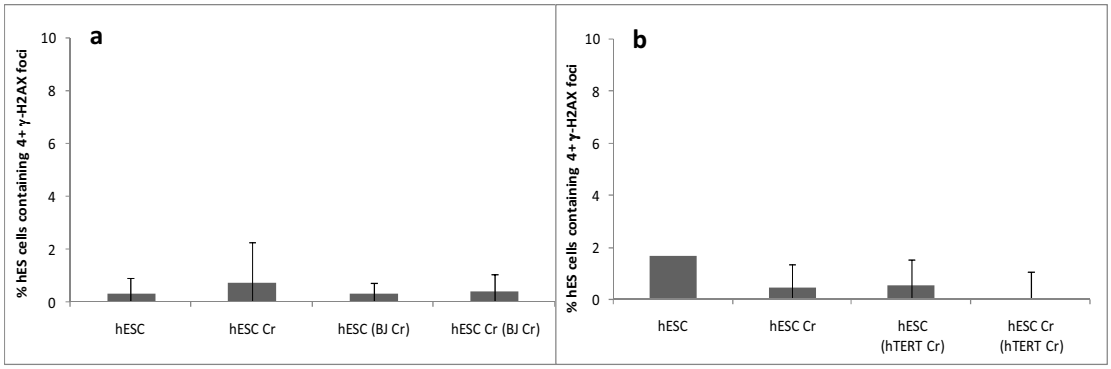

Figure 8.10 hES cells containing 4+ γ-H2AX foci following 24 hours co-culture with BJ (a) or hTERT (b) feeder cells 5 days post Cr treatment

It was thought that the ES cells may be more likely to differentiate when they were co-cultured with human feeders than with mEFs. This is why it was decided to use the stem cell marker Oct4 to discriminate between pluripotent embryonic stem cells and differentiated embryoid bodies in which the Oct4 signal was not present. Another factor of interest was whether Cr-exposure in feeder cells would influence differentiation or affect γ-H2AX levels in differentiated cells. In order to elucidate this, unexposed differentiated stem cells were scored for their γ-H2AX foci in the presence of either Cr exposed or unexposed BJ or hTERT feeder cells. Only the cells containing 4+ foci were counted as being positive for DNA double strand breaks. Figure 8.11 shows that differentiated cells contained more γ-H2AX foci than non-differentiated ES cells. Another factor of interest was whether BJ or hTERT feeders were more likely to induce stem cell differentiation. Therefore, quantification of differentiation was obtained for untreated ES cells with BJ or hTERT cells that were either Cr-exposed or unexposed. For this study, random samples of stem cells were counted and scored as being either differentiated (no Oct4 staining) or normal (staining positive for Oct4). A minimum of

one hundred cells were scored from at least three independent coverslips. Figure 8.12a shows that over 62% of ES cells were normal i.e. undifferentiated when co-cultured with control BJ feeder cells. Of the 37% that were differentiated, around a quarter of these (just under 9% of the total) contained 4+ γ-H2AX foci. Figure 8.12b shows that there was very little difference in differentiation and γ-H2AX focus formation in differentiated cells between ES cells co-cultured with control BJ cells and those co-cultured with Cr-exposed BJ cells. When ES cells were co-cultured with control hTERT feeder cells (Figure 8.12c), 77% of ES cells were differentiated. This is a lot higher than when BJ cells were feeders. Just over 6% of these differentiated cells contained 4+ γ-H2AX foci, around 5% of the total stem cells. In the presence of Cr-exposed hTERT feeder cells (Figure 8.12d), ES cells were even more likely to differentiate than in the presence of control hTERT cells. 84% of stem cells were differentiated and of these, 17% (15% of the total stem cells) contained 4+ γ-H2AX foci. For hTERT feeder cells, Cr treatment was linked to differentiation in stem cells and moreover these differentiated cells were more likely to contain γ-H2AX foci. However, it cannot be ascertained whether the Cr exposure caused in feeder cells caused the increase in focus formation in differentiated cells or whether differentiation was the causative factor. Figure 8.13 shows an image of differentiation within the ES cell colony, with differentiated cells lacking the green Oct4 signal.

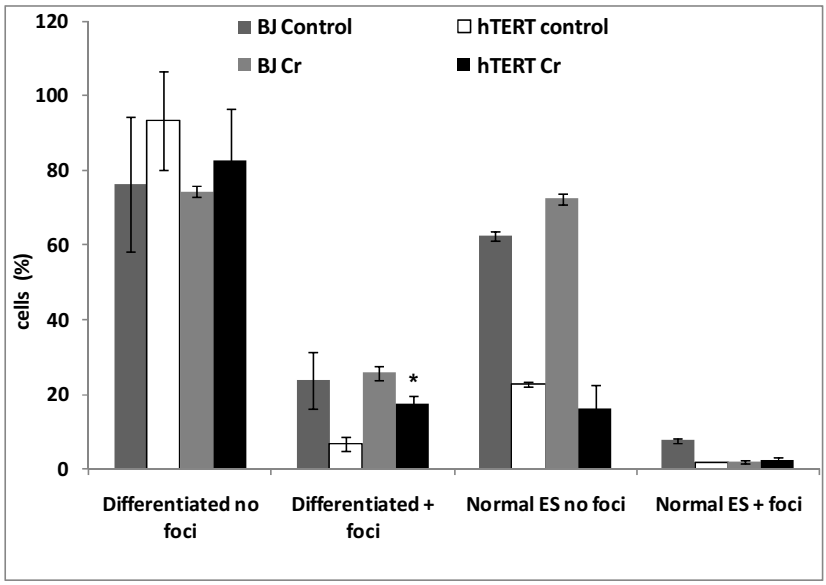

Figure 8.11 Differentiation in untreated hES cells containing either no foci or 4+ γ-H2AX foci following 24 hours co-culture with BJ or hTERT feeders treated with Cr or control

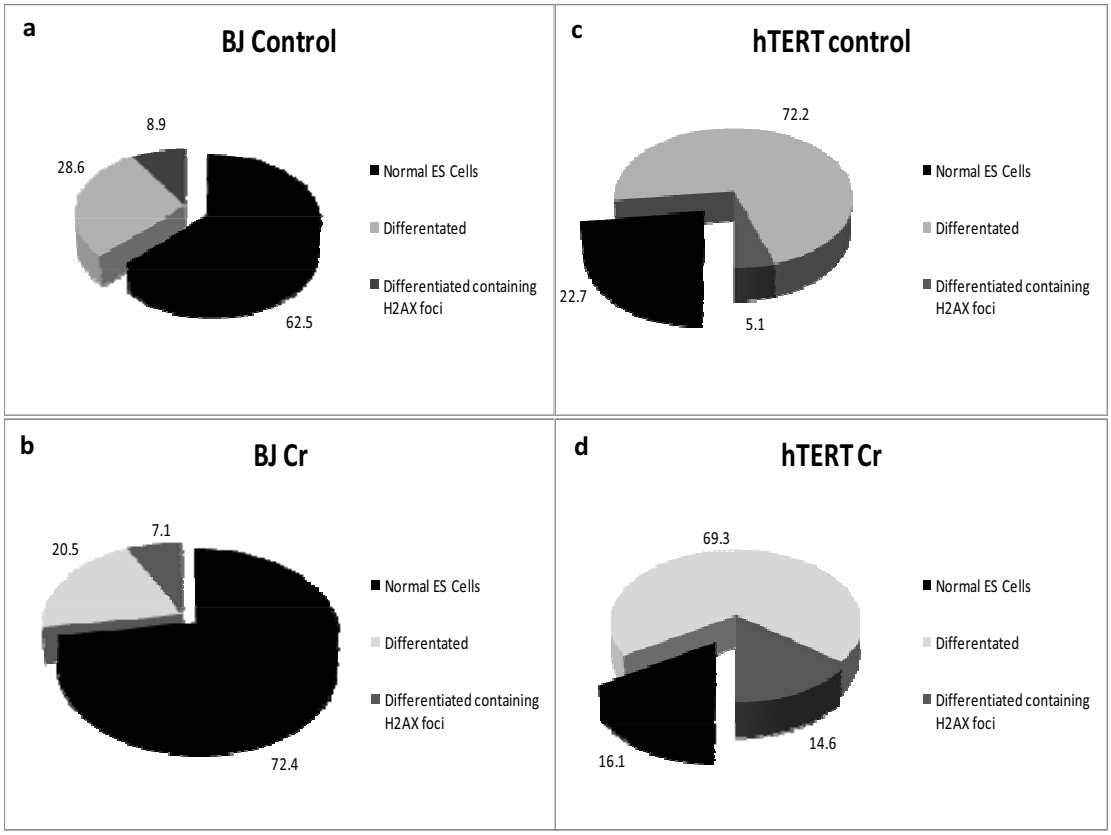

Figure 8.12 Fractions of normal and differentiated hES cells with γ-H2AX foci following 24 hours co-culturing with control BJ cells (a), Cr-treated BJ cells (b) control hTERT cells (c) or Cr-treated hTERT feeder cells (d)

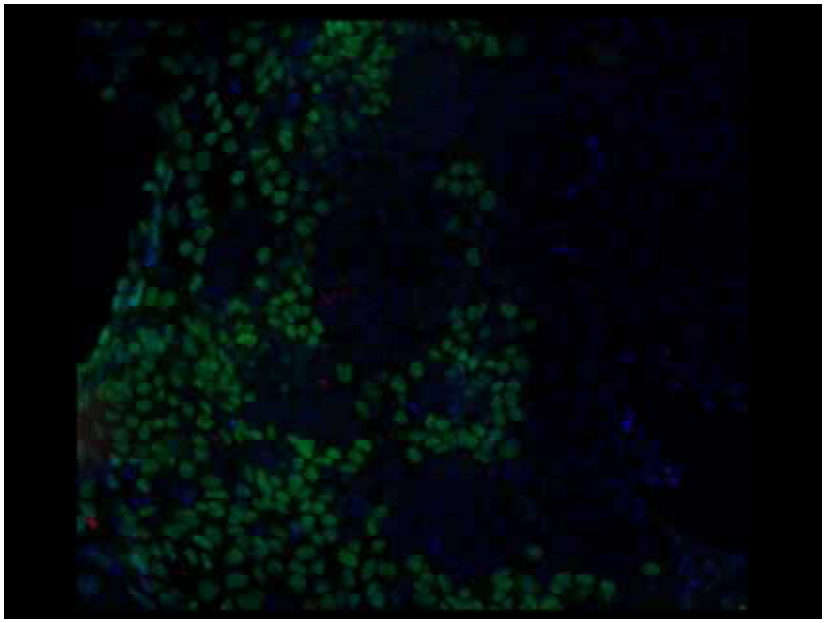

Figure 8.13 Differentiation within an ES cell colony in the presence of hTERT feeder cells at 10x magnification

8.3.2 hES cell Summary: Human Feeders

The hES cells were more likely to differentiate in the presence of human feeder cells than mouse feeder cells. Differentiated cells may no longer be telomerase positive nor have long telomeres and would therefore not provide a useful comparative study for hTERT cells. It was therefore very valuable to carry out Oct4 staining in order to distinguish differentiated from non-differentiated stem cells. There was no significant bystander effect between stem cells and human fibroblast cells to induce γ-H2AX foci in any cell type, unless stem cells had differentiated and were co-cultured with Cr-exposed hTERT cells.

CHAPTER 9 DISCUSSION

9.1 General Genotoxicity Evaluation:

9.1.1 Direct Treatment and Medium Transfer

Chapter 4 outlines the results obtained for induction of micronuclei, nucleoplasmic bridges, γ-H2AX foci and chromosome breaks in BJ and hTERT fibroblasts in response to direct exposure to Cr (VI) or exposure to conditioned medium from a Cr-treated cell. Genotoxicity assays were carried out immediately after treatment on day 0, 5 days or 30 days post direct 24 hour exposure to potassium dichromate. Bystander studies were carried out by transferring conditioned medium from cells treated with potassium dichromate for 24 hours to recipient bystander cells immediately on day 0, 5 days or 30 days post direct metal treatment in donor cells of the same type. For BJ fibroblasts, direct Cr exposure caused a significant increase in MN, NPBs, γ-H2AX foci and chromosome breaks that persisted for at least 30 days, increasing over time. This result is consistent with previous reports of the effects of Cr-induced DNA damage in fibroblasts (Glaviano et al. 2006; Tamblyn et al. 2009). For hTERT cells, Cr-induced DNA damage was not as pronounced as in BJ cells. For example, although MN induction in Cr-exposed hTERT cells was higher than in controls, it was not as high as that observed in BJ cells. Consistent with the observations of Glaviano et al (Glaviano et al. 2006), there was no significant Cr-induced formation of NPBs, a measure of dicentric chromosomes and ring chromosomes (Thomas et al. 2003) in hTERT cells at any time point. For BJ cells, there was a Cr-induced bystander effect propagated via transfer of conditioned medium from directly exposed cells to recipient bystander cells that induced a significant increase in MN, NPBs and γ-H2AX foci over time. However, hTERT cells were resistant to this bystander effect and no bystander DNA damage was

induced in medium transfer recipient hTERT cells at any time point. This may be related to reports that hTERT protects against genomic instability (Glaviano et al. 2006). For BJ cells, both direct Cr treatment and conditioned medium caused a significant induction of γ-H2AX foci typically in at least 10% of cells. This indicates that the micronuclei, which were observed in a smaller percentage of cells, were indeed due to clastogenic chromosome breakage events rather than aneugenic events resulting in chromosome loss. For hTERT cells however, there was no significant induction of DNA double strand breaks immediately post direct metal treatment, but a large peak on day 5 that persisted for at least 30 days post treatment. This could be related to the persistent induction of tetraploidy that occurs in hTERT cells over time following exposure to potassium dichromate (Glaviano et al. 2006), as more chromosomes were present to exhibit breakage foci. Cytogenetic analysis showed that direct Cr treatment in BJ cells caused chromosome breaks that persisted for at least 30 days, consistent with results for MN and DNA double strand break induction. There was a particularly high peak observed on day 30, which could have been due to the stress of cell culture over this long period of time. Cytogenetic analysis of medium transfer recipient cells also indicated that there was a metal-induced bystander effect to cause chromosome breaks in BJ cells at all three time points with a particularly high peak on day 5. Day 5 appears to be a significant time point for transfer of conditioned medium as donor cell chromosome breaks and DNA double strand breaks were observed to peak at this point for both BJ and hTERT cells respectively. This may be due to the expansion of donor cell populations to select for cells that will release more bystander factors or it could be due to failures in the DNA damage response in donor cells resulting in accumulation of damage that facilitates secretion of more bystander factor(s). Cytogenetic analysis in hTERT cells revealed that again there was a direct effect of Cr (VI) to induce

chromosome breaks at day 0 and day 5. However, the results for day 30 were not conclusive, large control values were observed for chromosome breaks, which may again be due to the induction of high levels of damage over time in culture. No bystander effects were observed for cytogenetic analysis in hTERT cells, indicating that hTERT cells are in some way protected against the induction of medium transmissible bystander effects.

Analysis of conditioned medium revealed that any residual chromium levels remaining after washing donor cells were negligible and therefore, the bystander effects observed in medium transfer recipient cells did not occur as a result of any residual chromium present in conditioned medium.

A crucial difference between BJ and hTERT cells is the ectopic expression of TERT in hTERT cells. STELA telomere length analysis revealed that hTERT cells did indeed have longer telomeres than the telomerase negative BJ cells. Glaviano et al (Glaviano et al. 2006) reported that telomerase activity in hTERT cells was significantly higher than in BJ cells and that direct exposure of cells to Cr had no effect on telomerase activity or telomere length. This difference in telomerase expression and telomere length between the two cell lines may play a role in their differing responses to metal-induced bystander effects.

A possible explanation for the protection of hTERT cells against Cr-induced bystander effects could be that telomerase positive cells have more stable mitochondria, which restrict their signalling responses after Cr-induced DNA damage. It is therefore of interest with respect to the results presented here that hTERT in higher eukaryotes such as plants and mammals has an N-Terminal leader sequence on the catalytic subunit of telomerase reverse transcriptase that facilitates its translocation from the nucleus to

the mitochondrion (Santos et al. 2006; Passos et al. 2007b). These presequences are characterised by the presence of 20-60 predominantly positively charged amino acids, hydroxylated amino acids and the tendency to fold into an amphipathic α-helix; and are usually cleaved upon import of the protein into an organelle (Neupert 1997). This may confer stress resistance to cells with improved antioxidant defense and mitochondrial protection (Ahmed et al. 2008). Indeed, Nugent et al (Nugent et al. 2007) showed that there are also increased mitochondria in bystander cells, which is indicative of a stress response to mitochondrial dysfunction.

Emerging evidence suggests that telomerase has additional extratelomeric roles in mediating cell survival and anti-apoptotic functions against various cytotoxic stresses. For example, hTERT protects against oxidative stress-induced apoptosis in human lens epithelial cells (Huang et al. 2005). Telomerase is also linked to p53-induced apoptosis and mediates inhibition of p53-induced apoptosis. The hTERT gene is down regulated upon activation of wt p53 (Xu et al. 2000) and inactivation of p53 in telomerase negative human mammary epithelial cells leads to reactivation of telomerase (Stampfer et al. 2003). However, telomerase activity is not required, as a catalytically inactive dominant negative hTERT is just as efficient as the wild-type (Rahman et al. 2005). This is an example of hTERT promoting cell survival independently of its enzymatic activity. Cao et al (Cao et al. 2002) hypothesised that the difference between the enzymatic activity of telomerase and its roles in telomere maintenance lie at least partly in complex molecular interactions of the hTERT protein.

Ectopic overexpression of hTERT prevents TRAIL-induced apoptosis independent of telomere maintenance (Dudognon et al. 2004). Work by Dudognon et al (Ashkenazi and Dixit 1998) hints at functions of telomerase distant from the telomeres,

even on the periphery of the plasma membrane. The receptor-mediated pathway referred to as the 'extrinsic' apoptotic pathway, involves members of the tumour necrosis factor (TNF) family of death receptors (DR), including TNF-related apoptosis-inducing ligand (TRAIL) receptors DR4 and DR5, which activate caspase-8. Acute hTERT inhibition facilitates apoptosis induction through the mitochondrial pathway, suggesting a novel role for hTERT as an endogenous inhibitor of mitochondrial apoptosis (Massard et al. 2006).

Protection of hTERT cells against the metal-induced bystander effect could be a product of the mitochondrial functions of telomerase in both donor and recipient cells. However, mitochondrial protection could be more important in donor cells, as Chen et al (Chen et al. 2009b) showed that using normal and mitochondria-depleted cells, mitochondria-dependent signalling derived from donor cells contributes to ROS-mediated genotoxicity in bystander cells. The role of donor cell mitochondrial function in bystander signalling has also been implicated by Tartier and colleagues (Tartier et al. 2007), who co-cultured normal (ρ^+) HeLa cells with pseudo ρ^0 cells (HeLa cells treated with ethidium bromide to deplete mitochondrial DNA to less than 5% of the normal level). Bystander induction of 53BP1 foci, which parallel DNA DSB levels did not occur in ρ^+ HeLa cells if ρ^0 cells were donors, irradiated through the nucleus or the cytoplasm but did occur in ρ^0 cells if ρ^+ HeLa cells were irradiated donors.

However, hTERT cells were not always protected against the metal-induced bystander effect. Medium transfer carried out from metal-exposed BJ cells to recipient hTERT cells and vice versa revealed that conditioned medium from Cr-exposed BJ cells was capable of inducing MN and γ-H2AX foci in recipient hTERT cells for at least 30 days post Cr exposure in donor cells. In addition, conditioned medium from Cr-exposed

hTERT cells induced MN and γ-H2AX foci in recipient BJ cells for at least 30 days post Cr exposure in donor cells. Taken together, these experiments suggest three things. Firstly, that both telomerase positive and negative cells can secrete a bystander signal. Secondly, that there is a difference in this signalling since the telomerase positive cells are susceptible to the signals from the telomerase negative BJ cells but are resistant to the signal from their own cell type, even though they can produce a signal that causes biological effects in telomerase negative cells. Thirdly, that hTERT cells are resistant to some aspects of genomic instability as they never show NPBs either from direct exposure to Cr (VI) or hTERT conditioned medium, perhaps due to effective telomere capping (Blackburn 2001). These complex results might be explained by a hypothesis that assumes that the hTERT negative BJ cells can produce (at least) two bystander signals whilst the hTERT positive cells are more restricted in their repertoire.

There is evidence that the mitochondrial role of hTERT is not wholly beneficial to a cell. For example, Santos et al (Santos et al. 2003) demonstrated that telomerase-expressing normal diploid fibroblasts are more vulnerable to H_2O_2-induced mitochondrial DNA (mtDNA) damage than other cell types and under the same conditions, the nuclear DNA (nDNA) of these cells seemed completely resistant to H_2O_2-induced damage. Perhaps hTERT cells would have been susceptible to metal-induced bystander effects but any mitochondrial DNA damage was not assayed for here and would have remained undetected. Santos et al (Santos et al. 2006) observed variable levels of telomerase activity in mitochondria but notably, hTERT ectopic expression resulted in 2-3-fold higher levels of mtDNA lesions in hTERT positive fibroblasts compared to hTERT negative parental fibroblasts in response to short H_2O_2 exposures. hTERT may be implicated in apoptosis by increasing the oxidative burden in the mitochondria, resulting in mtDNA damage. In accordance with this idea, according to

Pritchard et al (Pritchard et al. 2001), hTERT-expressing BJ fibroblasts were more sensitive than the parental BJ cells to apoptosis (mediated by mitochondrial membrane depolarisation) induced by Cr (VI). No apoptosis was measured during this PhD project but again, any Cr- or bystander- induced apoptosis in hTERT cells would have remained undetected. Santos et al (Santos et al. 2006) revealed that mutations in the N-terminal leader sequence that governs telomerase entry into the mitochondrion significantly reduced levels of mtDNA damage following hydrogen peroxide treatment, but strikingly also do not show any loss of viability or cell growth. Thus, localisation of hTERT to the mitochondria renders cells more susceptible to oxidative stress-induced mtDNA damage and subsequent cell death, whereas nuclear-targeted hTERT, in the absence of mitochondrial localization, is associated with diminished mtDNA damage, increased cell survival and protection against cellular senescence (Santos et al. 2006). Therefore, perhaps hTERT cells were not protected against metal-induced bystander effects and any signalling to result in apoptosis or mitochondrial DNA damage remained undetected here and may account for the differences observed in bystander responses between hTERT and BJ cells.

Here, the metal-induced bystander effect was induced following exposure to low doses of potassium dichromate. Radiation-induced bystander effects are also thought to be more important at low doses. For example, Belyakov et al (Belyakov et al. 2001) observed radiation-induced bystander effects in non-hit cells when only a single cell in a population was targeted with a single helium ion, independent of the dose delivered to the target cell. Sokolov et al (Sokolov et al. 2005) observed only a slight increase in bystander γ-H2AX foci when directly-irradiated target cells had been exposed to 20 rather than 2 α-particles, indicating that radiation dose was not a critical parameter. No investigation was carried out to determine the effects of dose on the metal-induced

bystander signalling, but as low doses were effective, it could be expected that it would saturate above a certain dose of metal, as is the case in general for radiation.

Another factor to consider when comparing radiation-induced bystander effects and the effects of metal treatment is the duration of bystander signalling from directly exposed cells. Here, metal-induced bystander signalling was effective for at least 30 days post donor cell exposure and did not diminish over time. In general, the later conditioned medium was harvested post metal treatment, the higher the level of bystander DNA damage. In the radiation literature, bystander effects seem to predominate sooner post irradiation rather than later. For example, Chen et al (Chen et al. 2009b) reported that conditioned medium harvested 10 minutes post irradiation was optimal for upregulation of bystander ROS and γ-H2AX in human-hamster hybrid cells following irradiation with α-particles, the signal dropping back down to background level if conditioned medium was harvested at 1 hour. However Sokolov et al (Sokolov et al. 2005) reported that there was more significant bystander γ-H2AX induction at 18 hours co-culture of α-particle irradiated human fibroblasts than at 30 minutes. As both of these studies used α-particles but different cell types, there may be a cell-type dependency for optimal incubation time prior to harvesting of conditioned medium. Prise et al (Prise et al. 1998) irradiated a small percentage of cells in a population with an α-particle microbeam and reported that 3 days incubation post irradiation was optimal for bystander MN and apoptosis induction.

The duration of medium conditioning reported here for metal-induced bystander effects was always 1 hour. No investigation was carried out that varied the incubation time. One hour was selected as the incubation period because studies of radiation-induced bystander effects frequently report one hour as being sufficient for harvesting

medium capable of inducing biological effects in recipient bystander cells (Vines et al. 2008; Vines et al. 2009).

Radiation-induced bystander signals do not appear to be as long-lived as those reported here following metal treatment, which persist for at least 30 days. The differences between radiation and metal-induced bystander effects may be due to the fundamental differences between these types of treatment. Metals may have more long-term effects on a target cell to cause long-lasting DNA cross-links and adducts that may be responsible for the longevity of the bystander signal.

The metal-induced bystander effect appeared to be cell-type specific, induced in normal human BJ fibroblasts after exposure to conditioned medium but not telomerase positive fibroblasts if donor cells were also telomerase positive. The radiation literature also reports the cell type specificity of bystander signalling. For example, in a study by Vines et al (Vines et al. 2008) using the human keratinocyte cell line HPV-G; and the hamster cell line CHO-K1, one hour of medium conditioning was sufficient to induce a significant reduction in surviving fraction of medium transfer recipient cells of either type when donor cells were irradiated at a dose of 0.5 Gy or 5 Gy. However, conditioned medium harvested from the E89 hamster cell line 1 hour post irradiation at doses of either 0.5 Gy or 5 Gy was not able to induce any significant reduction in surviving fraction in E89, CHO-K1 or HPV-G recipient cells. The E89 cell line is null for glucose-6-phosphate dehydrogenase, which is responsible for the generation of NADPH from reduction of $NADP^+$ in the pentose phosphate pathway. NADPH is required for cellular biosynthesis of many macromolecules and has been implicated in the production of and responses to bystander effects (Azzam et al. 2003). Vines et al (Vines et al. 2008) reported that the magnitude of the bystander effect is dependent on the source of conditioned medium as medium from irradiated HPV-G cells reduced the

clonogenic survival of HPV-G and CHO-K1 cells significantly more than conditioned medium from irradiated CHO-K1 cells but had no effect on the clonogenic survival of E89 cells. So bystander signals have varying toxicity depending on the source of conditioned medium. Some investigations have shown that fibroblasts do not contribute to bystander signalling at all, especially after low LET radiation. For example, medium harvested from human fibroblasts irradiated with ^{60}Co gamma-rays did not reduce the clonogenic survival of unirradiated cells unlike the medium from irradiated epithelial cells, which did reduce the cloning efficiency of unirradiated cells (Mothersill and Seymour 1997). However, Prise et al (Prise et al. 1998) reported cell-to-cell transmissible induction of MN and apoptosis in bystander human fibroblasts following α-particle irradiation of just 4 cells in a population. These differences could be due to differences in the type of radiation used as Mothersill et al used low LET gamma-rays and Prise et al used α-particles. Any genetic differences between the fibroblast cell strains cannot be ignored and may also contribute to the heterogeneity in bystander responses reported in the literature.

Perhaps one of the most useful tools for studying bystander effects is the microbeam which is able to irradiate certain subsets of cells within a population, crucially, allowing specific cells to remain unirradiated. Another difference between the two examples given above for the varying responses to bystander signalling reported for fibroblasts is the mode of transmission of bystander responses. Prise et al (Prise et al. 1998) used a microbeam in which bystander signalling could occur between irradiated fibroblasts and the neighbouring non-irradiated cells that were in close contact; whereas, Mothersill et al (Mothersill and Seymour 1997) used a medium transfer technique to show that medium from irradiated fibroblasts could not induce biological effects in unirradiated cells. The role of close cell-to-cell communication in the study of metal-

induced bystander effects was examined by microinjecting small fractions of cells in a population with 0.4 μM potassium dichromate in order to mimic the microbeam concept using metal exposure. hTERT cells appeared to show bystander induction of γ-H2AX foci when a small percentage of cells of the same type in a population was injected even though they did not show any induction of γ-H2AX foci in response to treatment with conditioned medium from Cr-exposed hTERT cells. Unfortunately, when injected BJ cell populations were viewed under a microscope after injection and antibody staining, it became clear that the cells tended to become detached from the coverslip, making any analysis difficult as it would be impossible to compare treatment and control. However, hTERT cells seemed to survive the injection and staining process and cells did not appear to lose confluence. Therefore, only hTERT cells were studied post microinjection. So far, there was no evidence of intra cell type metal-induced bystander effects for hTERT cells. However, following microinjection of small numbers of cells on a coverslip, a larger percentage of cells than could be expected based on the numbers microinjected, stained positive for γ-H2AX foci. Although this observation was based on qualitative observations rather than quantitative data, this could indicate that close cell-to-cell contact is more important than medium-borne effects for transmission of bystander factors in hTERT cells. However, it may also suggest that there had been some leakage of chromium during the injection process to cause direct Cr treatment rather than bystander treatment. However, this is unlikely, given the dilution of metal ions that would have occurred in the 5 ml total volume of the medium in the Petri dish containing the coverslip at the time of injection. This value is difficult to calculate as the injection volume is unknown and was not constant but the dilution may be in the region of up to 1000- fold. The effects of this concentration of potassium dichromate are probably negligible if compared to the results of the low dose, residual Cr assays

described in chapter 4, where direct exposure of cells to 0.003 μM Cr (VI) did not cause any significant induction of γ-H2AX foci or miconuclei.

Proteomic analysis of conditioned medium revealed no differences in secreted proteins between BJ and hTERT cells in response to either Cr or control treatment, indicating that the bystander factor was perhaps too small to be detected by 2D gel analysis or that no 'new' factor was secreted. Perhaps there was just a change in the balance of what is normally secreted. In a study of radiation-induced bystander effects, a change in proteome of bystander cells was reported using mass spectrometry (Gerashchenko et al. 2007). This study identified that nucleophosmin, a nucleolar protein, and enolase-α, a glycolytic enzyme were more highly expressed in bystander cells than unirradiated or directly irradiated cells (Gerashchenko et al. 2007). Perhaps mass spectrometric analysis of the peptides derived from the spots shown in this study could have enabled identification of the proteins present in conditioned medium perhaps alluding more closely to any subtle differences between treatments and cell type. This could aid in understanding the molecular events occurring in metal-induced bystander responses.

9.1.2 Antioxidants

Low concentrations of the antioxidant ascorbate were added to directly Cr-exposed cells after treatment and to conditioned medium in order to determine the role of oxidative stress in bystander responses. Significantly higher levels ($p<0.001$) of DNA double strand breaks were induced in BJ recipient cells that had received conditioned medium from Cr-exposed cells of either type (hTERT or BJ). Incubation of donor cells with ascorbic acid resulted in a considerable reduction in DNA double strand break induction caused by Cr-conditioned medium from either cell type in BJ recipient cells.

Ascorbic acid treatment in hTERT donor cells did not affect the level of γ-H2AX foci induced in hTERT recipient cells. This was expected, as hTERT conditioned medium had so far not led to any induction of DNA double strand breaks in hTERT conditioned medium-recipient cells (Chapter 4 (4.2)). However, in response to conditioned medium from BJ donor cells that had been exposed to both Cr (VI) and ascorbic acid, there was a significant increase in γ-H2AX foci. This increase in foci may have been due to a pro-oxidant activity of ascorbic acid in BJ cells that was triggered by the presence of some residual Cr (VI). Hydroxyl radical formation has been observed in the reaction of Cr (VI) with ascorbic acid resulting from auto-oxidation of ascorbic acid and H_2O_2 formation (Poljsak and Raspor 2008). These reactive oxygen species could be responsible for an increase in double strand breaks in the presence of ascorbic acid and Cr (VI) (Su et al. 2006). The reducing agent activity of ascorbic acid may have reacted with Cr^{6+} to yield Cr in lower oxidation states, which could then be oxidized by H_2O_2 resulting in the production of more hydroxyl radicals. Notably, based on the results from Chapter 4 (section 4.5), it would be expected that significantly more DNA double strand breaks would be induced in hTERT recipient cells that had been exposed to conditioned medium from Cr-exposed BJ cells compared to control than were seen here. However, the percentage of hTERT cells containing a high level of γ-H2AX foci (4+ foci per cell) following exposure to control medium from BJ donor cells exposed to PBS (rather than Cr (VI)) was unusually high at around 33%. Perhaps the ascorbic acid treatment just did not prevent the DNA double strand breaks caused by Cr-conditioned medium from BJ cells, even though this effect was masked by the high level of double strand break foci in control cells. There was a significant ($p<0.01$) difference between the percentage of BJ cells containing high levels of DNA double strand breaks following direct treatment with Cr and those cells that were also exposed to ascorbic

acid. In this case, the antioxidant completely abrogated the DNA damaging effects of direct Cr exposure in BJ cells. This may explain why the percentage of BJ recipient cells containing 4+ DNA double strand break foci per cell was much lower when Cr treatment in donor BJ fibroblasts was combined with antioxidant treatment. However, this does not explain the result observed in hTERT recipient cells that had been exposed to conditioned medium from Cr treated fibroblasts with or without ascorbic acid (hTERT rec BJ Cr ±vit C). In that case, the antioxidant did not eradicate any DNA damaging factors present in conditioned medium from Cr-exposed BJ donor cells. Direct Cr exposure in hTERT cells induced a high level of DNA double strand breaks and that this effect was completely diminished following ascorbic acid treatment ($p<0.001$), reducing γ-H2AX focus induction to similar levels to that observed in control cells. There even appeared to be a decrease in γ-H2AX focus formation caused by ascorbic acid in control cells, which may be interesting as cells in culture develop more 'cryptogenic' DNA double strand break foci, which may have been associated with oxidative stress caused by culture conditions. These experiments were all carried out in the absence of CO_2, which may have caused stress to the cells as stable pH values could not be ensured. This may have resulted in higher levels of DNA double strand breaks in control cells that became reduced in the presence of the antioxidant, l-ascorbic acid.

In summary, antioxidant treatment significantly reduced DNA double strand break induction following direct Cr treatment in BJ cells and transfer of conditioned medium from Cr exposed BJ donor cells or Cr exposed hTERT donor cells to BJ recipient cells. These results are consistent with reports of Harada and colleagues (Harada et al. 2008), who found that ascorbic acid suppressed micronucleus induction in bystander fibroblasts following co-culture with 1 Gy X-ray irradiated donor cells.

Ascorbic acid is thought to scavenge long-lived radicals induced by irradiation (Koyama et al. 1998) that may also be responsible for some of the detrimental effects of exposure to chromium ions, as intracellular reduction of Cr (VI) to Cr (III) gives rise to relatively long-lived chromium(V) and chromium(IV) species (Mattagajasingh et al. 2008).

These data suggest that perhaps there are multiple DNA damaging signals originating from Cr-exposed donor cells that differ between BJ and hTERT human fibroblasts. BJ donor cells may produce more than one bystander signal. One of these signals may not have an oxidative mode of action. This could explain why antioxidant treatment did not reduce the DNA double strand breaks that BJ-conditioned medium caused in hTERT recipient cells. This may also explain why DNA damage was seen at all in hTERT cells, as no DNA double strand breaks or micronuclei had been observed in hTERT recipient cells before BJ-conditioned medium was used. Perhaps hTERT donor cells produce a signal that sets in motion an oxidative-stress-related-mechanism that hTERT recipient cells are able to efficiently and effectively repair by virtue of their stable telomere structure and BJ recipient cells are not. It may be possible that the BJ donor cells also produce this oxidative stress factor as it caused damage in BJ recipient cells that was removed by antioxidant treatment. It is thought that hTERT expression and particularly the presence of the telomere associated proteins, especially TRF2 are protective against oxidative stress (Oh et al. 2003). Telomere structure rather than length is the prerequisite for normal telomere function (Blackburn 2001). Telomere structure depends on a collection of telomere associated proteins, including TRF2. TRF2 is involved in formation of the T-loop at the 3’ telomeric single stranded overhang. Around twelve mammalian proteins involved in DNA DSB repair have been implicated in telomere length homeostasis and chromosome end protection (De Boeck

et al. 2009). This suggests extensive functional interactions between telomere maintenance and DNA damage response mechanisms. In response to DNA damage, activation of the ATM protein kinase pathway leads to the rapid phosphorylation of TRF2. Phospho-TRF2 no longer binds telomeres but translocates to DNA damage sites (Tanaka et al. 2005). Therefore, it could be expected that the hTERT positive fibroblasts are more resistant to oxidative stress and DNA double strand breaks than BJ fibroblasts. Very little is known about telomeric proteins in telomerase negative cells, but according to Shen et al (Shen et al. 1997), the telomere associated proteins Pin2 and TRF1 are localised to only a few telomeres in telomerase negative cells. This suggests that the differential distribution of telomere associated proteins between telomerase positive and negative cells could be responsible for differing reactions to oxidative stress between BJ and hTERT cells.

9.1.3 p38 MAP Kinase

Radiation-induced bystander studies have revealed the importance of gap junctional intercellular communication for transmission of bystander signals between cells in close contact. For example Harada et al (Harada et al. 2009) irradiated 25 cells in a confluent monolayer with 5 carbon ions and observed that the bystander surviving fraction of cells cultured with lindane, a GJIC-suppressing agent, was significantly enhanced when compared to cells cultured with 8-Br-cAMP, a GJIC stimulating agent. However, it is still possible that medium transmissible factors still contribute to any bystander effects observed in gap junction studies as both the directly irradiated and bystander cells grow in the same culture medium. In this current study, GJIC was altered by manipulating p38 MAP kinase in an attempt to determine whether the differences in bystander signalling between BJ and hTERT cells could be attributed to differences in the structure of their gap junctions. That is, perhaps BJ cells have a more

open permissive gap junctions that allow bystander signalling to both cell types and hTERT cells had a more restrictive gap junction structure, only allowing bystander signalling to BJ cells. With this hypothesis in mind, anisomycin, an antibiotic produced by *Streptomyces griseolus* was used to reduce GJIC in BJ cells. Anisomycin activates p38 MAP Kinase leading to enhanced Connexin 43 phosphorylation and reducing GJIC (Ogawa et al. 2004). Treatment of BJ donor cells with anisomycin may induce a less permissive more 'closed' gap junction structure enabling the secretion of fewer bystander factors from BJ donor cells. The pyridinyl imidazole inhibitor SB203580 inhibits p38 MAP Kinase and is able to restore GJIC (Ogawa et al. 2004). There appeared to be a slight increase in the percentage of binucleated BJ recipient cells containing micronuclei in response to treatment with conditioned medium from SB203580-treated hTERT donor cells compared to the DMSO control-treated hTERT donor cells, however, this change was not significant. There was no difference in the percentage of hTERT recipient cells containing γ-H2AX foci between Cr exposed donors with or without SB203580. This suggests that either the gap junctions did not change with the use of this additive or that the metal-induced bystander effect is not dependent on gap junctional intercellular communication in telomerase positive fibroblasts. This does not completely agree with the observations made of hTERT fibroblasts after microinjection, where it appeared that communication between these cells in close contact was responsible for induction of γ-H2AX foci in uninjected cells. However, perhaps the effects observed following microinjection were medium transmissible effects, as the cells all grew in the same culture medium and the 6 hours of co-culture between injection and fixation was required to propagate bystander responses, indicating that the one hour of medium conditioning time used in this study for medium transfer may not have been sufficient to induce bystander responses in

hTERT cells. When hTERT recipient cells were treated with conditioned medium from BJ donor cells, anisomycin significantly ($p<0.05$) reduced the percentage of hTERT recipient cells containing a significantly high (4+ foci per cell) level of DNA double strand breaks. On average in the presence of anisomycin, over 37% of the cells were normal i.e. containing no DNA DSBs compared to an average of less than 31% of normal hTERT recipient cells exposed to conditioned medium from Cr-exposed BJ cells that lacked anisomycin treatment. This suggests that BJ cells were affected by anisomycin treatment and its effect on their gap junctions may have caused a less open structure and perhaps secretion of fewer bystander factors. The effect of anisomycin on cells is the activation of p38 MAP kinase, which may have played more of a role than just in 'blocking' of gap junctions, as members of the MAP Kinase family have been implicated as mediators of bystander responses (Azzam et al. 2002; Zhou et al. 2005; Lyng et al. 2006b). P38 is known to be activated in bystander cells (Azzam et al. 2002) and may have played a role in the reduction of DNA double strand breaks observed in recipient hTERT cells as the p38 pathway has been shown to promote cell growth and survival (Juretic et al. 2001). Lyng et al (Lyng et al. 2006b) investigated the role of MAP Kinase signalling in bystander cell death and showed that ERK and JNK pathways but not p38 were activated in bystander HPV-G cells exposed to conditioned medium. Inhibition of the ERK pathway appeared to increase bystander apoptosis whereas JNK inhibition led to a decrease in bystander cell death. Indeed Chen et al (Chen et al. 2009a) showed that the different MAP kinases have contrasting effects on Cr (VI)-induced cytotoxicity. p38 acted to reduce cell survival in response to Cr exposure and JNK reduced cell death. Therefore, a dynamic balance exists between these pro-apoptotic and pro-survival pathways.

9.1.4 TNF-alpha neutralization

Differential bystander signalling has been observed between BJ and hTERT cells. It was considered that there may be a range of bystander signals produced by Cr-treated fibroblasts that differ between these two cells. It was determined that the conditioned medium obtained from either cell type in response to Cr treatment could contain a factor capable of causing oxidative damage as the antioxidant l-ascorbic acid was able to influence bystander signalling (Chapter 6.1). hTERT cells appeared to be protected against this oxidative damage but BJ cells may produce an additional factor that could induce DNA damage in recipient hTERT cells. This second factor may be a cytokine or any molecule that is not associated with oxidative stress.

ROS are known to be released from irradiated cells, possibly into growth medium or transferred between cells in close contact via gap junctional intercellular communication (Lyng et al. 2000; Lyng et al. 2002; Rugo et al. 2002; Yang et al. 2005; Tartier et al. 2007; Chen et al. 2009b). However, since free radicals, ROS and NO have relatively short half lives, they are unlikely candidates for the longer term signalling factors present in conditioned medium derived from directly irradiated cells in medium transfer studies. Given that ROS and free radicals are relatively short-lived, perhaps they induce production of a more long-lived factor such as a cytokine.

TNF-alpha is a cytokine involved in systemic inflammation and is known to induce apoptotic cell death and inflammation and to inhibit tumourigenesis (Tracey and Cerami 1993). TNF-alpha has been implicated to play a role in bystander responses (Shareef et al. 2007) and addition of inhibitory anti-TNF-α to cell culture medium substantially decreased levels of NF-$\kappa\beta$ and COX-2 expression in both irradiated and especially, in bystander cells, leading to a significant increase in bystander clonogenic

survival (Zhou et al. 2008). Although 2D gel electrophoresis could not detect TNF-alpha in conditioned medium from either cell type following treatment with either Cr or PBS; to investigate whether the metal-induced bystander effect could be influenced by TNF-alpha signalling, a neutralizing antibody, anti-TNF-alpha was used to sequester functional TNF-alpha from the conditioned medium of both BJ and hTERT cells.

Neutralizing anti-TNF-alpha in conditioned medium caused a significant ($p<0.001$) increase in induction of DNA double strand breaks in BJ and hTERT recipient cells that were treated with control medium from PBS-treated donor cells of either cell type compared to conditioned medium from control donor cells lacking anti-TNF-alpha. However, when neutralizing anti-TNF-alpha was added to conditioned medium from Cr-exposed BJ donor cells, there was a 1.5-fold decrease in the percentage of BJ recipient cells containing 4+ γ-H2AX foci per cell compared to that caused by conditioned medium from Cr-exposed BJ donor cells lacking anti-TNF-alpha. When neutralizing anti-TNF-alpha was added to conditioned medium from Cr exposed hTERT donor cells, the resulting percentage of damaged BJ recipient cells decreased more than two-fold compared to the DNA damage induced by Cr-conditioned medium from hTERT donor cells that lacked anti-TNF-alpha neutralization. The same was true for hTERT recipient cells exposed to Cr conditioned medium from BJ donor cells. Anti TNF-alpha caused a reduction in DNA damage. There was a 2-fold increase in γ-H2AX foci in hTERT recipient cells exposed to conditioned medium from Cr exposed hTERT donors in the presence of anti TNF-alpha, compared to conditioned medium from Cr-exposed hTERT cells without anti TNF-alpha, which was low, as expected. Puzzlingly, the antibody increased DNA damage in controls but significantly decreased DNA damage in BJ recipients caused by conditioned medium from both Cr-treated BJ donor cells ($p<0.001$) and Cr-treated hTERT donor cells ($p<0.05$). A possible explanation for

these results could be that addition of a high concentration of anti-TNF-alpha to conditioned medium is toxic to recipient cells but that its neutralization of excess TNF-alpha in Cr-conditioned medium is beneficial to recipient cells resulting in reduced DNA damage. Therefore, in summary TNF-alpha did play a role in metal-induced bystander signalling.

9.1.5 K1 Thyroid Carcinoma Cell Line

In order to determine whether the lack of medium transmissible metal-induced bystander signalling observed with hTERT donor cells was representative of other telomerase positive cell lines; cells that are naturally telomerase positive were used. One of these was the K1 thyroid carcinoma cell line. In addition to this telomerase positive K1 cell line, a DN K1 line was also used, in which telomerase was inhibited through overexpression of a dominant negative, mutated in the reverse transcriptase motif. Overexpression of this dominant negative was effective in reducing telomere length. Chromium exposure had no further effect on telomere length for either cell type.

In chapter 4.4, it was shown that conditioned medium harvested from Cr-exposed hTERT cells caused a significant induction of γ-H2AX foci in bystander medium transfer recipient BJ cells. Like the signalling from hTERT donors to BJ recipient cells, conditioned medium from Cr-exposed telomerase positive K1 cells caused a significant ($p<0.01$) induction of γ-H2AX foci in BJ recipient cells. In addition, just like the lack of medium transmissible bystander signalling between hTERT cells, medium from metal exposed telomerase positive K1 cells did not induce any γ-H2AX foci in recipient hTERT fibroblasts. This demonstrates that hTERT cells and another telomerase positive cell line behave in the same way in metal-induced bystander signalling, at least for medium transmissible factor(s).

Inhibition of telomerase through overexpression of a dominant negative did not cause these cells to behave in the same way as telomerase negative BJ cells, as conditioned medium from Cr-exposed DN K1 cells did not induce γ-H2AX foci in BJ recipient cells. This is unlike the response for BJ cells described in chapter 4, in which medium transfer from Cr-exposed cells caused significant induction of biological effects in recipient cells.

No bystander γ-H2AX foci were observed in hTERT recipient cells exposed to conditioned medium from the telomerase positive K1 cell line, which shows that hTERT cells had behaved like another telomerase positive cell line, as in chapter 4, there was no effective bystander signalling between hTERT cells. Again, the DN cells did not behave in the same way as telomerase negative BJ cells, which could secrete a medium transmissible bystander factor to cause biological effects in recipient hTERT cells because there was no effective bystander signalling from DN cells to hTERT cells showed by a lack of induction of γ-H2AX foci.

Interestingly in view of the non telomere extending functions of telomerase (Passos et al. 2007a), expression of the dominant negative actually reduced the bystander effect and did not lead to any effective bystander signalling between the DN K1 cells and either BJ or hTERT fibroblasts. However, dominant negative TERT expression is sufficient to inhibit apoptosis and promote cell survival independently of its enzymatic activity (Dudognon et al. 2004; Rahman et al. 2005), so perhaps the DN donor cells were protected against Cr-induced damage, implicating differential roles for enzymatically functional telomerase and the dominant negative in transmission of bystander signalling. DN K1 cells may still have had an adequate leader sequence to facilitate mitochondrial entry and protection. Given that DN hTERT is catalytically

inactive, resulting in telomere shortening, perhaps the mitochondrial functions of telomerase are responsible for the lack of bystander signalling observed in hTERT cells. These results hint that the different roles of telomerase, i.e. its functions at the telomere and its non-telomeric activities may be responsible for the differential bystander signalling between telomerase positive and negative cells.

9.1.6 Human Embryonic Stem Cells

A second set of experiments using telomerase positive cell lines involved the study of metal-induced bystander effects between co-cultured mouse embryonic fibroblasts (mEFs) and human embryonic stem (hES) cells, both of which are telomerase positive. This set-up is ideal for the study of bystander effects as the cells are co-cultured, enabling the transmission of bystander signalling between cells in close contact and are morphologically different so if one population is treated with Cr, determination of bystander cells from directly exposed cells is very simple.

It is notable that baseline levels of γ-H2AX foci were very low in hES cells as fewer than 5% of control cells contained 4+foci per cell. In mEFs, baseline levels of damage were much higher at around 30%, which was probably due to the fact that these cells were mitomycin-C inactivated. Direct Cr treatment caused induction of γ-H2AX foci in both cell types as up to 10% of hES cells contained 4+foci per cell and around 60% for mEFs following direct Cr exposure. There was a significant bystander induction of γ-H2AX foci in untreated mEFs that had been co-cultured with Cr exposed stem cells. mEFs exposed to both direct Cr treatment and a bystander signal from hES cells contained more γ-H2AX foci than when only exposed to the hES cell bystander signal. This indicates a cumulative effect of Cr exposure when it came from both sources and it was more potent than just bystander treatment. There was no significant

bystander induction of γ-H2AX foci in hES cells co-cultured with Cr-exposed mEFs. As for hTERT fibroblasts, stem cells which are naturally telomerase positive were protected against metal-induced bystander effects, as both of these cell lines are telomerase positive. However, mEFs showed induction of bystander γ-H2AX foci, but TERT function in these cells may have been impaired by mitomycin-C inactivation, which is known to affect telomere length (Wick and Gebhart 2005).

Again, just as observed for microinjected hTERT cells, metal-induced bystander effects were observed between telomerase positive cells that were in close contact, which highlights the importance of the mode of transfer of bystander signalling. The time of medium conditioning during medium transfer must be an important factor for the study of metal-induced bystander effects between telomerase positive cells. In the microinjection and stem cell co-culture investigations, metal-exposed cells were in contact with bystander cells either immediately after treatment or within a very short period of time. This observation suggests two things; firstly that telomerase positive cells are sensitive to bystander factors that are secreted by other telomerase positive donor cells very rapidly after metal treatment, and secondly that production of this factor(s) does not persist for 24 hours because this was the time point for harvesting of conditioned medium in medium transfer studies, which yielded no bystander responses between hTERT cells.

In an additional study, BJ and hTERT human fibroblasts were used as feeder cells for hES cell culture. No metal-induced bystander effects were observed in BJ, hTERT or hES cells for induction of γ-H2AX foci. However, the hES cells were more likely to differentiate in the presence of human feeder cells than mouse feeder cells. Differentiated cells may no longer be telomerase positive nor have long telomeres and

would therefore not provide a useful comparative study for hTERT cells. It was therefore very valuable to carry out Oct4 staining in order to distinguish differentiated from non-differentiated stem cells. Although no bystander effects were observed, the experiment using human feeder cells was not as clear-cut as that using mEF feeders. Any potential bystander effects may have been masked by the differentiation of stem cells. Fewer embryonic stem cells were present, which may have contributed to the differences observed from the mEF system.

These results confirm a role for physiological telomerase in limiting bystander signalling and suggest that stem cells are particularly resistant to induction of DNA double strand breaks despite their close proximity to mitomycin C- and Cr-damaged feeder cells. Interestingly, significantly high levels of γ-H2AX foci were observed in differentiated ES cells after co-culture with Cr-exposed hTERT cells compared to controls. However, it is not clear whether the differentiated embryoid bodies presented more foci because they were more sensitive to bystander signalling than non-differentiated hES cells or whether any bystander signalling from Cr-exposed hTERT cells caused them to differentiate. Indeed a combination of both factors could be responsible for this observation.

hES cells have therapeutic potential in regenerative medicine, which lies in the transplantation of differentiated cell types for various degenerative diseases. Stem cells remain karyotypically and phenotypically stable for long periods in vitro so are a good source material for tissue replacement therapies. (Thomson et al. 1998; Amit et al. 2000; Reubinoff et al. 2000). For this reason, it is important to study any intercellular interactions including bystander effects that may take place between stem cells and feeders and also with their stroma after transplantation. Quality assurance systems will

have to be in place for the production of clinical grade human embryonic stem cells for cell transplantation treatment (Unger et al. 2008). Subsequent steps once clinical grade ES cell lines are established will include the induced differentiation of stem cells into other cell types. In the foreseeable future, until feeder-free systems can be established, clean human fibroblast-derived feeder cells could provide important start-up material for new hES cell lines. It is this stage where potential bystander reactions between human embryonic stem and feeder cells could have their most significant impact on the introduction of new therapeutic practices.

mEFs are traditionally used as feeders for maintenance of hES cells but this situation is not ideal and development of mEF-free feeder systems is underway. A problem with using mouse cells as feeders for human stem cells is the possibility of potential xenogeneic contamination. Concerns include contamination of stem cells with feeder-derived viruses and transmission of animal pathogens, and the potential immune rejection of xeno-proteins by human ES cells, which could affect future applications in cell based therapies (Martin et al. 2005). In order to support the long-term culture of stem cells and stably maintain their undifferentiated state, mEFs, which provide an extracellular matrix and growth factors must be cultured for the whole duration. This means there is a need to continuously sacrifice mouse embryos (Odorico et al. 2001). Mouse feeders can only be used for a limited passage number (usually between passage 4 and passage 6), after which they lose their stem cell supportive capacity (Richards et al. 2002). Therefore, other feeder systems and feeder-free systems are under investigation (Xu et al. 2001; Richards et al. 2002). Another reason to avoid the use of mEFs as feeder cells is presented by the results of this study, which show that feeder-derived bystander signalling can be responsible for DNA damage in stem cells, which is

an important factor to consider especially during therapy of metal-exposed patients or using metal-containing matrices.

Although no metal induced bystander effects were observed between human feeder cells and stem cells, there could be a concern if radiotherapy is involved in treatment as any radiation-induced bystander effects may reduce the efficacy of stem cell transplantation. According to Richards et al (Richards et al. 2003), who evaluated 11 different human adult, foetal and neonatal feeder cell lines for ES cell culture, not all human feeders support ES growth equally. Foetal and embryonic tissues perform better in vitro than adult tissues. This evaluation demonstrates why in this project BJ and hTERT could not support the undifferentiated growth of human ES cells as well as mEFs did. Firstly, feeder cells are required to be at a low passage number in order to support stem cell maintenance and the cells used here were around passage 7. Also as embryonic tissues perform better; it is no wonder that these adult human cells did not prevent the early differentiation of the stem cells. This differentiation may have caused a masking of any bystander effects as factors secreted solely by embryonic stem cells may be lost upon their differentiation.

The evaluation of possible bystander interactions between stem cells is also important, as the potential for use of ES cell–derived human feeders is under investigation. Stojkovic et al (Stojkovic et al. 2005) used hES cell line H1 as an autogeneic (isogenically related) feeder system to efficiently support the undifferentiated growth and maintenance of pluripotency of autogenic and allogeneic undifferentiated human ES cells. An advantage of using feeders derived from differentiated human ES cells is that they provide a more secure autogeneic or genotypically homogenous system for prolonged growth of undifferentiated stem cells. Feeders differentiated from the first clinical grade stem cell line could be universally

used, which could eliminate the transfer of pathogens. It is this potentially universal feeder cell line that would be the focus for ensuring that no deleterious cell-to-cell interactions including bystander effects between feeders and stem cells occur. The possibility of bystander signalling from stem cells to feeders and vice versa must be considered. Production of the first clinically used stem cell-derived feeder cell line would have to be tightly monitored to ensure no transference of bystander factors which could potentially damage the progeny of that feeder population. Likewise, a damaged feeder system being used to propagate the stem cells from which the feeders would be derived could also have disastrous consequences. Cell-to-cell interactions may be among the microenvironment factors influencing success of stem cell transplantation therapy and could be responsible for possible transdifferentiation of implanted stem cells.

Effective bystander signalling was observed between mEFs and ES cells. The mouse feeder cells used in this study were mitomycin-C inactivated The mouse feeder cells used in this study were mitomycin-C inactivated. The effects of mitomycin C on the feeder cells cannot be ignored, especially as mitomycin C is known to induce micronuclei in conditioned medium recipient bystander cells (Asur et al. 2009). Mitomycin C has cytostatic actions, inhibiting DNA synthesis and nuclear division and causes telomere shortening (Wick and Gebhart 2005) but no reduction in telomerase activity in human cancer cell lines (Akiyama et al. 1999). When the human fibroblasts were used as feeder cells, mitomycin C treatment affected BJ and hTERT cells in much the same way, causing over a 2-fold increase in DNA double strand breaks in the absence of Cr-exposure. The increase in DNA double strand breaks in cells that were both mitomycin C- and Cr-exposed compared to those cells that were just Cr exposed (in the absence of mitomycin C) was more than 4-fold for both BJ and hTERT cells,

which indicates that the effects of Cr exposure were dominant over mitomycin-C exposure. Here bystander signalling has been demonstrated to increase DNA double strand breaks between stem cells and mouse feeder cells. These results again show that (physiologically) telomerase positive cells are able to generate bystander signals after Cr (VI) exposure.

Therefore, this thesis has highlighted the importance of studying bystander interactions in stem cell culture, as feeder cells provide an environment for the interplay of signalling networks that regulate the fate of embryonic stem cells.

9.1.7 Bystander Effects in Telomerase Positive Cell Lines

In this thesis, when conditioned medium from metal-exposed normal human BJ fibroblasts was transferred to telomerase positive thyroid carcinoma cells, no significant induction of bystander γ-H2AX foci was observed. In addition, co-culture of metal-exposed BJ fibroblasts with telomerase positive human embryonic stem cells was unable to induce γ-H2AX foci in bystander stem cells. In both cases, physiological telomerase expression appears to play a key role in protection against bystander DNA damage induction comparable to that achieved by ectopic expression in the hTERT cells. This phenomenon has also been observed in studies of radiation-induced bystander effects. For example, conditioned medium derived from bystander experiments using exposure of the immortal (telomerase positive (Morgan et al. 1991)) human fibroblast cell line MSU-1 to ^{60}Co gamma-rays when transferred to (telomerase positive (Harle-Bachor and Boukamp 1996)) HaCaT immortal but non-transformed human keratinocytes caused no reduction in bystander cell survival between these two different telomerase positive cell lines. However, when HaCaT cells were exposed to conditioned medium from gamma-ray-exposed cells of their own type, a 61% reduction

in surviving fraction was observed. The human colon carcinoma cell line SW48 showed much lower percentage survival than HaCaT cells when exposed to bystander medium from an irradiated donor cell, this may be related to the high radiosensitivity of this cell line. Medium from irradiated HaCaT cells was able to induce cell killing in either fibroblasts or epithelial cells but medium from irradiated fibroblasts had no effect on epithelial cells (Mothersill and Seymour 1997). Evidence for the telomerase positive status of these cell lines is reviewed by Shay et al (Shay and Bacchetti 1997), who stated that the presence of telomerase is almost always required for immortalization. The work reported by Mothersill et al (Mothersill and Seymour 1997) indicates that the sensitivity of cells in responding to bystander signals may depend on intrinsic cellular characteristics and that bystander effects can be transferred between two different telomerase positive cell lines but perhaps the conditioned medium harvested from irradiated fibroblasts had limited effects compared to the medium of other cell lines. This is in agreement with the results presented in this thesis. hTERT positive cells were unable to respond to a medium-mediated bystander signal harvested from metal-exposed cells of the same type but could both induce a response and respond to a signal from BJ cells. When the normal telomerase negative human fibroblast AG01522 cell line was co-cultured with the (telomerase positive (Pennarun et al. 2005)) T98G glioblastoma cell line after direct α-particle microbeam irradiation, a 2-fold increase in micronucleus induction was observed in bystander fibroblasts (Shao et al. 2005). This was independent of the number of T98G cells irradiated and the number of nuclei traversed by a charged particle. However, when this system was reversed and the normal human fibroblasts were directly irradiated, only a one fold increase in micronucleus induction was observed in the glioblastoma cells. This again hints at the limited ability of fibroblasts to induce bystander effects in other cell types and also at

the protective effect of telomerase status against induction of bystander effects. Although the telomerase positive T98G cells exposed to direct α-particle microbeam irradiation could induce a bystander effect in unirradiated fibroblasts after co-culture, they were more resistant than fibroblasts to the bystander effect. In contrast, when normal human astrocytes directly irradiated with a 2 Gy dose of 250 kVp X-rays were co-cultured with T98G tumour cells, a significant induction of DNA DSBs in bystander tumour cells was observed. When the system was reversed, co-culture of irradiated T98G cells with normal astrocytes resulted in a significant induction of DNA DSBs in the astrocytes (Burdak-Rothkamm et al. 2007). Therefore, it is difficult to draw any firm conclusions regarding the effects of telomerase status on the transmission and receipt of radiation-induced bystander signals. However, in general for medium transfer studies, telomerase positive cells appear protected against bystander effects (Mothersill and Seymour 1997) but this is not always true for co-culture studies where directly exposed cells (whether to metal or radiation) and bystander cells are in close contact very soon after treatment (Shao et al. 2005). This indicates that bystander factors may be released very rapidly from hTERT cells and do not persist long enough to be harvested with conditioned medium.

9.2 Evaluation of Genotoxicity Assays

Recently published guidelines by Albertini et al (Albertini et al. 2000) advise that more than one endpoint should be assayed in order to provide a comprehensive evaluation of genotoxicity. However, there is yet no consensus as for which combination of genotoxic assays to use in each case.

9.2.1 Micronucleus Assay

The Micronucleus-Cyt B Assay was used as an initial genotoxic screening test, because it is considered by many both the easiest validated genotoxic assay to perform and the fastest method to obtain results. Results obtained from the MN Assay need to be examined carefully. First of all, not all acentric fragments and/or mal-segregated chromosomes result in the formation of MN, as a large number of them will inevitably exist within one of the main nuclei and are therefore will not be evaluated with this assay (Fenech 2000). Hence, the number of micronucleated binucleate cells can only serve as an index of chromosomal lesions rather than provide an accurate genotoxic evaluation largely because of the danger of under-estimating the levels of genotoxic damage that hazardous substances may induce. In criticism, this study relied on the recognition of micronuclei using Giemsa staining, which allows visualization of nuclei (including micronuclei) as purple against the background of a pink cytoplasm under a light microscope (Leitz). In some instances, it was difficult to distinguish the small micronuclei from crystals of the Giemsa stain. This may put the reliability of these results in question. However, presumably, the same problems would occur for all treatment combinations and therefore any mis-recognition of micronuclei would be fairly balanced for all dose points. A possible solution for this problem could be the use of the nuclear stain diamidino-2-phenylindole (DAPI), which stains double-stranded DNA blue with little background from the cytoplasm when viewed under an epifluorescence microscope. This may have resulted in easier identification of micronuclei also increasing the speed by which the assay could be carried out. Another criticism of this study lies in the inability to determine whether the micronuclei observed were centromere positive and therefore the result of an aneugenic (chromosome mal-segregation) event; or whether the micronuclei were centromere

negative and therefore the result of a clastogenic (fragmentation) event. A possible solution would be to combine the micronucleus assay with pan-centromeric labelling via fluorescence in situ hybridization (FISH) enabling the distinction of micronuclei as being either centromere positive or centromere negative. As no centromere labelling was carried out with the micronucleus assay, it was not possible to determine whether the MN observed were the result of a clastogenic event involving a chromosome break or an aneugenic event involving chromosome loss. For this reason, the γ-H2AX focus formation assay was carried out to determine the level of DNA double strand break induction. For the micronucleus assay, the way in which it has been performed here is limited. Using a human pancentromeric DNA probe, it can reveal whether a micronucleus contains a whole chromosome chromatid. Those that do not contain centromeres contain acentric fragments, which could suggest a clastogenic action.

9.2.2 γ-H2AX Focus Formation Assay

γ-H2AX foci quantification is not a validated genotoxic assay yet, however a number of groups have used this assay for genotoxic evaluations after studies demonstrated that the phosphorylation of histone H2AX is one of the first events following the formation of DSBs (Rogakou et al. 1998; Ha et al. 2004). γ-H2AX is involved in the recruitment of several DNA repair factors at sites of DNA DSBs. Thus, in essence the occurrence of DNA repair serves as a readily measured indicator of DNA damage, i.e. the abundance of γ-H2AX foci (which were visualized via immunofluorescence) served as a measure of DSB levels. In this project, low levels γ-H2AX foci were detected in control cells, which is in agreement with studies which have revealed that small numbers of gamma-foci are present in cells, even without the purposeful introduction of DNA DSBs. This indicates that the DSBs are a common aspect of normal metabolism. These ‘cryptogenic’ γ-H2AX foci increase in number

during senescence in culture and aging in mice (Sedelnikova et al. 2004). An important artefact that can be associated with γ-H2AX focus quantification is non-specific DNA damage during cell processing or associated with cell death (mediated through apoptosis and/or necrosis); such processes would result in the induction of DSBs. Scientists have reported that γ-H2AX foci appear during apoptosis concurrently with the initial appearance of high molecular weight DNA fragments, indicating that gamma-H2AX formation is an early chromatin modification following initiation of DNA fragmentation during apoptosis (Rogakou et al. 2000). In this study, γ-H2AX foci in untreated cells were minimal and were taken into account when conducting all statistical analyses.

Results from the γ-H2AX focus formation assay provide a plausible explanation for the differences in the genotoxic potential seen with the micronucleus assay between chromium conditioned medium and control medium. The comet (single cell gel electrophoresis) assay could be used as an alternative genotoxicity assay, providing sensitive detection of DNA breaks at the single cell level. Electrophoresis of lysed cells leads to migration of any broken DNA strands producing a 'tail' that when fluorescently labelled can be calculated by manual scoring or automatically using imaging software. According to Fairbairn et al (Fairbairn et al. 1995), as the comet assay provides a visual endpoint, comets can be scored based on their appearance as either damaged, undamaged or with graduations. However, this approach may be limited by observer bias.

9.2.3 Cytogenetics

It appears that there is no metal-induced bystander effect to cause chromosome breaks in hTERT cells 30 days after metal exposure in donor cells. There did appear to

be a direct metal treatment effect in hTERT cells on day 0 and day 5, however the result for day 30 was anomalous. It was expected that results for chromosome breaks would relate to those observed for the percentage of cells containing DNA double strand break foci visualised using anti-γ-H2AX. For example, in directly-treated hTERT cells on day 30 after chromium exposure, the γ-H2AX assay showed that there was an approximate 40% drop in the number of cells containing no γ-H2AX foci. However, it does not quite correlate with cytogenetic results. It has been proposed that although DSBs may result in visible chromosomal abnormalities, most would be probably present but not detectable by cytogenetic analysis (Sedelnikova et al. 2004). However, the results did correlate for day 0 in BJ cells, as the γ-H2AX assay shows that following direct treatment, there was an approximate 20% increase in the percentage of cells containing DNA double strand breaks. Likewise, for the metaphase spreads, there was an approximate 4.9-fold increase in metaphases containing a chromosome break. Other than for direct treatment of hTERT cells on day 30, all the results for γH2AX staining and chromosome breaks were comparable. For example, on day 30 in hTERT cells treated via medium transfer, there was no change in the percentage of cells containing γ-H2AX and there was no change in the level of chromosome breaks observed in the metaphase spreads. In general, the levels of chromosome breaks observed correlated with the number of γ-H2AX foci observed.

In addition, some experiments were carried out during this project for γ-H2AX detection on metaphase spreads. As shown in chapter 4, the individual chromosomes were not well defined and it was therefore not possible to link cytogenetic data to γ-H2AX focus formation. However, the images obtained did indicate that foci were present on the chromosomes and were not due to any background artefacts.

Other cytogenetic aberrations were also observed; these were dicentric chromosomes, tetraploidy (twice the normal diploid chromosome number) and aneuploidy (loss or gain of a chromosome). However, the results obtained for these observations were not repeatable, which put the reliability of the data into question. There were large overlapping errors in the data. Observing any changes in chromosome number required evenly spread metaphase preparations. However, the chromosomes were often bunched together and this made them difficult to count, rendering quantification of changes in chromosome number unreliable. Another challenge in determining the number of chromosomes in a metaphase was the fact that the metaphases could be spread out and it was difficult to determine whether some chromosomes belonged to one metaphase or another. Dicentric chromosomes were also difficult to identify. This could be because they occurred in very low numbers. If the level of nucleoplasmic bridges observed in the micronucleus assay (Figure 4.3) is an indicator of dicentric chromosomes (as stated by Thomas et al (Thomas et al. 2003)), they occurred in fewer than 1.5% of binucleate cells, which could therefore contribute to the inability to identify these aberrations. Another explanation for these difficulties could be that the fixative was added too quickly, which can result in clumping of metaphase chromosomes, making each chromosome difficult to distinguish from another. Also, perhaps some of the cell pellet was lost at each centrifugation, which would render metaphases difficult to score as there would be fewer metaphases to study.

Some modifications to the experimental technique could improve the cytogenetic data. The metaphase spreads were solidly stained with Giemsa. This renders it impossible to view any translocations and therefore limits the depth of knowledge that can be obtained from the assay. The method for bursting the cells with hypotonic solution could have been carried out too slowly, which may explain why there was a

cloud of cytoplasm surrounding the metaphases on the slide, making scoring slightly more difficult. It is also essential to ensure that the hypotonic solution is thoroughly mixed with the cell suspension whilst adding the first few drops. Also, the dropping of fixed cell suspension on to the microscope slides could be carried out closer to the slide rather than further away to aid in the spreading of the chromosomes. To carry out more detailed cytogenetic analysis, the three-colour FISH method could be used in which chromosome specific probes could aid in the identification of trisomies and monosomies.

9.2.4 Microinjections

During this study, a set of microinjection experiments was carried out. This experimental set up has the potential to provide lots of answers in the study of metal-induced bystander effects. To study the bystander effect, the loci of directly metal injected cells must be determined in order to study the induction of any biological effects in neighbouring bystander cells. In this study, the injected cells were stained green with a FITC conjugated dextran molecule. However, it could not be determined if an injected cell had been in a certain position and had subsequently become detached from the coverslip. If this occurred, we may see biological effects in cells that did not appear to be neighbouring a Cr-injected cell, when in fact they were in close proximity, resulting in false negative results. If this project were investigated further, to overcome this problem, cells targeted specifically for injection could be identified using a grid-etched microscope slide and the injection positions recorded with respect to the grid markings. Alternatively, cell tracker vital dyes could be used to label the injected cells and computer software employed to record the exact locations of injected cells. Another problem encountered with the microinjection method was that it could not be determined whether chromium ions were able to leak from the injected cell or the

injection buffer during the injection process and thus be present in the medium of neighbouring uninjected cells. If this were the case, any biological effects may not be attributed to bystander factors but to the direct action of chromium ions on these cells. A method that could be employed to determine chromium ion uptake by non-injected cells is inductively coupled plasma-atomic emission spectroscopy (ICP-AES) (Traore and Meyer 2001). ICP-AES is a fast multi-element technique that uses an ICP source to dissociate a sample into its constituent atoms or ions exciting them to a wavelength characteristic of a particular element - in this case chromium. The injected cells could easily be geographically isolated from uninjected cells by culturing them on separate coverslips, carrying out the injections in a Petri dish with both sets of coverslips sharing the same medium and then separating them to perform analysis after the injection process has taken place. Or, perhaps the dose of chromium in the injection could cause cells to lose membrane integrity resulting in the leakage of metal ions into the culture medium. To look at this possibility in more detail, sham injections could have been carried out in which the metal ion solution was injected in to the culture medium but not directly into the cells. Subsequent observations could have been carried out to determine whether the same level of γ-H2AX focus formation was induced as with microinjections.

A technique could have been developed to quantify the microinjection process more thoroughly, using coverslips etched with a grid pattern. These grids could have been used to pin-point the exact locations of microinjected cells. Therefore, if an injected cell became detached from the coverslip during the washing and staining process, any neighbouring cells that received bystander signals to induce γ-H2AX focus formation would appear as though they were not in contact with a chromium-exposed cell. To rectify this using grid-etched coverslips to pinpoint the exact location of

injected cells, it would still be known that a microinjection occurred in that locus, thus accounting for any DNA double stranded breaks observed in the cells neighbouring the injected cell even if that cell was no longer present. Injection sites can therefore be noted and accounted for whether or not the injected cell is still present. It would also be possible to observe how far, linearly, the signal can travel, to create a radius. The duration of microinjection depended on the amount of pressure exerted on the injection release and the amount of time that the release button was depressed for. This made the microinjection process fairly difficult to standardize as cells were injected with indefinable amounts of injection buffer.

Further study using the microinjection technique could enable the understanding of the nature of cell-to-cell interactions of cells in close contact and removes any deleterious effects of carrying out medium transfer. For example, a bystander signal could be only secreted for a short time and therefore not be present at the time medium is transferred, or a molecule or bystander factor could be lost during the medium transfer process.

9.3 Conclusion

The results presented here show for the first time that there is a bystander effect induced by metals. This is supported by studies using transfer of conditioned medium from metal-exposed cells and co-culture of exposed and non-exposed cells in close contact after microinjection. Although bystander effects have been widely reported following exposure to ionizing radiation, chemical-induced bystander effects are not well characterised and this work could pave the way for a new area of study into the non-targetted effects of exposure to toxic metals and also the role of telomerase status in cellular responses to bystander signalling. The significance of this is clear as so many

humans are exposed to metals through industry, agriculture, and medical or dental prostheses. The varying responses to metal-induced bystander effects in cells with different telomerase expression status could also have implications for the way cancers are treated, as most cancer cells are telomerase positive and could determine the fate of normal healthy tissue surrounding a tumour, which could be detrimentally affected by bystander signalling.

The biological endpoints investigated were the micronucleus assay, γH2AX focus formation assay and cytogenetic analysis. Initial results suggested that BJ cells were more susceptible to such a bystander effect that hTERT cells, which appeared to be protected from this effect. There was no significant induction of micronuclei, nucleoplasmic bridges or DNA double strand breaks in hTERT cells treated with conditioned medium via medium transfer. In contrast, genomic instability was induced by conditioned medium at a level comparable to direct treatment in BJ cells and persisted for at least 30 days. However, results obtained from microinjection studies and medium transfer between the two cell types in which biological effects were induced in bystander hTERT cells suggested that this was not the case.

The early initiation events occurring in metal-induced bystander effects could provide a key to understanding the mechanism of these responses. In general, telomerase positive cells did not respond to bystander signalling when exposed to conditioned medium harvested later than 24 hours post metal treatment. However, when exposed and non-exposed telomerase positive cells were cultured in close contact either immediately or within minutes after treatment (microinjection or mEF and hES cell co-culture), bystander effects characterised by γ-H2AX focus formation were observed. This indicates that any bystander factor(s) released by hTERT cells are secreted very

rapidly post treatment in a mechanism that may be mitochondrially mediated. As the response was generally abrogated by antioxidants, the initiation events probably involve secretion of ROS into the culture medium. These ROS could then set in motion cellular signalling cascades mediated by cytokines such as TNF-α or TGF-β and changes in calcium signalling to result in the induction of biological effects in bystander cells. The key difference between BJ and hTERT cells is the presence of catalytically active telomerase in hTERT cells. hTERT cells may be protected at the telomeres by effective telomere capping (Blackburn 2001) but telomerase is also known to have functions in the mitochondria, which could also contribute to the lack of delayed bystander signalling in telomerase positive cells. Medium transfer between hTERT and BJ cells suggested that the telomerase negative BJ cells are sensitive to factors that hTERT cells are not, as γ-H2AX foci and micronuclei were induced in bystander medium transfer recipient BJ cells. The telomerase negative cells may secrete additional factors capable of inducing biological effects in hTERT cells. Additional study into the differences in conditioned medium between these two cell lines could indicate whether additional factors such as cytokines are present in BJ conditioned medium. Here, a significant reduction of bystander effects was observed using a neutralizing antibody to TNF-alpha. TGF-β could also be a likely candidate for further study, as this is known to play a role radiation-induced bystander effects mediated by reactive species (Shao et al. 2008a; Shao et al. 2008b).

A more detailed toxicity analysis to include apoptotic and mitochondrial DNA responses could reveal even more about the response of telomerase positive cells to metal-induced bystander effects.

9.4 Implications

Consideration of metal-induced bystander effects in risk assessment is controversial as it requires modification of the existing concepts of heavy metal toxicity. It may no longer be possible to relate metal exposure risks to a given metal dose as even an unexposed cell is at risk. Further work is required to compare the metal-induced bystander effect to radiation-induced bystander effects and study in greater detail the effects of varied metal ions, concentrations, duration of medium conditioning and longevity of the bystander factor. In addition, combining metal and radiation treatment to look at both effects together may be important for considering risks of radiation exposure either therapeutically or otherwise in patients with metal prostheses or individuals that have experienced occupational exposure to metals.

Given that hTERT cells are susceptible to radiation-induced bystander effects (Ponnaiya et al. 2004), metal-induced bystander effects may occur via a different pathway or mechanism to those observed following radiation exposure because at least for medium transfer from other telomerase positive cells, hTERT fibroblasts showed no induction of biological effects. However, further study is required to characterise any apoptotic responses and mitochondrial DNA damage that may have remained undetected in this study. Also the role of close cell-to-cell contact in transmission of metal-induced bystander signalling must be further explored and may reveal that secretion of these factors is an early event in cellular responses to toxic metals.

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