RADIATION-INDUCED GENOMIC INSTABILITY AND CELLULAR COMMUNICATION: MECHANISTIC INVESTIGATIONS

Ammar Hassan Jasim Al-Mayah

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Abstract

Communication between irradiated and un-irradiated (bystander) cells can cause damage in cells that are not directly targeted by ionizing radiation (IR); a process known as the bystander effect (BE). BE can also lead to genomic instability (GI) within the progeny of bystander cells, similar to the progeny of directly irradiated cells. The molecular factors that mediate this cellular communication can be transferred between cells via gap junctions or be released into the extracellular media/ microenvironment of cells and tissue following irradiation. Although GI is thought to be a critical step in the onset and progression of cancer, BE response contributions in such processes are still not clear. Therefore, this study was designed to investigate the risks or benefits associated with the induction of non-targeted effects especially BE following exposure to low LET X-ray radiation using two different cell types. Additionally, the project aims to achieve an increased understanding of the mechanisms of non-targeted effects of ionizing radiation by examining the molecular signalling via exosomes within the irradiated, bystander and progeny of irradiated and bystander cell population.

Different cell combinations were established between tumour (MCF7) and non-tumour (HMT-3522S1) human breast epithelial cells using a 6-well plate co-culture system. The cells were irradiated with two doses of X-ray; 0.1 Gy (a diagnostic procedure relevant dose) and 2 Gy (therapeutic dose) and a sham-irradiation dose of 0 Gy (for control groups of experiment). The co-culturing time was 4 hours for all cell combination, whereupon a media transfer approach was used to induce BE within the cells in the exosome part of this study. The early and late cellular damage responses were evaluated by the following biological endpoints: cytogenetic/chromosomal analysis, apoptotic analysis, telomere length and telomerase activity measurements. In addition to these biological endpoints, the comet assay was utilised to estimate the initial and delayed DNA damage within the cells that had been treated with exosomes, previously extracted from the irradiated, bystander and control cell media.

The results showed that 2 Gy direct irradated MCF7 and HMT cells were both able to induce early and late chromosomal damage in the bystander MCF7 and HMT cells. Furthermore, these bystander cells exhibited early and delayed telomeric instability, which could prompt further GI at later time-points. In comparison, 0.1 Gy direct

irradiated MCF7 cells were only able to induce initial and delayed chromosomal damage within the bystander MCF7, which also demonstrated a high level of telomeric instability at early and late time-points. While, bystander HMT cells did not show chromosomal damage after 1, 12 and 24 generations/population doublings following co-culture with 0.1 Gy direct irradiated MCF7 or HMT cells. 0.1 Gy bystander HMT cells did reveal a high level of apoptosis at early and late time points, which might be due the removal of cells with a high level of chromosomal damage. Interestingly, the 0.1 Gy bystander HMT cells exhibited significant levels of telomeric instability at early and late time points, which could contribute to chromosomal instability at later time-points.

The investigation in to the mechanisms of molecular signalling via exosomes showed that the exosomes of irradiated cell conditioned media (ICCM) from MCF7 cells had the ability to induce BE within MCF7 and HMT cells similar to the effects of ICCM following 2 Gy X-ray. The exosomes that were isolated from the MCF7 bystander cell media had a similar effect as the ICCM on the MCF7 and HMT bystander cells. These exosome-bystander cells also showed GI within their progeny after 24 generations and retained the ability to induce cellular damage to fresh un-irradiated MCF7 cells, demonstrating an underlying mechanism for propagating the delayed damage responses. The inhibition of the exosome's cargo molecules by RNase treatment and protein denaturating (boiling of exosmes) significantly abrogated BE and GI in both MCF7 and HMT bystander cells following 2 Gy X-ray. Thus data demonstrated crucial roles for exosome RNA and protein molecules in the non-targeted effects of IR induction.

In summary, our investigations demonstrate that BE has detrimental consequences within the tumour and non-tumour breast epithelial cells (MCF7 and HMT3522S1) following low and high doses of X-ray irradiation, and these detrimental consequences are frequently mediated by exosomes that contain RNA and protein molecules. Inhibition of these molecules can abrogate BE and GI following a radiotherapy dose, which can potentially have an application in clinical radiotherapy.

Publications

1. **Al-Mayah AH**, Irons SL, Pink RC, Carter DR, & Kadhim MA. 2012. Possible role of exosomes containing RNA in mediating nontargeted effect of ionizing radiation. Radiation Research. 177(5): 539-45.

2. Chapman KL**, Al-Mayah AH**, Bowler D, Irons S. & Kadhim MA. 2012. No influence of serotonin levels in foetal bovine sera on radiation induced bystander effects and genomic instability. International Journal of Radiation Biology (In press).

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Abbreviations

BE Radiation induced-bystander effects
BEIR Committee on Biological Effects of Ionizing Radiation
BSA Bovine serum albumin
°C degrees Celsius
cGy centigray (1 centigray = 0.01 gray)
CHAPS 3-[(3Cholamidopropyl) dimethyl ammonio]-1-propanesulfonate
Da Dalton
dH2O Distilled water
DMSO Dimethyl Sulfoxide
DNA Deoxyribonucleic Acid
DSB DNA double strand breaks
dsDNA Double stranded DNA
EDTA Ethylenediaminetetraacetic
EGF Epidermal growth factor
FCS Foetal calf serum
FISH Fluorescence in-situ hybridization
GI Radiation induced-genomic instability
Gy Gray
HR Homologous recombination
hr Hour
IL Interleukin
IR Ionizing Radiation
KCl Potassium chloride
LET Linear Energy Transfer
M Molar
MEM Minimum Essential Medium
mg Milligram
miRNA MicroRNA
mins Minutes
ml Millilitre
mm Millimetre
mM Millimolar
MW Molecular weight
µg Microgram
µl Microlitre
µm Micrometer
ng Nanogram
NHEJ Non-homologous end joining
nm Nanometre
NO Nitric oxide
OH Hydroxyl group
p p-value
PBS Phosphate Buffered Saline
PD Population doubling
PNA Peptide nucleic acid
Q-FISH Quantitative in-situ hybridization

RNA Ribonucleic acid
rpm Revolutions per minute
ROS Reactive oxygen species
S Significant
SEM Standard error of mean
N/S insignificant
SSC Sodium chloride sodium citric acid
TAU Telomerase arbitrary unit
TFI Telomeric fluorescence intensity
TGF-β Transforming growth factor-beta
TNF-α Tumour necrosis factor-alpha
UV Ultra violet
v Volts
v/v Volume per volume
w/v Weight per volume

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CHAPTER 1

INTRODUCTION

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Chapter 1: Introduction

1.1 Direct and indirect action of ionizing radiation

Ionizing radiation (IR) is part of human environment, discharges from the medical, environmental, occupational and radioactive sources. IR includes high linear energy transfer (LET) radiation such as α- particles (nuclei of helium atoms) and low LET radiation such as x and γ- rays (electromagnetic radiation), which emit from natural sources (Hall and Giaccia, 2006). LET widely varies depending on the speed and charge of the particle involved (Nias, 1990, Grosch and Hopwood, 1979). It has been well documented that IR induces DNA damage in biological material either by direct or indirect actions of radiation (Burdak-Rothkamm *et al*., 2009, Folle, 2008, Koturbash *et al*., 2006b, Abraham *et al*., 2003, Suzuki *et al*., 2003, MacDonald *et al*., 2001, Bebb *et al*., 1998, Ikushima, 1987). IR produces a fast recoil electron that causes either direct damage by interaction with DNA directly or indirectly through free radicals production, which induce DNA damage (Hall and Giaccia, 2006).

Cellular damage may occur directly when the radiation interacts with the critical target (DNA) directly, for example α– particles (Hall and Giaccia, 2006). In contrast, indirect action of radiation occurs when radiation interacts with other atoms of molecules in the cell particularly water, leading eventually to production of the free radicals such as $OH^{\bullet}$ (Nias, 1990). Free radicals can induce DNA damage *in vitro* (Rao *et al*., 2008, Jagetia *et al*., 2003) and *in vivo* (Tanito *et al*., 2007, Mendiola-Cruz and Morales-Ramirez, 1999). In Summary, 'direct' action of IR refers to DNA hit directly by IR tracks, whilst 'indirect' action of IR occurs through production of free radicals that induce DNA damage. Biological effects can be induced by x or γ- rays directly or indirectly. About two-third of the biological damage by x-rays occurs by indirect action (Hall and Giaccia, 2006).

1.2 Targeted effects of IR

The target theory assumes that cells must have at least one critical site or target that is hit by radiation to induce cellular damage or cell death (Marshall *et al*., 1970). The nuclear DNA is considered as the principle critical target for IR induced cell death, chromosomal or chromatid aberrations, mutation and cell transformations (Kraft *et al*., 1992, Radford *et al*., 1988). However, some evidence postulate that cell membranes might be a target for biological effects of IR, which induce cell death (Mishra, 2004, Ross, 1999, Alper, 1977).

According to the target theory DNA damage occurs during or very shortly after irradiation. It can be also explained as potential biological consequences within one or two cell generations (Ward, 2002). The major types of DNA damage that are induced by IR are: i). Single-Strand Break (SSB), in which, only one of the two strands of DNA double helix is broken (Bryant *et al*., 2003, Bryant, 1998). SSB is not usually observed/visualised, because it is rapidly repaired using the opposite strand as a template (Bailey and Bedford, 2006, Bryant, 2004), ii) Double-Strand Breaks (DSB), these occur in both strands of DNA (Mozdarani and Bryant, 1987, Bryant, 1984), iii) DNA base damage, caused by IR damaging effect on purine and pyrimidine DNA bases (Klunglanda and Bjellandb, 2007, Ward, 1988) and iv) DNA-DNA and DNA-protein crosslink, which causes DNA replication arrest and cell death if the crosslink is not repaired (Ward, 1988). Ionizing radiation can also produce combinations of these lesions within a few base pairs, these are known as clustered or complex lesions (Goodhead, 1994)

The critical lesion leading to chromosomal and chromatid aberrations or cell death is DSB which can be induced by both IR (Bryant, 1984) and free radicals (Frankenberg-Schwager *et al*., 2008, Han *et al*., 2007). The DNA DSB can be repaired by two repair

mechanisms depending on the phase of the cell cycle: Non- homologous end joining (NHEJ), which usually occurs in the G1 phase of cell cycle, when such template is not available (Klug and Cummings, 2003), or by homologous recombination repair (HRR), this occurs predominantly during S and G2 phase of the cell cycle, when undamaged sister chromatid act as a template (Griffin and Thacker, 2004). Non-repair of DSB or misjoining of broken chromosomes leads to chromosomal aberrations, which fall into two types: a) Unstable chromosomal aberrations, which are considered lethal to cells such as dicentric chromosome (Hall and Giaccia, 2006) and b). Stable chromosomal aberrations, which are usually non- lethal, for example reciprocal translocation (Klug and Cummings, 2003).

1.3 Non- targeted effects of IR

As well as targeted effects of IR, which can be induced by deposition of energy in the nucleus of irradiated cells, recent evidence has demonstrated that IR can cause biological effects in the un-irradiated cells, which have been in the vicinity of irradiated cells, in a response known as the bystander effect (BE) (Mothersill and Seymour, 2001, Mothersill *et al*., 2000), or in the progeny of irradiated cells radiation-induced genomic instability (GI) (Kadhim *et al*., 1995). Both BE and GI have been reported as a non-targeted effect of IR (Morgan, 2003). In addition, IR can induce another non- targeted effect known as the adaptive response, in which, cells that are exposed to a very low, priming dose, of IR prior to being challenged by a high (acute) dose of radiation, have increased protection compared to cells that are exposed to a challenged dose alone. The priming dose is called the adaptive response which induces cell resistance against high doses of IR (Mothersill and Seymour, 2006, Bonner, 2003, Barquinero *et al*., 1995, Khandogina *et al*., 1991, Schmid *et al*., 1989). As well as abscopal effect is considered as a non-targeted effect of IR. Abscopal

effect is defined as the biological reaction within the un-irradiated cells that are far away (outside the field/zone of irradiation) from the irradiated cells following irradiation (Peter *et al*., 2007).

1.3.1 Bystander effect (BE)

The bystander effect (BE) is defined as the induction of biological effects in the cells that are not directly hit by radiation, but are neighbours to irradiated cells (Little, 2006, Mothersill and Seymour, 2001, Seymour and Mothersill, 1999, Mothersill and Seymour, 1998, Prise *et al*., 1998) (Figure 1.1). BE amplifies the consequences of IR and exaggerates the cellular damage in un-irradiated cells, such as sister chromatid exchange, gene mutation (Nagasawa and Little, 2002), apoptosis (Zhu *et al*., 2005), transformation (Weber *et al*., 2005) and chromosomal aberrations (Schollnberger *et al*., 2006, Lorimore *et al*., 1998). BE was initially demonstrated by Nagasawa and Little in 1992 (Nagasawa and Little, 1992). They exposed Chinese hamster ovary cells to a dose of α- particles where only 1% of cells population was traversed by an α- particle track. However, 30% of the cell population showed chromosomal damage, with a significant increase in the frequency of sister chromatid exchange.

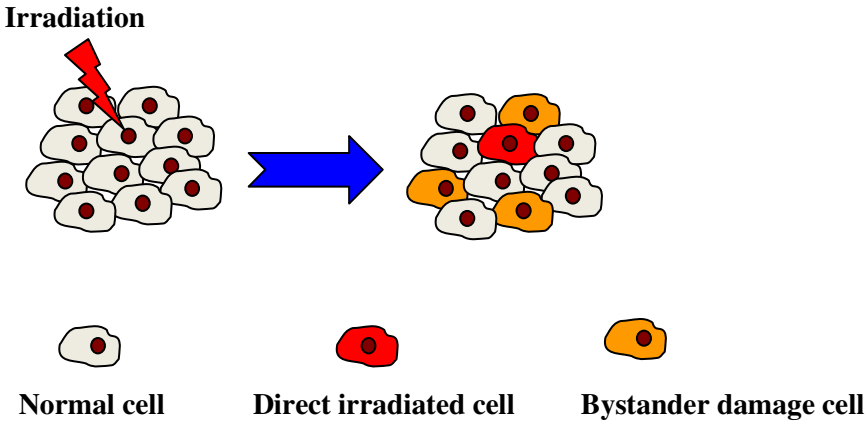


Figure 1.1: Scheme of radiation- induced bystander effect.

BE does not demonstrate a linear relationship to IR dose; that means BE is maximally induced by very low doses, suggesting a switch-on mechanism for its activation (Ding *et al*., 2005). Hickman *at al.,* have reported that rat lung epithelial cells showed BE post- exposure with low doses (as low as 0.6 cGy) of α- particles, indicating a number of cells having increased level of p53 protein. There was no evidence of threshold α-particle caused an elevation in the level of p53 protein in bystander cells. This increase in the level of p53 also occurred in X-irradiated cells. However, no increase was observed in cells that were hit with less than 10 cGy of X- ray, indicating the existence of a higher DNA damage threshold for sparsely IR (Hickman *et al*., 1994). Lehnert and Goodwin have similarly irradiated human fibroblast cells with relatively low dose of α- particles, excessive sister chromatid exchanges were observed in bystander cells (Lehnert and Goodwin, 1997). Other studies have showed that radiation- induced bystander effect can be induced by a low dose of IR such as X-ray and α-particle (Lewis *et al*., 2001, Zhou *et al*., 2000, Deshpande *et al*., 1996).

Generally, it has been demonstrated that BE can be induced very rapidly after irradiation. It has been well established that signal(s) from hit reporter cells can induce damage in non-hit cells (Nagasawa and Little, 1999, Mothersill and Seymour, 1998), depending on the cell type (Hickman *et al*., 1994), and the cell density (Ballarini *et al*., 2006). Mothersill and Seymour demonstrated that media from irradiated human epithelial cell line can induce BE in un-irradiated cells. However, media from irradiated human fibroblast had no effect on un-irradiated epithelial cells (Mothersill and Seymour, 1997). Another experimental evidence has suggested that cell density is important in bystander mutagenesis and the oncogenic transformation frequency between irradiated and un-irradiated cells. BE was observed to be higher in

high density than low density cultures afterα- particle irradiation (Mitchell *et al*., 2004).

There is strong evidence to demonstrate that BE has a non- linear dose dependence (Brenner and Sachs, 2002, Brenner *et al*., 2001). Studies suggest that IR can give a no- threshold effect, whereas a low dose threshold was able to cause BE which, was maximally induced by very low doses (0.2Gy α- particle, and between 0.3- 0.5 Gy X-ray) (Schettino *et al*., 2005, Brenner *et al*., 2001). These studies have confirmed a binary all-or-nothing model of triggering the bystander response (Smilenov *et al*., 2006, Schettino *et al*., 2005, Brenner *et al*., 2001), as shown in figure 1.2.

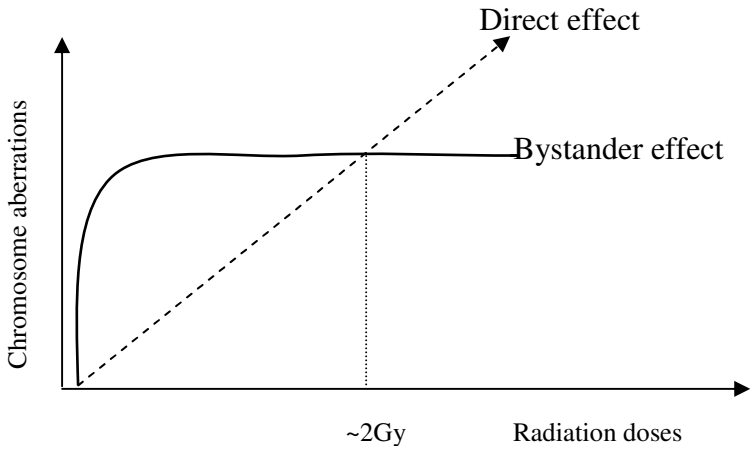


Figure 1.2: Diagram of bystander all or nothing model.

However, Studies by Portess *et al.* suggested that apoptotic levels were dose-dependent event in the rat bystander fibroblast cells using γ-ray and α-particles (Portess *et al*., 2007). Moreover, normal human blood lymphocytes showed a dose-dependent apoptotic responses following different doses of γ-ray irradiation (Pandey *et al*., 2011). Much evidence has proved that BE responses can by dose dependent by measuring apoptotic levels as shown in figure 1.3 (Vinnikov *et al*., 2012, Buonanno *et al*., 2011).

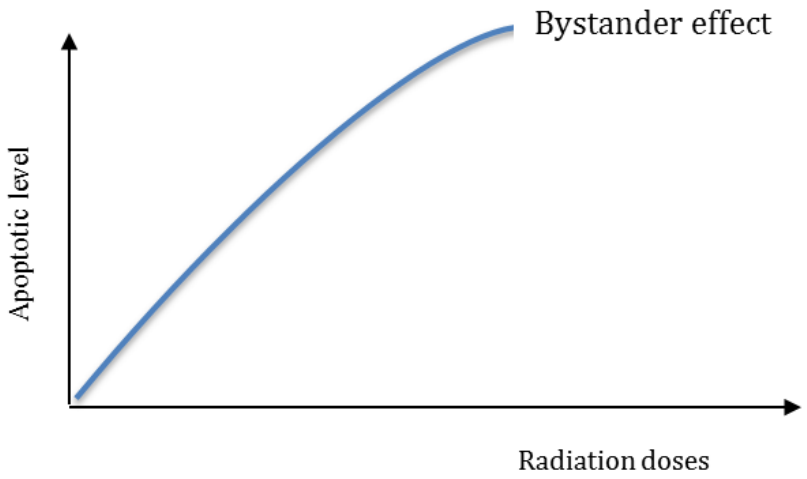


Figure 1.3: Diagram of bystander effect is a dose-dependent phenomenon.

1.3.1.ii Bystander- induced apoptosis

Apoptosis or programmed cell death occurs in multicellular organisms (Potten and Wilson, 2004). It is well documented that apoptosis can be induced by bystander factors (Albanese and Dainiak, 2000, Banerjee *et al*., 2005, Ding *et al*., 2005, Brochu *et al*., 1999). Lyng *et al*. have shown that IR can cause loss of mitochondrial membrane and increase calcium level and ROS in human keratinocytes (HPV-G) post gamma irradiation. The latter events can initiate apoptosis in un-irradiated HPV-G cells, which are fed with media from irradiated HPV-G cells (Lyng *et al*., 2002). Other publications have suggested that the induction of similar bystander signalling using the microbeam can induce apoptosis in HPV-G cells (Lyng *et al*., 2006a). Belyakov *et al.* demonstrated that BE can induce apoptosis and micronucleated in primary urothelial explants (Belyakov *et al*., 2002). Recent publications have postulated that human normal blood lymphocytes showed different significant levels of apoptotic induction following low and high doses of γ-ray irradiation. The study suggested these differential responses of apoptosis were significantly associated with

the levels of intracellular reactive oxygen species (Pandey *et al*., 2011). Moreover, Vinnikov and other authors have reported that bystander primary human peripheral blood mononuclear cells displayed dose dependent apoptotic responses following γ-ray irradiation (Vinnikov *et al*., 2012)

1.3.1.i Mechanisms of BE

Experimental evidence has indicated that irradiated cells secret toxic factors that are transmissible to recipient un-irradiated cells through GJIC or irradiated cell cultured media. Several mechanisms to regulate BE, have been proposed, these include: secreted soluble factors and oxidative metabolism. However, the mechanisms of bystander signalling are still unclear (Ballarini *et al*., 2006, Azzam *et al*., 2004).

Lyng *et al.,* used human papilloma virus- immortalized keratinocytes (HPV-G), which were cultured with irradiated cell-conditioned media (ICCM), to induce BE. They showed that ICCM caused a rapid increase in calcium, which was found to be an important modulator of bystander response (Lyng *et al*., 2006b). Lyng *et al*. also showed that pathways of the mitogen- activated protein kinase (MAPK) have been associated with growth factor- mediated regulation of cellular events such as proliferation, senescence, differentiation and apoptosis after cell exposure to ICCM (Lyng *et al*., 2006b). Another study has reported that calcium can modulate cell cycle functions, cell cycle regulation and can lead to apoptosis (Bygrave and Roberts, 1995). Furthermore, Shao *et al.,* working with T98G glioma cells and AG01522 fibroblasts, exposed to ICCM, have demonstrated that the early response in radiation-induced BE, may be due to calcium signalling. Moreover, they observed that calcium fluxes and BE were inhibited when the irradiated T98G were treated with amino guanidine (an inhibitor of nitric oxide (NO) synthase), and when the irradiated

AG01522 cells were treated with Dimethyl sulphoxide (DMSO), a scavenger of reactive oxygen species (ROS). They reported that NO and ROS may be linked in the bystander response in their system (Shao *et al*., 2006) and it is well documented from many studies that both ROS and NO are involved in bystander responses (Little, 2007, Shao *et al*., 2003b, Shao *et al*., 2002, Bishayee *et al*., 2001). Shao *et al*. have suggested that the concentration of NO in the co- culture media depends on the LET and dose of radiation. They showed a low concentration of NO can enhance cell proliferation, which has an important role in media- mediated BE (Shao *et al*., 2003a). Azzam and *et al.* have reported that micronucleus formation in bystander population from a confluent culture of irradiated normal human diploid fibroblast cells can be induced by superoxide and hydrogen peroxide (Azzam *et al*., 2002). However, other published data has suggested that increased ROS production in irradiated cells is not a substantial trigger of a bystander signal(s) (Kashino *et al*., 2007a). Much evidence has demonstrated that cytokines are implicated in the bystander response (Facoetti *et al*., 2006, Lorimore *et al*., 2003, Lorimore and Wright, 2003). Shao *et al*. observed that TGF- beta 1 can be released from irradiated T98G cells and can cause BE through production of free radicals leading to DNA damage in un-irradiated cells (Shao *et al*., 2008a). One such study reported an increase in the levels of expression of both replication protein A (RPA), which is involved in the DNA replication, repair and recombination, and apurinic/ apyrimidnic endonuclease (APE), which is implicated in the base excision repair pathway, in the bystander cells. The increased expression of RPA and APE might be due to DNA strand break and oxidised base lesion in the DNA of bystander cells (Balajee *et al*., 2004). More recent study has linked the mitochondrial DNA and induction of BE. The study suggested that BE is an energy dependent process. The authors used mitochondrial inhibitors rotenone and

oligomycin treated ICCM and ICCM without inhibitors with lymphoblastoid cells. They proved that cells treated with inhibitors ICCM did not exhibit an induction of BE compared to the ICCM without inhibitors treated cells. They suggested that mitochondrial ATP synthesis and entirely mitochondria function are necessary for BE generation (Rajendran *et al*., 2011). Furthermore, Kostyuk and other workers have postulated that extracellular DNA can play an important role in BE induction in human umbilical vein endothelial cells underlying ROS and NO-mediated BE (Kostyuk *et al*., 2012).

As yet, the exact mechanism of BE is not fully known. However, interaction between hit and non- hit cells may happen in at least two separate pathways: through gap junction intercellular communication (GJIC) (Azzam *et al*., 1998) or by cell culture mediated factors (Hickman *et al*., 1994).

a. Gap junction- mediated BE

The gap junction or nexus is common to many types of cells, especially epithelial, cardiac, and smooth muscle cells and some nerve cells (Telford and Bridgman, 1995). Gap junction consists of a hexamer of multipass transmembrane proteins with a central 1.5nm hydrophilic pore which, form a unit called a connexon (Paulsen, 2000, Ross *et al*., 1995). Connexons extend across a 2nm gap between cell membranes like a small pipes (Vaughan, 2002) allowing small molecules with molecular weight below 1500 Daltons to pass between cells (Junqueira *et al*., 1995).

There is much experimental evidence suggesting that BE can be induced through GJIC. Azzam *et al.*, proposed that GJIC regulates radiation-induced BE (Azzam *et al*., 2003). Bishayee *et al.* used Chinese hamster V79 cells, which were labelled with tritiated thymidine and mixed with unlabelled cells. They hit the labelled cells by

short- range β- particles. Cells were treated with dimethyl sulphoxide (DMSO), which is hydroxyl scavenger and lindane, a GJIC inhibitor. The results demonstrated that DMSO and lindane significantly protected unlabelled or bystander cells. In addition, they have suggested that BE can be induced through GJIC by free radicals (Bishayee *et al*., 2001). Zhou *et al.* have reported that the mutant yield significantly decreased in cells treated with lindane, post- irradiation. They confirmed that GJIC plays a critical role in the bystander phenomenon (Zhou *et al*., 2002). Other evidence using rat liver epithelial cells showed that the spatial proximity of cells is a crucial element for transmitting growth stimulation signals from irradiated cells to neighbouring un-irradiated cells (Gerashchenko and Howell, 2003).

b- Secreted transmissible factors-mediated BE

As well as GJ- mediated BE evidence, several approaches such as media transfer and co-culture experiments have established that BE can be induced in un-irradiated cells by media from irradiated cells. Anzenberg and other authors have documented that media from irradiated DU-145 human prostate carcinoma cells are able to cause bystander phenomenon in both un-irradiated DU-145cells and AG01522 human fibroblast. The bystander response of both types of cells was different to the same media-mediated signal(s) (Anzenberg *et al*., 2008). Basker, *et al*. showed that irradiated media from different cell types, can induce BE in un-irradiated cells depending on radiation quality (Baskar *et al*., 2007). Yang *et al.* suggested that media from irradiated cells has toxic factors that can cause BE in un-irradiated cells (Yang *et al*., 2005). Kanasugi, *et al*. observed that media from normal human fibroblast cells (post- exposure to low and LET radiation) can induce BE in un-irradiated cells through nitric oxide (NO) production. They have concluded that irradiated cells secrete NO and other molecules, which transmit radiation effects to un-irradiated

cells, leading to chromosomal aberrations (Kanasugi *et al*., 2007). Furthermore, media- mediated bystander events have been also well documented in other publications (Azzam and Little, 2004, Coates *et al*., 2004, Mothersill and Seymour, 2004). Some data has suggested that GJIC is not very important in mediating the bystander effect. Princen *et al*., observed that BE was not abolished in two type of cell lines (DHD/K12 and 9L), when they treated these cell lines with 18 alpha-glycyrrhetinic acid or 1- octanol (GJIC inhibitor). 9L cell line exhibited an extreme BE response, whilst, DHD/K12 showed a moderate BE. Some data suggests that BE is mediated by soluble factors from the cell line's media (Princen *et al*., 1999). Recent evidence has reported that irradiated human melanoma cells could induce BE in the un-irradiated human fibroblast cells co-culture system following irradiation. The authors suggested that the irradiated cells could increase the ROS level in the bystander cells, leading to produce high level of MN and apoptosis (Widel *et al*., 2012)

1.3.1.ii Bystander effect in vivo

Several studies demonstrated that BE can be induced *in vivo* (Azzam *et al*., 2003, Brooks, 2004, Mancuso *et al*., 2008), Kassis observed that specific irradiation of human tumour cells *in vivo* can cause BE in subcutaneously growing tumours (Kassis, 2004). Watson *et al.* transplanted a mixture of irradiated and labelled un-irradiated bone marrow cells of CBA/H mouse into female recipients; cytogenetic analysis results demonstrated a significant induction in the level of chromosome aberrations in the labelled un-irradiated cells. These results confirmed the induction of bystander effects *in vivo* (Watson *et al*., 2000). Similarly, BE has been observed in lead-protected medical grade shield mouse spleens, following cranial X-irradiation in an investigation to determine levels of DNA damage, cellular proliferation, apoptosis and

p53 protein in bystander spleen tissue (Koturbash *et al*., 2008). The study demonstrated that cranial irradiation was able to increase DNA damage, p53 expression, apoptosis and altered levels of cellular proliferation in bystander spleen tissue (Koturbash *et al*., 2008).

1.3.2 Radiation- induced genomic instability (GI)

It is well accepted that IR, when passed through biological tissue, induces cellular damage as a consequence of the deposition of energy (Hall and Giaccia, 2006, Dewey *et al*., 1995, Hickman *et al*., 1994). Additionally, there is evidence of an elevation in appearance of *de novo* chromosomal aberrations (Hofman-Huther *et al*., 2006), gene mutations (2005) and reproductive cell death (Belyakov *et al*., 1999) in the progeny of irradiated cells, which is defined radiation- induced genomic instability (GI), as shown in figure 1.4

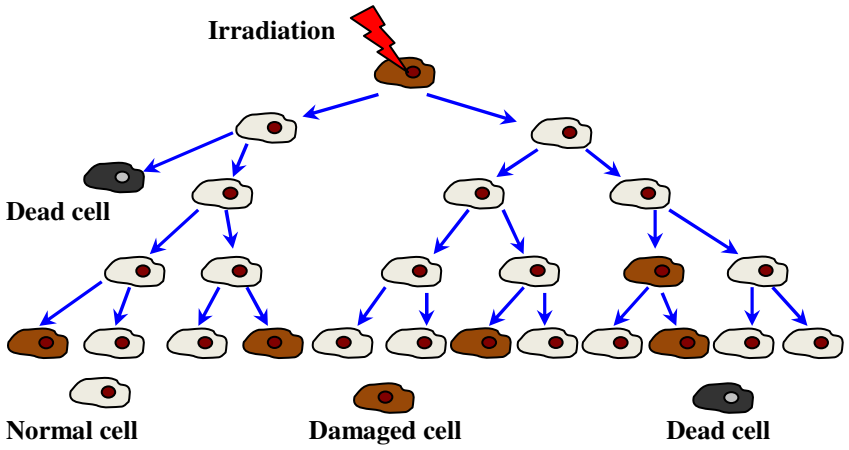


Figure 1.4: Scheme of radiation- induced genomic instability.

Kadhim *et al.* reported that type of radiation exposure, cell type and cell susceptibility, are factors which play important roles in the induction of chromosomal instability (Kadhim *et al*., 1995). Furthermore, it has been shown that duration of irradiation also has an important effect on GI in V- 79 Chinese hamster cells,

irradiated by gamma- ray in dose of 0.5 Gy at powers of 0.48 Gy/min (an acute irradiation) and 0.0485 Gy/min as a prolonged irradiation. Micronuclei (MN) formation was employed as an endpoint and measured after 20 doublings in both acute and prolonged population cells. Frequency of MN in the prolonged population cells was high and remained at this high level during 40-60 generations. In contrast, the number of MN started to reduce after 20 generations following acute irradiation (Antoshchina *et al*., 2005). Chang *et al.* observed a significant elevation in the percentage of MN in the immature reticulocytes (RET) and the mature normo-chromatic erythrocytes (NCE) of three types of mice (wild- type, hemizygotes and nullizygotes) post- exposure to an acute dose of highly charged and energetic (Ohzeki *et al*.) iron radiation. The study also showed a significant increase in p53 levels in both cell types (RET and NCE) of all three types of animals compared to control groups. In addition, the elevation in the percentage of MN in RET of wild- type and hemizygotes returned to the control level after 9 days post- radiation. However, the MN level in the nullizygotes mice persisted for 56 days. The authors demonstrated that persistence of elevation of micronuclei number depends on the p53 genetic background of animals. They suggested that p53 gene function may impact in the iron particle radiation- induced genomic instability (Chang *et al*., 2000). Nevertheless, Kadhim *et al.* have concluded that α-particle-induced chromosomal instability is independent of the p53 status of the human lymphoblast cells (Kadhim *et al*., 1996). Other data showed that genomic stability can be maintained by telomeres; i.e. genomic instability and gene amplification can be implicated in the shortening or loss of telomeric repeats or altered telomere chromatin structure and this can be correlated with telomere dysfunction such as chromosome end-to-end associations (Misri *et al*., 2008). Furthermore, folic acid deficiency can be involved in genomic instability

through increasing the cell sensitivity to radiation- induced genome damage (Fenech, 2006, Beetstra *et al*., 2005).

1.3.2.i Genomic instability and carcinogenesis

Most publications in the last two decades have suggested that GI can lead to initiation of cancer *in vitro* (Yan *et al*., 2006, Tawn, 2005, Preston, 2005, Chow and Choudhury, 2005). In this regard, Sabatier *et al.* suggested that GI can lead to genetic alterations that have an effect on chromosome structures, particularly telomeres. Telomeric instability has been shown in non-senescent cells from patients predisposed to cancer (Sabatier *et al*., 1995). Data from other evidence demonstrated that IR-induced ROS can cause DNA base modification in genomic DNA of lymphocytes of cancer patients exposed to radiotherapy. Some of these base modifications could lead to mutagenesis in critical genes and ultimately to secondary cancer such as leukaemia (Olinski *et al*., 1998). Piechowski suggested that GI is an important factor implicated under the rubric of malignant transformation (Piechowski, 2005). Some of the mechanisms, which are linked in the development of sporadic cancers, are linked with DNA DSB- induced gene translocation and GI, conferred by loss of DNA repair (Allan and Travis, 2005).

1.3.2.ii Mechanism of radiation-induced genomic instability

The expression of GI is observed in a large proportion of irradiated cells (about 10-20%) with low to moderate doses, compared to targeted mutation frequency which is typically less than 10^{-4} per Gy (Stewart *et al*., 2007). The observed high frequency of instability, as well as the lack of significant evidence of the involvement of DNA DSBs per cell in the initiation of instability, has led to the speculation that alterations in expression that disrupt cellular homeostasis may underlie induced instability (Baverstock, 2000). Evidence also points to the possible involvement of indirect, untargeted interactions between cells and complex cytokine-like signal transduction

processes (Natarajan *et al*., 2007, Moore *et al*., 2005). Instability might additionally arise through epigenetic mechanisms (Little, 1998), for example DNA methylation of a cytosine residue located within CpG dinucleotides. (Latchman, 1998). Methylation of cytosine is controlled by DNA methyltransferase (Turner, 2001), this molecular process regulates gene expression without DNA sequence changes (Lee, 2007) and has an important role in tumour formation (Gaudet *et al*., 2003). Several studies have been conducted in order to investigate the possible involvement of DNA methylation in the mechanism of genomic instability induction (Kovalchuk and Baulch, 2008, Kovalchuk *et al*., 2004, Gaudet *et al*., 2003). Dodge *et al*. observed that DNA hypomethylation cells showed aneuploidy, polyploidy and chromosomal breakage. These results thus suggest that DNA hypomethylation can induce genomic instability, which in turn leads to spontaneous immortalization (Dodge *et al*., 2005). Another study found evidence for spread dysregulation of CpG methylation persisting up to 20 population doublings following irradiation within the surviving progeny of directly irradiated cells and bystander cells, using an arbitrarily primed methylation sensitive PCR (Kaup *et al*., 2006). Fenech has suggested that DNA methyltransferase inhibition can lead to micronucleus formation either via chromosome breakage or chromosome loss (Fenech, 2006). Koturbash has investigated transgenerational genomic instability i.e. transgenerational carcinogenesis in offspring upon parental exposure. The offspring showed profound changes in DNA methylation, which led to genome instability and often served as a precursor for transgenerational carcinogenesis (Koturbash *et al*., 2006a). As well as epigenetic mechanisms and the role of miRNA can maintain the non-targeted effects of IR (Ilnytskyy and Kovalchuk, 2011).

1.3.3.ii Telomeres and genomic instability

Telomeres are nucleoprotein complexes consisting of specialised non-coded DNA sequences (TTAGGG in vertebrates) at the ends of eukaryotic chromosomes

(Blackburn, 1991). The main function of telomeres is to protect the chromosomes from degradation and from fusion by capping the ends of chromosome (Song *et al*., 2009, Misri *et al*., 2008), and also to provide the necessary templates for DNA polymerase during DNA lagging strand replication. The length of telomeres in human somatic cells is approximately 10 to 15 kb (Kipling, 1995), but they can shorten by 50 to 200 base pairs (bp) with each cell division (Huffman *et al*., 2000), because of end-replication problems (Levy *et al*., 1992). Thus, telomeres limit the replicative capacity of somatic cells, as well as playing a critical role in cancer-suppressor mechanisms through cell senescence; they therefore serve as a mitotic clock (Campisi, 2001, Campisi *et al*., 2001, Wright and Shay, 1992). Telomeres are synthesised and maintained by a ribonucleoprotein enzyme called telomerase. The main sub-units of telomerase enzyme are telomerase reverse transcriptase (Bryant *et al*., 2002) and telomerase RNA(TR), which work to add TTAGGG repeats to 3' end of DNA strands (Chan and Blackburn, 2004).

Much evidence has reported that telomeric instability and telomerase activity disorder frequently lead to chromosomal instability and can predispose to cancer formation (Keller *et al*., 2009a, Faure *et al*., 2008, Salin *et al*., 2008, Nasir *et al*., 2001, Dhaene *et al*., 2000, Norrback and Roos, 1997). Svenson *et al.* have reported from a study of 711 female participants, using real-time PCR to measure telomere length, that long telomeres of peripheral blood cells can be involved in increased risk of breast cancer (Svenson *et al*., 2008).In contrast, other studies have shown that telomere shortening, due to loss of capping function, can be implicated in chromosomal instability and cancer risk (Boukamp *et al*., 2005). The lack of capping function of telomeres can lead to end-to-end chromosomal fusion resulting in the formation of anaphase bridges, translocations, deletions and/or amplifications (Stewenius *et al*., 2007, Boukamp *et*

al., 2005, Stewenius *et al*., 2005). Jang *et al*. observed that lung cancer can be associated with telomere shortening and genomic instability in humans (Jang *et al*., 2008). Furthermore, by using a telomere/centromere-fluorescence in situ hybridisation (T/C-FISH) technique, Lange *et al*. have suggested that telomere shortening relates to the generation of chromosomal instability (Lange *et al*., 2010). Moreover, telomere shortening causes non-reciprocal translocation and aneuploidy, and links with high rates of malignant diseases in humans (Calado, 2009). Evidence also suggests that telomeric instability can promote structural and numerical chromosome aberrations in human mammary epithelial cells (Dickey *et al*., 2009).

Other studies have documented that high telomerase activity mediates genomic imbalance and cancer formation (Artandi and Cooper, 2009, Gumus-Akay *et al*., 2009, Akimcheva *et al*., 2008, Al-Wahiby and Slijepcevic, 2005). In mice, deficient telomerase activity can lead to telomere shortening, which can initiate tumour formation by induction of chromosomal instability (Djojosubroto *et al*., 2003). In contrast, Brachner *et al.,* have reported that excessive activation of telomerase, can result in a rapid elevation in the number of telomere sequence repeats. Additionally, aggressive growth in the number of telomere sequences can be induced by high hTERT (human telomerase reverse transcriptase) expression without changing the level of chromosomal instability (Brachner *et al*., 2006).

a. Telomerase can be implicated in cancer

Abundant evidence has proved the involvement of telomerase in cancer formation (Asai *et al*., 1998, Albanell *et al*., 1997). Bednarek *et al.* induced a premalignant papilloma in mouse skin using chemical carcinogenesis. A high induction level of telomerase activity was observed in the mouse skin papilloma cells after 30 weeks of treatment. In addition, aneuploidy and abnormal growth were detected as a delayed response that might be linked to an increase in the telomerase activity (Bednarek *et*

al., 1995). Other researchers have suggested that the catalytic telomerase sub-unit, hTERT, expression can relate to human squamous cell carcinomas (Boldrini *et al*., 2003). Moreover, it is well documented that telomerase activity is associated with hTERT gene expression in the ovarian cancer cell (Sun *et al*., 2007). In contrast, one study proved that hTERT can stimulate vascular endothelial growth factor (VEGF) in telomerase activity- independent, which might play an important role in aging and cancer (Zhou *et al*., 2009).

b. Targeting telomerase as a cancer therapy

Because of resistance of particular cancer cells to radiotherapy (Frosina, 2009), many researchers have targeted the activity of telomerase in cancer therapy as a substitute for radiotherapy (Ahmed and Tollefsbol, 2003, Akiyama *et al*., 2002, Newbold, 1999). Zhao *et al*. have reported that inhibition of hTERT using anti- hTERT siRNA can significantly hinder the proliferation and invasiveness of human glioma cells, through its effect on the telomere length (Zhao *et al*., 2007). Many *in vivo* studies using MCF7 cells that were incubated into the mice, have demonstrated a decrease in tumour weight and reduction in the metastasis following inhibition of telomerase by melatonin. Melatonin has also been shown to can inhibit TERT and TR sub-units *in vitro* (Leon-Blanco *et al*., 2003). Interestingly, *in vivo* research on breast cancer epithelial cells (MDA-MB-2231) demonstrated a high sensitivity to radiation following the inhibition of telomerase by GRN136L, a specific telomerase inhibitor (Gomez-Millan *et al*., 2007).

1.3.2.iv The link between bystander effect and genomic instability

There is irrefutable evidence to suggest that ionizing radiation induces GI (Barber *et al*., 2006, Kadhim *et al*., 2004, Mazurik and Mikhailov, 2001). Furthermore, there is strong evidence of GI in the bystander cells. Bowler *et al*. have reported that after 10-13 population doublings post- irradiation, GI was observed in the progeny of

bystander cells and the delayed chromosomal damage in this population was similar to the level of initial radiation- induced chromosomal damage (Bowler *et al*., 2006). Much evidence has confirmed that IR can induce GI in the progeny of un-irradiated cells (bystander cells) (Lorimore *et al*., 2005, Lorimore *et al*., 1998). Hu and other workers observed GI within the progeny of bystander human hamster hybrid cells using multicolour banding technique (Hu *et al*., 2012). Moreover, GI had been observed within the bystander un-irradiated haemopoietic cells *in vivo* using bone marrow transplantation method (Watson *et al*., 2000)

Aims and objective of thesis

The objective of this study is to investigate the risks or benefits associated with the induction of non-targeted effects, particularly BE following exposure to low LET X-ray radiation. Cell combinations between tumour (MCF7) and non-tumour (HMT-3522S1) human breast epithelial cells are established using the well defined cell communication approaches such as co-culture and media transfer systems. BE is considered beneficial if the bystander signals can induce apoptosis or auto-killing in the cancer cells, and these signals do not affect normal calls. Additionally, the project aims to achieve an increased understanding of the mechanistic link between radiation-induced genomic instability in irradiated and non-irradiated bystander cells by examining the molecular signalling via exosomes within the irradiated, bystander and progeny of irradiated and bystander cell population.

In order to fulfil these aims, the objectives of this project will be:

1. Establish the induction of bystander effects (BE) using a 6 well-plate co-culture system and media transfer technique.

2. Assess BE in bystander cells by chromosomal damage analysis.

3. Evaluate apoptosis in bystander cells, which could lead to elimination the damaged cells.

4. Assess telomere length and telomerase activity in order to determine telomere stability of bystander cells.

5. Examine molecular signalling through the purification, identification and characterisation of exosomes released from irradiated and bystander cells into conditioned media, utilising several approaches including electron microscopy.

8. Determine the role of exosomes in cell communication underlying the mechanism of non-targeted effects of ionizing radiation using relevant biological endpoints.

CHAPTER 2

MATERIALS AND METHODS

2

Chapter 2: Materials and Methods

2.1 Cell lines and tissue culture

2.1.1 Cell lines:

Two types of cell lines were employed in this study: non-tumour human epithelial cells (HMT-3522S1) and tumour metastatic human epithelial (MCF7) cells. Both of these cells were derived from breast epithelial cells of a female adult.

2.1.1.i HMT-3322S1 cell line

Cells were purchased from the HPA tissue culture bank/repository at passage number: 10 (p10). HMT-3522S1 is a subline derived from HMT-3522 after sub-culturing to passage 34 (Ohlsson *et al*., 1998). Originally, these cells were derived from normal breast epithelium of a 48 year old woman who had undergone malignant transformation (Ohlsson *et al*., 1998). Upon receipt, the cells were screened for mycoplasma- a negative test result was obtained.

G-banding karyotype demonstrated that HMT-3522S1 had a deletion in chromosome 1; translocations in chromosomes 8, 12, 14 and 17, as well as nullisomy of chromosome 6 (one copy of chromosome 6 was missing) i.e. the karyotype of HMT-3522S1 was found to be 45XX,-6, 1p-,8p+,12p+,14p+,17p+.

Cells were grown in DMEM/ Ham's F12 (1:1) (Gibco, 21331) supplemented with 2 mM L-Glutamine (Gibco, 25030); 250 ng/ml insulin (Sigma, I0516); 10 µg/ml holo-transferrin bovine (Sigma, T1283); 10 E-8M sodium selenite (Sigma, S5261); 10 E-10M 17 β-estradiol (Sigma, E4389); 0.5 µg/ml hydrocortisone (Sigma, H0888); 5 µg/ml ovine prolactin (Sigma, L6520); 10 ng/ml epidermal growth factor, EGF (Sigma, E9644) and 1% (v/v) penicillin/ streptomycin solution (Sigma, P0781). Tissue culture flasks were coated with 10 µg/ml collagen IV (Sigma, C5533), were used to aid cell adherence. Cells were incubated in a humidified 5% CO_2 incubator at 37 ºC. Cells were sub-cultured when the confluence was 80% - 90%. Approximately

1.4 x 10^6 cells were seeded in 75 cm^2 tissue culture flasks (T75). HMT-3522S1 cells grew as a mono-layer and had a population doubling time of 30 hours.

a. Coating procedure

To obtain 10 µg/ml collagen IV as a final working solution, 5 mg of collagen IV was dissolved in 500 ml 1X of Dulbecco's phosphate buffered saline, PBS (Gibco, 14190); 10 mM acetic acid was added. The working solution can be kept long-term refrigerated at 2-8 ºC. When required, 4 mls of collagen solution was aliquot into each T75 flask, the flask cap was replaced loosely and the flask set aside for 1 hr at room temperature in a Class 2 safety cabinet. Excessive collagen solution was then removed from the flasks and the caps were tightly replaced; the collagen coated flasks were able to be kept for several days, although if preferred the flasks could have been coated just prior to their use in cell culture.

2.1.1.ii MCF7 cell line

MCF7 cells were kindly provided by Dr Joestin Dahle (Oslo University, Norway). MCF7 cells are a metastatic breast cancer epithelial cell line (Gelmann *et al*., 1992), and have a negative test result for mycoplasma.

The number of chromosomes in any one particular metaphase preparation, ranged from hypertriploidy to hypotetraploidy. Although several marker chromosomes were observed, no double minutes were detected.

Cells were cultured in DEMEM/ Ham's F12 media supplemented with 13% inactivated foetal bovine serum, FBS (Sigma, F7524); 2 mM L-Glutamine (Gibco, 25030) and 1% (v/v) penicillin/ streptomycin solution (Sigma, P0781) in a humidified 5% CO_2 incubator at 37 ºC. For cell sub-culture, 1.4 x 10^6 cells were seeded per each T75. MCF7 cells grew as mono-layer and had a population doubling time of 25-26 hours.

a. Foetal bovine serum inactivation procedure

The purpose of heat inactivation (Liao *et al*., 2011) of FBS is to destroy the complement system of immunoassay that may affect the parameters of tissue culture system (Johnson *et al*., 1975). Briefly, the FBS bottle was completely thawed from it's storage at -20 °C. It was then inverted several times to mix before being completely submerged in a 56 ºC water bath for 30 minutes (e.g. the level of water in the water bath was higher than the level of FBS). The FBS was then aliquot into labelled 50 ml sterile tubes and stored in a -20 °C freezer.

2.1.2 Recovery of cells from liquid nitrogen

The cell line cryovials were routinely thawed by hand after sterilising cryovial with 70% ethanol. Once cells had defrosted they were aseptically transferred to 15 ml falcon tubes containing 10 ml pre-warmed culture media to reduce the concentration of dimethyl sulfoxide (DMSO) in the freezing media. The tubes were inverted several times for mixing and centrifuged at 259 X *g* for 7 minutes. Supernatant was aspirated and each cell pellet was re-suspended in 7 ml pre-warmed culture media and then transferred to 25 cm^2 tissue culture flasks (T25). Collagen IV coated tissue culture flasks were used for HMT-352S1 cell culture.

The flasks were incubated in a humidified 5% CO_2 incubator at 37 ºC. Routinely, after 2 hours incubation, the flasks were checked for cell attachment using an Olympus inverted phase contrast field microscope; if cells were viable they were shown to have successfully attached to the T25 base. Irrespective of cell attachment, all flasks were re-incubated for a further 24 hours; whereupon, flasks with no adherent cells, were safely discarded and new cell samples were set up from liquid nitrogen storage.

2.1.3 Maintenance of cells and cell sub-culturing / passage

Cells were cultured in either T25 or T75 with their specific media; the media was refreshed every 48 hours to eliminate the cells' metabolic waste and also to provide a supply of fresh nutritional media. Cells were subjected to sub-culturing when the cell confluence was 80% - 90%, as follows:

2.1.3.i MCF7 cells sub-culturing

Cells were cultured to 80% - 90% confluence. Media was removed from the tissue culture flask; attached cells were washed with sterile 1X PBS (2 ml for T25 and 5 ml for T75) for 1 minute to remove residual media/serum. PBS was discarded and cells were rinsed with 0.05% (w/v) trypsin-EDTA solution (0.05 gm 0f 1:250 trypsin (Gibco, 27250-180) and 100 ml of 0.02% ethylene diaminetetraacetic acid solution, EDTA (Sigma, E8008)), for 30-60 seconds. After trypsinisation, cells were incubated in a humidified 5% CO_2 incubator at 37ºC for 5-10 minutes to allow cells to dissociate from flask base. Cells were re-suspended in 10 ml culture media to inactivate trypsin and to enable collection of detached cells from flask. Approximately, 1.5 x 10^6 cells were transferred to a new labelled flask which was then topped up to 15 ml with appropriate culture media. Flasks were re-incubated in a humidified 5% CO_2 incubator at 37ºC.

2.1.3.ii HMT-3522S1 cells sub-culturing

Cells were grown to 80% - 90% confluence. Media was aspirated from flask and cells were washed with sterile 1X PBS. Because of the extreme adhesion of HMT-3522S1, cells were incubated with 0.02% EDTA for 10 minutes at 37ºC prior to trypsinisation. EDTA was removed and cells were rinsed with 0.05% (w/v) trypsin-EDTA solution for 30-60 seconds. Cells were then re-incubated for 5-10 minutes and flask gently tapped to detach the cells. To inactivate the trypsin, cells were treated with 2 ml of 0.025% (w/v) trypsin inhibitor-PBS solution (Trypsin inhibitor was from Sigma,

T0256) and 8mls of culture media were added to cells. The entire flask contents were then collected into a sterile 15ml falcon tube and centrifuged at 259 X *g* for 8 minutes. The supernatant was aspirated and the pellet was re-suspended in 10 ml pre-warmed culture media; approximately, 1.5 x 10^6 cells were transferred to a collagen IV coated T75.

2.1.4 Testing of mycoplasma contamination

Mycoplasma contamination has long been considered one of the main problems for basic scientific research; due to the ability of mycoplasma to affect cell culture system parameters (McGarrity *et al*., 1985, Hay *et al*., 1989). Therefore, mycoplasma tests were routinely performed for all of the cells lines used in this project.

Firstly, cells were grown with antibiotic-free media for 2 sub-cultures (passages). The cells were trypsinised and seeded onto a 4 multi-well chamber slide (Sigma, C6932) at an optimum dilution depending on cell type (4 x 10^4 / well for the experimental cells). When the cell confluence of the slide chamber wells became 70% - 75%, media was discarded from the wells and the cells were washed three times with sterile 1X PBS. The cells were then 'fixed' for 10 minutes in 25% glacial acetic acid in methanol and washed three times with sterile 1X PBS. The slide chamber was incubated in the dark at room temperature with 0.05 µg/ml Hoechst 33258 (Sigma: H6024) in FBS-free and antibiotic- free growth media for 10 minutes. The cells were then washed three times with distilled water and the chamber was carefully removed from the slide. The slide was then mounted with citifluor anti-fade mounting media (Agar Scientific) and the cover-slip sealed with clear nail polish.

A Zeiss Axioplan Pol Universal fluorescent microscope fitted with UV filter was employed to locate the cells. Once located, an X40 objective was used to analyse the presence of Hoechst 33258 labelled materials i.e. the presence of mycoplasma

infection. When Hoechst 33258 is bound to DNA, it is excited at 352 nm (UV filter) and emits at 461 nm. Mycoplasma testing demonstrated that all the cell lines to be used experimentally were negative for mycoplasma.

2.1.5 Cell counts using Erythrosin B viability stain

A stock solution of erythrosin B stain was first prepared: 0.4 g erythrosin B (Sigma, E9259); 0.81 g sodium chloride, NaCl (Sigma); 0.06 g potassium phosphate monobasic (Sigma) and 100 ml Hank's balanced salt solution, HBSS (Sigma) were placed in a glass beaker with a magnetic stirrer on a hotplate. The solution was brought to boil and 100–200 µl of 10 M NaOH (Sigma) was added until all compounds had dissolved. The solution was then left to cool at room temperature.

To make up working solution of erythrosin B stain, 1ml of erythrosin B stock solution was added to 4 ml de-ionized water; therefore the final concentration of erythrosin B working solution was 0.8 mg/ml. Both stock and working solution of erythrosin B were stored in refrigerator at 4–8ºC.

A Neubauer Haemocytometer (haemocytometer) with its associated cover-slip was utilised to count the number of cells in the experimental cell samples. The haemocytometer has two counting areas; each of which has a grid layout comprised of different size squares. For cell counting, the four large 1 mm X 1 mm corner squares (area of each is 1 mm^2), each subdivided into 16 small squares, are used (Figure 2.1). When the cover-slip is securely placed onto the haemocytometer, a 0.1 mm height is left between the cover-slip and the haemocytometer; therefore, the total volume of each large square is 1 mm X 1 mm X 0.1 mm = 0.1 mm^3 = 0.1 µl.

After cell trypsinisation, cells were collected in 10ml pre-warmed culture media in a 15 ml falcon tube. A 100 µl aliquot of cell suspension (well mixed) was added to 100 µl aliquot of erythrosin B working solution in an eppendorf tube; thereby making a

dilution factor of 2 (1 volume to 1 volume). From this well mixed 200 µl sample, a 10 µl aliquot was loaded underneath the haemocytometer cover-slip of one of the chambers and an Olympus inverted phase contrast field microscope was used to count the cells. As mentioned previously, cells were counted within the four large 1 mm X 1 mm corner squares; dead cells were observed as red and live cells were clear in appearance. The number of cells in 1ml was calculated using the following formula:

(Number of cells in the four large corner 1 mm^2 squares / 4) X dilution factor X 10^4

[(Number of cells in the four large corner 1 mm^2 squares / 4) X dilution factor] represents the number of cells in 0.1 µl of sample, which is then multiplied by 10^4 (1 ml = 1000 µl) to obtain the number of cells in 1 ml.

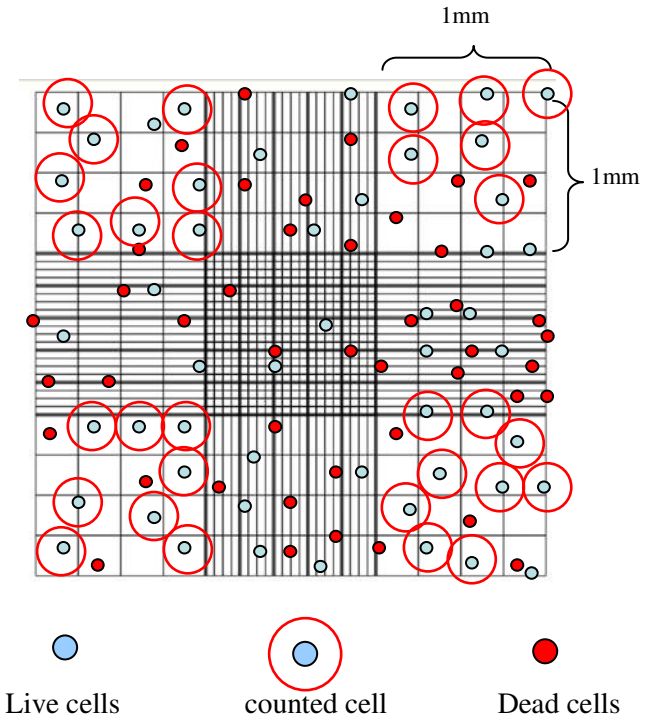


Figure 2.1: Scheme of one of the counting chambers from a Haemocytometer demonstrating 'live' and 'dead' cells in the counting procedure.

2.1.6 Cryopreservation of cells

The concept of cryopreservation is to maintain the longevity of cells by long-term storage in liquid nitrogen. Cells were prepared for liquid nitrogen storage by

preparing T75 cell cultures to 80% - 90% confluence. Cell suspensions for each cell type were prepared as previously described and these were then centrifuged at 259 X *g* for 10 minutes. The supernatants were aspirated and the pellets re-suspended in freezing media, depending on the number of cells. Ideally, 2-3 x 10^6 cells were re-suspended in 1ml freezing media and this was then transferred to a pre-labelled sterile cryovial, which was quickly moved to a -20 ºC freezer for 1-2 hours. The cryovials were then transferred to a -80 ºC freezer for 4-12 hours and subsequently transferred to liquid nitrogen storage.

Freezing media was prepared by adding 10% dimethal sulfoxide, DMSO (Sigma, D2650) to 90% culture media.

2.1.7 Cell karyotyping

Cells were subjected to a G-banding technique, to ascertain the exact karyotype of the experimental cells.

Cells were harvested for metaphase preparation (see chapter 2, section 2.1.11), when the confluence of cells was 60%. The cells were 'dropped' onto clean slides (see chapter 3, section 3.2.2), and left to air-dry at room temperature. The solutions required for trypsin G-banding method was prepared as following:

- 0.005% (w/v) trypsin-EDTA solution: 35 mg trypsin 1:250 (Difco) dissolved in 70 ml of PBS. The solution was left for approximately one day to stabilize.
- Phosphate buffered saline 1X (Gibco, 14190)
- Phosphate buffer (pH 6.8): One tablet buffer (pH 6.8, VWR, 363112P) was dissolved in 1 litre distilled water.
- Phosphate buffered saline 1X (Gibco, 14190)

- 0.005% (w/v) trypsin-EDTA solution, which was made up by dissolving 35 mg trypsin 1:250 (Difco) in 70 ml of PBS. The solution was left for approximately one day to stable.
- Giemsa stain: 2.5 ml Giemsa stain (Gurr's) added to 45 ml phosphate buffer (pH 6.8).
- De-ionized water

Slides were incubated for 20-40 seconds in 0.005% (w/v) trypsin-EDTA solution. They were then rinsed thoroughly with cold / refrigerated phosphate buffered saline 1X (PBS) and placed in the Giemsa stain for 5 minutes. Finally they were rinsed in de-ionized water and left to dry overnight at room temperature. The slides were then mounted with entellan mounting media (VWR, 1.07961.0100). A transmitted bright field microscope was used to analyse each cell's karyotype. Details of each experimental cell's karyotype can be found in section 2.1.1.

2.1.8 Cell co-culturing and irradiation of cells

A tissue culture 6 well-plate co-culture system (Falcon, 353502, 353092) was utilised in the experiments. This co-culture system consisted of a 6-well cell culture plate (Falcon, 353502) and associated cell culture insert (Falcon, 353092) (Figure 2.2). Cells were seeded in both the 6-well cell culture plates (herein referred to as 'Base dishes') and associated cell culture inserts, (herein referred to as 'Insert dishes'). The two cell populations were physically separated but communication was allowed between them via a porous translucent polyethylene terephthalate membrane (pore size diameter of 3.0 μm) which formed the bottom of the Insert dish (Hill *et al.*, 2006).

All the irradiations were performed at the Gray Institute for Radiation Oncology & Biology, Department of Oncology, University of Oxford, utilising MXR321 X-ray machine operating at 250 kV constant potential and 14 mA was used for irradiation.

Due to the nature of the Low LET ionizing radiation X-ray irradiation, every cell in the ionization track path receives some irradiation, although at a reduced dose as the energy deposited is sparse and uniform, just prior to X-ray irradiation/sham-irradiation, the companion Insert dishes were incubated separately from their associated Base dishes and temporarily placed onto media only Base dishes (without cells) and left in a humidified 5% CO_2 incubator at 37ºC. The cells in the Base dishes were meanwhile exposed to X-ray irradiation/sham-irradiation and were thus considered as 'direct irradiated' or 'direct sham-irradiated' cells. Immediately, after X-ray irradiation, the Insert dishes were then carefully placed into their associated sham/irradiation companion Base dishes so allowing communication between the two populations; i.e. the Insert cells, thus were able to receive soluble factors from the X-irradiated / sham-irradiated cell's media; these Insert cell population are considered as 'bystander cells'.

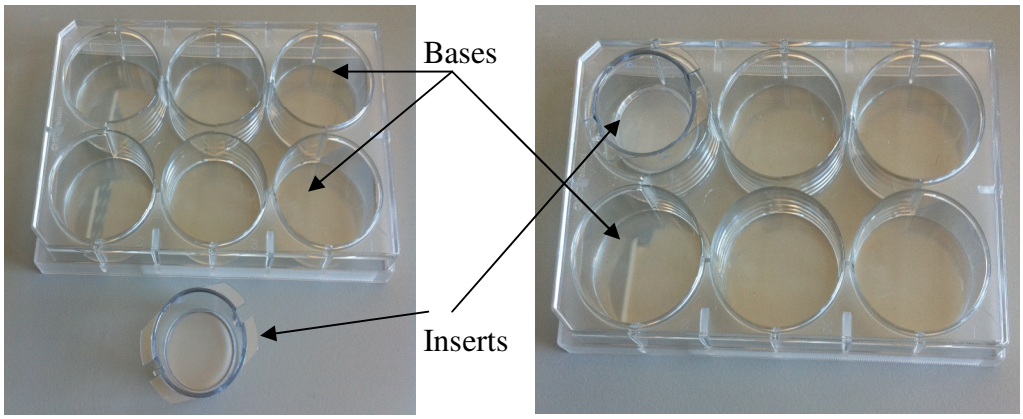


Figure 2.2: 6 well plate base and insert of co-culture system.

2.1.9 Experimental cell combinations

As described above, only cells in the Base dishes were sham/X- ray irradiated. Two doses of ionizing radiation were used: 0.1 Gy X-ray (a relevant dose for diagnostic procedures) and 2 Gy X-ray (a therapeutic dose).

Four cell combinations were established between MCF7 (tumour cells) and HMT-3522S1 (non-tumour cells). The first combination was between 'direct irradiated' tumour (Base dish cells) and 'bystander' non-tumour (Insert dish cells). The second cell combination was between 'direct irradiated' tumour cells and 'bystander' tumour cells. The third cell combination was between 'direct irradiated' non-tumour cells and 'bystander' tumour cells and the fourth cell combination was between 'direct irradiated' non-tumour cells and 'bystander' non-tumour cells. Control groups were established in parallel for all combinations. From these four cell combinations, several experimental groups were set up as shown in table 2.1:

Table 2.1: Experimental groups

Cell combi-nations	Experimental groups	Definition
First combination	1st MCF7 Dir Irr control	Tumour MCF7 cell were irradiated by 0Gy X-ray and considered as a control group of irradiated MCF7 cells of the second combination.
	1st MCF7 Dir 0.1Gy	MCF7 cells were directly exposed to 0.1 X-ray irradiation.
	1st MCF7 Dir 2Gy	MCF7 cells were directly irradiated with 2Gy X-ray.
	1st HMT BE control	Non-tumour HMT-3522S1 cells were incubated with 1st MCF7 Dir Irr control for 4 hrs, to be the control group of HMT-3522S1 of the first combination.
	1st HMT BE 0.1Gy	HMT-3522S1 cells were communicated with 1st MCF7 Dir 0.1Gy cells for 4 hrs (insert was placed in base).
	1st HMT BE 2Gy	HMT-3522S1 cells were incubated with 1st MCF7 Dir 2Gy.
Second combination	2nd MCF7 Dir Irr control	MCF7 cell were irradiated with 0Gy X-ray and considered as a control group of irradiated MCF7 cells of the second combination.
	2nd MCF7 Dir 0.1Gy	MCF7 cells were directly exposed to 0.1 X-ray irradiation.
	2nd MCF7 Dir 2Gy	MCF7 cells were directly irradiated with 2Gy X-ray.
	2nd MCF7 BE control	MCF7 cells were incubated with 2nd MCF7 Dir Irr control for 4 hrs, to be the control group of HMT-3522S1 of the second combination.
	2nd MCF7 BE 0.1Gy	MCF7 cells were communicated with 2nd MCF7 Dir 0.1Gy cells.
	2nd MCF7 BE 2Gy	MCF7 cells were incubated with 2nd MCF7 Dir 2Gy.
Third combination	3rd HMT Dir Irr Control	HMT-3522S1 cells were exposed to 0Gy X-ray irradiation and considered the control group of direct irradiated HMT-3522S1 groups of the third combination.
	3rd HMT Dir 0.1Gy	HMT-3522S1 cells were directly irradiated with 0.1Gy X-ray.
	3rd HMT Dir 2Gy	HMT-3522S1 cells were directly hit with 2Gy X-ray.
	3rd MCF7 BE control	MCF7 cells were incubated with 3rd HMT Dir Irr control cells for 4 hrs and considered a control groups of 3rd MCF7 bystander cells.
	3rd MCF7 BE 0.1Gy	MCF7 cells were communicated with 3rd HMT Dir 0.1Gy cells for 4 hrs.
	3rd MCF7 BE2Gy	MCF7 cells were incubated with 3rd HMT Dir 2Gy for 4 hrs.
Fourth combination	4th HMT Dir Irr Control	HMT-3522S1 cells were exposed to 0Gy X-ray irradiation and considered the control group of direct irradiated HMT-3522S1 groups of the fourth combination.
	4th HMT Dir 0.1Gy	HMT-3522S1 cells were directly irradiated with 0.1Gy X-ray.
	4th HMT Dir 2Gy	HMT-3522S1 cells were directly hit with 2Gy X-ray.
	4th HMT BE control	HMT-3522S1 cells were incubated with 4th HMT Dir Irr control cells for 4 hrs and considered the control groups of 4th HMT-3522S1 bystander cells.
	4th MCF7 BE 0.1Gy	HMT-3522S1 cells were communicated with 4th HMT Dir 0.1Gy cells for 4 hrs.
	4thMCF7 BE2Gy	HMT-3522S1 cells were incubated with 4th HMT Dir 2Gy for 4 hrs.

Immediately post- sham/irradiation of Base dishes; the companion Insert dishes were co-cultured with the Base dishes and incubated for four hours to allow the 'bystander' cells (insert cells) to receive the short and the long-lived bystander signals from the 'direct irradiated' / direct 'sham-irradiated cells. Cells from both direct and bystander groups were separately cultured and analysed for cytogenetics, apoptosis, telomere length (Q-FISH) and telomerase activity measurement (TRAP assay) at time-points. For each passage 1.4 million cells were seeded in a T75 flask using fresh (unconditioned) media and grown to 80% confluence (the 12 passages represented approximately 24 cell-doublings).

2.1.10 Experimental design

One cryovial from each cell line was propagated in T75s for several passages (see below) to enable sufficient number of cells for experiment. The experimental design is shown in figure 2.3. Briefly, HMT-3522S1 cells were propagated until passage 34 (p34) when they entered the experiment; 7 x 10^5 cells in 2ml culture media, were seeded into each Base dish and Insert dish, the two dishes were then co-cultured and re-incubated in a humidified 5% CO_2 incubator at 37ºC until they reached 80% confluence. In contrast, MCF7 cells were propagated until p29 when they entered the experiment and 6 x10^5 cells were seeded per Base and Insert dish. For both cell lines, Base dishes were then either sham / X-irradiated (as above), and insert dishes added to allow communication for 4 hours in a humidified 5% CO_2 incubator at 37ºC. Samples of cells from both Insert and Base for all experimental groups, were then either subjected to apoptotic analysis or put into individually labelled T25s and re-incubated in a humidified 5% CO_2 incubator at 37ºC, cells were now considered to be at passage 0 of experiment.

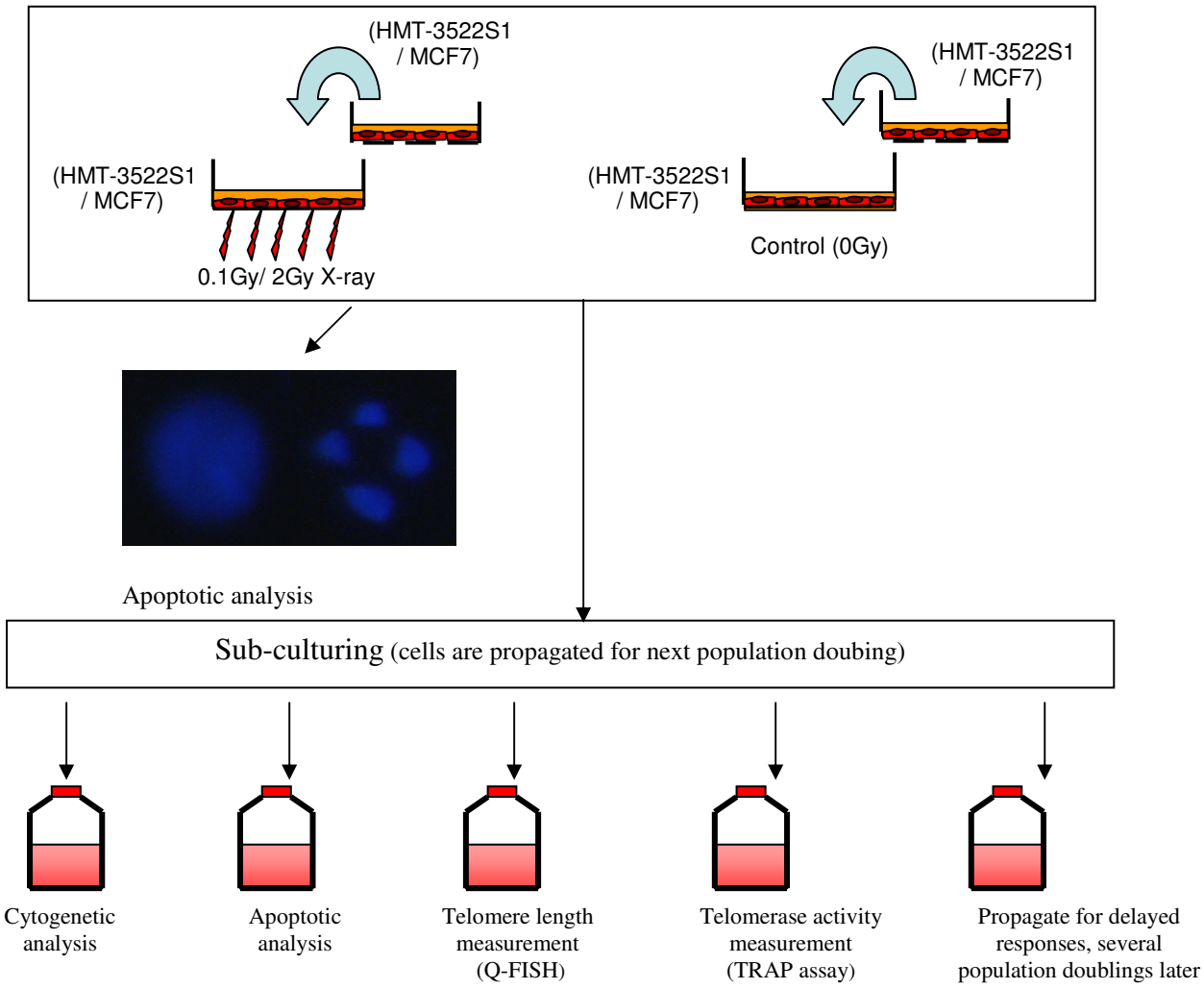


Figure 2.3: Schematic of the experiment, showing communication between 'direct irradiated' and 'bystander' populations.

Cells were irradiated with 0 Gy (for control/sham irradiated), 0.1 Gy (diagnostic dose) and 2 Gy (therapeutic dose) X-ray using 6-well plate co-culture system. Whilst bystander cells were incubated with irradiated cells for 4 hours. Both direct irradiated and bystander cells were analysed for apoptosis after 4 hours (before cell sub-culturing) following irradiation. Cells were then sub-cultured for the first population doubling and subjected to the cytogenetic, apoptotic analyses, telomere length and telomerase activity measurements. Irradiated and bystander cells were then propagated for late time points (12 and 24 generations following irradiation) and subjected to the cytogenetic, apoptotic analyses, telomere length and telomerase activity measurements.

As described previously, samples of cells from all the experimental groups were initially analysed for apoptosis after 4-6 hours post irradiation. They were also assayed for apoptosis at first population doubling following irradiation, and in addition all experimental groups were analysed for indications of early damage response by cytogenetic analysis, telomere length measurement and telomerase activity measurement. Intermediate analysis to enable the subsequent propagation of cell damage, was undertaken after 12 population doublings and incidence of genomic

instability and delayed response was assessed after 24 population doubling following direct X-ray irradiation and co-cultured condition.

2.1.11 Cytogenetic analysis

Cytogenetics is branch of genetics, deals with chromosomes using light microscopy study (Wolstenholme, 1992). In 1956 Tjio and Levan reported that human chromosome number was 46 not 48, which urged several laboratories to engage in human chromosome study (Shaffer *et al*., 2009). Aneuploidy, which is gain or loss of whole chromosome(s), was reported by Jacobs and other authors who described the abnormal numerical karyotype in human lymphocytes (Jacobs *et al*., 1961). The invention of cytogenetic staining techniques such as Giemsa, quiacrine and fluorescence microscopy technique (Fluorescence in situ hybridisation) makes the cytogenetic analysis a robust technique to assess and determine the structural and numerical abnormalities of chromosomes (Baker *et al*., 2009, Schrock *et al*., 1996).

It is very well documented that IR can induce chromosomal aberrations (Toyokuni *et al*., 2009, Gowans *et al*., 2005, Kadhim *et al*., 1992). As well as chromosomal analysis was widely used to assess the effect of IR directly and indirectly on cells (Li *et al*., 2008, Kadhim *et al*., 1998).

More recent chromosomal analysis evidence has utilised cytogenetic analysis to evaluate the effect of IR on peripheral blood lymphocyte chromosomal and the chromosomal instability following therapy. Cytogenetic analysis in this study included numerical and structural chromosomal aberrations and showed structural chromosomal damage in the progeny of irradiated cells (Salas *et al*., 2012). Moreover, chromosome aberrations are well documented to be one of the manifestations of the non-targeted radiation exposure, including BE and GI (Kadhim *et al*., 1992). Ponnaiya and co-workers detected chromosomal aberrations in irradiated and un-

irradiated human bronchi epithelial cells using cytogenetic analysis. In addition, they verified that chromosomal instability was observed in the progeny of irradiated and un-irradiated epithelial cells after 50 population doublings using chromosomal analysis (Ponnaiya *et al*., 2011). Therefore, chromosomal analysis was used in this study to determine the early and delayed effects of IR and bystander signals on experimental cells.

2.1.11.i Preparation of cells for harvesting of metaphases

Chromosomal preparation for Giemsa solid staining technique was carried out as described by Kadhim and other authors (Kadhim *et al*., 2004). At specific time-points post sham/irradiation, experimental cells were routinely cultured in T75s to 60% confluence. Flasks were then checked for the required presence of rounded/dividing cells, using an inverted phase contrast field microscope (Nikon TS 100). Cells were treated with 20 ng/ml demecolcine (Sigma, D0125) for 1.5 hours in a humidified 5% CO_2 incubator at 37 ºC. Demecolcine is a mitotic spindle fibre formation inhibitor (Brinkley *et al*., 1967), therefore its addition arrested cells in the metaphase stage of the cell cycle so enabling later visualisation of the fixed chromosomes with a transmitted bright field light microscope

Post-incubation with demecolcine, each experimental flask had its media collected in an individually labelled, sterile 30 ml universal tube. Cells were then washed with 1X PBS for 1 minute; this PBS was then added to its corresponding labelled universal tube. Cells were dissociated from each flask by addition of 0.05% trypsin-EDTA solution for 30 – 60 seconds. The trypsin-EDTA solution was then collected in the associated universal tubes. Detached cells were collected using 10 ml of the corresponding universal tubes contents (flask's media, PBS and trypsin-EDTA solutions) and returned to their corresponding universal tubes. All universal tubes were centrifuged at 259 X *g* for 10 minutes; the supernatants were discarded and the

remaining pellets re-suspended by flicking tubes. 10ml of hypotonic solution, ((75 mM potassium chloride solution (KCl, Sigma, P5405)) was subsequently added to each universal tube in a drop- wise manner for the first 1ml. The hypotonic solution's role was to make the cells swell and consequently fragile, therefore, that helped in the observation of the chromosomes spread easily. The cell - KCl suspensions were all transferred to 15ml falcon tubes and these were incubated in a 37°C water bath for 20 minutes. Three drops of 25% glacial acetic acid in methanol (3:1 fixative) was then added to each tube; all tubes were inverted once and centrifuged at 180 X *g* for 10 minutes. The supernatants were discarded, pellets re-suspended by flicking tubes and 10 ml of 3: 1 fixative were added to each tube (drop-wise). The fixed cells were incubated for 10 minutes at room temperature prior to centrifugation at 259 X *g* for 10 minutes. Supernatants were removed and the pellets re-suspended, 10 ml of 3: 1 fixative were added to each tube (drop-wise) and tubes were left at room temperature for 30 minutes. Cells were further centrifuged at 259 X *g* for 10 minutes; supernatants discarded and pellets re-suspended in 1- 2 ml of 3: 1 fixative, depending on the pellet size. Samples from all experimental groups were then 'dropped' (metaphase slides prepared) onto individually labelled, clean/degreased microscope slides (see 3.2.2).

Note: The fixed cell suspension was additionally able to be kept at -20ºC for long term storage after addition of 10 ml of 3: 1 fixative, to enable metaphase slide preparation at a later date.

2.1.11.ii Chromosomal slide preparation

Metaphase chromosomes were prepared from most of the experimental groups immediately after the harvesting procedure; for those groups that did not, the fixed samples were removed from -20 ºC storage and warmed to room temperature for approximately 1 hour.

The samples were centrifuged at 259 X *g* for 10 minutes, supernatant removed and pellets re-suspended in 1- 2 ml of 3: 1 fixative, depending on the pellet size.

Clean/de-greased microscope slides were obtained by soaking clean, frosted microscope slides in 1:1, diethyl ether: methylated spirit (or methanol) for 24 hrs prior to use. Residual dirt was removed by wiping the slides with a 'Kimwipe' tissue. Individual Fine-tip mini pastettes (alpha laboratories, LW4231) were used to drop each experimental sample onto individual de-greased slides from a height of 15-20 cm. Metaphases preparations were checked after each sample drop, using a transmitted bright field light microscope. The slides were left to dry/age overnight at room temperature.

2.1.11.iii Staining of slides

Giemsa solid staining is a simple technique used to stain chromosomes; it is a preferable method for detecting chromosome/ chromatid type aberrations.

A phosphate buffer (pH 6.8) solution was prepared by dissolving one buffer tablet (pH 6.8, VWR, 363112p) in one litre of de-ionized water. Giemsa solid stain solution was made up by adding 4 ml Giemsa stain solution (Gurr's, VWR, 350864X) to 60ml phosphate buffer (pH 6.8) in a Coplin jar. Two Coplin jars were filled with phosphate buffer (pH 6.8).

Aged metaphase slide preparations were placed in the Coplin jar containing Giemsa stain – phosphate buffer 6.8 solution, for 3 minutes. The slides were then agitated in the Coplin jars filled with phosphate buffer, each for few seconds and left to dry for 12- 24 hours at room temperature.

2.1.11.iv Mounting of slides with cover-slips

This procedure was performed in an Astecair 3000E cabinet. Briefly, the slides were placed into Coplin jars containing xylene (Fisher Scientific, X/0250/17) for a minimum of 10 minutes, maximum 1 hour, to remove the excessive stain from slides

and to aid the dispersal of mounting media onto the slides. Slides were removed from xylene and laid flat onto a filter paper; two drops of entellan mounting media (VWR, 1.07961.0100) were dropped onto each slide from a glass pipette. A 22 x 50 mm cover-slip was then carefully placed onto each microscope slide and the cover-slip gently blotted with filter paper to remove air bubbles and excessive entellan mounting. The slides were left 12 - 24 hours to dry at room temperature before analysis.

2.1.11.v Analysing of slides

A Zeiss Axioskop transmitted bright field light microscope was used to analyse the mounted experimental slides at 100X oil immersion objective. At least, 100 metaphases were scored for each group.

2.1.12 Preparation of cells for apoptotic analysis

The method used to determine the presence of apoptotic cells has been described by Schwartz and co-authors (Schwartz *et al*., 1995). Cells were cultured in T75 flasks to 80% confluence. The media was collected in 30 ml universal tubes (to ensure that all the cells, floating and detached cells, were collected). The cells in the flask were then washed with 5 ml of sterile 1X PBS for 1 minute and the PBS was collected in the same universal tube. The cells were disassociated from the flask by rinsing them with 2 ml of 0.05% (w/v) trypsin-EDTA solution for 30-60 seconds and this was then collected within the same universal tube. The cells were then collected with 10 ml of the saved media, PBS and trypsin-EDTA solution and all was returned to the universal tube. The tube was centrifuged at 259 X *g* for 8 minutes. The supernatant was aspirated and 10 ml of 25% acetic acid in methanol (fixative) solution was added to the re-suspended cell pellet in a drop-wise manner. The cell-fixative suspension was incubated for 10 minutes at room temperature. A further centrifugation was carried out at 259 X *g* for 10 minutes. The supernatant was discarded, whilst the cell

pellet was re-suspended in 10 ml of fixative (drop-wise manner) for 15 minutes. Then the cell suspension was centrifuged at 259 X *g* for 10 minutes. The supernatant was removed and 1-3 ml fixative (depending on the pellet size) was added to the cell pellet. The cell suspension was dropped onto clean labelled slide and left 10-15 minutes to dry at room temperature.

2.1.12.i Staining and analysing of slides

When the slides were fully dried, they were laid flat on the bench in the dark. The slides were then stained and mounted by adding 25 µl Prolong Gold anti-fade reagent with DAPI (Invitrogen, P36931) onto each slide and a cover-slip was gently placed on top. To check the cells for apoptosis, a Zeiss Axioplan Pol Universal fluorescent microscope fitted with UV filter was used. One thousand cells were analysed for each group. The normal cell nucleus was uniformly stained with DAPI (figure 4.1), whilst the apoptotic nuclei were recognised as pycnotic (apoptotic) bodies as shown in figure 2.4.

Cells were analysed for apoptosis after 4-6 hours, first population doubling, 12 population doublings and 24 population doublings following X-ray irradiation and co-cultured condition.

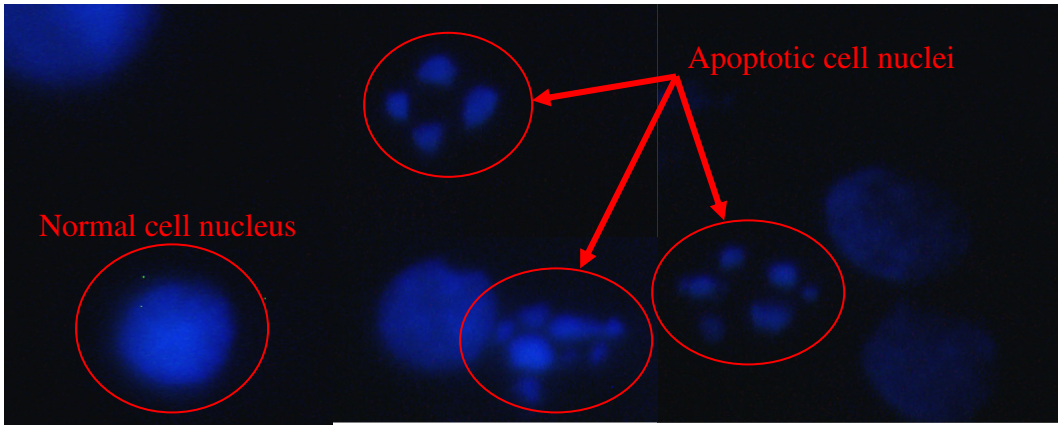


Figure 2.4: Normal and apoptotic human epithelial cell nuclei (stained with Prolong Gold reagent with DAPI).

2.1.13 Telomere stability analysis

As very well documented that telomere length has a crucial role in the chromosomal stability maintenance (Berardinelli *et al*., 2011, Salin *et al*., 2008, Skrobot Vidacek *et al*., 2007). Short telomeres can cause chromosomal fusion, instigating chromosomal instability (Murnane, 2012). As well as, telomerase dysfunction frequently lead to telomere shortening and then causing GI (Meyer and Bailis, 2008). Therefore, telomere stability analysis in this project included telomere length measurement (Q-FISH) and telomerase activity measurement (TRAP assay).

2.1.13.i Telomere length measurement, Q-FISH (quantitative fluorescence in-situ hybridisation)

The technique of Q-FISH or quantitative fluorescence *in-situ* hybridisation has been established from FISH (fluorescence *in-situ* hybridisation) and was used to measure telomere length in chromosomes by means of a PNA (peptide nucleic acid) probe. PNA is synthetic oligonucleotides, which has a high affinity for telomere repeat sequences. Synthetic DNA mimics (PNA and telomere repeat sequences) can be labelled by a fluorescent dye (routinely with FITC), and these can be easily detected by using fluorescence microscopy and analysis software (Poon and Lansdorp, 2001). Q-FISH is a reliable and accurate method for measuring chromosomal telomere length in interphase or metaphases of cell cycle (Slijepcevic, 2001).

a. Preparation of cells for Q-FISH assay

Preparation of cells for Q-FISH was similar to the method used for preparation of cells for harvesting of metaphases in chapter 2, sections 2.1.11. In brief, for each experimental group, cells were routinely cultured in T75 flasks to 60% - 70% confluence. They were then incubated with 20 ng/ml colcemid for 1.5 hours and subsequently collected in universal tubes. The tubes were centrifuged at 259 X *g* for 10 minutes. A hypotonic solution (75 mM potassium chloride) was added to each cell

pellet for 20 minutes. Then cells were fixed twice with 25% acetic acid in methanol solution prior to being dropped onto clean labelled microscope slides.

Slides were left to dry at room temperature for 30 minutes (to give the sample time to spread). Then the slides were aged by baking them on a hot plate at 55ºC for 3 days. Slides were aged in order to make the sample more resistant to the harsh Q-FISH melting agents such as formamide.

b. Rehydration of slide

A heating block was set up to a temperature of 70ºC (it was important that the temperature of the heating block did not fall below 70ºC or exceed 80ºC).

Preparation of solutions:

- Formaldehyde solution (4%): 4 mls of formaldehyde solution for molecular biology 36.5% (Sigma, F8775) was added to 96 ml double distilled water (ddH_2O), this gave enough for 2 Coplin jars.
- Acidified water (pH 2): 0.5 ml HCl (1N) was added to 50 ml ddH_2O.
- Pepsin solution (1 mg/ml): This was prepared by adding 0.5 ml stock solution (100 mg/ml) pepsin (Sigma, P7000) to 49.5 ml acidified water (pH 2). The solution was freshly prepared in the Coplin jar and put into the water bath at 37 ºC.
- 20X Salt sodium citrate (SSC): 1.752 g sodium chloride (VWR, 10241AP) and 0.882 g tri-sodium citrate (VWR, 102424L) were dissolved in 20 ml dH_2O.
- Formamide solution (70%): 70 ml formamide (Fisher scientific, F1550/ PB17) was added to 10 ml 20X SSC and 20 ml ddH_2O.

Note: A maximum of six slides were prepared at a time.

Slides were rehydrated by placing them in the Coplin jar, filled with 1X PBS. The Coplin jar was placed on shaker (200/minutes) for 15 minutes. The PBS was

discarded and the slides were fixed with 4% formaldehyde solution for 2 minutes without shaking. The formaldehyde solution was aspirated. Then the slides were washed three times with 1XPBS for 5 minutes per wash with shaking (using shaker). The slides were then transferred to the pepsin solution Coplin jar for 10 minutes at 37ºC water bath. Pepsin solution was disposed and the slides were washed twice with 1X PBS for 2 minutes with shaking. The slides were fixed again with 4% formaldehyde solution for 2 minutes without shaking. Formaldehyde solution was discarded and the Coplin jar was filled with 1X PBS to wash for 5 minutes with shaking. The 1X PBS was aspirated and the wash procedure repeated twice more.

c. Dehydration of slide

Slides were laid flat and dehydrated in a series of ethanol concentrations (70%, 90% and 100%) i.e. 1 ml of each ethanol concentration (starting with 70% and finishing with 100%) was put onto each slide for 30-60 seconds. The slides were then air-dried at room temperature.

d. Hybridisation

Once slides were dry, they were laid flat. A 20 µl aliquot of hybridisation probe, (peptide nucleic acid FITC (PNA FITC, Sigma)) was put on each slide. Then coverslips (22 X 50 mm) were used to spread the hybridisation probe on the slides. The slides were heated at 70ºC on hot block for 2 minutes and then incubated in a dark humid chamber for 2 hours at room temperature. Post-hybridisation, the cover-slips were carefully removed and the slides were placed in a dark Coplin jar (i.e. the Coplin jar and lid were covered completely with foil). The Coplin jar was filled with 70% formamide solution to wash the slides for 15 minutes without shaking. The formamide was aspirated and another wash was performed with formamide for 15 minutes without shaking. The formamide was discarded and the slides were washed three times with 1X PBS for 5 minutes per wash with shaking.

e. Dehydration

This procedure was identical to the dehydration procedure carried out in section 5.2.1.iii, in which the slides were dehydrated in different series of ethanol concentration (70%, 90% and 100%) by adding 1ml of each ethanol concentration onto each slide for 30 to 60 seconds. The slides were air-dried at room temperature.

f. Mounting of slides with DAPI mounting media

The slides were laid flat and 20 µl of Vectorshield mounting media with DAPI (Vector Laboratories, H-1200) was placed onto each. Cover-slips (24 x 50 mm) were then carefully placed on top of each slide.

g. Image capture and analysis

The analysis work was performed in Brunel University, using the smart capture software, IP-Lab software was additionally used to analyse the telomere fluorescent signals. The software had been supplied by digital scientific Cambridge. At least 20 cells were analysed per group.

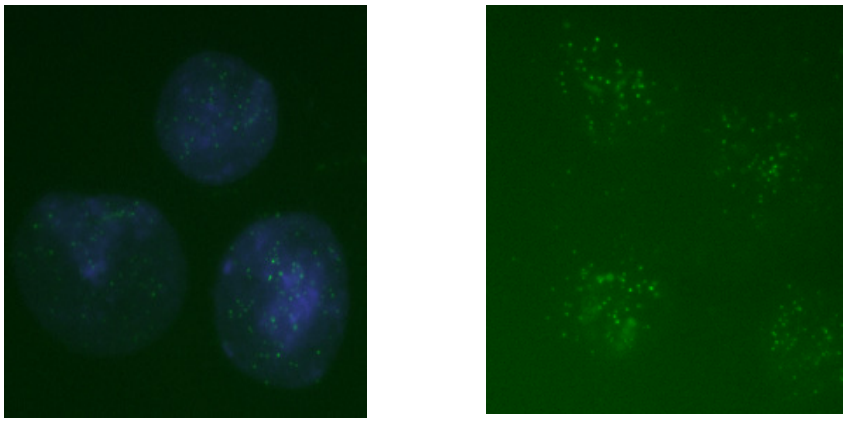


Figure 2.5: Telomere fluorescent images of tumour MCF7 cell nuclei (DAPI is on the left and FITC is on the right).
Cell nuclei were first imaged with DAPI, selecting separate individual nuclei (non-clumped nuclei). Then FITC images were utilised to measure telomeric fluorescence intensity (Telomere length).

2.1.13.ii Telomere repeat amplification protocol (TRAP assay)

The TRAP assay is a very accurate technique used to measure telomerase enzymatic activity, described by Fajkus (Fajkus, 2006). In this technique, telomerase (from each experimental cell extraction) extends TS primer with telomere repeat sequences by adding deoxyribonucleotide triphosphate (dNTP) to the 3' end of the TS primer. The

extended products of telomerase-TS primer are then amplified by real-time PCR with ACX reverse primer as shown in figure 2.5.

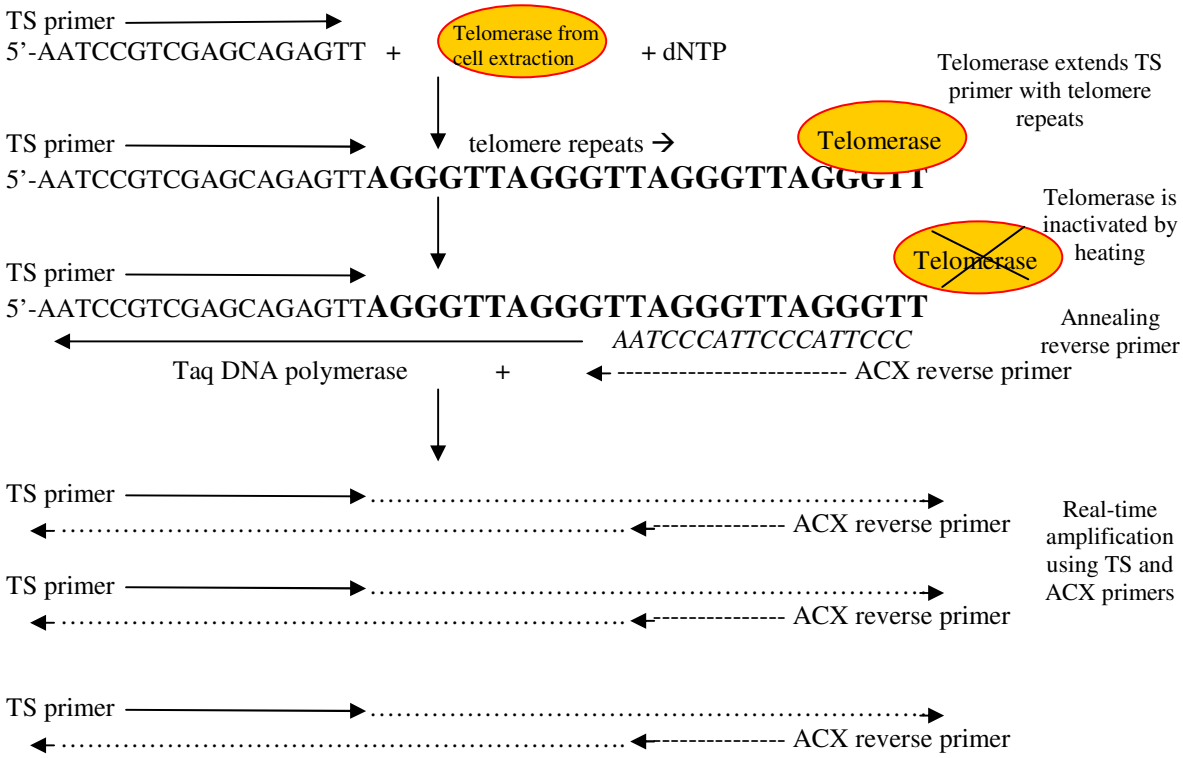


Figure 2.6: Scheme of TRAP assay for telomerase activity measurement (adapted from Fajkus J, 2006: Clinca chimica Acta, 371).

a. Preparation of samples for TRAP assay

Experimental cells were cultured in T75 flasks to 80% confluence. Then the cells were trypsinised and collected in 15ml falcon tubes. Counting of cells was carried out and 2 x 10^6 cells were collected in RNase free eppendorf tubes (0.5 ml). The eppendorf tubes (with cell suspension) were centrifuged at 3220 X *g* for 6 minutes. The supernatant was removed, whilst the pellet was re-suspended in 50µl CHAPS lysis buffer (Sigma, C5070) containing 500 IU RNase inhibitor (Invitrogen, 10777-019). The cell pellet was pipette up and down several times with CHAPS lysis buffer and incubated for 30 minutes at 4 ºC. Then the suspension (cell pellet and lysis buffer) was centrifuged at 259 X *g* for 30 minutes at 4 ºC. The supernatant (containing cell proteins) was then collected and stored at -80ºC.

b. Estimation of protein concentration for TRAP assay

Before starting the procedure, the bench, gloves, racks, glassware and surfaces were all wiped with RNase away (Molecular Bioproducts, 7003) to ascertain that there was no RNase contamination. In addition, RNase- and DNase-free filtered Gilson tips and eppendorf tubes were also used. For protein estimation assay, the CB-X™ protein assay kit with albumin was used. In brief, routinely triplicate standards of bovine serum albumin (BSA) were prepared at the following concentrations: 0, 0.2, 0.4, 0.6, 0.8 and 1 µg/ml with CHAPS lysis buffer in 1.5ml eppendorf tubes. 1ml of pre-chilled CB-X™ was added to each tube to precipitate proteins and to remove contamination. The tubes were mixed using a bench top vortex machine. The tubes were then centrifuged at 16000 X *g* for 5 minutes at 12 ºC. The supernatant was discarded and 50 µl CB-X™ solubilisation buffer I and 50 µl CB-X™ solubilisation buffer II were added to each tube (to pellet) and vortexed to dissolve the protein pellet. The tubes were incubated for 5 minutes at room temperature. To each tube, 1ml of CB-X™ dye was added and the tubes were then incubated for 5 minutes at room temperature.

Alongside this procedure, the experimental samples and a telomerase positive cell extraction (PC3 prostate cancer cell line) were analysed to ascertain their protein concentrations. Briefly, 5µl of each sample was put into a 1.5 ml RNase-free eppendorf tube. Then 1 ml of pre-chilled CB-X™ was added to all of eppendorf tubes and the samples were mixed by vortex. The eppendorf tubes were then centrifuged at 16000 X *g* for 5 minutes at 12 ºC. The supernatant was discarded and 50 µl CB-X™ solubilisation buffer I and 50 µl CB-X™ solubilisation buffer II were added to each tube, tubes were mixed by vortex. Then 1ml of CB-X™ dye was added and the tubes incubated for 5 minutes at room temperature.

For estimation of protein concentration, samples were read by micro-plate reader (LT-4000, Labtech) at 600 nm wave length.

The results were loaded onto an excel sheet. The standard curve was generated from the reading of standard samples, whilst the protein concentration of samples was read off against the standard curve.

c. TRAP assay

For each reaction well, a SYBR green master-mix was prepared as follows:

- 12.5 µl SBER green 2 X (Applied Biosystems Europe: 4364344).
- 1 µl TS primer (0.1 µg (19.96 pmol) / µl, 40nM) (5'-AATCCGTCGAGCAGAGTT-3', Sigma)
- 1 µl ACX primer (0.05 µg (5.53 pmol) / µl, 10nM) (5'-GCGCGG(CTTACC)3CTAACC-3', Sigma)
- 1-4 µl protein sample (containing 250 ng protein), routinely 2 µl of each sample was used.
- DEPC water (Millipore, 90411), each sample was approximately 8.5 µl (these were topped up to 25 µl with DEPC water)

The assay was run in triplicate for each sample. Prostate epithelial cancer cells (PC3), which are positive for telomerase activity (high telomerase activity), were used for standard samples (standard curve calculating). The standard, (PC3 cell extraction), was carried out at the following concentrations: 2.4 µg/2 ml, 1.2 µg/2 ml, 0.6 µg/2 ml, 0.3 µg/2 ml, 0.15 µg/2 ml, 0.075 µg/2 ml and 0.0375 µg/2 ml. A negative control (PC3 cell extraction) was prepared by heating the cell extract on a heating block at 98ºC for 10 minutes and then cooling it with ice. The standards, experimental samples, negative and positive (PC3 cell extraction) controls were loaded into a white 96 well plate (Thermo scientific, AB-2800/w). In addition, three wells were loaded with DEPC water alone, (non-template controls) and CHAPS lysis buffer was loaded in a further three wells as extra non-template controls. The TRAP assay was

performed using a real time PCR machine (CFX96 real-time system, C1000 thermal cycle, Bio RAD).

The real time PCR programme was 25ºC/20min, 95ºC/10min, (95ºC/30sec, 60ºC/90sec) 40 cycles. The reaction mixture was first incubated at 25ºC for 20 minutes (stage one) allowing the telomerase in the cell extraction to extend the TS primer by adding TTAGGG (telomere repeats). Then the telomerase was denaturised by heating in the second stage (95ºC/10 minutes) of real-time PCR programme, this was followed by reverse primer annealing and real time PCR amplification for forty cycle at 95ºC/30sec and 60ºC/90sec (stages 3 and 4) as shown in figure 2.6.

Telomerase arbitrary units were calculated from the raw data as shown in appendix I. In brief, the logarithm of the protein concentrations of the standard samples was used to calculate standard curve of the real-time PCR data, utilising Microsoft Excel. Then the sample values were calculated using R-squared value equation.

2.1.14 Comet assay

Single-cell gel electrophoresis or comet assay is a sensitive method to quantify total DNA damage (double-strand breaks, single-strand breaks and base damage) in individual cells (Chandna, 2004, Collins, 2004). The comet assay was performed as described by (Natarajan *et al*., 2007, Olive, 2009). Microscope slides were coated with 1% normal melting point agarose (NMPA, Sigma A9539) by dipping the clean slide in agar and wiping the back of the slide. The slides were allowed to dry at room temperature overnight. An 80% confluent flask was trypsinised, and cell count was carried out. A 1 X 10^4 /50 µl cell suspension was placed in 1.5 ml eppendorf tube and located in ice. The coated slides were then placed on a metal tray on ice. The 1 x 10^4 cell suspension was re-suspended with 200 µl of 0.6% low melting point agarose (LMPA, FisherBiotech BP165) and placed immediately onto chilled pre-coated slides.

The cell-LMPA suspensions were flattened with cover slips, which were removed after 5-10 minutes. The slides were then transferred to a Coplin jar, which was filled with cold lysis buffer (2.5 M NaCl, 100 mM EDTA pH 8.0, 10 mM Tris-HCl pH 7.6, and 1% Triton X-100, pH >10). The jar was kept at 4 ºC over night in the dark. The slides were then moved to a horizontal electrophoresis tank filled with electrophoresis buffer (0.3 M NaOH and 1 mM EDTA, pH 13) at 4ºC for 40 minutes. The electrophoresis was run for 30 minutes, at 19V, 300A. Slides were neutralised with neutralising buffer (0.4 M Tris-HCl, pH 7.5), washed with distilled water, and immediately stained with a 1:10,000 dilution of SYBR Gold (Molecular Probes/Invitrogen, Carlsbad, CA). The slides were analysed using Komet 5.5 Image Analysis Software (Kinetic Imaging Technology/Andor, Germany).

2.1.16 Statistical analysis

Samples and slides were coded and analysed in a 'blind fashion' (i.e. slides were coded by a colleague in the research group). Raw data from all experimental groups was used to compare and calculate p values. Standard error of mean was calculated to generate Y error bars for all experimental groups. For cytogenetic results; data was subjected to Fisher's exact test. Two proportions z-test (Minitab 15) was used to calculate p value of apoptotic and telomerase activity data. Whilst p value of telomere length assay was calculated by student *t* test (GraphPad Instat 3). Comet data was subjected to Mann-Whitney test (SPSS statistics 17.0) to measure p value.

CHAPTER 3

EARLY AND DELAYED EFFECTS OF IR ON TUMOUR AND NON-TUMOUR CELLS

3

Chapter 3: Early and delayed effects of IR on tumour and non-tumour cells

3.1 Introduction

It has been well established that IR can induce cellular damage in track of ionizing radiation. This cellular damage can be caused directly by high speed electrons such as alpha particles (Hall and Giaccia, 2006) or through indirect action by ionizing water molecules generating free radicals. These free radicals are predominant with low linear energy transfer (LET) radiation such as X-ray and gamma ray (Nias, 1990). The main target of IR that lead to cellular damage is DNA, and the main effect of IR is DNA breaks (Desai *et al*., 2005).

More recent evidence has documented that a very low dose of X-ray can cause metaphase chromosomal aberrations and DNA damage in the oral mucosa cells using comet assay (Ribeiro, 2012). Drissi and other authors have suggested that IR can induce DNA damage in normal human fibroblast, and this damage was telomere length dependent. They speculated that short telomeric fibroblast cells are more susceptible to IR than long telomeric fibroblast cells (Drissi *et al*., 2011). Another study showed that high LET α-particles and high dose of γ-ray can lead to DNA double strand breaks (DSB) in the SW-1573 lung tumour cells. These DSB detected by γ-H2AX scoring (Franken *et al*., 2012). Moreover, high LET α-particles can increase cell reproductive death ration in the SW-1573 lung tumour cells (Franken *et al*., 2011).

In addition, much evidence has proved that chromosomal instability can be observed within the progeny of irradiated cells (Amato *et al*., 2009, Noda *et al*., 2007, Kadhim *et al*., 1995). Recent evidence has demonstrated that IR can generate short DNA fragments, which might significantly play an important role in responses of non-malignant human mammary epithelial MCF10A cells to IR. Furthermore this study

suggested that these short fragments of DNA can affect non-homologous end-joining DNA repair mechanism leading to GI post irradiation (Pang *et al*., 2011). As well as GI had been detected in skin of Hiroshima and Nagasaki atomic bombs survivors from 1968 to 1999. Findings of 53BP1 expression showed significant increase in the rate of basal cell carcinoma as a delayed response of atomic bomb radiation *in vivo* (Naruke *et al*., 2009).

In this study, it was first necessary to evaluate the effect of low and high X-ray doses on tumour MCF7 and non-tumour HMT-3522S1 (HMT) cells to confirm that IR could cause cellular damage in the MCF7 and HMT cell. Therefore, early and late direct effects of X-ray on the MCF7 and HMT cells were measured using chromosomal analysis, apoptosis analysis, telomere length and telomerase activity measurements following 0.1 and 2 Gy X-ray irradiation.

3.2 Materials and methods

3.2.1 Cell culture

Tumour human breast epithelial cells (MCF7) and non-tumour human breast epithelial cells (HMT-3522S1) were utilised in the study. Both cell types were cultured and maintained as described in sections 2.1.1.i and 2.1.1.ii. In brief, cells were grown in their media for several population doublings in T75 tissue culture flasks. For MCF7 and HMT-3522S1 cell sub-culture, 1.4 x 10^6 cells were seeded per each T75, and sub-cultured at 80% confluence using unconditioned (fresh) media (One passage represented approximately 2 cell population doublings).

3.2.2 Cell irradiation

Cells were cultured in 6-well plate dishes as described in section 2.1.10. Cells were exposed to 0.1 Gy and 2 Gy X-ray irradiation and incubated for 4 hours in their media. After 4 hours, a fraction of cells were subjected to apoptotic analysis, whilst

other fractions were sub-cultured and incubated to further population doublings. Cells were analysed for chromosomal, telomere length and telomerase activity after 1, 12 and 24 population doublings. Control groups (sham/0 Gy irradiated cells) were established in parallel.

3.2.3 Chromosomal analysis

Direct irradiated (0, 0.1 and 2 Gy) MCF7 and HMT-3522S1 cells were analysed for early chromosomal damage after 1 population doubling post irradiation, and after 12 and 24 population doublings for the perpetuation and delayed damage responses respectively. Giemsa solid staining technique was used to determine chromosomal aberrations in the 'direct irradiated' cells as described in section 2.1.11. Briefly, cells were arrested at metaphase of the cell cycle using 20 ng/ml demecolcine. Cells were collected and treated with 75 mM potassium chloride solution for 20 minutes. Then cells were washed twice with 25% acetic acid in methanol and dropped onto clean microscope's slides. Slides were stained with Giemsa and covered by cover slipp.

3.2.4 Apoptotic analysis

Apoptotic levels were measured in the direct irradiated cells after 4 hours, 1, 12 and 24 population doublings for initial, perpetuation and delayed responses following irradiation respectively. The method of apoptosis was described in section 2.1.12, using prolong gold anti-fade reagent with DAPI. The normal cell nucleus uniformly stains with DAPI, whilst the apoptotic cell nucleus shows apoptotic bodies down the fluorescent microscope.

3.2.5 Telomere length measurement

Tumour MCF7 and non-tumour HMT-3522S1 telomeres were measured by utilising Q-FISH technique as described in section 2.1.13. Briefly cells were collected, fixed and dropped onto clean slides. The slides were rehydrated, fixed with formaldehyde,

then incubated with pepsin and hybridised using peptide nucleic acid FITC. The slides were then mounted with Vectorshield mounting media with DAPI. The smart capture and IP-Lab software was used to analyse the telomere fluorescent signals.

3.2.6 Telomerase activity measurement

Initial and delayed telomerase activity of MCF7 and HMT-3522S1 cells were measured after irradiation as illustrated in section 2.1.14 using real-time PCR. Concisely, cells were lysed by CHAPS lysis buffer, cell extractions were loaded with master mix into 96 well plates. Then 96 well plates were placed in real-time PCR to measure the activity of telomerase.

3.3 Results

In order to establish the responses of Tumour and non-tumour breast epithelial cells (MCF7 and HMT cells respectively) to ionizing radiation X-ray, cells were exposed to 0.1 Gy (low dose) and 2 Gy (high dose) X-ray. Dose responses were measured by chromosomal and apoptotic analysis, telomere length and telomerase activity estimating initial and delayed responses following irradiation.

3.3.1 Direct irradiated tumour breast epithelial MCF7 cells following 0.1 Gy X-ray

Low dose of X-ray (0.1 Gy) is considered as a diagnostic relevant dose, a full body CT scan (BER, 2010), as well as, 0.1 Gy X-ray is established as a fractionated dose to a high therapeutic dose of radiation in cancer treatment (Joiner, 1987). Therefore, cellular damage such as chromosomal aberrations, apoptosis and telomeric instability was evaluated; in order to estimate the effect of low dose of X-ray (0.1 Gy) at different time points, after 4hours, 1, 12 and 24 population doublings (early and late cellular responses to IR exposure).

Direct irradiated MCF7 cells showed a significant increase in the number of chromosome and chromatid aberrations ($p \leq 0.0001$) after 1 population doubling following 0.1 Gy X-ray irradiation (Figures 3.1, panel A) compared to the corresponding control groups. The mean aberrations per 0 Gy irradiated MCF7 cells (control) was 0.28±0.068, which significantly increased to 1.07±0.79 following 0.1 Gy irradiation. However, apoptotic level was observed only after 4 hours (before sub-culturing) in the 0.1 Gy direct irradiated cells (2.8%±0.02), which was statistically higher ($p \leq 0.0001$) than the control (1.12%±0.02). Direct irradiated MCF7 cells did not demonstrate induction of apoptosis after 1 generation post irradiation, suggesting that the apoptotic cells could be removed during the first cell sub-culture process. The level of apoptosis in the direct irradiated cells (0.79%±0.012) returned the normal level (0.88%±0.012) as shown in figure 3.1 panel A. Moreover, low dose of X-ray (0.1 Gy) was significantly able to shorten telomeres of 0.1 Gy direct irradiated MCF7 cells ($p \leq 0.005$). The intensity of telomeric fluorescence in the MCF7 direct irradiated control cells was 24.87±2.7, which was reduced to 15.52±1.7 by 0.1 Gy X-ray after 1 population doubling (figure 3.1, panel B). Furthermore, direct irradiated MCF7 cells exhibited a significant reduction in the telomerase activity ($p \leq 0.0001$) after 1 generation post 0.1 Gy X-ray irradiation (see figure 3.1, panel B). Low dose of X-ray reduced telomerase arbitrary unit (TAU) of direct irradiated MCF7 cells from 86636.7±2252.65 to 52003.3±825.29. Data suggested that low dose of IR can cause insufficient telomerase activity leading to telomeric shortening, which can instigate chromosomal aberrations as initial responses in the MCF7 cells.

Propagation of delayed damaged responses was monitored after 12 and 24 population doublings following irradiation. Data showed that progeny of 0.1 Gy direct irradiated MCF7 cells exhibited a significant chromosomal damage ($p \leq 0.005$) after 12

population doublings. The mean chromosomal aberrations of these progeny was 0.52±0.085, whilst the control mean chromosomal aberrations was 0.24±0.47 (figure 3.2, panel A). Nevertheless, these progeny did not demonstrate a significant induction of apoptotic level compared to the control (Figures 3.1, panel A). Although the progeny of 0.1 Gy direct irradiated MCF7 cells did not show a significant decrease in the telomerase activity, Telomere shortening was significantly observed in these progeny ($p \leq 0.05$) as shown in figures 3.1, panel B. Findings suggested that active telomerase can keep cell dividing even with short telomeres, leading to high chromosomal instability, with absence of apoptosis.

After 24 population doublings following irradiation, the progeny of 0.1 Gy direct irradiated MCF7 cells continued to demonstrate significant chromosomal and chromatid damage ($p \leq 0.05$) compared with the corresponding controls (Figures 3.1, panel A). The mean chromosomal aberrations of the progeny of sham/control direct irradiated MCF7 cells was 0.23±0.054, which significantly elevated to 0.50±0.096 following 0.1 Gy X-ray irradiation. However, apoptotic level was not significantly observed in these progeny after 24 population doublings following irradiation (figure 3.1, panel A). The Telomeres were significantly shorter ($p \leq 0.001$) in the progeny of direct irradiated MCF7 cells than the control cells after 24 population doublings post 0.1 Gy X-ray irradiation. However, these progeny showed a normal level of telomerase activity as a delayed response as shown in figures 3.1, panel B. Hence, findings suggested that the telomeric instability in the 0.1 Gy direct irradiated MCF7 cells can trigger chromosomal instability in the absence of apoptotic induction.

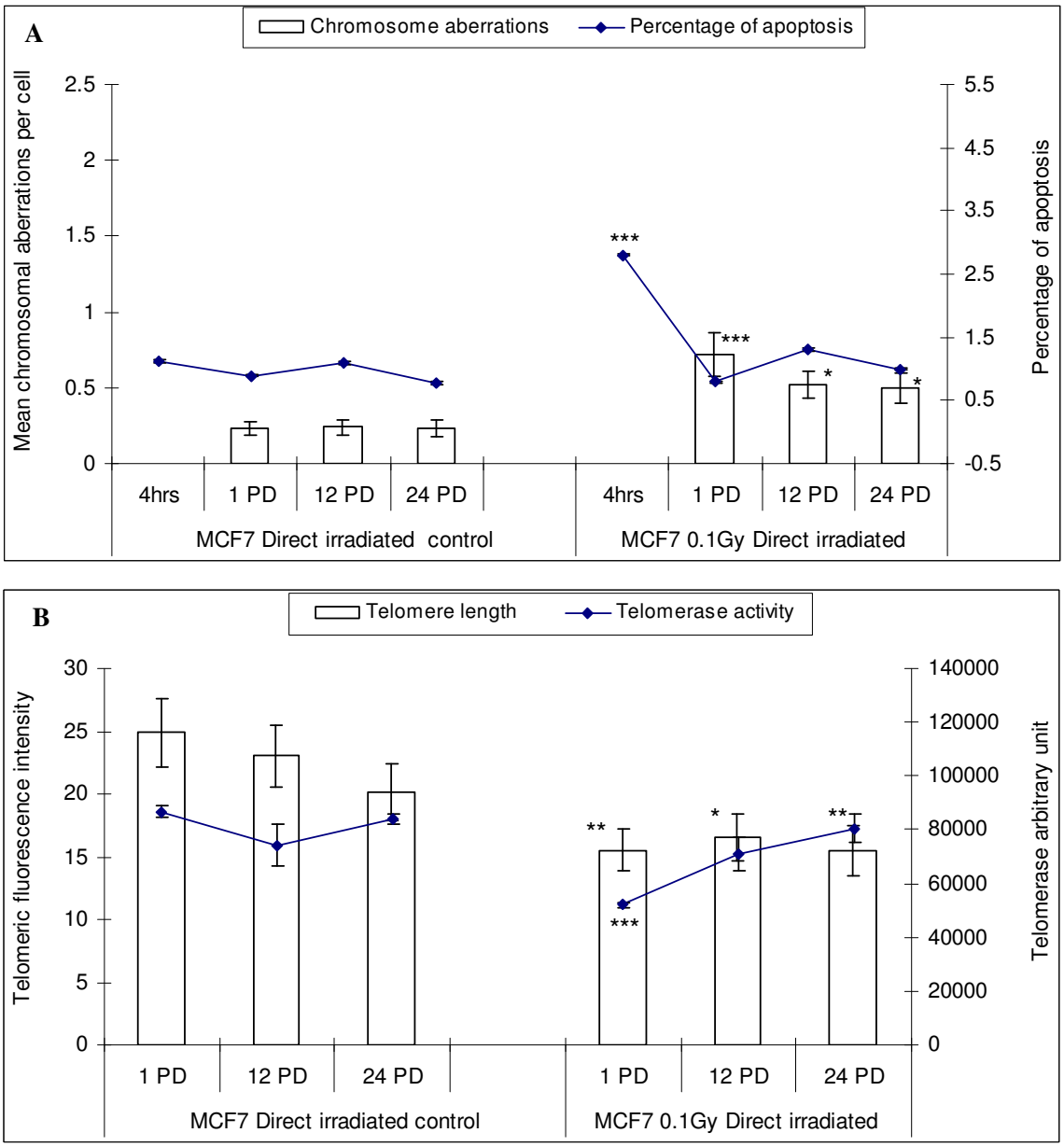


Figure 3.1: Chromosome aberrations, apoptosis, telomere length and telomerase activity in tumour breast epithelial MCF7 cells after 4 hours, 1, 12 and 24 population doublings following 0.1 Gy X-ray irradiation.

Cells were exposed to 0.1 Gy X-ray at 80% confluence. Then cells were harvested to chromosomal analysis, apoptotic analysis, telomere length and telomerase activity measurement. Panel A showed a significant induction of apoptosis (***$p \leq 0.0001$) only after 4 hours post irradiation, compared to the control this level of apoptosis returned to the normal level after 1 generation. However these cells revealed a high chromosomal damage (***$p \leq 0.0001$) after 1 generation following irradiation compared to the control. MCF7 cells continued to displayed chromosomal instability (*$p \leq 0.05$) after 12 and 24 generations ($p \leq 0.05$) following irradiation, in absence of apoptosis. Data reported that low dose of X-ray can induce initial and delayed chromosomal damage in the tumour MCF7 cells, if there is no delayed induction of apoptosis. Panel B: represent telomere instability in 0.1 Gy direct irradiated MCF7 cells, involving telomere length and telomerase activity. Cells revealed a significant telomerase insufficiency (***$p \leq 0.0001$) after one generation following 0.1 Gy X-ray irradiation. Moreover these cells showed a significant telomeric shortening (**$p \leq 0.005$) at the same time point. Although 0.1 Gy direct irradiated MCF7 cells exhibited a normal level of telomerase activity, telomere length was significantly shorter thank the controls after 12 (*$p \leq 0.05$) and 24 (**0.005) generations delayed telomere shortening. Hence, evidence that active telomerase could stimulate cell to proliferate even with short telomeres, which could lead to chromosomal instability.

Experiment was performed in 3 technical repeats.

3.3.2 Direct irradiated tumour breast epithelial MCF7 cells following 2 Gy X-ray

To evaluate the cellular response and sensitivity of MCF7 cells to therapeutic dose, the cells were exposed to 2 Gy X-ray irradiation and analysed at early and delayed time post irradiation for chromosomal analysis, apoptotic analysis, telomere length and telomerase activity measurement.

A significant chromosome and chromatid aberrations ($p \leq 0.0001$) were observed in MCF7 cells after 1 population doubling following 2 Gy X-ray irradiation compared to the control as shown in figure 3.2, panel A). Mean chromosomal aberrations of 2 Gy direct irradiated MCF7 cells was 1.38±0.14, which was significantly higher than 0.1 Gy direct irradiated MCF7 (0.72±0.14). Hence, the chromosomal damage responses were dose-dependent in MCF7 cells, i.e. high level of chromosomal damage associates with high dose of IR, and vice versa. Direct irradiated MCF7 displayed induction of apoptosis (($p \leq 0.0001$) only after 4 hours following 2 Gy irradiation. The percentage of apoptosis in the sham/control irradiated cells was 1.12±0.02, which reached to 2.6±0.03 post 2 Gy irradiation. However, the apoptotic level returned to the normal level after 1 generation (after one cell sub-culture) as shown in figure 3.2, panel A. Telomere shortening was significantly detected in 2 Gy direct irradiated MCF7 cells after 1 generation as an initial response IR. The telomere length of the sham/control irradiated cells was 24.87±2.7, which decreased to 10.13±0.92 in irradiated cells (Figure 3.2, panel B). Moreover, MCF7 cells demonstrated a significant decrease in the telomerase activity ($p \leq 0.0001$) after 1 population doubling following 2 Gy X-ray irradiation compared to the control. The sham/control irradiated MCF7 cells exhibited 86636.7±2252.65 TAU of telomerase activity, which significantly reduced to 39170±3980.37 (See figure 3.2, panel B). The findings

hypothesized that IR can cause telomerase insufficiency, leading to telomere shortening and chromosome aberrations, in the absence of apoptosis.

Results of delayed responses after 12 population doublings reported that MCF7 cells continued to exhibit a high induction of chromosome aberration ($p \leq 0.005$) in their progeny following 2 Gy X-ray irradiation. These progeny showed 0.59±0.09 mean chromosomal aberrations, which was significantly higher than the control mean chromosomal aberrations (0.24±0.047). However, these progeny did not exhibit induction of apoptosis compared to the control after 12 generations. The percentage of apoptotic level of direct irradiated MCF7 progeny cells (0.95±0.011) was very closed to the control progeny cells (0.88±0.12) as shown in figure 3.2, panel A. After 12 generations following irradiation, telomerase activity level of progeny of irradiated MCF7 cells returned to normal level compared to the control, nevertheless, these progeny showed a significant telomere shortening (12.43±2.34, $p \leq 0.05$) compared to the control (23±2.46) as shown in figure 3.2, panel B. Data showed that progeny cells with active telomerase and instable telomeres could display chromosomal instability in absence of apoptosis.

Delayed chromosomal damage within progeny of 2 Gy direct irradiated MCF7 was significantly observed ($p \leq 0.005$) after 24 population doublings following irradiation compared with the control. However, these progeny did not display a significant apoptotic level compared to the control (Figure 3.2, panel A). Although, normal level of telomerase activity was detected in the progeny of irradiated MCF7 cells, these progeny consistently continued to exhibit a short/instable telomere length. The telomere length of direct irradiated MCF7 progeny cells was 10.74±1.32, which was significantly shorter ($p \leq 0.0001$) than telomeres of the control progeny cells (Figure

3,2, panel B). A similar speculation was reported that instable telomeres can cause chromosomal instability in the absence of apoptosis and active telomerase.

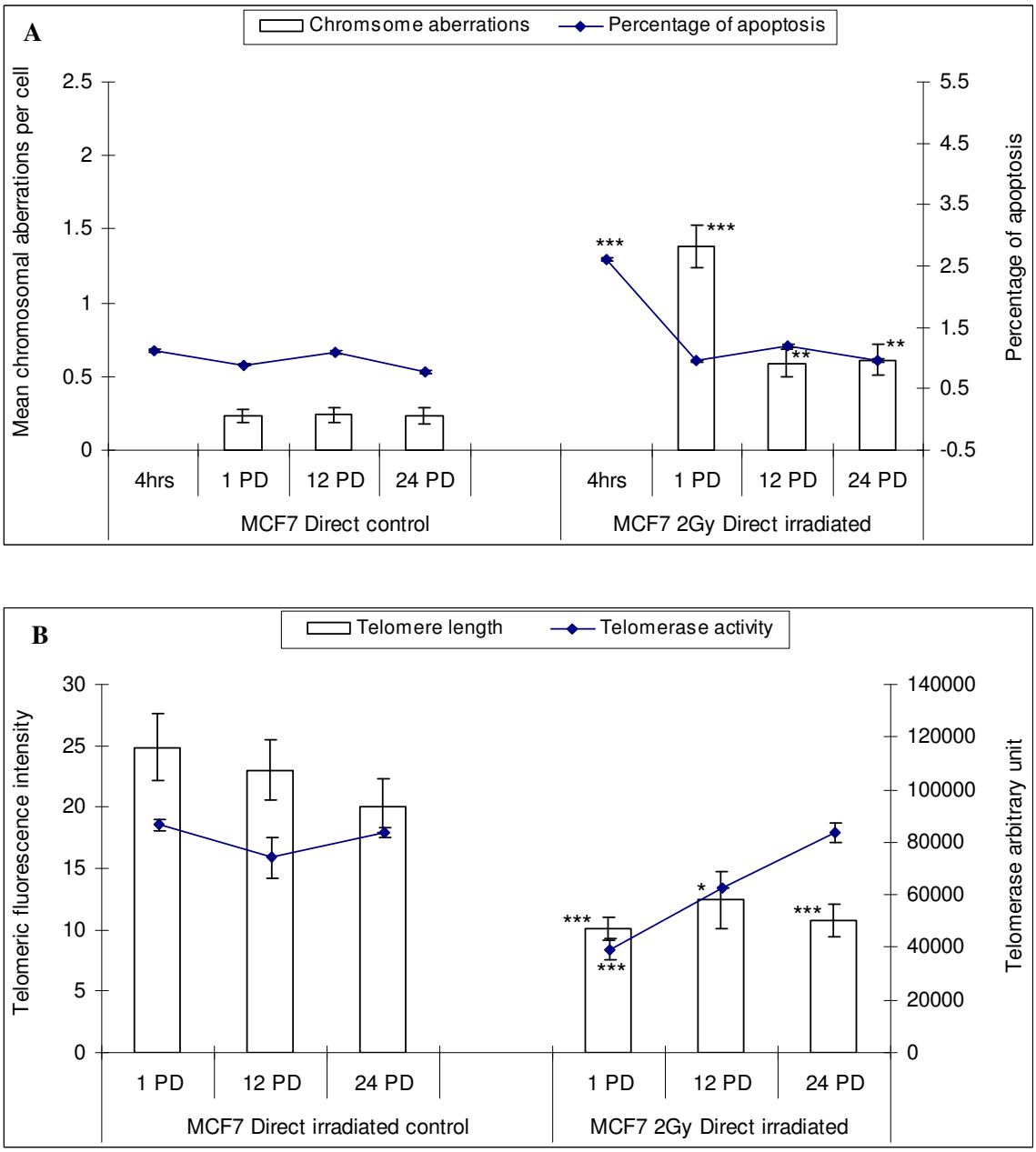


Figure 3.2: Initial and delayed chromosome aberrations, apoptosis, telomere length and telomerase activity in MCF7 cells following 2 Gy X-ray irradiation.

MCF7 cells at 80% confluent were irradiated by 2 Gy X-ray and harvested at dearly and delayed time points for chromosomal damage analysis, apoptotic analysis, telomere length and telomerase activity measurement. Panel A: High dose of X-ray (2 Gy) induced significant initial chromosomal damage and apoptosis (***$p \leq 0.0001$) in MCF7 cells. Chromosome aberrations were observed after 1 generation following irradiation; whilst induction of apoptosis was detect only after 4 hours following irradiation. MCF cells also showed chromosomal instability (**$p \leq 0.005$) after 12 and 24 population doublings post irradiation. However, delayed apoptotic response was insignificant in these cells compared to the controls. Panel B: The ability of 2 Gy X-ray to cause significant telomeric shortening and insufficient telomerase activity (***$p \leq 0.0001$) in MCF7 cells after 1 generation following irradiation. Although telomerase activity returned to the normal levels in 2 Gy MCF7 cells after 12 and 24 generations, These cells continued to show a significant telomeric shortening after 12 (*$p \leq 0.05$) and 24 (***$p \leq 0.0001$) generations post irradiation. Active telomerase could promote cell proliferation instigating chromosomal instability due to telomeric shortening and absence of apoptosis.
Experiment was performed in 3 technical repeats.

3.3.3 Direct irradiated non-tumour breast epithelial HMT-3522S1 cells following 0.1 Gy X-ray

Normal/non-tumour cells are frequently exposed to low doses of IR during diagnostic procedure (BER, 2010). As well as, normal cells, which are in the track of IR beam during radiotherapy, can obtain a low dose of IR (Joiner, 1987). Therefore, non-tumour breast epithelial HMT-3522S1 cells (HMT) were analysed for initial and delayed chromosomal damage≤ apoptotic response and telomeric instability following 0.1 Gy X-ray irradiation.

Direct irradiated HMT cells showed induction of chromosome aberrations ($p \leq 0.05$) after 1 generation post 0.1 Gy X-ray irradiation compared with the control (Figure 3.3, panel A). The mean chromosomal aberrations of sham/control cells was 0.14±0.037, which became 0.26±0.042 following irradiation. Apoptotic level was significantly ($p \leq 0.0001$) observed in the direct irradiated HMT only after 4 hours following irradiation. The percentage of apoptosis in the sham/control cells was 1.09±0.01, which elevated almost three and a half folds (3.6±0.028) after irradiation. Nevertheless, these cells did not reveal induction of apoptosis after 1 population doubling (Figure 3.3, panel A). Conversely, the progeny of 0.1 Gy direct irradiated HMT cells did not exhibit significant chromosomal aberrations after 12 and 24 population doublings compared to the corresponding control. Whilst the apoptotic levels were significantly observed ($p \leq 0.05$) within these progeny after 12 and 24 generations as delayed responses as shown in figure 3.3 panel A. Hence, findings reported that there is an inverse relationship between apoptosis and chromosomal instability. i.e. high level of apoptosis can eliminate the damaged cells.

Direct irradiated HMT cells demonstrated a significant telomere shortening ($p \leq 0.005$) after 1 generation post 0.1 Gy irradiation. These cells showed 9.47±0.96 telomeric

fluorescence intensity, which significantly shorter than the control (12.71±1.14). Moreover, telomerase activity was significantly reduced ($p \leq 0.0001$) from 60266.7±3530.97 TAU in the control cells to 33833.3±2598.29 TAU in the 0.1 Gy direct irradiated cells after 1 population doubling (Figure 3.3, panel B). The progeny of 0.1 Gy direct irradiated HMT cells continued to reveal shortening ($p \leq 0.05$) in their telomere length after 12 and 24 population doublings (10.59±1.1 and 9.89±0.93 respectively) compared to the corresponding controls. Nonetheless, these progeny did not display a significant telomerase insufficiency after 12 and 24 population doublings post irradiation compared with the controls (Figure 3.3, panel B). Thus, active telomerase could lead to cell division, even with short telomeres. Due to the short telomeres and active telomerase, findings suggested there is a potential risk of chromosomal/genomic instability; although these progeny did not reveal a significant chromosomal damage. Data suggested that it might be with further population doublings, cells can produce chromosomal instability later.

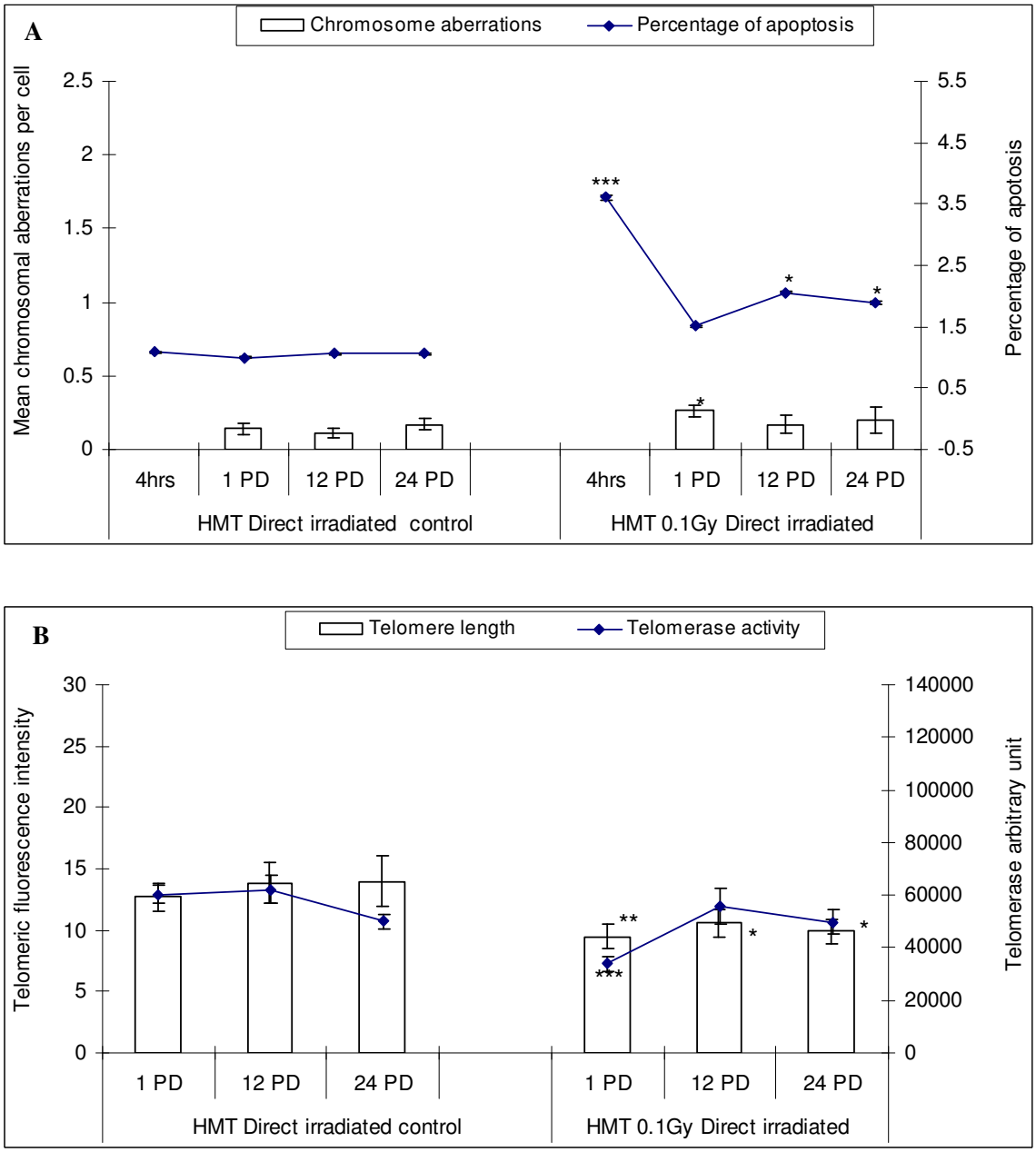


Figure 3.3: Initial and delayed chromosome aberrations, apoptosis, telomere length and telomerase activity in non-tumour breast epithelial HMT-3522S1 cells following 0.1 Gy X-ray irradiation.

HMT cells were irradiated by 0.1 X-ray at 80% confluence. Cells were then analysed for chromosomal damage analysis, apoptotic analysis, telomere length and telomerase activity measurement at early and late time points. Panel A illuminated the chromosomal damage and apoptotic responses in HMT cells to the low dose of X-ray. Cells demonstrated a significant initial chromosomal damage (*$p \leq 0.05$) after 1 generation and initial induction of apoptosis (***$p \leq 0.0001$) only after 4 hours following irradiation compared too the control. Surprisingly 0.1 Gy HMT cells did not reveal a significant delayed chromosomal damage after 12 and 24 generations post irradiation. Nonetheless, these cells exhibited a high induction of apoptosis (*$p \leq 0.05$) after 12 and 24 generations following irradiation. High apoptotic level could eliminate the damaged cells leading to a reduction in chromosomal instability. Panel B: Direct irradiated HMT cells showed initial and delayed induction of telomeric shortening (**$p \leq 0.005$ and $p \leq 0.05$ respectively) following 0.1 Gy X-ray irradiation. Nevertheless, these cells displayed a significant reduction in the telomerase activity (***$p \leq 0.0001$) only after 1 generation following 0.1 Gy irradiation. This level of telomerase activity returned to the normal level after 12 and 24 generations post irradiation as a delayed response. Due to the short telomeres and active telomerase, there is a potential risk of chromosomal/genomic instability. i.e. progeny of 0.1 Gy direct irradiated HMT cells can reveal chromosomal/genomic instability with further population doublings. Experiment was performed in 3 technical repeats.

3.3.4 Direct irradiated non-tumour breast epithelial HMT-3522S1 cells following 2 Gy X-ray

Non-tumour cells or tissue, which adhere or are in vicinity of cancer cells or tissues, can receive high doses of IR during radiotherapy. Therefore, to estimate the response and sensitivity of non-tumour cells to high dose of IR, HMT cells were exposed by 2 Gy X-ray, then early and delayed chromosomal damage, apoptosis and telomeric instability were measured.

Similarly to 0.1 Gy direct irradiated HMT cells, HMT cells were showed a high induction of chromosomal damage ($p \leq 0.0001$) after 1 population doubling following 2 Gy X-ray irradiation compared to the control. The mean chromosomal aberrations of sham/control HMT cells was, 0.14±0.037, which significantly increased to 0.94±0.16 post irradiation (Figure 3.4, panel A). Chromosomal damage response was dose-dependent in HMT cells. The mean chromosomal aberrations of HMT cells was 0.26 following 0.1 Gy X-ray irradiation, and (0.94) post 2 Gy irradiation. High induction of apoptosis ($p \leq 0.0001$) was detected in 2 Gy direct irradiated HMT (3.45±0.026) only after 4 hours following irradiation compared to the control (1.09±0.01). However, the apoptotic level returned to the normal level after 1 generation following irradiation compared to the control as shown in figure 3.4, panel A). Moreover, the 2 Gy direct irradiated HMT cells demonstrated a significant telomeric shortening ($p \leq 0.005$) and reduction in the telomerase activity ($p \leq 0.0001$) after 1 population doubling post irradiation compared with the controls (Figure 3.4, panel B). Telomeric fluorescence intensity and telomerase activity were 12.71±1.14 and 60266.7±3530.97 TAU respectively in sham/control HMT cells, which decreased to 8.84±0.99 and 31066.7±2305.31 following 2 Gy X-ray irradiation.

After 12 population doublings following irradiation, interestingly HMT cells did not exhibit induction of chromosome aberrations. However, these cells showed an elevation in apoptotic level, which was statistically insignificant ($p \leq 0.061$) compared to the control (See figure 3.4, panel A). Data suggested that the high level of apoptosis could remove the damage cells, which can be another confirmation of inverse relationship between apoptosis and chromosomal instability. Although the progeny of 2 Gy direct irradiated cells did not display a significant telomerase insufficiency. These progeny continued to show a significant induction ($p \leq 0.05$) of telomeric shortening (9.53 ± 1.12) after 12 generations post irradiation compared to the control (13.82 ± 1.66) as shown in figure 3.4, panel B.

After 24 generations post irradiation, the progeny of 2 Gy direct irradiated HMT cells demonstrated a high induction of chromosomal instability ($p \leq 0.0001$) compared to the control. These progeny showed 0.57 ± 0.097 mean chromosomal aberrations; whilst the mean chromosomal aberrations of sham irradiated progeny cells was 0.17 ± 0.042 (figure 3.4, panel A). The progeny of 2 Gy direct irradiated did not expressed induction of apoptosis after 24 generations following irradiation, which can be considered another evidence of inverse relationship between apoptosis and chromosomal instability (figure 3.4, panel A). A consistently telomeric shortening was significantly observed ($p \leq 0.05$) in the progeny of 2 Gy direct irradiated HMT cells after 24 population doublings following irradiation, although these progeny did not reveal induction of telomeric insufficiency (Figure 3.4, panel B). A similar suggestion was reported that 2 Gy X-ray could cause telomeric instability instigating to chromosomal instability, in the absence of apoptosis and active telomerase.

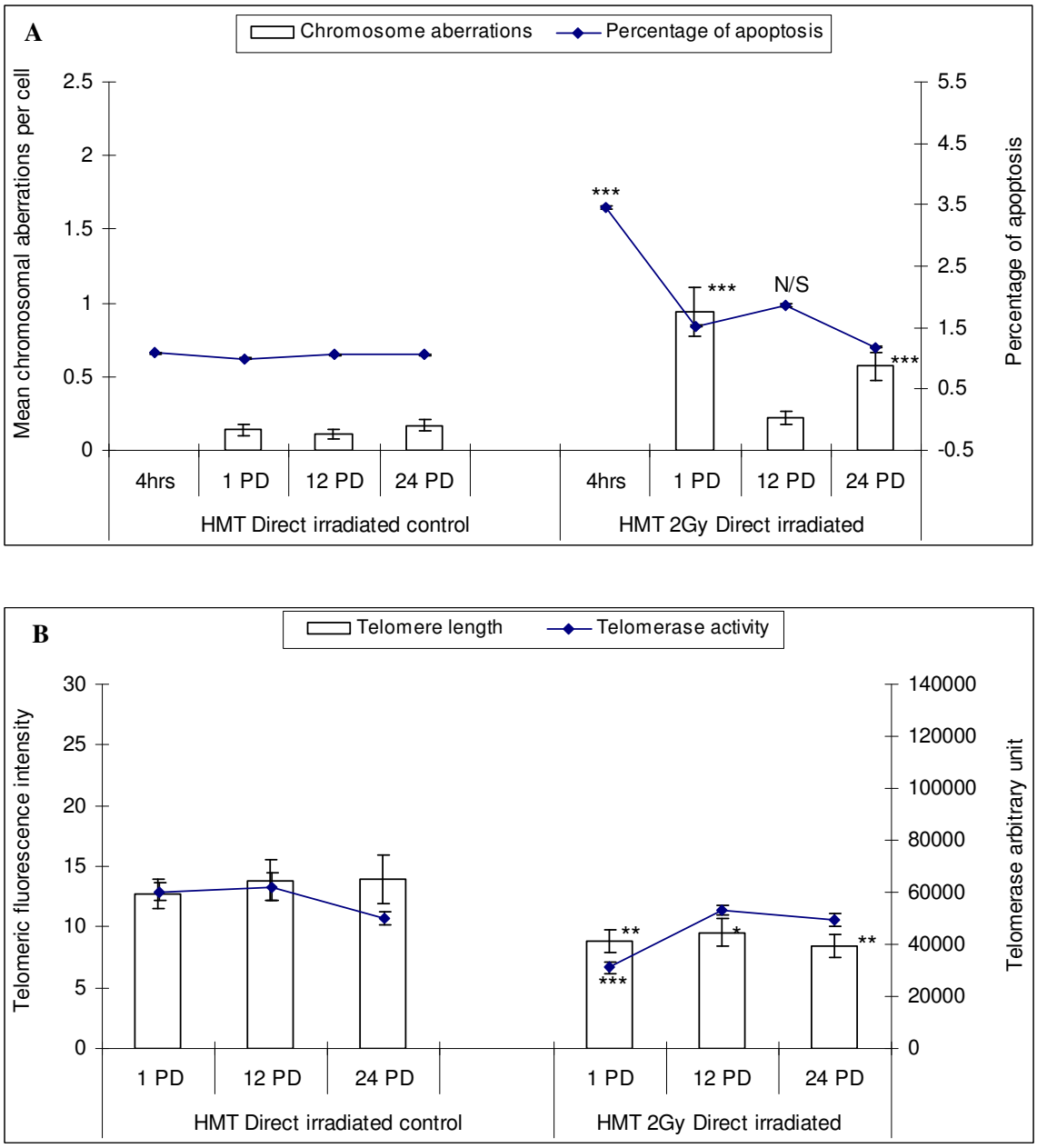


Figure 3.4: Effect of high dose (2 Gy) X-ray irradiation on HMT-3522S1 cells.

At 80% confluence, HMT cells were exposed to 2 Gy X-ray irradiation. Cells were subjected to chromosomal analysis, apoptotic analysis, telomere length and telomerase activity measurement at early and late time points. Panel A: Chromosome aberrations were significantly observed (***p≤0.0001) in HMT cells after 1 generation following 0.1 Gy X-ray irradiation. However, these cells showed a high induction of apoptosis (***p≤0.0001) only after 4 hours following irradiation as an early response. After 12 population doublings following irradiation, cells did not demonstrate a significant chromosomal damage; nevertheless, cells exhibited an elevation in apoptosis, which was statistically insignificant (p=0.061). Data suggested that the high level of apoptosis could remove the cells with high chromosomal damage. HMT cells showed a high induction of chromosomal instability (***p≤0.0001) with insignificant level of apoptosis after 24 generations following 2 Gy irradiation. Panel B illuminated the ability of 2 Gy X-ray to induce an early induction of telomeric shortening (**p≤0.005) and insufficient telomerase activity (***p≤0.0001) in HMT cells compared to the controls. Cells continued to reveal a significant telomeric shortening after 12 (*p≤0.05) and 24 (**p≤0005) generations following irradiation. However, telomerase activity returned to the active level after 12 and 24 generations post irradiation, which could stimulate cells proliferation even with short telomeres. Hence, cells could exhibit more chromosomal/genomic instability in absence of apoptosis.

Experiment was performed in 3 technical repeats.

3.4 Discussion and conclusions

Low and high doses of X-irradiation can cause chromosomal damage in normal and tumour cells by depositing energy, directly or indirectly, in the nuclei of cells (Bryant, 1988, Hall and Giaccia, 2006, Guerci *et al*., 2004). Our results have revealed high initial chromosomal damage in both tumour MCF7 and non-tumour HMT-3522S1 cell population following direct X-irradiation with 0.1 Gy and 2 Gy. Similar results, i.e. chromosomal and chromatid aberrations post X-irradiation, have been reported in normal and cancer bronchial epithelial cells by Konopacka and Rogolinsk (Konopacka and Rogolinski, 2010). In addition, a recent study has suggested that low and high LET can interrupt NHEJ pathway enhancing chromosomal radio-sensitivity in human cancer epithelial cells (MCF10A). In this study, micronucleus (MN) formation was used as an endpoint to evaluate radio-sensitivity of chromosomes (Vandersickel *et al*., 2010).

Our study has demonstrated that different doses of irradiation induced differing levels of initial chromosomal and chromatid aberrations in the irradiated groups. The 2 Gy irradiated MCF7 cells significantly demonstrated higher induction of early chromosomal and chromatid damage compared to the 0.1 Gy irradiated MCF7 cell group. Similar results were also observed in directly irradiated HMT-3522S1 cells (Figures 3.1 A, 3.2 A, 3.3 A and 3.4 A). Therefore, our data suggests that radiation-induced initial direct damage can be a dose-dependent response; i.e. the level of chromosomal and chromatid aberrations significantly elevate with increased radiation dose as an initial direct response. However, more studies at different doses are needed for this suggestion. This data is supported by the work of Mosesso *et al.* who have demonstrated that high doses of IR increase the duration of chromosomal aberration recovery (Mosesso *et al*., 2010).

Apoptotic analysis study showed that IR frequently induced apoptosis in both MCF7 and HMT-3522S1 direct irradiated cells after 4 hours following 0.1 and 2 Gy X-irradiation (Figures 3.1 A, 3.2 A, 3.3 A and 3.4 A). Much evidence has reported that non-lethal and lethal doses of radiation can make cells more amenable to commit apoptosis (Ifeadi and Garnett-Benson, 2012, Portess *et al*., 2007). Vogelstein and co-authors have suggested that DNA damage can activate p53, which frequently cause apoptosis by activating Bcl-2 family members (pro-apoptotic factors) such as Puma, Noxa, Bim, etc. (Vogelstein *et al*., 2000). Moreover, Protein phosphatise 2 (PP2A) plays an important role in p53 dephosphorylation by IR leading to apoptosis (Mi *et al*., 2009). In addition, Lyng, *et al.* have suggested that irradiation can decrease in mitochondrial membrane potential causing an increase in the Bcl-2 and cytochrome c secretion (Lyng *et al*., 2006a), which can lead to intrinsic apoptosis or mitochondrial pathway of apoptosis (Czerski and Nunez, 2004). In this apoptotic pathway (mitochondrial apoptosis) can happen in 2 pathways. The first pathway is that caspase 8 cleavs Bcl-2 interacting protein (BID) leading to enhance the mitochondria to increase cytochrome c release. Cytochrome c binds to apoptotic protease activating factor 1 (APAF 1), which with dATP activates caspase 9. Caspase 9 cleaves pro-caspase 3 leading to activate caspase 3 then commit the cells to apoptosis. In the second pathway, caspase 8 directly cleaves pro-caspase 3, and then activates caspase 3 causing apoptosis (Kim *et al*., 2006, Alladina *et al*., 2005). Abundant evidence supported our apoptotic findings, which suggested that IR can induce apoptosis in direct irradiated tumour and non-tumour cells ((Ilyenko *et al*., 2011, Wang *et al*., 2008). However, apoptosis returned to the normal level after one cell sub-culturing (one population doubling) in both MCF7 and HMT-3522S1 direct irradiated cells as shown in figures 3.1 A, 3.2 A, 3.3 A and 3.4 A). The data suggested that the damaged

and unhealthy cells with non-transmissible chromosomal damage had been eliminated by cell sub-culture; or the time point was inadequate to catch the apoptotic cells after one population doubling following irradiation.

Telomeric data demonstrated that IR could significantly perturb telomerase activity and telomere length in both MCF7 and HMT-3522S1 direct irradiated cells after one generation following irradiation (See figures 3.1 B, 3.2 B, 3.3 B and 3.4 B). The findings suggested that the dysfunction of telomerase causing by IR led to shortening in the telomere length for irradiated MCF7 and HMT-3522S1 cell. Our investigation has been supported by Kovalenko and co-workers. They have documented that targeting and disorder hTERT (the catalytic sub-unit of telomerase) can cause DNA damage at telomeric and extra-telomeric sites. These telomeric DNA damage frequently increase in the sensitivity of prostate cancer epithelial (LnCaP)cells and Human epithelial carcinoma (Gaytan *et al*., 1996) cells (Kovalenko *et al*., 2010).

In addition to investigate the initial effects of IR on the MCF7 and HMT cells, it well documented that radiation induces GI in the irradiated cells (Kadhim and Wright, 1998, Kadhim *et al*., 1992). Delayed chromosomal damage was significantly observed within the progeny of MCF7 direct irradiated cells after 12 and 24 population doublings following 0.1 and 2 Gy X-ray irradiation compared to the controls. However, the apoptotic results did not reveal induction level of apoptosis in this progeny (Figure 3.1 A and 3.2 A). These data was supported by abundant evidence that pointed to ionizing radiation-induced chromosomal instability (Huang *et al*., 2007, Kadhim *et al*., 2004). Drissi and other authors have demonstrated that telomeric shortening within cells can lead to histone acetylation and methylation (Drissi *et al*., 2011) leading to genomic instability as a delayed response of IR (Aypar *et al*., 2011). Moreover, alternative lengthening of telomeres (ALT) can enhance cancer stem cells'

ability to continue long term proliferation, as a result of telomerase activity (Silvestre *et al*., 2011). The results illustrated that telomerase activity can be increased within survival cells following IR (Aravindan *et al*., 2011). In addition, the increase in telomerase activity can be linked to the increased GI levels (Bednarek *et al*., 1995). Our data showed the ability of telomerase of progeny of direct irradiated MCF7 cells to maintain cell proliferation instigating chromosomal/genomic instability, due to short telomeres. Furthermore, uncapped (dysfunctional) telomere can lead to telomere-telomere fusion and ionizing radiation-induced telomere-DSB fusion causing GI. Dysfunctional telomeres frequently affected non-homologous end-joing (MHEJ) DNA repair mechanism leading to instability following irradiation (Williams *et al*., 2009, Williams *et al*., 2002). The lack of capping function of telomeres can produce end-to-end chromosomal fusion resulting in the formation of anaphase bridges, translocations, deletions and/or amplifications as a delayed response (Stewenius *et al*., 2007). Moreover, non-reciprocal translocation and aneuploidy can be observed in cells with short telomeres, and associates with high rates of malignant diseases in humans (Calado, 2009). Although, the high chromosomal damage was detected within the progeny of MCF7 direct irradiated cells; these cells did not demonstrate a significant delayed response of apoptosis compared to the controls (Figures 3.1 A and 3.2 A). The experimental findings suggested that there is an inverse relationship between chromosomal instability and apoptosis. In other words, a high level of apoptosis will eliminate and diminish the level of chromosomal damage and vice versa. This suggestion was clearly proved by the late results of progeny of HMT-3522S1 0.1 Gy direct irradiated cells. This group did not show induction of chromosomal aberrations. However, significant levels of apoptosis were observed after 12 and 24 generations ($p \leq 0.05$) following 0.1 Gy X-ray irradiation. The high

level of apoptosis in this progeny might eliminate the damaged cells with high rate of chromosomal aberration, and that proved our suggestion above. Furthermore progeny of HMT-35 22S1 2 Gy irradiated cells demonstrated insignificant chromosomal aberrations and high level of apoptosis (it was not quite significant, p=0.061), which can be considered another prove that apoptosis and chromosomal instability have an inverse relationship. The high level of apoptosis in the irradiated HMT-3522S1 cells could be instigated by the short telomeres. Ilyenko and other authors illustrated that blood lymphocytes from Chornobyl radiation workers showed a short telomeres compared to healthy donors. The authors proved that cells with short telomeres after low doses displayed induction level of apoptosis (Ilyenko *et al*., 2011).

The progeny of HMT-3522S1 2 Gy direct irradiated cells showed the same scenario of progeny of MCF7 direct irradiated cells, which is high level of chromosomal damage and insignificant induction of apoptosis (Figure 3.4 A). As well as, a significant telomeric shortening ($p \leq 0.005$) and normal level of telomerase activity were observed in this progeny as shown in figure 3.4 B.

Moreover, MCF7 cells were particularly more susceptible to ionizing radiation (IR) than HMT-3522S1 cells, which it can be two reasons behind the high sensitivity of MCF7 cells to IR in comparison with HMT-3522S1 cells. The first reason is HMT-3522S1 cells showed higher induction of apoptosis than MCF7 following 0.1 and 2 Gy X-ray irradiation (Figures 3.1, 3.2, 3.5 and 3.6). Thus, apoptosis might eliminate the cells with high chromosomal damage, to restore the homeostasis status of cell culture (Tesfaigzi, 2006). In addition, apoptotic bodies can link with inflammatory mediators leading to decrease in pro-inflammatory cytokines and controlling inflammation (Ren *et al*., 2008, Tesfaigzi, 2006), causing less chromosomal aberrations as delayed responses to the IR (Martin *et al*., 2011). The second reason is

MCF7 cell is aneuploidy (from 70 to more than 100), whilst HMT-3522S1 cell nucleus has 45 chromosomes. Therefore, the number of chromosomes in the ionization track path per each MCF7 cell nucleus is higher than the HMT cell nucleolus. Consequently, this could explain the high chromosomal damage in MCF7 cells following irradiation compared to HMT-3522S1 cells.

Data demonstrated that delayed chromosomal damage was a non-dose-dependent response in both direct irradiated MCF7 and HMT-3522S1 cells. However, other genomic instability manifestations, which are apoptosis, telomere length and telomerase activity, were dose-dependent responses as shown in figures 3.1 A and B, 3.2 A and B, 3.3 A and B and 3.4 A and B. These data suggested cells can survive and proliferate with a limitation in the number of chromosomal damage. In other words, cells with high multi chromosomal aberrations will be removed by apoptosis. Conversely, cells with transmissible or non-lethal aberrations can move to another generation with these chromosomal aberrations without committing apoptosis. Thus to explain why chromosomal damage is a non-dose-dependent, and apoptosis is a dose-dependent reaction.

Conclusions

1. IR can induce initial cellular damage in both tumour (MCF7) and non-tumour (HMT-3522S1) breast epithelial cells following low and high doses of X-ray irradiation.

2. Delayed chromosomal damage was observed in direct irradiated MCF7 cells following 0.1 and 2 Gy irradiation and in 2 Gy direct irradiated HMT-3522S1 cells. However, 0.1 Gy direct irradiated HMT-3522S1 cells did not show a delayed induction of chromosomal damage.

3. Delayed apoptotic induction was detected only in the 0.1 Gy direct irradiated HMT-3522s1 cells.

4. There was an inverse relationship between apoptosis and chromosomal instability within direct irradiated MCF7 and HMT following 0.1 and 2 Gy X-ray irradiation.

5. Telomerase activity level returned to the normal in both types of cells after few generations following the low and high doses of irradiation. Thus to suggest that these active telomerase enzymes could maintain cell dividing even with short telomeres causing more instability in the progeny.

6. Tumour MCF7 cells have been documented more susceptible to IR than non-tumour HMT-3522S1 cells.

7. Findings demonstrated that initial chromosomal damage was a dose-dependent. However, delayed chromosomal aberrations were in a non-dose-dependent manner in direct irradiated MCF& and HMT-3522S1 cells following 0.1 and 2 Gy irradiation.

8. Initial and delayed apoptotic levels, telomere length and telomerase activity were dose-dependent responses.

CHAPTER 4

RADIATION-INDUCED BYSTANDER EFFECTS IN TUMOUR AND NON-TUMOUR BREAST EPITHELIAL CELLS

4

Chapter 4: Radiation-induced bystander effects in tumour and non-tumour breast epithelial cells

4.1 Introduction

Communication between irradiated and un-irradiated cells can cause damage in cells that are not directly targeted by ionizing radiation (IR) underlying the requisite of Radiation-induced bystander effects (BE) (Morgan and Sowa, 2007). BE can also lead to chromosomal/genomic instability within the progeny of bystander cells, similar to those observed in the progeny of direct irradiated cells (Bowler *et al*., 2006). The factors that mediate this cellular communication can be transferred between cells via gap junctions (Hu *et al*., 2012) or by their release into the extra-cellular media (Mothersill *et al*., 2006).

Cell type, cell density and irradiation dose and quality can play important roles in BE induction (Buonanno *et al*., 2011, Ballarini *et al*., 2006, Hickman *et al*., 1994). Mothersill and Seymour have shown that bystander signals in irradiated cell conditioned media (ICCM) from human epithelium can induce BE in neighbouring un-irradiated human fibroblast cells, but have no effect on human epithelial cells (Mothersill and Seymour, 1997). Other studies have also demonstrated that ICCM from irradiated normal fibroblasts can increase the level of micronuclei (MN) in un-irradiated tumour giloma cells implying that this bystander response could have beneficial consequences in radiotherapy treatment. The researchers also suggest that reactive oxygen species (ROS) and nitric oxide (NO) could be involved in this bystander induction (Shao *et al*., 2005). Conversely, Konopackaand and Rogolinski have proved that ICCM from irradiated bronchial epithelial cells can induce chromosomal aberrations and MN in the normal bronchial epithelial cells following X-ray irradiation in an attempt to mimic the radiotherapy of cancer (Konopacka and Rogolinski, 2010).

Therefore, the purpose of our study was to investigate BE consequences between tumour and non-tumour breast epithelial cells following low (diagnostic) and high (therapeutic) doses of X-ray.

4.2 Materials and methods

4.2.1 Cell culture

Tumour (MCF7) and non-tumour human breast epithelial cells (HMT-3522S1) were grown and maintained as described in sections 2.1.1.i and 2.1.1.ii. In brief, cells were cultured in their media for several generations in T75 tissue culture flasks. For each cell type sub-culture, 1.4 x 10^6 cells were seeded per T75, and sub-cultured to 80% confluence using unconditioned (fresh) media. (One passage represented approximately 2 cell population doublings).

4.2.2 Experimental design

The experiment was designed as described in sections 2.1.8, 2.1.9 and 2.1.10. In brief, cells (MCF7 and HMT) were cultured in a 6-well plate system (base and insert dishes) until 70%-80% confluence. Cells in base dishes only, were exposed to 0.1 Gy and 2 Gy X-ray irradiation; immediately following the irradiation the insert dishes were placed inside the base dishes and incubated as a co-culture for 4 hours. As a result of co-culture system cell-cell communications, four cell combinations were established: 1) Direct irradiated MCF7 (base) - bystander HMT (insert). 2) Direct irradiated MCF7 (base) - bystander MCF7 (insert). 3) Direct irradiated HMT (base) - bystander MCF7 (insert) and 4) Direct irradiated HMT (base) - bystander HMT (insert). Each cell combination was irradiated with either 0.1 Gy or 2 Gy X-ray irradiation. Following the 4 hour co-culture time, samples of each irradiated and bystander cell populations were subjected to apoptotic analysis, whilst the remaining cells were sub-cultured to further population doublings. Cells from all groups were analysed for chromosomal,

telomere length and telomerase activity after 1, 12 and 24 population doublings. Control groups (sham-irradiated/0 Gy) were established in parallel.

4.2.3 Chromosomal analysis

Chromosomal analysis was performed as described in section 2.1.11. Briefly, cells were arrested at metaphase of the cell cycle using 20 ng/ml demecolcine. Cells were then collected and treated with 75 mM potassium chloride solution for 20 minutes causing the cells to burst and release their chromosomes thus allowing them to subsequently spread out onto microscope slides. Cells were then fixed twice with 25% acetic acid in methanol and dropped onto clean microscope slides. A Giemsa solid method was utilised to detect chromosome aberrations as described in sub-section 2.1.11.iii. Slides were then mounted with a cover slip and analysed using, a bright field light microscope.

4.2.4 Apoptotic analysis

Analysis of apoptotic levels was performed using prolong gold anti-fade mounting media with DAPI as described in section 2.1.12. Cells were collected with their media and fixed by 25% acetic acid in methanol prior to being dropped onto microscope slides and stained by mounting media. Direct irradiated and bystander cells were analysed after 4 hours and 1 population doubling for the initial apoptotic response and then after 12 and 24 generations for the delayed apoptotic response.

4.2.5 Telomere length measurement

Q-FISH technique was used to measure telomere length as described in section 2.1.13. In brief, cells were collected, fixed and dropped onto clean microscope slides. The slides were rehydrated, fixed with formaldehyde and then hybridised using peptide nucleic acid FITC. They were then mounted with Vectorshield mounting

media with DAPI and analysed for telomeric fluorescence signals using smart capture and IP-Lab software.

4.2.6 Telomerase activity measurement

Real-time PCR technique was utilised to determine initial and delayed telomerase activity as described in section 2.1.14. In brief, cells were lysed by CHAPS lysis buffer; cell extractions were then loaded with the master mix into 96 well plates and then placed in real-time PCR machine to measure the activity of telomerase at 25ºC/20min, 95ºC/10min, (95ºC/30sec, 60ºC/90sec) 40 cycles programme.

4.3 Results

We have previously determined the targeted effects of low and high dose X-ray within MCF7 and HMT cells (Chapter 3) and now we evaluate the non-target effects of X-ray irradiation in these cells from the results of the various cell combinations in the co-culture system (Figure 2.2) which was able to generate direct and bystander groups. Direct irradiated cells were seeded in the 6-well cell culture base, whilst the bystander cells were cultured in the insert dish. The two cell populations were physically separated; but a porous translucent polyethylene terephthalate membrane (3.0 μm diameter pore size), which formed the bottom of the insert dish, (Hill *et al*., 2006) enabled media between the two vessels to be shared and thus communication to be established.

4.3.1 Bystander responses in the non-tumour HMT cells through communication with 0.1 Gy direct irradiated tumour MCF7 cells

To investigate the induction of BE in HMT cells from MCF7 cells, MCF7 cells were exposed to 0.1 Gy X-ray and co-cultured with un-irradiated HMT cells. This cell combination would also be used to determine the effect of irradiated tumour cells on neighbouring un-irradiated non-tumour cells in terms of mimicking the low dose

radiotherapy fraction treatment of cancer cells and could thus explain BE consequences in non-tumour cells in comparison with other cell combinations following low and high irradiation doses. Additionally, data from the bystander response could be compared with the direct irradiated response under the same conditions.

Chromosome aberrations, apoptotic induction, telomere length and telomerase activity results from the 0.1 Gy direct irradiated MCF7 cells was all were shown to be significant at the early time-point. However, apoptotic levels and telomerase activity returned to normal after 12 and 24 population doublings, although chromosomal instability and telomeric shortening were maintained at significant levels at the delayed time-point. (For more detail see section 3.3.1).

In order to investigate and ascertain that the short lived and long lived damaging signals from irradiated cells can be received by neighbouring bystander cells, the un-irradiated insert dish containing HMT cells was immediately incubated (co-cultured) with the base dish, containing direct irradiated MCF7 cells, for 4 hours following 0.1 Gy irradiation. The two dishes (base and insert) were then separated, cells were taken for apoptotic analysis and the remaining cells were incubated to allow subsequent chromosomal and apoptotic analysis, telomere length and telomerase activity measurement at 1, 12 and 24 population doublings. Results for the chromosomal analysis revealed an increase in the mean number of chromosome aberrations after 1 population doubling in the HMT bystander cells compared to their control, 0.21±0.045 and 0.15±0.038, respectively however, the difference was statistically insignificant (Figure 4.1 A). Interestingly these bystander cells exhibited a significant high apoptotic induction ($p \leq 0.0001$), with levels of 2.7±0.018 at the 4 hour time-point, which was higher than the control (1.16±0.008), although the levels decreased

to 1.27±0.021 after 1 population doubling, statistically insignificant compared to the control (0.79±0.012), as shown in figure 4.1 A. Telomere length and telomerase activity were also both significantly reduced ($p \leq 0.005$ and $p \leq 0.0001$ respectively), in these bystander cells compared to their corresponding controls at this time-point. Telomeres were shortened from 13.18±1.49 (control) to 9.14±0.89 (bystander); whilst the telomerase activity measured 94130±1357.69 TAU in the control cells, which diminished to 65096.7±5247.96 TAU in the bystander cells (Figure 4.1 B). Although the bystander HMT cells displayed initial telomeric shortening and telomerase deficiency, the degree of chromosomal damage (aberrations) was shown to be insignificant. This could have been the result of apoptosis as a significant raised level was observed, indicative of elimination cells with high chromosomal damage.

After 12 population doublings, the number of chromosome aberrations remained low and insignificant in the bystander HMT cells compared to the control although a high induction of apoptosis was maintained ($p \leq 0.05$) as shown in figure 4.1 A. Data thus suggest an inverse relationship between high levels of apoptosis and a reduction in chromosomal damage. Moreover, 0.1 Gy bystander HMT cells did not show a significant reduction in either telomerase activity or reduced telomere length (10.22±0.92), compared to their control (13.03±1.91) as shown in figure 4.1 B, implying that the signals of 0.1 Gy direct irradiated MCF7 cells were not effective at inducing BE in the HMT cells.

At the 24 population doublings time-point, the bystander HMT cells exhibited an elevation in chromosome aberrations (0.23±0.054) although statistically not significant compared to the control (0.16±0.039). The reduction in the apoptotic level, observed in these bystander cells could explain the increase in chromosomal instability (Figure 4.1 A). Interestingly, the bystander cells showed significant

telomeric shortening ($p \leq 0.05$) as a delayed response, although telomerase activity had returned to normal levels (Figure 4.1 B). Our findings suggest that bystander HMT cells exhibited a high potential for chromosomal/genomic instability at initial time-points due to reductions in telomere length/apoptosis and active telomerase, but this was not observed at our delayed time-point, although the data suggest that the number of delayed chromosomal aberrations could increase with further population doublings i.e. at a much later time-point.

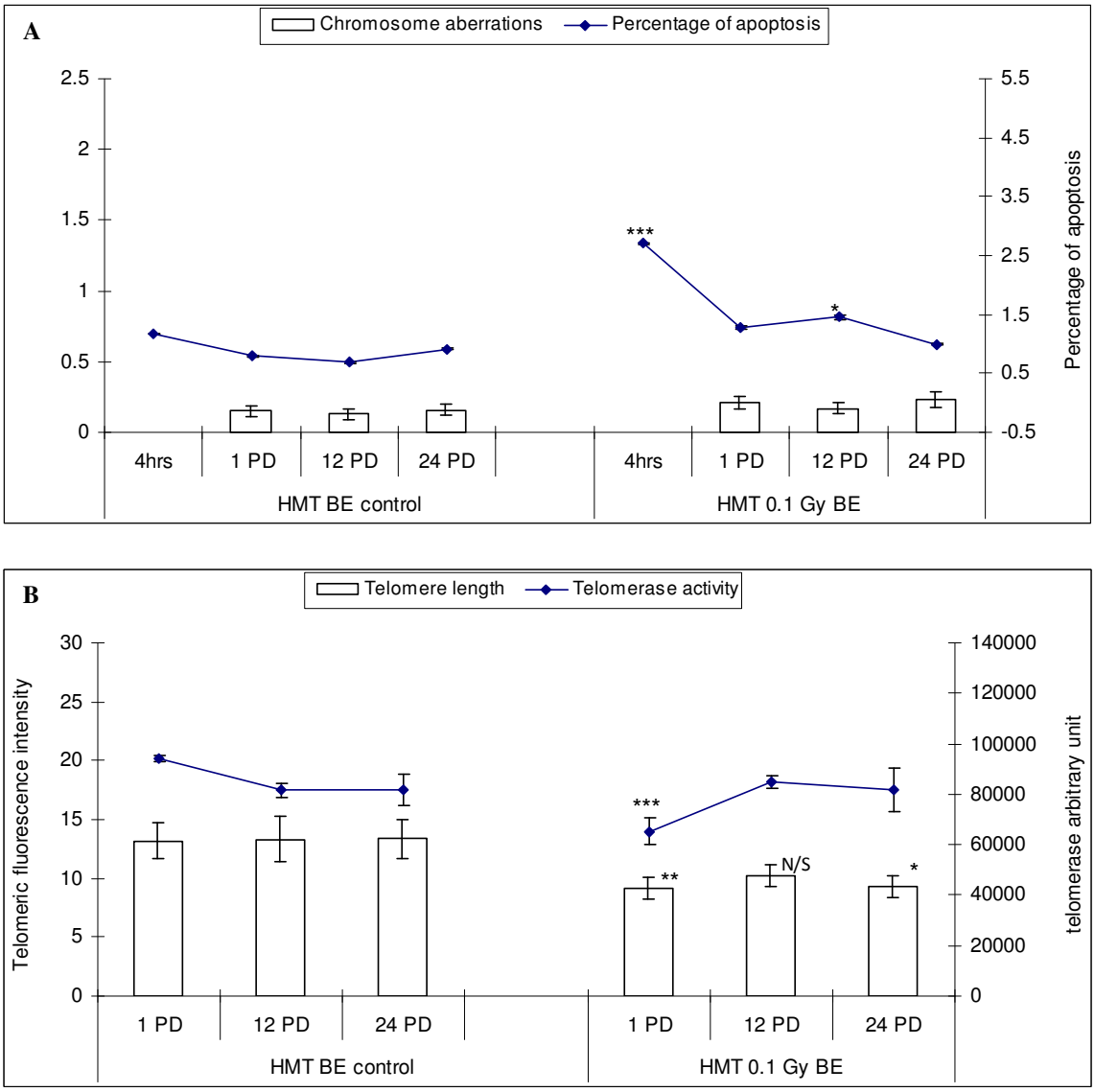


Figure 4.1: Initial and delayed bystander consequences in un-irradiated, non-tumour (HMT) cells following co-culture with 0.1 Gy direct irradiated tumour (MCF7) cells.

Un-irradiated non-tumour HMT cells were immediately incubated for 4 hours with irradiated tumour MCF7 cells following 0.1 Gy X-ray irradiation. Cells were analysed for apoptosis after 4 hours, 1, 12 and 24 population doublings (PD); whilst, chromosomal analysis, telomere length and telomerase activity measurements were performed after 1, 12 and 24 PD.

Panel A represent early and late chromosomal data and apoptotic induction; interestingly the HMT cells did not show a significant induction of initial and delayed chromosome aberrations. However, these cells demonstrated a high induction of apoptosis (***$p \leq 0.0001$) after 4 hours post co-culturing which returned to normal levels after 1 PD, but significantly increased for a second time at 12 PD (*$p \leq 0.05$) and then returned to normal levels at 24 PD. These results suggest that the high level of apoptosis observed at the initial time-point had eliminated the bystander cells with high chromosomal damage.

Panel B showed the ability of bystander signals of 0.1 Gy direct irradiated MCF7 cells to induce telomeric shortening (**$p \leq 0.005$) and telomerase activity reduction (***$p \leq 0.0001$) compared to their controls at 1 PD. Although, the telomerase activity of bystander HMT cells returned to normal levels after 12 PD, there was still a reduction in the telomere length albeit statistically insignificant ($p=0.07$) compared to the control which remained the case at 24 PD. Despite the fact that bystander HMT cells did not show a significant induction of chromosomal instability, there is a possibility that chromosomal/genomic instability could be observed at much later time-points (25 PD plus), due to telomeric shortening, maintained telomerase activity and absence of apoptosis.

Experiment was performed in 3 technical repeats.

4.3.2 Bystander responses in the non-tumour HMT cells through communication with 2 Gy direct irradiated tumour MCF7 cells

A direct irradiated MCF7-bystander HMT cell combination was established to determine the effect of bystander signals of tumour cells on neighbouring non-tumour cells following a radiotherapy dose (2 Gy X-ray). The targeted effects of a radiotherapy dose, has been quantified in section 3.3.2. Briefly, the bystander cells showed significant initial and delayed chromosomal damage responses and increased apoptotic levels at 4 hours following irradiation. We have previously demonstrated that direct irradiated MCF7 cells showed significant early responses of telomeric shortening and reduction in telomerase activity, the former had persisted at delayed time-points although telomerase activity levels were shown to have returned to normal levels. (For more details see section 3.3.2).

The effects of bystander signalling from 2 Gy direct irradiated MCF7 were shown to induce initial chromosomal damage ($p \leq 0.05$) in the HMT cells (Figure 4.2 A), which was higher (0.35 ± 0.06) than that observed following 0.1 Gy, suggesting of BE (chromosomal damage manifestation) is a dose-dependent. There was also significant induction of apoptosis ($p \leq 0.0001$) after 4 hours; nonetheless, the levels decreased becoming insignificant after the first cell sub-culture (1 population doubling) compared to the control (Figure 4.2 A). Furthermore at this time-point, these cells additionally demonstrated a significant induction of initial telomeric shortening ($p \leq 0.005$), which could have been mediated by the significant low levels of telomerase activity ($p \leq 0.0001$) as shown in figure 4.2 B. Telomerase activity was shown to be 94130 ± 1357.69 TAU in the control cells but had decreased to 6436.3 ± 3637.92 in the bystander cells. As mentioned above, this reduction could have initiated telomeric shortening, thus instigating chromosome aberrations in the bystander cells.

Following 12 population doublings, the progeny of the 2 Gy bystander HMT cells were shown not to exhibit chromosomal damage, nor did they interestingly show an induction of apoptosis (Figure 4.2 A). Although, these cells were shown to have shortened telomeres ($p \leq 0.005$) and normal levels of telomerase activity at this time-point. Therefore in summary, the bystander HMT cells demonstrated similar cellular damage responses as the HMT cells that were directly irradiated with 2 Gy X-ray (Chapter 3). Data for the HMT bystander cells, suggest that the manifestation of delayed genomic instability may be observed at later time-points, i.e. 25 population doublings plus; a suggestion supported by the results for chromosomal damage, where a high induction of chromosome aberrations ($p \leq 0.0001$) was observed at 24 population doublings (Figure 4.2 A). The mean number of chromosomal aberrations in the control cells was 0.16 ± 0.039, which increased more than 4 folds to 0.76 ± 0.12 in the bystander cells. There was no induction of apoptosis (Figure 4.2 A); data therefore again suggest that HMT bystander cells demonstrate an inverse relationship between apoptosis and chromosomal/genomic instability. Similarly, the bystander HMT cells continued to exhibit short telomeres ($p \leq 0.005$) after 24 population doublings, although normal levels of telomerase activity were measured (Figure 4.2 B). Data suggest that the sustained levels of telomerase activity could maintain cell division even when the cells have short telomeres, and this led to chromosomal instability, especially with the absence of apoptotic induction.

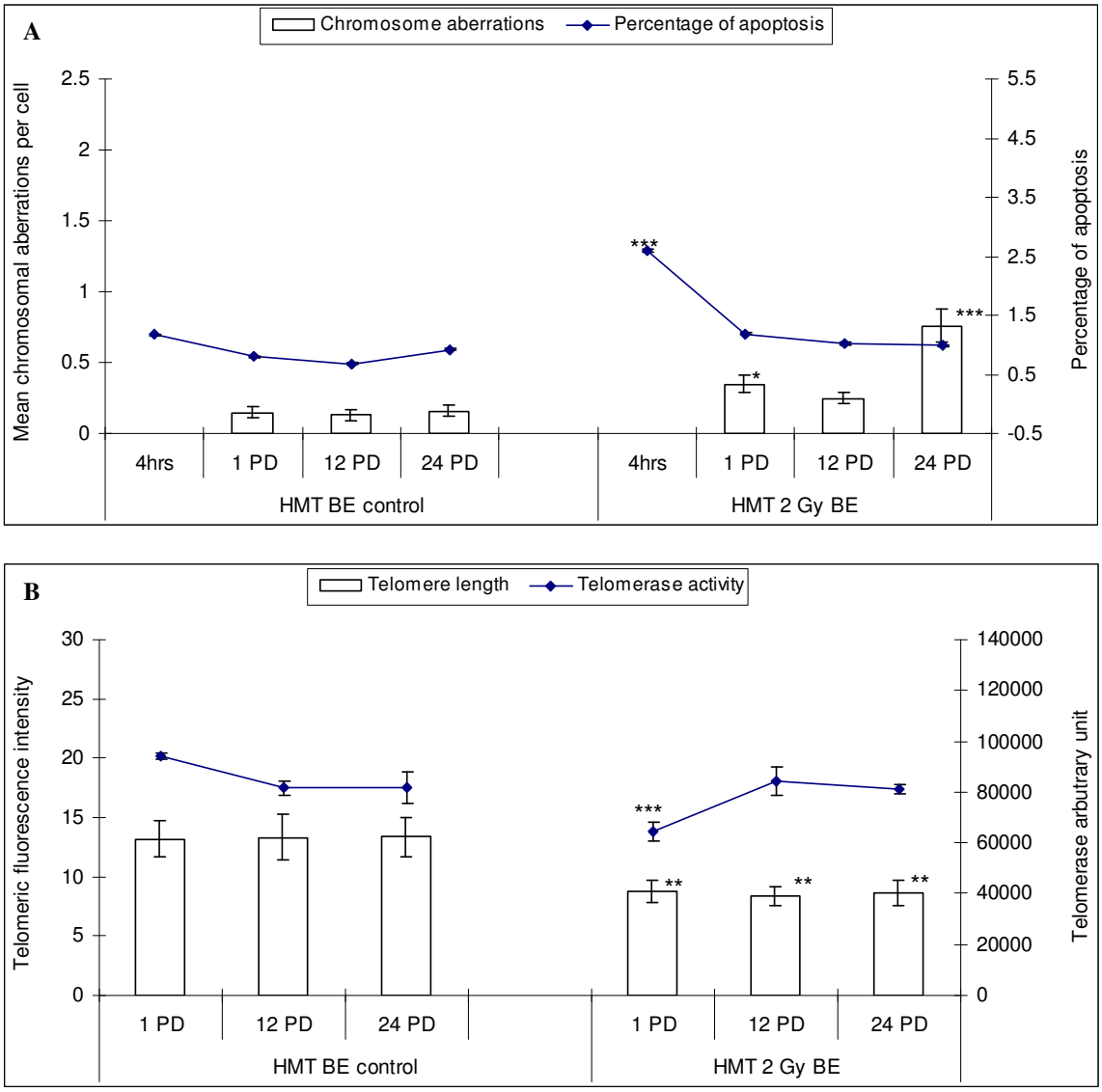


Figure 4.2: Bystander effects and genomic instability in un-irradiated HMT cells following co-culture with 2 Gy direct irradiated MCF7 cells.

Un-irradiated bystander HMT cells were immediately incubated (co-cultured) with direct irradiated MCF7 cells for 4 hours following 2 Gy X-ray irradiation. Cells were collected for apoptosis after 4 hours, 1, 12 and 24 population doublings (PD), whilst chromosomal analysis, telomere length and telomerase activity were performed after 1, 12, and 24 PD following co-culture.

Panel A demonstrate significant initial chromosomal damage (*$p \leq 0.05$) in the bystander HMT cells compared to the control. In addition, these cells revealed a high induction of apoptosis after 4 hours (***$p \leq 0.0001$) although levels returned to normal after 1 PD. At 12 PD, there was no significant evidence of delayed chromosomal instability moreover, cells continued to show insignificant apoptotic levels at this and later time-points. Interestingly though, these bystander cells exhibited a high induction of chromosomal instability (***$p \leq 0.0001$) at 24 PD. Thus, chromosomal instability and apoptosis data demonstrate an inverse relationship.

Panel B represent telomeric instability, including telomere length and telomerase activity. Telomerase activity was significantly reduced (***$p \leq 0.0001$) in the bystander cells after 1 PD. However, this reduction was not maintained at the later time-points (12 and 24 PD), although the cells demonstrated significant telomeric shortening (**$p \leq 0.005$) at all time-points. These finding therefore suggest that telomerase activity maintained cell proliferation although the cells had unstable short telomeres, which could instigate chromosomal instability, especially in the absence of apoptosis.

Experiment was performed in 3 technical repeats.

4.3.3 Bystander responses in the tumour MCF7 cells through communication with 0.1 Gy direct irradiated tumour MCF7 cells

To complete the investigation into non-targeted effect/consequences of 0.1 Gy X-irradiation, a direct irradiated-bystander MCF7 cell combination was set up. Sections 4.3.2 and 4.3.2 have shown that the bystander signals of 0.1 Gy direct irradiated MCF7 cells were only capable of inducing small biological effects in non-tumour (HMT) bystander cells compared to those observed following 2 Gy X-ray. These results could have major implications for warranting the use of low doses of IR in cancer radiotherapy.

In contrast, the effect of using a low dose of X-ray (0.1 Gy) on the tumour cell type (MCF7), frequently induced early and delayed cellular damage (Section 3.3.1). Interestingly, these signals could also initiate early induction of chromosomal damage in bystander MCF7 cells ($p \leq 0.005$), as shown in figure 4.3 A. The bystander signals were shown to have increased the mean number of chromosomal aberrations in the un-irradiated MCF7 cells from 0.21±0.049 to 0.55±0.09 and in addition, induced a high level of apoptosis ($p \leq 0.0001$) at the 4 hour time-point (3.64±0.028) compared to the control (1.27±0.012), however, the high apoptotic levels were abolished after 1 generation and remained at low levels at the later time-points (12 and 24 population doublings), as shown in figure 4.3 A. Moreover, at 1 population doubling, it appears that the bystander signals of irradiated MCF7 had disturbed telomeric status, prompting telomeric shortening ($p \leq 0.05$) and telomerase activity reduction ($p \leq 0.0001$) in the un-irradiated MCF7 cells (Figure 4.3 B). Data suggest disturbance of telomeres could instigate chromosomal instability, which was seen to be the case in the bystander MCF7 cells after 12 and 24 population doublings. Mean chromosomal aberrations in the 0.1 Gy bystander MCF7 cells was 0.55±0.98 and 0.52±0.11 (12 and 24 population doublings, respectively), which were significantly higher ($p \leq 0.05$) than

the controls' mean chromosomal aberrations (0.29±0.05 and 0.2±0.047 respectively) as shown in figure 4.3 A. In summary, these cells demonstrated an inverse relationship between apoptosis and chromosomal instability as a delayed response. At 12 population doublings, the bystander cells displayed a reduction in telomere length although not significant ($p=0.062$) compared to the control; whilst telomerase activity returned to normal levels (Figure 4.3 B). In contrast, at 24 population doublings, there was significant telomere shortening ($p\leq0.05$) despite normal levels of telomerase activity in these bystander cells (Figure 4.3 B). In summary, the bystander MCF7 cells demonstrated similar delayed responses/consequences to the MCF7 cells that had been directly irradiated with 0.1 Gy X-ray irradiation. Hence, the data confirms that genomic instability needs many generations to manifest as chromosomal aberrations and telomeric shortening.

Our findings suggest that bystander signalling is beneficial as it can induce initial multi-chromosomal damage in cancer cells; however, it can also have detrimental delayed response consequences (GI), with resultant tumour aggressiveness within the progeny of bystander cells.

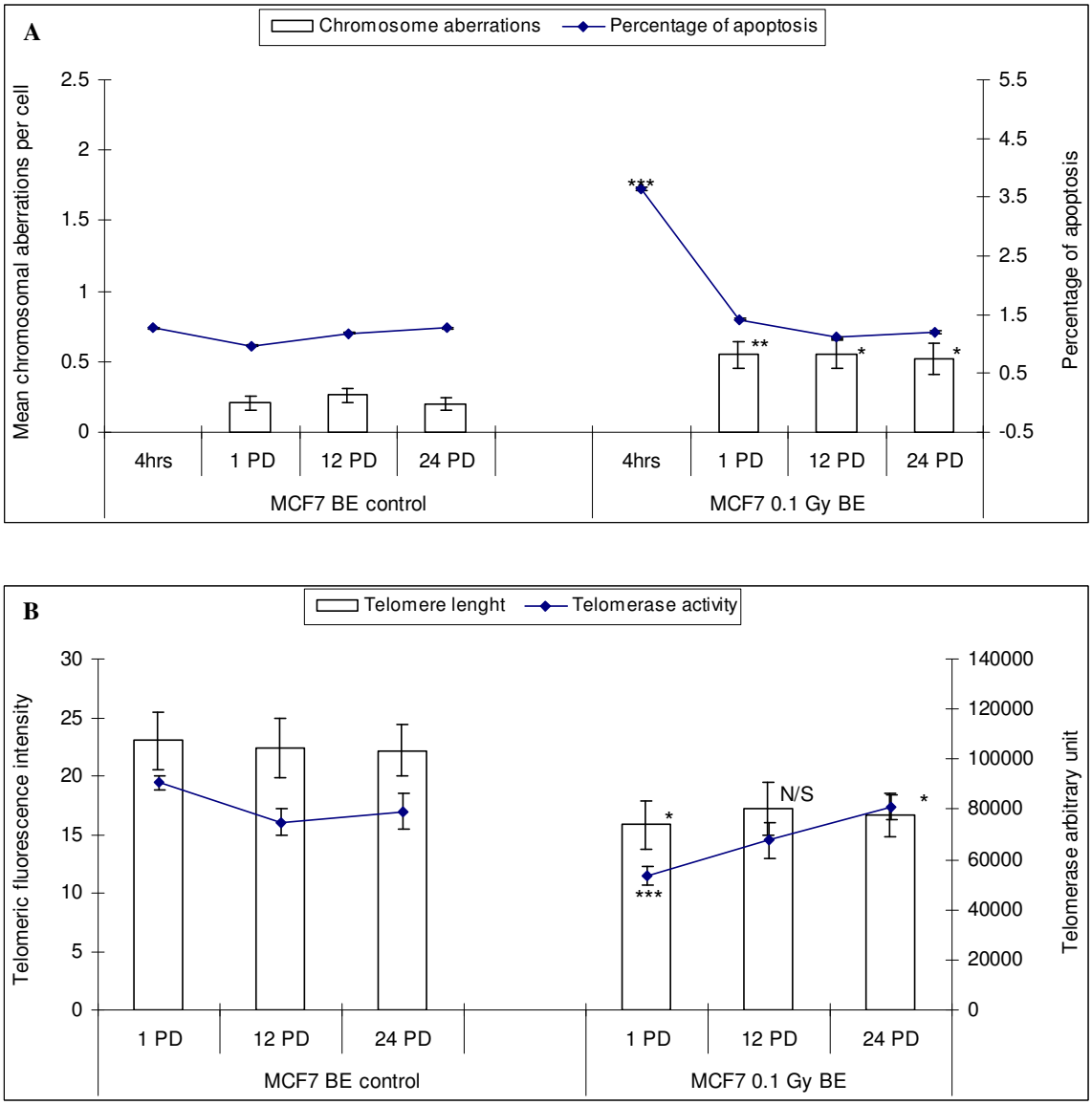


Figure 4.3: Early and late induction of chromosomal instability, apoptosis and changes to telomere length and telomerase activity within the bystander MCF7 cells following co-culture with 0.1 Gy direct irradiated MCF7 cells.

The un-irradiated bystander MCF7 cells were immediately incubated (co-cultured) with direct irradiated MCF7 cells following 0.1 Gy X-ray irradiation. After 4 hours, cells were subjected to apoptotic analysis; in addition, they were analysed for chromosomal damage, apoptosis, telomere length and telomerase activity measurements at 1, 12 and 24 population doublings (PD).

Panel A represent mean aberrations per cell and apoptotic levels. Chromosomal damage was significantly observed (**$p \leq 0.005$) in these cells after 1 PD. The cells also showed a high induction of apoptosis (***$p \leq 0.0001$) after 4 hours however, levels returned to normal after 1 PD and continued up to 24 PD. The reduction in apoptosis could have initiated the observed significant chromosomal instability (*$p \leq 0.05$) at 12 and 24 PD.

Panel B illustrate telomere instability within 0.1 Gy bystander MCF7 cells. The bystander cells showed a reduction in telomere length (*$p \leq 0.05$) and telomerase activity (***$p \leq 0.0001$) after 1 PD compared to the controls. Although normal levels of telomerase activity was observed at 12 and 24 PD, the telomere length was shortened within this progeny after 12 PD ($p = 0.62$) and 24 PD (*$p \leq 0.05$). Data suggest that telomeric instability could lead to chromosomal instability. In addition, we speculate that this trend would be maintained at later time-points (25 PD plus).

Experiment was performed in 3 technical repeats.

4.3.4 Bystander responses in the tumour MCF7 cells through communication with 2 Gy direct irradiated tumour MCF7 cells

Bystander responses of cancer cells were studied following a radiotherapy clinical dose, in order to estimate the effect of bystander signalling on tumour (MCF7) and normal cells (HMT). In addition, this cell combination was established to investigate the delayed responses (GI) in the bystander population cells, which can lead to increased tumour aggression.

We have previously shown that direct irradiated MCF7 cells demonstrate initial and delayed damage responses following 2 Gy X-ray irradiation. (For more details, see section 3.3.2).

In this recent investigation, bystander cells of 2 Gy MCF7-MCF7 cell combination showed a chromosomal damage response ($p \leq 00.05$) after 1 population doubling compared to the control. Initial mean chromosomal aberrations in the control cells was 0.21 ± 0.049, which increased to 0.52 ± 0.082 in the bystander group, (Figure 4.4 A). A significant induction of apoptosis ($p \leq 0.0001$) was additionally observed in these bystander cells at 4 hours compared to the control however, this was short lived as levels returned to normal after 1 population doubling (Figure 4.4 A). Moreover, additionally at this time-point, a significant initial telomeric shortening response was observed ($p \leq 0.005$) in these bystander cells along with a significant reduction in telomerase activity ($p \leq 0.0001$), as shown in figure 4.4 B. Data proved that bystander signals of MCF7 cells could induce initial cellular damage in the bystander MCF7 cells; cellular responses including chromosomal damage, telomere length disruption and telomerase activity reduction, whilst the apoptotic response was only increased at the 4 hour time-point.

After 12 population doublings, the 2 Gy bystander MCF7 cells were shown to exhibit significant chromosome aberrations ($p \leq 0.005$) compared to the control but levels of

apoptosis remained low (Figure 4.4 A). The bystander cells were additionally observed to have significant telomeric shortening ($p \leq 005$) but normal levels of telomerase activity, suggesting maintained cell division and subsequent initiation for chromosomal instability (Figure 4.4 B).

The 2 Gy bystander MCF7 cells persisted in exhibiting an induction of chromosomal damage ($p \leq 0.05$) after 24 population doublings compared to the control. These cells also continued to show a normal of level of apoptosis as a delayed response (Figure 4.4 A). These robust findings confirmed the inverse association between apoptosis and chromosomal instability. Furthermore, the data again suggest that chromosomal instability could be as a result of active telomerase and telomeric shortening (Figure 4.4 B); telomerase activity was shown to be normal at this time-point and this would have therefore maintained cell proliferation, despite significantly shortened telomeres, which could ultimately lead to chromosomal instability.

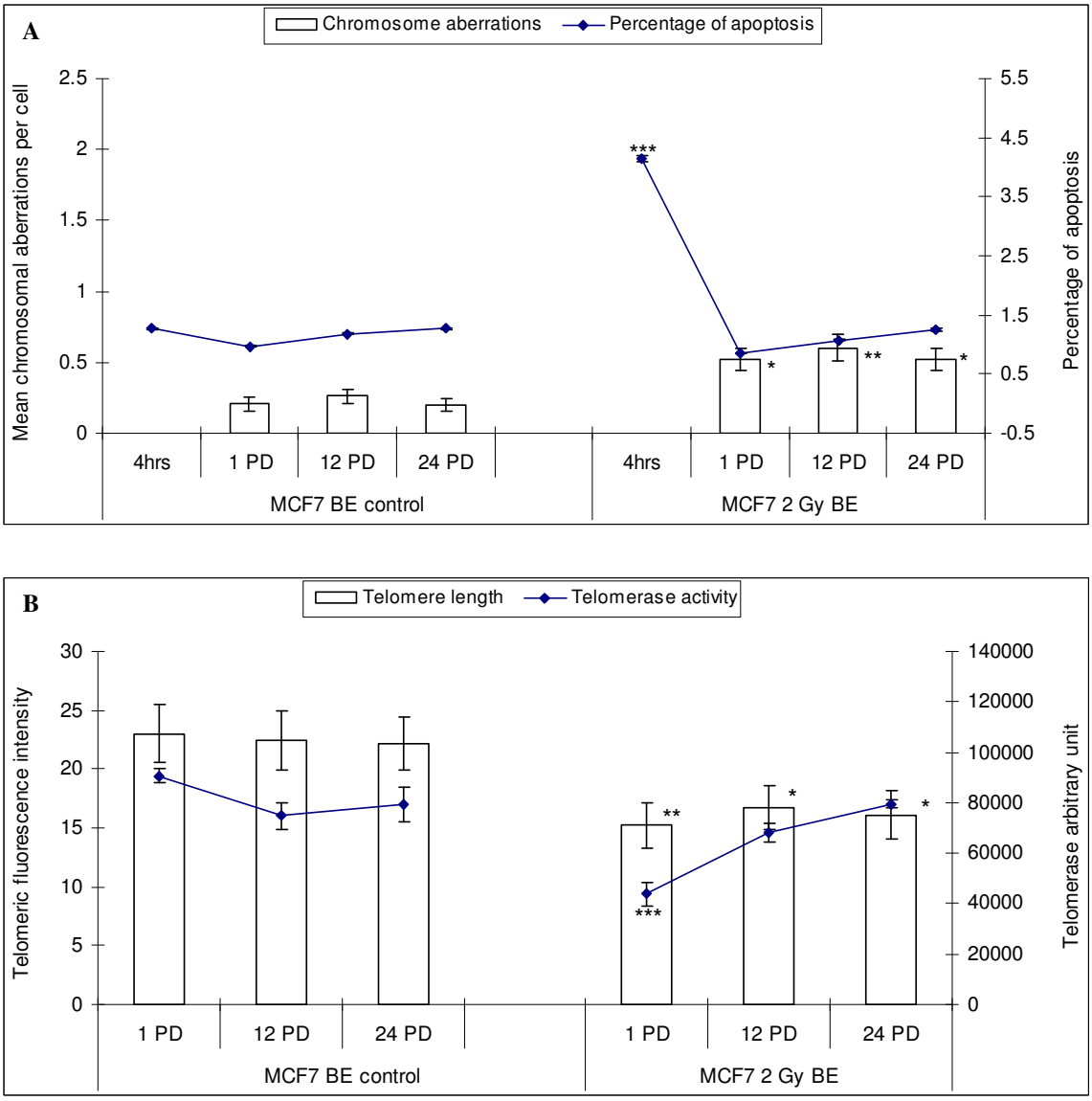


Figure 4.4: Bystander consequences and genomic instability within the bystander MCF7 cells following co-culture with 2 Gy direct irradiated MCF7 cells.

Tumour MCF7 cells were exposed to 2 Gy X-ray irradiation. Un-irradiated MCF7 cells were immediately incubated with the 2 Gy irradiated MCF7 cells for 4 hours to induce BE, where upon apoptotic analysis was performed. Further apoptotic measurements along with those for chromosomal analysis, telomere length and telomerase activity were made after 1, 12 and 24 population doublings (PD).

Panel A illustrate values for early and late mean aberrations per cell and apoptotic percentage within the 2 Gy bystander MCF7 cells. The cells demonstrated an early induction of genomic instability (*$p \leq 0.05$) compared to the control similar to that observed for the 0.1 Gy bystander MCF7 cells. Moreover, a high induction of apoptosis (***$p \leq 0.0001$) was observed in these cells after 4 hours compared to the control; however, levels returned to normal after 1, 12 and 24 PD. Absence of apoptosis could have led to the significant chromosomal instability observed within these cells after 12 PD (**$p \leq 0.005$) and 24 PD (*$p \leq 0.05$).

Panel B show the telomere length and telomerase activity by Q-fish and TRAP assay respectively. Short telomeres (**$p \leq 0.005$) and decreased telomerase activity (***$p \leq 0.0001$) were detected in the bystander cells after 1 PD compared to the controls. However, there was a dramatic rise in telomerase activity in these bystander cells after 12 and 24 PD, although the progeny consistently exhibited significant telomere shortening (*$p \leq 0.05$) at this time-point. Hence, we suggest that the significant chromosomal/genomic instability observed was due to reduction in telomere length, sufficient telomerase activity and absence of apoptosis.

Experiment was performed in 3 technical repeats.

4.3.5 Bystander responses in the tumour MCF7 cells through communication with 0.1 Gy direct irradiated non-tumour HMT cells

It has been well documented that normal/non-tumour cells are frequently exposed to low doses of IR during diagnostic procedures and radiotherapy treatment (BER, 2010, Joiner, 1987). Evaluation of bystander effects from direct irradiated normal cells is therefore important to allow estimation of cellular damage in the neighbouring un-irradiated tumour or cancer cells. Thus, the HMT-MCF7 cell combination was established to investigate the effect of bystander signals on tumour MCF7 cells following irradiation of non-tumour HMT cells by 0.1 Gy X-ray. As with the other experimental combinations, the un-irradiated MCF7 (bystander) cells were co-cultured with 0.1 HMT cells for 4 hours and subjected to chromosomal analysis, apoptotic analysis, telomere length and telomerase activity measurement.

The initial and delayed responses are explained in section 3.3.3. (For full details see the section above).

The un-irradiated bystander MCF7 cells showed significant chromosomal damage ($p \leq 0.0001$) after 1 population doubling following co-culture. The mean number of chromosomal aberrations in the control cells measured 0.09±0.032, but levels were significantly elevated to 0.84±0.12 in the bystander cells, (Figure 4.5 A). Furthermore, these bystander cells had previously displayed a significant induction of apoptosis ($p \leq 0.0001$) at the 4 hour time-point, (1.05±0.011 and 4.02±0.03, for control and bystander cells respectively); although after 1 population doubling, the level had decreased to control values (Figure 4.5 A). Measurements for the bystander MCF7 cell's telomeric fluorescence intensity, indication of telomere length, gave readings of 17.94±2.11, which was importantly lower ($p \leq 0.05$) than the control (23.84±2.59) at this time-point, as shown in figure 4.5 B. Moreover, telomerase activity was significantly reduced ($p \leq 0.0001$) in these bystander cells, 31735±1797.450 compared

to control values of 43718.3±2648.73 (Figure 4.5 B). These findings thus proved that bystander signals from 0.1 Gy direct irradiated HMT cells could cause high initial cellular damage responses in the bystander MCF7 cells.

The progeny of the bystander MCF7 cells also revealed a high induction of chromosomal instability ($p \leq 0.0001$) after 12 and 24 population doublings compared to their controls (Figure 4.5 A). The mean number of chromosomal aberrations in these bystander cells was 0.68±0.12 and 0.65±0.11 after 12 and 24 generations respectively compared to the controls, which were 0.19±0.05 and 0.22±0.056. However at these time-points, delayed apoptotic response in the bystander progeny was insignificant compared to the control (Figure 4.5 A). The absence of apoptosis could explain the high induction of chromosomal damage as a delayed response. In addition, the bystander cells were also shown to have significantly shortened telomeres ($p \leq 0.05$), with values of 17.9±1.95 and 18.18±2.02 after 12 and 24 generations respectively, although telomerase activity was shown to have returned to normal levels at these time-points (Figure 4.5 B). The findings suggest that despite normal telomerase activity of this progeny, which may have played a critical role in maintaining cell proliferation, the telomere lengths remained compromised, thus leading to the induction of chromosome instability. Additionally, with the absence of apoptosis this could result in greater tumour aggression.

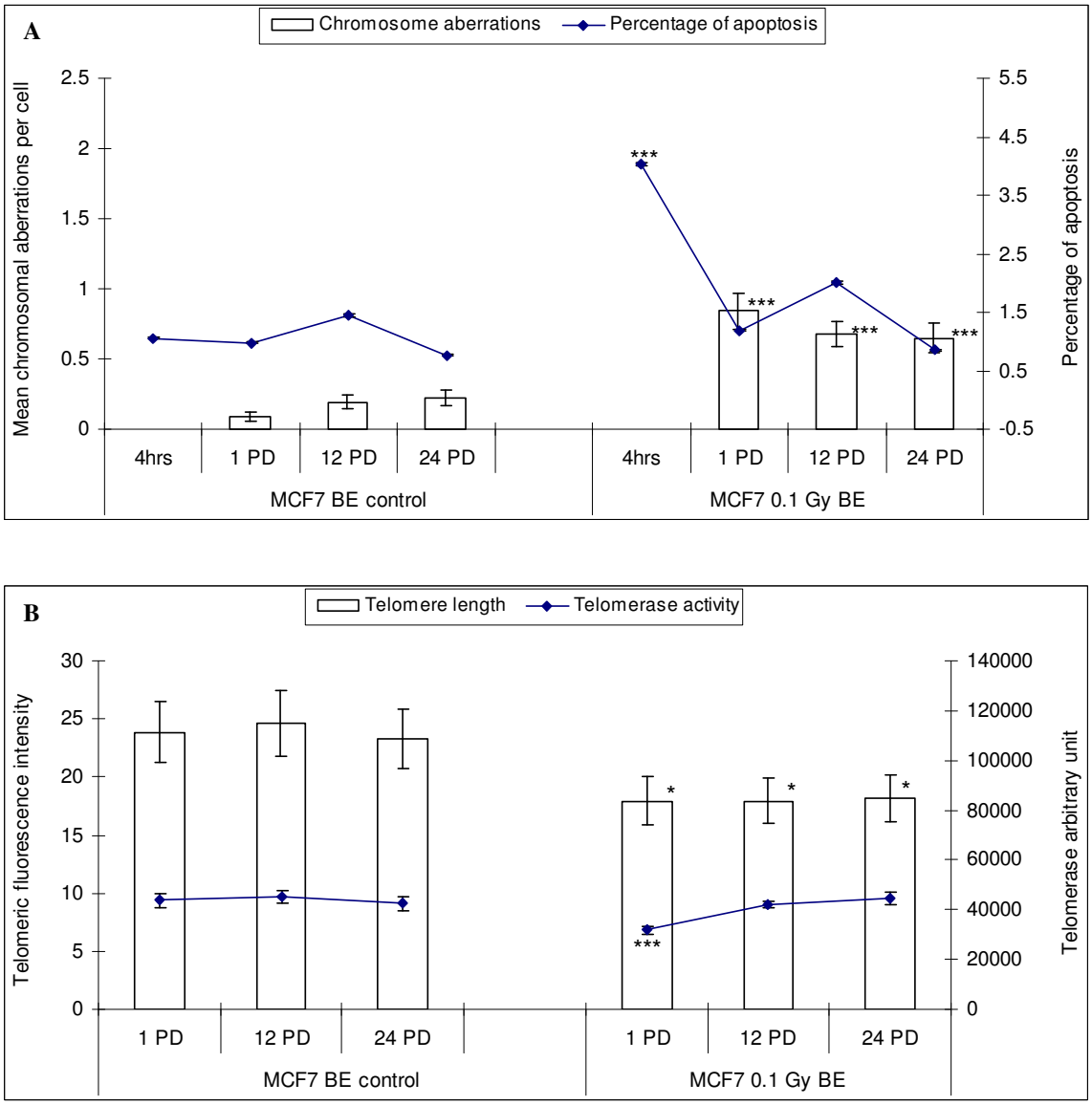


Figure 4.5: Initial and delayed cellular damage response within un-irradiated bystander MCF7 cells following co-culture with 0.1 Gy direct irradiated HMT cells

Non-tumour HMT cells were irradiated with 0.1 Gy X-ray irradiation. Immediately following irradiation un-irradiated tumour MCF7 cells were incubated with the irradiated cells for 4 hours to induce BE. Cells were then propagated up to 24 population doublings (PD) for genomic instability estimation.

Panel A illustrate the incidence of early and late chromosome aberrations and percentage of apoptosis. The 0.1 Gy direct irradiated HMT cells induced significant chromosomal damage (***$p \leq 0.0001$) in the bystander MCF7 cells after 1 PD following co-culture compared to the control. However, bystander MCF7 cells had previously shown a high induction of apoptosis (***$p \leq 0.0001$) at the 4 hour time-point, although there was no sustained significant induction after 1, 12 and 24 PD. Conversely, the bystander cells continued to exhibit significant chromosomal instability (***$p \leq 0.0001$) after 12 and 24 PD; thus due to the absence of apoptosis, cells maintained unstable chromosomes.

Panel B show measurements of telomere length and telomerase activity as early and delayed responses within the 0.1 Gy bystander MCF cells. A significant reduction of telomere length (*$p \leq 0.05$) and telomerase activity (***$p \leq 0.0001$) was observed within the bystander cells after 1 PD. Although telomerase activity returned to normal levels after 12 and 24 PD, these bystander cells consistently revealed significant telomeric shortening (*$p \leq 0.05$) at the same time-points. Data suggest that active telomerase activity could not compensate for the reduction in telomere length, which we suggest might instigate chromosomal instability.

Experiment was performed in 3 technical repeats.

4.3.6 Bystander responses in the tumour MCF7 cells through communication with 2 Gy direct irradiated non-tumour HMT cells

In radiotherapy treatment, normal cells surrounding the targeted tumour cells could receive a high dose of IR. Therefore, to mimic this scenario, non-tumour (HMT) cells were directly irradiated with 2 Gy X-ray irradiation (radiotherapy dose) and subsequently co-cultured with tumour (MCF7) cells to evaluate early and late cellular damage. Additionally the results would provide estimation as to whether bystander signals are beneficial by inducing multi-chromosomal damage in the cancer cells leading to auto-killing, or detrimental by increasing the rate of GI and cancer aggression. The data of direct irradiated HMT cells was represented in section 3.3.4. In brief, a significant amount of chromosomal damage was observed after 1 population doublings in the directly irradiated cells although high levels of apoptosis had been was observed at the initial 4 hour time-point. However, subsequent measurements of apoptosis (1 population doubling time-point), found levels returned to normal although they were again significantly elevated at 12 population doublings in conjunction with absence of chromosomal instability, indicative of removal of heavily damaged cells by apoptosis. In contrast, delayed chromosomal damage was significantly revealed within the progeny of the irradiated cells at 24 population doublings in the absence of a significant apoptotic response (Figure 3.4 A). Moreover, the irradiated HMT cells exhibited a high induction of telomeric shortening after 1, 12 and 24 population doublings; however, telomerase activity was maintained at all time-points with exception of the first time-point as shown in figure 3.4 B. (For more detailed see section 3.3.4).

Turning now to the results of the bystander responses of the un-irradiated tumour (MCF7) cells following communication with the 2 Gy directly irradiated non-tumour (HMT) cells, it was shown that there was a significant initial induction of

chromosomal damage in the bystander MCF7 cells ($p \leq 0.0001$) at the 1 population doubling time-point, as shown in figure 4.6 A. The mean number of chromosomal aberrations increased from 0.09±0.032 (control) to 0.76±0.12 (bystander) cells. A significant ($p \leq 0.0001$) early (4 hour time-point), apoptotic response was observed in these bystander cells (4.29±0.037) compared to the control (1.05±0.011). However, levels returned to normal within the bystander cells after 1 population doubling. Hence, data suggest that cell-cell communication between 2 Gy irradiated HMT and bystander MCF7 cells could disrupt the apoptotic mechanism in the bystander cells (Figure 4.6 A). This disrupting effect could be a long-lived response, as evidenced within the progeny of 2 Gy bystander cells after 12 and 24 population doublings significantly ($p \leq 0.0001$) demonstrating high levels of chromosomal instability, although apoptotic levels had returned to normal at these delayed time-points compared to their controls (Figure 4.6 A). The mean number of chromosomal aberrations was shown to be 0.71±0.1 and 0.61±0.1 after 12 and 24 population doublings respectively compared to the controls, which showed values 0.19±0.05 and 0.022±0.056.

Moreover, the 2 Gy direct irradiated HMT cells caused significant telomeric shortening ($p \leq 0.05$) and telomerase activity reduction ($p \leq 0.0001$) in the bystander MCF7 cells after 1 population doubling following co-culture (Figure 4.6 B). Telomere length data at the same time-point, showed the bystander cells to have reduced lengths (16.91±2.18) compared to the control (23.84±2.56) and also reductions in their telomerase activity, 27685±826.13 TAU (bystander), compared to 43718.3±2684.73 TAU (control), as shown in figure 4.6 B.

Although the telomerase activity in the bystander cells returned to normal levels after 12 and 24 population doublings, significant reduced telomere lengths remained

($p \leq 0.05$) as shown in figure 4.6 B. Hence, the findings supported the conjecture above (Section 4.3.5), that telomerase played an important role in cell proliferation leading to chromosomal/genomic instability due in part to shortened telomeres and absence of apoptosis.

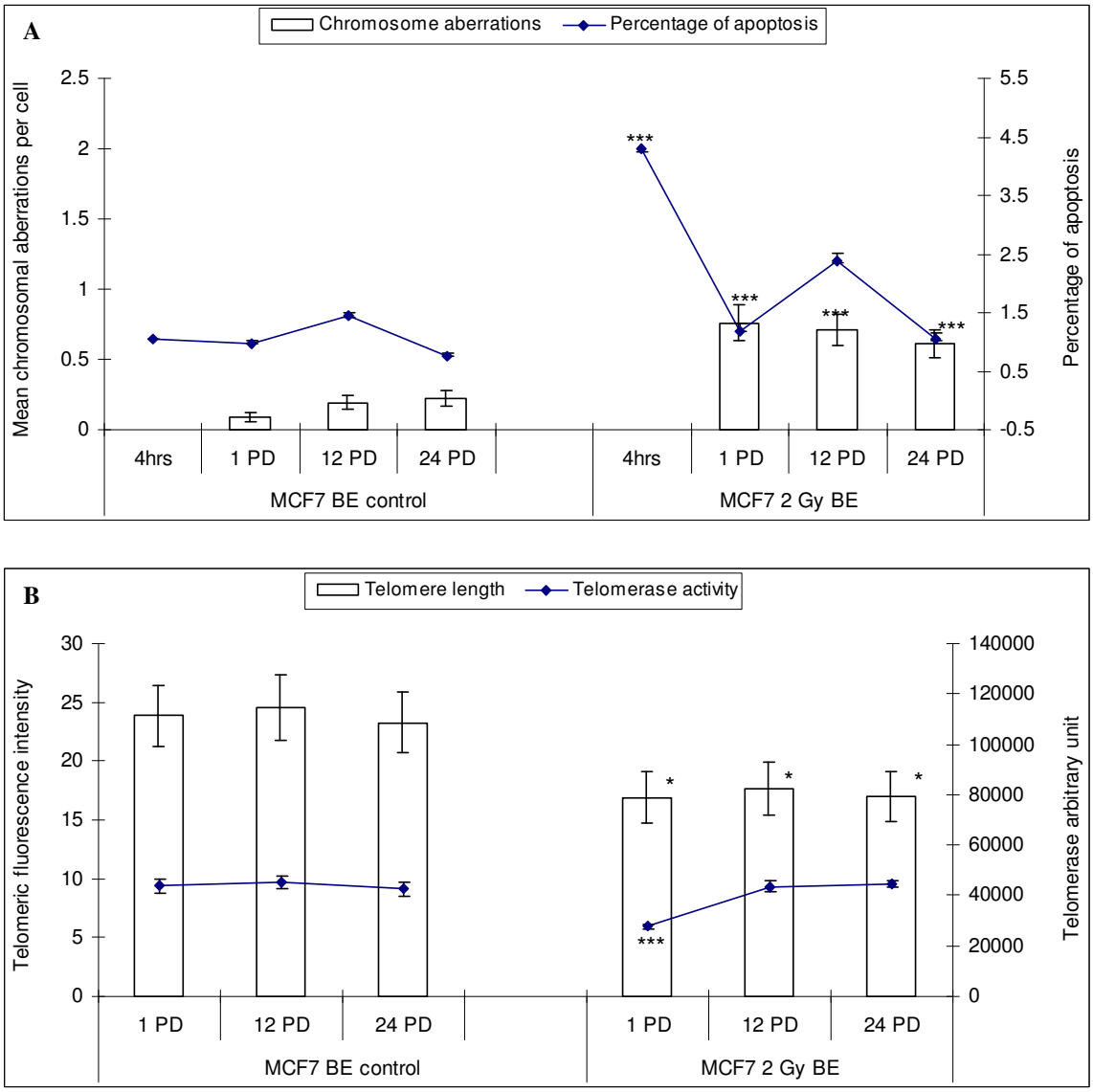


Figure 4.6: Early and late damage responses within the un-irradiated bystander MCF7 cells following co-culture with 2 Gy direct irradiated HMT cells.

Immediately, following irradiation of HMT cells with 2 Gy X-ray, un-irradiated MCF7 cells were co-cultured/incubated with them for 4 hours to induce BE. The cells were propagated for several population doublings (PD) for delayed damage response evaluation. The cells were subjected to chromosomal analysis, apoptotic analysis, telomere length and telomerase activity measurements.

Panel A illustrate early and late chromosomal aberrations and apoptotic levels within the bystander MCF7 cells. Chromosome aberrations were significantly observed (***$p \leq 0.0001$) within the bystander MCF7 cells after 1, 12 and 24 PD following co-culture with 2 Gy direct irradiated HMT cells compared to the controls. However, an apoptotic response was only significantly detected in these bystander cells at the 4 hour time-point. The bystander cells showed normal levels of apoptosis after 1, 12 and 24 PD compared to the controls. The induction of delayed chromosomal damage could be due to the absence of apoptosis in these cells, which suggest an inverse relationship between chromosomal instability and apoptosis.

Panel B illustrate telomere instability within 2 Gy bystander MCF7 cells by telomere length and telomerase activity measurements. Bystander cells showed a significant initial decrease in telomere length (*$p \leq 0.05$) and telomerase activity (***$p \leq 0.0001$) compared to the controls. Telomerase activity levels returned to normal levels after 12 and 24 PD. However, this progeny continued to exhibit significant telomere shortening (*$p \leq 0.05$) after 12 and 24 PD compared to the controls. Similar to the 0.1 Gy MCF7 cells, data suggest that sustained telomerase activity could not compensate for the reduction in the telomere length, which could lead to chromosomal instability.

Experiment was performed in 3 technical repeats.

4.3.7 Bystander responses in the non-tumour HMT cells through communication with 0.1 Gy direct irradiated non-tumour HMT cells

Our previous data has unmistakably shown that direct irradiated non-tumour cells cause early and delayed cellular damage to the bystander cancer (MCF7) cells following low doses of IR. Therefore, a non-tumour - non-tumour cell combination was set up to estimate non-targeted effects in the bystander non-tumour cells after a low dose of IR.

Section 3.3.3 illustrated early and delayed responses of HMT cells following 0.1 Gy X-ray irradiation. (For more detail see the section above).

Interestingly, in this latest cell combination, there was no initial significant evidence of chromosomal damage in bystander HMT cells following their co-culture with 0.1 Gy direct irradiated HMT cells (Figure 4.7 A). However, a bystander effect did manifest as apoptotic induction, telomeric shortening and telomerase activity reduction. The bystander cells demonstrated a highly significant ($p \leq 0.0001$) induction of apoptosis (4 fold) after 4 hours following co-culturing, with values of 1.3±0.008 (control) and 5.13±0.02 (bystander) as shown in figure 4.7 A. Nonetheless, the trend was short-lived as levels significantly decreased at 1 population doubling, although they remained above control levels (1.51±0.018 bystander, compared to 1.05±0.013 control). Additionally at this time-point, there was significant telomeric shortening ($p \leq 0.05$) and telomerase activity reduction ($p \leq 0.0001$) exhibited within these bystander cells; with values of 10.24±0.96 observed in the bystander cells and 14.16±1.76 in the control. Whilst the telomerase activity was significantly reduced from 51666.7±1770.44 TAU (control) to 36666.7±1941.08 TAU (bystander) as showed in figure 4.7 B. Thus, data showed that 0.1 Gy direct irradiated HMT cells could not cause chromosomal damage within bystander HMT cells, which was most probably due to the high level of apoptosis observed at the initial time-point.

The 0.1 Gy bystander HMT cells continued to demonstrate insignificant chromosomal damage/instability after 12 and 24 population doublings as shown in figure 4.7 A. Conversely though, the cells exhibited a significant induction of apoptosis ($p \leq 0.05$) after 12 population doublings; however, this level was not maintained for the delayed time-point. Thus we suggest that the bystander cells showed a type of resistance to the 'bystander signals' from the direct irradiated cells by inducing apoptosis, thereby removing cells with high chromosomal damage/instability.

Although the level of telomerase activity in the bystander cells was shown to have returned to normal levels at the 12 and 24 population doublings time-point, these cells consistently showed significant telomeric shortening ($p \leq 0.05$) as shown in figure 4.7 B. The findings suggest that cells with sort telomeres could proliferate for several population doublings, because of satisfactory telomerase activity. Despite that fact that 0.1 Gy bystander HMT cells did not demonstrate chromosomal/genomic instability as a delayed response at our time-points, there is potential for chromosomal instability in later progeny (25 generations plus), because of sustained telomeric shortening and absence of apoptosis.

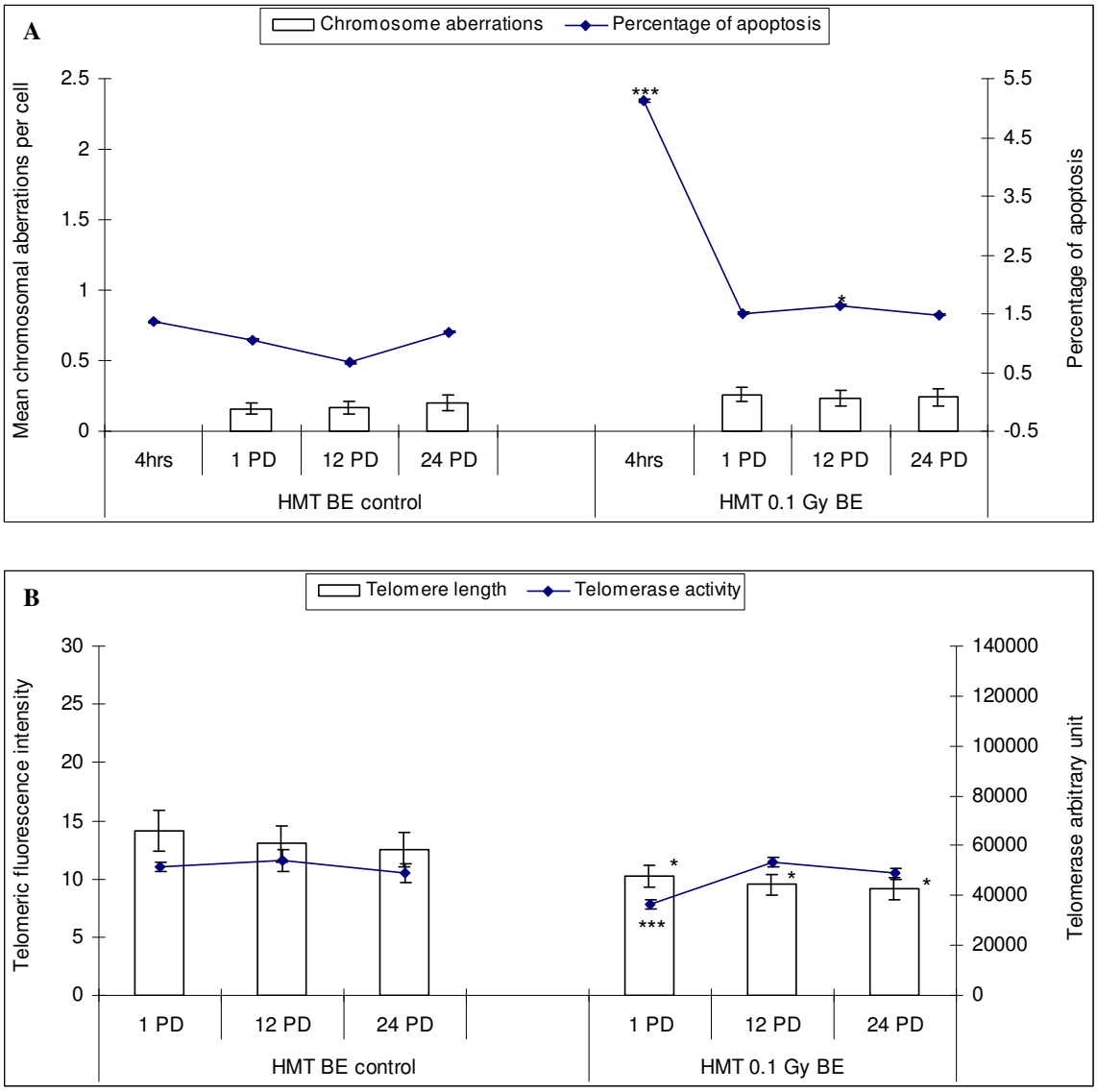


Figure 4.7: Initial and delayed damage response within the un-irradiated bystander HMT cells following co-culture with 0.1 Gy direct irradiated HMT cells.

The un-irradiated HMT cells were immediately incubated with the direct irradiated HMT cells following 0.1 Gy X-ray irradiation. The cells were subjected to apoptotic analysis after 4 hours, 1, 12 and 24 population doublings (PD) following co-culture; whilst chromosomal analysis, telomere length and telomerase activity measurements were carried out after 1, 12 and 24 PD.

Panel A demonstrate the early and late chromosomal damage and apoptosis within the 0.1 Gy bystander HMT cells. The bystander cells did not show significant initial or delayed chromosomal damage at any time-points. Nevertheless, the bystander cells did exhibit a high induction of apoptosis (***$p \leq 0.0001$) after 4 hours compared to the control, although this was shown to have decreased after 1 PD following co-culture. Delayed apoptotic response was significantly elevated (*$p \leq 0.05$) within the progeny of 0.1 Gy bystander HMT cells after 12 PD, which could explain the insignificant chromosomal instability. i.e. the high level of apoptosis could remove the cells with high chromosomal instability. After 24 PD following co-culture, the apoptotic response in the bystander cells was insignificant; however, chromosomal instability was increased although statistically insignificant. Data suggest that chromosomal instability in the bystander cells could significantly be associated with the increase in the number of population doublings. Panel B illustrate the telomere length and telomerase activity measurements using Q-FISH and TRAP assay respectively. The bystander HMT cells revealed significant telomeric shortening (*$p \leq 0.05$) and telomerase activity reduction (***$p \leq 0.0001$) after 1 PD compared to the controls. After 12 and 24 PD the bystander cells showed normal levels (sufficient) telomerase activity, however, telomere shortening was significantly observed in this progeny at these time-points (*$p \leq 0.05$). The findings demonstrated that sufficient telomerase activity could maintain cell proliferation even with telomere instability, which could lead to chromosomal instability. Although the progeny of 0.1 Gy HMT cells did not show significant chromosomal/genomic instability after 24 generations, there was a potential risk of chromosomal instability with more population doublings. Experiment was performed in 3 technical repeats.

4.3.8 Bystander responses in the non-tumour HMT cells through communication with 2 Gy direct irradiated non-tumour HMT cells

We have previously shown that 2 Gy X-ray could induce cellular damage within direct irradiated non-tumour (HMT) cells as illustrated in section 3.3.4, and additionally cause early and late damage response within un-irradiated bystander cancer cells (Section 4.3.6). Therefore, a direct irradiated-bystander non-tumour cell combination was established to investigate the bystander consequences within bystander non-tumour cells following 2 Gy X-ray irradiation.

We have demonstrated that chromosomal damage was significantly induced within 2 Gy direct irradiated HMT cells after 1 and 24 population doublings. However, these cells showed insignificant chromosomal aberrations after 12 population doublings. We suggest that the reduction in the chromosomal damage could have been due to the high level of apoptosis that was exhibited by the cells. Moreover, the telomeres were significantly shortened in the direct irradiated cells at the all time points (early and delayed); however, a significant reduction in telomerase activity was only observed at the early time point. (For more details see section 3.3.4).

Estimation of bystander signalling bystander cells in this latest combination, were determined as in previous investigations, by early and late chromosomal analysis, apoptosis analysis, telomere length measurement and telomerase activity evaluation.

The results demonstrated a high induction of initial chromosomal damage ($p \leq 0.0001$) in the bystander HMT cells as shown in figure 4.8 A. The mean number of chromosomal aberrations ranged from 0.16±0.039 for the control to 0.49±0.088 for the bystander cells at 1 population doubling (Figure 4.8 A). In addition, these bystander cells had shown a high induction of apoptosis ($p \leq 0.0001$) after 4 hours following co-culture compared to the control. However, the apoptotic response sharply decreased to normal levels after one the first cell sub-culture (Figure 4.8 A).

The Telomeric status of these bystander cells, including telomere length and telomerase activity at this time-point, showed that the cells had undergone a significant reduction in their telomere length ($p \leq 0.005$) compared to the control, 14.16±1.7 (control cells) and 9.15±1 (bystander). Similarly, telomerase activity was reduced from 51666.7±1770.4 TAU in the control to 37100±3939.9 in the bystander HMT cells (Figure 4.8 B). Thus, we suggest that the 2 Gy direct irradiated HMT cells caused a significant initial cellular damage response within the recipient bystander HMT cells through media soluble factors.

Interestingly, the 2 Gy bystander HMT cells did not show induction of chromosomal instability after 12 population doublings compared to the control; however, these cells demonstrated an elevation in apoptotic response at the same time-point, although not statistically significant ($p=0.08$) compared to the control. Data once more confirmed the inverse relationship between apoptosis and chromosomal instability as a delayed response (Figure 4.8 A). Although telomerase activity of the progeny of 2 Gy bystander HMT cells returned to normal levels after 12 generations, these cells continued to exhibit significant telomeric shortening ($p \leq 0.05$) compared to the control, (9.15±108 and 12.99±1.52, for bystander and control respectively), as shown in figure 4.8 B. These findings thus show that normal levels of telomerase activity maintained cell proliferation even though the cells sustained reduction in their telomere length.

After 24 population doublings, the progeny of 2 Gy bystander HMT cells displayed a high induction of chromosomal instability ($p \leq 0.0001$) compared to the control. However, no significant induction of apoptosis was detected at the same time-point (Figure 4.8 A). The mean number of chromosomal aberrations within the progeny of bystander HMT cells measured 0.33±0.06 compared to 0.52±0.18 (control). The

progeny of the 2Gy bystander HMT cells additionally showed significant telomeric shortening ($p \leq 0.05$) although normal levels of telomerase activity, after 24 generations (Figure 4.8 B). Data thus confirmed a positive correlation between increased cell population doublings and chromosomal instability which could be due to sustained reduction in telomere length, maintained telomerase activity and absence of apoptosis.

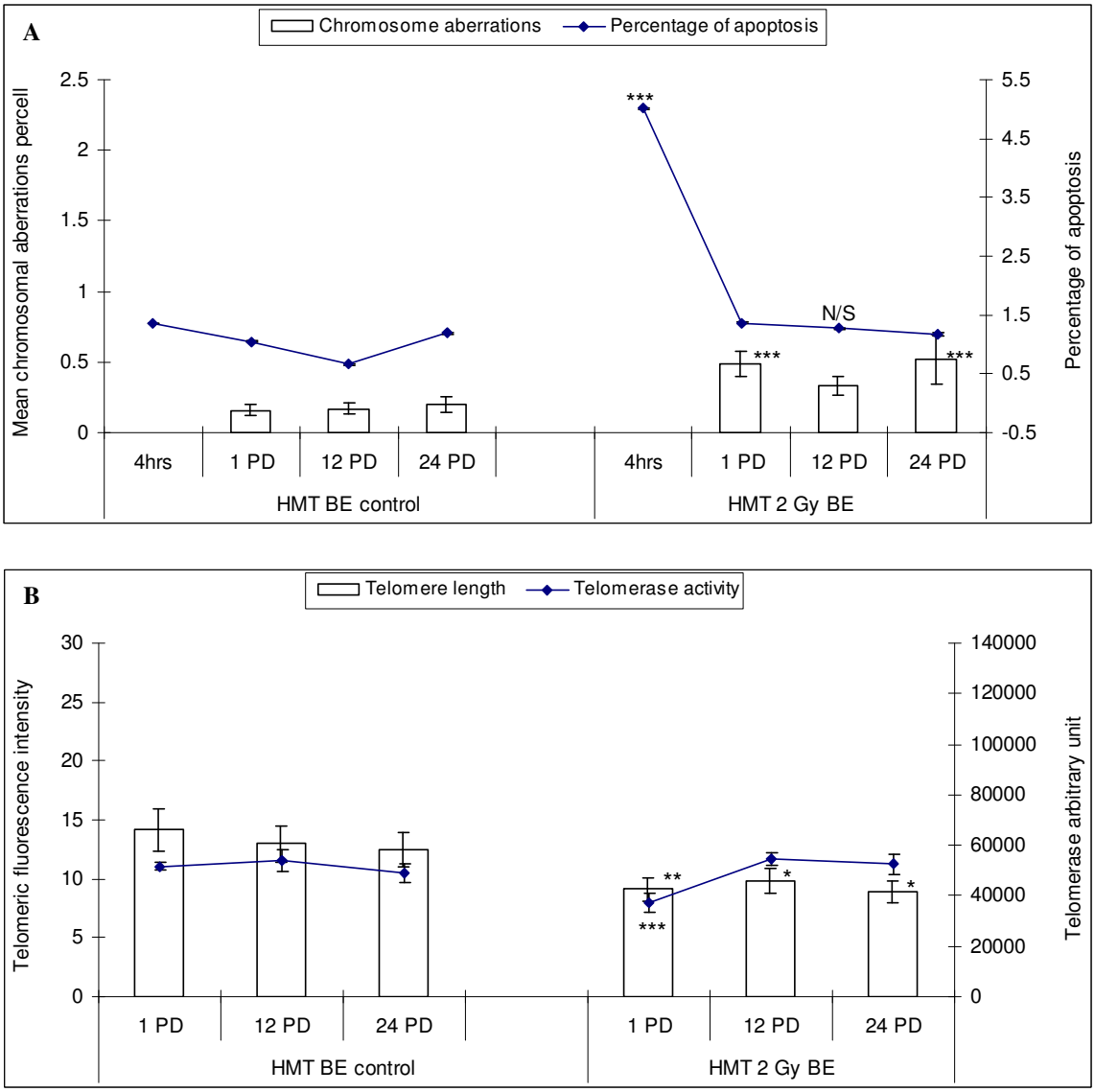


Figure 4.8: Early and late cellular damage response within un-irradiated bystander HMT cells following co-culture with 2 Gy direct irradiated HMT cells.

The non-tumour HMT cells were exposed to 2 Gy X-ray irradiation, and co-cultured with un-irradiated HMT cells to induce BE. The cells were propagated up to 24 population doublings (PD) for genomic instability estimation. Early and late chromosomal analysis, apoptotic analysis, telomere length and telomerase activity measurements were performed to evaluate the bystander consequences and genomic instability within the 2 Gy bystander HMT cells.

Panel A illustrate the mean number of chromosome aberrations and apoptotic levels within bystander HMT cells following co-culture with 2 Gy direct irradiated HMT cells. Chromosomal damage was significantly observed (***$p \leq 0.0001$) within the bystander cells after 1 PD compared to the control. In addition, these cells showed a high induction of apoptosis (***$p \leq 0.0001$) after 4 hours following co-culture compared to the control. However, the level of apoptosis returned to normal after the initial cell sub-culturing. Interestingly the bystander HMT cells did not demonstrate significant chromosomal instability after 12 PD, which could be due to the high level of apoptosis detected at the same time point. However, this apoptotic level was statistically insignificant ($p \leq 0.6$) compared to the control. Additionally, these cells revealed a high induction of chromosomal instability (***$p \leq 0.0001$) after 24 PD compared to the control. Nonetheless, apoptotic induction within this progeny was insignificant compared to the control at the same time-point. Data thus suggest that there was an inverse relationship between apoptosis and chromosomal instability. Moreover, a positive correlation was observed between chromosomal instability and an increase in population doublings.

Panel B illustrate the telomere length and telomerase activity measurements within the 2 Gy bystander HMT cells using Q-FISH and TRAP assay respectively. Bystander cells showed significant telomeric shortening (**$p \leq 0.005$) and telomerase activity reduction (***$p \leq 0.0001$) after 1 PD following co-culture compared to the controls. Although telomerase activity returned to normal levels after 12 and 24 PD within the progeny of bystander cells, these cells continued to exhibit significant short telomeres ($p \leq 0.05$). Data suggest that although telomerase activity had not been reduced it was not sufficient to repair the shortened telomere length; however, it could maintain cell proliferation, which could lead to chromosomal instability.

Experiment was performed in 3 technical repeats.

Cell combinations	Bystander HMT / Irradiated MCF7				Bystander MCF7 / Irradiated MCF7				Bystander MCF7 / Irradiated HMT				Bystander HMT / Irradiated HMT			
X-ray dose	0.1 Gy		2 Gy		0.1 Gy		2 Gy		0.1 Gy		2 Gy		0.1 Gy		2 Gy	
Bystander groups	HMT received signals from MCF7		HMT received signals from MCF7		MCF7 received signals from MCF7		MCF7 received signals from MCF7		MCF7 received signals from HMT		MCF7 received signals from HMT		HMT received signals from HMT		HMT received signals from HMT	
Time-points	Early	Late	Early	Late	Early	Late	Early	Late	Early	Late	Early	Late	Early	Late	Early	Late
Chromosomal damage	N/S	N/S	S	S	S	S	S	S	S	S	S	S	N/S	N/S	S	S
Apoptotic level	S (4 hrs)	S (12 PD)	S (4 hrs)	N/S	S (4 hrs)	N/S	S (4 hrs)	N/S	S (4 hrs)	N/S	S (4 hrs)	N/S	S (4 hrs)	S (12 PD)	S (4 hrs)	N/S
Telomere shortening	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
Telomerase activity reduction	S	N/S	S	N/S	S	N/S	S	N/S	S	N/S	S	N/S	S	N/S	S	N/S

Table 4.1: Summary data of bystander groups, illustrating the early and late responses following 0.1 and 2 Gy X-ray
S=significant, N/S= insignificant, PD= population doubling, hrs= hours

4.4 Discussion and conclusions

Results from the previous experiment (Chapter 3) demonstrated the induction of early and delayed damage in MCF7 (tumour) cells and HMT (non-tumour) cells following low and high doses of X-ray exposure. This experiment was established with the purpose of mimicking the consequences of low dose irradiation from diagnostic procedures and also those of high doses from radiotherapy fractions. In order to investigate the non-targeted effect/responses of IR in the non-hit bystander cells, responding from signals emitted from neighbouring irradiated cells, several experimental cell combinations were set up between the tumour (MCF7) and non-tumour (HMT) cells, using both low and high doses of X-ray, as described in the materials and methods section (Section 4.2.2). The instability of delayed responses was subsequently measured within the progeny of bystander cells, thus enabling comparisons to be made between the direct and the non-targeted effects of low and high doses of IR within tumour and non-tumour cells.

The 6-well plate co-culture system was used to facilitate BE in these cells, set up in opposing combinations, i.e. HMT cells seeded in insert co-culture vessels and MCF7 cells seeded in the co-culture base and vice versa. The co-culture system allows bystander cells to receive the signals that have been secreted by the irradiated cells (Hill *et al*., 2006). Briefly, base dishes are irradiated or sham-irradiated in the absence of the insert dishes but immediately following sham/irradiation, the insert dishes are placed within the base vessels and the entire co-culture plate incubated for 4 hours. Thus the two cell populations are physically separated but communication is allowed between them via the porous translucent polyethylene terephthalate membrane of the insert vessel.

The duration of the co-culture time, was designed to ascertain that short lived and long-lived signals from the sham/irradiated cells would be received by bystander

cells. Other studies, using a similar co-culture system, have suggested the involvement of short-lived bystander signals, such as superoxide, in the induction of BE in human prostate cancer cells (Wang and Coderre, 2005). Similarly, BE has also been shown to be induced by long-lived radicals, whilst, nitric oxide (NO) has been implicated in the formation of bystander signals although not in the bystander effect itself (Harada *et al*., 2008).

The different cell combinations between tumour and non-tumour cells following low and high doses of IR in this study provided a wide range of evidence to confirm whether BE has detrimental or beneficial consequences. Apoptotic analysis and chromosomal damage estimation enabled measurement of the early negative and positive bystander effects. BE was considered beneficial when the bystander signals induced high level of apoptosis or multi-chromosomal damage leading to auto-killing within the cancer cells but not the normal cells.

Telomere length and telomerase activity were measured to investigate GI and potential risk of second malignancies and to this end we were able to show that telomeric instability within bystander cells could instigate chromosomal instability, which could lead to cancer, as reported by Williams and co workers (Williams *et al*., 2009). Furthermore, evaluation of BE in our different cell combinations (tumour-normal; tumour-tumour; normal-tumour and normal-normal cell communications) presented a valuable comparison study between tumour and non-tumour cells following low and high doses of IR. These cells combinations provided robust evidence that suggest BE in the HMT and MCF7 cells was a dose-dependent phenomenon and cell specific.

Other studies have demonstrated that the occurrence of BE is higher within high density cell cultures than those of low cell density (Mitchell *et al*., 2004). Therefore,

with this in mind, only culture vessels (insert/base/culture flasks) of equivalent cell densities (80% confluence), were chosen for our experimental groups. Additionally, both experimental HMT cells and MCF7 cells were each propagated from one cryovial, ensuring the cells were age matched. Experimental cells were sub-cultured and maintained by seeding approximately 1.5 x 10^6 cells per T75 tissue culture flask, and then incubated until 80% confluence. Thus, probable bystander effects and delayed responses were allowed to manifest under identical conditions.

Both HMT and MCF7 cells demonstrated bystander responses following co-culturing in all cell combinations and irradiation conditions (irradiated MCF7-bystander HMT, irradiated MCF7-bystander MCF7, irradiated HMT-bystander MCF and irradiated HMT-bystander HMT, 0.1/2 Gy respectively). Thus, our results are in accordance with other studies that show cell-cell communication can induce BE following low and high doses of IR (Singh *et al*., 2011, Fleishman *et al*., 2008, Zhu *et al*., 2008). In all our cell combinations, bystander HMT and MCF7 cells both showed a high level of apoptosis after the 4 hour co-culturing/incubation time (Figures 4.1 A; 4.2 A; 4.3 A; 4.4 A; 4.5 A; 4.6 A; 4.7 A and 4.8 A). Vorob'eva and co-authors have suggested that 1 Gy γ-ray irradiated lymphocytes could cause apoptosis within bystander lymphocytes, due to a reduction in DNA repair resulting in increases in DNA DSB thus instigating apoptosis (Vorob'eva *et al*., 2011). Moreover, Kovalchuk and co-authors have suggested that miRNA (microRNA) could play an important role in bystander apoptotic induction. They suggest that miRNA could change BCl2 gene expression leading to apoptosis (Kovalchuk *et al*., 2010). Surprisingly, apoptotic levels within the bystander HMT and MCF7 cells of all cell combinations returned to normal levels after 1 population doubling i.e. after the first cell sub-culture. Our findings suggest that the process of cell sub-culture eliminate cells that are highly

damaged. Many studies have demonstrated apoptotic induction in bystander cells after 24-72 hours (Lyng *et al*., 2006a, Belyakov *et al*., 2005). However, it is unknown in these studies if apoptosis had been measured following cell sub-culture.

The bystander HMT and MCF7 cells in all cell combinations exhibited shortened telomeres and reduction in telomerase activity after 1 population doubling (Figures 4.1 B; 4.2 B; 4.3 B; 4.4 B; 4.5 B; 4.6 B; 4.7 B and 4.8 B). Belloni and co-authors have shown the ability of 0.1 Gy X-ray irradiation to induce apoptosis and reductions in telomere length in bystander human peripheral lymphocytes. They suggest that the observed high levels of reactive oxygen species (ROS) and hydrogen peroxide, could have major roles in both of these observations (Belloni *et al*., 2011). However, the mechanism for bystander telomeric shortening is not fully understood. Data suggest the bystander signals could down-regulate the genes of telomerase enzyme as an early response (Gorman *et al*., 2009); however, DNA microarray is required to support this suggestion.

In our studies, the bystander cells showed different chromosomal damage responses following 0.1 and 2 Gy X-ray irradiation. Interestingly, bystander HMT cells did not show significant chromosomal damage after 1 population doubling following co-culture with 0.1 direct irradiated MCF7 cells (Figure 4.1 A). However, these bystander cells did show a high level of apoptosis, which could have subsequently removed cells with high chromosomal damage and been due to the inability of the low X-ray dose to cause effects on these cells. Sowa and co-workers have reported that low-LET IR was unable to induce DNA damage in either bystander primary human fibroblast or epithelial colon carcinoma cells (Sowa *et al*., 2010).

Conversely, we have been able to demonstrate the ability of 2 Gy direct irradiated MCF7 cells to induce early high levels of chromosome aberrations in the bystander

HMT cells (Figure 4.2 A). This data supports research by Gow and co-authors who observed different responses following different irradiation doses and thereby suggest that bystander effects are a dose-dependent phenomena (Gow *et al*., 2008). Moreover, it has been previously reported that high doses of IR can diminish the antioxidant enzymes activity in the bystander cells leading to high levels of oxidation and lipid peroxidation, which can cause a high induction of chromosome aberrations. Radiation dose-dependent chromosomal damage has additionally been demonstrated in bystander cells. Buonanno and fellow authors reported that high doses of IR showed higher bystander chromosomal damage than low doses of IR (Buonanno *et al*., 2011). Whilst Groesser and co-workers have reported that human colon epithelial cancer cells (SW48) did not show significant bystander DNA damage following high doses of IR, as detected by γ-H2AX (Groesser *et al*., 2008), thus confirming that bystander responses can also be cell line-dependent (Vines *et al*., 2008). Aside from DNA damage, bystander effects have been shown to manifest in other responses, as shown in 0.1 Gy irradiated MCF7-bystander HMT cell combination. In HMT bystander cells, the BE response was observed by significant apoptotic induction, telomeric shortening and reduction in telomerase activity respectively. There was an absence of any early chromosomal damage as a bystander manifestation.

In contrast, the MCF-MCF7 cell combination demonstrated significant chromosomal damage in the bystander population following both 0.1 and 2 Gy X-ray irradiation (Figures 4.3 A and 4.4 A). This data supports work by He and co-authors who in addition reported that cytochrome-c can increase NO production in bystander human hepatoma cells cause DNA damage (He *et al*., 2012). There has also been much evidence documenting the major role that ROS plays in bystander DNA damage induction (Widel *et al*., 2012, Pandey *et al*., 2011, Lyng *et al*., 2011).

The bystander MCF7 exhibited a high induction of chromosomal damage after 1 population doubling following co-culture with both 0.1 and 2 Gy direct irradiated HMT cells (Figures 4.5 A and 4.6 A). Whilst direct irradiated MCF7 cells were only shown to induce chromosomal damage within bystander HMT cells following 2 Gy X-ray irradiation. The data suggest that bystander signals could be cell line-dependent. Other studies have also shown that the bystander cell response from signals emitted from irradiated cells could be variable between cell lines (Vines *et al*., 2008). Moreover, ICCM from irradiated human epithelial cells have only been shown to induce BE in human fibroblasts but not vice verse (Mothersill and Seymour, 1997). Interestingly in our study, bystander HMT cells at the early time-points did not demonstrate significant chromosomal damage following co-culture with 0.1 Gy direct irradiated HMT cells (Figure 4.7 A) although the bystander HMT cells did exhibit high levels of apoptosis, telomeric shortening and reduction in telomerase activity. In contrast significant chromosome aberrations were observed within bystander HMT cells following co-culture with 2 Gy direct irradiated HMT cells (Figure 4.8 A). The data thus suggest that bystander-damage responses could be dose-dependent.

The 0.1 Gy irradiated MCF7-bystander HMT cell combination demonstrated that bystander HMT cells showed a high induction of apoptosis after 12 generations following co-culture (Figure 4.1 B). We suggest that this induction of apoptosis could by instigated by the presence of short telomeres (Figure 4.1 B). A recent study has shown a sustained reduction in telomere length in the lymphocytes of Chernobyl clean workers, 20 years following initial exposure to low doses of IR. Moreover, a high induction of apoptosis was detected in these cells, which the authors suggest were as a result of telomeric shortening (Ilyenko *et al*., 2011). The homeostatic status of cell culture is frequently maintained by apoptosis i.e. removal of cells with high

chromosomal damage (Tesfaigzi, 2006). Apoptotic bodies have additionally been linked with inflammatory mediators leading to decrease in pro-inflammatory cytokines and inflammation control (Ren *et al*., 2008, Tesfaigzi, 2006), causing less chromosomal aberrations in delayed responses to the IR (Martin *et al*., 2011). The absence of chromosomal instability within bystander HMT cell after 12 generations following co-culture with 0.1 Gy irradiated MCF7 cells thus confirms the inverse correlation between apoptosis and chromosomal instability (Figure 4.1 A). Telomerase activity of these cells returned to the normal levels after 12 and 24 population doublings following co-culture. Bednarel and co-authors report that telomerase activity positively correlate to increased GI levels (Bednarek *et al*., 1995). Thus, we suggest that the active telomerase could maintain cell proliferation even with short telomeres, as observed in the 0.1 Gy bystander HMT cells after 24 generations (Figure 4.1 B). Consequently, there was a potential risk of chromosomal instability within the progeny of these bystander cells, due to short telomeres (Gorman *et al*., 2009). Data thus suggest that bystander HMT cells could reveal chromosomal instability with increased population doublings, i.e. 25 population doublings plus.

Conversely, bystander HMT cells showed a high induction of chromosomal instability ($p \leq 0.0001$) after 24 population doublings following co-culture with 2 Gy direct irradiated MCF7 cells compared to the control (Figure 4.2 A). Bystander cells have been frequently shown to demonstrate chromosomal instability within their progeny (Lorimore *et al*., 2008, Bowler *et al*., 2006). Short telomeres were reported to have a crucial role in GI induction within both normal and cancer cells (Hills and Lansdorp, 2009). Transforming growth factor-β (TNF-β), ROS and NO has additionally been shown to increase DNA DSB and inflammatory responses in both normal (non-

tumour) and cancer bystander cells leading to subsequent GI and oncogenic transformation (Dickey *et al*., 2009, Lorimore *et al*., 2001). Toyokuni and co-authors have reported that X-chromosomes with large deletions can instigate GI (Toyokuni *et al*., 2009). Recently, there has been much evidence implicating epigenetics, including DNA methylation and miRNA, in the GI pathway. Up-regulation of miRNA can be induced by IR leading to suppression of the expression of lymphoid-specific helicase (LSH), which is important for DNA methylation maintenance. Decrease LSH expression frequently instigates DNA hypomethylation of retroelements ((long interspersed nuclear elements 1 (LINE1) and short interspersed nuclear elements B2 (SINE B2)) causing GI (Kovalchuk *et al*., 2011). LINE1 aberrant methylation has also been shown to be associated with GI and chromosomal aneuploidy (Zeimet *et al*., 2011). Moreover, Aypar and co-workers have demonstrated that X-ray irradiation (low LET) causes increased epigenetic changes compared to those observed following Fe ions irradiation (high LET) (Aypar *et al*., 2011). They have shown that 6 miRNA types are involved in the epigenetic pathway leading to aberrant epigenetic changes following X-ray irradiation (Aypar *et al*., 2011) initiating chromosomal/genomic instability (Tamminga and Kovalchuk, 2011).

Our studies have demonstrated the inability of 2 Gy direct irradiated MCF7 cells to induce chromosomal instability in bystander HMT cells after 12 population doublings, although high levels of chromosomal damage was observed in the bystander progeny at 24 population doublings. Thus indicating that the high level of apoptosis observed in these cells at 12 population doubling time-point had most likely eliminated highly damaged cells (Figure 4.2 A). These findings also suggest that there is a threshold of signals that can induce GI. Furthermore, these signals might

positively correlate to increases in cell population doubling as discussed in chapter 5 below.

The progeny of bystander MCF7 cells showed chromosomal and telomeric instability after 12 and 24 population doublings following co-culture with 0.1 and 2 Gy direct irradiated MCF7 and HMT cells. However, apoptotic levels and telomerase activity were shown to have returned to normal levels in these cells at the same time-points (Figures 4.3 A and B; 4.4 A and B; 4.5 A and B and 4.6 A and B). The findings suggest that the absence of apoptosis induction and active telomerase could maintain cell proliferation even with a reduction in telomere length, which ultimately led to chromosomal instability. Additionally we propose the involvement of inflammatory responses and epigenetics to be a contributory factor in GI induction (Moore *et al*., 2005, Averbeck, 2010).

The progeny of bystander HMT cells of 0.1 Gy direct irradiated HMT-bystander HMT cell combination showed a similar pattern/response as the progeny of bystander HMT cells of 0.1 Gy direct irradiated MCF7-bystander HMT cell combination (Figures 4.7 A and B). This bystander HMT progeny continued to exhibit reduced telomere length as a delayed response and high level of apoptosis after 12 population doublings. However, these cells failed to demonstrate significant chromosomal instability at 12 and 24 population doublings time-points. Moreover, these cells showed an ability to maintain proliferation, which we propose was due to the maintained normal telomerase activity levels. Data again suggest that the high induction of apoptosis could have played a crucial role in the absence of chromosomal instability until the 24 population doubling time-point. Nevertheless, we propose that chromosomal instability could be exhibited at later time-points (25 population doublings plus), due to short telomeres.

Various delayed cellular responses within progeny of bystander HMT cells were significantly detected following co-culture with 2 Gy direct irradiated HMT cells (Figures 4.7 A and B and 4.8 A and B), although chromosomal instability was only observed at the 24 population doublings time-point. Consequently, data suggest that the level of effective GI signals correlates to increased population doublings as discussed in chapter 5.

We have shown that bystander HMT cells exhibit a higher apoptotic response than bystander MCF7 cells (Figures 4.1 A, 4.2 A, 4.3 A, 4.4 A, 4.5 A, 4.6 A, 4.7 A and 4.8 A). Thus the findings confirm that HMT cells were more resistant to the bystander signals than MCF7 cells. As discussed above, apoptosis could eliminate the cells with high chromosomal damage, or could decrease the inflammatory responses (Tesfaigzi, 2006), which can decrease chromosomal damage in the bystander cell populations (Lorimore *et al*., 2001) .

As to the thinking as to whether BE is detrimental or beneficial, our data has shown that bystander signals of 2 Gy cell combinations exhibited detrimental responses within both bystander tumour and non-tumour cells. Results of the 2 Gy bystander MCF7 cells showed a high induction of chromosomal damage as an initial response to the bystander signals, and additionally the progeny of these cells revealed significant chromosomal instability, which can lead to second malignancies (Buonanno *et al*., 2011). Furthermore 2 Gy bystander HMT cells similarly, demonstrated significant early and delayed chromosomal damage. Hence, the findings confirm that high doses of X-ray can induce damaging BE.

In contrast, initial bystander responses following 0.1 Gy X-ray were shown to have beneficial consequences by inducing high chromosomal damage within the bystander tumour cells alone. Multi-chromosomal damage is known to cause auto-killing in

tumour cells (Abdelrazzak *et al*., 2011). However, the delayed responses (chromosomal damage and telomeric shortening) that were observed within the progeny of 0.1 Gy bystander tumour cells (MCF7) suggest that BE was detrimental. In addition, the progeny of 0.1 Gy bystander non-tumour cells (HMT) exhibited unstable telomeres but normal telomerase activity, which could promote chromosomal instability in future generations i.e. at time-points later than 24 population doublings. Chromosomal/genomic instability has been frequently shown to generate second malignancies at later periods (Salas *et al*., 2012), Consequently, the findings suggest that BE has detrimental consequences following low and high doses of X-ray irradiation.

Conclusions

1. Both non-tumour (HMT) and tumour (MCF7) cells demonstrated bystander responses following both low and high doses of X-ray irradiation. The bystander signals of direct irradiated MCF7 cells frequently induced BE within un-irradiated non-tumour cell and tumour cells and vice versa. Moreover, the bystander response was seen to manifest in the form of chromosome aberrations, apoptosis or telomere dysfunction and/or all of these manifestations.

2. Bystander responses were dose-dependent in the bystander HMT and MCF7 cells. For example, the 0.1 Gy bystander HMT cells demonstrated different biological responses from 2 Gy bystander HMT cells following co-culture with direct irradiated MCF7 cells.

3. Bystander signals can be cell-dependent, i.e. the bystander signals from 2 Gy direct irradiated non-tumour cells (HMT) induced bystander responses in the un-irradiated tumour cells (MCF7) that were different from those observed from the 2 Gy direct irradiated MCF7 cells.

4. Bystander signals of direct irradiated MCF7 and HMT cells could induce GI within MCF7 and HMT cells following 0.1 and 2 Gy X-ray irradiation.

5. All the experimental bystander cells (HMT and MCF7) revealed genomic instability within their progeny. Genomic instability was manifest as chromosomal damage, telomeric instability or apoptosis or all of these expressions.

6. GI was dose and cell-dependent within bystander HMT and MCF7 cells.

7. Apoptosis showed an inverse relationship with GI in the bystander cell populations of HMT and MCF7 cells.

8. Telomerase activity positively correlated to increase in GI and population doublings.

9. Bystander HMT and MCF7 cells both maintained their ability to proliferate despite sustained reductions in their cell's telomere length.

10. BE was shown to have detrimental consequences due to GI within progeny of 0.1 and 2 Gy bystander non-tumour (HMT) and tumour (MCF7) cells.

CHAPTER 5

EXOSOMES MEDIATED-NON-TARGETED EFFECTS OF IR

5

Chapter 5: Exosomes mediated- non-targeted effects of ionizing radiation

5.1 Introduction

Exosomes are nano-membrane vesicles, between 40–100 nm in diameter that are released by a wide range of cells into the extracellular environment after fusion of multivesicular endosomes with the plasma membrane (Simons and Raposo, 2009). Exosomes can be secreted by both normal, non-tumour cells (van Niel *et al*., 2001, Segura *et al*., 2005) and cancer cells (Hong *et al*., 2009, Keller *et al*., 2009b) and have been identified in a number of body fluids such as blood plasma (Caby *et al*., 2005), urine (Pisitkun *et al*., 2004), human saliva (Palanisamy *et al*., 2011), bronchoalveolar lavage fluid (Admyre *et al*., 2008) and amniotic fluid (Keller *et al*., 2007).

Exosomes formation starts with endocytosis; the proteins of the cells surface are engulfed as endocytic vesicles, creating early endosomes, which can be recycled back to the plasma membrane or transferred to late endocytic vesicles (van Niel *et al*., 2006), which are recycled to the extracellular environment through the plasma membrane (Figure 5.1). Exosomes' cargo originates from endosomes, plasma membrane and the cytosol; however, there is no specific marker of exosomes (van Niel and Heyman, 2002).

Exosomes have a lipid membrane, enriched in sphingomyelin (Subra *et al*., 2007). Depending on the type and condition of cell, exosomes contain a number of different proteins such as chaperone, cytoskeletal (actin, tubulin and moesin), tetraspanin (CD9, CD63, CD81 and CD82), transport and fusion proteins (Rab2, Rab7, Rab11 and annexines) (Escola *et al*., 1998, Caby *et al*., 2005, Keller *et al*., 2006). Moreover, exosomes have been reported to contain mRNA and microRNA (miRNA) molecules (Valadi *et al*., 2007, Yang *et al*., 2011).

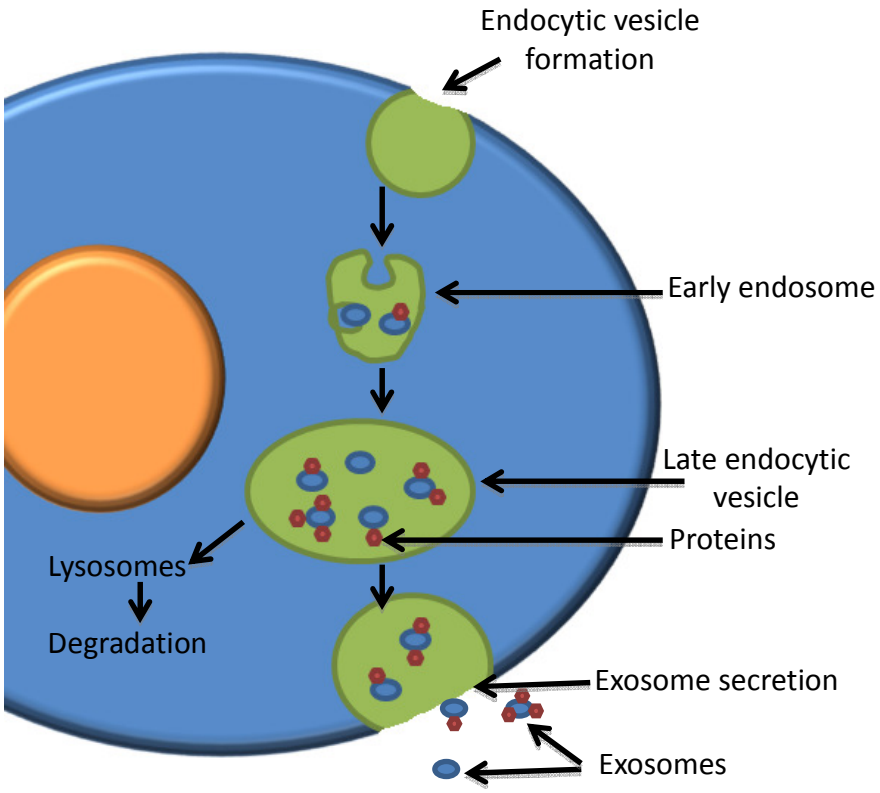


Figure 5.1: Diagram of exosome formation and secretion.

5.2 Function of exosomes

Exosomes' function depends on the cells that they are released from and under which condition they are created (e.g. healthy, disease or stress etc.). They offer one form of cell-cell communication (Fevrier *et al.*, 2005), through their attachment/fusion with plasma membrane of target cells resulting in delivery of exosomal surface proteins and the exosomes' cargo into the recipient cell (Denzer *et al.*, 2000a, Caby *et al.*, 2005). These nanovesicles can, furthermore, interact with recipient plasma membrane cells through receptors-ligand interactions (Clayton *et al.*, 2004).

B cell derived exosomes of both human and mouse can stimulate T-lymphocyte responses (Raposo *et al.*, 1996). In addition, Zitvogel and co-worker have demonstrated that exosomes from dendritic cells (professional antigen presenting cells) can induce T-lymphocyte stimulation *in vivo* (Zitvogel *et al.*, 1998).

Immunological effects have also been observed in cells that received exosomes from mast cells; these under IL-4 stimulation, can increase lymphocyte activation and production of IL-2 and IFN-gamma *in vitro* and *in vivo* (Skokos *et al*., 2001, Skokos *et al*., 2003). Additionally, mast cell derived exosomes can activate endothelium to release Plasminogen Activator Inhibitor Type-I, causing properties of pro-co-coagulant (Al-Nedawi *et al*., 2005).

Cancer cells shed exosomes that appear capable of inducing oncogenic properties in recipient cells, including increased cell division or metastatic behaviour (Higginbotham *et al*., 2011, Keller *et al*., 2009b, Skog *et al*., 2008). Exosome-mediated signalling may underlie the cancer 'field effect', in which tumour cells have been shown to influence the phenotype of nearby cells (Chai and Brown, 2009), and this is consistent with findings that exosome levels are raised in the blood of cancer patients (Keller *et al*., 2009b, Taylor and Gercel-Taylor, 2008). Cancer cell derived exosomes can change immune responses in terms of activation (Wolfers *et al*., 2001) and inhibition (Clayton *et al*., 2007).

Bystander cells exhibit a wide range of biological responses, with many phenotypic similarities to GI. The nature of the soluble transmitting factor(s) is yet to be fully understood, but cytokines including IL-8 (Facoetti *et al*., 2006), TGF-beta (Burr *et al*., 2011), and TNF-alpha (Moore *et al*., 2005, Kadhim *et al*., 2006), as well as calcium fluxes, NO (Shao *et al*., 2008b) and ROS (Matsumoto *et al*., 2007) have been suggested as mediators of bystander responses. A role for plasma membrane-bound lipid rafts has also been indicated (Hamada *et al*., 2007). Recently, miRNA has been shown to be a potential mediator of BE (Aypar *et al*., 2011). Interestingly, miRNA molecules have been found in exosome multi-protein complexes, which are known to be one of the cell-cell communication signals (Mathivanan *et al*., 2011), secreted by

healthy and non-healthy cells. In addition, exosomes have been found to be associated with the process of senescence (Lehmann *et al*., 2008).

Therefore, this study was established to test the hypothesis that exosomes mediate non-targeted effects of ionizing radiation, and that RNA and protein molecules of exosomes play a critical role in this process.

5.3 Materials and Methods

5.3.1 Cell culture

Breast epithelial cancer and non-tumour cells (MCF7 and HMT 3522S1 respectively) were utilised in this study. They were cultured as described in sections 2.2.1.i and 2.2.1.ii.

5.3.2 Experimental design

The experiment was set up to study the induction of non-targeted effects of X-irradiation and comprised three aims.

1) Part 1: to investigate the role of exosomes using tumour (MCF7) cells.

2) Part 2- experiment 1: to investigate the possible role(s) of exosomes' cargo (RNA and protein molecules) using irradiated MCF7 and bystander MCF7 cells.

3) Part 2- experiment 2: to identify of the role of exosomes' cargo in the induction of BE in non-tumour (HMT) cells following a radiotherapy dose (irradiated MCF7 and bystander HMT cell combination).

A media transfer technique was used in all experiments after 4 hour incubation following irradiation.

a. Part 1: MCF7 cells were grown in T75 flasks. At 70% confluence, four flasks were irradiated with 2 Gy X-ray irradiation. One irradiated flask was incubated for 24 hours as a direct irradiated group, whilst the remaining irradiated flasks were incubated for 4 hours, after which, the irradiated cell conditioned media (ICCM) were pooled and

filtered through 1% BSA treated 0.2 µm filter (Section 5.3.3). Fresh MCF7 cells were treated with 15 ml filtered ICCM at 70% confluence and considered as a bystander group (ICCM bystander group). The remaining 30 ml filtered ICCM was ultra-centrifuged (Section 5.3.3) and the supernatant transferred to fresh MCF7 cells (70% confluence) to induce ICCM supernatant bystander group. The exosome pellet was re-suspended in 400 µl PBS, 200 µl was processed for electron microscopy (Section 5.3.4) and 200 µl was transferred to fresh MCF7 cells (70% confluence) to induce ICCM exosome bystander group. Control cell conditioned media (CCCM) was established in parallel by repeating the procedure above without irradiation. All groups were analysed for total DNA damage after 24 hour incubation (at first population doubling). Cells were propagated until p10 (approximately 20 cell-doublings) for assessment of delayed chromosomal/genomic instability. For each passage 1.4 million cells were seeded in a T75 flask using fresh (unconditioned) media and grown to 80% confluence. All experimental groups were analysed using comet assay (Section 5.3.5) for total DNA damage estimation. After 20 population doublings following irradiation, exosomes from progeny media of irradiated, bystander and exosome bystander cells were purified and transferred to fresh MCF7 cells in order to investigate whether the exosomes of these groups are able to induce DNA damage in fresh un-irradiated cells (Figure 5.2).

b. Part 2- experiment 1: MCF7 cells were seeded in T75 flasks until 70% confluence. Seven T75 flasks were irradiated with 2 Gy X-ray irradiation. One flask was incubated for 24 hours as direct irradiated cells. After 4 hour incubation, ICCM of the remaining 6 irradiated flasks were pooled and filtered through 1% BSA treated 0.2 µm filter (Section 5.3.3). Bystander effects were induced by transferring 15 ml of ICCM filtered media to flasks of fresh MCF7 cells (70% confluence). The remaining

ICCM filtered media was subjected to exosomes purification process. Exosomes' pellet was re-suspended in 1000 µl PBS and divided into 5 fractions (200 µl/fraction); 1 fraction was processed for electron microscopy (Section 5.3.4) imaging whilst the remaining 4 fractions were processed as follows to create:

1: Exosome bystander group (Exo bystander) by adding the fraction to fresh MCF7 cells (70% confluence)

2: RNase treated exosome bystander group (RNase bystander) by treatment of fraction with 30 µg/ml RNase A (Sigma, R6513) for 30 minutes at 37ºC in order to digest exosomes' RNA molecules, prior to adding to fresh MCF7 cells (70% confluence)

3: Exosome inactivated proteins bystander group (Boiled Exo bystander) by boiling fraction at 98 °C for 10 minutes prior to adding to fresh MCF7 cells (70% confluence)

4: Exosome's inactivated RNA and protein group (Boiled Exo-RNase bystander) by adding 30 µg/ml RNase A to the fraction for 30 minutes at 37ºC, and then boiling it at 98 °C for 10 minutes prior to adding to fresh MCF7 cells (70% confluence).

Control cell conditioned media (CCCM) was generated in parallel by repeating the procedure above without irradiation. At 24 hours and p12 (approximately 24 cell-doublings), all groups were subjected to chromosomal analysis, apoptotic analysis, telomere length and telomerase activity measurements for assessment of initial and delayed chromosomal/genomic instability (Figure 5.3). For each passage, 1.4 million cells were seeded in a T75 flask using fresh (unconditioned) media and grown to 80% confluence.

Part 2- experiment 2: This was performed similarly to experiment 1 above, using irradiated MCF7 and bystander HMT cell combination. Briefly, MCF7 cells were irradiated with 2 Gy X-ray irradiation; bystander HMT cells received ICCM from

these irradiated MCF7 cells. For this experiment, the exosome pellet was divided into 2 fractions to create:

1: Exosome bystander group (Exo bystander) by adding the fraction to fresh HMT cells (70% confluence)

2: Exosome's inactivated RNA and protein group (Boiled Exo-RNase bystander) by adding 30 µg/ml RNase A to the fraction for 30 minutes at 37ºC, and then boiling it at 98 ºC for 10 minutes prior to adding to fresh HMT cells (70% confluence).

Control groups were set up in parallel and all groups were subjected to the same time-points and end-points as experiment 1 (figure 5.4).

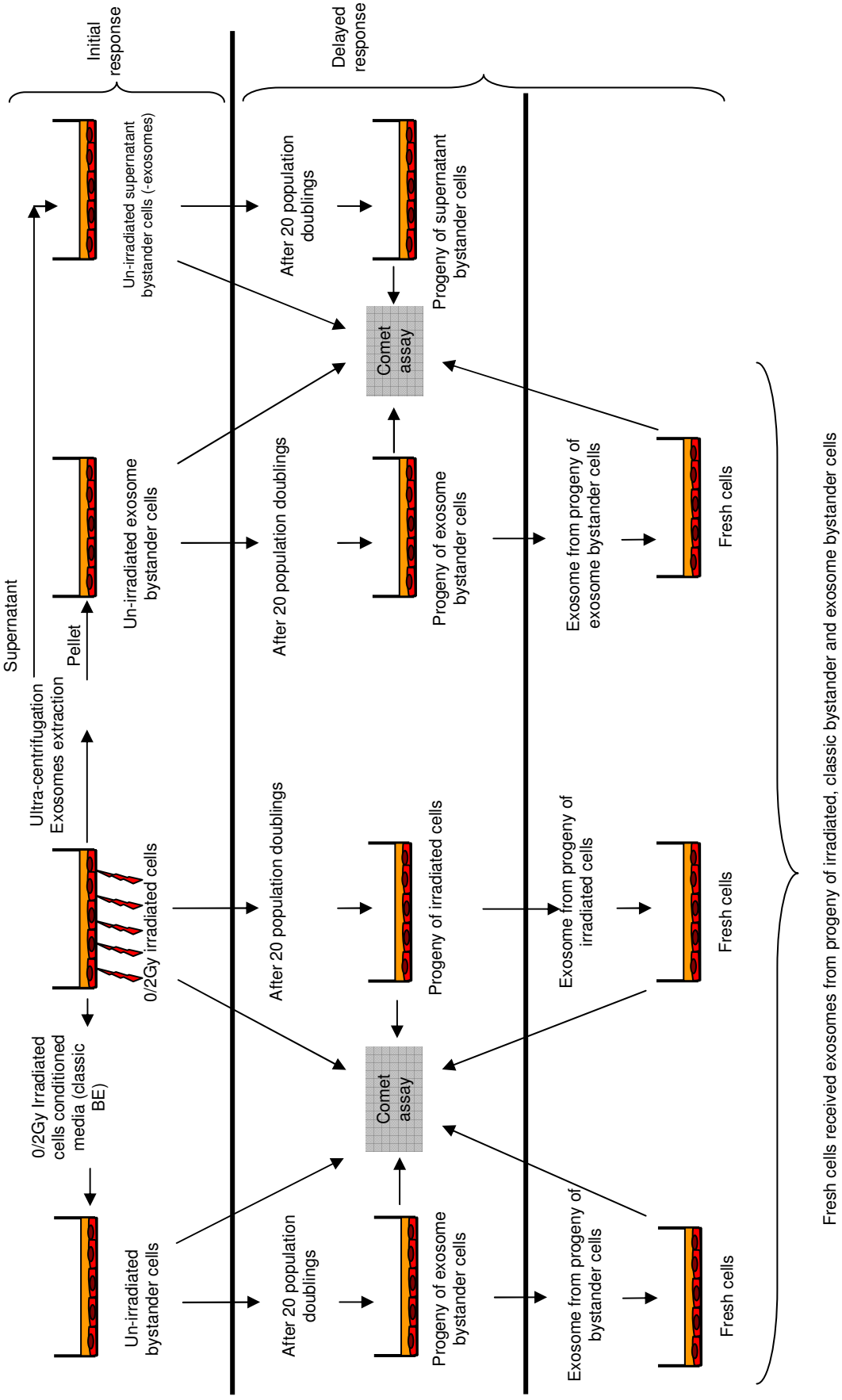


Figure 5.2: Exosomes' experimental schematic, part 1.

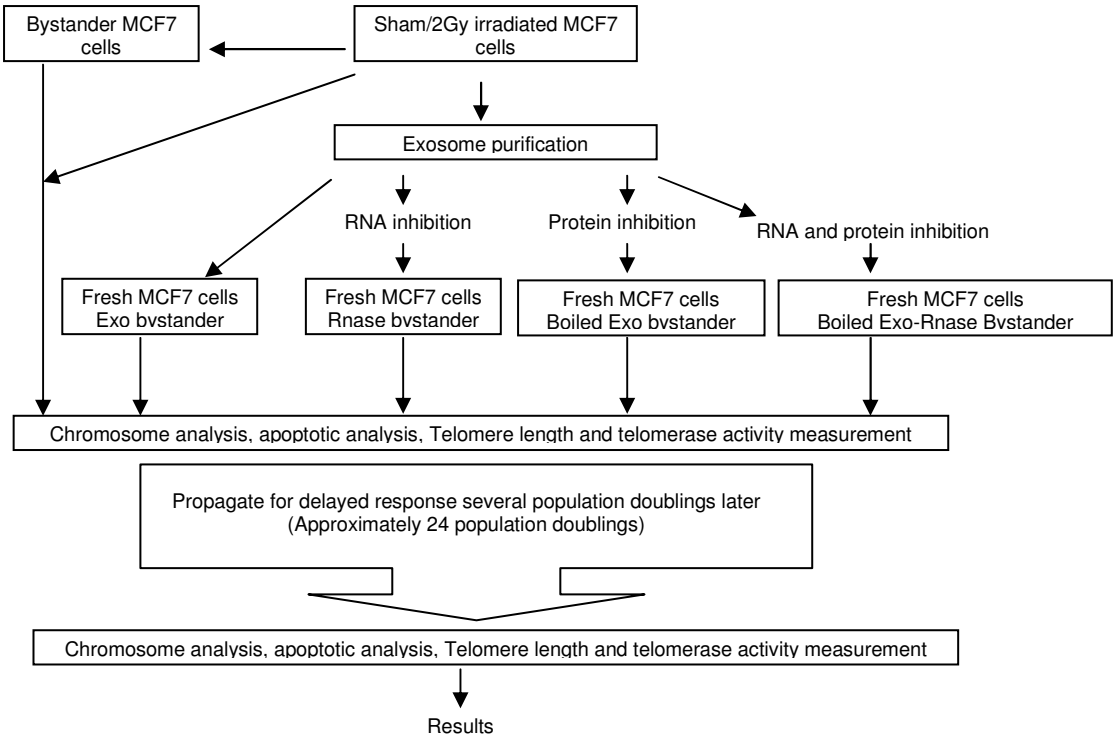


Figure 5.3: Exosome's experimental schematic, part 2, experiment 1

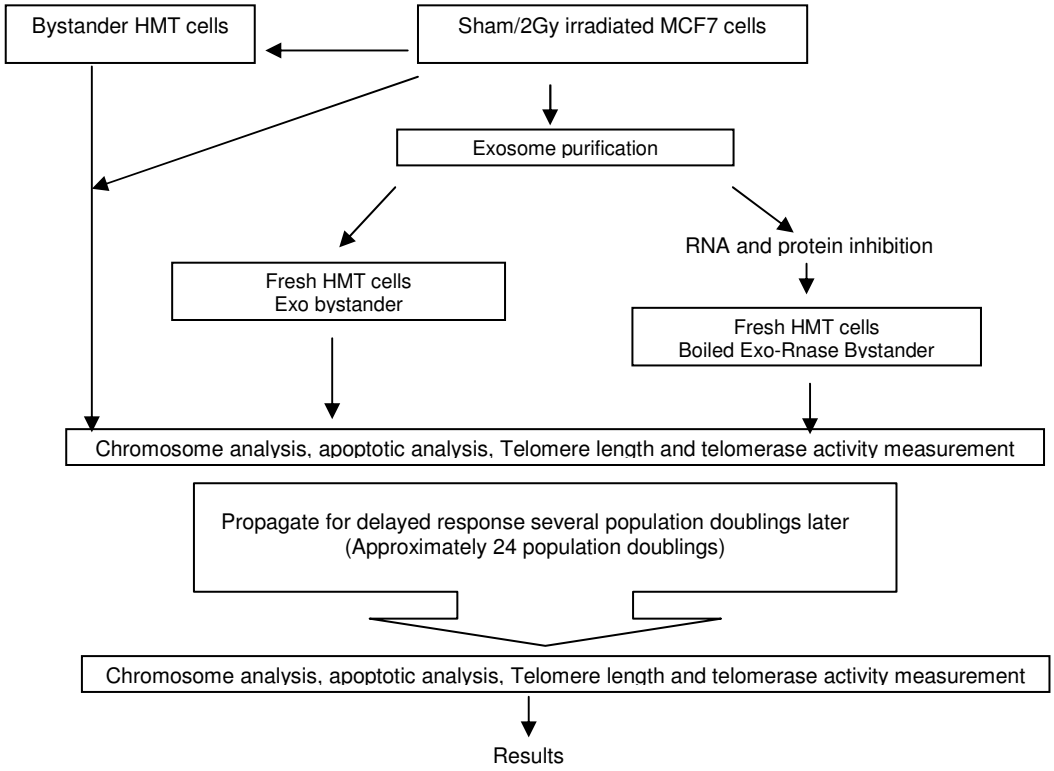


Figure 5.4: Exosomes' experimental schematic, part 2, experiment 2

5.3.3 Exosome purification

The exosome extraction method was adapted from (Lehmann *et al*., 2008). Briefly, exosomes were isolated from the collected irradiated cell conditioned media (ICCM), control (un-irradiated) cell conditioned media (CCCM), bystander cell conditioned media and progeny of irradiated and bystander cell conditioned media. All were filtered through 1% BSA treated 0.2 µm filters (Sartorius, 16532) and then centrifuged at 14000 X *g* for 15 minutes (Eppendorf 5417R), to eliminate cell debris. The exosome vesicles were pelleted by ultra-centrifugation at 100,000 X g for 90 minutes at 4°C (Beckman Coulter LE-80K). Both supernatant and exosome pellet were used to induce BE in cells.

5.3.4 Electron microscopy

Exosome fractions in PBS were incubated on formvar coated nickel grids (Agar scientific S138N3) and negative stained with 3% aqueous uranyl acetate; excess stain was removed and the grids were allowed to air dry prior to observation using a Hitachi H7650 transmission electron microscope at 120 kV.

5.3.5 Comet assay

This was described in section 2.1.14; briefly, microscope slides were coated with 1% normal melting point agarose (NMPA) and allowed to dry over night. The coated slides were then placed on a metal tray on ice. 2 x 10^4 cells were re-suspended with 200 µl of 0.6% low melting point agarose (LMPA) and placed immediately onto chilled pre-coated slides. The cell-LMPA suspensions were flattened with cover slips, which were removed after 5-10 minutes. The slides were then transferred to a Coplin jar, which was filled with cold lysis buffer (2.5 M NaCl, 100 mM EDTA pH 8.0, 10 mM Tris-HCl pH 7.6, and 1% Triton X-100, pH >10). The jar was kept at 4ºC over night. The slides were then moved to a horizontal electrophoresis tank filled with

electrophoresis buffer (0.3 M NaOH and 1 mM EDTA, pH 13) at 4ºC for 40 minutes. The electrophoresis was run for 30 minutes, at 19V, 300A. Slides were neutralised with neutralising buffer (0.4 M Tris-HCl, pH 7.5), washed with distilled water, and immediately stained with a 1:10,000 dilution of SYBR Gold (Molecular Probes/Invitrogen, Carlsbad, CA). The slides were analysed using Komet 5.5 Image Analysis Software (Kinetic Imaging Technology/Andor, Germany).

5.3.6 Chromosomal analysis

Cells were analysed for chromosomal damage after 1 and 24 population doublings for initial and delayed damage response respectively as described in section 2.1.11. In brief, cells were treated with 20 ng/ml demecolcine for 1.5 hours. They were then collected and treated with 75 mM potassium chloride solution for 20 minutes prior to fixation with 25% acetic acid in methanol (twice). Cells were then dropped onto clean microscope's slides, aged, stained with Giemsa and mounted with cover slips.

5.3.7 Apoptotic analysis

Apoptotic levels were determined as described in section 2.1.12, after 1 and 24 population doublings for initial and delayed responses following irradiation. Prolong gold anti-fade reagent with DAPI was utilised to detect the apoptotic bodies. Cells were collected into universal tubes and washed twice with 25% acetic acid in methanol. Cell were dropped onto slides and mounted with prolong gold anti-fade reagent with DAPI. The normal cell nucleus uniformly stains with DAPI, whilst the apoptotic cell nucleus shows apoptotic bodies using fluorescent microscope.

5.3.8 Telomere length measurement

Q-FISH technique was used to measure the telomere length of chromosomes as described in section 2.1.13. Briefly, cells were collected, fixed and dropped onto clean slides. The slides were washed with PBS and then fixed with formaldehyde. The

slides were incubated with pepsin and hybridised using peptide nucleic acid FITC. The slides were then mounted with Vectorshield mounting media with DAPI. The slides were analysed for telomere length (telomeric fluorescence intensity) using smart capture and IP-Lab software.

5.3.9 Telomerase activity measurement

Cells were analysed for telomerase activity by TRAP assay as described in section 2.1.14. In brief, cells were lysed by CHAPS lysis buffer. Cell extractions were mixed with master mix and loaded into 96 well plates. Telomerase activity was measured using real-time PCR.

5.4 Results

5.4.1 Exosome-mediated non-targeted effects of IR (part 1)

5.4.1.i Exosomes of ICCM-induced BE

In order to investigate the role of exosomes in BE induction and in cell-cell communication signals, exosomes were extracted from ICCM of direct irradiated MCF7 cells and transferred to fresh un-irradiated MCF7 cells to induce ICCM-exosomes bystander. Additionally, ICCM after exosome extraction was placed on fresh un-irradiated MCF7 cells to induce ICCM supernatant bystander.

Total DNA damage (double-strand breaks, single-strand breaks and base damage) in individual cells of all the experimental groups was measured using the sensitive comet assay (Collins, 2004). Preliminary work confirmed that MCF7 cells were capable of eliciting a response after direct irradiation and as a bystander population. The subsequent experimental results also confirmed that exosomes could be involved in BE induction. The direct irradiated MCF cells showed significant DNA damage ($p \leq 0.0001$) after 1 population doubling following 2 Gy X-ray irradiation, with median percentage values of 18.65±0.61 and 13.47±0.5 for direct irradiated and control,

respectively (Figure 5.5 A). Moreover, MCF7 ICCM caused a high induction of DNA damage ($p \leq 0.0001$) in the un-irradiated MCF7 (22.33±0.61) compared to its corresponding control (CCCM) (9.9±0.37) as shown in figure 5.5 A. These results confirmed that whole ICCM could induce BE in the fresh (un-irradiated) MCF7 cells after 24 hours communication. A high induction of DNA damage ($p \leq 0.0001$) was also observed in fresh un-irradiated bystander MCF7 cells treated with the exosome pellet of ICCM following 100000 X g (MCF7 ICCM-exosomes) after 24 hours communication compared to its control (MCF7 CCCM-exosomes) as shown in figure 5.5 B. Interestingly, the supernatant of ICCM following 100000 X *g* was unable to induce DNA damage in the fresh un-irradiated bystander MCF7 cells (MCF7 ICCM-supernatant) at this time-point (Figure 5.5 B). These findings suggest that ICCM exosomes could mediated BE in the MCF7 cells through exosome cargo molecules, including RNA and protein molecules (Lasser *et al*., 2012), or at least one of them following 2 Gy X-ray irradiation. Nevertheless, ICCM Supernatant (without exosomes) did not show a significant effect on the bystander MCF7 cells.

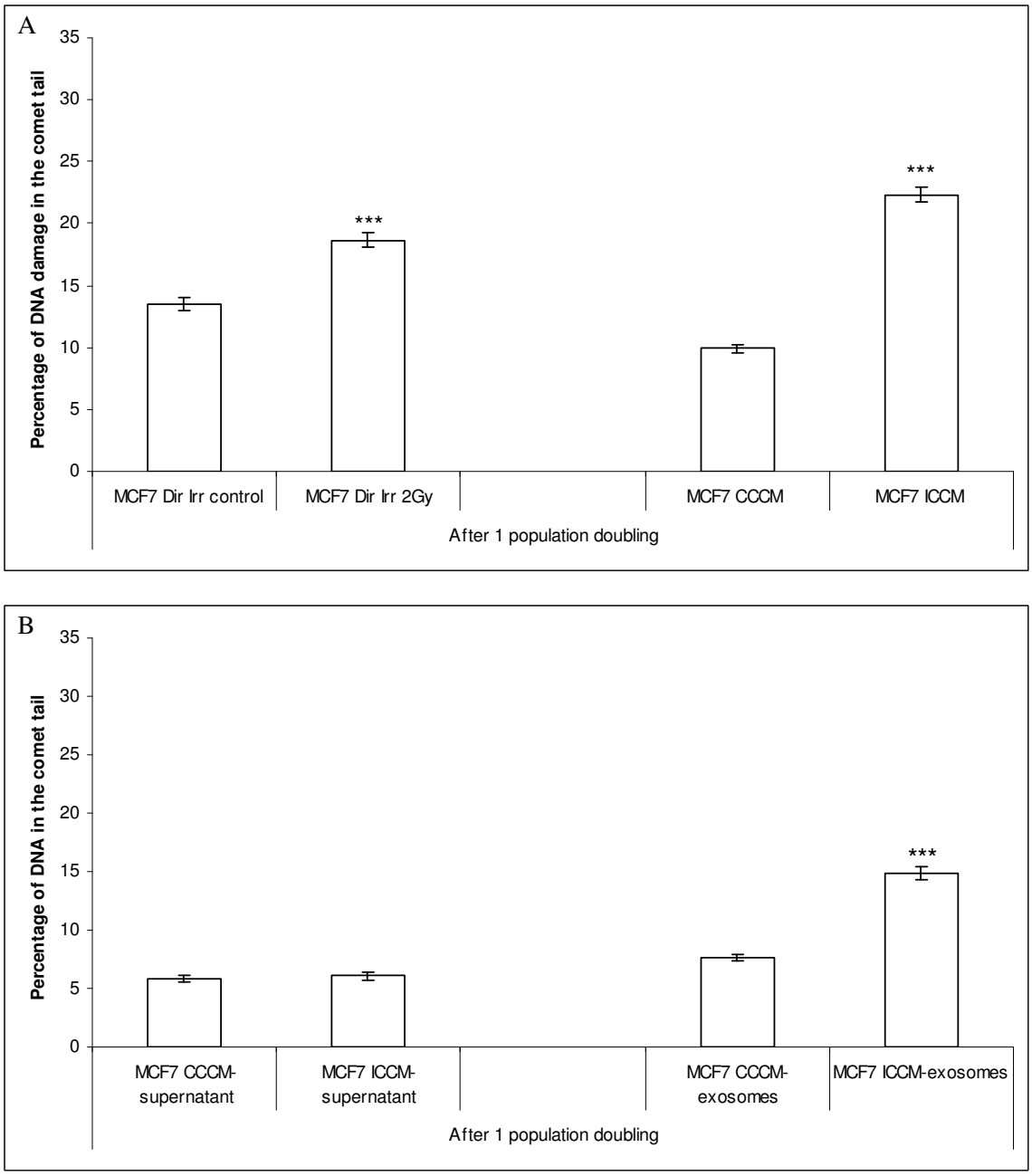


Figure 5.5: Percentage of DNA in the comet tail within direct irradiated and bystander MCF7 cell populations after 1 generation following 2 Gy X-ray irradiation.

MCF7 cells were irradiated with 2Gy X-ray and subjected to the comet assay after 1 generation following irradiation. The ICCM were transferred to fresh un-irradiated MCF7 cells to induce BE. The bystander cells were also analysed for total DNA damage using comet assay. Panel A illustrated the non-targeted effects of 2 Gy X-ray irradiation within MCF7 cells after 1 population doubling. . Both 2 Gy direct irradiated and bystander MCF cells showed significant DNA damage (***$p \leq 0.0001$). Panel B showed the ability of exosomes of ICCM to induce DNA damage in un-irradiated MCF7 cells. The CCCM and ICCM were ultra-centrifuged, the exosomes' pellets and the supernatant were separately transferred to un-irradiated cells. Interestingly, the exosome pellet of ICCM caused DNA damage (***$p \leq 0.0001$) in the cells compared to the control; however, the supernatant bystander cells did not demonstrate a significant DNA damage compared to the control. The finding showed that exosome pellet caused BE compared to the supernatant of ICCM.
Experiment was performed in 3 technical repeats.

5.4.1.ii Exosomes of ICCM-induced GI

The cells (MCF7 direct irradiated, MCF7 ICCM, MCF7 ICCM–supernatant and MCF7 ICCM-exosomes and their controls) were propagated for approximately 20 population doublings following irradiation for delayed DNA damage response (GI) estimation. A high induction, approximately 4.5 folds higher, of DNA damage ($p \leq 0.0001$) was observed in the progeny of direct irradiated (22.17±0.9) and ICCM MCF7 cells (24.3±0.85) compared to the controls (5.11±0.39 and 4.98±0.25 respectively), as shown in figure 5.6 A. Moreover, there was also a similar (4.5 folds higher) significant induction of DNA damage ($p \leq 0.0001$) observed within the progeny of MCF7 ICCM-exosomes (28.79±1.03) after 20 generations following exosome pellet transfer compared to the control (6.41±0.35), as shown in figure 5.6 B. Interestingly, the progeny of MCF7 ICCM supernatant (without exosomes) were unable to induce a significant delayed DNA damage compared with the control (Figure 5.6 B), thereby confirming that ICCM exosomes induced GI within MCF7 cells following 2 Gy X-ray irradiation. Data suggested that exosomes could be either: long-lived signals; secreted from the progeny of irradiated/bystander cells or from cells that were treated with ICCM exosomes.

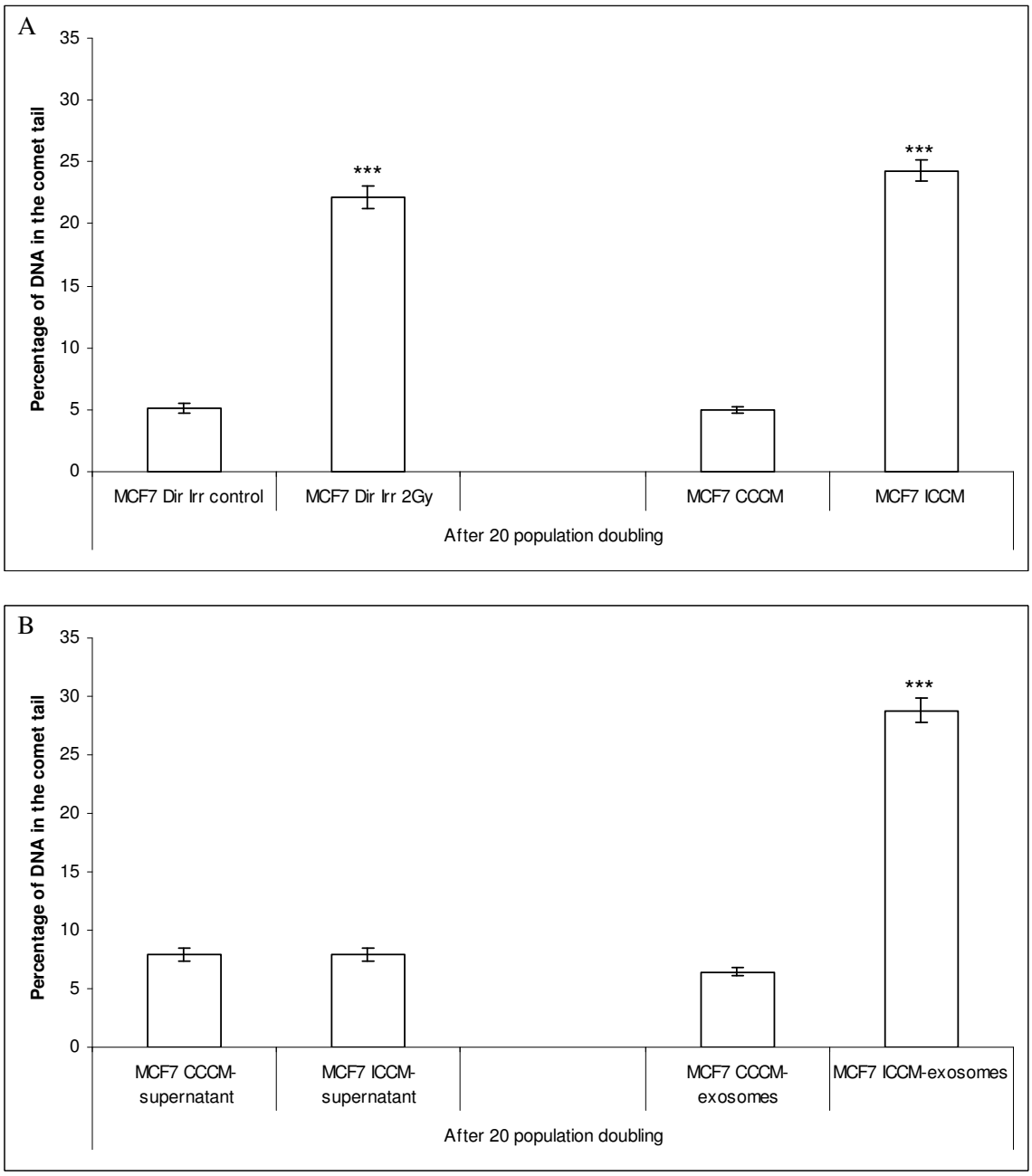


Figure 5.6: Delayed responses within the progeny of direct irradiated and bystander MCF7 cell populations after 20 population doublings following 2 Gy X-ray irradiation.

The direct irradiated and bystander cells were propagated until about 20 population doublings for delayed response. Panel A showed similar results as those of the early time-point, i.e. both treatment groups showed a significant induction of DNA damage (***p≤0.0001) compared to the controls. Panel B demonstrated the delayed responses within the progeny of supernatant (without exosomes) and exosome treated cells following irradiation. The progeny of supernatant bystander cells did not exhibit induction of delayed DNA damage after 20 generations. Nonetheless, the progeny of exosome bystander cells revealed a high induction of delayed response in terms of total DNA damage (***p≤0.0001) compared to the control. Data demonstrated that the exosomes of ICCM could cause delayed DNA damage compared to the exosomes of CCCM.
Experiment was performed in 3 technical repeats.

5.4.1.iii Progeny displayed exosome-induced DNA damage

The data clearly showed that irradiated cells secrete exosomes and these played a significant role in bystander signalling, further endorsed by evidence in section 5.4.1, of exosomes ability to induce BE by cell-cell communication. We have also observed that MCF7 cells treated with exosomes from the progeny of direct irradiated, ICCM and ICCM-exosomes cells expressed DNA damage underlying the delayed response. All demonstrated a high induction of DNA damage ($p \leq 0.0001$) compared to their corresponding controls (Figure 5.7). The percentage of DNA damage was 22.9±1.49, 22.14±0.91 and 20.87±1.08 in these groups, compared to their controls, which exhibited 5.33±0.81, 4.43±0.33 and 7.54±0.74 percentage of DNA damage respectively, indicating that exosomes may have an important role in GI.

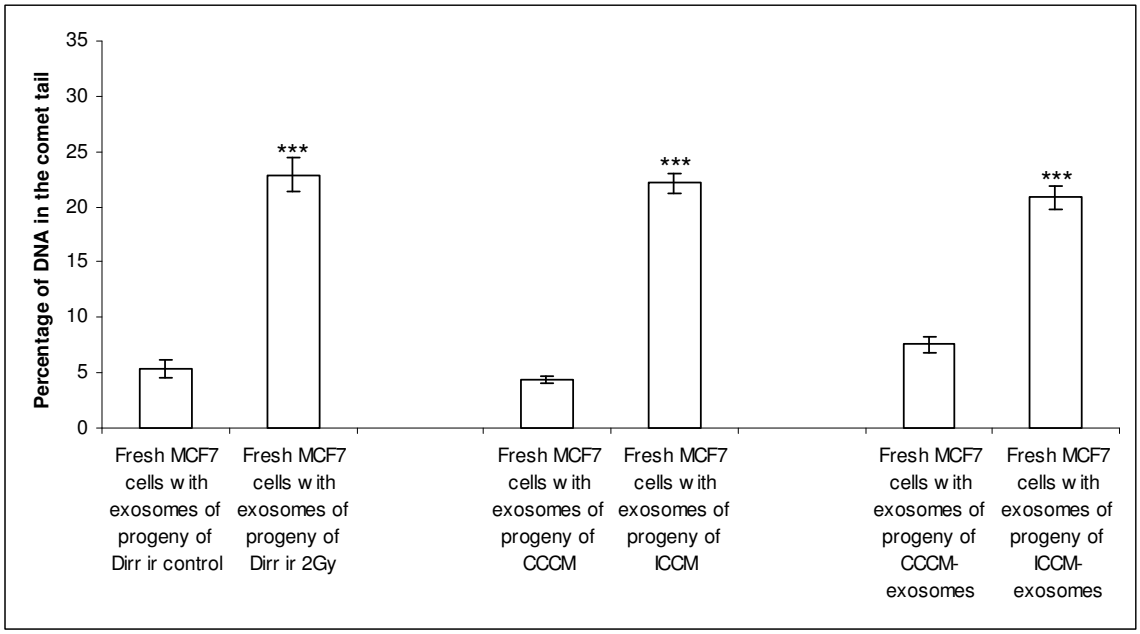


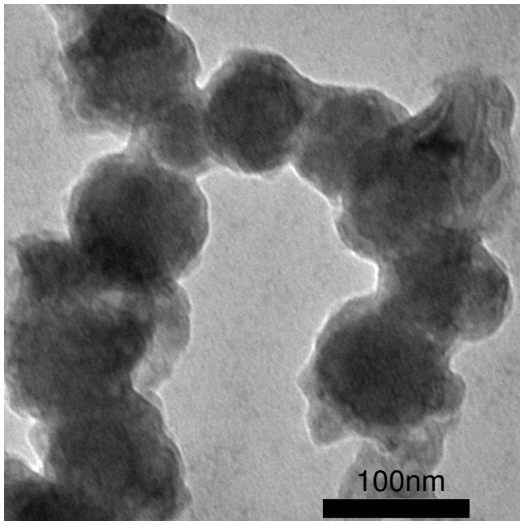
Figure 5.7: Induction of DNA damage in cells treated with exosomes from the progeny of irradiated, bystander and exosome bystander cells.

The media of progeny of irradiated, bystander, exosome bystander cells and their controls were collected separately, each was then purified. The exosome pellets were added to fresh un-irradiated cells to investigate the ability of progeny exosomes to induce delayed DNA damage. Interestingly, the cells treated with exosome pellets of the progeny showed a high induction of DNA damage (***$p \leq 0.0001$) compared to the controls. This suggests that exosomes secreted by the direct irradiated and bystander progeny cells could mediate delayed DNA damage responses.

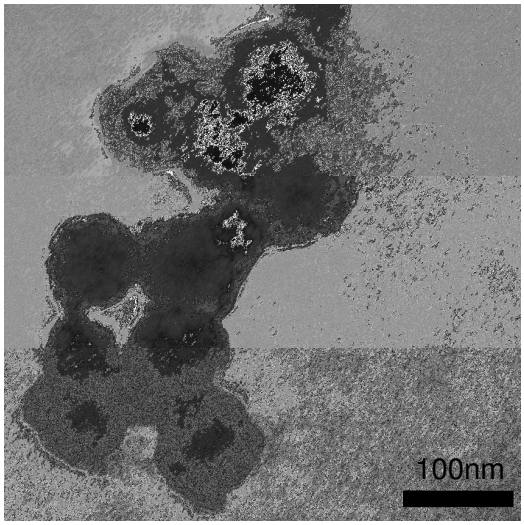
Experiment was performed in 3 technical repeats.

5.4.1.iv Electron microscopy analysis

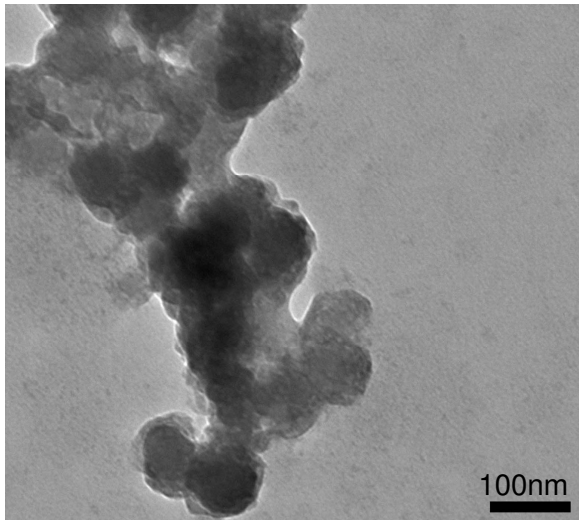
Exosome pellets of irradiated, bystander and the progeny of irradiated, bystander and bystander-exosome MCF7 cells were processed for electron microscopy imaging in order to visualise and characterise their morphology to determine if the various treatments conferred differences. However, the data did not show any morphological distinction (qualitative and quantitative differences) between any of the groups but confirmed them to be of a size between 40-140 nm (Figure 5.8). Therefore, it was necessary to perform further studies/specific methods to in order to qualify and quantify if differences did actually exist between the exosomes of CCCM, ICCM and bystander cells.



CCCM-exosomes



ICCM-exosomes



Exosomes of bystander cell-conditioned media

Figure 5.8: Electron microscope images of exosomes from the media of shame/control, direct irradiated and bystander MCF7 cells.

Exosomes were isolated from the media of sham/control, direct irradiated and bystander cells. The exosomes of these cells were shown to be between 40-140 nm in diameter. There was no morphological difference between any of the treatment groups. Further, qualitative and quantitative studies were therefore needed to investigate possible differences between the exosomes from CCCM, ICCM and bystander-exosomes, respectively.

5.4.2 Exosome cargo molecules and non-targeted effects of IR (part 2)

As shown in section 5.4.1, data demonstrated that exosomes could mediate non-targeted effects of IR. In order to investigate which of the exosome cargo molecules were involved in the non-targeted effects induction, exosomes were purified from ICCM and divided into 5 fractions (Section 5.3.2). Briefly, the first fraction was used for electron microscopy imaging. The second fraction was transferred to fresh MCF7 cells to investigate the induction of BE by exosomes (EXO BE). The third was treated with RNase A, to digest exosome RNA molecules; thus it was considered an exosomes-bystander without RNA molecules (EXO-RNase BE). The fourth fraction was boiled to denature exosome proteins and considered as exosome-bystander without exosome proteins (boiled EXO-BE). The last fraction was treated with RNase and then boiled to digest and stop the actions of exosome RNA and protein molecules together. Thus it was considered an exosome-bystander without exosome RNA and protein molecules (boiled EXO-RNA BE). The control groups were established in parallel. Chromosomal analysis, apoptotic analysis, telomere length and telomerase activity measurements were utilised as biological end points to estimate BE and GI consequences in the all groups to enable comparisons with the previous results of chapters 3 and 4. Hence, the investigations were set up specially to understand whether the exosome effects were from RNA molecules or protein molecules or both.

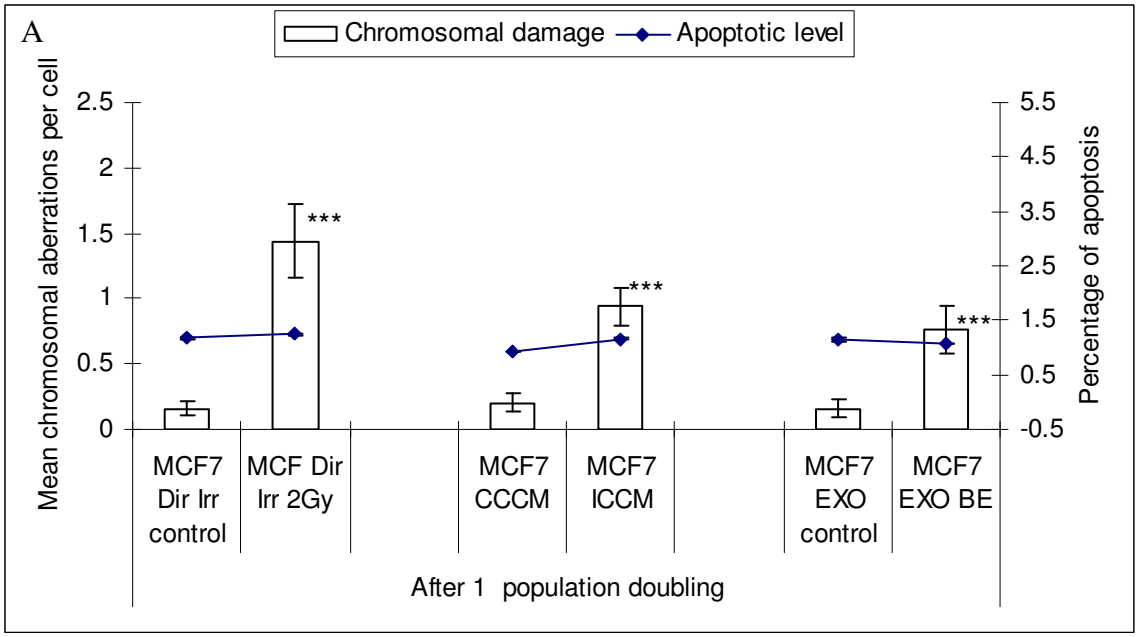
5.4.2.i Exosome-induced BE and GI within MCF7 cells

It was first necessary to confirm the existence of BE following IR and communication via exosomes from ICCM in MCF7 cells. Thus, MCF7 cells were directly irradiated with 2 Gy X-ray (MCF7 Dir Irr 2Gy). Bystander populations were created by CCCM and ICCM transfer (MCF7 CCCM and MCF7 ICCM), while the exosome pellet was added to fresh un-irradiated MCF7 to generate exosome bystander (MCF7 EXO BE). Cells were analysed for BE and GI after 1 and 24 generations.

Early chromosomal damage was significantly induced ($p \leq 0.0001$) in the direct irradiated, bystander and exosome bystander MCF7 cells compared to their corresponding controls (Figure 5.9 A). The mean number of chromosomal aberrations observed in MCF7 Dir Irr 2Gy cells was 1.44±0.28 compared to 0.16±0.05 of the MCF7 Dir Irr control group. In contrast MCF7 CCCM cells showed values of 0.2±0.06 which increased to 0.94±0.14 after ICCM transfer (MCF7 ICCM cells). Similar numbers of chromosomal aberrations (0.76±0.18), were observed in the exosome bystander (MCF7 EXO BE cells) compared to the control (MCF7 EXO control) which only showed 0.16±0.06 aberrations. The data thus demonstrated the efficiency of 2 Gy X-ray, ICCM and the whole component of the exosomes of ICCM to cause initial chromosomal damage within MCF7 cells. However, no induction of apoptosis was observed in any of these groups (Figure 5.9 A). Conversely, all demonstrated a significant reduction in the telomerase activity ($p \leq 0.0001$) at the same time-point (Figure 5.9 B), although statistically significant telomeric shortening was only observed ($p \leq 0.05$) in the MCF7 Dir Irr 2Gy compared to the control.

Cytogenetic analysis from the delayed time-point (24 generations) exhibited significant damage ($p \leq 0.0001$) within the progeny of MCF7 Dir Irr 2Gy, MCF7 ICCM and MCF7 EXO BE cells and the media transfer compared to the corresponding controls (Figure 5.9 C), however, there was no induction of apoptosis (Figure 5.9 C), which would have contributed to the expression of a high induction of chromosomal instability, according to the inverse relationship between apoptosis and chromosomal instability. Furthermore, progeny of MCF7 Dir Irr 2Gy cells continued to demonstrate significant telomeric shortening ($p \leq 0.05$) as shown in figure 5.9 D and interestingly, the progeny of MCF7 ICCM and MCF7 EXO BE cells also revealed significant telomeric shortening ($p \leq 0.05$), which had previously been absent in the

earlier time-point. Moreover, telomerase activity was now shown to have returned to control levels, suggesting that the initial increase in the telomerase activity positively correlated with GI. These findings indicate that short telomeres, sufficient telomerase activity and absence of apoptosis frequently led to chromosomal instability within the progeny of irradiated, bystander and exosome bystander progeny.



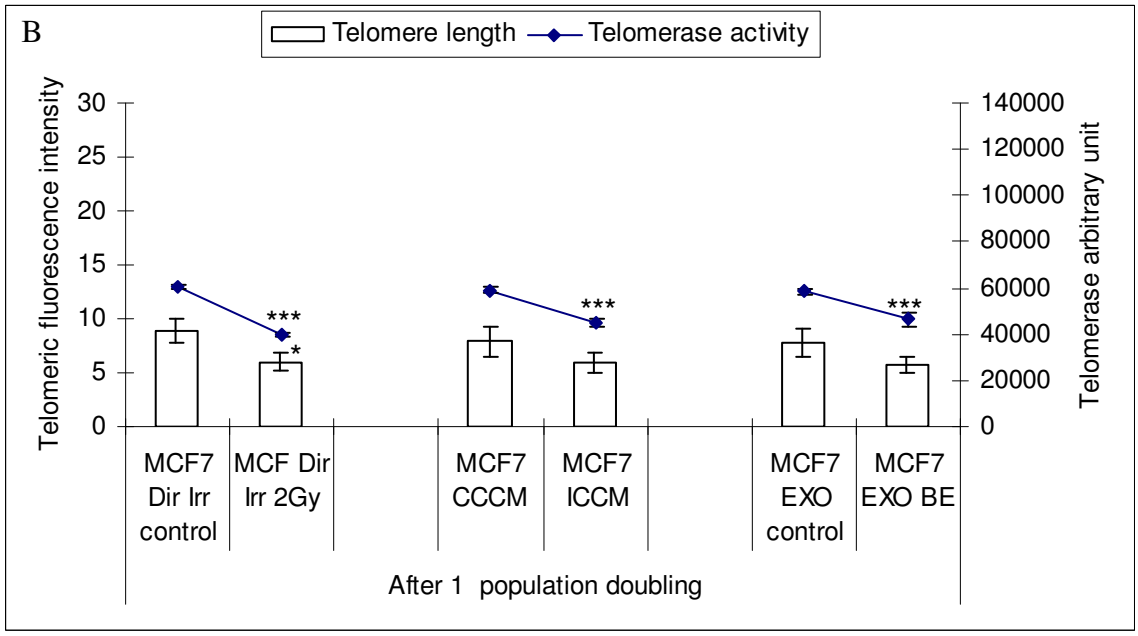
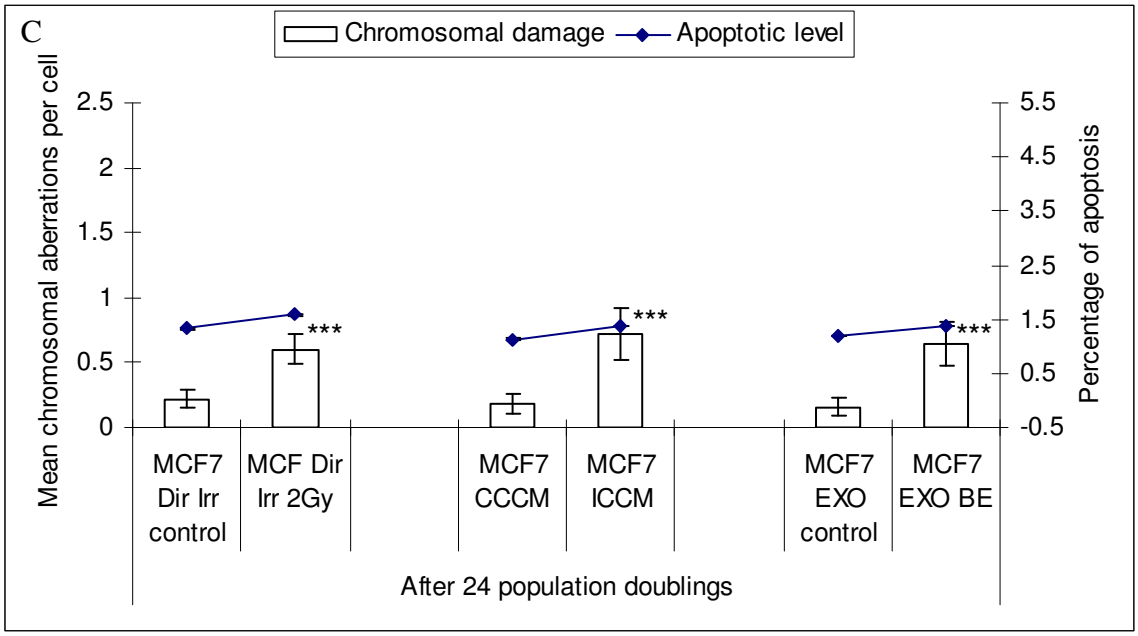


Figure 5.9: Cellular damage in MCF7 direct irradiated, bystander and exosome bystander cell after 1 and 24 population doublings following 2 Gy X-ray irradiation.

MCF7 cells were irradiated with 2 Gy X-ray irradiation. The CCCM and ICCM were transferred to un-irradiated MCF7 cells to induce BE (MCF7 CCCM and ICCM). The exosomes were extracted from CCCM and ICCM and placed onto fresh un-irradiated MCF7 cells to induce exosome-BE (MCF7 EXO control and BE). Cells were analysed for initial responses after 1 generation. Cells were then propagated until 24 population doublings for delayed responses. Panel A represented chromosomal data and apoptotic levels within the cells after 1 population doubling. Chromosomal damage was significantly induced (***$p \leq 0.0001$) within the direct irradiated, bystander and exosome bystander cells compared to the controls. However, the cells did not show a significant apoptotic induction at the same time-point. Panel B illustrates telomeric instability including telomere length and telomerase activity estimation in the irradiated and bystander MCF7 cells. The irradiated cells exhibited significant telomeric shortening (*$p \leq 0.05$) compared to the control. The MCF7 ICCM cells were shown to exhibits short telomeres following bystander induction; nevertheless, the telomeric shortening of these bystander cells was statistically insignificant compared to the control. Similarly, MCF7 EXO BE cells revealed short telomeres although statistically insignificant. Moreover, direct irradiated, ICCM and EXO BE MCF7 cells demonstrated a significant reduction of telomerase activity (***$p \leq 0.0001$) compared to the controls. Thus data showed that exosome pellet of irradiated MCF7 cells elicited a similar effect to ICCM on MCF7 bystander cells underlying BE. Experiment was performed in 3 technical repeats.



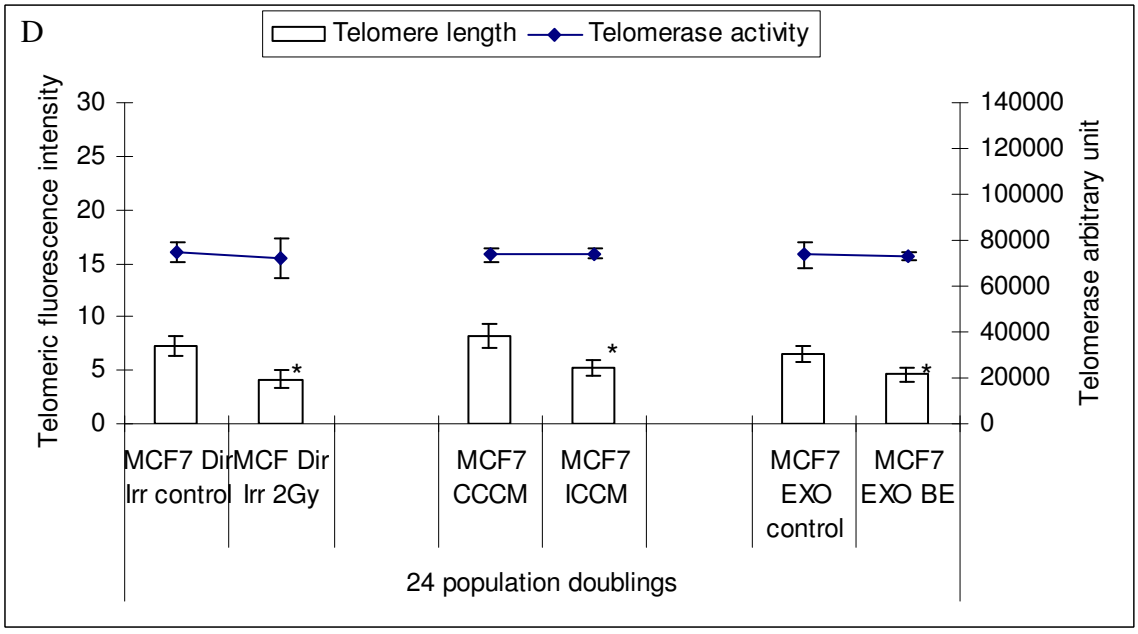


Figure 5.9:

Panel C represent the delayed chromosomal damage and apoptotic levels in the progeny of direct irradiated, bystander and exosome bystander cells after 24 population doublings. The chromosome aberrations were significantly observed ($p \leq 0.0001$) within the progeny of direct irradiated, bystander and exosome bystander cells. However, there was no significant induction of apoptosis; thus, results confirmed the inverse relationship between apoptosis and chromosomal instability.

Panel D exhibit telomere length and telomerase activity within the progeny of direct irradiated, bystander and exosome bystander cells after 24 generations. Telomerase activity was shown to have returned to control levels although, there was significant telomeric shortening ($p \leq 0.05$), which suggest that sufficient telomerase activity can maintain the cell proliferation even when chromosomes have short telomeres, which can lead to chromosomal instability especially in the absence of apoptosis. Moreover, data suggested that exosomes could mediate GI in MCF7 cells following 2 Gy X-ray irradiation.

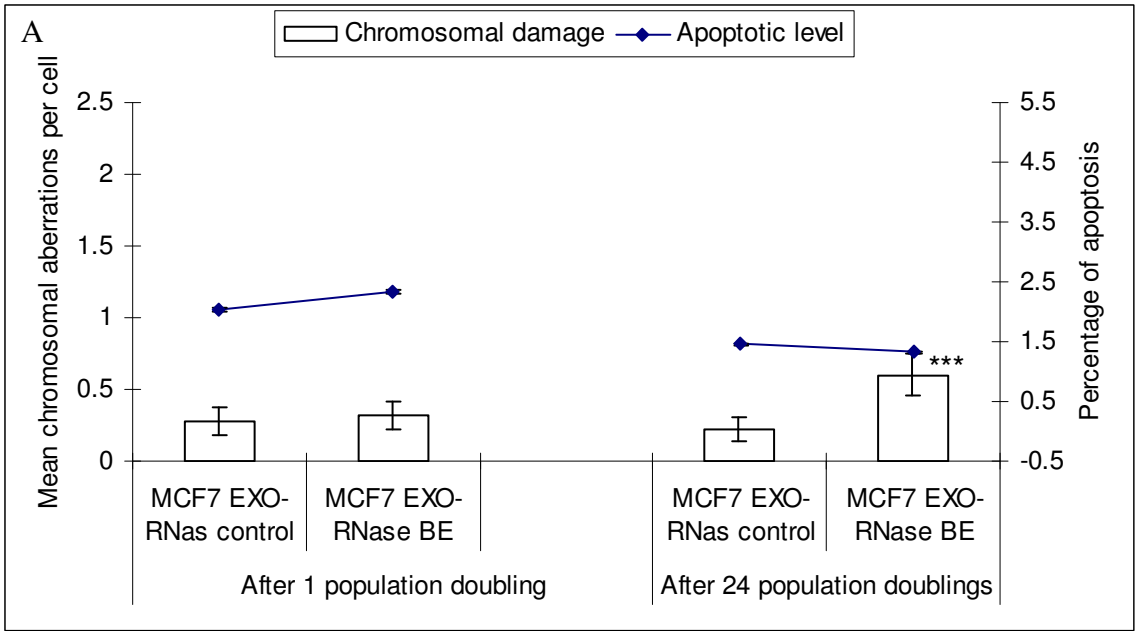
Experiment was performed in 3 technical repeats.

5.4.2.ii Exosome RNA molecules and non-targeted effects of IR

In order to investigate the role of exosome RNA molecules following irradiation, exosome pellets (ICCM and CCCM exosomes) were separately treated with RNase A for 30 minutes to digest RNA molecules within the pellets. The pellets were then transferred to fresh un-irradiated MCF7 cells to induce RNase exosome BE and control (MCF7 EXO-RNase BE and MCF7 EXO-RNase control cells). Cells were then analysed for chromosomal damage, apoptosis telomere length and telomerase activity measurements after 1 and 24 population doublings in order to measure early and late cellular responses.

Interestingly, the MCF7 EXO-RNase BE cells did not show induction of chromosomal following digestion of exosome RNA molecules by RNase A indicating that RNase abrogated the effect of ICCM exosomes to induce initial chromosome aberrations within MCF7 cells. However, the mean chromosomal background damage in the MCF7 EXO-RNase control group (0.28±0.09), was higher than other experimental control levels (MCF7 Dir Irr control, MCF7 CCCM and MCF7 EXO control) which had mean aberrations levels of between 0.16-0.20 (Figure 5.10 A); this increase could have been caused by RNase treatment. Apoptotic levels were also insignificant in MCF7 EXO-RNase BE cells compared to the control cells. Moreover, MCF7 EXO-RNase BE cells did not show a significant telomeric shortening or reduction in the telomerase activity compared to the control (Figure 5.10 B). However, the levels of telomerase activity in both MCF7 EXO-RNase BE and MCF7 EXO-RNase control cells were low, suggesting that RNase may have digested the telomerase RNA subunit whereby instigating a reduction in telomerase activity. Overall, the results showed that RNase increased the damage in the control cells and slightly decreased the effect of exosome treatment within the treated cells.

Interestingly, chromosomal instability was significantly observed ($p \leq 0.0001$) within the progeny of MCF7 EXO-RNase BE cells after 24 generations (Figure 5.10 A). The mean chromosomal aberrations in the control cells was 0.22±0.08, which increased to 0.6±0.14 in the progeny of MCF7 EXO-RNase BE cells. These results suggest that inhibition of exosome RNA molecules in the parent cells partly abolished initial chromosomal damage but not delayed damage, the latter may be due to other molecules e.g. exosome proteins and/or long-lived molecules. Apoptotic levels in the progeny of MCF7 EXO-RNase BE cells were insignificant different after 24 population doublings compared to the control (Figure 5.10 A). Additionally, data did not show a significant difference in telomerase activity levels between the progeny of MCF7 EXO-RNase BE and the control cells at the same time-point. Nonetheless, the level of telomerase activity was significantly higher ($p \leq 0.0001$) in the progeny cells (after 24 generations) than parent cells (after 1 generation) we think due to RNase treatment affecting the function of telomerase in the parent cells (Figure 5.10 B). Interestingly, the progeny of MCF7 EXO-RNase BE cells showed short telomere length after 24 generations although insignificant compared to the control (Figure 5.10 B). Data suggest that instable telomeres led to chromosomal instability.



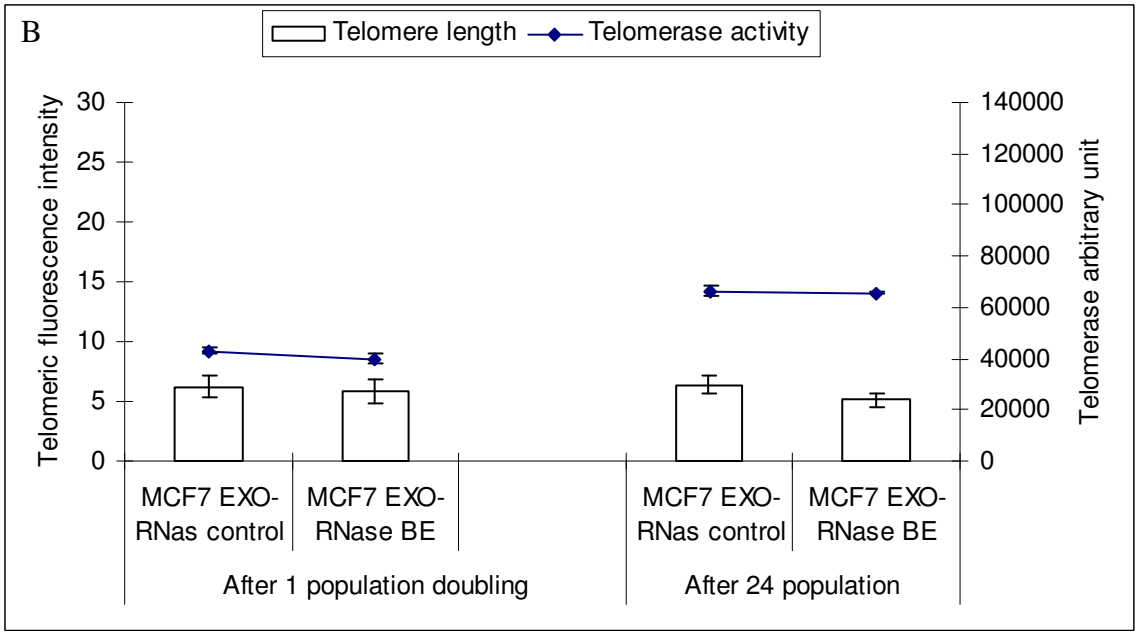


Figure 5.10: Early and delayed cellular responses within the RNase treated-exosome bystander cells.

Exosomes were separately isolated from CCCM and ICCM of MCF7 cells and treated with RNase to digest exosome RNA molecules. The RNase treated exosomes were then transferred to fresh un-irradiated MCF7 cells to induce BE. i.e. BE was induced within MCF7 cells by RNA deficient exosome molecules. Panel A: represent initial and delayed chromosomal damage and apoptotic induction within the cells. RNase abrogated chromosomal damage within MCF7 EXO-RNase BE cells after 1 generation following transferring compared to the control. Surprisingly, these cells showed a significant chromosomal damage (***$p \leq 0.0001$) after 24 generations compared to the control. Data suggest that in addition to the exosome RNAs another molecule mediated the delayed chromosomal aberrations. Similarly in the direct irradiated, bystander and exosome bystander apoptotic responses, the MCF7 cells that were treated with RNase treated-ICCM exosomes did not exhibit early or delayed induction of apoptosis. Panel B demonstrates telomere length and telomerase activity data within the MCF7 EXO-RNase control and BE cells. These cells did not demonstrate significant telomeric shortening or reduction in the telomerase activity after 1 generation compared to the control. These cells continued to show normal levels of telomerase activity after 24 population doublings although they exhibited shortened telomeres but these were not statistically significant. Overall, the data suggest that exosome RNAs could play a role in the non-targeted effects of IR, although we postulate that chromosomal instability was caused by another long-lived molecule.
Experiment was performed in 3 technical repeats.

5.4.2.ii Exosome proteins and non-targeted effects of IR

Exosome protein molecules were additionally studied to investigate the correlation between them and the induction of non-targeted effects of IR. In order to do this, the pellets from ICCM and CCCM were boiled at 98°C for 10 minutes to denature the proteins and to abolish their action. The pellets were then added to the fresh un-irradiated cells to investigate bystander response of MCF7 cells and their control group (MCF7 boiled EXO BE and Control cells, respectively). Chromosomal and, apoptotic analysis, telomere length and telomerase activity measurements were performed to evaluate the early and late effects.

The MCF7 boiled EXO BE cells showed induction of chromosomal damage ($p \leq 0.05$) after 1 population doubling compared to the control (Figure 5.11 A). A significant reduction in telomerase activity ($p \leq 0.05$) was also observed at the same time-point (Figure 5.11 B). However, the levels of chromosomal damage and telomerase activity (0.4±0.11 and 54515±1102.6 TAU respectively) in the MCF7 boiled EXO Control group were significantly ($p \leq 0.05$) lower than those from the MCF7 EXO cells (0.76±0.18 and 46365±2744.2TA). Moreover, the MCF7 boiled EXO BE cells failed to demonstrate induction of apoptosis and significant telomeric shortening as early responses compared to the controls (Figures 5.11 A and B respectively). Data suggest that inactivation of exosome protein could partially reduce bystander cellular damage within MCF cells following irradiation.

After 24 population doublings, the progeny of MCF7 boiled EXO BE cells revealed unstable chromosomal damage ($p \leq 0.05$) compared to its control (Figure 5.11 A). Conversely, there was no significant difference in apoptotic levels between the groups and telomerase activity had returned to normal despite shortened telomeres, although statistically insignificant, in the treated group compared to its control (Figure 5.11 B).

We speculate that short telomeres could play a crucial role in chromosomal instability in MCF7 cells treated with boiled exosomes (MCF7 boiled EXO BE).

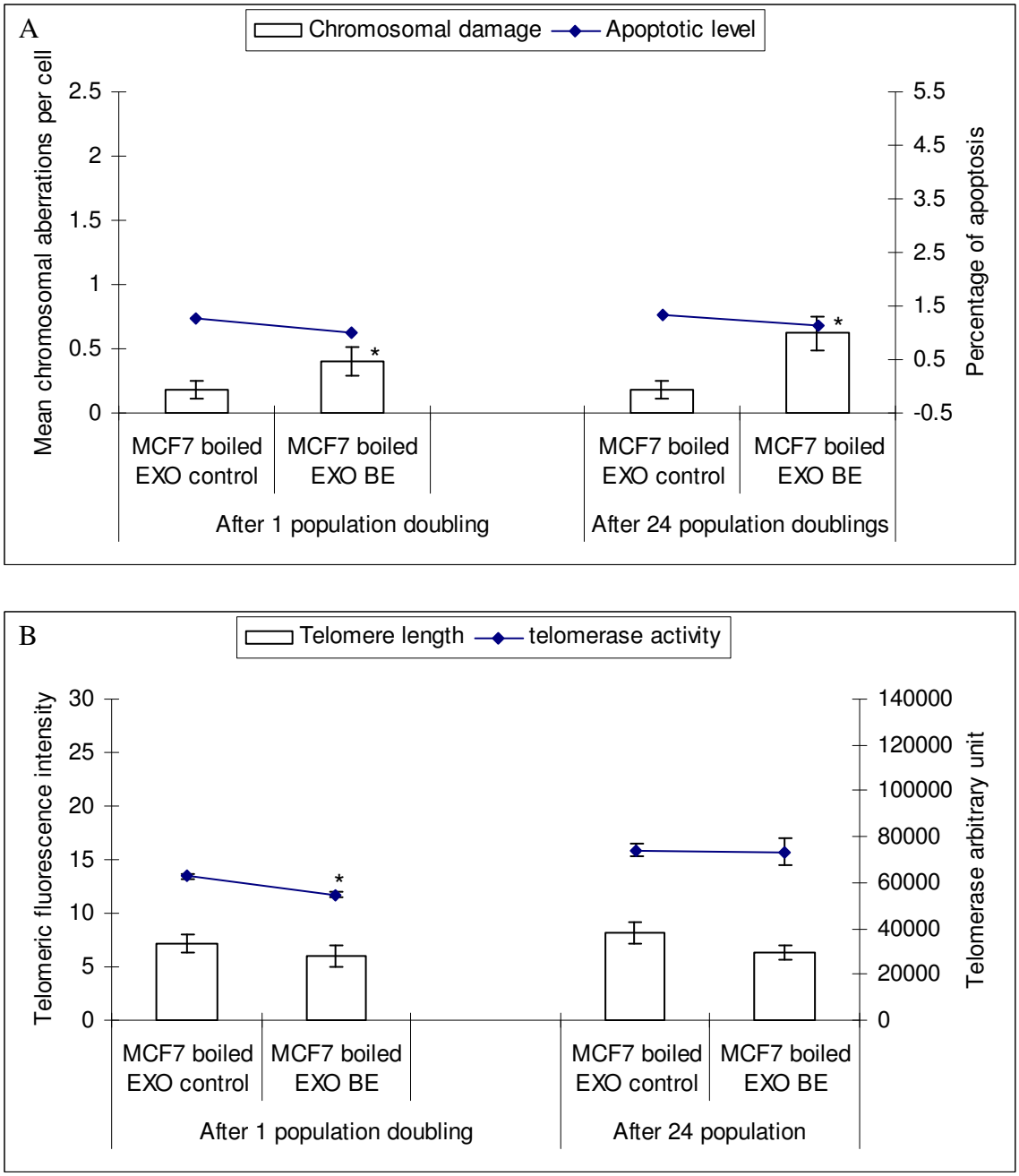


Figure 5.11: Initial and delayed cellular response within MCF7 cells treated with boiled exosomes of irradiated MCF7 cells.

Exosomes were extracted from the CCCM and ICCM of MCF7 cells and separately boiled at 98°C for 10 minutes to denature and inhibit the exosomes' proteins. The boiled exosomes were added to fresh un-irradiated MCF7 cells in order to investigate the role of exosomes' protein in bystander effect induction. Panel A illustrate chromosomal damage and apoptotic levels after 1 and 24 generations following treatment. The boiled EXO BE cells showed significant initial chromosomal damage (*$p \leq 0.05$) compared to the control. However, the induction of chromosomal damage within these control cells was lower than the exosome BE cells suggesting that exosomes' protein inhibition could reduce bystander chromosomal damage. The boiled EXO BE cells exhibited a delayed chromosomal damage (*$p \leq 0.05$) compared to the control, although they did not demonstrate significant early and late induction of apoptosis, an observation similar to other bystander groups. Panel B represent telomere length and telomerase activity data. The MCF7 boiled EXO BE cells showed initial and delayed telomeric shortening although statistically insignificant. The results suggest that exosome, protein inhibition could reduce the exosome BE. Telomerase activity reduction was significantly observed in the MCF7 boiled EXO BE cells after 1 generation following boiled exosome transfer. Nevertheless, the telomerase activity returned to normal levels after 24 generations. These findings suggest that exosomes' protein molecules could partly contribute to induce BE following X-ray irradiation within MCF7 cells.

Experiment was performed in 3 technical repeats.

5.4.2.iii Exosome RNA and protein molecules inhibition abrogates non-targeted effects of IR

To investigate whether RNAs and proteins of exosomes both associate with the induction of non-targeted effects of IR, exosome pellets of ICCM were treated with RNase A to digest exosome RNAs, boiled to denature the proteins of exosomes prior to being added to fresh un-irradiated MCF7 cells (MCF boiled EXO-RNase BE). In parallel a control group was established for comparison. Cells were analysed for chromosomal damage measurement, apoptotic analysis, telomere length estimation and telomerase activity measurement after 1 and 24 population doublings.

Interestingly, the MCF7 boiled EXO-RNase BE cells did not show significant initial (after1 population doubling) or delayed (after 24 population doubling) any cellular damage responses (chromosomal damage, apoptosis, telomere shortening, and telomerase activity reduction) compared to the controls (Figures 5.12 A and B). These results thereby suggest that both RNA and protein molecules of exosomes work together in a synergistic manner to infer the non-targeted effects of IR. We realise that the exosomes endured a long preparation time (3 hours) and that the short-lived bystander signals from ICCM would subsequently have been lost; our data thus suggests that exosomes mediated non-targeted effects of IR through long-lived signals.

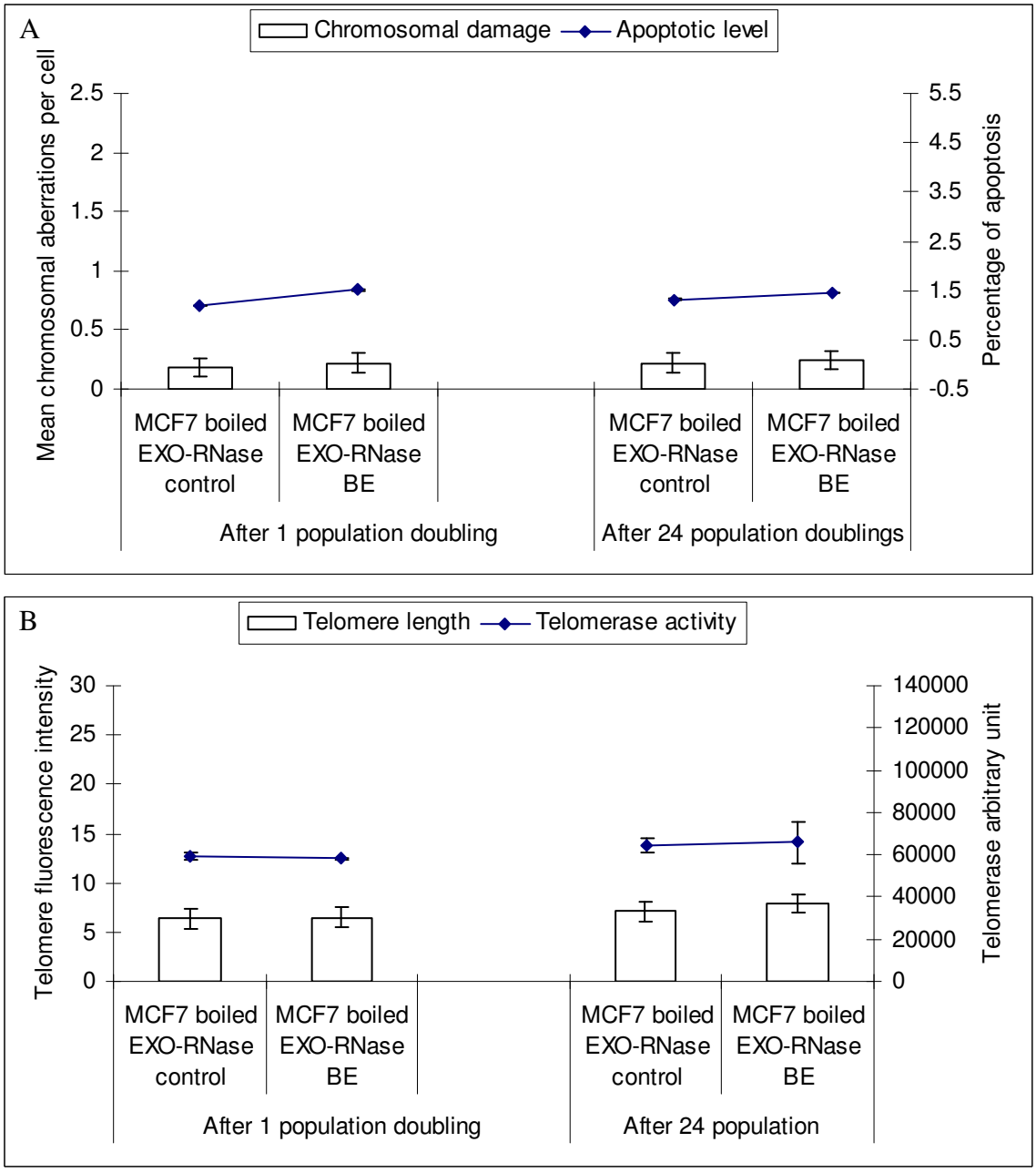


Figure 5.12: Early and late cellular responses within irradiated MCF7 cells following exosome RNA/ protein inhibition treatment.

Exosomes were purified from CCCM and ICCM of MCF7 cells. The exosomes' pellets were treated with RNase to digest the RNA molecule, they were then boiled to denature and inhibit the exosomes' proteins prior to being added to fresh un-irradiated MCF7 cells to induce free RNAs/proteins exosomes BE. Panel A show the early and delayed responses of chromosomal damage and apoptotic induction. Interestingly, the cells that were treated with RNase treated/boiled exosomes did not show a significant induction of initial and delayed chromosomal damage or apoptotic responses compared to the corresponding controls. Moreover, these cells did not demonstrate a significant reduction in the telomere length or telomerase activity at either time-point. Consequently, data suggest that both exosome RNA and proteins are required to induce BE in the MCF7 cells following 2 Gy X-ray irradiation.

Experiment was performed in 3 technical repeats.

5.4.3 Exosomes of irradiated tumour cells mediate non-targeted effects of IR within non-tumour cells following a radiotherapy dose of X-ray (part 2)

It was shown that irradiated MCF7 (tumour) cells can induce BE in the HMT (non-tumour) cells following 2 Gy X-ray (Section 4.3.2). We have also shown that exosomes from irradiated MCF7 cells are involved in the induction of BE in the MCF7 cells (Section 5.4.1.i). We were therefore interested to investigate if these exosomes would facilitate communication between MCF7 and HMT cells following 2 Gy X-ray. Therefore, experiments were set up (Section 5.3.2, part 2, experiment 2) which enabled exosomes of ICCM and CCCM of 2 Gy and direct sham-irradiated MCF7 to be collected, purified and added to un-irradiated HMT cells to induce exosome BE.

The results demonstrated that this indeed was the case, thus, in brief, after 1 population doubling, chromosomal damage was significantly observed ($p \leq 0.0001$) in the bystander HMT cells (HMT ICCM) following ICCM media transfer compared to the control (Figure 5.13 A). Nevertheless, these cells did not show an early induction of apoptosis (Figure 5.13 A). However, their telomere length was significantly shortened ($p \leq 005$) as shown in figure 5.13 B; moreover telomerase activity within these bystander cells was significantly reduced ($p \leq 00001$) compared to the control (Figure 5.13 B).

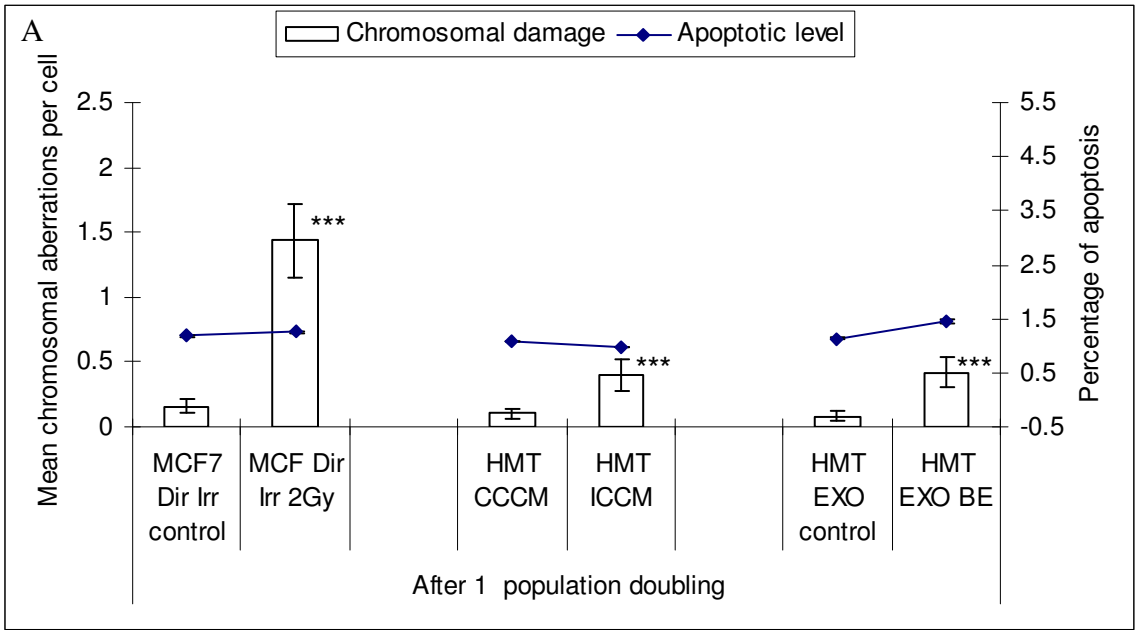
After 24 generations, the progeny of the HMT ICCM cells demonstrated a high induction of chromosomal instability ($p \leq 0.0001$) and no significant levels of apoptosis (Figure 5.13 C). Additionally, although the telomerase activity returned to normal levels within this progeny there was significant telomere instability ($p \leq 0.05$, figure 5.13 D), which could instigate chromosomal instability.

The ICCM exosomes' pellet of the 2 Gy direct irradiated MCF7 cells was also shown to cause initial chromosomal damage ($p \leq 0.0001$) within HMT EXO BE cells compared to the HMT EXO control cell; however, there was no significant induction of apoptosis (Figure 5.13 A). In addition, telomeric shortening ($p \leq 0.01$) and telomerase activity reduction ($p \leq 0.005$) were also significantly demonstrated in these cells compared to the control (Figure 5.13 B). In summary, all these results gave similar bystander cellular responses as those seen in the HMT ICCM (bystander cells) and thus we can surmise that exosomes of 2 Gy direct irradiated MCF cells initiate the underlying bystander signals.

Similarly after 24 generations, the progeny of HMT EXO BE cells demonstrated comparable results to those of the HMT ICCM (bystander) cells i.e. chromosomal instability was significantly detected ($p \leq 0.0001$) compared to the control (Figure 5.13 C). Moreover, these cells did not exhibit a significant induction of apoptosis. However, telomeric shortening was significantly observed whilst telomerase activity returned to normal levels (Figure 5.13 D), thus suggesting telomerase activity maintained cell proliferation, although the presence of short telomeres and absence of apoptosis led to chromosomal instability.

To confirm that BE and GI could be mediated by exosome RNAs and proteins from the irradiated tumour (MCF7) cells, the exosome pellets of the 0 and 2 Gy cells respectively, were treated with RNase for 30 minutes at 37°C in order to digest exosomes' RNAs. The pellets were then boiled at 98°C for 10 minutes to denature the exosomes' proteins and stop their activities and finally they were transferred to un-irradiated HMT cells (HMT boiled EXO-RNase control and HMT boiled EXO-RNase BE) cells respectively.

The findings showed that inactivation of exosome RNA and protein molecules abolished the non-targeted bystander effects of 2 Gy X-ray i.e. direct irradiated MCF7 cells were unable to induce damage in the bystander (HMT) cells (Figures 5.14 A and B) suggesting that exosomes have crucial roles in the non-targeted effects of IR, specifically long-lived signals, as the exosome inactivation process took a long time (3 hours).



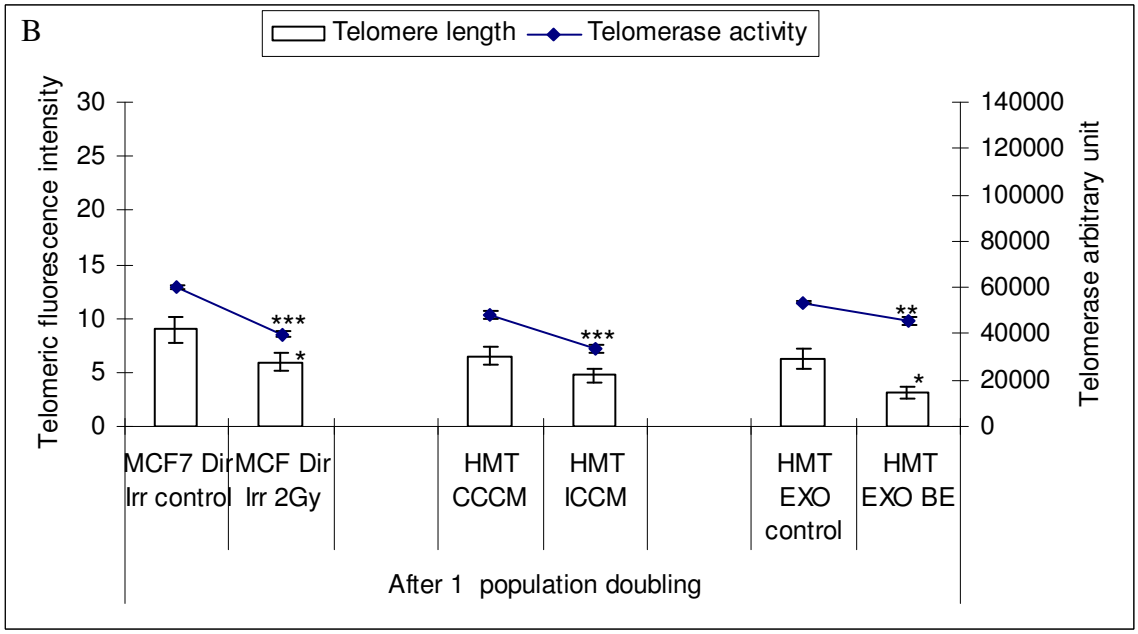
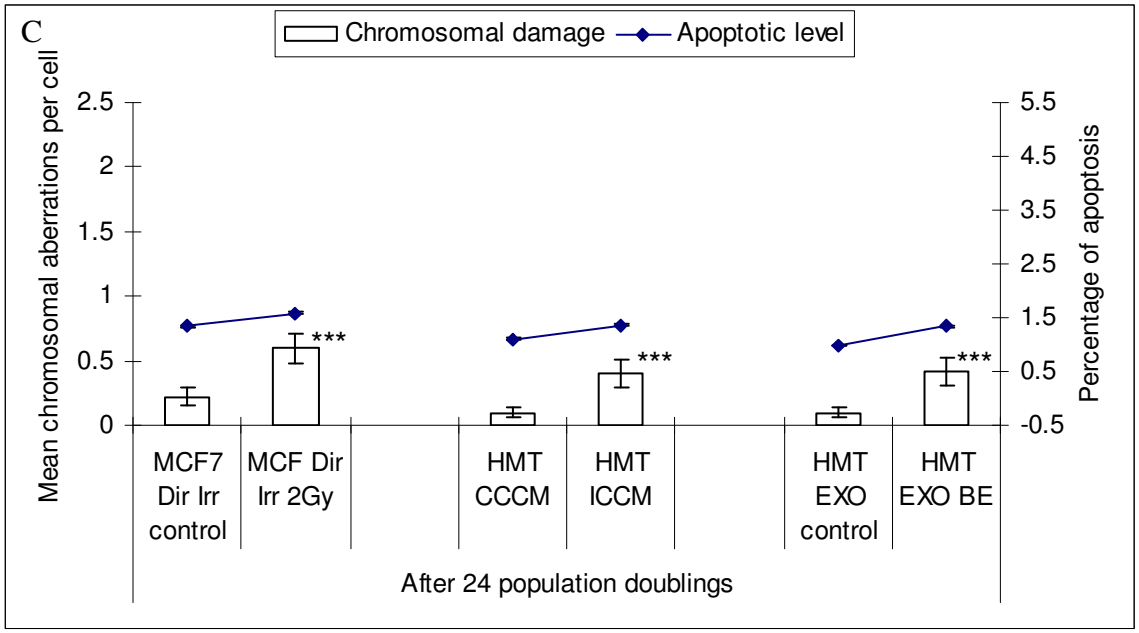


Figure 5.13: Early and late cellular responses within direct irradiated MCF7 cells, bystander and exosome bystander HMT cells following 2 Gy X-ray irradiation.

MCF7 cells were irradiated with 2 Gy X-ray irradiation. The CCCM and ICCM were transferred to un-irradiated HMT cells to induce BE (HMT CCCM and ICCM). The exosomes were extracted from CCCM and ICCM and placed onto un-irradiated HMT cells to induce exosomes-BE (HMT EXO control and BE). Cells were analysed after 1 and 24 population doublings to asses early and late damage responses. Panel A represent chromosomal data and apoptotic levels within the cells after 1 population doubling. Chromosomal damage was significantly induced (***$p \leq 0.0001$) within the direct irradiated MCF7, bystander and exosome bystander HMT cells following irradiation and treatment compared to the controls. However, these groups did not show significant apoptotic induction. Panel B illustrate telomeric instability including telomere length and telomerase activity in the irradiated MCF7, bystander and exosome bystander HMT cells after 1 population doubling. The irradiated cells exhibited a significant telomeric shortening (*$p \leq 0.05$) following irradiation compared to the control. The HMT ICCM cells demonstrated short telomeres following bystander induction; nevertheless, the telomeric shortening of these bystander cells was statistically insignificant compared to the control. However, HMT EXO BE cells revealed a significant initial telomeric shortening compared to the control. Moreover, direct irradiated MCF7, ICCM and EXO BE HMT cells demonstrated a significant reduction of telomerase activity (***$p \leq 0.0001$; **$p \leq 0.005$) compared to the controls. Thus data showed that exosomes pellet of irradiated tumour MCF7 cells had the similar effect of MCF7 ICCM on non-tumour HMT bystander cells underlying BE induction i.e. exosomes of tumour cells could play crucial roles in BE within non-tumour cells following radiotherapy dose (2 Gy X-ray). Experiment was performed in 3 technical repeats.



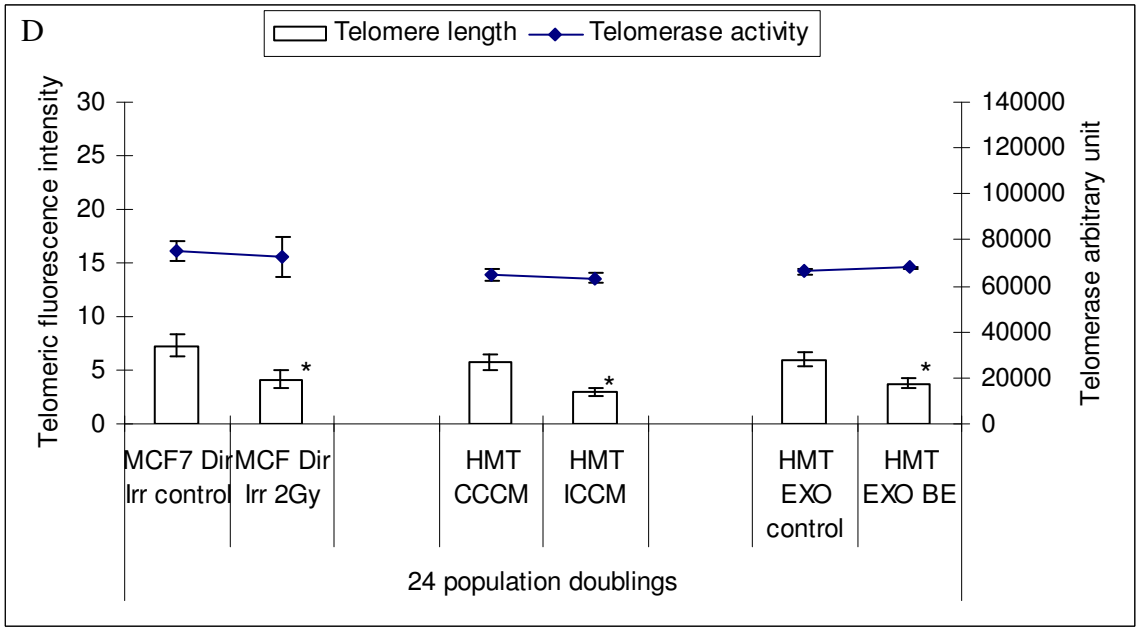
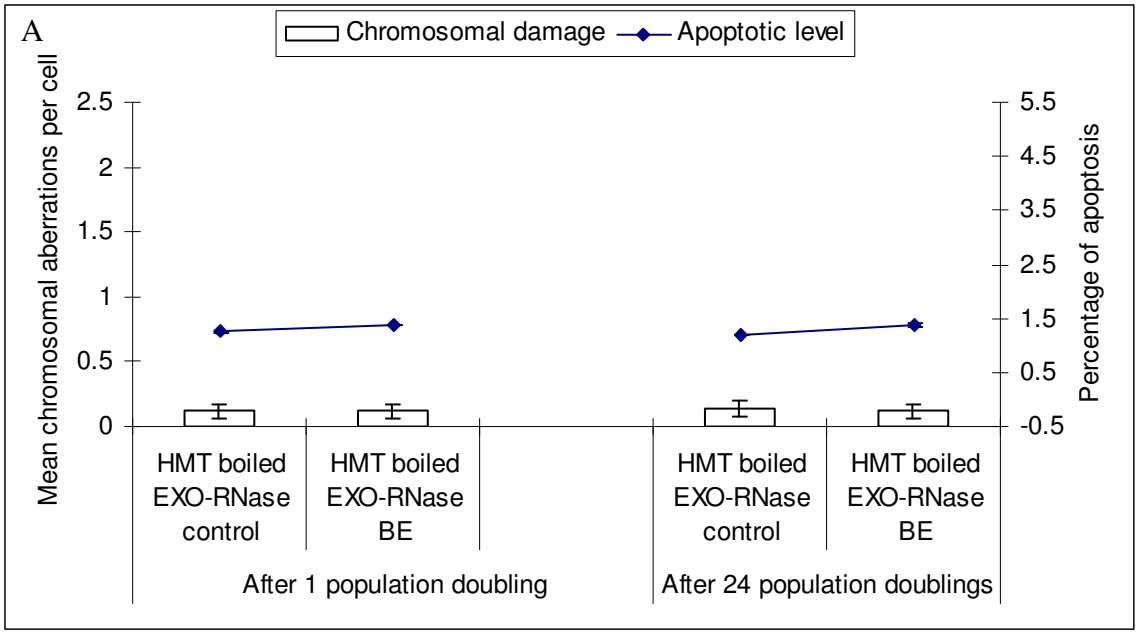


Figure 5.13:

Panel C represent the delayed chromosomal damage and apoptotic levels within the progeny of direct irradiated MCF7, bystander and exosomes bystander HMT cells after 24 population doublings. The chromosome aberrations were significantly induced (***p≤0.0001) within the progeny of direct irradiated MCF7, bystander and exosomes bystander HMT cells. However, there was no significant induction in apoptosis. These results suggest there was an inverse relationship between apoptosis and chromosomal instability.
Panel D illustrate telomere length and telomerase activity within the progeny of direct irradiated MCF7, bystander and exosomes bystander HMT cells after 24 generations. Although telomerase activity returned to normal levels, the progeny showed significant telomeric shortening (*p≤0.05). These results suggest that the telomerase activity could maintain the cells proliferation even with short telomeres, which could lead to chromosomal instability especially with lack of apoptosis. Moreover, data suggest that exosomes could mediate GI following 2 Gy X-ray irradiation.
Experiment was performed in 3 technical repeats.



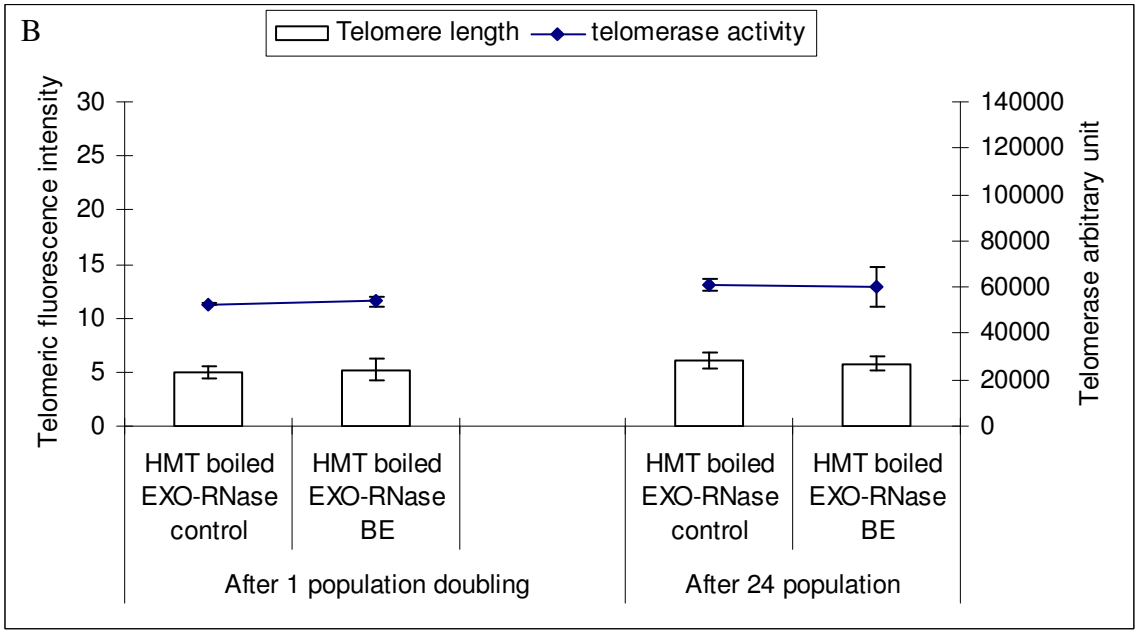


Figure 5.14: Effects of exosomes of ICCM from MCF7 cells on HMT cells following exosomes' RNA/ protein inhibition.

In order to confirm the role of irradiated MCF7 exosomes in BE induction of HMT cells, exosomes from CCCM and ICCM of MCF7 cells were purified and treated with RNase to digest the exosomes' RNA molecules. They were then boiled to denature and inhibit the exosomes' proteins producing RNAs-free and protein-free exosomes, which were added to fresh un-irradiated HMT cells. Panel A illustrate the early and delayed responses of chromosomal damage and apoptotic induction. Interestingly, the cells that were treated with RNase treated/boiled exosomes did not show a significant induction of initial and delayed chromosomal damage or apoptotic responses compared to the corresponding controls. Moreover, these cells did not demonstrate a significant reduction in the telomere length or telomerase activity. Thus the data suggest that inhibition of irradiated MCF7 exosomes could abrogate the non-targeted effect responses within non-tumour HMT cells.
Experiment was performed in 3 technical repeats.

5.5 Discussion and conclusions

In order to investigate the signals that mediate BE, it was first necessary to prove that radiation could cause direct cellular responses and BE within MCF7 cells following 2 Gy X-ray irradiation. Our data successfully demonstrated this phenomenon at both early and late time-points (Chapter 3 and 4 respectively). It is has been well established that transfer of irradiated cell conditioned media (ICCM) induces BE in un-irradiated cells (Belloni *et al*., 2011, Herok *et al*., 2010, Bowler *et al*., 2006). Therefore, in our study, a media transfer technique was utilised to induce BE and also to induce exosome-mediated BE following irradiation, i.e. ICCM was collected and then transferred to fresh un-irradiated cells following exosome extraction. Therefore, in summary, all the bystander cell populations (bystander and exosome-bystander cells) were treated under the same conditions.

The findings showed that exosomes from the ICCM of 2 Gy direct irradiated MC7 cells induced early and late DNA damage ($p \leq 0.0001$) within the un-irradiated MCF7 cells. However, a similar effect was not observed following media transfer i.e. exosome-free ICCM of 2 Gy direct irradiated MCF7 cells transferred onto un-irradiated MCF7 cells (Figures 5.5 B and 5.6 B), however, these cells did exhibit similar initial and delayed cellular responses as the bystander cells, implying that non-target effects of IR could be mediated by exosomes. Other data also suggest cell-cell communications by exosomes are the means by which bystander signals are delivered to recipient bystander cells (Porto-Carreiro *et al*., 2005). Denzer and other workers have proposed that exosomes fuse with the plasma membrane of the target cells thus enabling delivery of the exosomes' cargo into recipient cells (Denzer *et al*., 2000b). Furthermore, it is known that exosomes frequently transfer miRNAs between dendritic cells (Montecalvo *et al*., 2012).

Recent evidence has shown that exosomes contain RNA and protein molecules (da Silveira *et al*., 2012, Lasser *et al*., 2012), which can play crucial roles in the mechanism of the non-targeted effects of IR. Dickey *et al*. have reported that miRNA has an indirect role in BE induction; they suggest that miRNA can be considered as a non-primary bystander signal leading to DNA double strand breaks (Dickey *et al*., 2011). Additional studies have shown that miRNA can play an important role in the manifestation of delayed BE by affecting DNA methylation and apoptosis through changes in the expression of BCL-2 (Kovalchuk *et al*., 2011, Koturbash *et al*., 2007). Whilst, McCabe and others have also reported that miRNA can associate with DNA methylation (McCabe *et al*., 2009), which can lead to GI (Zeimet *et al*., 2011). Moreover, it has been shown that GI in the parental germline can be caused by miRNA and DNA methylation, inducing GI within the progeny (Filkowski *et al*., 2011). Much evidence has confirmed that exosomes of colon cancer epithelial cells can transfer a cytokine-like pro-inflammatory protein, which may increase inflammation between cells (LiuLiu *et al*., 2006). Furthermore, cytokines, such as TNF-α, are localised within exosomes of fibroblast cells (Liu *et al*., 2010, Johnson *et al*., 1975). All these results therefore suggest that exosome proteins could mediate non-targeted effects of IR; however, the exact mechanism needs to be quantified for more accurate results.

In our study's data, we were able to demonstrate the ability of the progeny exosomes of direct irradiated, bystander and exosomes bystander cells to induce DNA damage in un-irradiated MCF7 recipient cells underlying the delayed damage response following irradiation (Figure 5.7). In general, the results of the first part of our study (Section 5.4.1) suggest that irradiated cells released exosomes molecules into the microenvironment (the media) consequently inducing BE responses in the un-

irradiated cells through one and/or two possible mechanisms. The first mechanism could involve inflammation, possibly mediated by the exosomes proteins, and the second mechanism could involve exosome RNAs (miRNAs). Moreover, bystander cells and the progeny of direct irradiated and bystander cell populations also showed the ability to secret exosomes into the microenvironment, which could contribute to the delayed responses (GI) as illustrated in figure 5.15.

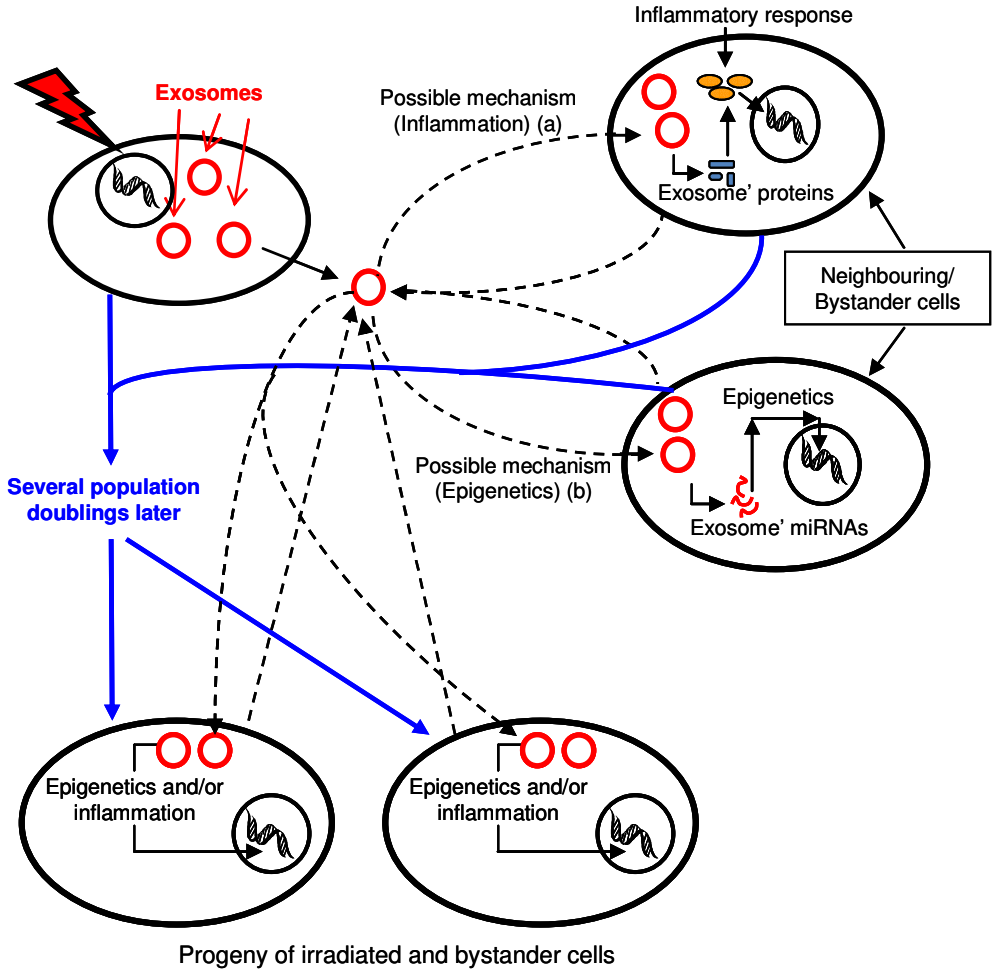


Figure 5.15: Scheme of possible mechanisms of exosome-mediated non-targeted effects.

Irradiated cells release exosomes into the microenvironment providing two possible mechanisms of their involvement in inducing BE in the un-irradiated cells, either through (a) exosomes' protein molecules, which can lead to inflammation and chromosomal damage and (b) through epigenetics, which could be mediated by exosomes' RNA molecules. Delayed damage could be mediated by one or both of these mechanisms together. The progeny of irradiated and bystander cells could release exosomes, which could affect the progeny and increase delayed responses.

To confirm the existence of two possible mechanisms of exosome-mediated non-targeted effects of IR, we set up experiments were the ICCM exosomes' pellet was

divided into 4 fractions (Section 5.3.2). The first fraction was directly added to fresh cells to induce exosome BE; as we believed exosome RNA and protein molecules contribute to induce BE. The second fraction was treated with RNase thus producing RNA-free exosomes, to investigate the role exosome RNAs in the induction of non-targeted effects of IR. The third exosome pellet fraction was boiled at 98°C for 10 minutes to denature and inhibit the exosomes' protein molecules, to allow investigation of the role of exosomes' proteins in the non-targeted effects of IR. However, because non-targeted effects of IR could be abrogated by inhibiting both RNAs and proteins of exosomes, the fourth exosome pellet fraction was treated with RNase A for 30 minutes at 37ºC and then boiled at 98°C for 10 minutes, resulting in both the exosome RNAs and proteins inhibition.

As shown in section 5.4.2.ii, treatment of exosome pellet with RNase significantly abrogated its ability to induce bystander chromosomal damage and apoptotic induction after 1 population doubling (Figure 5.10 A), suggesting that the initial signals mediating BE include a RNA molecule. These cells exhibited an elevation in chromosomal damage; nevertheless this elevation was statistically insignificant. Moreover, MCF7 exosome cells that were treated with RNase did not show a significant reduction in telomere length and telomerase activity at the same time-point compared to their controls (Figure 5.10 B). However, telomerase activity levels within MCF EXO-RNase BE group and their control were lower than MCF7 direct control, MCF7 CCCM and MCF7 exosome control (Figure 5.9 B). These findings suggest that RNase A digested telomerase RNA (TR or TERC, telomerase template) causing telomerase dysfunction. Surprisingly, the progeny of MCF7 EXO-RNase cells demonstrated a high induction of chromosomal damage ($p \leq 0.0001$) after 24 generations compared to the control (Figure 5.10 A). Our data suggest that exosome

RNA molecules are partly involved in the signals of non-targeted effects of IR. Furthermore, the progeny were shown to exhibit short telomeres although statistically insignificant (Figure 5.10 A) and normal levels of telomerase activity. These findings suggested that RNase A did not completely abolish BE and GI within MCF7 cells following 2 Gy X-ray irradiation, implying that exosome RNAs could be in part, involved in the induction of non-targeted effects of IR in the MCF7 cells following 2 Gy X-ray. Other results from our investigations additionally suggest that signals mediating BE and GI were not solely due to RNA molecules but that exosome proteins played a role since when the ICCM containing exosomes were boiled causing their subsequent inhibition, the level of initial chromosomal damage in the MCF7 cells (MCF7 boiled EXO BE, figure 5.11 A) was significantly diminished ($p \leq 0.05$) compared to the MCF7 that had not been boiled (MCF7 EXO BE, figure 5.9). However, early chromosomal damage was significantly observed ($p \leq 0.05$) in the MCF7 boiled EXO BE cells compared to the corresponding control. Moreover, these cells demonstrated a high induction of delayed chromosomal damage ($p \leq 0.05$) after 24 population doublings (Figure 5.11 A). MCF7 boiled EXO BE cells also showed a lower reduction in telomere length and telomerase activity (Figure 5.11 B) compared to MCF7 EXO BE cells (Figure 5.9 B) after 1 population doubling. These results are similar to those observed by the MCF7 boiled EXO BE group suggesting that the exosome proteins were partly involved in mechanisms of non-targeted effects of IR.

Thus in summary, both RNA and protein molecules of ICCM exosomes were shown to play a crucial role in the induction of non-targeted effects of IR. This was confirmed by the results of the bystander cells treated with RNase and boiled (MCF7 boiled EXO-RNase BE), where no significant early or late cellular responses following treatment (Figures 5.12 A and B) were observed; thus implying that

inhibition of RNA and proteins molecules of ICCM exosomes abrogated the consequences of BE and GI in the bystander MCF7 cells following 2 Gy X-ray.

Our investigations also found that communication between irradiated tumour MCF7 and non-tumour HMT cells following radiotherapy dose (2 Gy X-ray) could be also mediated by exosomes. Our data showed that HMT cells, treated with ICCM exosomes of 2 Gy direct irradiated MCF7 cells, exhibited similar responses to HMT cells that were treated with ICCM (Figures 5.13 A, B, C and D). These findings suggest that exosomes were involved in the signals mediating BE and GI in the HMT cells following irradiated MCF7-bystander HMT cell communication. Moreover, inhibition of exosomes molecules (RNAs and proteins) abrogated the induction of BE and GI in HMT cells following irradiated MCF7-bystander HMT cell communication (Figure 5.14 A and B). These results could have potential consequences in the genesis of secondary cancer following radiotherapy.

Due to the duration of exosome preparation (almost 3 hours), our findings suggest that exosomes are frequently associated with the long-lived signals of non-targeted effects of IR. However, for more robust data, exosomes and their molecules would need to be further quantified.

Conclusion:

1. Exosomes are nanovesicles (40-140nm), which are secreted by sham-irradiated/control and 2 Gy direct irradiated MCF7 cells into the microenvironment (media). Exosomes of sham-irradiated cells are morphologically similar to the exosomes of 2 Gy irradiated cells.

2. Both sham-irradiated and 2 Gy direct irradiated MCF7 cell exosomes can communicate with un-irradiated bystander cells. However, exosomes of 2 Gy irradiated cells only can cause BE.

3. Bystander cells are also able to release exosomes, which can induce cellular damage. Moreover, the progeny of irradiated and bystander cells showed the ability to secret exosomes which were able to induce cellular damage in fresh un-irradiated cells underlying delayed damage response. It is this mechanism that is most likely for the propagation of GI.

4. Exosome RNA and protein molecules play crucial roles in the mechanism of non-targeted effects of IR. Inhibition of exosome RNAs and proteins, can abrogate the induction of BE and GI in cells.

5. Exosomes can mediate the non-targeted effects of IR in the non-tumour HMT cells following cell communication with 2 Gy irradiated MCF7 cells.

6. Exosomes associate with the long-lived signals of the non-targeted effects of IR. However, further studies are required to assess whether exosomes are also involved in short-lived signals of BE. Further investigations are also needed to enable quantification of the exosomes that are released following irradiation.

CHAPTER 6

DISCUSSION

6

Chapter 6: Discussion

Our understanding of radiation effects is evolving from a mechanism driven exclusively by direct damage to DNA, to one where secondarily induced DNA damage and instability, as well as intra and particularly inter-cellular communication become integral components. The development of ideas around non-targeted effects has highlighted the important role of intercellular signalling in the development of bystander effects and the triggering of genomic instability (GI). Key understanding of these effects has derived from several advances in the field of radiation biology research. These include numerous *in vitro* and *in vivo* studies that indicate in addition to the targeted effects/damage response induced directly in cells by irradiation, a variety of non-targeted effects may make important contributions to determining the overall outcome after radiation exposure. Research studies in this field were reviewed in chapter one. However, the non-targeted effects response is not universally expressed, and this could be due to several factors including: cell/tissue types, radiation quality and dose as well as genetic predisposition factors (Kadhim *et al*., 2004, Kadhim, 2003). Also, studies of the non-targeted responses of radiation suffer from a gap in our understanding of the likely mechanisms associated with non-DNA targeted effects, particularly with respect to human health consequences at low and intermediate doses of ionizing radiation such as those used for radiation therapy. In addition, other outstanding questions that need to be addressed include: the direct/indirect crossmechanistic links between the different non-targeted responses, and if the observed variation in non-targeted response between individuals and cell lines are linked to genetic background or epigenetic effects. Furthermore, whilst the initial target and early interactions in cells that give rise to non-targeted responses in neighbouring or descendant cells are still unknown, numerous studies point towards an epigenetic mechanism.

This study was set up to address some of the above questions and provide answers to the conflicting views and reports of the implication of the non-targeted effects for health risk and therapy, especially the question of whether BE is beneficial or detrimental. Therefore this study was set up to:

1- Investigate whether the bystander effect is potentially detrimental or beneficial in normal versus tumour cells. Linked to this, the mechanistic relationship between bystander effects and the GI response in normal and tumour cells will be explored.

2. Achieve an increased understanding of the mechanistic link between radiation-induced genomic instability in irradiated and un-irradiated bystander cells including the molecular signalling involved.

As described in the introduction, radiation-induced bystander effect (BE) is the biological effect observed in un-irradiated cells that received signals from nearby irradiated cells (Nagasawa and Little, 1992). Furthermore, it has been well documented that BE frequently induce chromosomal damage (Lorimore *et al*., 2005), micronuclei (Kashino et al., 2007b), mutations, apoptosis (Hamada *et al*., 2008) and GI within the progeny of bystander cells (Bowler *et al*., 2006). Thus, BE could be considered as harmful or beneficial depending on the consequences of the biological effects. Therefore, this study first aimed to investigate the beneficial BE consequences by using different cell communications between tumour MCF7 and non-tumour HMT cells. The cell communication included irradiated MCF7-bystander HMT, irradiated MCF7-bystander MCF7, irradiated HMT-bystander MCF7 and irradiated HMT-bystander HMT cells following low (0.1 Gy) and high (2 Gy) doses of X-ray irradiation; mimicking the effects of diagnostic (0.1 Gy) and radiotherapy doses (2 Gy) on the human body respectively. A full body CT scan emits 0.1 Gy X-ray (BER, 2010), additionally this dose has been established as a fractionated dose to a high

therapeutic dose of radiation in cancer treatment. Moreover, it is known that normal cells, in the track of IR beam during radiotherapy, can receive a low dose of IR (Joiner, 1987). In contrast, 2 Gy X-ray was chosen to investigate the effects of bystander signals secreted from tumour cells to the normal surrounding cells following equivalent radiotherapy doses and also to mimic conditions of the normal cells, which adhere or are in vicinity of cancer cells, which thus frequently receive high doses of IR during radiotherapy. Our investigations could be potentially considered as important issues in the radiotherapy process.

Chromosomal damage, apoptotic induction and telomeric instability, including telomere length and telomerase activity measurement, were measured as biological end-points, and these responses are strongly correlated. Telomeric instability can instigate chromosomal instability (Berardinelli *et al*., 2011), and short telomeres frequently cause apoptosis (Merle *et al*., 2011). A study by Meznikova and co-authors showed telomerase dysfunction can lead to telomeric shortening (Meznikova *et al*., 2009) causing chromosome aberrations (Song *et al*., 2012). Moreover, a previous study by Kadhim and colleagues has suggested there is an inverse relationship between apoptosis and chromosomal instability, in which a high level of apoptosis can remove cells with high levels of chromosomal damage, consequently resulting in a decrease in chromosomal instability (Kadhim et al., 1995). Such mechanisms could contribute to the possibility of the beneficial effects of non-targeted effects of radiation, for example BE is considered beneficial if the bystander signals are able to induce apoptosis and multi-damage leading to auto-killing within bystander cancer cells (Abdelrazzak et al., 2011) but not in normal cells. On the other hand, detrimental consequences of bystander effects can cause more genomic instability in the progeny of bystander cancer or normal cells, which can lead to more aggressiveness in the

cancer cells or cause cancer in the normal tissue (Raynaud *et al*., 2008, Naruke *et al*., 2009).

The second aim of this study was to increase the understanding of the mechanism of non-targeted effects of IR underlying cell-cell communication signals. The study tested the hypothesis that exosomes of ICCM mediate the BE and GI, and that RNA and protein molecules play an important role in this process.

The experimental results of chapter 3 and 4 addressed the first aim where the direct irradiated and bystander responses in both MCF7 and HMT cells were discussed. The tumour MCF7 cells exhibited different responses from non-tumour HMT cells, i.e. HMT cells were shown to be more resistant to IR and bystander signals than MCF7 cells. The results showed that both direct irradiated and bystander MCF7 cells demonstrated early and late chromosomal damage and telomere shortening following 0.1 and 2 Gy X-ray. However, apoptosis and telomerase dysfunction were observed only at the 4 hours and 1 population doubling time-point respectively. Data suggest that both direct IR and/or bystander signals induced DNA damage within MCF7 cells. This DNA damage could lead to apoptosis and chromosomal aberration as early responses. Also IR and bystander signals could cause telomerase dysfunction, which could instigate telomere shortening in the MCF7 cells. The short telomeres frequently led to chromosomal aberration as early responses. MCF7 cells could produce high frequency of chromosomal instability during DNA misrepair. The results also showed that both direct irradiated and bystander MCF7 cells exhibited delayed short telomeres, which could cause chromosomal instability. Active telomerase could also maintain cell proliferation even with short telomeres. Therefore, BE within MCF7 cells could be considered detrimental, due to the high level of GI, which can lead to more cancer cell aggressiveness as shown in figure 6.1.

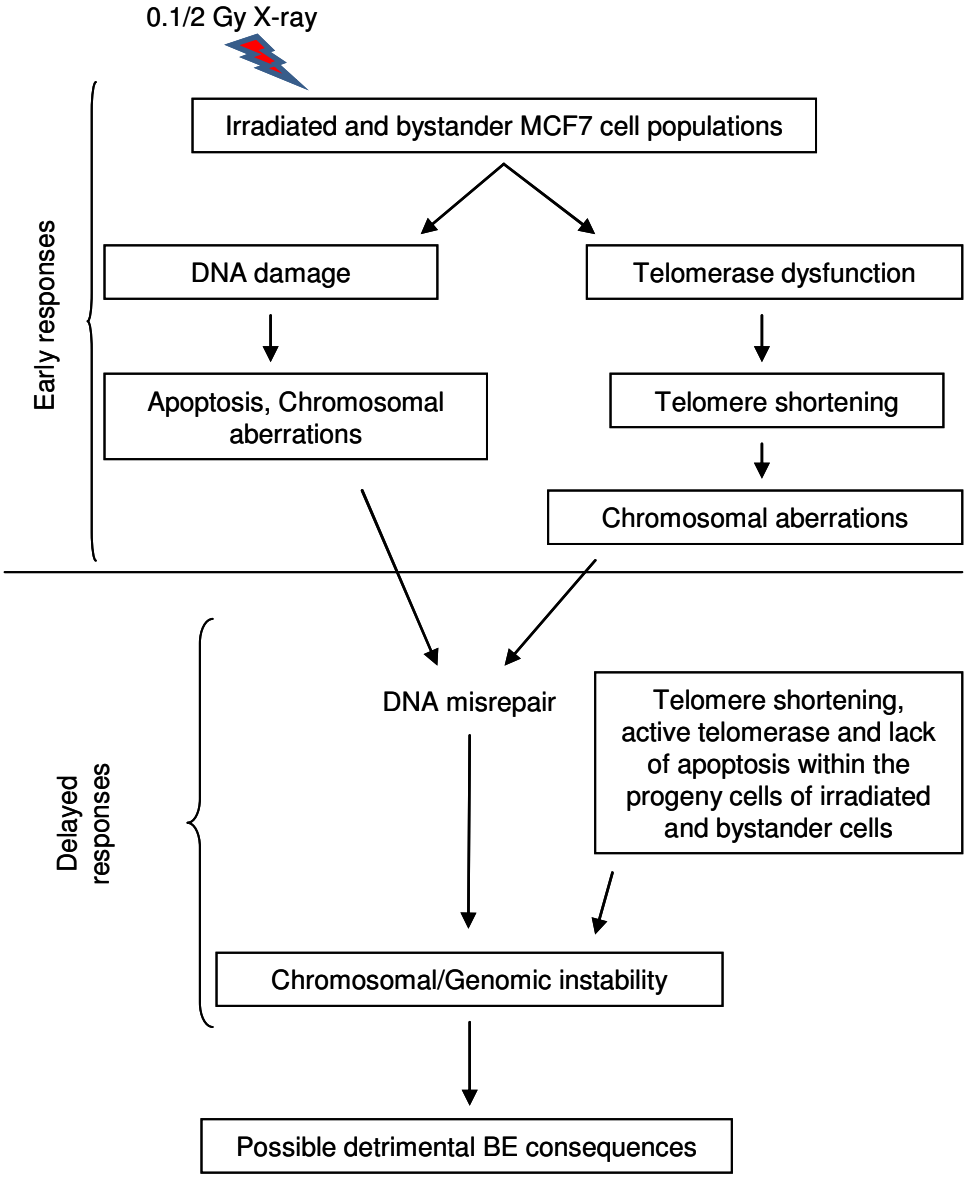


Figure 6.1: The possible cellular responses in the direct irradiated and bystander MCF7 cells following 0.1 and 2 Gy X-ray irradiation.

Whilst the cellular responses of HMT cells were shown to be similar to the MCF7 cells following 2 Gy X-ray in both direct irradiated and bystander cells, the 0.1Gy direct irradiated and bystander cells showed different responses, as shown in figure 6.2. The direct irradiated HMT cells demonstrated significant chromosomal damage after 1 population doubling following 0.1 Gy X-ray. The results suggest that IR induced DNA damage that caused initial apoptosis and chromosomal damage following 0.1 Gy direct radiation exposure. Moreover, the results showed early

telomerase dysfunction response, which could lead to telomere shortening as early responses in these cells. Interestingly these cells did not reveal chromosomal instability; however, a high level of apoptosis was observed after 12 population doublings (delayed response). This suggested that the high level of apoptosis eliminated the cells with high chromosomal damage, demonstrating an underlying mechanism for the inverse relationship between apoptosis and chromosomal instability, as shown in chapter 3 and 4. Although the 0.1 Gy direct irradiated HMT cells did not show chromosomal instability after 24 population doublings, there was a potential risk of chromosomal instability because of the short telomeres within these cells. Data suggest that at later generation doublings (more than 24 generations) these cells might express a significant level of chromosomal instability. The 0.1 Gy bystander HMT cells did not show significant chromosomal damage after 1 population doubling. However, these cells exhibited a significant induction of early apoptosis, telomere shortening and telomerase activity reduction. Moreover, these cells continued to show insignificant chromosomal instability and a significant apoptotic level and telomere shortening as late responses. However, telomerase activity returned to the normal level (See chapter 4). Similarly, due to the telomeric instability within these cells, there was a potential risk of chromosomal instability, which might be observed at later population doublings.

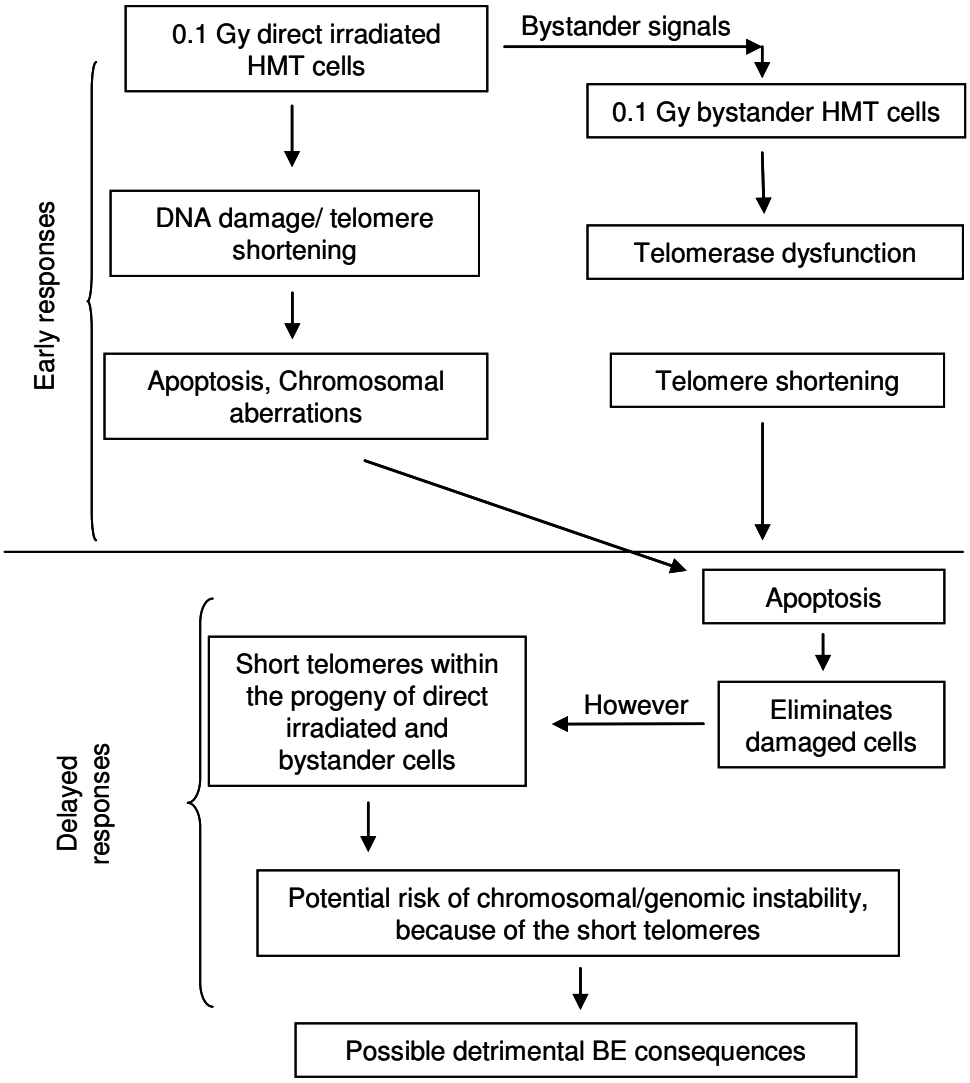


Figure 6.2: The possible cellular responses in the direct irradiated and bystander HMT cells following 0.1 Gy X-ray irradiation.

The mechanisms of BE and GI induction were not clear from our first experiments (Figure 6.2 - the direct irradiated and BE experiments). There were still outstanding questions: 1) How were the signals of non-targeted effects of IR released from the irradiated cells? 2) How were these signals received by un-irradiated cells? 3) Were these signals short or long-lived and were they transmissible from generation to generation causing damage and delayed response? 4) Could the progeny of the direct irradiated and bystander cells release different or similar signals, which could induce delayed damage responses? These important questions needed to be addressed in order to understand the molecular mechanisms of cell communications following

irradiation and bystander responses which could have potential application in radiotherapy, especially between direct irradiated tumour-bystander tumour and direct irradiated tumour-normal cell combination following 2 Gy (the radiotherapy dose). These issues/questions were investigated in chapter 5 from the work of exosome cell-cell communication signals following irradiation. Data showed that exosomes from ICCM of MCF7 cells induced early DNA damage in bystander MCF7 cells following 2 Gy X-ray irradiation. In contrast, the ICCM without exosomes could not cause significant damage within the bystander MCF7 cells (exosomes bystander cells). These results suggest that irradiated cells secrete exosomes and these acted as delivery vesicles instigating DNA damage within the bystander cells. The results also demonstrated that the progeny of bystander cells were able to release exosomes, which could mediate GI as discussed in chapter 5. Additional to the transmissible damage that can lead to GI (Glaviano *et al*., 2006), our data suggest that delayed damage responses could be caused by exosomes, in which RNA and protein molecules of exosomes play an important role in this process. The data also demonstrate that exosome RNAs and proteins of direct irradiated MCF7 cells were responsible for producing the initial and delayed cellular responses in the MCF7 and HMT cells. Inhibition of exosome RNA and protein molecules frequently abrogated the non-targeted effects of IR, as shown in chapter 5. Thus, the possible cellular responses observed in the 2 Gy direct irradiated MCF7 and bystander MCF7 and HMT cells could be due the exosomes. We propose that these exosomes, molecules/signals were received by bystander cells (MCF7 and HMT cells) causing cellular damage responses such as DNA damage and reduction in telomere lengths. Moreover, bystander cells released exosomes, which could increase cellular damage within the bystander un-irradiated cells. Additional to possible DNA misrepair, the

progeny of direct irradiated and bystander cells secreted exosomes that frequently increased cellular damage underlying the delayed damage responses as shown in figure 6.3.

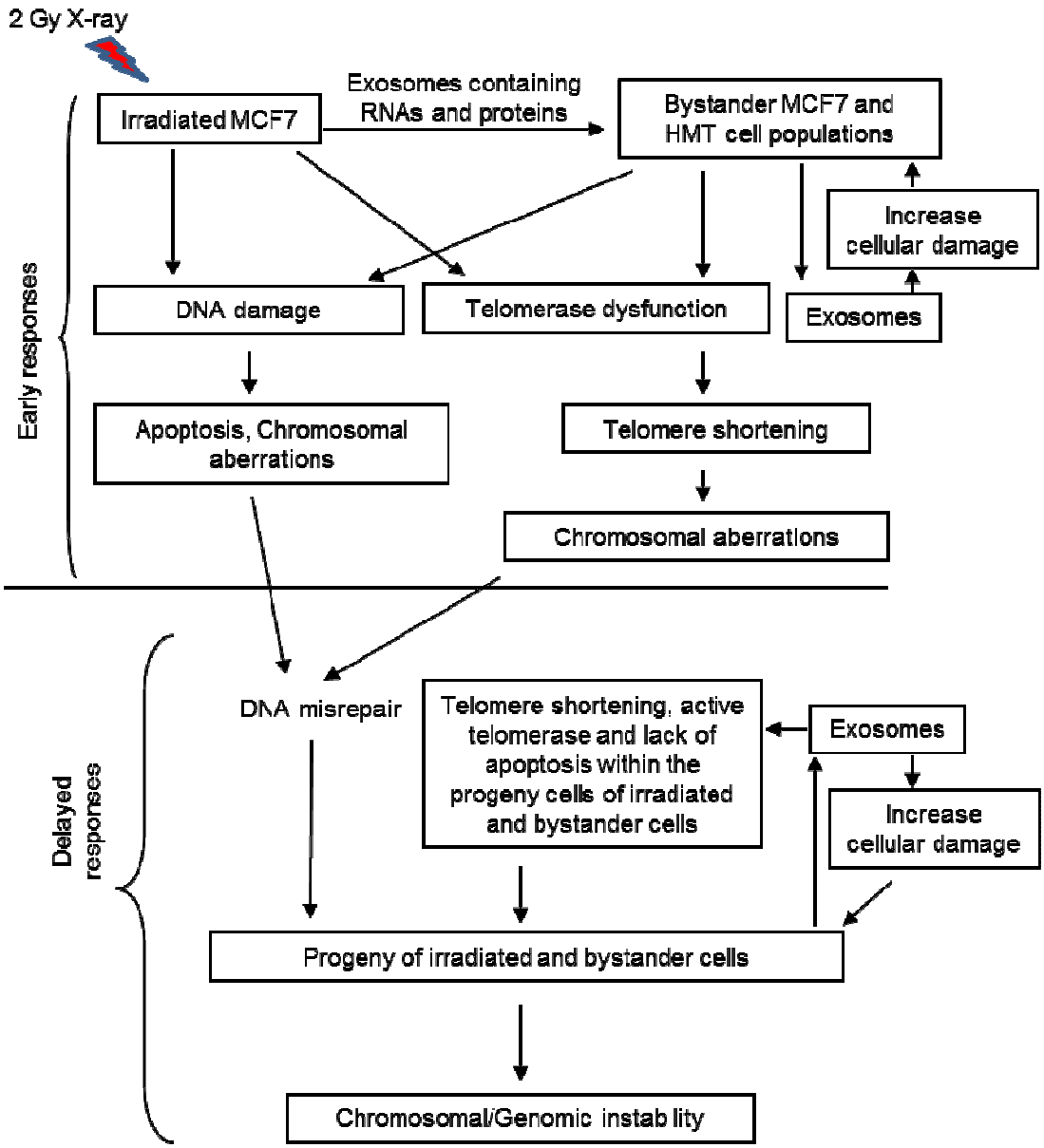


Figure 6.3: The possible cellular responses in the direct irradiated MCF7 and bystander MCF7 and HMT cells following 2 Gy X-ray irradiation.

Conclusion and future work

The findings concluded that BE most likely has detrimental consequences within the tumour (MCF7) and non-tumour (HMT-3522S1) breast epithelial cells following both low (0.1 Gy) and high (2 Gy) doses of X-ray irradiation, because of the induction of chromosomal and telomeric instability. These detrimental consequences are frequently mediated by exosomes that contain RNA and protein molecules. Inhibition of these molecules can abrogate BE and GI following radiotherapy dose, which can potentially have an application in the clinical radiotherapy.

These findings provide a window of opportunity for further investigations in to the exact characteristic of exosomes in samples by demonstrating the presence of specific exosomes' marker using for example immunofluorescence. In addition, quantifying exosomes is important in irradiated/bystander population vs. control and further functional tests to investigate the origin of the RNAs and proteins involved in mediating BE are also needed. Another potential important area for future study is to investigate how exosomes and their cargo (RNA and protein molecules) are inhibited?

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APPENDICES

Appendices

I. Activities arising from this work

1. Posters

- Radiation-induced chromosomal instability and intercellular communication: The role of radiation doses and genetic predisposition. Research student symposium, Oxford Brookes University, 2011.

- Exosomes' RNA and proteins can mediate Non-Targeted Effect of Ionizing Radiation. 5th International System Radiation Biology Workshop, Oxford, 2nd-5th Sptempter 2012

2. Oral presentation

- Radiation-induced bystander effect: detrimental or beneficial? ARR meeting (conference), University of Oxford, 28-30/06/2010.

- Do the bystander effects have beneficial consequences? United Kingdom Environmental Mutagen Society (ukems) and ARR meeting (conference), The University of Nottingham, 29-30/06-01/07/2011.

- Non-targeted effect of ionising radiation; possible mechanisms and implications. Research student forum, Oxford Brookes University, 23/02/2012.

- Possible role of Exosomes cargo in mediating Non-Targeted Effect of Ionizing Radiation. ARR meeting (conference), University of Brunel 25-28/06/2012