

THE ROLE OF RETROTRANSPOSITION AND EXOSOMAL DNA IN RADIATION- INDUCED GENOMIC INSTABILITY

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Abstract

Radiation-induced genomic instability (RIGI) is characterized by an increased rate of genomic alterations including delayed mutations, chromosomal aberrations, and micronucleus formation in the descendants of irradiated cells many generations after the initial radiation insult.

At present the mechanism of RIGI remains elusive, however it is possible that the random insertion nature of the LINE-1 retrotransposons could be a cause of RIGI. The work in this thesis has aimed investigate the role of retrotransposable elements in the mechanism of RIGI. Three different cell lines (HF19, Caco-2, and MCF7) were used to investigate if clinically relevant doses of 0.1 Gy and 2 Gy X-rays activated LINE-1 retrotransposition, and if this caused an instability within the genome. 48 hours post exposure to 0.1 Gy X-rays the HF19 fibroblast cell line showed an increase in LINE-1 levels, which correlated with an increase in micronuclei formation. While speculative, this is suggestive of an early activation of LINE-1 retrotransposition thereby indicating a possible link to radiation-induced damage.

Recent studies show an abundance of retrotransposon sequences within the DNA of extracellular vesicles (EVs). EVs, including exosomes, have become of keen interest due to their widespread implications in intercellular communication, and have been suggested as a likely means of transmission for retrotransposons between cells. Therefore, the work in this thesis also aimed to characterize the exosomes released from sham and irradiated MCF7 cells. Furthermore, we aimed to extract and characterize exosomal DNA using whole-genome sequencing to identify any copy number variation by comparing exosomes excreted from sham and 2 Gy irradiated MCF7 cells. The results herein suggest that exosomes excreted from MCF7 cells following a 2 Gy X-ray dose have a different composition and DNA contents compared to those excreted from sham-irradiated cells, including high levels of LINE-1 found in exoDNA in irradiated populations. Additionally, results from the copy number variation (CNV) analysis show a genetic variation between the sham and irradiated exoDNA samples.

In summary this study has contributed to the knowledge in the mechanism of RIGI and the role of exosomes in the IR response.

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Abbreviations

°C	Degrees Celsius
ΔΔCt	Delta-delta CT
μl	Microliter
μM	Micromolar
3'	Three prime
5'	Five prime
ALIX	ALG-2 interacting protein X
ANOVA	Analysis variance
B2M	Beta-2-microglobulin
BE	Bystander effect
bp	Base pair
Caco-2	Colon cancer cell line
CGH	Comparative genomic hybridization
CNV	Copy number variation
CO ₂	Carbon dioxide
DDR	DNA damage response
DNA	Deoxyribonucleic acid
DSBs	Double strand breaks
EGFR	Epidermal growth factor receptor
EVs	Extracellular vesicles
exoDNA	Exosomal dna
FCS	Fetal Calf Serum
g	Gram
gDNA	Genomic DNA
GI	Genomic instability
GJIC	Gap junction intercellular communication
GPTases	Guanosine triphosphatases
Gy	Gray
H ₂ O ₂	Hydrogen peroxide
HaCaT	Human keratinocyte cells
HF19	Primary lung fibroblasts
HR	Homologous recombination
HVL	Half value layer
IR	Ionizing radiation
kb	Kilobase
KCL	Potassium chloride
kVp	Kilovoltage peak
LET	Linear energy transfer
LINE-1	Long Interspersed Element-1
lncRNAs	Long non-coding RNAs
LTRs	Long terminal repeats
M	Molar
mA	Milliamps
mbar	Millibar
MCF7	Breast cancer cell line
MEM	Minimal essential medium
mg	Milligram

miRNAs	mircoRNA
mm	Millimeter
MMEJ	Microhomology-mediated end joining
MN	Micronuclei
mRNAs	Messenger RNA
NaOH	Sodium Hydroxide
NaCl	Sodium chloride
NE	Nuclear envelope
NGS	Next-generation sequencing
NHEJ	Non-homologous end-joining
NHEK	Normal human epidermal keratinocytes
nm	nanometers
non-LTRs	Non-long terminal repeats
NOS	Nitric oxide species
NTA	Nanoparticle tracking analysis
NTEs	Non-targeted effects
ORFs	Open reading frames
pA	Polyadenylation
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PDAC	Pancreatic ductal adenocarcinoma
PEG	Polyethylene glycol
PFA	Paraformaldehyde
PGLs	Paragangliomas
PI	Post-irradiation
QC	Quality check
qPCR	Quantitative PCR
RIBE	Radiation-induced bystander effects
RIGI	Radiation-induced genomic instability
RNAi	RNA interference
RNA	Ribonucleic acid
RNP	Ribonucleoprotein
ROS	Reactive oxygen species
RPM	Revolutions per minute
RT	Room temperature
SD	Standard deviation
SINE	Short interspersed nuclear elements
SNP	Single nucleotide polymorphism
SSA	Single strand annealing
SSBR	Single strand break repair
SSBs	Single strand breaks
ssDNA	Single-stranded DNA
STING	Stimulator of interferon genes
TEM	Transmission Electron Microscopy
TEs	Transposable Elements
TPRT	Target Primed Reverse Transcription
TPT	Topotecan
tRPS	Tunative resistive pulse sensing
TSAP6	tumour suppressor-activated pathway 6

TSD	Target site duplications
UTR	Untranslated region
UV	UltraViolet
WB	Western blot
WGS	Whole genome sequencing
X-ray	X-ray
α-particle	Alpha particle
γ-ray	Gamma ray

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Chapter 1 Introduction

1.1 Ionizing Radiation

'Radiation' specifies the movement of energy from one source as it travels as a wave or particle to another source. Naturally, the most notable form is radon, which arises from the ground and can be found in all rocks and soils. Artificially, radiation can occur from nuclear power stations, nuclear weapons testing, and medical imaging (such as X-rays). Radiation can be described as either non-ionizing or ionizing and the electromagnetic spectrum, shown below, covers these sources (Figure 1.1). Ionizing radiation (IR) is distinguished from non-ionizing radiation by the higher energy, which has the capability to break the chemical bonds of a molecule, thus removing (or gaining) electrons (Hall and Giaccia, 2006). This project will focus on the mechanisms of ionizing radiation in the induction of non-targeted effects (NTEs) (see section 1.2.2).

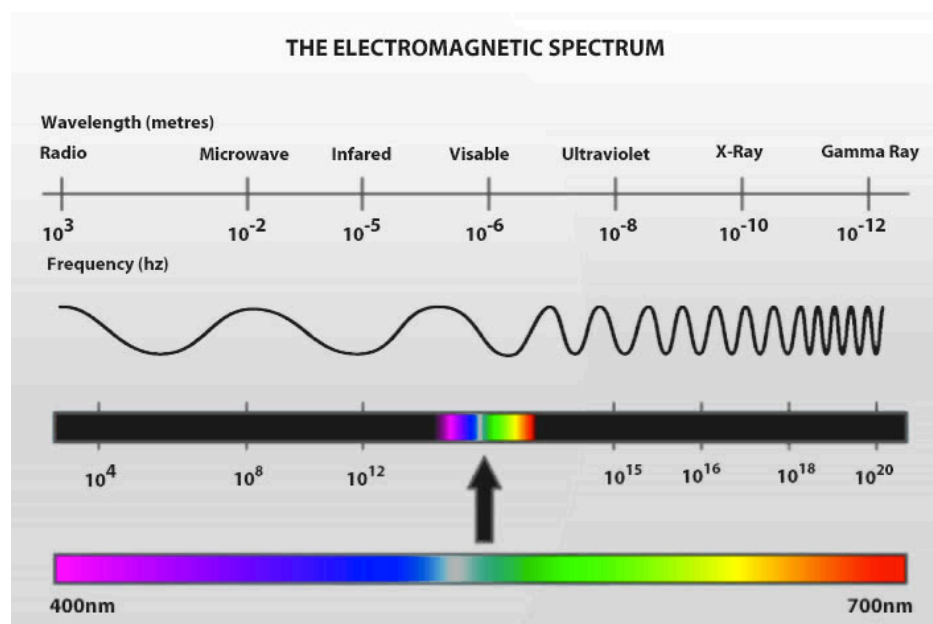


Figure 1.1 The electromagnetic spectrum covering the ionizing/non-ionizing sources of radiation. (Dean et al., 2011).

IR occurs in two forms (either as a particle or an electromagnetic ray), and both can be distinguished from each other according to the average energy transferred per unit length of track (linear energy transfer, LET) (Kadhim, 2003; Goodhead, 1994). High LET radiation (such as α -particles) leads to

straight and dense ionizations along their path. Low LET radiation (includes X-rays and gamma rays) leads to sparse ionizations, resulting in scattered energy along several tracks (as shown in Figure 1.2).

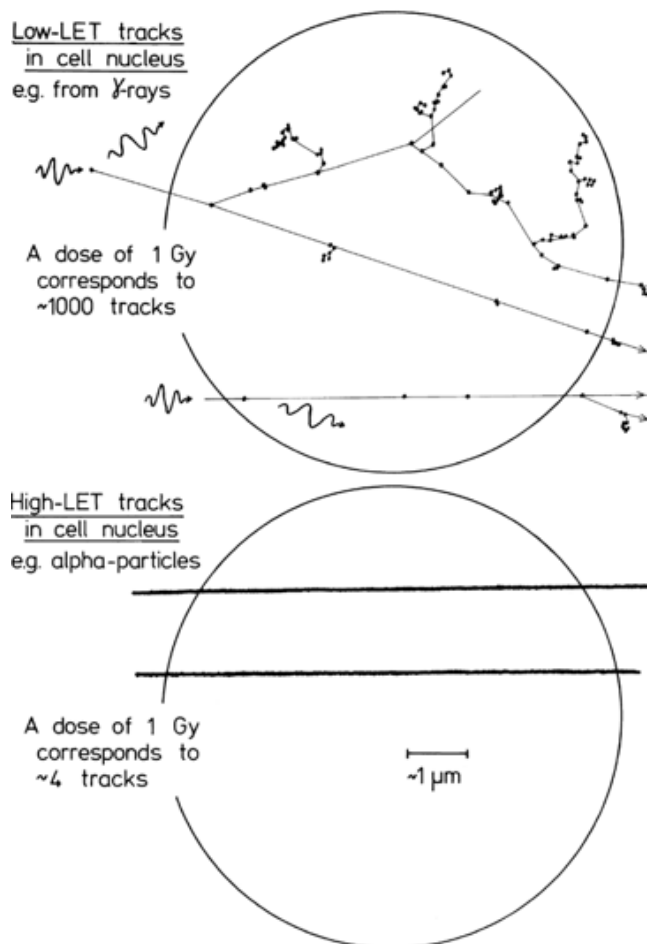


Figure 1.2 Visualization of the differences in the transfer of energy along High and Low LET tracks (Goodhead, 1988).

1.2. Damage Induced by Ionizing Radiation (Targeted and Non-Targeted Effects)

1.2.1. Targeted Effects of Ionizing Radiation

There are two mechanisms by which ionizing radiation can cause cellular damage, either directly or indirectly. Direct damage refers to the interaction with nuclear deoxyribonucleic acid (DNA), and could affect the ability of the cell to replicate and survive. Indirect damage refers to the production of free radicals through the hydrolysis of water. Free radicals are highly reactive atoms with an

unpaired number of electrons and can be formed from the ionization of water (H_2O^+). If H_2O^+ interacts with another water molecule, hydroxyl radicals ($\text{OH}^\cdot$) could form. Hydroxyl radicals are dangerous as they are able to diffuse through a cell and attack DNA, thus causing indirect damage (as shown in Figure 1.3) (Riley, 1994).

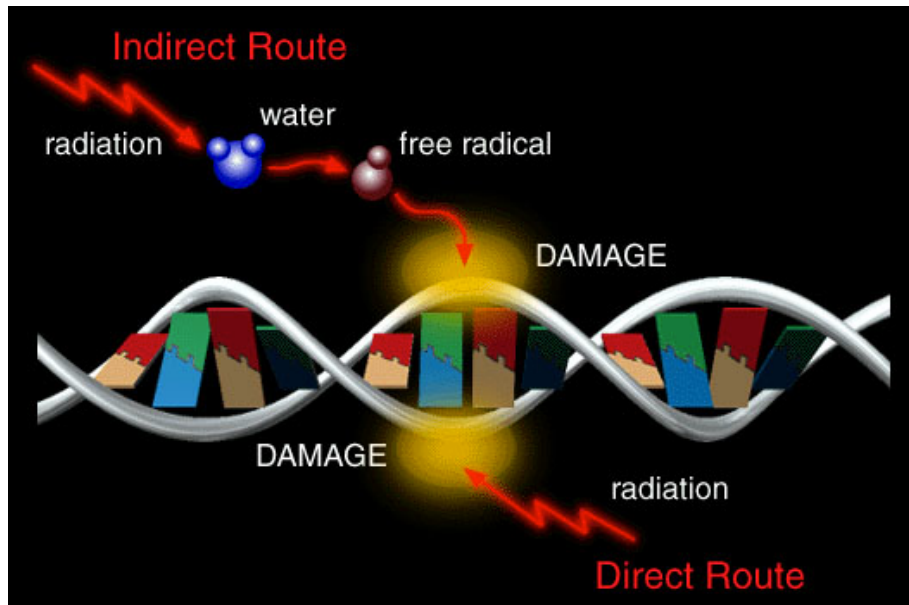


Figure 1.3 Schematic diagram demonstrating how radiation either can damage DNA either directly or indirectly through the production of free radicals (Russell, 2008).

IR is capable of producing lesions in DNA such as base damages, crosslink formation, single strand breaks (SSBs) and double strand breaks (DSBs). DSBs are the most severe form of damage and lead to chromosomal aberrations, causing gene malfunctions that in turn lead to cell cycle arrest (Goodhead, 2006). Low LET is more likely to cause scattered DNA damage such as SSBs, oxidation of bases, and the rare DSB, while high LET is expected to cause clustered damage with many DSBs (Goodhead, 1994). The initial damage to the DNA (whether it be a SSB or DSB) activates a cascade of events in the DNA repair system in an effort to prevent cellular death. A SSB is fixed quite quickly by the cell due to the fact that one strand is used as a template to guide the repair of the damaged strand. The single strand break repair (SSBR) process can be separated into four basic steps: SSB detection, DNA end processing, DNA gap filling, and finally DNA ligation (Caldecott, 2008).

SSBs are primarily detected by the protein poly (ADP-ribose) polymerase (PARP), which promotes the binding and activation of downstream repair factors. Depending on if the SSB is a consequence of a direct or indirect break certain molecules will assist in the next step of end processing. In an indirect break AP endonuclease (APE1) is used to remove the damaged DNA, while in a direct break APE1 is also used in combination with a molecular scaffold protein known as XRCC1 (Caldecott, 2003). XRCC1 is a crucial protein as it contributes much in the recruitment/coordination of polypeptides during the entire process of SSBR (Caldecott, 2003). The DNA polymerase known as pol-beta ($\text{pol}\beta$) is then used in the gap-filling step to form a template section of the DNA by either inserting a single nucleotide or up to 2-15 nucleotides. The final step (DNA ligation) is the restoration of the phosphodiester backbone and is completed by DNA ligase III α (Lig3 α) during a single nucleotide repair and DNA ligase I (Lig1) when a longer nucleotide repair is needed (Caldecott, 2003). This repair system highlighting direct and indirect single strand breaks can be seen in Figure 1.4.

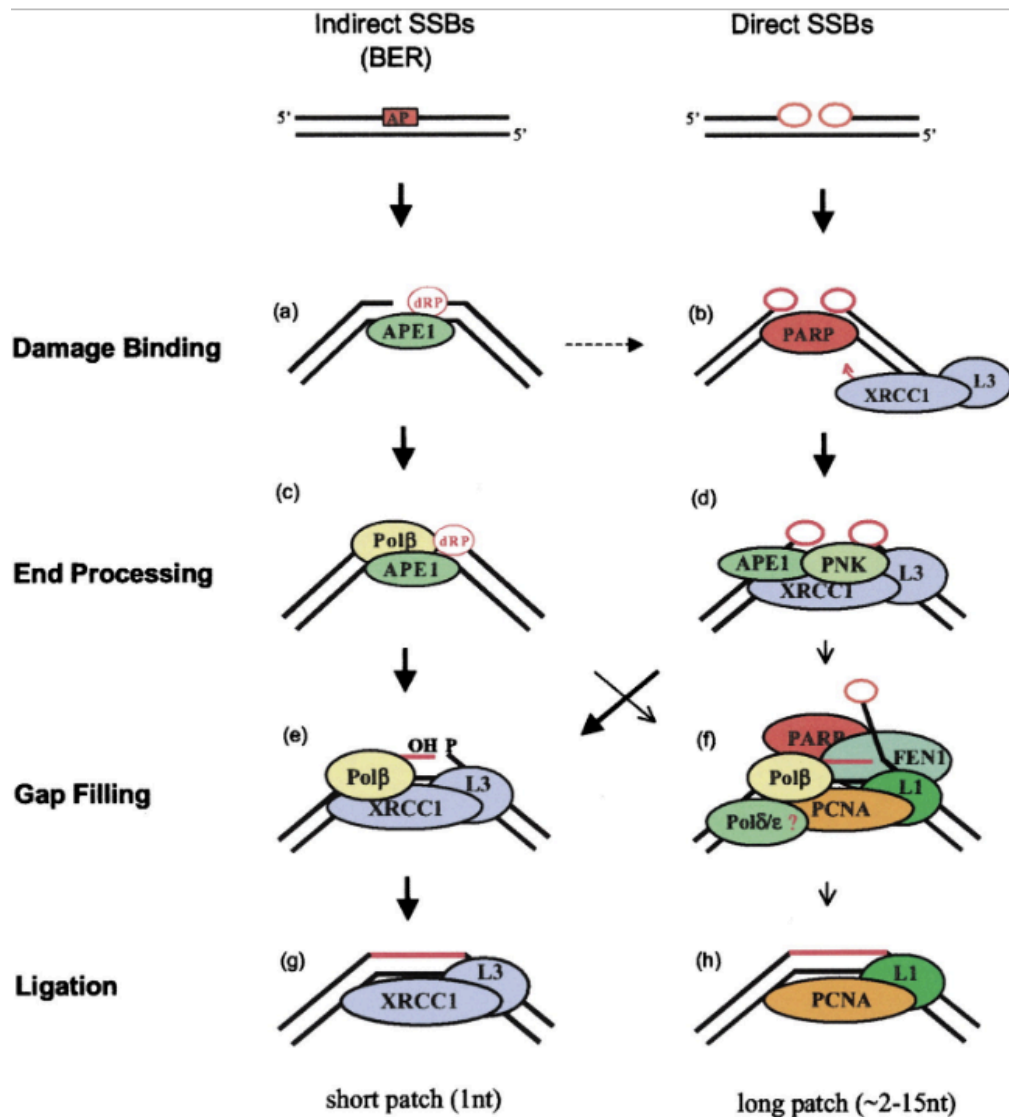


Figure 1.4 Diagram illustrating the proteins and steps involved in the repair process of a SSB of DNA (Caldecott, 2003).

Double strand repair is more complex, and in mammalian cells two repair pathways are predominantly used known as non-homologous end-joining (NHEJ) or homologous recombination (HR). The two pathways differ as they use different proteins for repair systems, occur during different stages of the cell cycle, and NHEJ is prone to errors while HR is more reliable. Both pathways are similar as they utilize the MRE11-RAD50-NBN (MRN) complex that uses the signalling proteins ataxia telangiectasia (ATM) and RAD3-related (ATR) to halt the cell cycle in order for the

repair system to begin (Vignard et al., 2013). The repair processes of both systems can be visualized in Figure 1.5.

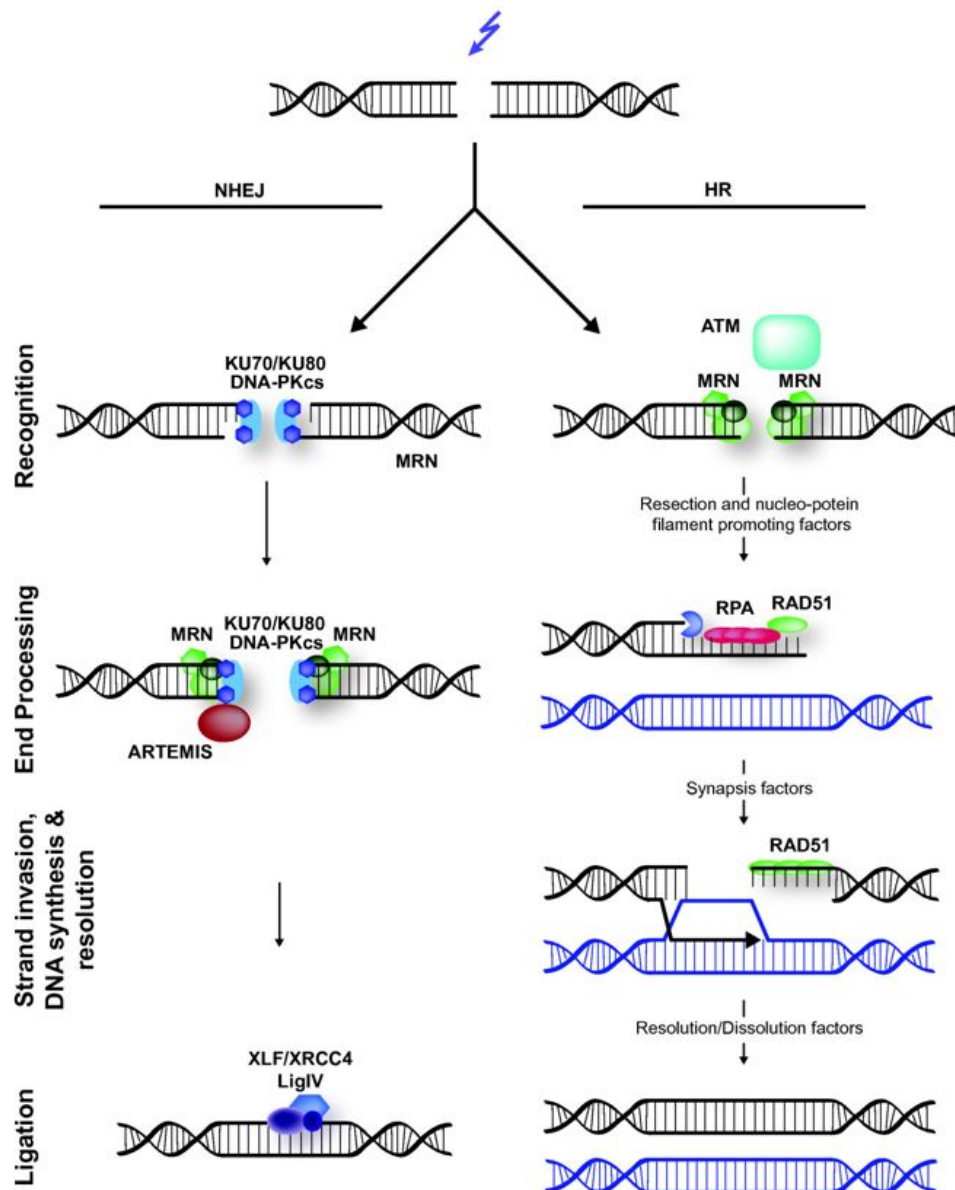


Figure 1.5 Schematic diagram of NHEJ and HR repair pathways, each requires different proteins and machinery to repair damaged DNA (Lans et al., 2012).

NHEJ occurs during all stages of the cell cycle and requires the trimming of DNA before ligation can take place, which results in errors from the loss of genetic information. It begins with the incorporation of proteins Ku70 and Ku80, which bind to the broken ends of the DNA and form a heterodimer. Then multiple other repair proteins are recruited such as phosphatidylinositol 3-kinase DNA-PKcs (O'Driscoll

and Jeggo, 2006). Once phosphorylated by the DNA-PKcs, the processing of the ends is done by the ARTEMIS nuclease. Polymerases and a complex formed from DNA ligase IV and XRCC4 join the DNA end by the process of ligation (Lans et al., 2012).

HR occurs only in late S and G2 phases of the cell cycle and functions by using a sister chromatid as a template. This repair system is initiated by end-resection with utilization of the MRN complex and several other nucleases. The single-stranded DNA that is formed from DNA end-resection is then bound by the DNA binding protein, RPA, and is then replaced by RAD51 (O'Driscoll and Jeggo, 2006). RAD51 then polymerizes the strand invasion by the DNA to a template strand, resulting in the formation of a DNA crossover that is known as a Holliday junction. This leads to a synapsis, DNA synthesis by DNA polymerase using the intact strand as a template, and repair by migration (Lodish, et al., 2000). DSBs are the most severe form of DNA damage as they can ultimately lead to genomic instability (GI) (Mladenov et al., 2016).

Radiation-induced damage has followed a classical target theory for many years, suggesting that the biological effects produced after radiation is a direct result of energy from IR. However, there is now a considerable amount of evidence that challenges the target theory as there have been numerous effects observed post irradiation many generations after the radiation insult. This model has been well established in the past two decades and is known as the non-targeted effects (NTEs) of IR (Kadhim et al., 2013).

1.2.2. Non-Targeted Effects (NTEs)

The effects that are present in the progeny of unirradiated cells many generations after the initial radiation event are known as NTEs. NTEs refer to genomic instability (GI, genetic changes detected in the descendants of the cells which were originally irradiated) and the bystander effect (BE, non-irradiated cells that have had some communication with irradiated cells and as a consequence they exhibit similar cellular affects) (Kadhim et al., 2013).

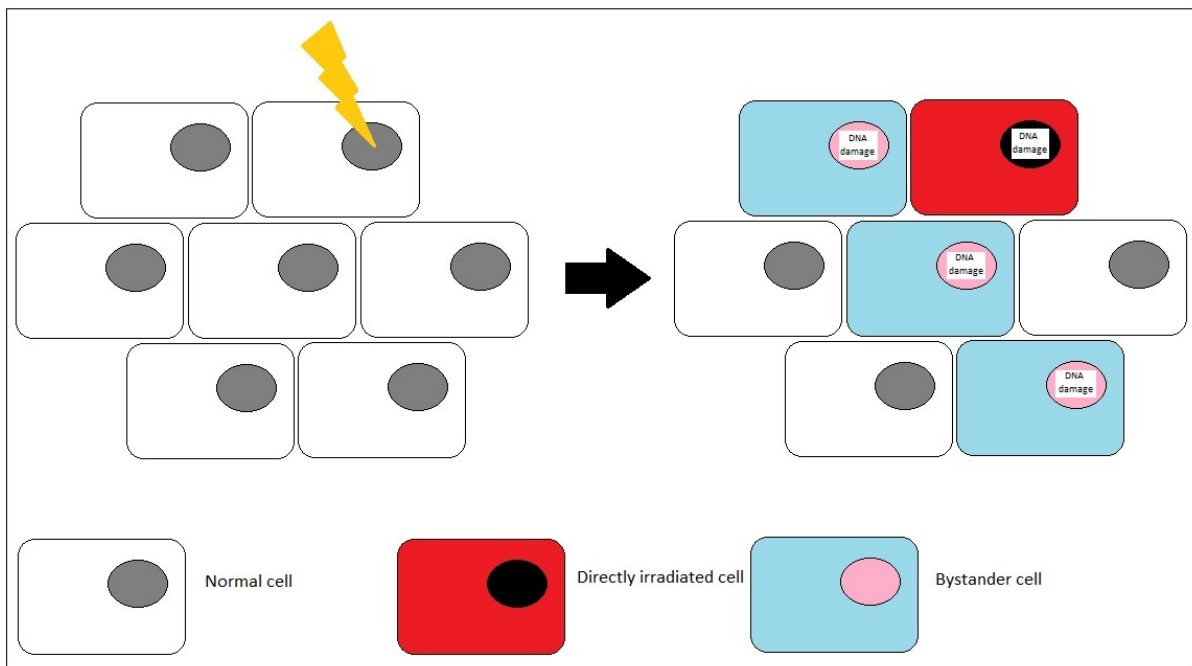
1.2.2.1 Radiation-Induced Bystander Effects (RIBE)

RIBE are defined as the phenomenon in which non-irradiated cells exhibit radiation type effects as a result of having some form of communication with those that have had exposure to radiation (Blyth and Sykes, 2011). Cell type, dose, radiation type, and time of analysis post irradiation influence the biological responses shown within bystander populations as they can vary from chromosomal aberrations to the level of mutations observed. Interestingly, most of the work on RIBE has shown that the phenomenon is induced maximally at low doses of radiation (Prise et al., 2003).

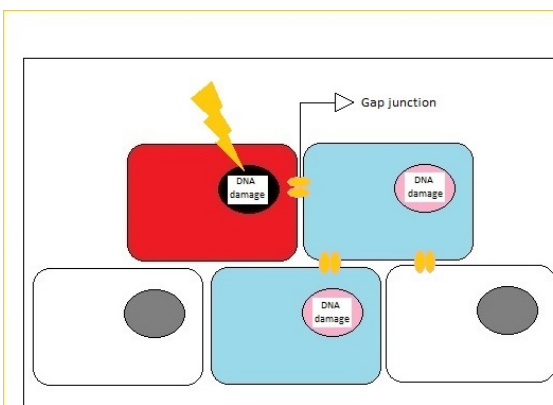
1.2.2.1.1 Mechanisms of RIBE

The two proposed mechanisms underlying RIBE are 1) Cell to cell contact via gap junction intercellular communication (GJIC) and 2) The transfer of soluble and diffusing factors within the cells/tissue microenvironment and medium. Gap junctions are the transmembrane channels that connect the cytoplasm of cells that are in contact with one another. GJIC is regulated by connexin proteins, which form channels that permit the transfer of signals from irradiated to unirradiated cells (Hamada et al., 2011). These mechanisms can be visualized in a schematic diagram of the RIBE in Figure 1.6.

A



B



C

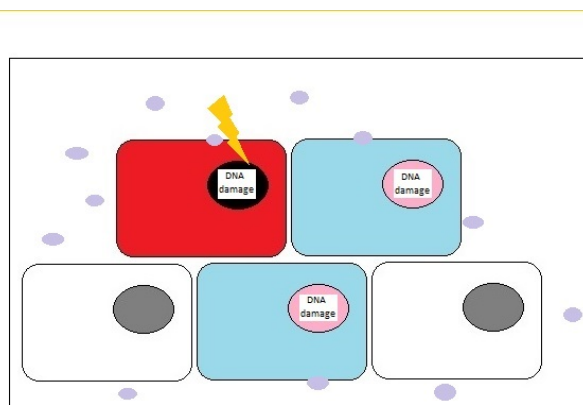


Figure 1.6 Illustration of RIBE and the two proposed mechanisms of RIBE

A) After an initial radiation insult cells that are directly hit by X-rays (red) demonstrate DN damage as expected, however some bystander cells (blue) not directly hit by radiation exhibit DNA damage effects as though they had been. B) Cell to cell communication via gap junction. C) Signals from medium transferred from irradiated cells.

Cells that are recipients of culture media from irradiated cells are also capable of secreting a signalling molecule. It has been shown in numerous *in vitro* studies that after media transfer unirradiated cells have damage that is similar to that seen after irradiation. Examples of signals that are produced as a result of media transfer include reactive oxygen species (ROS), nitric oxide species (NOS) (Shao et al., 2006), and cytokines (Hei et al., 2011). Matsumoto *et al.* (2001) demonstrated

that NOS produced an increase in protein p53 and hsp72 in p53 glioblastoma cells (unirradiated) after co-cultivation and exposure to medium from irradiated p53 cells, thus highlighting NOS as a mediator of the bystander effect. Moreover, recent studies have shown that microvesicles/exosomes could be accountable for the communication between unirradiated and irradiated cells involved in BE (Al-Mayah et al., 2012; Jella et al., 2014).

1.2.2.2 Radiation-Induced Genomic Instability (RIGI)

GI describes the increased rate and changes that lead to a loss of stability in the mammalian genome. The manifestations of RIGI include changes in ploidy, chromosome aberrations and rearrangements, micronuclei formation or cellular death (Baulch, 2019). In particular, IR is capable of inducing *de novo* copy number variation (CNV) mutations (Arlt et al., 2014). Radiation-induced genomic instability (RIGI) is recognized by the presence of biological affects that occur in the progeny of irradiated cells many generations after the initial irradiation of parental cells (highlighted in Figure 1.7) (Kadhim et al, 1992; Baulch, 2019). RIGI leads to a variety of structural abnormalities and modifications and has therefore been labelled as a hallmark of tumourigenic progression or cancer (Pikor et al., 2013). While the unstable phenotypes produced from NTEs are well characterized, the cellular processes that initiate RIGI remain elusive (Morgan, 2003; Kadhim et al., 2013).

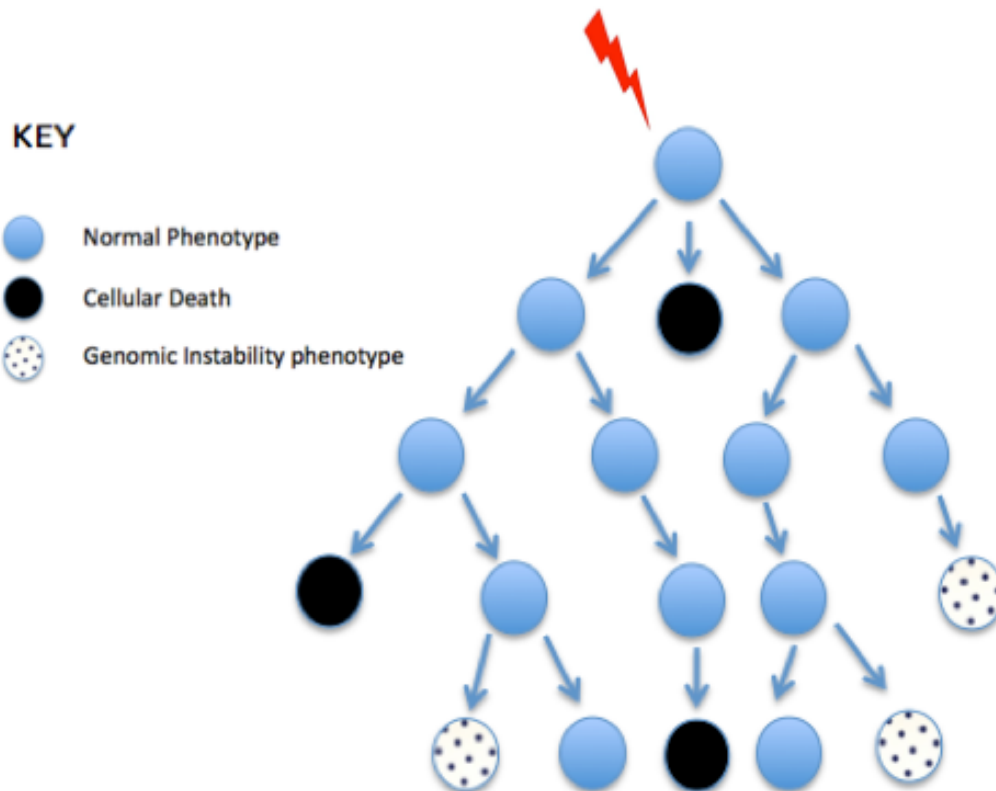


Figure 1.7 Schematic diagram showing possible phenotypes that could arise after exposure to ionizing radiation.

1.2.2.2.1 Mechanisms of RIGI

RIGI is potentially one of the pathways that exposure to radiation can lead to cancer. Therefore, unveiling the mechanistic properties that govern the response could further the understanding of the development of radiation-induced carcinogenesis. While IR is capable of producing double-strand breaks and mutations in DNA, extensive research has been gathered that suggests these are not the initiating factors of GI (Morgan, 2003; Snyder and Morgan, 2005). Alternatively, an epigenetic origin has been suggested as several studies have shown delayed changes in DNA methylation, chromatin remodelling, and expression of miRNA when analyzed post-irradiation (Aypar et al., 2011). Another explanation implicates the rise in intracellular oxidative stress, involving either direct communication between ROS and DNA, or the effects of ROS with intracellular compartments and organelles (Suzuki et al., 2003). Cytokines (signalling molecules possibly liable for the pathogenesis of several effects post-irradiation) have also been found to become upregulated by

heightened ROS levels, as well as become produced after exposure to radiation (Kim et al., 2006). It has also been recently shown that RIGI could be partially mediated by exosomes/microvesicles (Al-Mayah et al., 2012; Al-Mayah et al., 2015). IR is also known to trigger transposable elements, thus causing instability within the genome (Tanaka et al., 2012). However, the mechanism behind the transcriptions and insertions into new areas in the genome is not clear.

1.3. Introduction to Transposable Elements

The haploid human genome is approximately 3.2 billion base pairs long. It is comprised of both protein-encoding genes and non-coding DNA, where protein-coding regions only account for 1.5% of the total genome. The remaining non-coding regions are associated with the transcriptional and translational regulation of protein-coding sequences, non-coding RNA molecules, introns, pseudogenes, repetitive sequences, mobile elements, and other sequences without a known role within the genome.

About 45% of the human genome is comprised of Transposable Elements (TEs) (Cordaux and Batzer, 2009), and they are unique as they are capable of moving from one location in the genome to another. There are two classes of TEs, and they are distinguished from each other according to their mechanism of transposition. Class I TEs depend upon reverse transcription (the transcription of RNA into DNA) to transpose, while the transposition of Class II TEs does not require an RNA intermediate (Fernandez-Medina et al., 2012).

Class II TEs make up less than 2% of the human genome, while the remaining TEs (Class I) are known as retrotransposons and can be further categorized into two subclasses, known as non-long terminal repeats (non-LTRs) and long terminal repeats (LTRs). Non-LTRs occur discontinuously throughout the genome as singularly dispersed copies. In comparison, long terminal repeats (LTRs) comprise about 8% of the human genome and encode reverse transcriptase (similar to retroviruses). Long Interspersed Element-1 (LINE-1) is a non-LTR, comprises about 17% of the human genome, and is an

active and autonomous family. Another 11% of the human genome is comprised of Alu (short interspersed nuclear elements (SINE)) and SVA elements that are active and non-autonomous, relying on LINE-1 machinery for mobilization (Babatz and Burns, 2013). Figure 1.8 illustrates the transposable elements found within the human genome.

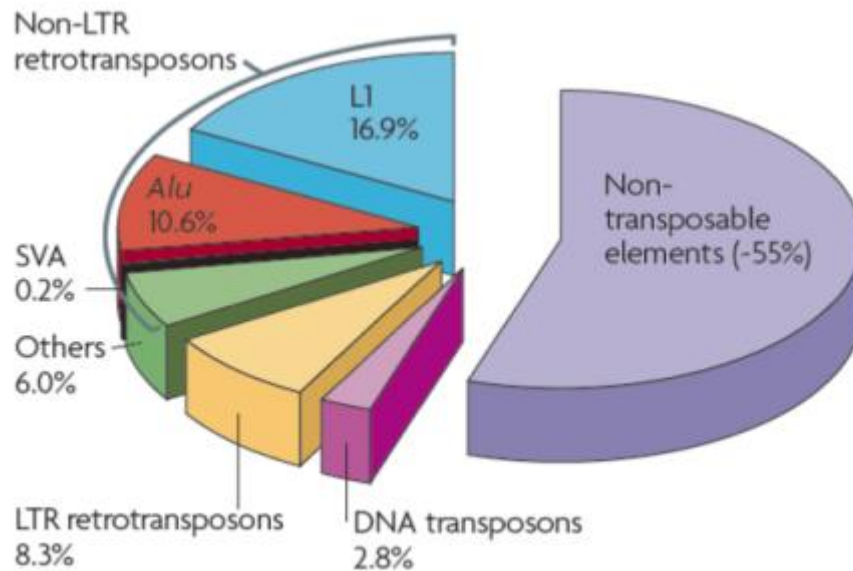


Figure 1.8 A pie chart representing the transposable elements within the human genome, the majority of which belong to the non-LTR retrotransposons (Cordaux and Batzer, 2009).

Although only a small proportion (less than 0.05%) of these retrotransposons are currently still active within the human genome, they are capable of altering the genomes of their hosts (Mills et al., 2007). They continue to develop human genomic variation today by insertions, deletions, mutations, and inversion polymorphisms (Babatz and Burns, 2013). In some cases retrotransposon insertions have been involved in Mendelian disorders such as cystic fibrosis, neurofibromatosis, haemophilia, breast cancers and colon cancers (Cordaux and Batzer, 2009; Hancks and Kazazian, 2012). Human genomes are therefore still susceptible to being disturbed by the ‘jumping’ actions of the retrotransposons.

1.3.1. Mechanism of Retrotransposons

The human LINE-1 element is 6 kb in its full-length form and contains a 5' untranslated region (UTR), two open reading frames (ORFs), and a 3' UTR. The LINE-1 promoter is found within the 5'UTR, while the 3' UTR consists of a polyadenylation (pA) signal. The first open reading frame (ORF1) encodes for a protein (ORF1p) that aids in RNA binding, while the second open reading frame (ORF2) encodes for a protein (ORF2p) with dual function as an endonuclease and reverse transcriptase. Figure 1.9 shows a schematic representation of the LINE-1 element.



Figure 1.9 LINE-1 element showing the promoter (PRO) region that begins with the 5' UTR and continues with the open reading frames ORF1 and ORF 2.

The 3' UTR consists of the pA signal and is completed by pA tails of various lengths. Grey arrows symbolize where the target-site duplications occur after retrotransposition (Belancio et al., 2008).

The process of LINE-1 replication begins with the transcription of the LINE-1 element and is most likely enforced by an RNA polymerase II promoter in the start of the 5' UTR (Belancio et al., 2008).

The end result of LINE-1 transcription is the creation of a full-length LINE-1 mRNA that contains both ORFs (Dombroski et al., 1991). LINE-1 transcripts are polyadenylated at their internally positioned pA sites, specifically the LINE-1 3' UTR. LINE-1 transcripts are then spliced, however the exact mechanism by which the LINE-1 RNA is exported from the nucleus and into the cytoplasm is obscure. LINE-1 proteins (ORF1p and ORF2p) are then translated by the ribosomes (Kozak, 1989).

LINE-1 insertion begins once the RNP localizes to an insertion site on the chromosomal DNA, then the ORF2p nicks the bottom strand at the target site (Belancio et al., 2008). The LINE-1 poly(A) tail of the mRNA then base-pairs with the 3' end of the cellular DNA, thus becoming a primer for the first-strand cDNA synthesis. ORF2p then reverse transcribes the LINE-1 RNA, forming the cDNA (this process is known as Target Primed Reverse Transcription (TPRT)). The rest of the mechanical details of the LINE-1 integration process remain elusive. The final result is a new LINE-1 element insert

within in genome with target site duplications (TSD) that vary in length (Jurka, 1997). The integration process is illustrated in Figure 1.10.

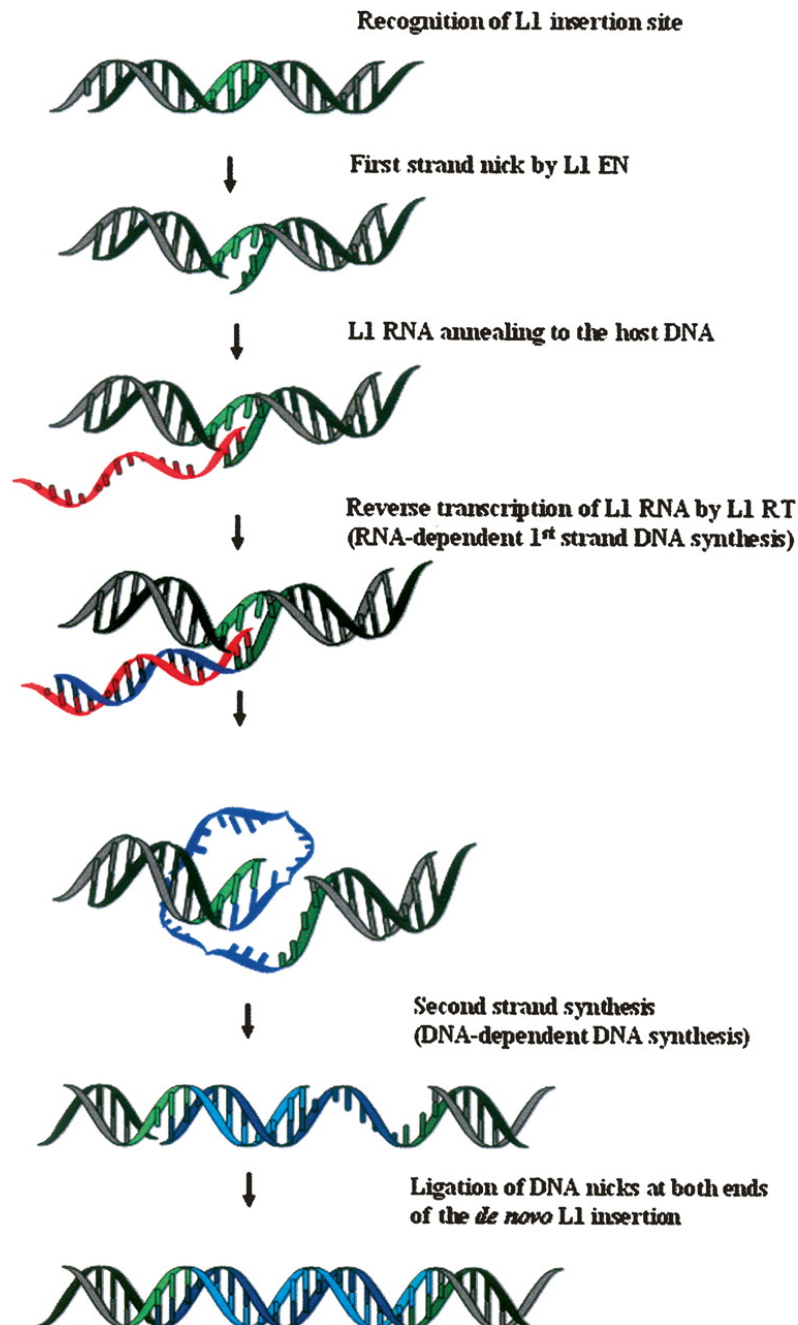


Figure 1.10 A detailed outline of the steps in the integration of LINE-1 elements.

The green represents 5'-TTTTAA-3' sequence in the genomic DNA, where the first-strand nick occurs in between the T and the A. The red represents the poly(A) tail of the L1 mRNA, where the 3' end of the host DNA will start to pair. The blue shows the cDNA synthesis by the L1 reverse transcriptase (Belancio et al., 2008).

1.3.2. Retrotransposons and Ionizing Radiation

As stated previously, IR is capable of inducing GI in a variety of circumstances in the irradiated cells and in their progeny through NTEs. IR also triggers mobile elements such as retrotransposons, however the mechanism behind the transcriptions and insertions into new areas in the genome is not certain (Tanaka et al., 2012).

Previous studies on yeast (*Saccharomyces cerevisiae*) showed that γ -rays and UV irradiation-induced transcription and transposition of the Ty retrotransposon with increasing doses of irradiation (Morawetz, 1987). Contrastingly, the human choriocarcinoma Jeg-3 cell line showed no increase in LINE-1 promoter activity when exposed to 2.5 Gy and 5 Gy doses of X-radiation (Morales et al., 2003). One other study demonstrated that γ -rays were capable of enhancing LINE-1 retrotransposition in 143B human osteosarcoma cells when exposed to 2 Gy and 4 Gy (Farkash et al., 2006). The NP-2 human glioma cell line was used in a study that focused on two types of ionizing radiation (X-rays and carbon-ion beams) and when exposed to various doses of irradiation they both produced altered LINE-1 retrotransposition (Tanaka et al., 2012). A more recent study by Miousse *et al.* (2017) used hematopoietic stem cells of CBA/J mice and low LET γ -rays, and results showed persistent loss of LINE-1 DNA methylation two months post exposure to IR, however there was no DNA damage detected in correlation.

These findings accentuate the need for further investigation of the effect of ionizing radiation on the mobilization of retrotransposons as the mutations that are caused could contribute significantly to genomic instability in cells that are directly affected as well as their progeny.

1.3.2.1 Activation of Retrotransposons

The process by which retrotransposon expression is activated depends on the physiological stress of the cell (Wilkins, 2010). When a retrotransposon is subjected to physiological stress or any damaging agents (such as IR), it can modify the expression of nearby genes by triggering them or intervening with their expression (Wilkins, 2010). For example, LINE-1 elements have a high GC content, thus

making then highly susceptible to DNA methylation (which is used by cells to suppress transcription). The methylation of LINE-1 embedded CpG islands therefore has the potential to silence the expression of nearby genes (Estecio et al., 2012). While it must be noted that retrotransposons are also transcribed in normal cells (not only due to stimulation from stress), the transcripts derived from pressure-induced situations would have a possibility to cause genomic damage.

The three steps by which LINE-1 is able to retrotranspose begins with the loss of DNA methylation in line LINE-1 5'UTR, followed by LINE-1 transcription, and LINE-1 transposition (as described in previous section). Regarding the first step, studies have shown that hypomethylation of the LINE-1 promoter is associated with an overexpression of LINE-1 transcripts, thereby suggesting a decrease in DNA methylation may play a role in LINE-1 retrotransposition (Wolff et al., 2010). Both biochemical and cell culture experiments have shown different ways in which Ten-eleven-translocation (TET) proteins take part and regulate transcription, and thereby potentially have an effect on TE expression (Deniz et al., 2019). In mammalian cells TET enzymes function by binding cis-regulatory regions (such as gene promoters and enhancers) where TET is involved in such processes as cell differentiation, oncogenesis, and neuronal function (Wu and Zhang, 2017). However, recent research implicates their roles as regulators of transposable element expression (Deniz et al., 2019). TET proteins have been shown to decrease 5-methylcytosine (5mC) levels at LINE-1 elements, SINEs, and DNA transposons (Kang et al., 2015; de la Rica et al., 2016; Zhang et al., 2017). Guallar *et al.* (2018) also found that TETs are capable of modifying RNA and therefore destabilizing TE transcripts such as murine endogenous retrovirus L (visual example shown in Figure 1.11). The modified formations of 5mC formed by TETs such as oxidized 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC), and 5-carboxylcytosine (5cacC) can also affect downstream pathways by prohibiting binding of certain proteins, such as transcription factors (Hashimoto et al., 2014). 5fC and 5cacC recruit various DNA repair proteins and TP53, and these modified versions by TETs have been shown to recruit modification-specific readers (Iurlaro et al., 2013; Xiong et al., 2016), however it is

unclear whether these mechanisms act at transposable elements. These proposed mechanisms can be visualized in Figure 1.11.

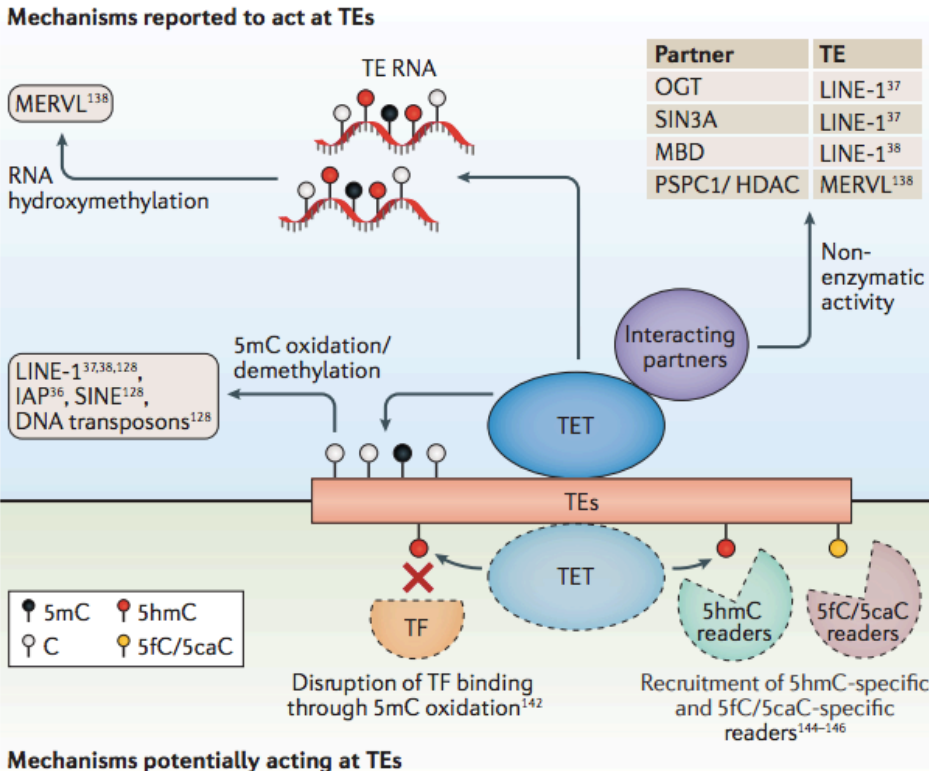


Figure 1.11 Diagram showing the proposed mechanisms by which TET proteins mediate regulation of various transposable elements, notably LINE-1. The top half of the diagram shows mechanisms which have been shown in various publications to be involved in the regulation of TE expression, while the bottom half of the diagram shows mechanisms that are known to act at other loci without any documented roles in TE regulation (Deniz et al., 2019).

1.4. Introduction to Extracellular vesicles and subtypes

Both normal and pathological cells are known to release membrane-bound vesicles that researchers have isolated from conditioned media as well as a variety of biofluids such as plasma, urine, saliva, ascites fluid, breast milk, and semen (Klein-Scory et al., 2014). These nano-sized EVs are important as they are involved in intercellular communication by transferring their biological content (such as proteins, lipids, and nucleic acids) between cells. EVs are an attractive method for delivery of functional and biological cargo due to their capability in protecting their contents beneath

membranes, ability to travel to cellular targets over long and short distances, and their inherent competency to transport biomolecules between cells (Zappulli et al., 2016).

These vesicles have been termed extracellular vesicles, and are primarily distinguished by their biogenesis, size, and content (Zhang et al., 2019). The three main classes are apoptotic bodies (1-5 μm), microvesicles (100-1,000 nm), and exosomes (30-150 nm). Apoptotic bodies are heterogeneous vesicles only produced by a cell undergoing cellular death by apoptosis, whereas microvesicles and exosomes are constantly released by normal, living cells (Akers et al., 2013). Apoptotic bodies are formed during the disassembly of apoptotic cells, when both nuclear and cytoplasmic parts are packaged into various membrane-bound vesicles in a wide range of sizes (1-5 μm). The contents found within apoptotic bodies therefore consist of the cellular components that were in the protrusion that produced it and can contain degraded nuclei, fragments of DNA, lipids, proteins, histones, and other cellular organelles (Hauser et al., 2017). Microvesicles are heterogeneous and produced through direct outward budding from the plasma membrane and into the extracellular space. While microvesicles have been shown to carry proteins and lipids, they may also carry DNA, mRNAs, microRNAs (miRNAs), and long non-coding RNAs (lncRNAs) (Liu et al., 2016). Exosomes are smaller, heterogeneous vesicles that arise from the inward budding of endosomal membranes, which leads to produce intracellular microvesicular bodies. Invaginations of the microvesicular body leads to the formation of exosomes, and when the bodies fuse with the plasma membrane exosomes are released to the extracellular environment (Zhang et al., 2017). Figure 1.12 illustrates the biogenesis of the three subtypes of EVs.

Currently it seems that all cell types form EVs, and while the biogenesis is a complex and versatile process the cargo found within EVs and release abundance can be altered by cellular stress and disease (Akers et al., 2013).

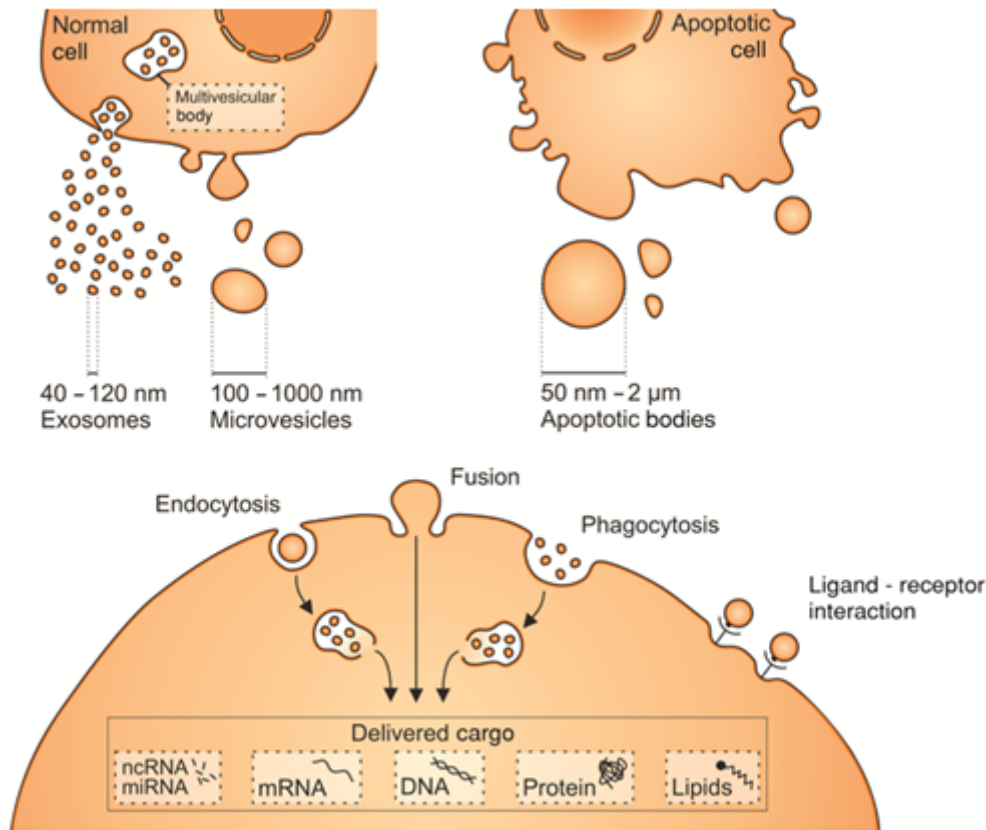


Figure 1.12 Illustration of biogenesis of three subtypes of EVs: exosomes, microvesicles, and apoptotic bodies. Adapted from (Zaborowski et al., 2015).

Both microvesicles and exosomes have been shown to contain proteins, microRNAs, mRNAs, lipids, mitochondrial DNA, and genomic DNA. The containments can be delivered to recipient cells by the fusion of the microvesicle/exosome with the target cell membrane. Due to their ability to deliver biological material, much research over the past three decades has been completed to better understand the roles EVs play in disease development, as well as their function as mediators and biomarkers of disease.

1.4.2. Exosomal isolation and characterization

Several different methods have been developed in the recent years in order to successfully isolate, purify, and characterize exosomes. Commonly used isolation techniques include separating vesicles based on the physical properties such as size and density such as ultracentrifugation (the 'gold

standard') and ultrafiltration (Cheruvanky et al., 2008). Other isolation techniques separate exosomes based on their differential solubility (chemical precipitation using polyethylene glycol (PEG)) (Zerlinger et al., 2015), and based on their immunoaffinity (using the micro-plate enzyme-linked immunosorbent assay (ELISA)) (Zarovni et al., 2015). However, such isolation procedures can coisolate mixed EV populations, and further characterization of exosome markers is warranted. Some unique proteins that are used as markers for exosomes include endosomal proteins (ALIX, Tsg101, flotillins), heat-shock proteins (HSP70, HSP90), and tetraspanins (CD9, CD63, CD81) (Zhang et al., 2018). Exosomes can be visualized using transmission electron microscopy, and their characteristic cup-shaped morphology is examined as well as their diameters ranging from 30 to 150 nm (Li et al., 2017). The concentration and size of vesicles can be measured using several technologies such as the nano-based tunable resistive pulse sensing (tRPS), nanoparticle tracking analysis (NTA), as well as with flow cytometry (Zhang et al., 2018).

1.4.3. Exosomes Secretion Modulation

Exosomes are the most commonly studied subtype due to their biological relevance regarding intercellular communication (Maia et al., 2018; Meldolesi, 2018) and immunological responses (Robbins and Morelli, 2014; Kurywchak et al., 2018). Their small size and presence of CD47 on exosomes is also appealing as it allows them to evade the phagocyte system (Kamerkar et al., 2017). The regulation of exosome secretion has been found to be controlled by the Rab guanosine triphosphatases (GTPases). For example, Rab27a/b proteins have been shown to affect the size and of EVs, as well as maintaining control of their secretion pathway (Ostrowski et al., 2010). An alteration in secretion of exosomes can be due to a multitude of factors including calcium signalling, where an increase in Ca^{2+} stimulates exosome secretion (Savina et al., 2003). Under stressed conditions p53-regulated product (TSAP6) demonstrated an increase of exosome production (Yu et al., 2006). Parolini *et al.*, (2009) also showed in a melanoma cell line that exosome secretion and uptake by recipient cells increases when the microenvironmental pH is low (low pH is a characteristic

of tumour malignancy). In general, much research has also shown that tumour cells produce high quantities of exosomes when compared to normal cells.

1.4.4. Exosomes and Ionizing Radiation

Ionizing radiation has been shown to increase release of exosomes in a variety of cell lines (Arscott et al., 2013; Yu et al., 2006; Al-Mayah et al., 2015). Research has also shown that exosome release is in accordance with time and dose of irradiation. For example, a study using a glioblastoma cell line (U87MG) irradiated with 2, 4, 6 and 8 Gy of X-rays showed an increase in exosome secretion in a dose-dependent manner 24 hours post-irradiation (PI) (Arscott et al., 2013). The same study showed a time dependency effecting the amount of exosomes released from the glioblastoma cells, with a significant increase 24 and 48 hours post-irradiation (PI), however insignificant at 12 hours PI (Arscott et al., 2013).

It has been proposed that exosome release is first activated by stress-inducible pathways in which increased expression of transmembrane protein tumour suppressor-activated pathway 6 (TSAP6) is stimulated by the damage-induced p53 transcription factor (Abramowicz et al., 2019). Once released from the donor cell, exosomes are transferred to and enter recipient cells through such processes as phagocytosis or membrane fusion. Once the radiation-induced exosomes have entered the target cell, specific processes in the target cell are activated (Jelonek et al., 2016). The irradiation of a

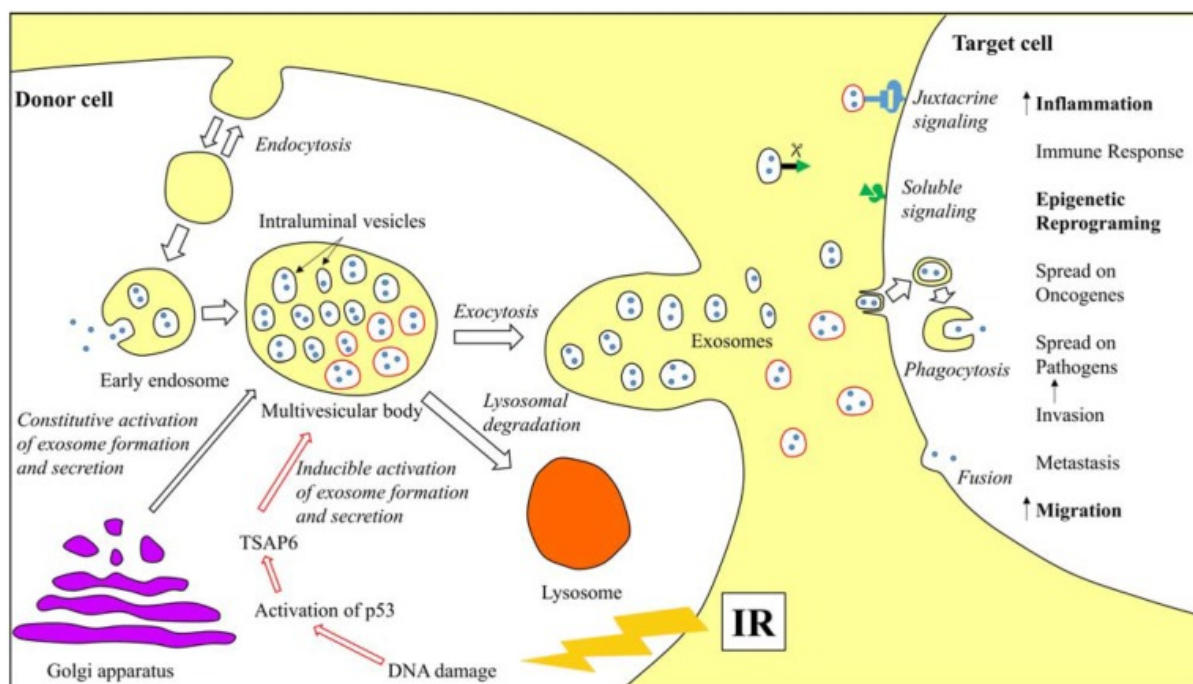


Figure 1.13 Illustration of actions following irradiation of a target cell that lead to the formation, release, and uptake of exosomes by another cell.

Processes affected in recipient cells are shown in **bold** (Jelonek et al., 2016).

target cell and the following events that lead to the formation, release, and uptake of exosomes by another cell can be seen in Figure 1.13.

As mentioned previously, the radiation-induced bystander effect (RIBE) is the phenomenon in which non-irradiated cells exhibit radiation type effects as a result of having some form of communication with those that have had exposure to radiation. Research has shown that exosomes from irradiated cells are able to deliver RNA and induce RIBE as well as delayed effects of GI (Al-Mayah et al., 2015). Medium from breast cancer cells (MCF7) and irradiated with 2 Gy X-rays as well as bystander progeny was then transferred and incubated with non-irradiated cells. DNA damage was apparent in early and delayed cell populations, showing that exosomes released by directly irradiated cells and exosomes secreted from bystander progeny play an important role in mediating the NTE of IR (Al-Mayah et al., 2015). Another study using human keratinocyte cells (HaCaT) were irradiated with 0.005, 0.05, and 0.5 Gy γ -rays also showed that exosomes are involved in RIBE signalling (Jella et al., 2014).

There is limited knowledge regarding IR and its effect on exosomal composition, and the information available focuses on their proteomes. In a study using 6 Gy γ -rays, exosomes were collected from the BHY cancerous cell line (irradiated and non-irradiated samples) and proteome analysis revealed 39 IR-upregulated and 36 IR-downregulated proteins. Not only were these proteins associated with a stress response and immunity, but to cellular adhesion and motility as well (Mutschelknaus et al., 2017). A more recent study by Abramowicz *et al.* (2019) aimed to establish the radiation-induced changes in exosome proteome composition by using UM-SCC6 cells derived from head-and-neck cancer. Exosomes were collected 24 hours after exposure to 0, 2, 4, and 8 Gy IR. Results showed a significance in 472 proteins affected by IR (at any dose), with 425 proteins being upregulated and 47 downregulated. Interestingly, some of their associated biological functions were those involved in response to IR such as DNA repair, ROS, chromatin packaging, and protein folding. Therefore, the exosomal protein content excreted by irradiated cells is indicative of their role in mediating the response to IR and suggests selective packaging. In addition, several 'exosomal marker' proteins such as tetraspanin CD63, CD81, ALIX, and 5 out of 8 heat shock proteins were found to be significantly upregulated in the exosome samples subjected to irradiation. Intriguingly, other markers such as CD9 and TSG101 (as well as the 3 other heat shock proteins) showed no alteration in exosomes released from cells exposed to IR. This is suggestive of cellular stressors affecting the packaging of exosomal contents.

1.4.5. Functions of exosomes

Through much research exosomes have proven to be critical facilitators of intercellular communication through their various biological cargo confined within or associated with their lipid bi-layer membrane (Meldolesi, 2018). They have become a primary focus in intercellular cross-talk studies as they are capable of transferring molecules between cancerous and other cancerous/non-cancerous cells in local and distant microenvironments (Tai et al., 2018). In addition, exosomes have been implicated in multiple biological and pathological processes such as cellular homeostasis

(Takahashi et al., 2017), tumour metastasis (Zhang et al., 2018), angiogenesis (Todorova et al., 2017), cardiovascular disease (Y. Zhang et al., 2017), embryonic development (Saadeldin et al., 2015), and their ability to modulate innate and adaptive immune responses (Greening et al., 2015).

In regards to cancer, exosomes are involved in various processes related to cancer progression. For example, epidermal growth factor receptor (EGFR)-containing exosomes released from gastric cancer cells are capable of being transported into the liver and then integrated on the plasma membrane of liver cells, thus promoting liver-specific metastasis (H. Zhang et al., 2017). In addition, exosomes derived from breast cancer cells and patients with breast cancer were shown to contain miRNA processing machinery and transport miRNAs that induced transformation and tumour formation in non-cancerous epithelial cells (Melo et al., 2014). Another study by Dioufa *et al.* (2017) showed that when priming the hepatic niche with exosomes exuded from MDA-231 breast cancer cells, it facilitated seeding of the cancer cells in the liver. Intriguingly, the same study also showed a difference in a certain set of miRNA contents in the tumour-derived exosomes and the exosomes from normal cells (which happened to be a set of miRNAs involved in epithelial cell differentiation) (Dioufa et al., 2017). It was also shown that bone-derived exosomes can stimulate proliferation in tumour cells, as well as mediate communication between cancerous and bone cells (Rossi et al., 2018). Thus highlighting how exosomes can aid in metastasis, by helping tumour cells with adapting and living in a different microenvironment.

Further, cancer-derived exosomes can trigger an immune response, which studies have revealed can be either suppressive or enhance immune activities. For example, Wen *et al.* (2016) determined that exosomes derived from breast cancer cells suppress an immune response by directly inhibiting T-cell proliferation and decreasing Natural killer cell cytotoxicity, therefore promoting metastasis.

Contrastingly, studies involving exposing tumour cells to stress have shown improvements in antitumour immunity. For example, Dai *et al.* (2005) showed that exosomes released from heat-stressed carcinoembryonic antigen (CEA)-positive tumour cells have high immunogenicity. Another

study showed that following exposure to X-irradiation, exosomes originating from tumour cells contain dsDNA that activated stimulating of Interferon signalling in recipient cells, thereby causing a protective antitumour T-cell response (Diamond et al., 2018).

1.4.6. Exosome DNA (exoDNA)

As previously stated, exosomes contain much cargo (RNA, proteins, lipids and DNA) which they are able to deliver to recipient cells. Recently, large fragments of genomic DNA has been isolated from exosomes (Thakur et al., 2014; Kahlert et al., 2014; San Lucas et al., 2016; Yang et al., 2017). Thakur *et al.*, (2014) used three cancer cell lines (human K-562, HCT116, and murine B16-F10) and were the first to show that a majority of the DNA found within exosomes is double-stranded. They also demonstrated (by pre-treating exosome samples with specific DNases) that a majority of external exoDNA is double stranded and is larger in size than internal exoDNA (Thakur et al., 2014). Research performed by Kahlert *et al.* (2014) utilized human pancreatic cancer cells (Panc-1 and T3M4) and were able to show that exosomes contain 10 kb (or larger) fragments of double-stranded genomic DNA, mutations in KRAS and p53 were found within the genomic DNA and exoDNA from both Panc-1 and T3M4, and serum exosomes from patients who have pancreatic cancer contain genomic DNA that is representative of all chromosomes. Therefore, in summary, this study showed that exoDNA is representative of the entire genome and it also reflects the mutational status of parental tumour cells.

Work by Yang *et al.* (2017) followed up on this discovery by studying the circulating exosomal DNA (KRAS and TP53 mutations) in clinical patients with pancreas-associated pathologies. They found that in 39.6% of patients a KRAS mutation was found within exosomal DNA, while in 4.2% of patients a TP53 was uncovered (Yang et al., 2017). It has been proposed from an accumulation of these findings that circulating exosomal DNA could be used in the detection of cancer-specific mutations as exosome isolation from blood and other bodily fluids has been readily established.

Furthermore, in another study next-generation sequencing (NGS) was utilized as exoDNA and matched tissue DNA derived from pancreaticobiliary cancers was subjected to whole genome, exome and transcriptome sequencing (San Lucas et al., 2016). The researchers found that many cancer-detecting biomarkers were found within the exoDNA, and further elucidated their potential for use as a quick clinical tool for cancer diagnosis in the future. Interestingly, this study also found that in one instance there were 100-fold more mutations found in the exosomal DNA when compared to the matching tumour tissue DNA (San Lucas et al., 2016). Contrastingly, previous research performed whole-genome sequencing on pancreatic ductal adenocarcinomas (PDACs) and found a much lower degree of mutational variation in the tissue samples (Waddell et al., 2015). The researchers hypothesize that this drastic increase of mutations in the exoDNA when compared to the tissue DNA is because exoDNA represents tumour heterogeneity at a degree that is not obtainable with tissue sequencing (San Lucas et al., 2016). However, very little research has been completed in this area and future work will be needed to understand the increase of mutations in exoDNA versus tumour tissue.

1.4.7. ExoDNA in cancer

The reasoning behind the packaging of DNA into exosomes is not fully understood, yet research thus far has shown that the transfer of tumour-derived exoDNA has a functional impact on recipient cells (Lee et al., 2016), and that exosomes remove damaged nuclear DNA from cells in order to maintain cellular homeostasis (Takahashi et al., 2017). Regarding the latter study, when Takahashi *et al.* (2017) restricted the production of exosomes an ROS-dependent DNA damage response (DDR) (mediated by the cytosolic DNA sensor, STING (stimulator of interferon genes)) was triggered by the aggregation of DNA in the cytoplasm. Additionally, another study treated breast cancer cells with the antitumour agent topotecan (TPT), an inhibitor of topoisomerase I, which led to a significant increase in exoDNA production and simultaneously triggered dendritic cell activation through the STING pathway (Kitai et al., 2017). Furthermore, a study previously mentioned by Diamond *et al.*

(2018) showed that only exosomes derived from X-irradiated cancer cells carried DNA that were capable of inducing interferon production by dendritic cells in a STING-dependent fashion (when compared to exosomes released from sham irradiated samples). Collectively, these studies indicate that exoDNA accumulation is possibly a product of DDR and other stress-induced signaling circumstances, as portrayed in Figure 1.14.

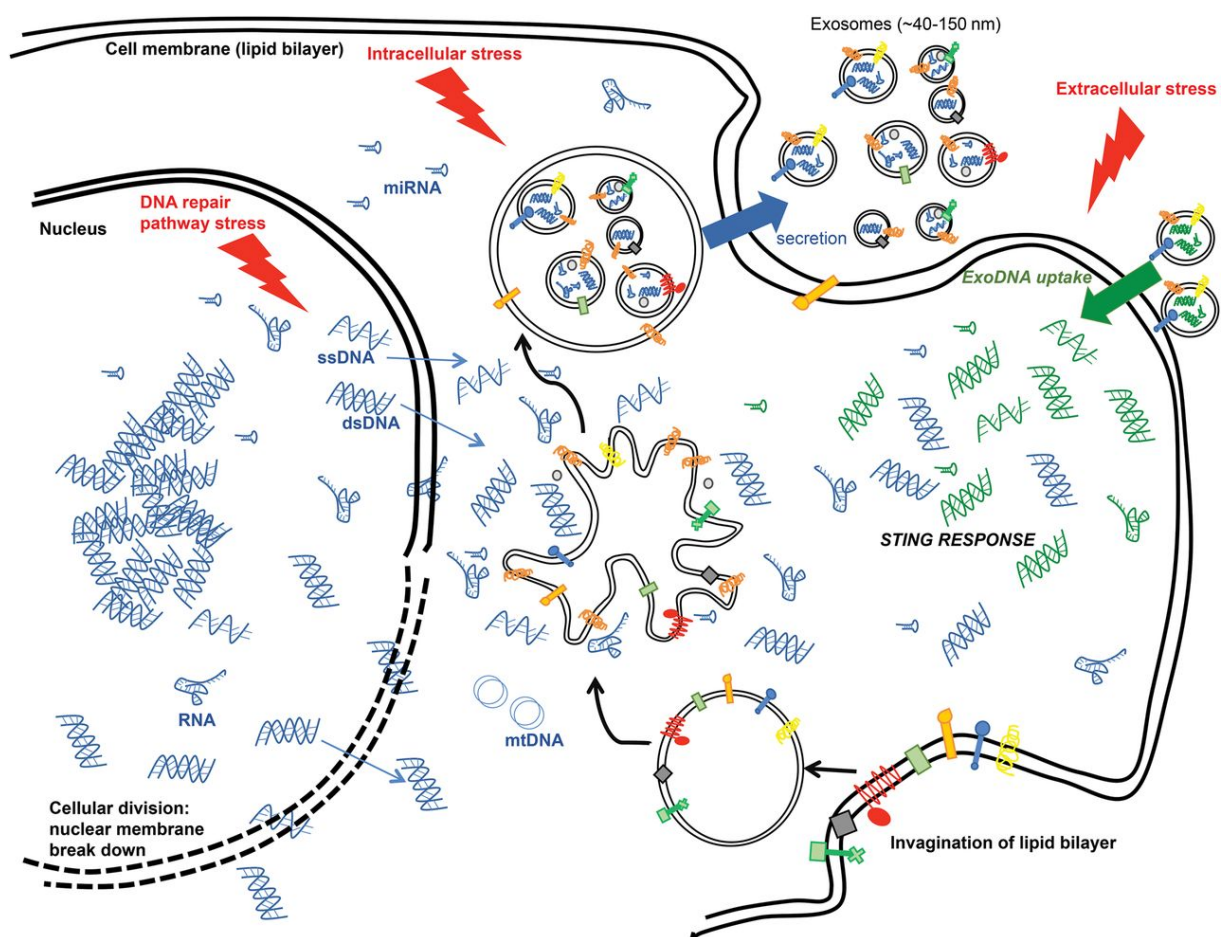


Figure 1.14 Illustration of actions following cellular stress and subsequent packaging of nucleic acids into exosomes.

An increase in cellular stress may lead to an increase of nucleic acids (mRNA, miRNA, mtDNA, ssDNA, and dsDNA) found in the cytosol which are eventually packaged into intraluminal vesicles (ILVs) before they are released as exosomes. Particularly, an increase in amount of cytosolic DNA, potentially via the uptake of exoDNA, could possibly lead to STING activation and elicit an immune response (Kalluri and LeBleu, 2016).

While little is known about the functional impact of the delivery of exoDNA to recipient cells, an *in vivo* study by Cai *et al.* (2014) indicates that exoDNA can help stimulate cancer-associated phenotypes. For example, upon injection of cancer-derived exosomes containing the BCR/ABL hybrid

gene (involved in the pathogenesis of chronic myeloid leukemia) into rats the results showed some varying characteristics such as weight loss, splenomegaly, and neutrophilia (Cai et al., 2014).

Interestingly, the study also found the BCR/ABL DNA, mRNA, and protein in the neutrophils of the treated rats, and treatment with actinomycin D (mRNA synthesis inhibitor) prevented the above mentioned symptoms from happening (Cai et al., 2014). While there is not much research into how the delivery of exoDNA can be incorporated or impact recipient cells, future studies are warranted to understand the functional impact exoDNA could possibly have in the role of tumourigenesis or metastasis.

1.4.8. ExoDNA and transposable elements

As mentioned previously, recent findings propose that microvesicles are a probable means of transmission for the transposable elements (LINE-1, Alu, and HERV) between cells (Balaj et al., 2011). The study by Balaj *et al.* (2011) used three medulloblastoma cell lines (D425, D385, and D458), one GBM (11/5), one melanoma (0106), and a primary human fibroblast line (HF19) to compare the amount of retrotransposon sequences found within their exosomal DNA. They discovered high levels of retrotransposon RNA transcripts, specifically LINE-1 and Alu retrotransposons within the tumour exosomes. They also demonstrated that HERV RNA expression levels were increased in tumour cells when compared to normal cells (Balaj et al., 2011). Graphs of transposable element DNA indicated contrasting levels of retrotransposon levels in exosomes compared to the cells from which they came, implying there is a different composition of exoDNA versus nuclear DNA (Balaj et al., 2011). This study highlights the abundance of genetic information within exosomal DNA and once again its potential as a use for a biomarker in cancer.

1.5. Aims of thesis

One of the key aims of this thesis are to investigate the role of retrotransposable elements in the mechanism of radiation-induced GI. In order to achieve this aim, the investigation will focus on the presence of retrotransposition at time points that are relevant to genomic instability. The hypothesis to be tested is that ionizing radiation may potentially cause an increase in LINE-1 retrotransposition, therefore leading to an instability within the genome. Another key aim of this study is to better characterize exosomes released from irradiated MCF7 cells and to profile their DNA contents to unirradiated cells in order to identify any IR-induced alterations in composition. The hypothesis will test if exoDNA is derived from specific locations within the genome, and upon cytogenetic analysis IR could induce a change in genetic composition.

Therefore, the following aims were addressed in this thesis:

1. To investigate LINE-1 retrotransposition in response to diagnostic and therapeutic doses of ionizing radiation at time points relevant to genomic instability in a normal fibroblast cell line, HF19 and two cancerous cell lines, Caco-2 and MCF7.
2. To determine a link between LINE-1 retrotransposition and RIGI, by means of micronuclei (MN) induction.
3. To characterize the exosomes released from sham and irradiated MCF7 cells, including morphology, biochemical composition, sizing, and quantification.
4. To extract and characterize exosomal DNA using whole-genome sequencing to identify any copy number variation by comparing exosomes excreted from sham and 2 Gy irradiated MCF7 cells.

Chapter 2 Materials and Methods

2.1. Cell Culture

All cell culture was performed in a laminar flow hood under appropriate aseptic conditions.

2.1.1. Cell culture of HF19 normal fibroblast cell line

HF19 cells were provided by Cox and Masson (Cox and Masson, 1975) from the Medical Research Council (MRC), Harwell Campus, Oxfordshire, UK as laboratory stock. The HF19 human fibroblast cells were maintained in minimal essential medium (MEM) (Gibco, 10370021) and supplemented with 10% (v/v) Fetal Calf Serum (FCS) (Sigma, F7524), 1% (v/v) L-glutamine (Fisher, VX15140122), and 1% (v/v) penicillin/streptomycin solution (Fisher, VX15140122).

2.1.1.1 Subculture of HF19 cells

HF19 cells were trypsinized at 80% confluence. Cells were washed twice with Phosphate-buffered saline (PBS) (Fisher, 10214733), followed by a wash with 5ml 0.025% trypsin (v/v) (Fisher, VX15400054) for 30 seconds. Once cells detached from the flask, cells were resuspended in 10 ml warm media and seeded into flasks at the appropriate density. HF19 cells were incubated at 37°C with 5% CO₂ in a humidified air incubator.

2.1.2. Cell culture of Caco-2 colon cancer cells

The Caco-2 cells were purchased by Professor Munira Kadhim while working at the Medical Research Council (MRC). Caco-2 cells were cultured in Dulbecco's Modified Eagle's Medium – high glucose (Sigma: D5671), and supplemented with 10% (v/v) FCS, 1% (v/v) penicillin/streptomycin, and 1% (v/v) MEM non-essential amino acids (Gibco, 11140050).

2.1.2.1. Subculture of Caco-2 cells

Caco-2 cells were trypsinized at 70-80% confluence. Cells were washed twice with PBS, followed by a wash with 0.25% trypsin for 1 minute. Cells were placed in the incubator for 2-3 minutes, and when detached they were resuspended in 10 ml warm media and seeded into flasks at the appropriate density. Caco-2 cells were incubated at 37°C with 5% CO₂ in a humidified incubator.

2.1.3. Cell culture of MCF7 breast cancer cells

MCF7 cells were donated by Dr. Joestin Dahle (University of Oslo, Norway). The MCF7 cell line was maintained in DMEM F-12 basal media (Gibco, 21311), supplemented with 10% (v/v) heat-inactivated FCS, 1% (v/v) L-glutamine, and 1% (v/v) penicillin/streptomycin.

2.1.3.1. Subculture of MCF7 cells

MCF7 cells were trypsinized at 70-80% confluence. Cells were washed once with PBS, followed by a wash with 5 ml trypsin (0.025%) for 30-45 seconds. Cells were placed in the incubator for up to 2 minutes, and when detached they were resuspended in 10 ml warm media and seeded into flasks at the appropriate density. MCF7 cells were incubated at 37°C with 5% CO₂ in a humidified air incubator.

2.2. Cell Counts using Viability Stain, Erythrosin B

In order to calculate cell viability to seed the desired amount of HF19, Caco-2, and MCF7 cells throughout all experiments, cell counts were performed using Erythrosin B (Sigma, E9259).

Erythrosin B stains only nonviable cells, and is compatible with automated cell counters (Kim et al., 2016). Erythrosin B solution was prepared by adding 0.4 g Erythrosin B powder (Sigma, E9259), 0.81 g sodium chloride, NaCl (Sigma, S5886), and 0.06 g potassium phosphate monobasic (Sigma, P5655) to 100 ml Hank's balanced salt solution (Sigma, H9269). The solution was then inserted into a glass beaker and put on a hot plate with a magnetic stirrer. Once the solution began to boil, 100-200 µl of 10 M Sodium Hydroxide (NaOH) (Sigma, 221465) was added until all the components dissolved. The concentrated stock solution then cooled to room temperature (RT). Once cooled, 1 ml of stock solution was added to 4 ml dH₂O, making the working solution. Both solutions were stored at 4°C. To calculate cell viability to seed the accurate amount of cells, cell counts were performed using a 1:1 cell suspension with Erythrosin B working solution (100 µl cell suspension, 100 µl Erythrosin B, 200 µl total volume) and a Neubauer haemocytometer counting chamber (figure 2.1). 10 µl of the

mixture was then pipetted at the edge under a coverslip on the Neubauer haemocytometer and allowed to run under the coverslip. Live cells appeared clear, while dead cells appeared red and were ignored. The live cells were counted from within the four outer corner squares (as shown in red in Figure 2.1), and averaged. Each large square, with coverslip on top, represents a total volume of 0.1mm^3 (the height of the chamber with cover glass is 0.1mm , so $1.0\text{mm} \times 1.0\text{mm} \times 0.1\text{mm}$) or 10^{-4}cm^3 . Multiplying by 10,000 gives the number of cells per mL. The following calculation was used to calculate the number of live cells per 1 ml of cell suspension:

$$(\text{Number of live cells in the four large red squares} / 4) \times \text{Dilution factor (2)} \times 10^4$$

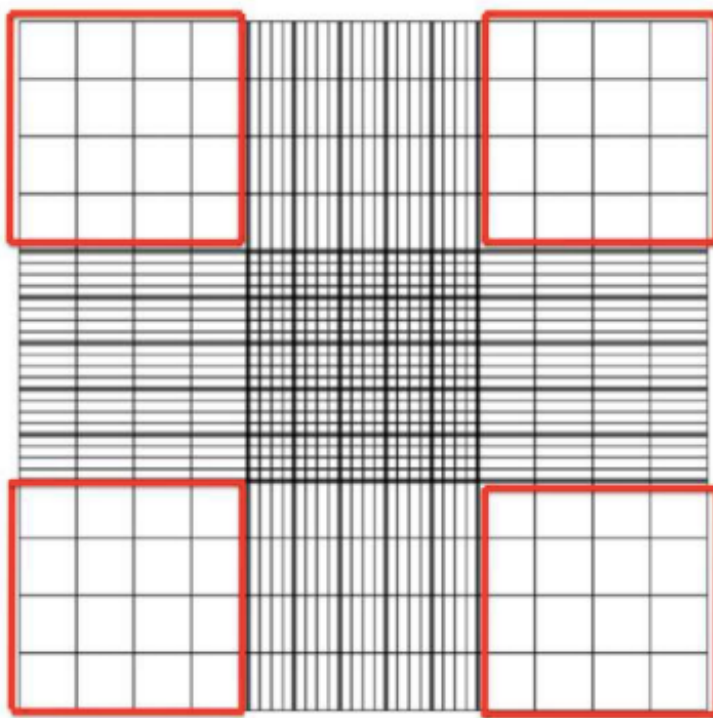


Figure 2.1 Schematic representation of the Neubauer haemocytometer counting chamber. Highlighted (in red) the four outer corner squares where the viable cells were counted. Viable cells appeared clear, while dead cells were stained with Erythrosin B, and appeared red.

2.3. X-rays

Cell irradiations were performed at the Oxford Institute for Radiation Oncology, Department of Oncology, University of Oxford. Irradiations were performed using a Comet MXR-321 x-ray tube with

a Gulmay MP1 controller producing X-rays with an energy of 250kVp, a current of 12.0mA and half value layer (HVL) of 0.997 mmCu. Cells were exposed to either 0.1 Gy or 2 Gy X-rays at a dose rate of 0.545 Gy/minute. Flasks were all placed horizontally on the irradiation platform and were exposed to the X-rays from beneath. Sham-irradiated flasks were taken to the oncology department as well, however they were not exposed to X-rays. After irradiation cells were taken back to Oxford Brookes University and incubated at 37°C, 5% CO₂ until any subsequent analysis.

2.4. Cell viability assay

A viability assay is used to measure the ability of cells to maintain or recover viability. The Muse™ Count & Viability Reagent (Millipore, MCH100102) was utilized to measure the viability of all cell lines throughout the time course experiments. The Muse™ Count & Viability Reagent differentially stains viable and dead cells based on their permeability to two DNA-binding dyes. The first, a DNA-binding dye that stains the nucleus of dead cells that have lost their membrane integrity, thus allowing differentiation from the non-stained live or ‘viable’ cells, as shown in Figure 2.2 (below). The second, a membrane-permeant DNA dye that stains all cells with a nucleus, thus labelling them as ‘nucleated’. The Muse™ Cell Analyzer then counts the stained nucleated events and uses the cellular size properties to differentiate free nuclei and cellular debris from cells in order to calculate an accurate cell count, also shown in Figure 2.2.

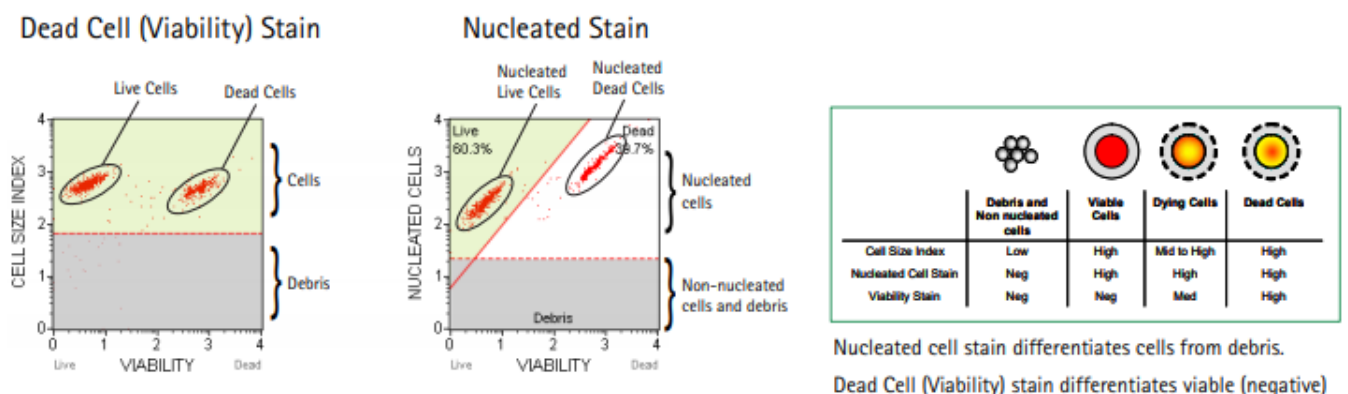


Figure 2.2 Plots as shown on the The Muse™ Cell Analyzer.

A) Muse plot that allows elimination of debris based on size using gate marker (Dashed red line). B) Muse plot that also allows elimination of debris/cells without a nucleus with gate marker, and also has an angled marker (viability discriminator, red angled line) that separates live cells from dead cells. (Figure 2.2 is taken from Millipore, 2013).

Flasks were seeded at 1.5×10^6 cells, and following trypsinization (at appropriate time point) 20 μ l of cell suspension was added to 180 μ l Count & Viability reagent (1:20 dilution) and left to sit for 5 minutes before running on the flow cytometry based Muse™ Cell Analyzer. To ensure the functionality of the system, one aliquot at the start of each replicate was taken from the control group and used as a positive control (treated with 1% hydrogen peroxide (H_2O_2) (Sigma, H1009), five minutes).

2.5. Genomic DNA extraction

All cellular genomic DNA extractions were completed using the Wizard Genomic DNA Purification Kit (Promega, A1125). DNA was extracted as per the Manufacturer's instructions. Flasks were seeded at 1.5×10^6 cells, and at appropriate time points, cells were harvested as usual and collected in 10 ml fresh media. Next, cell suspension was transferred to 15 ml falcon tube and centrifuged at RT for 5 minutes at 300 x g. Supernatant was then discarded and cells re-suspended in 5 ml PBS, followed by a second 300 x g centrifugation step for 5 minutes at RT. Next, supernatant was discarded and re-suspended in 1 ml PBS. Cell suspension was then transferred to a 1.5 ml Eppendorf using a 200 μ l pipette, followed by a centrifuge spin at RT for 20 seconds at 14,000 x g. Cell suspension was then vortexed to re-suspend cells, and 600 μ l of Nuclei Lysis Solution (from kit: A794) was added and vortexed thoroughly to lyse the cells. Next, 3 μ l of RNase solution (from kit: A797) was added, followed by vortexing and inverting 2- 5 times. The sample was then incubated on heating block for 15 minutes at 37°C. Following incubation, the sample was allowed to cool to RT for 10 minutes, followed by an addition of 200 μ l of Protein Precipitation Solution (from kit: A795). The sample was then vortexed vigorously at high speed for 20 seconds, mixed for 45 seconds in total (by flicking, inverting, and vortex). Sample then chilled on ice for 5 minutes. Next, sample was centrifuged at RT for 9 minutes at 14,000 x g. The precipitated protein forms a tight white pellet, and using a 200 μ l pipette the supernatant is then carefully transferred (contains the DNA) from the pellet to a clean 2 ml Eppendorf containing 600 μ l RT Ethanol, Absolute (200 Proof), Molecular Biology Grade (Fisher

BioReagents™: 10644795). Next, the solution was gently mixed by inversion until the white thread-like strands of DNA form a visible mass. Following inversion, the sample was centrifuged at RT for 3 minutes at 14,000 x g, and then the supernatant is carefully discarded. Next, 600 µl of RT Ethanol Solution 70%, Molecular Biology Grade (Fisher BioReagents™: 15420665) was added to the visible pellet and then the tube was gently inverted several times to wash the DNA. Next, the sample was centrifuged at RT for 2 minutes at 14,000 x g and the ethanol supernatant was carefully removed. The tube was then inverted on clean absorbent paper and subsequently the pellet was dried for 5 minutes on a heating block at 37°C. Finally, the DNA was eluted in 100 µl of DNA Rehydration Solution (from kit: A796), and incubated overnight at 4°C. Samples were then labelled and stored at -20°C until further analysis.

2.5 Extracellular vesicle extraction from cell media

Cells were grown in 175 cm² flasks (1T75) (6 x 10⁶ cells/flask, unless indicated otherwise) 24 hours prior to X-irradiation. Immediately before irradiation, supplemented media was removed from all flasks and was replaced with serum-free media. 48 hours post irradiation, media was harvested from irradiated or unirradiated cells. Following removal of cellular debris at 300 x g, the supernatant was then centrifuged at 2,000 x g for 15 minutes to separate apoptotic bodies. Next, the supernatant was centrifuged at 4°C at 10,000 x g to exclude larger vesicles (microvesicles). The supernatant was then centrifuged at 120,000 x g at 4°C in a 70 Ti fixed angle rotor (Beckman, 337922) in a Beckman Coulter Optima LE-80K ultracentrifuge for 90 minutes at 4°C. The exosome pellets of each group were pooled in 500 µL filtered PBS.

2.6. DNase I treatment of exosomes

To reduce external DNA contamination prior to DNA extraction, exosomes were treated with DNase I (Promega, M6101). Each pellet was incubated with 10 µL of DNase I (1 unit/µL) at 37 °C for 30 min. Subsequently, 50 µL of DNase stop solution (Promega, M199A) was added to each sample and they were then heated in a water bath at 65 °C in a water bath for 5 min. Next, the pooled exosome

pellet was washed in 30 ml sterile PBS, and a second step of ultracentrifugation was performed at 120,000 g at 4°C for 90 min. After aspiration of the supernatant, the exosome pellet was then thoroughly resuspended in 200 µL sterile PBS and the 120,000 x g spin repeated to wash the exosome pellet of contaminants.

2.7. Exosomal DNA extraction

DNA was extracted from exosomes using the QIAamp DNA mini kit (QIAGEN, 51304) following the manufacturer's protocol for cultured cells, with the following modifications: Lysis step was performed at 56°C for 1 hour, and the final elution step was performed in duplicate with 25 µL elution buffer (Buffer AE, from kit: 190777), and incubated for 5 minutes at room temperature before each centrifugation spin. DNA was then stored at -20°C until further analysis.

2.8. Statistical Analysis

Data were analyzed using the SPSS Software or Excel, and for all comparisons a significance level of $p < 0.05$ was adhered to. All irradiated groups were first statistically tested for normality using the Kolmogorov-Smirnov normality test. Error bars were calculated by using the standard error of the mean ($\pm$ SEM).

Chapter 3 The role of retrotransposition in the induction of Non-Targeted Effects of Ionizing Radiation

3.1. Introduction

Humans are exposed to radiation daily whether it is from natural background radiation (such as radon found in the air), or from man-made sources (such as medical imaging with X-rays). Ionizing radiation (such as X-rays, gamma rays, and alpha particles) is capable of altering the structural formation of an atom, and therefore contains much more energy than non-ionizing radiation sources (such as ultraviolet and visible light). When ionizing radiation is applied to the treatment of cancer (radiotherapy), these higher-energy sources are used as they are capable of damaging the targeted cancerous tissue, therefore leading to cell death or other types of damage such as chromosomal aberrations (structural alterations to the chromosome) and mutations (Baulch, 2019). However, as cells are studied further after the initial radiation exposure, an alternative model has arisen from the effects that are observed post-irradiation and as an indirect result of radiation damage. These effects are known as Non-Targeted Effects (NTE) and refer to genomic instability (GI, genetic changes, such as mutations and chromosomal aberrations, detected in the descendants of the cells which were originally irradiated) and the bystander effect (BE, non-irradiated cells that have had some communication with irradiated cells and as a consequence they exhibit cellular effects such as DNA-damage and chromosome alterations) (Kadhim et al., 2013).

About 45% of human DNA is comprised of transposable elements (TEs) (Cordaux and Batzer, 2009). TEs are referred to as “jumping genes” as they are DNA sequences that can move from one location of DNA to another. Although only a small proportion (less than 0.05%) of these transposable elements (which can be further classified based on size and structure as retrotransposons, specifically the retrotransposon known as LINE-1) are currently still active within human DNA they are capable of mutating the DNA of their hosts (Mills et al., 2007). Insertions, deletions, and mutations caused by retrotransposons have been found to lead to various types of disorders such as

cystic fibrosis, neurofibromatosis, haemophilia, breast cancers and colon cancers (Cordaux and Batzer, 2009; Hancks and Kazazian, 2012). Human DNA is therefore still highly susceptible to being disturbed by the 'jumping' actions of the retrotransposons.

Several studies have shown that ionizing radiation increases the insertion rates of transposable elements, thus highlighting the damaging potential of these agents (Morawetz, 1987; Farkash et al., 2006; Tanaka et al., 2012; Miousse et al., 2017). At present, the mechanism of induction of NTE by ionizing radiation remains elusive, however it is possible that retrotransposons could be the reason for the instability. The aims of this chapter are to investigate the role of retrotransposable elements in the mechanism of radiation-induced GI. In order to achieve this aim, the investigation was focused on the presence of retrotransposition at time points that are relevant to genomic instability. The hypothesis to be tested is if ionizing radiation could cause an increase in LINE-1 retrotransposition, therefore leading to a possible instability within the genome.

3.2. Materials and Methods

3.2.1. Cell lines and irradiation

The following assays were performed (and repeated 3 times each) in three mammalian cell lines HF19 (human primary fibroblasts) and MCF7 (breast adenocarcinoma), and Caco-2 (colorectal adenocarcinoma) following radiation exposure at therapeutic (2 Gy) and diagnostic (0.1 Gy) doses. Cells were seeded at 1.5×10^6 in a T75 flask 24 hours prior exposure to irradiation. Cells were then exposed to X-irradiation (as per section 2.3) and early time points (to assess immediate/early response to IR) were evaluated at 1 hour, 10 hour, 24 hour, 48 hour, and 1 week post-irradiation, while delayed time points for GI were assessed at 2 weeks, 4 weeks, and 6 weeks post-irradiation, unless stated otherwise (such as in micronuclei analysis).

3.2.2. Quantitative PCR (qPCR)

The quantitative PCR (qPCR) analysis was conducted in order to test a hypothesis that ionizing radiation would cause a response in LINE-1 retrotransposition that contribute to the mechanism of NTE/RIGI. An increased expression of LINE-1 elements may lead to genomic instability in cells that are directly affected as well as their progeny. Prior to qPCR, DNA samples were extracted from each time point (as per section 2.5) and quantified using the Nanodrop. For qPCR, SYBR Green mastermix (Bio-Rad, 1725271) was used with a 1:1 mastermix comprised of equal amounts of DNA from each sample and 10 uM of each primer. $\Delta\Delta C_t$ method was used to observe the differences as the target sample (sham and irradiated LINE-1) is relative to the reference sample (sham and irradiated beta-2-microglobulin), normalized to the reference gene (beta-2-microglobulin, B2M). Primers utilized throughout the experiments for qPCR amplification in following chapters are as follows: LINE1Orf1, 5'-ctcggcagaaacctacaag-3' and 5'-ccatgttagcgcttccttc-3' (Jones et al., 2013); B2M, 5'-gctgggtagctctaacaatgtattca-3' and 5'-ccatgtactaacaatgtctaaaatggt-3' (Phillips et al., 2014).

3.2.3. Mironuclei Assay

Micronuclei (MN) provide a convenient and reliable method to measure chromosome breakage and loss. MN are formed as a result of cell division whenever a chromosome or a fragment of a chromosome is not fused into either daughter nuclei in anaphase, and is a sign of chromosomal instability. The small nucleus is therefore termed a 'micronucleus' and analyzing chromosome aberrations by scoring MN is a common method used to measure radiation sensitivity *in vitro* (Jdey et al., 2017).

At each time point post-irradiation, cells were cultured into T25 flasks and grown to 70-75% confluency. Cytochalasin B (1 µg/ml) was then added to inhibit cytokinesis, and flasks were incubated for a further 40 hours. Cytochalasin B (Sigma, C6762) inhibits mitotic spindle fibre formation, thus halting cytoplasmic division. Cells are therefore identified by their binucleated appearance after their first nuclear division. After 40 hours incubation time with cytochalasin-B, the media from each sample per group was collected and saved in a sterile universal tube. Flasks were washed twice with PBS, the first wash added to the universal and the second wash discarded. Cells were then detached by trypsinization for 60 seconds, and the trypsin solution added to the universal contents. Dissociated cells were then collected with the universal contents, and all added collectively to the universal. Cells in universal tubes were then centrifuged at 200 x g for 10 minutes, and supernatants discarded. Remaining pellets were resuspended, and then 1ml of hypotonic solution (pre-warmed KCL: 0.55g potassium chloride (Sigma, P3911) and 100 ml ultrapure water kept at 37 °C in a water bath) was added drop-wise, and then another 10 ml KCl was added. Tubes were then incubated for 5 minutes in the 37 °C water bath, followed by treatment of 3 drops of 25% glacial acetic acid in methanol (a 3:1 fixative). Tubes were inverted once and then centrifuged at 200 x g for 10 minutes. Supernatants were then discarded, followed by a thorough resuspension of the cell pellet. 1 ml of 3:1 fixative was then added drop-wise to the tube, and topped up with an additional 10 ml of fixative. Cells were then left at RT for 10 minutes, followed by another centrifugation at 200 x g for 10 minutes; supernatants were discarded and pellets resuspended in 1 ml of 3:1 fixative. The

fixed cell suspension was then dropped onto labelled and de-greased microscope slides. Finally, slides were left to dry at RT for 24 hours prior to staining (Figure 3.1).

Slides were then stained with 0.00625% acridine orange (Sigma, A6014) by adding 0.0031g acridine orange to 50 ml pH 6.8 buffer in a falcon tube and allowing slides to stain for 25 seconds. Acridine orange is a cell-permeable nucleic acid selective fluorescent dye that allows for visualization of nuclear changes. After staining, the slides were moved for 5 seconds to a buffer jar. The slides were then left to dry at room temperature (RT) before the commencement of visualization and scoring with a fluorescent microscope. MN were scored only in binucleated cells, and 500 binucleated cells were scored per group. The data was presented as the percentage of binucleated cells with micronuclei (%Binucleated +MN). For statistical analysis, the MN data was shown to be normally distributed and was further subjected to t-test, p values ≤ 0.05 were considered statistically significant. Error bars were calculated by using the standard error of the mean ($\pm$ SEM).

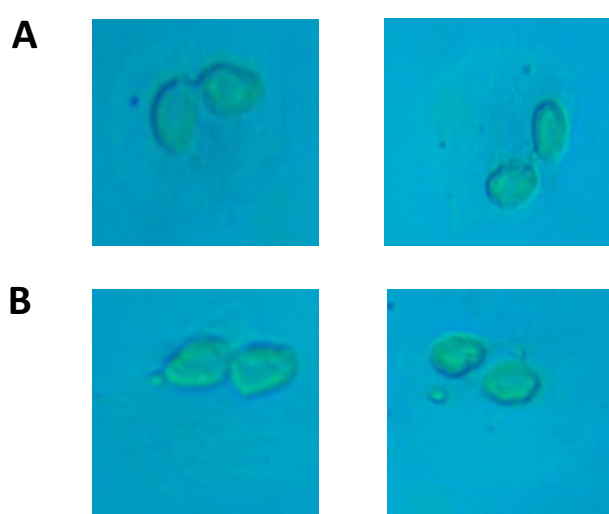


Figure 3.1 Images of binucleated cells for scoring:
A) Binucleated cells without micronucleus, B) Binucleated cells with micronucleus.

3.3. Results

3.3.1. HF19 cell line

3.3.1.2 Viability in HF19 cell line

The Muse™ Count & Viability Reagent (Millipore) was utilized to measure the viability of the HF19 cell line throughout the time course of the experiment (as per section 2.4). The statistics on the software show the percentage of viable cells as the reagent consists of a DNA-binding dye that stains the nucleus of dead cells that have lost their membrane integrity.

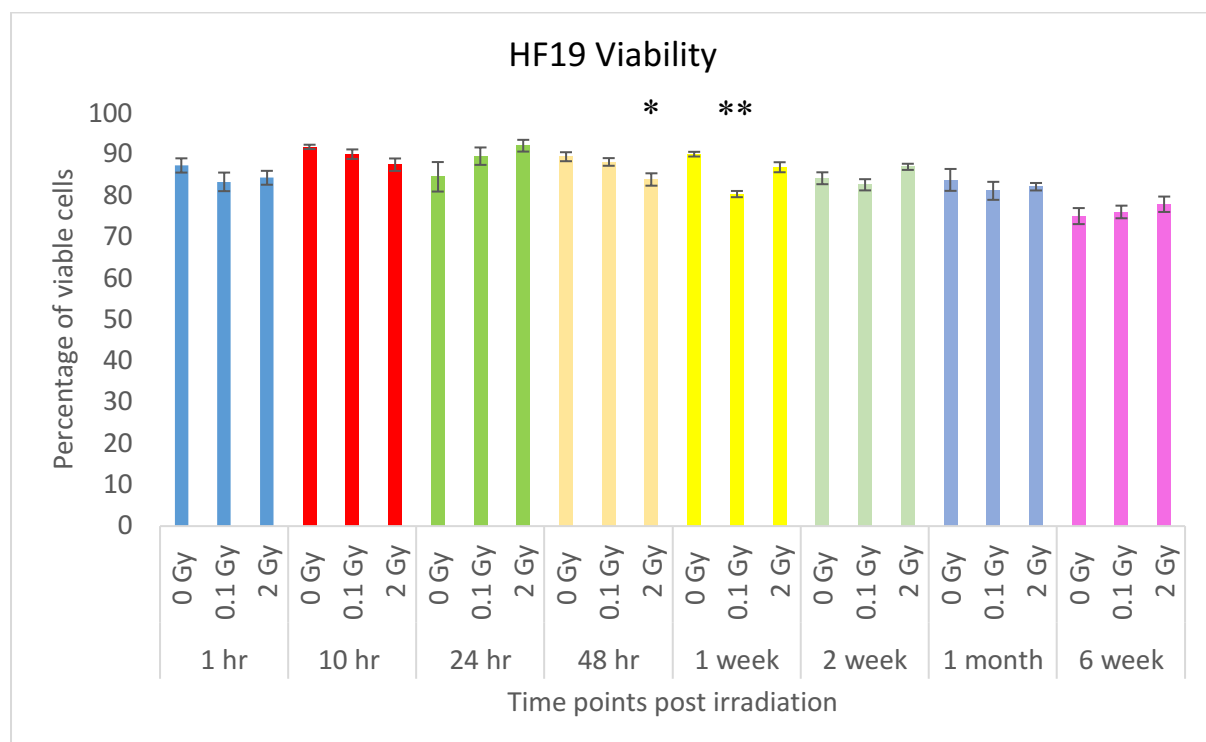


Figure 3.2 Percentages of viable HF19 cells 1 hr, 10 hr, 24 hr, 48 hr, 1 week, 2 week, 1 month, and 6 weeks after exposure to 0.1 Gy and 2 Gy X-rays. Cell viability was measured using the flow cytometry based Muse™ system, $n=3$ per group, $*p<0.05$, $**p<0.01$, t -test. Error bars $\pm$ S.E.M. In the HF19 cells significance was found after exposure to 0.1 Gy X-rays at 1 week, and significance was found PI to 2 Gy X-rays at 48 hrs.

Viability percentages of HF19 cells after exposure to X-rays at 0.1 Gy and 2 Gy at various time points can be visualized in Figure 3.2. A significance in cell viability was observed in both groups of irradiated cells throughout the time course of the experiment. In the 0.1 Gy group at 1 week post irradiation viability was significantly decreased ($p<0.01$) compared to the control. A significant

reduction in cell viability ($p < 0.05$) was observed in the 2 Gy irradiated cells at 48 hours. The 2 Gy X-irradiated cells showed an early response to IR in terms of viability, with the later time points showing no significance when compared to the control cells. This observation suggests that cell regrowth is occurring at 48 hours post irradiation.

3.3.1.2 LINE-1 Retrotransposition in HF19 cell line

The quantitative real-time PCR (qPCR) analysis was conducted in order to test a hypothesis that ionizing radiation would cause a response in LINE-1 retrotransposition that contribute to the mechanism of NTE/RIGI. An increased expression of LINE-1 elements may lead to genomic instability in cells that are directly affected as well as their progeny. Genomic DNA samples were collected from each time point (replicated 3 times) and qPCR was utilized to detect retrotransposition. Analyzation of the data employed the use of $\Delta\Delta C_t$ method and plotted results as fold change relative to the housekeeping gene B2M.

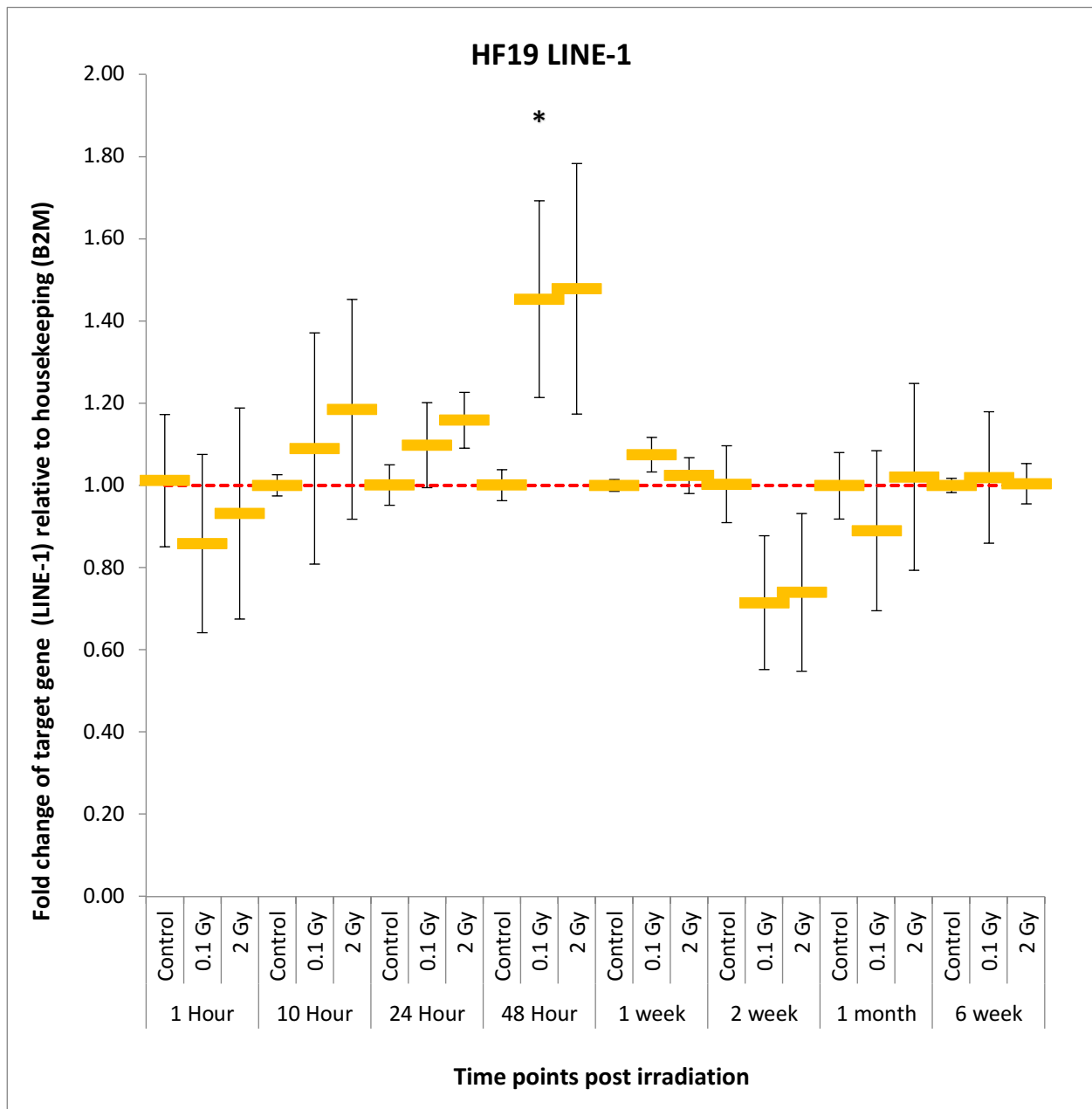


Figure 3.3 The HF19 cell line is assessed for LINE-1 levels at various time points following X-ray exposure at a diagnostic (0.1 Gy) dose therapeutic (2 Gy) doses. $n=3$ per group, LINE-1 levels are relative to B2M housekeeping gene, t-test, error bars $\pm$ S.E.M. * $p<0.05$, compared to the control group (0 Gy). In the HF19 cells significance was found at 48 hours in the 0.1 Gy group.

Figure 3.3 shows the changes in LINE-1 throughout the time course of the experiment in regards to the HF19 primary cell line. While there are some slight fluctuations in LINE-1 levels throughout the experiment, a significant increase ($p<0.05$) is found in the 0.1 Gy group 48 hours post exposure to IR.

However, after 1 week the LINE-1 levels returned to baseline without a continuation of increase in retrotransposition.

3.3.1.3 MN in HF19 cell line

In order to determine chromosomal damage in response to IR in HF19 cells, the micronucleus assay was utilized in this chapter. Early time points of 24 hours and 48 hours were assessed to determine any initial damage, while a late time point of 2 weeks was assessed to observe any prolonged effects in the progeny of the initially irradiated cells. Five hundred binucleate cells were scored in each experiment, and each experiment replicated three times. Data is presented as a percentage of binucleated cells containing micronuclei (%MN/Binucleated).

While there was no chromosomal damage 24 hours post IR detected in both the irradiated groups, 48 hours post IR shows a significant induction of micronuclei in both the 0.1 Gy and 2 Gy irradiated groups compared to the control ($p < 0.001$) (Figure 3.4). Interestingly, these results complement the statistically significant LINE-1 results from 48 hours in the previous section (section 3.3.1.2), and while speculative it can be suggested that the increase in retrotransposition could be the cause of the GI at this time point.

Two weeks post IR shows that there was a significant induction of chromosomal damage ($p < 0.01$) observed within the progeny of the 2 Gy irradiated cells, while the 0.1 Gy cells show no induction of micronuclei formation. These results suggest an enhanced and (delayed RIGI) response may be possible at a higher dose.

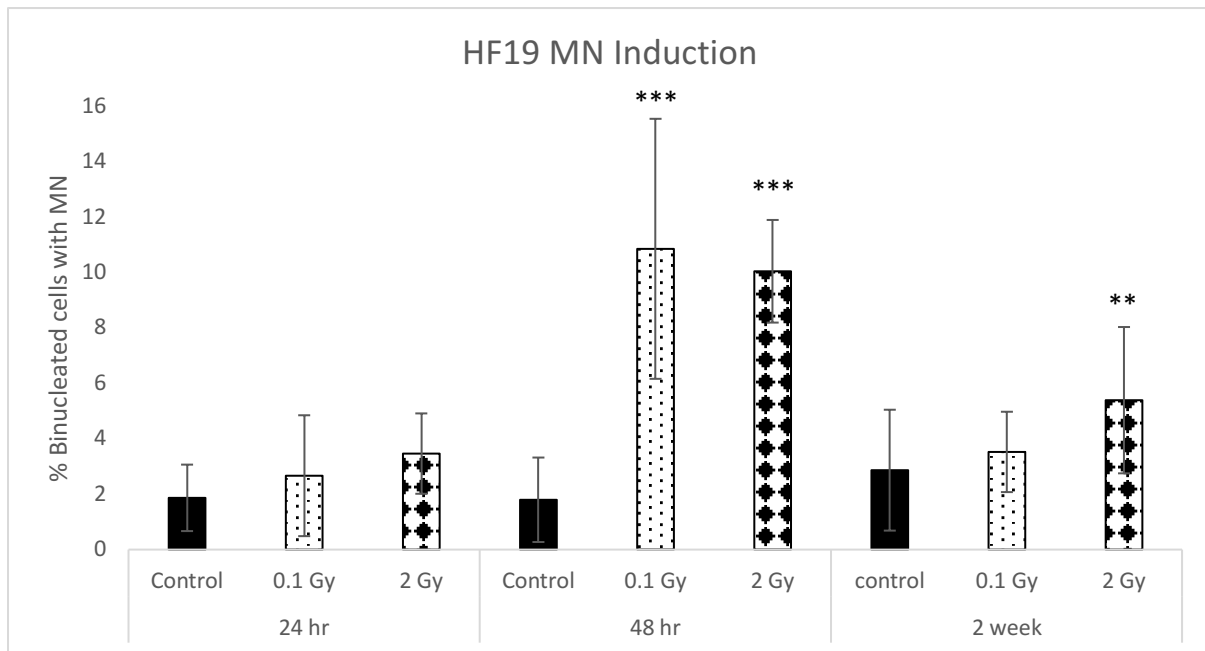


Figure 3.4 The HF19 cell line is assessed for MN induction at early time points (24 hours and 48 hours) and late time point (2 weeks) following X-ray exposure. $N = 3$ biological replicates per group, $**p < 0.01$ $***p < 0.001$, t test. Error bars $\pm$ S.E.M. In the HF19 cells significance was found at 48 hours post IR in the 0.1 Gy and 2 Gy groups, as well as 2 weeks post IR in the 2 Gy group.

3.3.2. MCF7 cell line

3.3.2.1 Viability in MCF7 cell line

The percentages of both dead and viable cells following 0.1 Gy and 2 Gy doses of X-irradiation were measured using The Muse™ Count & Viability Reagent (Millipore). Viability percentages of MCF7 cells throughout the time course of the experiment are shown in Figure 3.5. While slight fluctuations are apparent throughout the time course of the experiment, these results show the possible resistance of the MCF7 breast cancer cells (in terms of viability) immediately and several weeks post-irradiation.

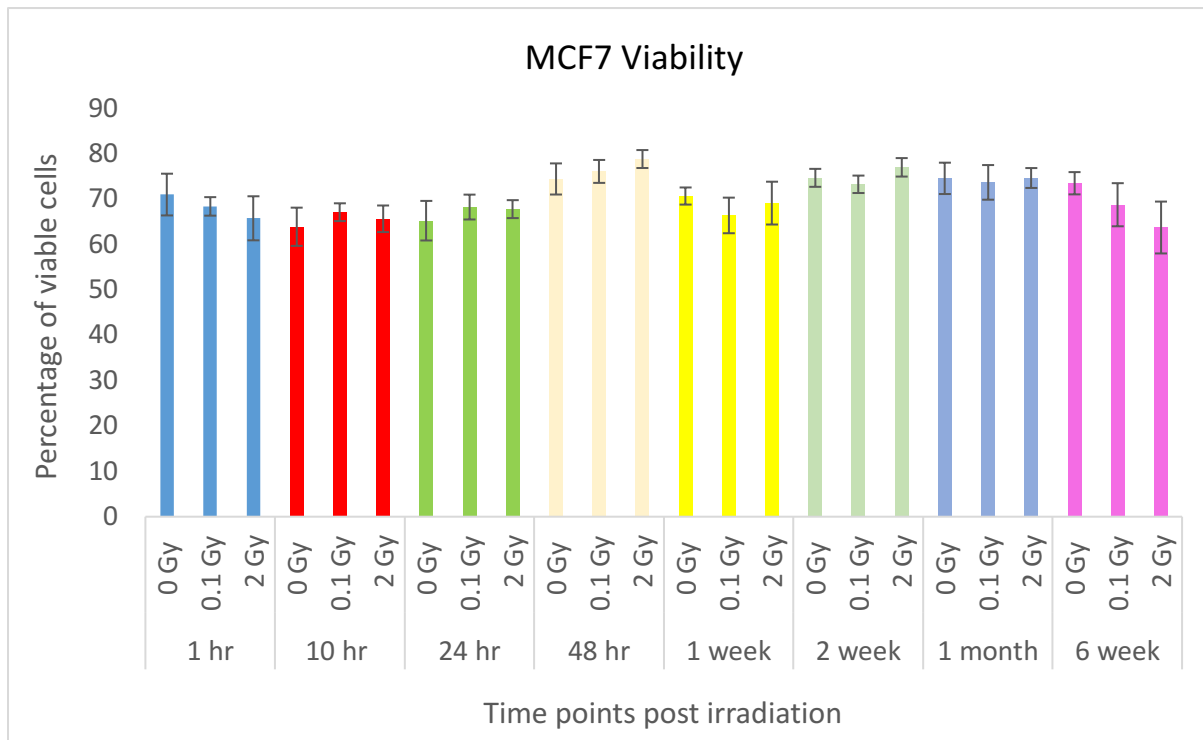


Figure 3.5 Percentages of viable MCF7 cells 1 hr, 10 hr, 24 hr, 48 hr, 1 week, 2 week, 1 month, and 6 weeks after exposure to 0.1 Gy and 2 Gy X-rays.

Cell viability was measured using the flow cytometry based Muse™ system, $n=3$ per group, t -test. Error bars $\pm$ S.E.M. In the MCF7 cells there was no significance found within the viability.

3.3.2.2 LINE-1 Retrotransposition in MCF7 cell line

Figure 3.6 shows the changes in LINE-1 throughout the time course of the experiment in regards to the MCF7 breast cancer cell line. Interestingly, all 0.1 Gy groups show a trend to increase in levels of LINE-1 throughout the time course of the experiment (with significance found at 1 month post IR), apart from the 1-week time point. The 2 Gy group seems to fluctuate throughout the time course of the experiment in regards to LINE-1 retrotransposition, however no significance was found within the data. The large error bars in Figure 3.6 are also quite prominent and are indicative of the variability of the data.

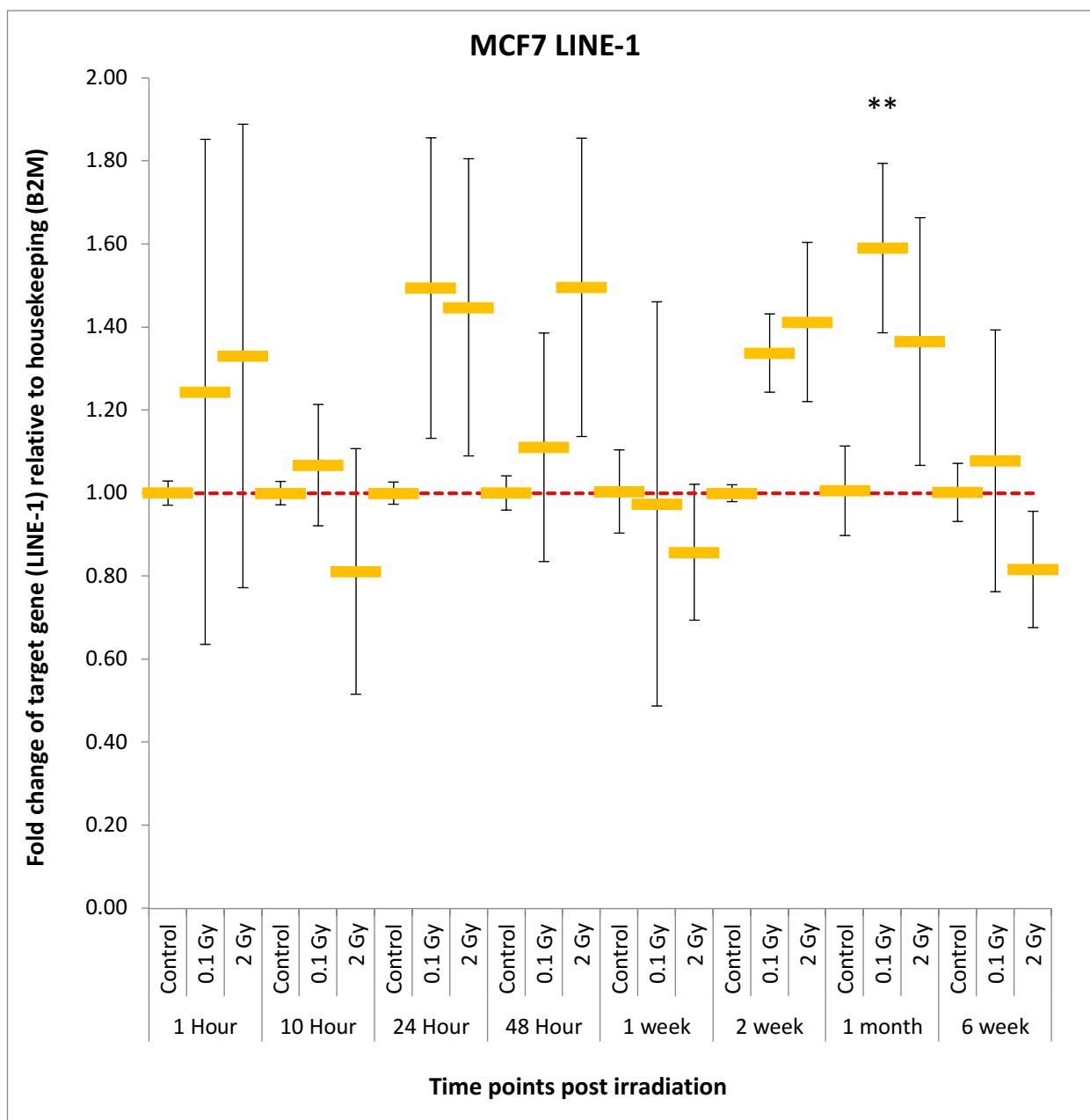


Figure 3.6 The MCF7 cell line is assessed for LINE-1 levels at various time points following X-ray exposure at therapeutic (2 Gy) and diagnostic (0.1 Gy) doses. $n=3$ per group, LINE-1 levels are relative to housekeeping B2M, t-test, error bars $\pm$ S.E.M., $**p<0.01$. In the MCF7 cell line significance was found in the 0.1 Gy X-irradiated group 1 month post IR (in terms of LINE-1 levels).

3.3.2.3 MN in MCF7 cell line

The MCF7 cell line was analyzed in response to a diagnostic dose of X-rays (0.1 Gy) and to a clinically relevant dose of X-rays (2 Gy), and compared to the sham-irradiated samples. The micronucleus assay allowed for the analysis of chromosomal damage at three separate time points (24 hours, 48 hours, and 2 weeks) post-irradiation to monitor the possible changes with the initial induction of genomic instability, the progression of GI induction, and the delayed response of GI. The data are displayed as a percentage of binucleate cells with micronuclei over total binucleate cells in sham and irradiated MCF7 cells.

Results shown in Figure 3.7 show that a dose of 2 Gy X-rays induces a manifestation of micronuclei 24 hours and 48 hours post IR ($p < 0.001$) when compared to the control. However, after 2 weeks no induction is observed and therefore there is no delayed GI induction observed. This observed reduction could be due to either DNA repair of the simple damage and/or eliminations of those cells that accumulated lethal damages by apoptotic processes.

The 0.1 Gy cells show no induction of micronuclei formation throughout the time course of this experiment in the MCF7 cell line. This indicates that the induction of chromosomal damage (MN, specifically) by low LET X-rays is dose dependent.

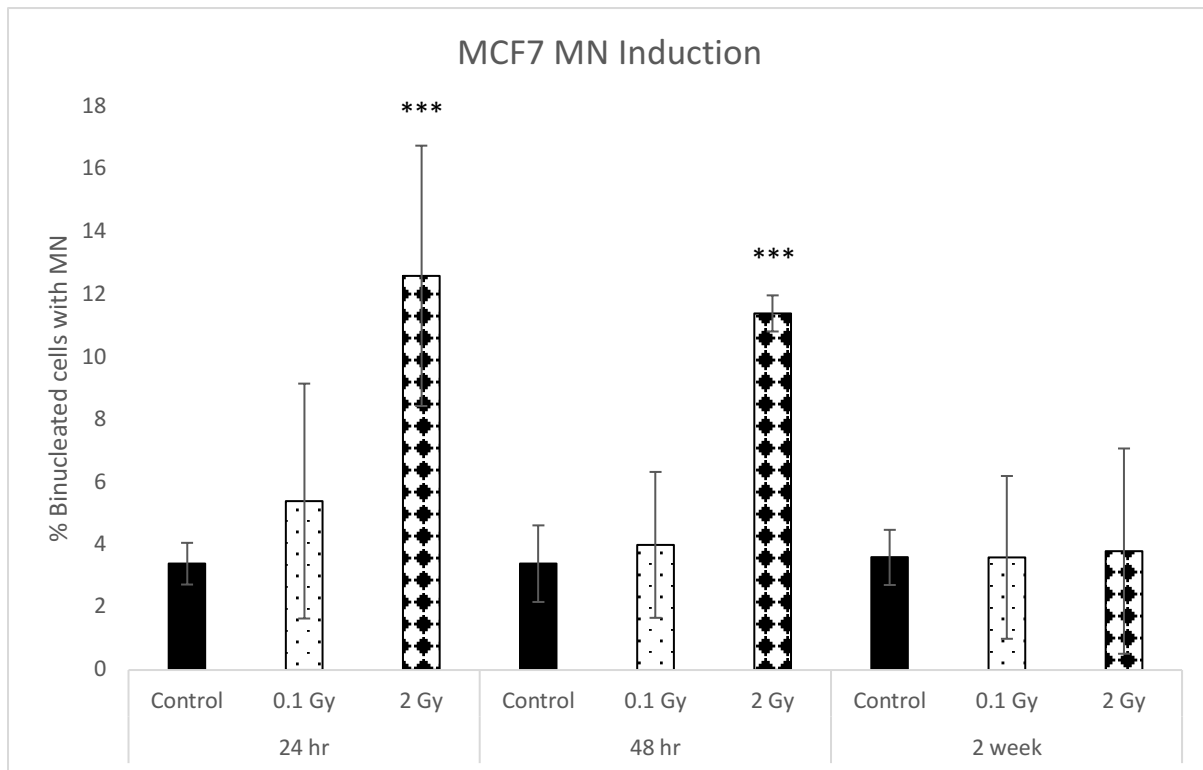


Figure 3.7 The MCF7 cell line is assessed for MN induction at early time points (24 hours and 48 hours) and late time point (2 weeks) following X-ray exposure.

$N = 3$ biological replicates per group, *** $p < 0.001$, t test. Error bars $\pm$ S.E.M. In MCF7 cells significance was found at 25 hours and 48 hours post IR in the 2 Gy X-irradiated groups.

3.3.3. Caco-2 cell line

3.3.3.1 Viability in Caco-2 cell line

The Muse™ Count & Viability Reagent (Millipore) was utilized to measure the viability of the Caco-2 cell line at 1 hour, 10 hour, 24 hour, 48 hour, 1 week, 2 weeks, 1 month, and 6 weeks post exposure to 0.1 Gy and 2 Gy doses of X-irradiation. The results of the Caco-2 cell line viability is shown in Figure 3.8. The results of the Caco-2 cell line viability time course experiment are shown in Figure 3.8. While there is a visual fluctuation of data (notably at 10 hours post IR in the 0.1 Gy group and 1 month post IR in both irradiated groups), results show no significance and highlight the radioresistance of the Caco-2 cell line in terms of viability.

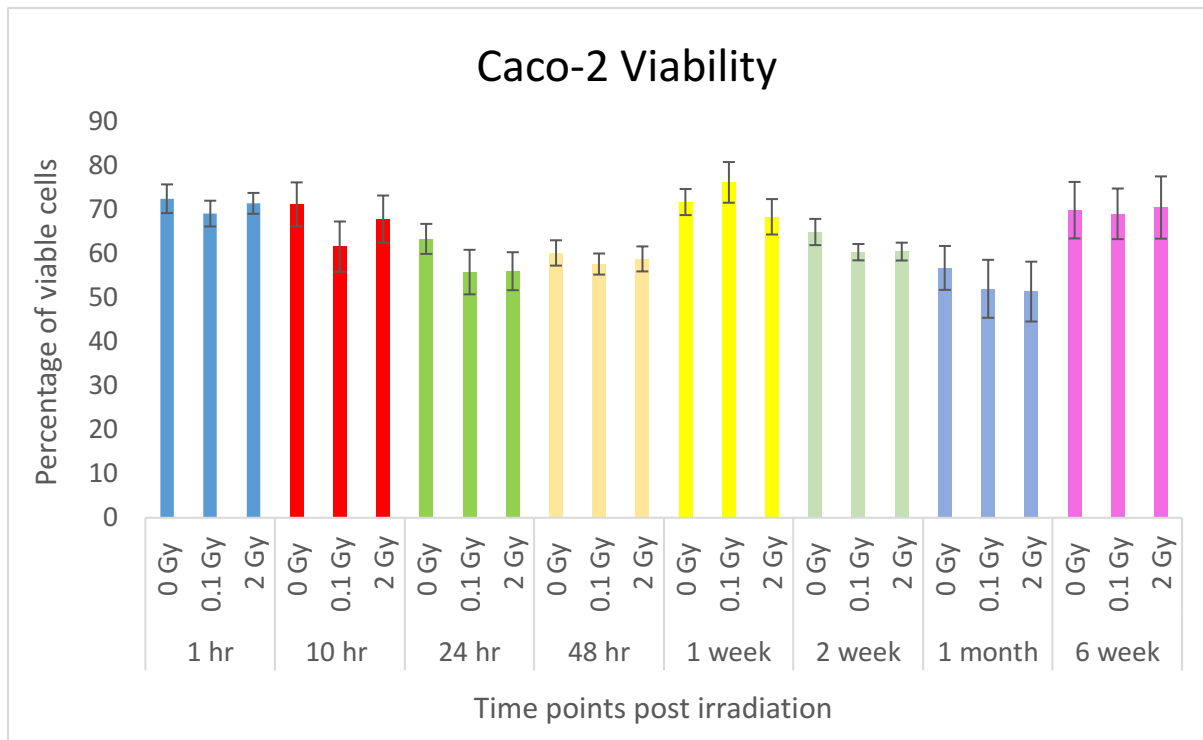


Figure 3.8 Percentages of viable Caco-2 cells 1 hr, 10 hr, 24 hr, 48 hr, 1 week, 2 week, 1 month, and 6 weeks after exposure to 0.1 Gy and 2 Gy X-rays. Cell viability was measured using the flow cytometry based Muse™ system, n=3 per group, t-test. Error bars $\pm$ S.E.M. In the Caco-2 cells there was no significance found within the viability.

3.3.3.2 LINE-1 Retrotransposition in Caco-2 cell line

Figure 3.9 shows the changes in LINE-1 throughout the time course of the experiment in regards to the Caco-2 colorectal adenocarcinoma cell line. A significant increase ($p < 0.05$) is found at 48 hours post exposure to 0.1 Gy X-rays. This indicates an early response of retrotransposition in the Caco-2 cell line. LINE-1 levels remain at baseline for the remainder of the time course experiment in the 0.1 Gy X-irradiated progeny. While LINE-1 levels fluctuate throughout the experiment for the 2 Gy group, no significance is found.

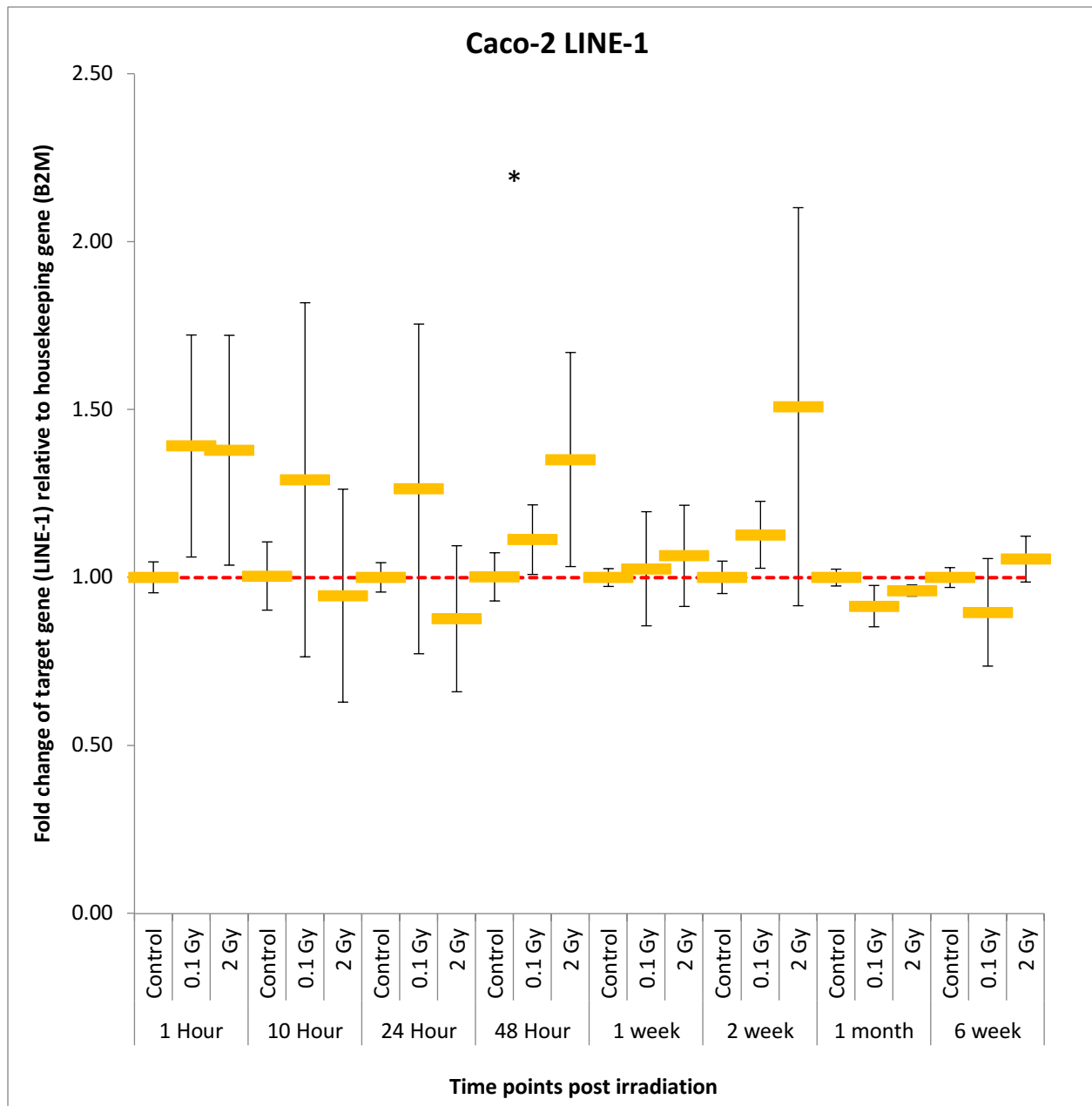


Figure 3.9 The Caco-2 cell line is assessed for LINE-1 levels at various time points following X-ray exposure at a diagnostic (0.1 Gy) dose therapeutic (2 Gy) doses. $n=3$ per group, LINE-1 levels are relative to housekeeping gene B2M, t-test, error bars $\pm$ S.E.M. * $p<0.05$, compared to the control group (0 Gy). In the Caco-2 cells significance was found at 48 hours post IR in the 0.1 Gy X-irradiated group (in terms of LINE-1 levels).

3.3. MN in Caco-2 cell line

Micronuclei induction post exposure to 0.1 Gy and 2 Gy X-rays at 24 hr, 48 hr, and 2 weeks post IR is shown in Figure 3.10. The graph shows that there is no radiation-induced MN in the Caco-2 cells at the respective time points when compared to the sham-irradiated samples. This highlights the radioresistance of the Caco-2 cell line in terms of MN induction. While the LINE-1 results showed an increase in retrotransposition in the 0.1 Gy group 48 hours post IR, the MN analyses showed no induction of MN. Therefore, it is likely that an increase in retrotransposition by IR does not cause a response in MN, leading to GI (in this cell line under these specific conditions).

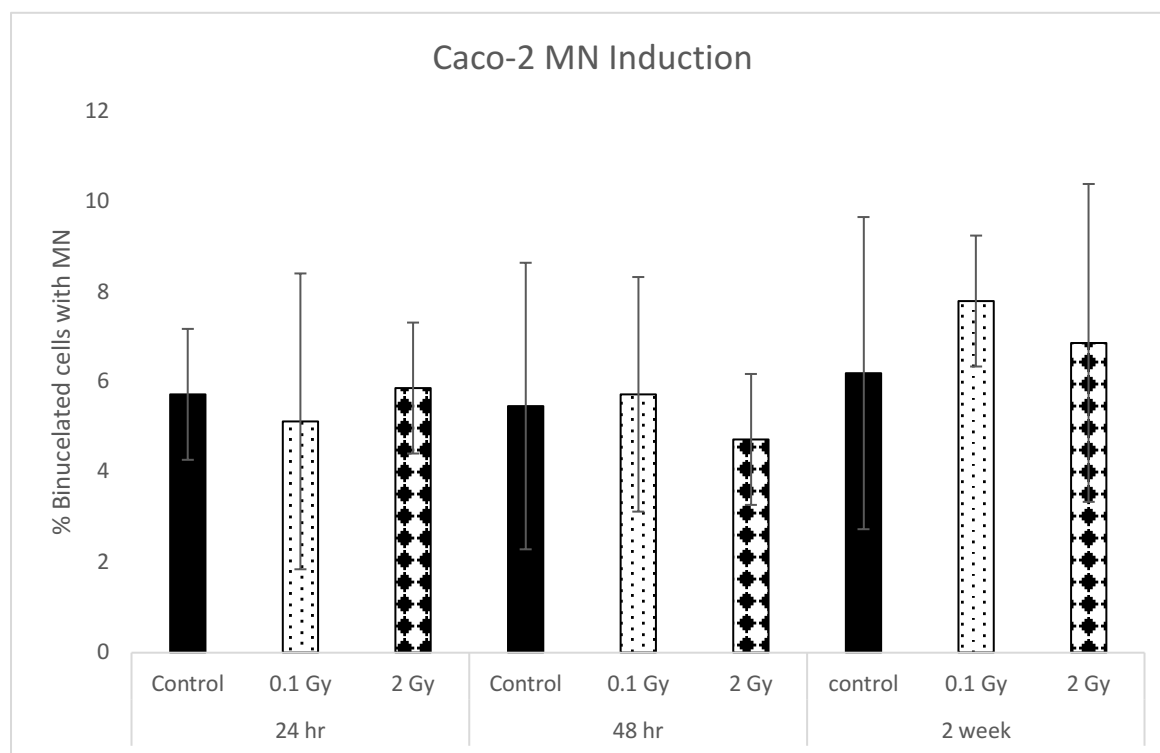


Figure 3.10 The Caco-2 cell line is assessed for MN induction at early time points (24 hours and 48 hours) and late time point (2 weeks) following X-ray exposure. $N = 3$ biological replicates per group, t test. Error bars $\pm$ S.E.M. In the Caco-2 cells there was no significance found within the induction of MN.

3.4. Discussion

Radiation-induced genomic instability (RIGI) refers to the increased acquisition rate of alterations or mutations in the genome being transferred to progeny cells and can manifest as delayed gene mutations, micronucleus formation, chromosomal aberrations, and gene amplifications (Kadhim et al., 2013). While these responses to IR in the progeny of cells have been well documented, the underlying mechanisms behind RIGI remain elusive. LINE-1 insertions are known to cause major genomic rearrangements that can lead to genomic instability (Lee et al., 2012). As yet a mechanism to account for RIGI is not completely understood, however it is possible that LINE-1 could be driving these phenomena promoting the destabilization of the genome due to its random insertion nature, and the appearance of GI. This chapter investigated the LINE-1 retrotransposition response to IR in three different cell types at time points relevant to GI, utilizing the cell viability assay (examining cytotoxicity) and micronucleus assay (assessing micronuclei induction). Therefore, this study was established to investigate if IR would cause an increase in LINE-1 retrotransposition, thereby possibly causing an instability within the genome.

3.4.1. Diagnostic and therapeutic doses of X-irradiation on HF19, MCF7, and Caco-2 cells has varying impacts on viability

As a reduction in cell viability induced by ionizing radiation is commonly used as a standard regarding radiation-induced cytotoxicity (Joo et al., 2012), the viability was assessed at both early and late time points in all three cell lines. Short-term cell viability (1 hour, 10 hour, 24 hour, 48 hour, and 1 week post-irradiation) and long-term viability (2 weeks, 4 weeks, and 6 weeks post-irradiation) were both examined through the employment of the flow cytometry based Muse™ system.

HF19 primary fibroblasts showed significant effects of cytotoxicity induced by X-radiation after exposure to 0.1 Gy at 1 week. At 48 hours results show a reduction in the percentage of viable HF19 cells compared to the control following 2 Gy X-irradiation. Previous research has shown conflicting results regarding the effects of IR on cell death in non-cancerous cell lines. For example, Qiao *et al.*

(2014) demonstrated a significant decrease in cell viability in normal human fibroblasts 24 hours post exposure to 0 – 2.5 Gy X-irradiated cells. However, another study by Maierhofer *et al.* (2017) shows no significance in cell viability compared to the controls in primary human fibroblasts 6 hr and 24 hr post exposure to 2 Gy and 4 Gy X-rays. In another study, normal human epidermal keratinocytes (NHEK) were exposed to 5 Gy and 10 Gy doses of γ -irradiation, as well as non-ionizing UVB irradiation (5 W/m²). The cytotoxic effects of γ -irradiation and UVB were evaluated 7-11 days post-exposure, and 5 Gy and 10 Gy doses of γ -irradiation generated more than 95% loss of survival, highlighting the impact that higher doses of ionizing radiation can have on cell survival (Petit-Frère *et al.*, 2000). While a higher dose of X-irradiation could potentially show a detrimental effect on cell viability, HF19 shows that both doses of low LET X-rays have significant effects on cell viability in both short and long term analyses in this chapter. Particularly, 2 Gy X-rays demonstrate cell regrowth 48 hours post irradiation with no significance in long term analyses in the HF19 primary cell line.

MCF7 cells also did not show any significant effects of cytotoxicity induced by 0.1 Gy and 2 Gy doses of X-radiation throughout the time course of the experiment. This is found in concordance with results from a previous study by Girdhani *et al.* (2009) where interestingly, 2 Gy γ -rays caused a dose-dependent decrease in viability of MCF7 cells after 72 hours of radiation exposure to doses 2, 3, 4, and 5 Gy. However, these results were insignificant when compared to the control. Another study evaluated the effects on cell death of 2 Gy, 4 Gy, and 6 Gy X-rays on MCF7 cells after 24hr, 48 hr, and 72 hours (Fard *et al.*, 2017). While 4 Gy and 6 Gy X-rays caused a significant increase in cell death in all time points, 2 Gy did not show any effect (Fard *et al.*, 2017). Another study showed that only at a high dose of X-irradiation (8 Gy) was radiation shown to significantly affect the viability of MCF7 cells (Sazgarnia *et al.*, 2013). These results are suggestive of a dose-dependent effect on viability in MCF7 cell line, and that they are possibly resistant to any responses below 2 Gy.

Caco-2 cells also did not show any significant effects of cytotoxicity induced by 0.1 Gy and 2 Gy doses of X-radiation throughout the time course of the experiment. Upon further investigation, recent studies have shown a weak effect of radiation on Caco-2 cells with up to 10 Gy X-rays (Morini et al., 2017; Babini et al., 2018). In both of these studies various techniques post exposure to IR were analyzed, and Caco-2 demonstrated a high radio-resistance. In terms of viability, the effect of 2 Gy, 5 Gy, and 10 X-rays after 24 hours and 48 hours was examined in Caco-2 cells, and in both study no increase in cell death was observed (Morini et al., 2017; Babini et al., 2018). Another recent study by Jalili-Firoozinezhad *et al.* (2018) implies that the Caco-2 cell line is not as radiosensitive as other cell lines. Therefore, in the future a cell line that is more susceptible to the effects of radiation would be appropriate in further studies.

Collectively, the viability results in this section suggest that the amount of energy transferred to the three cell lines at 0.1 Gy and 2 Gy X-rays was not sufficient enough for causing cell death.

3.4.2. LINE-1 changes post-irradiation found in all cell lines

As an increased expression of LINE-1 elements may lead to genomic instability in cells that are directly affected as well as their progeny, this experiment was set up in order to test a hypothesis if ionizing radiation would cause a response in LINE-1 retrotransposition, therefore potentially leading to an instability within the genome. Previous studies have suggested that even low levels of LINE-1 activity have the potential to contribute to GI, aging, and cancer, and therefore warrant further investigation (Belancio et al., 2010).

48 hours after exposure to 0.1 Gy X-rays the HF19 fibroblast cell line showed that LINE-1 retrotransposition was significantly increased when compared to the control. While this increase is exhibited at 48 hours post-irradiation, it is not permanent as the following time point (1 week post-exposure) shows LINE-1 levels have returned to baseline. This could be due to the cellular death and elimination of those cells exhibiting high levels of LINE-1. Previous studies have also shown that IR triggers LINE-1 insertion rates into the genome (Farkarsh et al., 2006; Tanaka et al., 2012). A more

recent study has shown that epigenetic alterations may serve as driving forces of radiation-induced carcinogenesis, as loss of LINE-1 DNA hypomethylation in the hematopoietic stem cells of CBA/J mice was observed two months after the original radiation insult (Miousse et al., 2017). Notably, Miousse *et al.* (2017) also showed that effects of IR on LINE-1 DNA methylation are dependent on the type of irradiated cell. While highly speculative, results from this chapter could suggest that 48 hours post exposure to IR stimulates LINE-1 retrotransposition, thus leading to an increase of insertions in the genome, and therefore may serve as driving forces of RIGI. Further experiments (including measuring LINE-1 activation in response to IR and GI assays) at the same time point would need to be conducted in order to prove any possible association. The complementing MN data (increase in chromosomal damage) at the 48-hour time point and its implications regarding RIGI will be discussed in the next section (section 3.4.3).

The MCF7 cell line showed elevated levels of LINE-1 throughout the duration of the experiment, however LINE-1 levels were only found significantly increased at 1 month post exposure to 0.1 Gy X-rays. Interestingly, a previous study used a fractionated dose of 10 Gy γ -irradiation in MCF7 cells which showed no increase in LINE-1 methylation levels 2 weeks and 24 days post exposure to IR (Kuhmann et al., 2011). While this data presented in this chapter represents the pooled means of 3 independent experiments, the error bars are very large, showing the variability of the data from the mean. As the data is highly variable, it suggests that perhaps qPCR is not the most appropriate technique to measure the sensitivity of LINE-1 retrotransposition. It could be possible that other methods of investigating LINE-1 activation, such as LINE-1 methylation or gene expression status, would be more appropriate to measure LINE-1 in response to IR.

Caco-2 cells irradiated with 0.1 Gy X-rays showed an increase in LINE-1 levels 48 hours post-irradiation. However, at the following time points the LINE-1 levels remain close to the baseline in the progeny of the 0.1 Gy X-irradiated cells. No complementing data is found regarding GI through investigation of MN induction.

Results show the activation of LINE-1 retrotransposons by ionizing radiation in HF19, MCF7, and Caco-2 cell lines. Investigation into the mechanisms behind radiation-induced retrotransposition are warranted, however, due to the variability of the LINE-1 results (within the 3 repeated experiments) future directions in this thesis will focus on the characterization of exosomal DNA (exoDNA). ExoDNA has been shown to contain an abundance of genetic information, and findings suggest EVs as a likely means of transmission for the TEs between cells (Balaj et al., 2011).

3.4.3. MN Induction in HF19 and MCF7 cell lines

The MN assay has evolved into a reliable and reproducible method for measuring damage caused by IR by analyzing fragments or whole centric chromosomes or chromosomes resulting from non-repaired or mis-repaired double-strand breaks in anaphase (Gutierrez-Enriquez et al., 2011). Several studies show that the number of radiation-induced micronuclei is correlated with the radiation dose and quality. Collectively, studies have shown that the dose-response curve in the range of 0 – 4 Gy for MN induction from low-LET X-rays or γ -rays is considered to be linear-quadratic (Müller et al., 1996), while high-LET radiations (such as neutrons, protons, α -particles, and heavy ions) show a linear relationship between dose and response and curves are likely to plateau at high doses (around 4 -5 Gy) (Durante and Formenti, 2018). Studies have also shown that induction of MN is more effective from high-LET radiations than sparsely ionizing low-LET X-rays when irradiated at the same dose (Snijders et al., 2015; Hirayama et al., 2015).

After exposure to 0.1 Gy and 2 Gy X-rays, the HF19 fibroblast cell lines exhibits early MN induction 48 hours in both irradiated doses, and after 2 weeks the 2 Gy X-irradiated progeny show induction of GI by manifestation of MN. While these results are not indicative of a linear-quadratic dose response curve, they suggest that a higher dose of low-LET X-rays will cause a delayed and enhanced response in the progeny of the directly irradiated cells. As mentioned previously, the results from the MN inductions at the 48 hour time point complement the statistically significant LINE-1 increase in retrotransposition after exposure to 0.1 Gy X-rays. While speculative, it can be hypothesized that the

increase in LINE-1 copy number is possibly associated with the instability at this time point by IR in the HF19 cell line. While there is no evidence of RIGI at later time points examined in this chapter, our study is only limited to measuring DNA damage by MN induction. It could be hypothesized that by examining other manifestations of DNA damage or indicators of cellular crisis (such as increases in ROS or cellular senescence) there may be evidence of IR-induced genomic instability, caused by the early increase of LINE-1 insertion. However, further studies are needed to demonstrate any direct link between LINE-1 retrotransposition and RIGI. While it has been speculated upon, no studies to date have shown evidence for the role LINE-1 plays in the development of RIGI. In a study by Farkash *et al.* (2006), LINE-1 retrotransposition was increased 4-fold in cultured cells after exposure to γ -irradiation, and the frequency of LINE-1 levels was proportional to the level of phosphorylated H2AX foci (another measurement of DNA damage, by measuring DSBs). Another study by Miousse *et al.* (2017) demonstrated a loss of LINE-1 DNA hypomethylation in stem cells of CBA/J mice two months post IR, however the epigenetic alterations were not associated with any DNA damage or indicators of cellular stress.

As mentioned previously in this section, further experimentation is warranted to determine if the increase in LINE-1 is the cause of RIGI. This could be accomplished by using RNA interference (RNAi) to repress LINE-1 at 48 hours post IR with 0.1 Gy X-rays, and investigating GI at early and late time points in terms of MN induction (and other measurement of DNA damage such as phosphorylated H2AX foci or the comet assay). It will then be determined if the repression of LINE-1 still results in an increase in chromosomal/DNA damage.

In this chapter MCF7 cells show MN induction in the 2 Gy X-irradiated groups 24 hr and 48 hr post IR, however after 2 weeks there is no delayed response in the progeny of these cells. This is indicative that at 24 and 48 hours as the MCF7 cells have attempted to go through mitosis, they have subsequently undergone mitotic catastrophe. The reduction at 2 weeks suggests the repair of DNA damage, as more cells are able to successfully complete mitosis. The reduction could also be due to

eliminations of those cells that accumulated lethal damages by apoptotic processes by the delayed time point. The lower dose of 0.1 Gy X-rays shows no induction of MN throughout the time course of the experiment, suggesting that chromosomal damage (by measurement of MN induction) is dose-dependent. A study used a fractionated dose of 5 x 2 Gy IR to MCF7 cells and monitored MN induction (Demasters et al., 2006). The study found an increase in binucleated cells containing MN 24 hours post IR, however after 7 days the initial irradiation insult the binucleated cells containing MN decreased, as more cells were able to complete mitosis and damage was repaired (Demasters et al., 2006), thus in agreement with the results in this chapter. The Caco-2 cell line demonstrated its inherent radio-resistance and displayed no induction of MN throughout the time course of the experiment.

3.5. Conclusions

The aim of this chapter was to investigate a link between an increase in LINE-1 retrotransposition and radiation-induced genomic instability. Results in this chapter have characterized LINE-1 retrotransposition in response to diagnostic and therapeutic doses of low LET X-rays in HF19, MCF7, and Caco-2 cell lines. Retrotransposition showed an increase in all three cell lines at various time points following x-irradiation exposure at a diagnostic (0.1 Gy) dose. The presence of the LINE-1 increase in the HF19 cell line 48 hours post IR was interestingly complemented by an increase in MN induction by 0.1 Gy X-rays. Further studies are warranted to determine if the increase in LINE-1 retrotransposition is associated with the instability within the genome at this time point and to investigate any direct link between LINE-1 retrotransposition and RIGI.

Interestingly, recent work shows an abundance of transposable element sequences within the DNA of extracellular vesicles (Balaj et al., 2011). Extracellular vesicles (EVs) including exosomes have emerged as an appealing subject matter as they are generated by all living cells, contain DNA, RNA, and proteins, and are composed of multiple markers that provide clinically useful information.

Recent findings also suggest EVs as a likely means of transmission for the TEs between cells (Balaj et al., 2011). Therefore ongoing work for this project also includes the characterization of exosomal DNA (exoDNA) within the MCF7 cell line.

Chapter 4 Extracellular vesicles and characterization of MCF7 exosomes and exosomal DNA

4.1. Introduction

Both normal and dying/cancerous cells excrete heterogeneous groups of extracellular vesicles (EVs) including apoptotic bodies, microvesicles, and exosomes (as described in section 1.4). Extracellular vesicles are distinguished from each other according to their size and biogenic pathways, and each subtype has a different cargo and function (Dai and Dais, 2018). As the different subtypes are highly heterogeneous, it is necessary to characterize them into the appropriate subtype in order to properly understand their content and functions in physiological and pathological processes. While there have been many efforts to characterize, isolate, and analyze distinct subtypes from each other, the field is lacking in a definitive and standardized method and is currently still being developed. In the meantime, researchers are to include information pertinent to physical characteristics of the extracellular vesicles (size/morphology/density), biochemical composition of EVs (protein content such as the tetraspanins, integrins, and heat shock proteins) and descriptions of experimental conditions or parental cells (large/small oncosomes, hypoxic EVs) to define extracellular vesicle subtypes (Théry et al., 2018).

As extracellular vesicles (exosomes, in particular) are known to play an important role in cell to cell communication, and their cargo (DNA, RNA, protein, etc) can deliver specific molecular messages to distal tissues and cells (Ramirez et al., 2018) it is necessary to get a better understanding of what components lie beneath the phospholipid bilayer. Stress level, duration of culture time, the size and amount of the small vesicles makes it difficult to obtain relatively pure preparations of each sample, and efforts have focused on isolating and collecting the vesicles without any contamination. This is also an obstacle when extracting the exosome DNA (exoDNA), and this chapter will aim to characterize the response of MCF7 and Caco-2 cell lines and their associated extracellular vesicle changes in response to different culture conditions and irradiations.

4.2. Materials and Methods

4.2.1. Cell culture and Irradiation conditions

MCF7 cell culture was performed as previously described in section 2.1.3 and section 2.1.3.1. Caco-2 cell culture was also performed as previously described in section 2.1.2 and section 2.1.2.1. Apoptotic bodies, microvesicles, and exosomes were extracted via serial centrifugation or ultracentrifugation as per section 2.5, unless stated otherwise. Irradiations were also performed as per section 2.3.

4.2.2. Transmission Electron Microscopy (TEM) analysis

Transmission Electron Microscopy (TEM) with negative staining was used to visualize the exosome samples. To fix exosome samples on grid they were combined 1:1 with 4% paraformaldehyde (PFA) (Sigma-Aldrich, 158127) and put on ice for 15 minutes. Carbon-formvar coated copper 200 mesh grids (TAAB, F077) were in contact with exosome samples droplets for 45 minutes. Grids were then washed on 20 μ L droplets of filtered ultrapure water 3 times, using filter paper to remove excess water between washes. Grids were next placed on a 20 μ L drop of 2% uranyl acetate for 10 minutes to stain, followed by another 3 washes with filtered ultrapure water. Grids were let to dry, stored sample-side up and then imaged using Hitachi H7650 TEM at 100kV at 15,000x magnification. Exosomes were sized and images captured using Advanced Microscopy Techniques software (Advanced Microscopy Techniques, Massachusetts, USA).

4.2.3. Characterizing exosome size and concentration by tunable resistive pulse sensing

Exosome size distribution and concentration was measured via tunable resistive pulse sensing (tRPS) using the qNano (Izone ScienceTM). tRPS works by suspending nanoparticles in electrolytes on a particle-by-particle basis as they flow through a nanopore. Before and after each sample a calibration is performed by using the CPC100 calibration particles in the Izon kit, as per the manufacturer's instructions. The stretch applied to the pore for all samples was 47 nm, voltage 0.62V, current 100-120 nA, pressure 7 mbar, with noise <10 pA. At least 500 particles recorded per

calibration and per sample. Izon software version 3.2.2.251 was used for data recording and for calculating exosome size ranges and concentrations.

4.2.4. Western blot (WB) analysis

Exosome pellets (extracted as per section 2.5) were vortexed and lysed for 20 minutes on ice in 100µL radioimmunoprecipitation assay (RIPA) buffer. Lysates were cleared by centrifugation (10,000 x g, 4°C, 5 min) and protein concentrations measured using a Bradford assay. 9 µl of each protein along with 1X NuPAGE Sample Buffer (Invitrogen, NP0007) and 5% β-mercaptoethanol (Sigma, M7154) was loaded into a 4-12% Bis-Tris gel (Invitrogen, NP0323BOX) and run using 1X MOPS running buffer (Invitrogen, NP0001) at 200V for 45 minutes. The proteins were then transferred to a nitrocellulose membrane (Sigma, GE10600003) using 1X NuPAGE transfer buffer (Invitrogen, NP0006) and run at 30V for 90 minutes. Membranes were blocked for 1 hour (room temperature) with 5% powdered milk in 1X PBS (Sigma-Aldrich, P4417) and 0.1% Tween-20 (Sigma-Aldrich, P9416). Membranes were then incubated overnight with primary antibodies in 1X PBS, 0.1% Tween-20, and 2% milk at 4°C. Membranes were washed 3x 5 minutes with 1X PBS and 0.1% Tween-20, then incubated with secondary antibodies for 1 hour at room temperature with 1X PBS and 0.1% Tween-20. For band visualization, membranes were washed 3x 5 minutes in 1X PBS and 0.1% Tween-20 and imaged using Bio-Rad ChemiDoc™ imaging scanner. A list of the antibodies used for western blot can be found in Table 1.

Primary antibodies					
Antigen	Host	Vendor	Catalog #	Application	Dilution
GAPDH	Mouse	Abcam	ab8245	WB	1:1000
ALIX	Mouse	Abcam	ab117600	WB	1:500
HSP70	Rabbit	Abcam	ab181606	WB	1:1000
Secondary antibodies					
Ab Type	Host	Vendor	Catalog #	Application	Dilution
IgG	Rabbit	Invitrogen	A21441	WB	1:1000
IgG	Mouse	Invitrogen	A21200	WB	1:1000

Table 1 Antibodies used for WB experiments.

Proteins GAPDH, ALIX, and HSP70 were used throughout the experiment for exosome verification.

4.2.5. Population Doubling Level (PDL) Calculations

PDL refers to the total number of times the cells in the population have doubled while in culture.

Cells were seeded at 1.5×10^6 cells at beginning of each incubation time, and the number of viable cells was determined via the The Muse™ Cell Analyzer (as per section 2.4). Population doubling was measured once cells reached 80-90% confluency, and examined for two weeks. A formula used for the calculation of PDL is as follows: $PDL = PDL^1 + (c) * (\log(Y) - (\log(i)))$ (Haylick, p.220). Where i is the cell number at the beginning of the incubation time, Y is the cell number at the end of the incubation time, PDL^1 is the starting PDL, and c is the constant (3.322).

$$PDL = PDL + (c) * (\log(Y) - (\log(i)))$$

4.3. Results

4.3.1. Cell culture media affects yield of exoDNA

FBS is used as a supplement in cell culture as it contains a multitude of nutritional and macromolecular factors essential for cell growth. However, FBS contains its own extracellular vesicles and other proteins, such as albumin, which can contaminate the serum used for exosome experiments. To avoid contamination, experiments involving exosomes will commonly use exosome-depleted media to culture cells prior to exosome isolation. However, while collecting preliminary exoDNA data, it was evident that the use of exosome-depleted media limited the quantity and purity of exoDNA we were able to extract from the exosome samples. Therefore, we aimed to determine how different types of media would affect the yield of exoDNA and a quality check of the media was assessed.

Experiments began with Caco-2 cells, and they were seeded in a T175 cell culture flask at 3.0×10^6 cells and grown for 24 hours in normal media (Dulbecco's Modified Eagle's Medium – high glucose (Sigma: D5671), 10% FBS, 1% penicillin/streptomycin, 1% MEM amino acids). Next, original normal media was removed and replaced with four different types of media for 48 hours: Normal media (10% FBS, 1% penicillin/streptomycin, 1% MEM amino acids), Exosome-depleted media (10% FBS-exosome depleted serum, 1% penicillin/streptomycin, 1% MEM amino acids), serum-free media (1% penicillin/streptomycin, 1% MEM amino acids), and high-glucose base media (Sigma: D5671) without supplements. After 48 hours, exosomes were isolated from each of the four different types of media by ultracentrifugation (as described in section 2.5). A table showing the ingredients of each type of media is shown in Table 2. Exosomal DNA extraction for Caco-2 was performed as per section 2.7.

Type of media	Ingredients
normal	DMEM high glucose, 10% FBS, 1% penicillin/streptomycin, 1% MEM amino acids
exo-depleted	DMEM high glucose, 10% exosome-depleted FBS, 1% penicillin/streptomycin, 1% MEM amino acids
serum-free	DMEM high glucose, 1% penicillin/streptomycin, 1% MEM amino acids
base	DMEM high glucose

Table 2 *The ingredients used in the four types of media in the exosome experiments.*

The four types of media proved to contain varying amounts (ng/ μ l) of exosomal DNA (as shown in Figure 4.1). After 48 hours, exosomes produced in normal media contained 15.5 ng/ μ l (average) of DNA, while the exosomes produced in exosome-depleted media contained 7.7 (average) of DNA. The serum-free media contained the highest amount of dsDNA, with an average of 82.2 ng/ μ l, while the base media contained 63 ng/ μ l (average) of exosomal DNA. To confirm that the DNA was unquestionably produced from the exosomes in the media originating from the cells, and not the media itself, another quality check was established. As a negative control, normal media and the serum-free media (directly from the bottle, without interaction with cells) were subjected to the exosomal DNA extraction protocol. Both produced negative results (amount of DNA was negligible (<1 ng/ μ l), and DNA qualities were far off from acceptable range; data not shown).

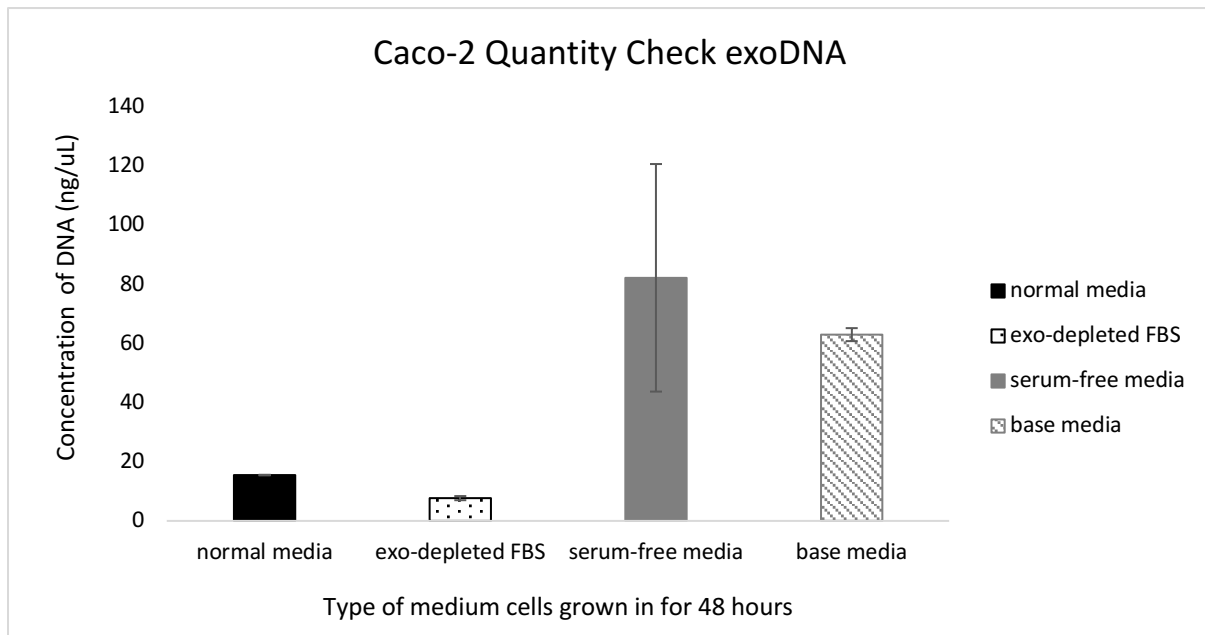


Figure 4.1 The quantification of DNA extracted from the Caco-2 cell line grown in different types of media. Quantity of exoDNA is expressed as nanogram per microliters, and two replicates have been performed. Error bars = SD.

4.3.2. DNA is abundant in cancer-derived exosomes

To investigate if exoDNA was ubiquitous across cell lines, exoDNA was extracted from exosomes released by MCF7, and two primary fibroblast cell lines (HF19 and FSF160414B). All cell types were seeded into 6 T175 cell culture flask at 3.0×10^6 cells and grown for 24 hours in normal media. Next, original normal media was removed and replaced with serum-free media for 48 hours prior to DNA extraction. ExoDNA was extracted as per section 2.7.

ExoDNA was found in all the exosomes examined, and found in abundance in the two cancerous cell lines. The amount of fibroblast exoDNA was 19-fold less than the amount of exoDNA derived from cancer cells (Figure 4.2). This could be due to the aggressive replication and highly proliferative nature of cancerous cells.

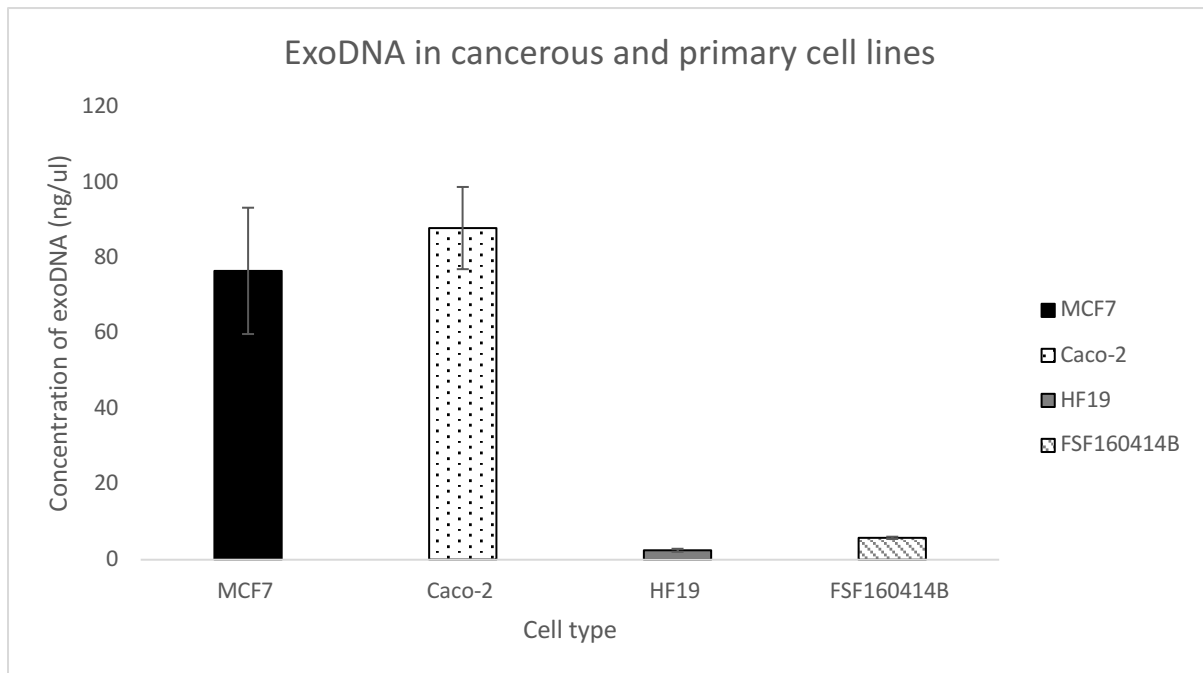


Figure 4.2 Comparison of the amount of exoDNA isolated from two cancer cell lines (MCF7 and Caco-2), and two primary fibroblast lines (HF19 and FSF160414B). Quantity of exoDNA is expressed as nanogram per microliter, and these experiments were performed in duplications. Error bars = SD.

4.3.3. DNA associated with EV subtypes in MCF7 cell line

We decided to continue investigations with the MCF7 breast cancer cell line due to its well-annotated genome.

As previous research has suggested that EV subtypes may package different parts of the genome (Lázaro-Ibáñez et al., 2014) and the release of EVs increases in response to IR (Jella et al., 2014; Al-Mayah et al., 2015), we investigated if IR would affect the DNA concentration in EV subtypes.

Apoptotic bodies (AB) and microvesicles (MV) were extracted via serial centrifugation and ultracentrifugation as per section 2.5, and DNA extracted as per section 2.7. Results show that apoptotic bodies contain an abundance of DNA when compared to microvesicles (as shown in Figure 4.3), suggesting a preference of packaging DNA into the larger-sized extracellular vesicles. When MCF7 cells were exposed to 2 Gy X-rays, apoptotic bodies also showed an increase in DNA concentration compared to sham-irradiated apoptotic bodies. These results help give a better understanding of the effect of IR on different EV subtypes and their cargo.

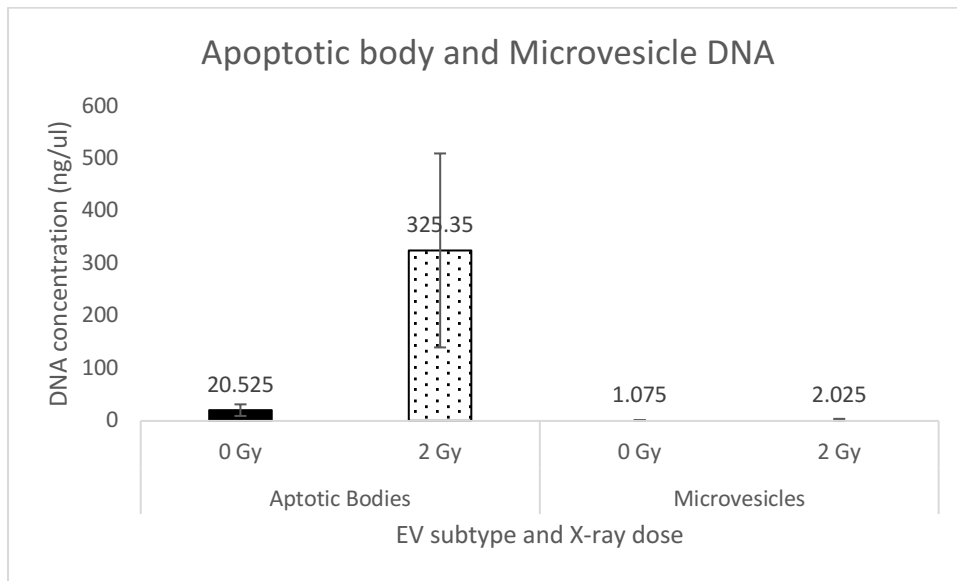


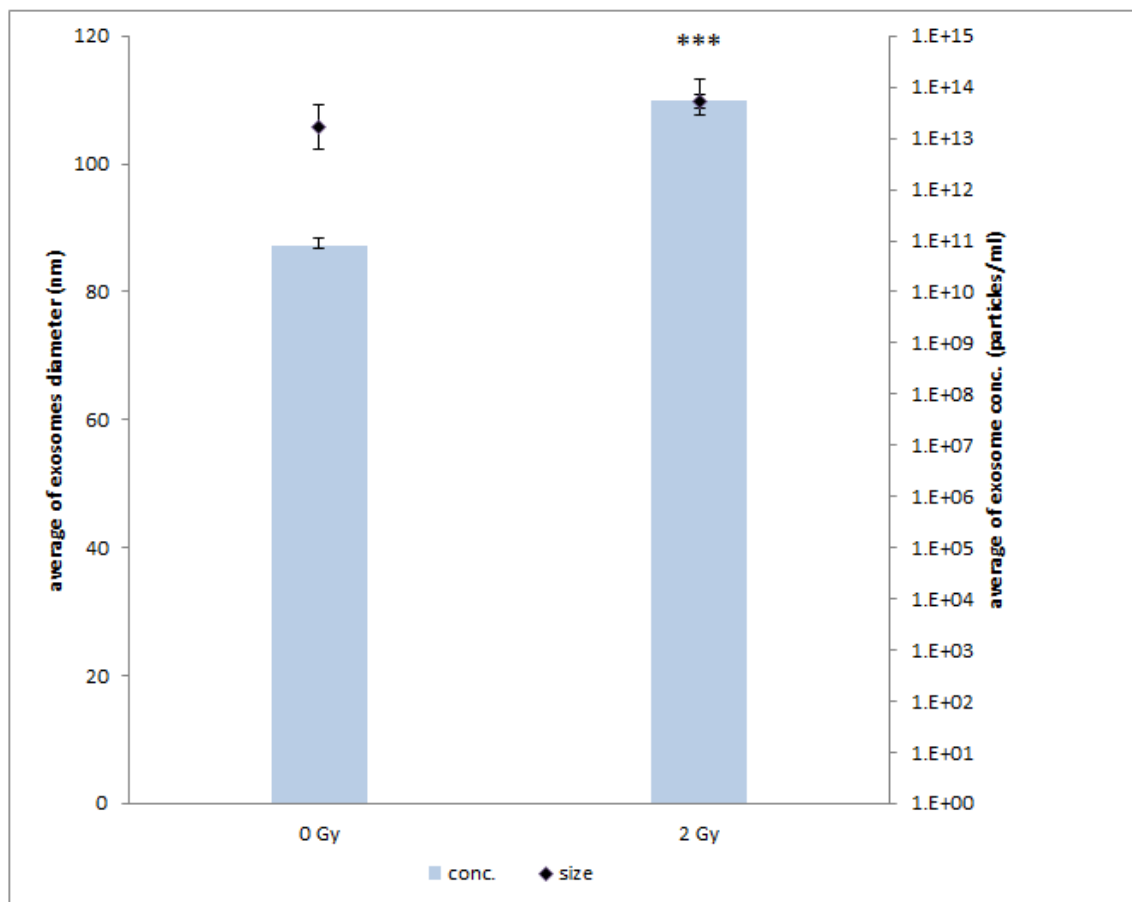
Figure 4.3 The quantification of DNA extracted from different EV subtypes derived from MCF7 cell line after exposure to 2 Gy X-rays. Quantity of exoDNA is expressed as nanogram per microliter, $n=4$, error bars = SD.

4.3.4. Characterization of exosomes released from irradiated and non-irradiated MCF7 cells

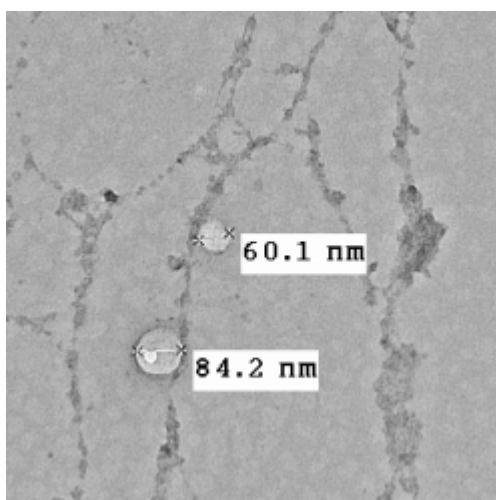
Measurement of sham-irradiated exosomes displayed a mean diameter of 106 nm, and 2 Gy exosomes displayed a mean diameter of 110 nm, thus ensuring the characterization of exosomes by their small size as opposed to the larger size of other EVs (Figure 4.4A). Quantification of exosome secretion concentrations after exposure to 2 Gy X-rays revealed that IR significantly increases the amount of exosomes excreted from MCF7 cells 48 hours post IR, thus indicating that IR enhances the overall amount of exosome excretion (Figure 4.4A). To further characterize the size, nanostructure, and physical properties of exosomes we employed the use of TEM (Figure 4.4B and Figure 4.4C). Analysis revealed the presence of round, cup-shaped structures that are characteristic of exosomes, as well as exosomes within the expected size range of approximately 30-150 nm in diameter. Western blotting confirmed the presence for markers generally used in exosome identification: ALG-2-interacting protein X (ALIX), involved in multivesicular biogenesis, and cytosolic protein heat shock 70 kDa protein 4 (HSP70) (Figure 4.4D). TEM and western blot analysis therefore successfully identify

the isolated samples as exosomes. All results are shown below in Figure 4.4.

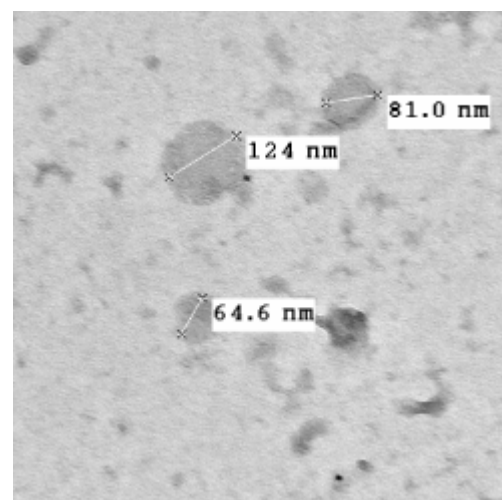
A



B



C



D

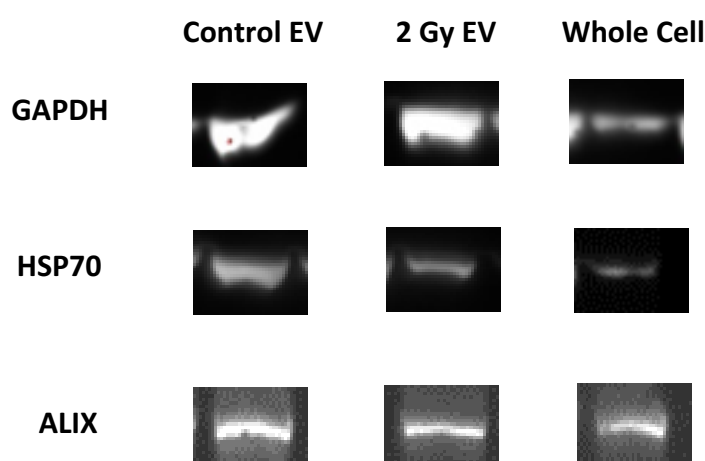


Figure 4.4 Characterization of exosomes derived from MCF7 cell line

A) Average size and concentration distribution of exosomes isolated 48h following irradiation with 0 Gy and 2 Gy X-rays determined by tRPS. Error bars represent SEM for three biological replicates. *** $p < 0.001$ B) TEM imaging of exosomes extracted from sham-irradiated MCF7 cells C) TEM imaging of exosomes extracted from 2 Gy X-irradiated MCF7 cells. D) Western blot analysis of exosome and cellular lysates from MCF7 cell line probed for exosome-specific markers HSP70 and ALIX, as well as housekeeping protein GAPDH.

4.3.5. Assessment of cell growth in serum-free media

In our exosome experiments cell cultures are incubated for 48 hours with serum free media, which could potentially starve the cells and initiate cell death. In order to examine the effect of serum on cell growth over time, the population doubling level (PDL) and viability of cells was examined and a growth curve was determined (Figure 4.5). PDL was calculated as per section 4.2.5. The cell viability did not decrease below 85% either with or without serum (Figure 4.6), and the growth rate of cells exposed to serum free media for 48 hours showed no change compared to the supplemented media, both growing at an exponential rate despite any serum deprivation. Results show there is no difference in the growth of cells that were starved of serum for 48 hours, and the lack of nutrients does not have an effect on the growth of the MCF7 cell line.

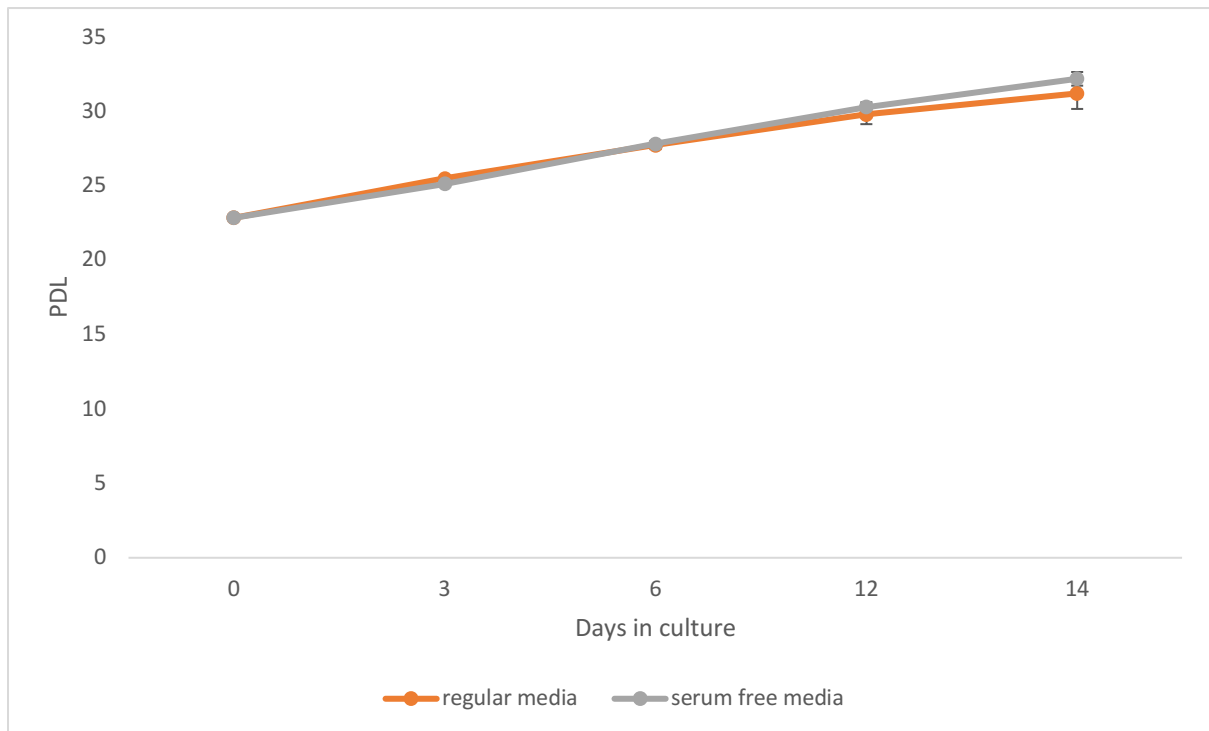


Figure 4.5 Population doubling levels of MCF7 cells following incubation with supplemented or serum-free media. Cells were incubated with supplemented media (containing FBS) or without FBS (serum free) for 48 hours. Population doubling was measured once cells reached 80-90% confluency and examined for up to two weeks for prolonged effects. Data are expressed as the mean +/- standard deviation, SD (n=3).

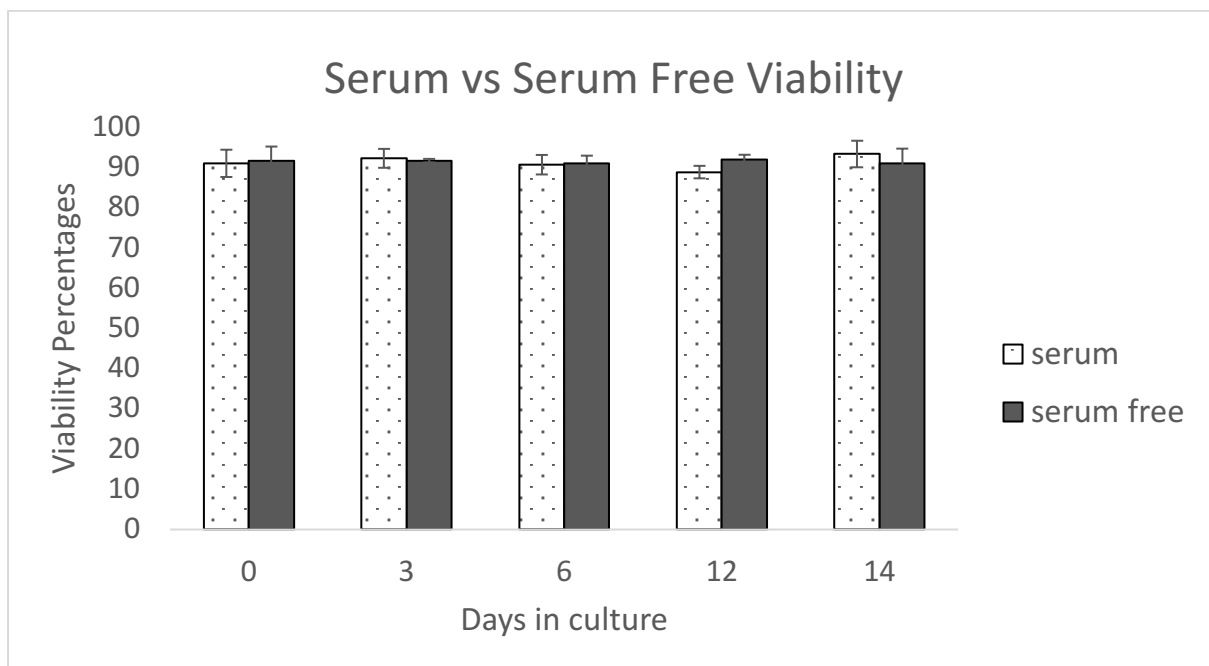


Figure 4.6 Viability Percentages of MCF7 cells following incubation with supplemented or serum-free media. Cells were incubated with supplemented media (containing FBS) or without FBS (serum free) for 48 hours. Viability was measured once cells reached 80-90% confluency and examined for up to two weeks for prolonged effects. Data are represented as the mean +/- standard deviation, SD (n=3), t test. There was no statistical change found in the viability of the cells following incubation with or without serum.

4.3.6. DNase treatment

To ensure the DNA that is extracted from the exosomes is coming from within the exosome, the freshly extracted exosome pellet is pre-treated with double-stranded DNase, dsDNase (0.15 units/ul) to digest any external double-stranded DNA that may be on the outer membrane.

However, when the pre-treated dsDNase exoDNA extracted from the MCF7 cells is run on a gel, there is a clear band at the bottom that shows RNA contamination in all of the samples (Lanes B, C, and D in the Figure 4.7). While the treatment of dsDNase may eradicate any external dsDNA on the outer membrane of the exosome, the single-stranded DNA (ssDNA) or possibly RNA may remain.

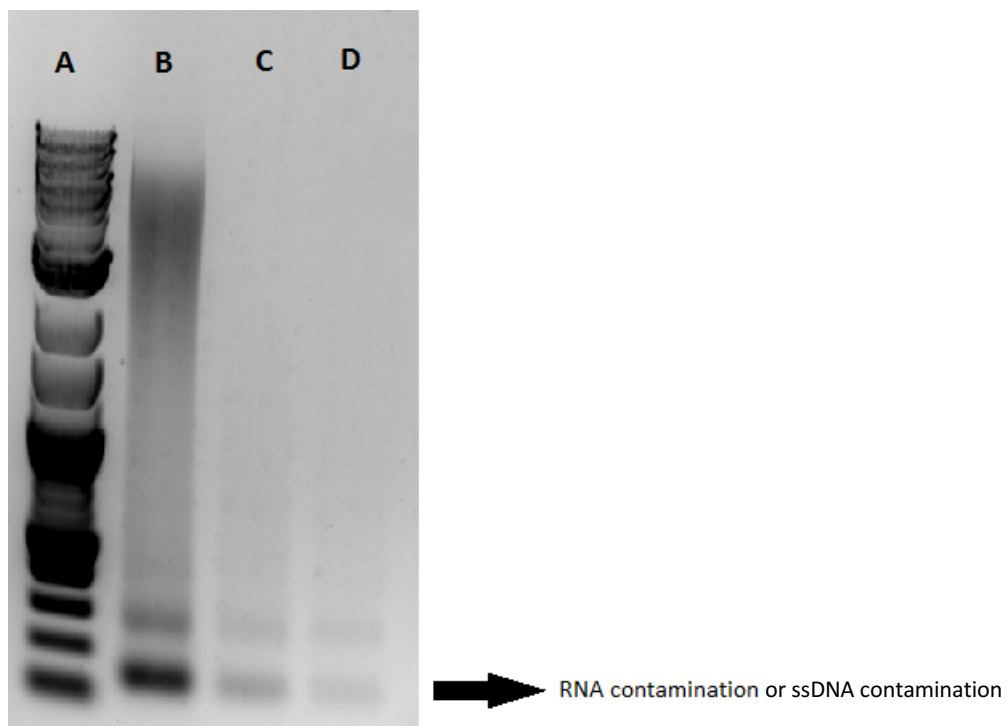


Figure 4.7 ExoDNA extracted from MCF7 exosome samples pre-treated with dsDNase and run on gel. A) 2log ladder. B) exoDNA extracted from a 2 Gy X-irradiated sample. C) exoDNA extracted from a sham-irradiated sample. D) exoDNA from a second sham-irradiated sample. All samples (Lanes B, C, and D) show a clear band distinguishing RNA or ssDNA contamination. 10 ng of exoDNA from each sample was loaded into the appropriate wells.

Therefore, to eradicate external DNA and eliminate RNA/ssDNA contamination from the exosome pellet prior to exoDNA extraction, DNase I (1 unit/ul), an endonuclease that digests both dsDNA and

ssDNA, was employed. Treatment of DNase I resulted in no ssDNA contamination or RNA contamination (many DNases are very purified and contain remnants of RNases), as well as a large reduction of the total amount of exoDNA, indicating that much of the exoDNA is located externally (as seen in Figure 4.8).

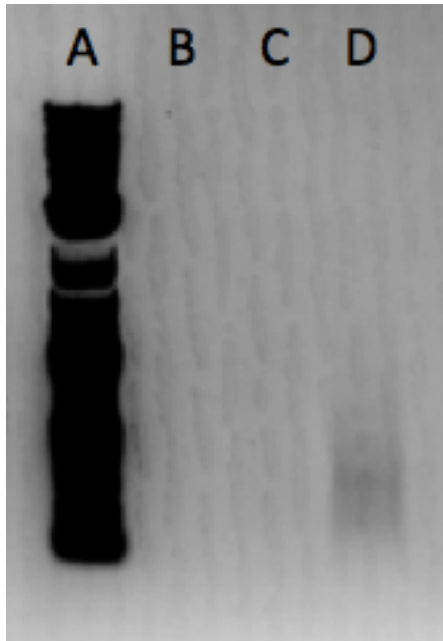


Figure 4.8 ExoDNA extracted from MCF7 exosome samples pre-treated with DNase I and run on gel. A) 2log ladder. B) N/A (empty) C) exoDNA extracted from a sham-irradiated sample. D) exoDNA from a 2 Gy X-irradiated sample.

4.3.7. DNA yield optimization for downstream analysis

As the application of DNase I results in a large loss of total concentration of exosomal DNA, it was essential to optimize the protocol for a higher yield and purity of double-stranded DNA that is destined for downstream sequencing analysis in the following chapter. In all previous experiments, unless otherwise noted, exosomes were collected from 6 T175 flasks seeded at 3.0×10^6 cells. In order to obtain a higher concentration suitable for sequencing, we hypothesized that by seeding with a larger quantity of cells (larger starting material) it would result in a higher and more favorable exoDNA yield. Figure 4.9 shows the DNA yield from exosomes pre-treated with DNase I and the original starting material from the sham and 2 Gy X-irradiated samples. The sham irradiated samples show an increase in exoDNA yield (from an average of 3.2 ng/ μ l to 8.2 ng/ μ l) by increasing the

number of cells seeded. 2 Gy X-irradiated samples show a yield of exoDNA (average 21.7 ng/ μ l), thus establishing a final method for exoDNA extraction in order to obtain a favorable exoDNA yield for downstream analysis (low-depth whole genome sequencing).

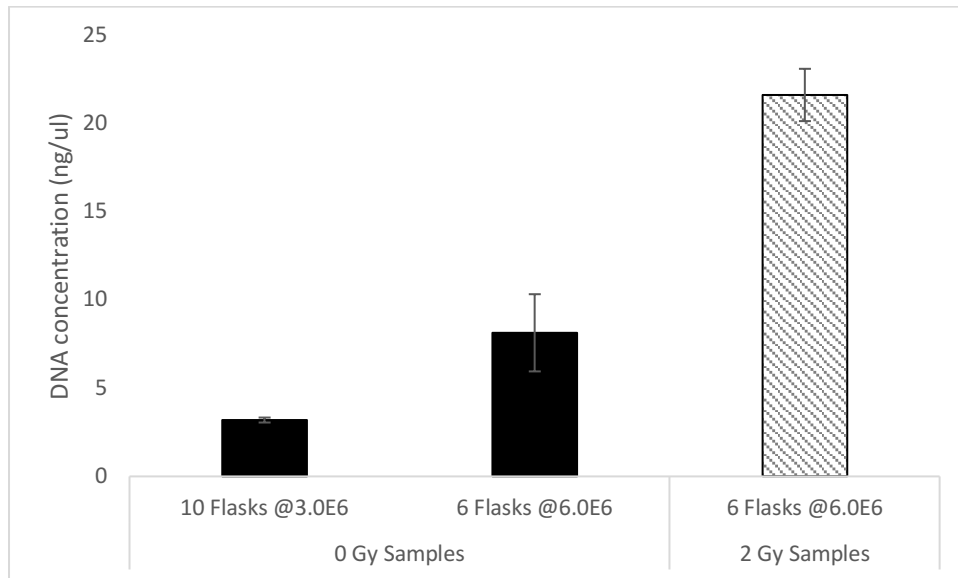


Figure 4.9 ExoDNA yield (from MCF7 exosomes) increases by increasing number of cells seeded. Cells were seeded at 3.0×10^6 or 6.0×10^6 and 48 hours post IR they were collected in serum-free media from the number of flasks labelled, and treated to DNase I prior to exoDNA extraction. Data are expressed as the mean $\pm$ SD ($n=3$).

4.4. Discussion

4.4.1. Exosomes excreted in response to IR are characteristic

In order to study exosomes they must be isolated from cell culture media or biofluids, and before any functional analysis it is crucial to ensure the purified nano-vesicles demonstrate the size and protein composition consistent with exosomes. Several technologies have been developed (and are constantly developing) to detect and characterize the unique and distinguishable properties of exosomes.

The small size distribution of exosomes is a defining characteristic that separates them from other EVs, one approach commonly used to visually determine the size distribution and morphology of exosomes is TEM. TEM combined with negative staining allows for detection from non-irradiated and irradiated groups in this chapter, and results show a size and cup-shaped morphology that is consistent with exosomes (Figure 4.4B and Figure 4.4C).

Size and concentration of a sample can be simultaneously measured via two commonly used methods: 1) nanoparticle tracking analysis (NTA), which uses principles of light scatter and Brownian motion (Szatanek et al., 2017); 2) tunable resistive pulse sensing (tRPS), which detects the passage of individual particles through a pore in a membrane, and as they pass through a transient change in ionic flow is identified and used to calculate the volume of the exosome (de Vrij et al., 2013). In this chapter tRPS measurements performed on the Izon qNano instrument revealed that exosomes excreted from irradiated MCF7 cells demonstrated an increase in exosome release following exposure to IR (Figure 4.4A). This is consistent with previous findings as MCF7 cells were used in another study that showed an increase in exosome concentration following exposure to 2 Gy X-rays (Al-Mayah et al., 2015), as well in a study by Jella *et al.* (2014) where human cancer cells were exposed to increasing doses of γ -rays, and resulted in increasing exosome concentration. Other cellular stressors such as heat stress (Clayton et al., 2005) and chemotherapy DNA-damaging drug (cisplatin) (Xiao et al., 2014) have been shown to result in increasing exosome excretion. It is unclear

why cells respond to stress by releasing an excess of exosomes, however it could be an adaptive mechanism by which cells communicate to neighboring cells, warning by increasing secretion. Another possibility is that cells secrete waste products through exosome release. A recent study by Takahashi *et al.* (2017) has shown that exosomes maintain cellular homeostasis by removing damaged DNA from cells. The study showed that by treating cells with DNA-damaging agent doxorubicin, exosomes react by preventing the cytoplasmic accumulation of nuclear DNA, and therefore prevent activation of DNA damage response (DDR) pathways (Takahashi *et al.*, 2017). Interestingly, the study also showed that by reducing exosome release it resulted in increased signs of the DDR in human cells (Takahashi *et al.*, 2017).

Exosomes contain various membrane and cytosolic proteins, and these proteins can be used to help distinguish exosomes from other EVs. Proteins used as markers for exosomes include the GTPases, heat-shock proteins (HSP70, HSP90), membrane and transport proteins like annexins, and the tetraspanins (CD9, CD63, CD81) (Li *et al.*, 2017). Exosomes can also be identified by certain protein markers that indicate their endocytic origin such as TSG101 and ALIX (Szatanek *et al.*, 2017). Results from this chapter confirm the presence of HSP70 and ALIX in the exosome samples. Therefore, analysis of samples by TEM, tRPS, and western blotting successfully characterize and identify the isolated samples as exosomes.

4.4.2. DNA in EV subtypes and exosomes

Previous research regarding the subtypes of extracellular vesicles and the DNA found within them has shown that apoptotic bodies, microvesicles, and exosomes from cell culture supernatants and plasma contain different DNA fragments (MLH1, PTEN, and TP53), leading to a conclusion that certain cargo (nucleic acids and protein) are selectively packaged into separate EV subpopulations (Lázaro-Ibáñez *et al.*, 2014). Results in this chapter are complementary to these findings, as the concentration of DNA found in apoptotic bodies was far more abundant than DNA found in microvesicles (Figure 4.3), thus suggesting that EV subtypes are more inclined to carry certain

material than others. Apoptotic bodies are excreted exclusively during apoptotic cell death, and this is apparent as the irradiated populations in this chapter show a higher concentration of DNA than the non-irradiated samples. The cargo of apoptotic bodies is comprised of the cellular components that happened to be in the cytoplasmic protrusion that created it, which in this case is likely damaged and fragmented DNA resulting from the irradiation insult. In general, studies have shown that apoptotic bodies help to prevent leakage of cellular content into the extracellular space, therefore prohibiting inflammation, autoimmune responses, and tissue damage (Wickman et al., 2012; Weigert et al., 2006). While the exact purpose of apoptotic bodies remains unclear, it is proposed that breakage into apoptotic bodies contributes to more efficient removal of apoptotic cells (Wickman et al., 2012). Notably, defects in the removal of apoptotic bodies have been implicated in the development of autoimmune disease (Hanayama et al., 2004). While it is well known that apoptotic bodies contain DNA, further investigation is warranted regarding the negligible amounts of DNA found within the irradiated and sham-irradiated microvesicle samples, and the molecular mechanisms of DNA packaging into certain EV subtypes.

The outer membrane of the exosome is comprised of lipids such as sphingomyelin, cholesterol, ceramide, and phosphatidylserine, and the adhesive properties of the lipids allow the EV to interact and bind to nucleic acids (Kawamura et al., 2017). While it is standard procedure to eliminate the external DNA on the surface of the exosomes via DNase I digestion, multiple studies have shown that DNA influencing horizontal gene transfer (Fischer et al., 2016), induction of autoimmunity (Sisirak et al., 2016), and cellular uptake (Shelke et al., 2016) have been found on the exofacial membrane. The DNA digested from the outer membrane surface may contain information that also explains the cell source, and generation of extracellular vesicles. It is unclear whether the exosomes leave the growing parental cells with DNA already on their surface, or if that takes place extracellularly. Conflicting arguments state that DNA from the cells or supernatant of the cell culture could have been attached to the exosomes in previous and early isolation steps, thus contributing to

contamination of the exosomes. In this case, it would explain the essential step of DNase I digestion, however the previously mentioned works show important roles external DNA may play in physiological processes of the exosomes.

There is a great understanding that exosomes are released by both normal and cancerous cells, with extensive research on cancer-derived exosomes suggesting that the vesicles are capable of promoting cancer (Kahlert and Kalluri, 2013; Yu et al., 2015; Kalluri, 2016). The DNA within the exosomes has been and is being actively investigated, as its encapsulation within the phospholipid bilayer has been found to be stable (Jin et al., 2016) and found in cell culture supernatant as well as a variety of human and mouse biological fluids such as blood, seminal fluid, and urine. As the research and interest in exosome DNA has been rapidly expanding, several researchers have found a common factor regarding the DNA in exosomes derived from non-cancerous cells: there is none. This, or it is negligible (as has been shown in this chapter). ExoDNA from primary fibroblasts has been described as 'virtually undetectable' (Balaj et al., 2011), or 'extremely low yield to extract DNA' (Thakur et al., 2014). As the biology and function of exosomes is still questionable, it is possible that exoDNA is a feature of cancerous/damaged exosomes, yet there has been little research on the subject and warrants further investigation. One study showed that tumour-derived EVs carried an abundance of the oncogene *c-Myc* as well as an abundance of transposable elements such as LINE-1, Alu, and HERV sequences (Balaj et al., 2011), suggesting they could be a carrier of deleterious cargo available for horizontal gene transfer. Another possible explanation is that damaged DNA is the source of exoDNA (for which from normal cells is not excessive), and this will be discussed further in the next chapter.

4.5. Conclusions

In conclusion, results in this chapter suggest selective packaging of DNA into EV subtypes (namely apoptotic bodies and exosomes, as per Figure 4.3), as well as exoDNA being a characteristic of cancer-cell derived exosomes, as opposed to non-cancerous cells. Results also show that exosomes excreted from unirradiated and irradiated populations portray both similar and different characteristics, such as a similarity in size and shape (as per TEM and tRPS analysis, Figure 4.4), and differences in quantities of exosomes release in response to IR (as per tRPS analysis, Figure 4.4). As exosome research is expanding, it is essential to understand what they are comprised of, and how external factors (such as IR) can manipulate their functions and content. The work in this chapter shows the only known effect of IR on exoDNA thus far in literature (an increase in concentration), and by optimizing the exoDNA extraction method for the highest yield and purity the next chapter can aim to enrich this knowledge and more sensitively explore the DNA contents/composition of exosomes by whole-genome sequencing.

Chapter 5 The DNA composition of exosomes released from MCF7 cells following X-ray exposure

5.1. Introduction

Exosomes are known to carry a multitude of heterogeneous molecules including proteins, DNAs, mRNAs, miRNAs, and lipids that collectively reflect their cell of origin. While the function of exosomes is still largely unknown, studies have shown that they play a pivotal role in intercellular communication due to their ability to transfer the genetic cargo of their parental cells, thus modulating the biological functions of their recipient cells (Jella et al., 2018). Tumour-derived exosomes have been shown to be involved in communication between tumour and normal cells, leading to changes that promote tumour progression. For example, Wang *et al.* (2018) demonstrated that exosomes released from hepatocellular carcinoma cells and added to adipocytes were actively internalized by the adipocytes. Tumour exosomes were capable of influencing the adipocytes and results showed an increase in tumour growth, enhanced angiogenesis, and other characteristics indicative of a suitable environment for tumours, thus aiding in the promotion of tumour progression. Other studies show how exosomes are able to modify immune responses within the tumour microenvironment. For example, exosomes derived from X-irradiated tumours were shown to contain double-stranded DNA (dsDNA) that activate stimulation of Interferon signaling in recipient cells, while the non-irradiated exosomes showed no activation (Diamond et al., 2018).

Exosomes are also an appealing biomarker as they can be noninvasively collected from nearly all kinds of bodily fluids including saliva, bile, amniotic fluid, urine, semen, ascites, tears, breast milk, and blood (Kalluri, 2016). Furthermore, because almost all types of cells secrete exosomes and they contain many biomolecules (mentioned above), there is possibility for simultaneous identification of various biomarkers to increase accuracy for differentiating between healthy and pathogenic states of a patient.

DNA within exosomes, in particular, can provide information regarding cancer-specific mutations and has been shown to reflect the mutational status of the parental cell (Kahlert et al., 2014). KRAS and p53 are the most commonly mutated genes in pancreatic ductal adenocarcinoma (PDAC), and exosomal DNA (exoDNA) was collected from the Panc-1 cell line and from the serum of two PDAC patients. The results revealed that the exoDNA contained the same mutations in KRAS and p53 that are apparent in the cellular DNA. In another study focusing on pheochromocytomas (PCCs) and paragangliomas (PGLs), Wang *et al.* (2018) found the same heterozygous mutations in four genes (RET, HIF2A, VHL, and SDHB) in the genomic DNA from cell line PC12 as well as in exoDNA collected from the serum of 12 PCC or PGL patients. This provides further evidence that exoDNA from serum is a suitable biomarker for detecting cancer risk.

As previously mentioned, increased retrotransposition is commonly observed in a number of different cancer cells and several studies have shown the role of TEs in human carcinogenesis. Retrotransposons can cause significant damage to the genome by insertions, deletions, and rearrangements. In a study by Balaj *et al.* (2011) EVs derived from glioblastoma and melanoma cell lines showed high levels of retrotransposon elements including LINE-1, Alu, and HERV. While there is much research that shows the ability of transposable elements to replicate and integrate into the host genome, there is limited knowledge on cell- to-cell transfer of transposable elements. As EVs are key mediators in intercellular communication and can transfer genetic material to neighboring and distant cells, it has been suggested that oncogenic sequences and retrotransposon elements could be engulfed and integrate their DNA into recipient cells, thereby causing phenotypic changes that lead to genomic expansions and tumour growth in neighboring cells or at distant locations (Kawamura et al., 2017). To theoretically explain this phenomenon, after activation of retrotransposons (via exogenous or endogenous stimuli) and translation, the RNP complex would be imported into the nucleus followed by the integration process possibly initiated by the LINE-1 encoded endonuclease. Cells derived from retrotransposon activation would then differentially

reproduce through cancer development. LINE-1 insertions into transcriptionally active sites that confer favourable traits would be expressed that may contribute to cancer progression through the clonal expansion of select cells (Doucet-O'Hare et al., 2016). Interestingly, a very recent study has shown that RNA transcripts derived from an active LINE-1 retrotransposon were packaged into EVs and initiated retrotransposition in recipient cells (Kawamura et al., 2019). Horizontal DNA transfer mediated by exosomes has been shown by others (Ono et al., 2019; Kurywchak 2018; Kawamura et al., 2019; Crow et al., 2017; Fischer et al., 2016) however it is still unclear how exoDNA is transferred into the nuclei. Imaging analysis in a study by Kurywchak (2018) frequently revealed exosomes overlying the cell nuclei, suggesting a possibility of direct exosome fusion to nuclei.

While the content of an EV generally depends on the type and state of a parental cell, it is capable of being influenced by stress conditions on which the cell is exposed. IR is one of the well-recognized conditions causing cellular stress and has been shown to induce changes in EV composition, however research is limited and primarily focuses on its proteome (Jelonek et al., 2016). In a study by Jelonek *et al.* (2015) exosomes isolated from irradiated FaDu cells (head and neck cell carcinoma) were shown to have substantially elevated levels of proteins involved in translation, transcription, cell signaling, and cell division. While this study and a few others have shown that exosomal cargo reflects specific changes induced by IR, there has been no literature to date no to date regarding the effects of IR on exosomal DNA content or composition. Therefore, the aim is to extract and characterize exosomes released from irradiated MCF7 cells and to profile their DNA contents in response to radiation. The hypothesis is that exoDNA could be derived from specific locations within the genome.

5.2. Materials and methods

5.2.1. Cell culture and Irradiation conditions

MCF7 cell culture was performed as previously described in section 2.1.3 and section 2.1.3.1.

Irradiations were also performed as per section 2.3.

5.2.2. PCR

Polymerase chain reaction (PCR) is a method used to amplify a specific DNA sequence. 100 ng DNA for both genomic DNA and exosome DNA was used as a template. Reactions using Q5 High-Fidelity DNA Polymerase (M0491) and were performed in a 25 ul reaction and 10 uM of each primer.

Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Alpha (PIK3CA) primer pairs are previously described (Simi et al., 2008) and were as follows: PIK3CA exon 9, 59-

CTAGCTAGAGACAATGAATTAAGGGAAA39 (forward), 59-CATTTTAGCACTTACCTGTGACTCCA39

(reverse). Amplification conditions consisted of: Initial denaturation at 98°C for 30 seconds followed by 30 cycles of 20 seconds at 60°C, and a final extension at 72°C for 2 minutes. All PCR products were then purified using NucleoSpin Gel and PCR clean-up kit (740609.250) before submission for Sanger Sequencing to Eurofins genomics.

5.2.3. Sample processing, extraction method, and library preparation of DNA sequencing samples

Exosomes samples were extracted and treated as per section 2.5 and 2.6. ExoDNA was extracted as per section 2.7. Libraries were then prepared and sequenced as shown in Figure 5.1 by the Next Generation Sequencing Facility at the University of Leeds.

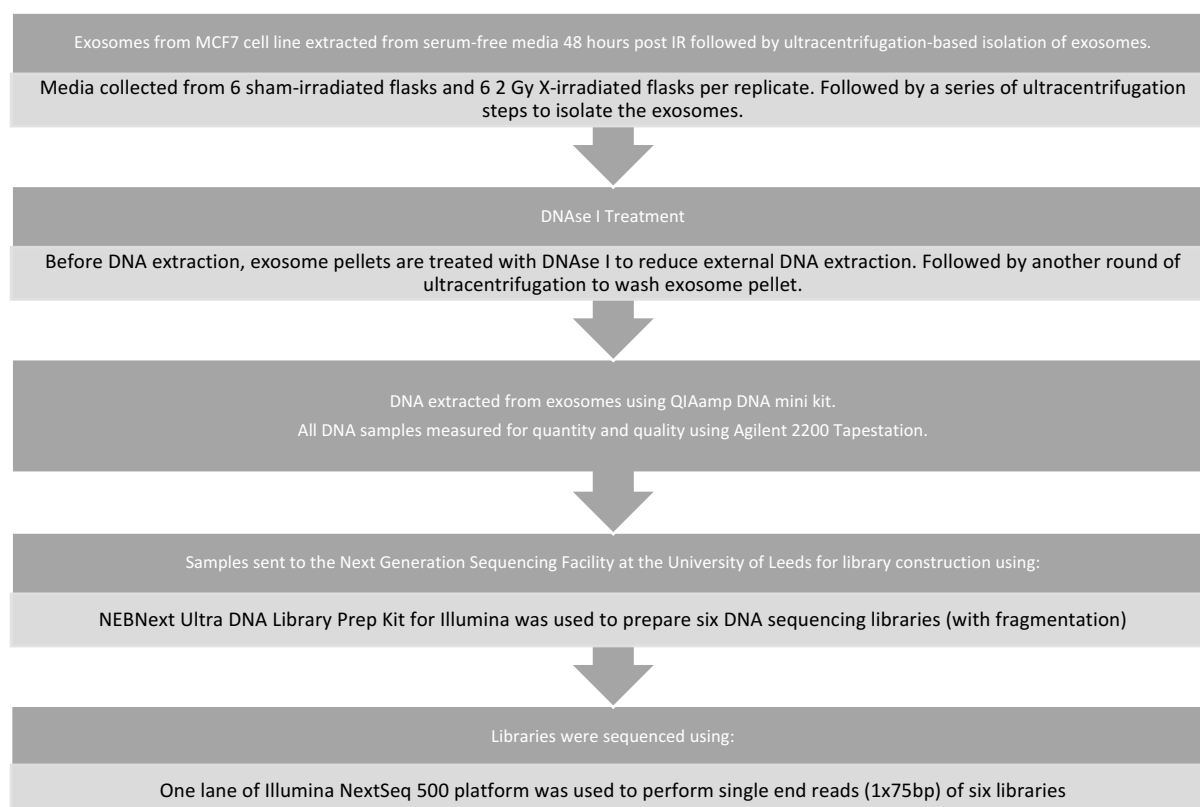


Figure 5.1 Workflow detailing sample generation and processing, DNase I treatment, exosome DNA extraction, library preparation, and platforms used to perform DNA sequencing. Exosomes were isolated from the MCF7 cell line, followed by DNA extraction. Libraries were prepared at the Next Generation Sequencing Facility at the University of Leeds. Libraries were then sequenced using the Illumina NextSeq 500, as detailed.

5.2.4. Initial Quality Check (QC)

FASTQC was used to perform an initial QC of the samples (before bioinformatics processing) to look for adaptor sequences in the data, as well as the spread of low quality base calls. Next, the tool CutAdapt was utilized to further clean the data by trimming low quality base calls from each end of the reads and any adapter sequences. Once this is finished, a second FASTQC was run to check the quality of the trimmed reads (.fastq file) and ensure the processing was sufficient for further bioinformatics processing. In Table 3 average read depth is shown for all six samples sent for whole genome sequencing (WGS). Read depth is the number of times that a given nucleotide in the genome has been read in a sequencing experiment. The aim was to examine copy number variants (genomic structural variation in 8 kb indels) from low-depth whole genome sequencing data. WGS was utilized as it aims to determine the complete DNA sequence of the MCF7 genome.

Sample	Total Reads (million)	Average read depth (x)
1	80.33	1.96685076
2	88.84	2.183355738
3	88.78	2.167231962
4	97.12	2.404107084
5	93.83	2.299388886
6	88.54	2.160788746

Table 3 Average read depth of exoDNA samples sent for WGS
Samples 1-3 are sham-irradiated (0 Gy) while samples 4-6 are 2 Gy X-irradiated.

5.2.5. Alignment of reads to the genome

The read data for each sample is aligned to the hg19 human genome reference sequence using the mem algorithm of the BWA aligner (Li and Durbin, 2009). BWA is a program designed for aligning sequencing reads against a large reference genome, and version 0.7.12 was used with this data set.

BWA was run with the following command:

```
bwa mem -t 4 $bwaIndex $read1 > $prefix.sam
```

Where:

Variable in command	Description
\$bwaIndex	The name and path of the bwa index (created with default options).
\$read1 \$read2	The name and path to the fastq read data.
\$prefix.sam	The name and path to the output file in the sam file format.

Table 4 Description of command used for BWA
Script used in the bioinformatics analysis of sequencing data concerning the alignment of the reads to the hg19 reference genome.

The position of each mapped to the autosomal, sex and mitochondrial chromosomes are noted and stored, ordered by chromosome and position in a text file (available upon request, information on website, <ftp://dna.leeds.ac.uk>, login required).

5.2.6. Data partitioning (Copy Number Variation (CNV) analysis)

The reads from the 6 samples along with the equivalent data derived from 50 patient control 'normal and healthy' individuals is used to determine how the data is partitioned along each

chromosome such that each partition contains a set number of reads. The set of 50 patient controls is used by the NHS (it is generated by the Regional DNA diagnostic lab in the Leeds Teach Hospital Trust), and is an internal dataset. The ratio of sample reads compared to the number of reference reads is determined for each partition and saved as a log2 value, which is saved to file along with the partition's chromosome, start and stop positions. The software that partitioned the data was a script written in-house (at the Next Generation Sequencing Facility at University of Leeds).

5.2.7. CNV analysis (chromosome images)

This data (from data partitioning) is then processed by the R package called DNACopy (Olshen et al., 2004) that compares the series of read count ratios and removes the noise inherent in the data to determine the corrected copy number for extended segments of a chromosome. This data is then saved as a series of images for each chromosome, plus a text file containing the corrected copy number data.

5.2.8. Origin of Reads

An empirical examination on the origin of reads in the data set was completed by manually blasting the first 1,000 reads of each data file against human sequences in the NCBI non-redundant DNA database using the blastn algorithm via the NCBI website. The sequenced reads were processed in set of 50 reads which were stored in a single text file in the fasta format and the results were returned formatted as a XML file.

5.2.8. LINE-1 repetitive mapping

The first 1,000 reads of each data file was blasted against the Genbank nr/nt database, and then counted for how many hits the first results produces. Sequences homologous to LINE1 sequence would produce multiple hits to genomic sequences, and sequences that were not homologous to LINE1 were found to identify a single hit per sequence.

5.2.9. Differences in DNA content/reads between irradiated and sham samples

In order to establish whether the DNA contents of sham or irradiated MCF7 exosomes reflected or differed from each other the first 1,000 reads of each sample was blasted against the Genbank nr/nt database. The reads with more than one hit were disregarded, as it would not be possible to determine which the correct sequence alignment was. Reads with more than 10 miss-matches (more than 10 nucleotides different between sample sequence and database) were also ignored as reads with an excess of miss-matches could be the result of misalignment of sequences present in the samples genome, but not found in the reference sequence. As each read was 76 base pairs long, a 'perfect match' should have 76 aligned bases and contain no gaps. A table of the results of each analysis is found in Table 6 and Table 7. The match rate (the number of bases in the reads that match the reference sequence) was calculated as:

$$\frac{\text{Number of aligned bases}}{\text{Total number of bases in the reads analyzed (76 x number out of 1000 that were not discarded)}}$$

The workflow of sequencing analysis of the WGS data is depicted in Figure 5.2.

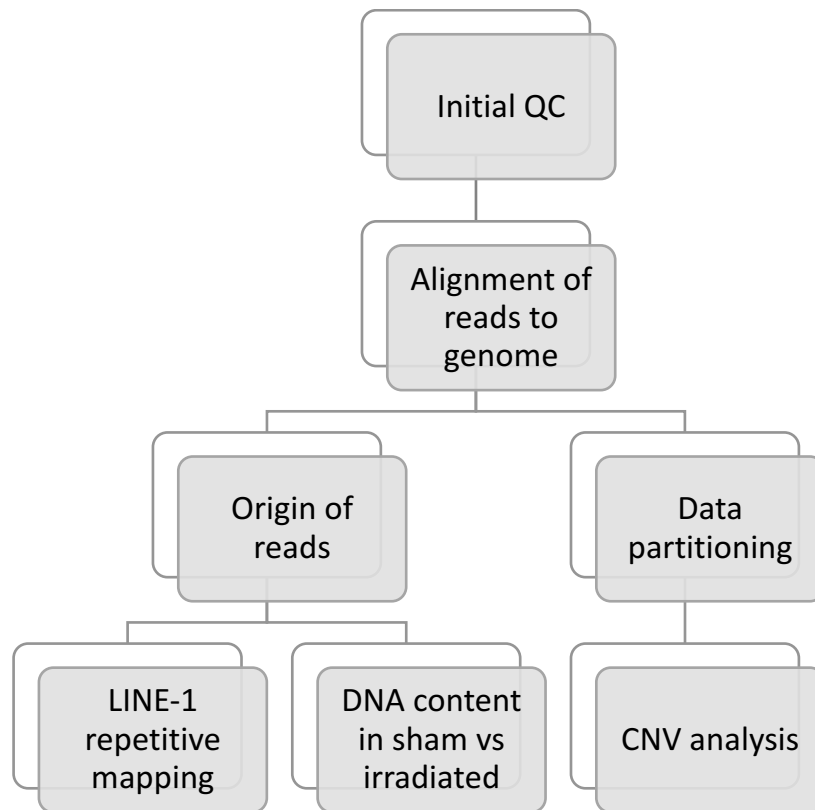


Figure 5.2 Workflow of the various sequencing analysis that was completed from the whole genome sequencing data.
Following the alignment of the reads to the hg19 reference genome, CNV analysis, LINE-1 repetitive mapping, and DNA content was all completed.

5.3. Results

5.3.1. Genomic DNA and exoDNA from MCF7 cell line contain PIK3CA mutation

The MCF7 cell line is known to have a heterozygous PIK3CA mutation in exon 9 that results in an amino acid change at position E545 where in codon GAG the G is replaced by an A resulting in AAG (Beaver et al., 2013). To confirm that the genomic DNA contained the same mutation as the exoDNA, PCR products from both genomic DNA and exoDNA (for PIK3CA mutation) were sent out for Sanger Sequencing. Figure 5.3 shows the mutation analysis of genomic DNA from MCF7 and corresponding exosomes, revealing the same heterozygous mutation in PIK3CA at position E545. Overlapping peaks for A and G can be seen in the chromatograms in all the samples at position E545, confirming the heterozygous mutation.

A**E545**

```

NC_000003   AGAACAGCTCAAAGCAATTTCTACACGAGATCCTCTCTGAAATCACTGAGCAGGAGAA
SYBR_g      -----GCTCAAGCAATTTCTACACGAGATCCTCTCTGAAATCACTGAGCAGGAGAA
Q5_g1       -----GCTCAGCATTCTACACGAGATCCTCTCTGAAATCACTGAGCAGGAGAA
Q5_g2.      -----CTCAAGCATTCTACACGAGATCCTCTCTGAAATCACTGAGCAGGAGAA
SYBR_exosome. -----AAGCATTCTACACGAGATCCTCTCTGAAATCACTGAGCAGGAGAA
Q5_exosome1 --- --ACGCTCAAGCATTCTACACGAGATCCTCTCTGAAATCACTGAGCAGGAGAA
Q5_exosome2 -----GCTCAGCATTCTACACGAGATCCTCTCTGAAATCACTGACAG---AAA

```

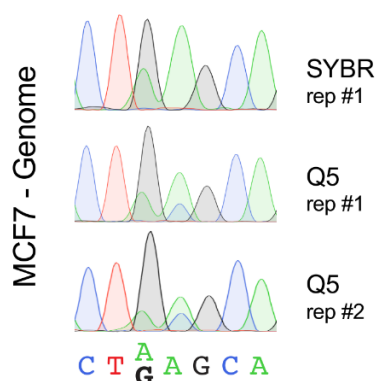
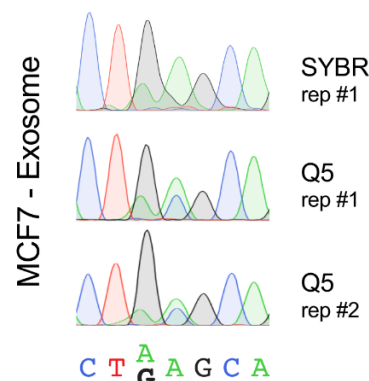
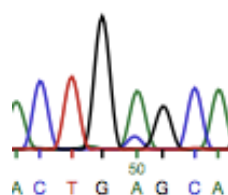
B**C****D**

Figure 5.3 Cellular DNA and exosomal DNA contain heterozygous *PIK3CA* mutation.

In figures B and C two different polymerases (1) SYBR Green Mastermix, primarily utilized for qPCR, and (2) Q5 High-Fidelity DNA Polymerase were used for the PCR reactions. A) Position E545 is highlighted in the sequenced PCR products. B) Chromatogram results for genomic DNA shows overlapping peaks for A and G in all three samples at position E545, confirming the heterozygous mutation. C) Chromatogram results for exoDNA shows the heterozygous mutation in all three samples at position E545, confirming the same mutation that is found within the genomic DNA. D) Chromatogram from the control genomic DNA sample (A2780 human ovarian cancer cell line), showing the same E545 position, without the heterozygous mutation (strong peak in G position, no overlapping peaks).

5.3.2. LINE-1 levels in MCF7 nuclear gDNA and exosomal DNA

The quantitative PCR (qPCR) analysis was conducted in order to analyse the levels of LINE-1 found within the nuclear gDNA and exosomal DNA in the MCF7 cell line following exposure to 2 Gy X-rays. Nuclear gDNA and exosomal DNA was collected 48 hours after exposure to 2 Gy X-rays (replicated 3 times), and $\Delta\Delta C_t$ is used to observe the differences as the target sample (LINE-1) is relative to the reference sample (housekeeping gene B2M, as described in section 3.2.2). The results (Figure 5.4)

demonstrate that there is a significant abundance of LINE-1 elements packaged into the DNA of exosomes when compared to nuclear gDNA, thus indicating a possible difference in composition of exoDNA compared with nuclear gDNA. This also suggests a selection of LINE-1 elements into the packaging of exosomal cargo.

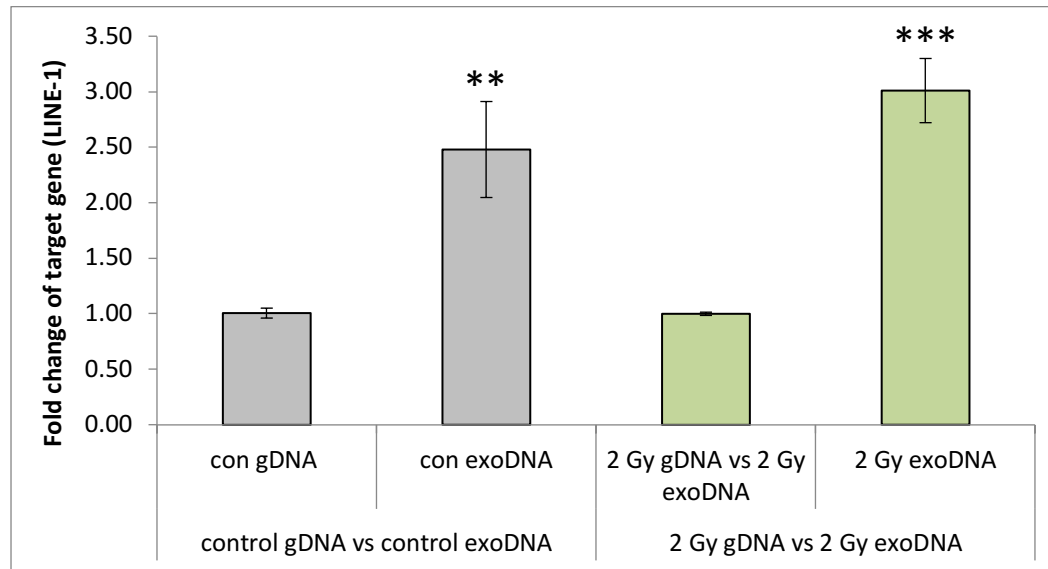


Figure 5.4 The MCF7 nuclear gDNA and exoDNA is assessed for LINE-1 levels by qPCR. Levels were quantitated 48 hours following X-ray exposure at a therapeutic dose (2 Gy). Fold change for LINE-1 corrected to housekeeping gene B2M. $n=3$ per group, t -test. Each bar represents the mean $\pm$ S.E.M. of 3 independent experiments. ** $P<0.01$, *** $P<0.001$.

5.3.3. DNA profile of EVs released from MCF7 cells

Prior to sequencing the samples were run on the Agilent 2200 TapeStation™ to assess size, quality, and quantification of the EV DNA. In order to evaluate the DNA profile the EVs were harvested from serum-free media 48 hours post irradiation, treated with DNase I to eliminate any external DNA contamination, and the total DNA was then extracted and analyzed on the Agilent TapeStation (Figure 5.5) Results demonstrated a variety of different sizes of DNA within the exoDNA samples, with every sample (both irradiated and unirradiated) showing a main peak at 200 bp fragments. While the only unirradiated sample showing another (minor) peak is Sample 1 at around 15000 bp, all of the irradiated samples show a variety of different DNA fragment sizes ranging from 250 to a steady decline around 3000 bp. While a majority of the fragment sizes are 200 bp, the irradiated EV

samples contain a variety of larger fragment sizes within their DNA when compared to the unirradiated EV samples. The electropherograms show that there are several different sized fragments of DNA from irradiated and unirradiated MCF7 EVs. The six samples sent for DNA sequencing were also run on a gel, and this is shown in Figure 5.6. Table 5 shows the DNA yield (which also varies from irradiated and unirradiated samples) of the six exoDNA samples.

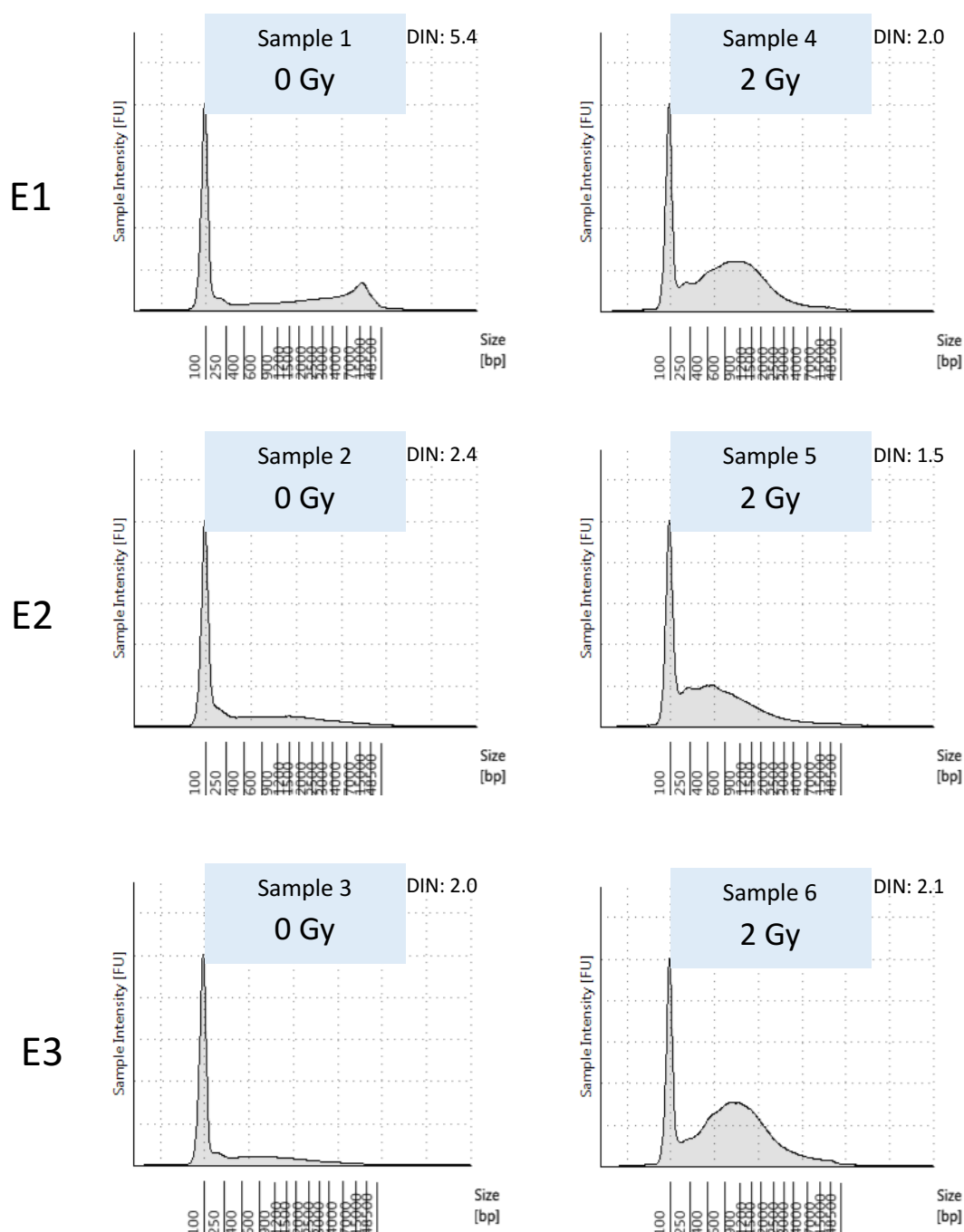


Figure 5.5 TapeStation profiles of total EV DNA.

Electropherograms of the three biological replicates of EV DNA used for DNA sequencing (E1, E2, and E3). Each EV DNA sample was harvested after 48 hours from 6 x T175 flasks of serum free MCF7 cell media.

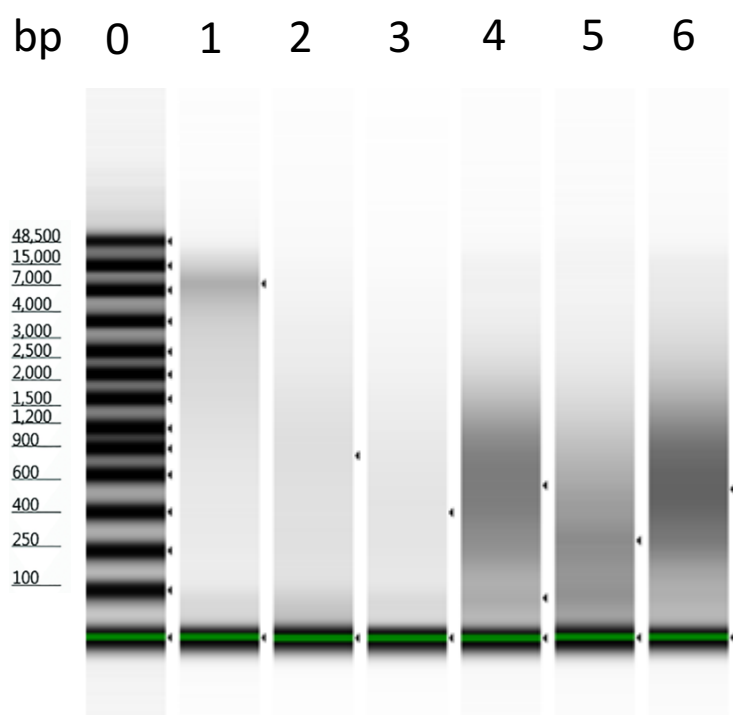


Figure 5.6 Image of TapeStation gel of total EV DNA.

The virtual gel image shows EV DNA extracted from MCF7 cells: Lane 0, ladder; lanes 1, 2 and 3, 0 Gy (Samples 1-3 in Figure 5.5); lanes 4, 5 and 6, 2Gy (Samples 4-6 in Figure 5.5).

Sample	0 Gy EV DNA (ng/ μ l)	2 Gy EV DNA (ng/ μ l)
E1	9.07	20.7
E2	6.28	14.5
E3	4.56	26.1

Table 5 EV DNA yield from MCF7 cells.

Three biological replicates. 10 μ l of each sample was sent to sequencing facility.

5.3.4. CNV Analysis

5.3.4.1 Whole genome sequencing shows that exoDNA is reflective of cellular DNA

In order to establish whether the DNA contents of sham and irradiated exosomes came from random or specific regions across the genome, a low depth whole genome sequencing experiment was conducted. The copy number analysis for all six samples was performed against the set of 50 patient controls (healthy individuals) that is used by the NHS, as described in section 5.2.6. Figure 5.7

was supplied by the Next Generation Sequencing Facility at the University of Leeds and shows an example of genomic DNA from a ‘normal and healthy’ patient. The average copy number remains constant at 1, without any chromosomal gains or losses, indicating a normal diploid genome. Figure 5.8 shows the sham irradiated (0 Gy) CNV sequencing results from one of the samples of MCF7 exosome DNA (replicate E2 shown, other 2 replicates in Appendix A, Appendix B, Appendix C, Appendix D). The exosomes from the MCF7 cell line clearly contain genomic DNA spanning all chromosomes, with several chromosomal gains/amplifications (regions above the baseline) and chromosomal losses (regions below the baseline) depicted throughout the profiles.

When comparing the CNV results from this exoDNA data to previously published MCF7 genomic DNA from array comparative genomic hybridization (CGH) copy number profiles (Shadeo and Lam 2006; Hampton et al., 2009) the pattern of copy number changes in the genome matches that of the exosome data. For example, four highly amplified regions (p arm of chromosome 1, p arm of chromosome 3, q arm of chromosome 17, and q arm of chromosome 20) in MCF7 genomic DNA, as determined by array CGH (Hampton et al., 2009) correspond exactly to the CNV results for MCF7 exoDNA found in this chapter. The majority of locations that change in copy number are due to the MCF7 cell line having a large number of rearrangements as part of its inherent genomic instability, and the exoDNA data reflects this as well. Thus indicating that exoDNA is of nuclear origin.

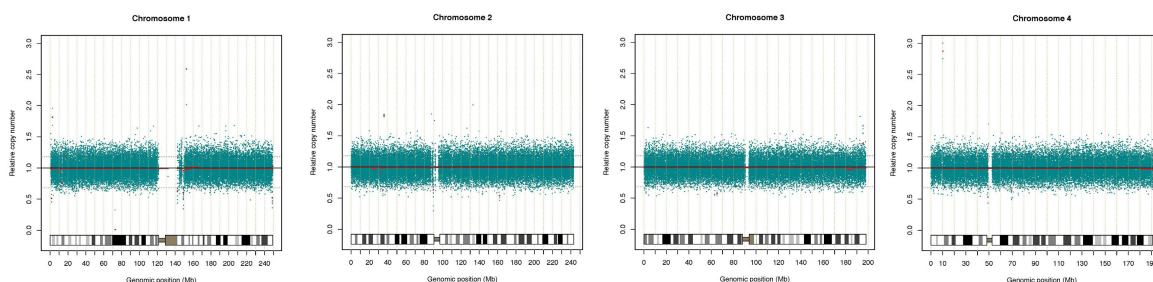


Figure 5.7 CNV Sequencing analysis of patient genomic DNA.

Chromosomes 1-4 from a ‘normal and healthy’ patient (i.e. no known genetic predisposition to any disease). The y-axis represents the log ratio of patient to control read counts (green) and the copy number call of each segment (red). The corresponding chromosome position (Mb) is shown beneath the CNV profile (x-axis). CNV-Seq analysis of ‘normal and healthy patient’ supplied by Next Generation Sequencing Facility at University of Leeds.

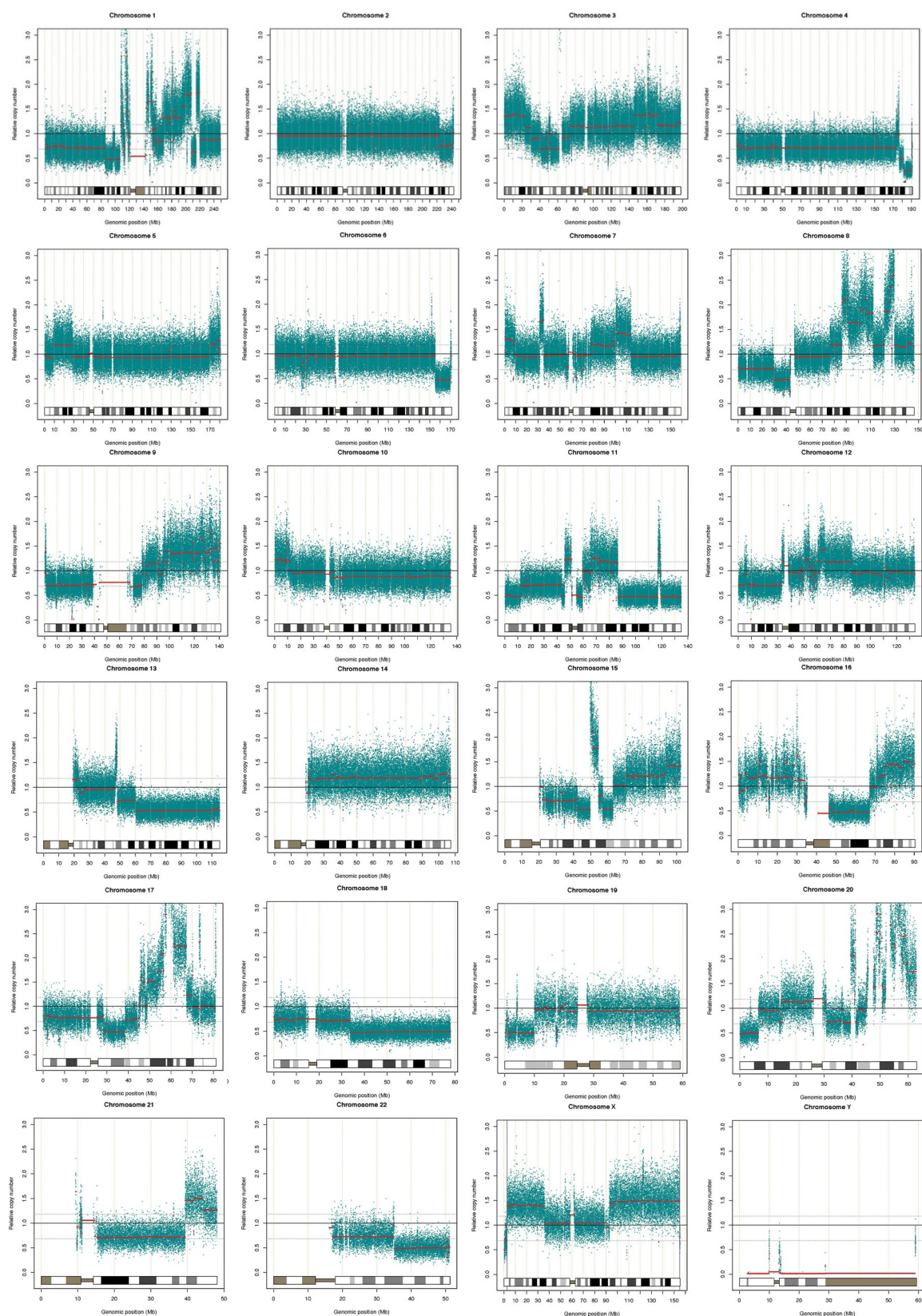
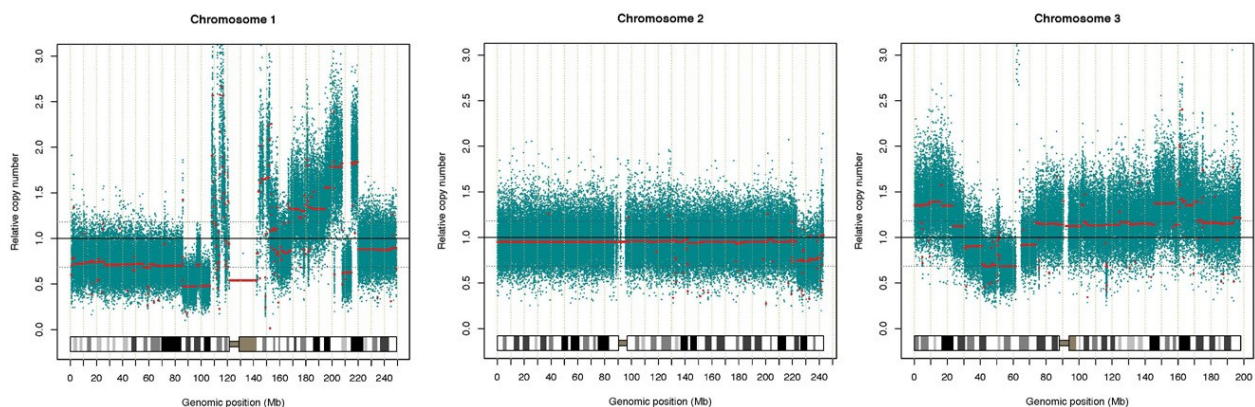


Figure 5.8 CNV Sequencing analysis of sham irradiated (0 Gy) MCF7 exosome DNA. Complete set of chromosome profiles (previously mentioned sample E2, sham) showing chromosomal gains/amplifications (above the baseline) and losses (below the baseline). The y-axis represents the log ratio of patient to control read counts (green) and the copy number call of each segment (red). The corresponding chromosome position (Mb) is shown beneath the CNV profile (x-axis).

5.3.4.2 Copy number variation in sham vs irradiated exoDNA

We then investigated if IR had an impact on the distribution of exoDNA between sham and irradiated samples to understand if this was a random or orchestrated process. After visual inspection of the CNV profiles, the irradiated samples are far more fragmented than the sham samples. A side-by-side comparison demonstrates this in Figure 5.9, as the 2 Gy irradiated exoDNA copy number has a high degree of variation, while in the sham samples the copy number is relatively constant. This difference led us to question what was causing the variation between the sham and irradiated exoDNA samples, and we decided to investigate the reads (inferred sequence of base pairs) generated from the sequencing experiment. A full set of the 2 Gy X-irradiated CNV analysis can be seen in Figure 5.10.

A) 0 Gy



B) 2 Gy

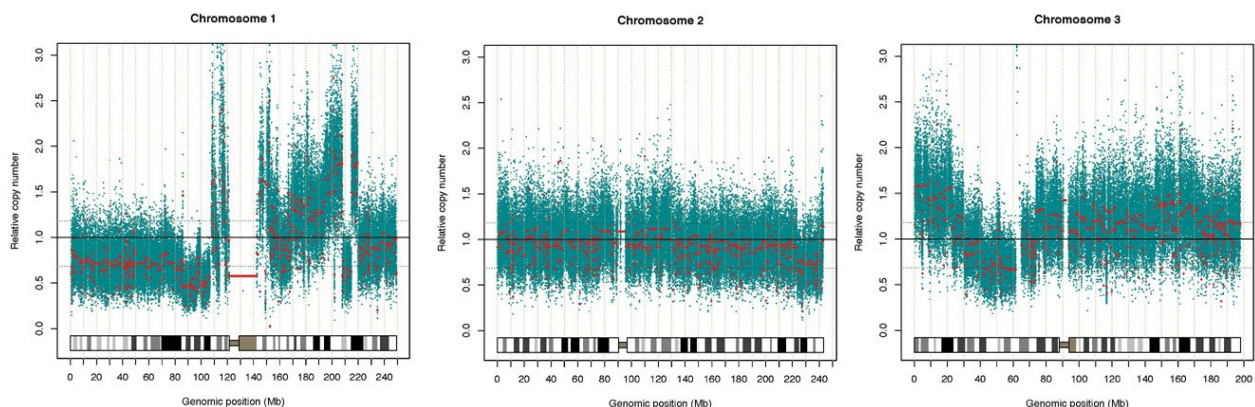


Figure 5.10 Comparing the CNV Sequencing analysis of 0 Gy (sham irradiated) and 2 Gy X-irradiated exosome DNA. The first three chromosomes are shown from each set in order to give a clear visual demonstration of the fragmentation between the (A) 0 Gy and (B) 2 Gy X-irradiated exoDNA samples. 2 Gy irradiated exoDNA copy number shows a high degree of variation, while the sham samples (0 Gy) show a relatively constant copy number.

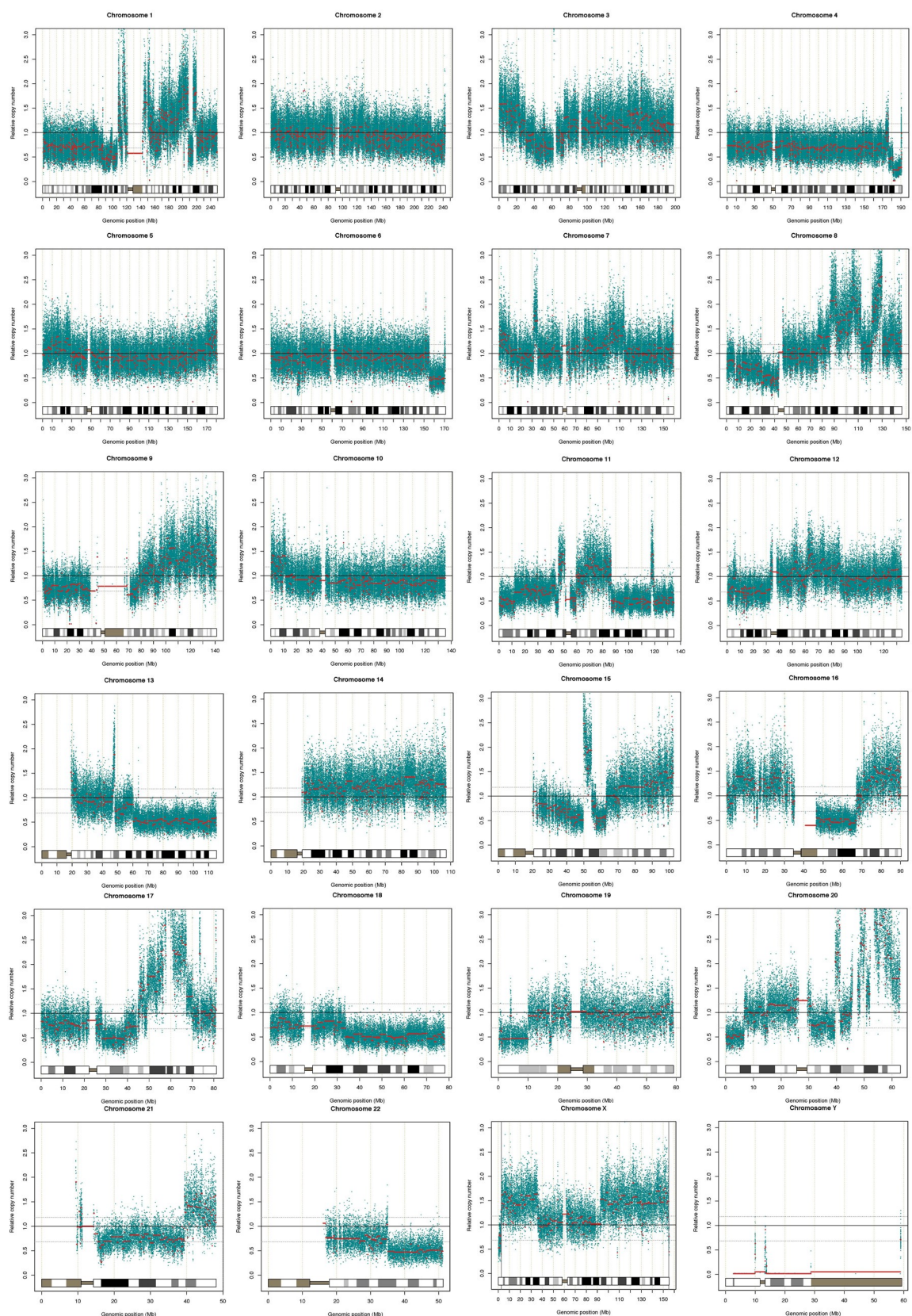


Figure 5.12 CNV Sequencing analysis of 2 Gy X-irradiated exosome DNA originating from MCF7 cell line. Complete set of chromosome profiles (sample E2, section 5.5) showing chromosomal gains/amplifications (above the baseline) and losses (below the baseline). The y-axis represents the log ratio of patient to control read counts (green) and the copy number call of each segment (red). The corresponding chromosome position (Mb) is shown beneath the CNV profile (x-axis).

5.3.4.3 Differences between DNA sequences in sham and irradiated exosomes

We then investigated if there was any difference in the sham and irradiated exoDNA reads generated from the sequencing experiment by blasting the first 1,000 reads of each sample against a database used for sequence analysis (Genbank nr/nt database, analysis as described in section 5.2.9). A table of the results of each analysis is found below in Table 6 and Table 7. The sham-irradiated samples (samples 1-3) gave values of 0.99578, 0.99431, and 0.99478 (average value of 0.99495), while the 2 Gy irradiated samples (samples 4-6) gave values of 0.99457, 0.99343, and 0.99563 (average value of 0.99454). As the two average values are very similar, and the values for each set of samples overlap, there is no difference between the irradiated and sham sequences in the data set, as per this CNV analysis. While there is an obvious visual variation between the sham and irradiated samples, it is possible that the difference is on the nucleotide level and cannot be accurately determined via the low depth sequencing in this experiment.

Sample	Total # of Bases	Total # of Aligned Bases	Match Rate	# of gaps in Alignment	Gaps per base	Avg match rate
1	55936	55700	0.995780892	4	0.005434783	
2	55024	54711	0.994311573	8	0.011049724	
3	53656	53376	0.994781571	8	0.011331445	
						0.994958012

Table 6 Results of differences between top 1,000 reads of sham (0 Gy) exoDNA sequences. Reads were filtered for multiple hits and >10 miss-matches.

Sample	Total # of Bases	Total # of Aligned Bases	Match Rate	# of gaps in Alignment	Gaps per base	Avg match rate
4	54340	54045	0.994571218	1	0.001398601	
5	54264	53908	0.993439481	11	0.015406162	
6	53428	53195	0.995638991	11	0.015647226	
						0.994549897

Table 7 Results of differences between top 1,000 reads of 2 Gy irradiated exoDNA sequences. Reads were filtered for multiple hits and >10 miss-matches.

5.3.5. LINE-1 repetitive mapping

As previous results in this chapter indicated elevated levels of LINE1 in irradiated exoDNA, we also investigated if the reads generated from the sequencing experiment displayed any sequence selection to LINE1 elements in sham or irradiated samples. However, results showed no excess in reads mapping to sequences homologous to LINE1 (results not shown).

5.4. Discussion

5.4.1. MCF7 exosomes contain DNA with PIK3CA mutation

The MCF7 cell line is distinguished as carrying a heterozygous mutation in PIK3CA at position E545 (E545 K) on exon 9, and has been previously shown within its genomic DNA (Daneshmand et al., 2012; Schneck et al., 2013). The results in this chapter (Section 5.3.1) confirm the previously known mutation within the cancer cell line, while also showing the same heterozygous mutation within the exoDNA. As exosomes circulate through the body and are easily and quickly collected from a variety of bodily fluids, exoDNA has the potential to serve as a noninvasive biomarker in early detection of certain types of cancers.

5.4.2. LINE-1 levels elevated in exoDNA

It has been well documented that increased retrotransposon activity and LINE-1 insertion correlates to tumourigenesis. For example, LINE-1 insertions have been found in the MYC gene in breast cancer (Morse, 1988) as well as in the APC gene in colorectal cancer (Bundo et al., 2014). It has been shown that EVs are able to transfer retrotransposon RNA to normal cells (Balaj et al., 2011). In the same study, Balaj *et al.* (2011) showed an abundance in retrotransposon elements (LINE-1, Alu, and HERV) originating from cancer cell-derived EVs. This discovery suggested an alternative method for retrotransposons to spread from one cell to another via horizontal transmission. If the exoDNA from cancer cells containing retrotransposons can be delivered to recipient cells they could potentially contribute to genomic instability.

The elevated LINE-1 levels in MCF7 exosomal DNA in this chapter is consistent with the study by Balaj *et al.* (2011), in both the sham and 2 Gy X-irradiated samples (Section 5.3.2). The significant increase in LINE1 from the exoDNA versus the cells from which they were derived indicates a difference in composition of exoDNA compared with nuclear DNA, and selective packaging of retrotransposons in exosomal DNA. While the results show an abundance of LINE-1 levels in cancer cell-derived exoDNA, it is unclear whether the functional impact of retrotransposon transfer would

have any effect on recipient 'normal' cells, and would require further investigation. However, as irradiation is known to cause DNA DSB and also that retrotransposons can act as insertional mutagens that can target DSB, it can be hypothesized that the effects of exoDNA containing active retrotransposons could exacerbate the spread of insertional mutagenesis in human diseases with deregulated retrotransposition events.

5.4.3. Large fragment size distribution in irradiated exoDNA

Previous research has shown that exosomes contain a range of DNA fragment sizes. Thakur *et al.* (2014) demonstrated that a majority of internal exoDNA within three cancerous cell lines was found to be between 100 bp and 2.5kb, while Kahlert *et al.* (2014) showed exoDNA fragments greater than 10,000 bp in size. The TapeStation plots in this chapter (Figure 5.5) are consistent with the literature that suggests exosomes contain DNA fragments within the size ranges of 100 bp-10,000 bp.

IR is a genotoxic stressor that causes DNA double-strand breaks, and activates a DNA damage response (DDR) that allows cells to sense and respond to damaged DNA by halting the cell cycle and repairing the damage (Vignard *et al.*, 2013). Studies have shown that genomic stress leads to an accumulation of DNA in the cytosol (Shen *et al.*, 2015; Ho *et al.*, 2016; Erdal *et al.*, 2017) and also that damaged nuclear DNA tends to move into the cytoplasm (Härtlova *et al.*, 2015; Lan *et al.*, 2014; Takahashi *et al.*, 2017). Micronuclei formation is a notable example of DNA damage and accumulation of cytoplasmic DNA as they are small, fragmented chromosomes that can result from errors by NHEJ repair. A study by Bakhoun *et al.* (2018) suggests that fragmented nuclear DNA is divided into micronuclei, and when ruptured they release nuclear DNA into the cytosol. All of the 2 Gy X-irradiated exoDNA in this chapter consist of larger DNA fragments ranging in size from 250-3000 bp. It is probable that damaged nuclear DNA is the source of the larger fragments of exoDNA. Furthermore, the presence of DNA in the cytosol is recognized by Stimulator of Interferon Genes (STING) and activates the cGAS-STING pathway, inducing a protective immune response that defends against a variety of DNA-containing pathogens (Chen *et al.*, 2016). Takahashi *et al.* (2017)

demonstrated in normal human diploid fibroblasts (HDFs) that exosome secretion prevents the aberrant activation of DDR pathways by excreting harmful cytoplasmic DNA originating from the nucleus from cells, thus maintaining cellular homeostasis. It can therefore be speculated that if tumour cells are exporting harmful cytoplasmic DNA into exosomes, they avoid activating cGAS-STING pathway.

Moreover, this alludes to a plausible explanation to the phenomenon that is known as the abscopal effect. The link between radiation therapy (RT) and immune therapy has recently become an important area of study, in particular cGAS-STING signaling has shown to be essential for response to both radiation and immune checkpoint blockade. For example, interferon production after radiation and the resulting antitumour-immunity was dependent on STING (Deng et al., 2014). If tumour cells are treated with RT and then excrete harmful cytoplasmic DNA into exosomes, they avoid activating anti-tumour immune components, then carrying and secreting the harmful DNA to distant sites outside the RT field. Thus leading to a plausible explanation as to why it is difficult to see abscopal effects, however further research is required to link this theory.

Interestingly, a recent study by Diamond *et al.* (2018) showed that only irradiated tumour-derived exosomes carried dsDNA that were capable of inducing interferon production in a STING-dependent fashion, while the untreated tumour-derived exosomes displayed no activation. The researchers suggested that the dsDNA present in the irradiated tumour-derived exosomes has characteristics distinct from the dsDNA present in the untreated tumour-derived exosomes. Taken into consideration with the results from this section, and the previously mentioned studies that have shown damaged nuclear DNA tends to move to the cytoplasm, therefore we hypothesize that it is size/abundance of the dsDNA within irradiated (or damaged) exosomes that regulates and activates the interferon stimulatory function. Further experimentation will be required to determine the amount of dsDNA required to activate the interferon pathway via cGAS-STING.

5.4.4. CNV Analysis

While there is a considerable amount of research regarding the role of exosomes in cell-cell communication and transfer of their cargo, there is not much research regarding how exosome cargo (DNA, RNA, proteins) is sorted into the exosomes. RNA content profiles within exosomes has been shown to be different compared to the RNA content of the parental cells (Skog et al., 2008; Pigati et al., 2010; Guduric-Fuchs et al., 2012), suggesting selective packaging rather than random sorting. Balaj *et al.* (2011) showed that tumour microvesicles were enriched for retrotransposon elements when compared to their cellular origins, indicative of selective packaging as well.

Interestingly, a thorough characterization of dsDNA derived from tumour exosomes by Thakur *et al.* (2014) revealed no bias for gene-coding versus intergenic regions and no amplifications/deletions in specific regions when compared to genomic DNA. Copy number variations (CNVs) are gains or deletions of genomic segments, and can be best identified by comparing the read distribution in a tumour genome with that of the matched 'normal' genome.

The revelations from Thakur *et al.* (2014) are consistent with the findings within this chapter (section 5.3.4.1), when comparing the CNV results from the exoDNA to previously published MCF7 genomic DNA from array CGH profiles (Hampton et al., 2009) it is apparent that the pattern of copy number changes in the genome matches that of the exosome data. The patterns of losses and gains of certain regions in the chromosomes is completely consistent throughout the genome, showing the highly reflective status of the DNA within exosomes of that to the parental cell. This observation supports the hypothesis that exoDNA originates from nuclear DNA.

While CNVs are important and are attributed to human genetic variation and diseases, not much is known about the molecular mechanisms and environmental factors that impact CNV formation.

Agents that cause replication stress and DNA damage (aphidicolin, hydroxyurea, and IR) have been shown to induce *de novo* CNVs in normal human fibroblasts, all within similar hotspot regions (Arlt et al., 2009; Arlt et al., 2011; Arlt et al., 2014). While a majority of regions in the exoDNA that change

are due to the MCF7 cell line having a large number of rearrangements as part of its inherent genomic instability, there is an obvious visual difference between the fragments in the sham and irradiated exoDNA samples in this chapter. Following exposure to 2 Gy X-irradiation the exoDNA shows a high degree of variation in copy number. This led to the question of what was causing the difference between the treated and untreated samples, where was this variation in the copy number coming from?

A recent study by Takahashi *et. al* (2017) suggests that exoDNA originates from damaged nuclear DNA. After inducing DNA damage with doxorubicin, exosomes react by preventing the cytoplasmic aggregation of nuclear DNA. The exosomes do this by removing the damaged DNA in order to maintain cellular homeostasis and prevent activation of DDR. A previously mentioned study by Balaj *et al.* (2011) showed an enrichment of transposable elements (LINE-1 and HERV) in exoDNA originating from tumour cells. When considering this report and the previous results from this chapter (increased levels of LINE-1 in exoDNA compared to nuclear DNA, section 5.3.2) it was hypothesized that the variation in copy number in exoDNA may be originating from sequence specific selection of transposable elements (LINE-1, in particular). However, analyzation of the sequences from the control and irradiated exoDNA samples showed no excess in reads mapping to sequences that were homologous to LINE-1 (data not shown), suggesting a different cause for the variation in copy number.

The quest for the variation in copy number continued and the comparison of the exoDNA of the sequenced samples to the reference sequence did not show any difference between the control and irradiated, as per this analysis. The genetic variation in the irradiated samples is visually evident, and it is plausible to suggest that the difference is on the nucleotide level, not at the variation in copy number.

This whole genome sequencing experiment was performed at a low depth (1.9x-2.4x), and at this level of read depth SNPs would not be detectable. For example, the 'standard' average depth used

to reliably identify SNPs has been 30x (Ahn et al., 2009; Wang et al., 2008). As the original intent of the CNV analysis was to investigate if the exoDNA was derived from random/specific regions in the genome, a low depth WGS was sufficient. However, upon reflection of the results it is clear that a sequencing experiment at a much higher resolution will need to be conducted in order to identify the cause of the genetic variation between the control and irradiated exoDNA samples. Sequencing errors can also be improved by high-coverage sequencing and result in reliable single nucleotide polymorphism (SNP) detection. It must also be noted that the variation could be due to 'noise' and 'technical' errors, however as all three irradiated (and control) samples follow the same trend this is unlikely. Regardless, further analysis is required in order to determine the cause of the variation.

5.5. Conclusions

The results in this chapter have led to a further understanding into the contents of exosomal DNA. We were able to show that the exoDNA from the cancerous MCF7 cell line is encapsulated within exosomes and spans the whole genome. The mutation characterizing the MCF7 cell line (PIK3CA) within the exoDNA reflects the mutational status of the parental cell lines, thus highlighting its potential use as a noninvasive genetic marker for cancer.

The effects of IR on exoDNA has not yet been reported in literature. Results from our study in this chapter is the first to show an increase in exoDNA fragment size after exposure to 2 Gy X-rays, suggesting that damaged nuclear DNA is the source of the larger fragments of exoDNA. Currently there are no studies directly connecting nuclear rupture with exosomal DNA content, and further work is required in this area. Results from CNV analysis show an obvious visual difference between the sham and irradiated exoDNA samples, however it is unclear what is causing the variation. Deeper sequencing could allow for higher-confidence calls of specific variants, such as SNPs.

Chapter 6 Discussion

The cellular effects of IR are serious and varying, and induce a multitude of cellular alterations such as chromosomal aberrations, genetic amplifications/deletions, micronuclei induction, karyotypic heterogeneity, mutagenesis, alterations in mutation rates, and subcloning efficiencies (Keszenman et al., 2015). IR directly affects DNA structure by causing base damage, DNA breaks, such as SSBs or DSBs, or indirectly by production of free radicals (such as ROS) (Hall and Giaccia, 2006). If the cells fail to repair these disturbances to the molecular structure it can cause apoptosis, chromosomal abnormalities, and mutations that induce cell death and mitotic failure. It is postulated that all damage responses occur during or very shortly after irradiation of the nuclei in targeted cells. The potential for biological consequences can be expressed within one or two generations (Kadhim et al., 2013). However, within the past two decades, it has been made apparent that the effects of radiation are not limited to the initial insult but can cause delayed effects that appear many generations after exposure. Thus, the non-targeted theory was established which highlights the imperative role of intercellular signalling and results in radiation-induced genomic instability (Kadhim et al., 1992; Kadhim et al., 1994) and radiation-induced bystander effects (Nagasawa and Little, 1992; Morgan and Sowa, 2007). Notably, GI is defined by its novel biological effects (such as delayed gene mutations, gene amplifications, chromosomal damage, micronucleus formation, and de novo chromosomal aberrations) that occur in the progeny of cells many generations after the original radiation event and is a hallmark of tumourigenic progression or cancer. While the unstable phenotypes produced from NTEs are well characterized, the cellular processes that initiate radiation-induced GI remain elusive (Morgan, 2003; Kadhim et al., 2013), however it is possible that the random insertion nature of the LINE-1 retrotransposons could be the reason for the instability. LINE-1 is the most active mobile element in the human genome, and can cause insertions and major genomic rearrangements that lead to GI (Lee et al., 2012). Several studies have shown that IR triggers transposable elements and increases the insertion rates into the genome (Morawetz, 1987;

Farkash et al., 2006; Tanaka et al., 2012), thus highlighting the damaging potential of these agents. As mentioned above, the mechanism for radiation-induced GI is not completely understood and remains elusive, however it is possible that the random insertion nature of the LINE-1 retrotransposons could be the reason for the instability. Therefore, we have investigated the hypothesis that retrotransposons are involved in the mechanism contributing to NTE induction. Interestingly, recent work has shown high levels of transposable elements (including LINE-1) within the DNA of extracellular vesicles (EVs) derived from cancerous cells (Balaj et al., 2011). EVs, including exosomes, have recently become of keen interest due to their widespread implications in intercellular communication across numerous fields. They have the ability to transfer their biological content (such as proteins, lipids, and nucleic acids) between cells, thus influencing the function of recipient cells (Jella et al., 2018; Al-Mayah et al., 2015). Exosomes are also able to travel to distant locations (Peinado et al., 2012), and have been shown to promote favourable environments for tumour growth and invasion (Li et al., 2018; Meehan and Vella, 2016; Zhao et al., 2016). The content of exosomes generally depends on the type and state of its parental cell, however it is capable of being influenced by cellular stressors as well. IR, in particular, has been shown to induce alterations in EV composition, however, there is not much research and mainly refers to its proteome (Jelonek et al., 2016). Interestingly, an increase in retrotransposon sequences and the oncogene *c-Myc* were found in the DNA of cancerous microvesicles, which could potentially be carried by the vesicles and deposited into non-cancerous areas, promoting tumour metastasis (Balaj et al., 2011). It has also been recently shown that RIGI could be partially mediated by exosomes/microvesicles (Al-Mayah et al., 2012; Al-Mayah et al., 2015; Al-Mayah et al., 2017). ExoDNA has also been shown to be upregulated in response to cellular stress (Takahashi et al., 2017). However, there is no literature to date regarding the effects of IR on exoDNA content or composition and further research must be conducted as there are several implications regarding radiotherapy and intercellular communication.

Therefore, in this study we aimed to characterize exosomes released from irradiated MCF7 breast cancer cells and to profile their DNA contents in response to irradiation. The hypothesis is that exoDNA could be derived from specific locations within the genome.

Based on the above knowledge this study was also designed to investigate a mechanistic cause of RIGI, particularly in the case of retrotransposon (LINE-1) insertion. Furthermore, the effect of IR on exoDNA (originating from MCF7 cancer cell line) was investigated to determine any alterations in exoDNA content or composition. Thus, the following aims were proposed and tested:

1. To investigate LINE-1 retrotransposition in response to low and moderate doses of ionizing radiation that are relevant to clinical diagnostic and therapeutic doses, at time points relevant to genomic instability in a normal fibroblast cell line, HF19 and two cancerous cell lines, Caco-2 and MCF7.
2. To determine potential links between LINE-1 retrotransposition and RIGI.
3. To characterize the exosomes released from sham and irradiated MCF7 cells, including morphology, biochemical composition, and sizing.
4. To extract and characterize exosomal DNA using whole-genome sequencing to identify any copy number variation by comparing exosomes excreted from sham and 2 Gy irradiated MCF7 cells.

The experiments described in this thesis aimed to address the above questions using various methods. In Chapter 3 0.1 Gy and 2 Gy X-ray doses, similar to those that may be encountered in diagnostic and therapeutic uses in radiotherapy, were used to investigate the susceptibility of HF19, Caco-2, and MCF7 cells to the induction of initial damage and genomic instability. DNA damage was assessed using the micronucleus assay, whereas copy number using qPCR assessed LINE-1 retrotransposition. Furthermore, cell viability was monitored at multiple time points, as described in section 2.4. The aim of chapter three was to investigate if diagnostic and therapeutic doses of IR will

cause an increase in LINE-1 retrotransposition, thereby causing an instability within the genome and if this correlated with the induction of micronuclei.

At 48 hours post exposure to 0.1 Gy X-rays the HF19 normal fibroblast cell line showed an increase in LINE-1 levels, and in correlation the prevalence of MN induction was also significant at this time point. While speculative, this data is suggestive of an early activation of LINE-1 retrotransposition thereby causing radiation-induced damage. Previous studies have also shown IR-induced LINE-1 retrotransposition results in correlating DNA damage (Farkash et al., 2006; Banaz-Yasar et al., 2012). However, two weeks post IR show no significant DNA damage, suggesting the increase in LINE-1 insertions did not contribute to the persistent and long-term effects causing RIGI. Nevertheless, the results in this chapter are only limited to investigations regarding copy number increase in the LINE-1 transposon. It could be possible that by utilization of other methods of investigating LINE-1 activation, such as LINE-1 methylation or gene expression status, it could show effects of IR on the retrotransposon. For example, a study by Luzhna *et al.* (2015) showed that early analysis revealed short-term hypomethylation of LINE-1 (96 hours post IR) was associated with long-term reactivation of LINE-1 (12 and 24 weeks post IR) by analysing LINE-1 ORF1 gene expression through qRT-PCR. While the methylation level of LINE-1 was back to control levels at the later time points, radiation exposure also contributed to the translation of the LINE-1 element as LINE-1 protein was increased after 24 weeks (Luzhna et al., 2015). The authors hypothesized that the activation of LINE-1 retrotransposon may trigger its migration and insertion into the genome, where a few copies could be inserted into a highly active genomic region and remain unmethylated, while still having a very high copying potential. Perhaps by analysing IR-induced LINE-1 activation and copying potential by other methods a difference in results in chapter three could be revealed, yet it is difficult to prove without further investigation.

Several studies have shown that cellular stressors increase exosome release from cells (Lehmann et al., 2008; Xiao et al., 2014; Beer et al., 2015; Kanemoto et al., 2016). The results in chapter four

support previous studies that show IR increases exosome secretion from cells (Lehmann et al., 2008; Jella et al., 2014; Al-Mayah et al., 2015). Notably, treatment of cells with cisplatin (Xiao et al., 2014) and under hypoxic conditions (King et al., 2012) has also resulted in an increase of secretion of exosomes from cells. It is unclear why cells exude more exosomes after exposure to stressful conditions, as our knowledge of the biogenesis of exosomes under various conditions is still relatively undeveloped. Initially exosomes were largely ignored (when discovered about 50 years ago) as they were thought to be disposals of cellular waste. This is a theory that could hold true, however it is now understood this material could elicit functional changes in neighboring cells and possibly promote pathological conditions (Rashed et al., 2017). Another possibility is that cells may communicate to each other regarding intracellular stress by increasing exosome excretion.

The results in chapter five that showed the increase in LINE-1 elements in exoDNA in response to IR are in concordance with previous research that shows an abundance of retrotransposon elements (LINE-1, Alu, and HERV) derived from EVs from cancerous cells (Balaj et al., 2011). The abundance of LINE-1 elements found within the exoDNA in chapter five is indicative of selective packaging of these retrotransposons, and suggests a different composition of exoDNA compared to genomic DNA. It has been well documented that increased LINE-1 activity contributes to tumour onset and progression (Sinibaldi-Vallebona et al., 2011). As previously mentioned, LINE-1 insertion has been shown to cause major genomic rearrangements that can lead to genomic instability (Symer et al., 2002; Gilbert et al., 2002). LINE-1 is also known to create and target double-strand breaks (Belgnaoui et al., 2006). Interestingly, research has also implicated exoDNA in horizontal gene transfer as recipient cells were able to receive genetic information via exoDNA (Fischer et al., 2016). Therefore, in theory, exoDNA could carry elevated levels of transposable elements, and by horizontal transmission deposit these elements into recipient cells, thereby exacerbating the spread of insertional mutagenesis and be the cause of GI and BE. While this is an intriguing theory, and results in this chapter reveal an abundance of LINE-1 levels in exoDNA originating from cancerous cells, further investigation is needed to

investigate if retrotransposon transfer via exoDNA would have any functional impact on recipient cells.

Results from chapter five showed the first effects of IR on exoDNA composition, including an increase in exoDNA fragment size after exposure to 2 Gy X-rays (0 Gy exoDNA samples showed a peak at 200 bp, while 2 Gy samples showed fragment sizes from 250-3000 bp). A plausible explanation resides in the theory that damaged nuclear DNA is the source of exoDNA, and mechanisms causing their generation will be discussed further below. Takahashi *et al.* (2017) supports this theory where treatment with the DNA-damaging agent doxorubicin significantly increased the amount of exoDNA within the doxorubicin-treated cells. Interestingly, this was accompanied by an increase of aggregation of nuclear DNA in the cytoplasm. The restriction of exosome release also resulted in cytoplasmic accumulation of nuclear DNA, demonstrating that exosome release prohibits the cytoplasmic accumulation of nuclear DNA (Takahashi *et al.*, 2017). Notably, this study also showed that exosome secretion prevents the aberrant activation of DDR pathways by removing the damaged DNA, thus maintaining cellular homeostasis.

As mentioned previously, damaged nuclear DNA results in the accumulation in the cytosol (Härtlova *et al.*, 2015; Lan *et al.*, 2014; Takahashi *et al.*, 2017), notably in the form of MN. MN are initially formed with a nuclear envelope (NE), but after mitosis more than half the MN lose compartmentalization as their NE is split apart (Hatch *et al.*, 2013). It is also a possibility that exoDNA originates from MN that have degraded. It has been shown that after DNA damage the NE of MN ruptures, and then cGAS is recruited to MN, thus activating the cGAS-STING pathway (Mackenzie *et al.*, 2017; Yang *et al.*, 2017). Relatedly, a recent study by Bakhoun *et al.* (2018) showed that continuous errors in chromosomal segregation (whereby measuring chromosomal instability as sustained MN induction) leads to the spilling of genomic DNA into the cytosol, then activating the cGAS-STING pathway. By genetically suppressing chromosomal instability, the study showed that the rate of metastasis significantly decreased, while inducing ongoing chromosome segregation errors

resulted in the promotion of tumour cell invasion and metastasis (Bakhoum et al., 2018). While this study does not implicate exoDNA production, it advocates that exoDNA is a consequence of chromosomal instability.

Another previously mentioned study by Diamond *et al.* (2018) showed that only irradiated exosomes from a cancerous cell line were able to induce IFN production in a STING-dependent way, while the unirradiated exosomes displayed no such activation. The researchers then hypothesized that the dsDNA in the irradiated exoDNA population has characteristics that differ from the sham irradiated samples, and theorized that the difference could lie in the amount (abundance) of dsDNA delivered to induce IFN activation. Moreover, in the study by Takahasi *et al.* (2017) (above) results showed that the DDR pathway activation was reliant on the amount of added exosomes, suggesting a quantitative-regulated response. Collectively, taken into consideration with the results from chapter 5 (increase in exoDNA fragment size in irradiated populations), the studies (and results from chapter 4) that show non-cancerous cells do not contain exoDNA (Balaj et al., 2011; Thakur et al., 2014), and the previously mentioned studies that show damaged nuclear DNA moves to the cytoplasm, we hypothesize that it is the size/abundance of the DNA within irradiated (or damaged) exosomes that is responsible for the activation of immune responses. Currently there is no link between chromosomal instability and exoDNA aggregation, however research in this study and previous studies alludes to a link. Further research is also warranted in regards to cytosolic DNA and exoDNA, if their production and exoDNA packaging is an orchestrated process or a result of cellular stress. Regarding the mechanisms behind the stress-induced exoDNA, a plausible explanation lies in error prone DNA repair processes such as microhomology-mediated end joining (MMEJ). IR is capable of inducing DNA damage in the form of single-strand breaks and double-strand breaks (DSBs). The processing and repair of such chromosome lesions can lead to mutations and chromosome rearrangements that lead to GI, cell death, or cancer. The most common and prevalent pathways to repair DSBs are non-homologous DNA end joining (NHEJ) and homologous recombination (HR),

however there is now evidence for additional repair mechanisms that can modify the DNA sequence and result in loss of genetic information. In MMEJ, repair starts by resection or unwinding dsDNA to reveal short, micro-homologies of 5-25 bp (Ma et al., 2003). The sequences then anneal, and repair is then achieved by the cleavage of overhanging 3' flaps, synthesis to fill single-stranded gaps, and finally ligation (Rodgers and McVey, 2016). MMEJ is inherently mutagenic and results in deletions of varying lengths (Rodgers and McVey, 2016). In theory, the loss of genetic information via this mechanism (or similar error prone DNA repair processes single strand annealing (SSA) and alternative end joining (ALT-EJ)) could be the cause of exoDNA formation, originating from deletions of error prone DNA repair mechanisms.

While literature regarding radiation-induced changes in exosome composition is quite limited, there are studies that show an increase in proteins found in exosomes treated with IR (Lehmann et al., 2008; Hurwitz et al., 2010; Jelonek et al., 2015) and unique proteins found exclusively in exosomes treated with radiation (Jelonek et al., 2015; Diamond et al., 2018; Mrowczynski et al., 2018). Analysis of RNA contents revealed upregulated levels of oncogenic miRNAs and oncogenic RNAs in radiation-derived exosomes as well (Mrowczynski et al., 2018). Moreover, the study by Mrowczynski *et al.* (2018) showed an increase in miR-889, an oncogenic miRNA that increases cancer cell proliferation, radiation resistance, and metastasis (Xu et al., 2015; Yang et al., 2016; Liao et al., 2015). The study also showed decreased levels of tumour-suppressive miRNAs (miR-516 and miR-365), where miR-516 is capable of decreasing metastasis (Takei et al., 2011). While there is currently no literature regarding IR-induced alteration in exoDNA composition, previously mentioned studies not involving IR show increases in retrotransposon sequences in EV DNA (Balaj et al., 2011) and different DNA fragments found within separate EV subpopulations (Lázaro-Ibáñez et al., 2014).

While the studies mentioned above provide limited information on EV/exosome DNA content, this is the first study (in this thesis) to report alterations in exoDNA composition as a result of IR.

Specifically, low-depth WGS revealed a high degree of variation in exoDNA copy number following

exposure to 2 Gy X-rays. However, the cause of the variation is not apparent. While several studies have shown that low-coverage WGS is effective for CNV detection in clinical samples (Liang et al., 2014; Liu et al., 2015; Dong et al., 2016), it is likely that the genetic variation in the irradiated samples in this study is due to differences on the nucleotide level, not at the variation in copy number. Deep-coverage WGS methods would detect SNPs, short insertions and deletions that are not able to be detected by low-coverage WGS. Additionally, recent studies have been able to detect structural variation due to TE insertion at x30 (Xing et al., 2013) and a mean depth of x68 (Yu et al., 2017), whereas the low-depth coverage in this study (1.9x-2.4x) revealed no apparent homology to LINE-1 sequences (data not shown).

Altogether the results in this thesis have characterized IR-induced LINE-1 retrotransposition in correlation with radiation-induced damage (MN induction) and characterized exosomes released in the radiation response of the MCF7 cell line to IR. This is the first study to investigate the effect of IR on exoDNA composition. The results show an increase in exoDNA fragment size following exposure to X-irradiation, thereby suggesting that damaged nuclear DNA is the source of exoDNA. A high degree of variation in exoDNA copy number is also apparent for the first time following exposure to IR, however the cause of the variation is unknown.

6.1. Conclusions and Future work

The findings presented in this thesis are suggestive (while highly speculative without further experimentation) of a link between the increase in IR-induced LINE-1 retrotransposition as a contribution to the DNA damage at 48 hours post IR in the HF19 cell line. The findings also show that exosomes excreted from MCF7 cells following a 2 Gy X-ray dose have a different composition and DNA contents compared to those excreted from sham-irradiated cells. Additionally, results from the CNV analysis show a genetic variation between the sham and irradiated exoDNA samples.

The overall results from this study have significantly contributed to the knowledge and effects of RIGI and the study of exosomes in the IR response. Advances in molecular biology techniques would allow us to repress LINE-1 and observe phenotypes post radiation. Horizontal transfer of retrotransposons by exosomes treated with IR is an interesting theory and investigation of their functional impact into recipient cells would provide an insight if they are exacerbating the spread of insertional mutagenesis and a contributing factor to the cause of genomic instability and possibly the bystander effect. An investigation into the origin of exoDNA is also intriguing, and by correlating nuclear rupture with exoDNA content and chromosomal instability it would provide insight if damaged DNA is the source of exoDNA.

Chapter 7 References

- Abramowicz, A., Wojakowska, A., Marczak, L., Lysek-Gladysinska, M., Smolarz, M., Story, M., Polanska, J., Widlak, P., Pietrowska, M. (2019). Ionizing radiation affects the composition of the proteome of extracellular vesicles released by head-and-neck cancer cells in vitro. *Journal of Radiation Research*, 60(3), pp.289-297.
- Ahn, S., Kim, T., Lee, S., Kim, D., Ghang, H., Kim, D., Kim, B., Kim, S., Kim, W., Kim, C., Park, D., Lee, Y., Kim, S., Reja, R., Jho, S., Kim, C., Cha, J., Kim, K., Lee, B., Bhak, J., Kim, S. (2009). The first Korean genome sequence and analysis: full genome sequencing for a socio-ethnic group. *Genome Research*, 19(9), pp.1622–1629.
- Akers, J., Gonda, D., Kim, R., Carter, B., Chen, C. (2013). Biogenesis of extracellular vesicles (EV): exosomes, microvesicles, retrovirus-like vesicles, and apoptotic bodies. *Journal of neuro-oncology*, 113(1), pp.1–11.
- Al-Mayah, A., Bright, S., Bowler, D., Slijepcevic, P., Goodwin, E., Kadhim, M. (2017). Exosome-Mediated Telomere Instability in Human Breast Epithelial Cancer Cells after X Irradiation. *Radiation Research*, 187(1), pp.98-106.
- Al-Mayah, A., Bright, S., Chapman, K., Irons, S., Luo, P., Carter, D., Goodwin, E., Kadhim, M. (2015). The non-targeted effects of radiation are perpetuated by exosomes. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis*, 772, pp.38–45.
- Al-Mayah, A., Irons, S., Pink, R., Carter, D., Kadhim, M. (2012). Possible role of exosomes containing RNA in mediating nontargeted effect of ionizing radiation. *Radiation Research*, 545(5), pp.539–545.
- Arlt, M., Mülle, J., Schaibley, V., Ragland, R., Durkin, S., Warren, S., Glover, T. (2009). Replication stress induces genome-wide copy number changes in human cells that resemble polymorphic and pathogenic variants. *American journal of human genetics*, 84(3), pp.339–350.
- Arlt, M., Ozdemir, A., Birkeland, S., Wilson, T., Glover, T. (2011). Hydroxyurea induces de novo copy number variants in human cells. *Proceedings of the National Academy of Sciences of the United States of America*, 108(42), pp.17360–17365.
- Arlt, M., Rajendran, S., Birkeland, S., Wilson, T., Glover, T. (2014). Copy number variants are produced in response to low-dose ionizing radiation in cultured cells. *Environmental and molecular mutagenesis*, 55(2), pp.103–113.
- Arcott, W., Tandle, A., Zhao, S., Shabason, J., Gordon, I., Schlaff, C., Zhang, G., Tofjlön, P., Camphausen, K. (2013). Ionizing Radiation and Glioblastoma Exosomes: Implications in Tumor Biology and Cell Migration. *Translational Oncology*, 6(6), pp.638–648.
- Aypar U., Morgan, W., Baulch, J. (2011). Radiation-induced genomic instability: Are epigenetic mechanisms the missing link? *International Journal of Radiation Biology*, 87(2), pp.179-191.
- Babatz T., Burns, K. (2013). Functional Impact of the Human Mobilome. *Current Opinion in Genetics and Development*, 23(3), pp.264-270.

Babini, G., Morini, J., Barbieri, S., Baiocco, G., Ivaldi, G., Liotta, M., Tabarelli de Fatis, P., Ottolenghi, A. (2018). A Co-culture Method to Investigate the Crosstalk Between X-ray Irradiated Caco-2 Cells and PBMC. *Journal of visualized experiments : JoVE*, (131), 56908.

Bakhoun, S., Ngo, B., Laughney, A., Cavallo, J., Murphy, C., Ly, P., Shah, P., Sriram, R., Watkins, T., Taunk, N., Duran, M., Pauli, C., Shaw, C., Chadalavada, K., Rajasekhar, V., Genovese, G., Venkatesan, S., Birkbak, N., McGranahan, N., Lundquist, M., LaPlant, Q., Healey, J., Elemento, O., Chung, C., Lee, N., Imielenski, M., Nanjangud, G., Pe'er, D., Cleveland, D., Powell, S., Lammerding, J., Swanton, C., Cantley, L. (2018). Chromosomal instability drives metastasis through a cytosolic DNA response. *Nature*, 553(7689), pp.467–472.

Balaj, L., Lessard, R., Dai, L., Cho, Y., Pomeroy, S., Breakefield, X., Skog, J. (2011). Tumour microvesicles contain retrotransposon elements and amplified oncogene sequences. *Nature Communications*, 2, 180.

Banaz-Yasar, F., Gedik, N., Karahan, S., Diaz-Carballo, D., Bongartz, B., Ergun, S. (2012). LINE-1 retrotransposition events regulate gene expression after X-ray irradiation. *DNA and Cell Biology*, 31(9), pp.1458-67.

Barber G. N. (2015). STING: infection, inflammation and cancer. *Nature reviews Immunology*, 15(12), pp.760–770.

Baulch, J. (2019). Radiation-induced genomic instability, epigenetic mechanisms and the mitochondria: a dysfunctional ménage à trois? *International Journal of Radiation Biology*, 95(4), pp.516-525.

Beaver, J., Gustin, J., Yi, K., Rajpurohit, A., Thomas, M., Gilbert, S., Rosen, D., Ho Park, B., Lauring, J. (2013). PIK3CA and AKT1 mutations have distinct effects on sensitivity to targeted pathway inhibitors in an isogenic luminal breast cancer model system. *Clinical cancer research*, 19(19), pp.5413-5422.

Beer, L., Zimmermann, M., Mitterbauer, A., Ellinger, A., Gruber, F., Narzt, M., Zellner, M., Gyongyosi, M., Madlener, S., Simader, E., Gabriel, C., Mildner, M., Ankersmit, H. (2015). Analysis of the secretome of apoptotic peripheral blood mononuclear cells: impact of released proteins and exosomes for tissue regeneration. *Scientific Reports*, 5, pp.16662.

Belancio, V., Hedges, D., Deininger, P. (2008). Mammalian non-LTR retrotransposons: for better or worse, in sickness and in health. *Genome Research*, 18(3), pp.343-358.

Belancio, V., Roy-Engel, A., Pochampally, R., Deininger, P. (2010). Somatic expression of LINE-1 elements in human tissues. *Nucleic Acids Research*, 38(12), pp.3909–3922.

Belgnaoui, S., Gosden, R., Semmes, O., Haoudi, A. (2006). Human LINE-1 retrotransposon induces DNA damage and apoptosis in cancer cells. *Cancer Cell International*, 6, pp.13.

Blyth, B., Sykes, P. (2011) Radiation-induced bystander effects: what are they, and how relevant are they to human radiation exposures? *Radiation Research*, 176(2), pp.139–157.

- Bundo, M., Toyoshima, M., Okada, Y., Akamatsu, W., Ueda, J., Nemoto-Miyauchi, T., Sunaga, F., Toritsuka, M., Ikawa, D., Kakita, A., Kato, M., Kasai, K., Kishimoto, T., Nawa, H., Okano, H., Yoshikawa, T., Kato, T., Iwamoto, K. (2014). Increased L1 retrotransposition in the neuronal genome in schizophrenia. *Neuron*, 81(2), pp.306–13.
- Cai, J., Wu, G., Tan, X., Han, Y., Chen, C., Li, C., Wang, N., Zou, X., Chen, X., Zhou, F., He, D., Zhou, L., Jose, P., Zeng, C. (2014). Transferred BCR/ABL DNA from K562 extracellular vesicles causes chronic myeloid leukemia in immunodeficient mice. *PloS One*, 9(8), e105200.
- Caldecott, K.W., (2003). DNA Single-Strand Break Repair and Spinocerebellar Ataxia. *Cell*, 112(1), pp.7-10.
- Caldecott, K.W., (2008). Single-strand break repair and genetic disease. *Genetics*, 9, pp.619-631.
- Chen, Q., Sun, L., Chen, Z. (2016). Regulation and function of the cGAS-STING pathway of cytosolic DNA sensing. *Nat Immunology*, 17(10), pp.1142-1149.
- Cheruvanky, A., Zhou, H., Pisitkun, T., Kopp, J., Knepper, M., Yuen, P., Star, R. (2008). Rapid isolation of urinary exosomal biomarkers using a nanomembrane ultrafiltration concentrator. *American Journal of Physiology-Renal Physiology*, 292(5), pp.1657–1661.
- Clayton, A., Turkes, A., Navabi, H., Mason, M., Tabi, Z. (2005). Induction of heat shock proteins in B-cell exosomes. *Journal of Cell Science*, 118(16), pp.3631–3638.
- Cordaux, R., Batzer, M. (2009). The impact of retrotransposons on human genome evolution. *Nature Reviews Genetics*, 10(10), pp.691-703.
- Costa, E., Pinto, I., Gonçalves, M., da Silva, J., Oliveira, L., da Cruz, A., Silva, D., da Silva, C., Pereira, R., da Cruz, A. (2018). Small de novo CNVs as biomarkers of parental exposure to low doses of ionizing radiation of caesium-137. *Scientific Reports*, 8(1), pp.5914.
- Cox, R., Masson, W. (1975). X-ray survival curves of cultured human diploid fibroblasts. In Alper, T. (ed.), in *Cell Survival after Proceedings of the 6th L. H. Gray Conference*. London: Institute of Physics/Chichester: John Wiley.
- Crow, J., Atay, S., Banskota, S., Artale, B., Schmitt, S., Godwin, A., (2017). Exosomes as mediators of platinum resistance in ovarian cancer. *Oncotarget*, 8(7), pp.11917-11936.
- Dai, S., Wan, T., Wang, B., Zhou, X., Xiu, F., Chen, T., Wu, Y., Cao, X. (2005). More efficient induction of HLA-A*0201-restricted and carcinoembryonic antigen (CEA)-specific CTL response by immunization with exosomes prepared from heat-stressed CEA-positive tumor cells. *Clinical Cancer Research*, 11(20), pp.7554-63.
- Dai, Y., Dias, P. (2018). Exosomes or Microvesicles, a Secreted Subcellular Organelle Contributing to Inflammation and Diabetes. *Diabetes*, 67(11), pp.2154-2156.
- Daneshmand, M., Hanson, J., Nabavi, M., Hilton, J., Vandermeer, L., Kanji, F., Dent, S., Clemons, M., Lorimer, I. (2012). Detection of PIK3CA Mutations in Breast Cancer Bone Metastases. *ISRN Oncology*, 2012, 492578.

de la Rica, L., Deniz, Ö., Cheng, K., Todd, C., Cruz, C., Houseley, J., Branco, M., (2016). TET-dependent regulation of retrotransposable elements in mouse embryonic stem cells. *Genome Biology*, 17(1), pp.234

de Vrij, J., Maas, S., van Nispen, M., Sena-Esteves, M., Limpens, R., Koster, A., Leenstra, M., Broekman, M. (2013) Quantification of nanosized extracellular membrane vesicles with scanning ion occlusion sensing. *Nanomedicine*, 8(9), pp.1443–58.

Dean, A., William, D., Rea, M., Smith, C., Barrier, A. 2011. *EMFs and Human Health*. [Online]. [Accessed 10 April 2019]. Available from: <http://nutritionalbalancing.org/center/environment/articles/emf-emr-health-effects.php#ref-01>

Demasters, G., Di, X., Newsham, I., Shiu, R., Gewirtz, D. (2006). Potentiation of radiation sensitivity in breast tumor cells by the vitamin D3 analogue, EB 1089, through promotion of autophagy and interference with proliferative recovery. *Molecular Cancer Therapeutics*, 5(11), pp.2786–97.

Deng, L., Liang, H., Xu, M., Yang, X., Burnette, B., Arina, A., Li, X., Mauceri, H., Beckett, M., Darga, T., Huang, X., Gajewski, T., Chen, Z., Fu, Y., Weichselbaum, R. (2014). STING-Dependent Cytosolic DNA Sensing Promotes Radiation-Induced Type I Interferon-Dependent Antitumor Immunity in Immunogenic Tumors. *Immunity*, 41(5), pp.843–852.

Deniz, Ö., Frost, J., Branco, M., (2019). Regulation of transposable elements by DNA modifications. *Nature Review Genetics*, 20(7), pp.417–431.

Desouky, O., Ding, N., Zhou, G. (2015). Targeted and non-targeted effects of ionizing radiation. *Journal of Radiation Research and Applied Sciences*, 8(2), pp.247–254.

Diamond, J., Vanpouille-Box, C., Spada, S., Rudqvist, N., Chapman, J., Ueberheide, B., Pilonis, K., Sarfraz, Y., Formenti, S., Demaria, S. (2018). Exosomes Shuttle TREX1-Sensitive IFN-Stimulatory dsDNA from Irradiated Cancer Cells to DCs. *Cancer Immunology Research*, 6(8), pp.910–920.

Dioufa, N., Clark, A., Ma, B., Beckwitt, C., Wells, A. (2017). Bi-directional exosome-driven intercommunication between the hepatic niche and cancer cells. *Molecular Cancer*, 16(1), 172.

Dombroski, B., Mathias, S., Nanthakumar, E., Scott, A., Kazazian, H. (1991). Isolation of an active human transposable element. *Science*, 254, pp.1805–1808.

Dong, Z., Zhang, J., Hu, P., Chen, H., Xu, J., Tian, Q., Meng, L., Ye, Y., Wang, J., Zhang, M., Li, Y., Wang, H., Yu, S., Chen, F., Xie, J., Jiang, H., Wang, W., Choy, K., Xu, Z. (2016). Low-pass whole-genome sequencing in clinical cytogenetics: a validated approach. *Genetics in Medicine*, 18(9), pp.940–8.

Doucet-O'Hare, T., Sharma, R., Rodić, N., Anders, R., Burns, K., Kazazian, H., Jr., (2016). Somatic Acquired LINE-1 Insertions in Normal Esophagus Undergo Clonal Expansion in Esophageal Squamous Cell Carcinoma. *Human Mutation*, 37(9), pp.942–54.

Durante, M., Formenti, S. (2018). Radiation-Induced Chromosomal Aberrations and Immunotherapy: Micronuclei, Cytosolic DNA, and Interferon-Production Pathway. *Frontiers in Oncology*, 8, 192.

- Durkin, S., Ragland, R., Arlt, M., Mulle, J., Warren, S., Glover, T. (2008). Replication stress induces tumor-like microdeletions in FHIT/FRA3B. *Proceedings of the National Academy of Sciences of the United States of America*, 105(1), pp.246-251.
- Erdal, E., Haider, S., Rehwinkel, J., Harris, A., McHugh, P. (2017). A prosurvival DNA damage-induced cytoplasmic interferon response is mediated by end resection factors and is limited by Trex1. *Genes & Development*, 31(4), pp.353–369.
- Estecio, M., Gallegod, J., Dekmezian, M., Lu, Y., Liang, S., Issa, J. (2012). SINE Retrotransposons Cause Epigenetic Reprogramming of Adjacent Gene Promoters. *Molecular Cancer Research*, 10(10), pp.1332-1342.
- Fard, A., Tavakoli, M., Salehi, H., Emami, H. (2017). Synergetic effects of Docetaxel and ionizing radiation reduced cell viability on MCF-7 breast cancer cell. *Applied Cancer Research*, 37, 29.
- Farkash, E., Kao, G., Horman, S., Prak, E. (2006). Gamma radiation increases endonuclease-dependent L1 retrotransposition in a cultured cell assay. *Nucleic Acids Research*, 34(4), pp.1196-1204.
- Fernandez-Medina, R., Ribeiro, J., Carareto, C., Velasque, L., Struchiner, C. (2012). Losing identity: structural diversity of transposable elements belonging to different classes in the genome of *Anopheles gambiae*. *BMC Genomics*, 13(272), pp.1-15.
- Fischer, S., Cornils, K., Speiseder, T., Badbaran, A., Reimer, R., Indenbirken, D., Grundhoff, A., Brunswig-Spickenheier, B., Alawi, M., Lange, C. (2016). Indication of Horizontal DNA Gene Transfer by Extracellular Vesicles. *PLoS One*, 11(9), pp.e0163665.
- Flunkert, J., Maierhofer, A., Dittrich, M., Müller, T., Horvath, S., Nanda, I., Haaf, T. (2018). Genetic and epigenetic changes in clonal descendants of irradiated human fibroblasts. *Experimental Cell Research*, 370(2), pp.322-32.
- Gilbert, N., Lutz-Prigge, S., Moran, J. (2002). Genomic deletions created upon LINE-1 retrotransposition. *Cell*, 110(3), 315-325.
- Girdhani, S., Ahmed, M., Mishra, K. (2009). Enhancement of Gamma Radiation-induced Cytotoxicity of Breast Cancer Cells by Curcumin. *Molecular and Cell Pharmacology*, 1(4), pp.208-217.
- Goodhead, D. T. (1988). Spatial and temporal distribution of energy. *Health Physiology*, 55(2), pp.231-240.
- Goodhead, D. T. (1994). Initial events in the cellular effects of ion-izing radiations: clustered damage in DNA. *International Journal of Radiation Biology*, 65, pp.7–17.
- Goodhead, D. T. (2006). Energy deposition stochastics and track structure: what about the target? *Radiation Protection Dosimetry*, 122(1-4), pp.3-15.
- Greening, D., Gopal, S., Xu, R., Simpson, R., Chen, W. (2015). Exosomes and their roles in immune regulation and cancer. *Seminars in Cell & Developmental Biology*, 40, pp.72-81.

- Guallar, D., Bi, X., Pardavila, J., Huang, X., Saenz, C., Shi, X., Zhou, H., Faiola, F., Ding, J., Haruehanroengra, P., Yang, F., Li, D., Sanchez-Priego, C., Saunders, A., Pan, F., Valdes, V., Kelley, K., Blanco, M., Chen, L., Wang, H., Sheng, J., Xu, M., Fidalgo, M., Shen, X., Wang, J., (2018). RNA-dependent chromatin targeting of TET2 for endogenous retrovirus control in pluripotent stem cells. *Nature Genetics*, 50(3), pp.443-451.
- Guduric-Fuchs, J., O'Connor, A., Camp, B., O'Neill, C., Medina, R., Simpson, D. (2012). Selective extracellular vesicle-mediated export of an overlapping set of microRNAs from multiple cell types. *BMC Genomics*, 13, 357.
- Gutierrez-Enriquez, S., Ramon, Y., Alonso, C., Corral, A., Carrasco, P., Cornet, M., Sanz, J., Ribas, M., Baiget, M., Diez, O. (2011). Ionizing radiation or mitomycin-induced micronuclei in lymphocytes of BRCA1 or BRCA2 mutation carriers. *Breast Cancer Research and Treatment*, 127(3), pp.611–622.
- Hall, E.J., Giaccia, A. J. (2006). *Radiology for the Radiologist*, Lippincott Williams & Wilkins.
- Hamada, N., Maeda, M., Otsuka, K., Tomita, M. (2011). Signaling pathways underpinning the manifestations of ionizing radiation-induced bystander effects. *Current Molecular Pharmacology*, 4(2), pp.79-95.
- Hampton, O., Den Hollander, P., Miller, C., Delgado, D., Li, J., Coarfa, C., Harris, R., Richards, S., Scherer, S., Muzny, D., Gibbs, R., Lee, A., Milosavljevic, A. (2009). A sequence-level map of chromosomal breakpoints in the MCF-7 breast cancer cell line yields insights into the evolution of a cancer genome. *Genome Research*, 19(2), pp.167–177.
- Hanayama, R., Tanaka, M., Miyasaka, K., Aozaka, K., Koike, M., Uchiyama, Y., Nagata, S. (2004). Autoimmune disease and impaired uptake of apoptotic cells in MFG-E8-deficient mice. *Science*, 304(5674), pp.1147-50.
- Hancks, D., Kazazian, H. (2012). Active human retrotransposons: variation and disease. *Current Opinion in Genetics & Development*, 22(3), pp.191-203.
- Härtlova, A., Erttmann, S., Raffi, F., Schmalz, A., Resch, U., Anugula, S., Lienenklaus, S., Nilsson, L., Kroger, A., Nilsson, J., Ek, T., Weiss, S., Gekara, N. (2015). DNA damage primes the type I interferon system via the cytosolic DNA sensor STING to promote anti-microbial innate immunity. *Immunity*, 4(2), pp.332-343.
- Hatch, E., Fischer, A., Deerinck, T., Hetzer, M. (2013). Catastrophic nuclear envelope collapse in cancer cell micronuclei. *Cell*, 154(1), pp.47–60.
- Hauser, P., Wang, S., Didenko, V. (2017). Apoptotic Bodies: Selective Detection in Extracellular Vesicles. *Methods in Molecular Biology*, 1554, pp.193-200.
- Haylick, L. (1973). Krause, P.F., Patterson, M.K. (eds.) *Tissue Culture Methods and Applications*. New York: Academic Press, pp.220-223.
- Hei, T., Zhou, H., Chai, Y., Ponnaiya, B., Ivanov, V. (2011). Radiation induced non-targeted responses mechanism and potential clinical implications. *Current Molecular Pharmacology*, 4, pp.96-105.

- Hirayama R, Uzawa A, Obara M, Takase N, Koda K, Ozaki M, Noguchi, M., Matsumoto, Y., Li, H., Yamashita, K., Koike, S., Ando, K., Shirai, T., Matsufuji, N., Furusawa, Y. (2015). Determination of the relative biological effectiveness and oxygen enhancement ratio for micronuclei formation using high-LET radiation in solid tumor cells: an in vitro and in vivo study. *Mutation Research – Genetic Toxicology and Environmental Mutagenesis*, 793, pp.41–7.
- Ho, S., Zhang, W., Tan, N., Khatoor, M., Suter, M., Tripathi, S., Cheung, F., Lim, W., Tan, P., Ngeow, J., Gasser, S. (2016). The DNA structure-specific endonuclease MUS81 mediates DNA sensor STING-dependent host rejection of prostate cancer cells. *Immunity*, 44, pp.1177–1189.
- Hurwitz, M., Kaur, P., Nagaraja, G., Bausero, M., Manola, J., Asea, A. (2010). Radiation therapy induces circulating serum Hsp72 in patients with prostate cancer. *Radiotherapy and Oncology : Journal of the European Society for Therapeutic Radiology and Oncology*, 95(3), pp.350-358.
- Iurlaro, M., Ficiz, G., Oxley, D., Raiber, E., Bachman, M., Booth, M., Andrews, S., Balasubramanian, S., Reik, W., (2013). A screen for hydroxymethylcytosine and formylcytosine binding proteins suggests functions in transcription and chromatin regulation. *Genome Biology*, 14(10), pp.R119.
- Jalili-Firoozinezhad, S., Prantil-Baun, R., Jiang, A., Potla, R., Mammoto, T., Weaver, J. C., ... Ingber, D. E. (2018). Modeling radiation injury-induced cell death and countermeasure drug responses in a human Gut-on-a-Chip. *Cell Death & Disease*, 9(2), 223.
- Jdey, W., Thierry, S., Popova, T., Stern, M., Dutreix, M. (2017). Micronuclei Frequency in Tumors Is a Predictive Biomarker for Genetic Instability and Sensitivity to the DNA Repair Inhibitor AsiDNA. *Cancer Research*, 77(16), pp.4207-4216.
- Jella, K., Nasti, T., Li, Z., Malla, S., Buchwald, Z., Khan, M. (2018) Exosomes, Their Biogenesis and Role in Inter-Cellular Communication, Tumor Microenvironment and Cancer Immunotherapy. *Vaccines*, 6(4), pp.69.
- Jella, K., Rani, S., O'Driscoll, L., McClean, B., Byrne, H., Lyng, F. (2014). Exosomes Are Involved in Mediating Radiation Induced Bystander Signaling in Human Keratinocyte Cells. *Radiation Research*, 181(2), pp.138-145.
- Jelonek, K., Wojakowska, A., Marczak, L., Muer, A., Tinhofer-Keilholz, I., Lysek-Gladysinska, M., Widlak, P., Pietrowska, M. (2015). Ionizing radiation affects protein composition of exosomes secreted in vitro from head and neck squamous cell carcinoma. *Acta Biochimica Polonica*, 62(2), pp.265-72.
- Jin, Y., Chen, K., Wang, Z., Wang, Y., Liu, J., Lin, L., Shao, Y., Gao, L., Yin, H., Cui, C., Tan, Z., Liu, L., Zhao, C., Zhang, G., Jia, R., Du, L., Chen, Y., Liu, R., Xu, J., Hu, X., Wang, Y. (2016). DNA in serum extracellular vesicles is stable under different storage conditions. *BMC Cancer*, 16(1), 753.
- Joo, H. M., Nam, S., Yang, K., Kim, C., Jin, Y., Kim, J. (2012). The effects of low-dose ionizing radiation in the activated rat basophilic leukemia (RBL-2H3) mast cells. *The Journal of Biological Chemistry*, 287(33), pp.27789–27795.
- Kadhim, M. (2003). Role of genetic background in induced instability. *Oncogene* 22, pp.6994-6999.

Kadhim, M., Lorimore, S., Hepburn, M., Goodhead, D., Buckle, V., Wright, E. (1994) Alpha-particle-induced chromosomal instability in human bone marrow cells. *The Lancet*, 344(8928), pp.987–8.

Kadhim, M., Macdonald, D., Goodhead, D., Lorimore, S., Marsden, S. Wright, E. (1992). Transmission of chromosomal instability after plutonium α -particle irradiation. *Nature*, 355(6362), pp.738–740.

Kadhim, M., Salomaa, S., Wright, E., Hildebrandt, G., Belyakov, O., Prise, K., Little, M. (2013). Non-targeted effects of ionising radiation-Implications for low dose risk. *Mutation Research*, 752, pp.84-98.

Kahlert, C., Kalluri, R. (2013). Exosomes in tumor microenvironment influence cancer progression and metastasis. *Journal of Molecular Medicine*, 91(4), pp.431–437.

Kahlert, C., Melo, S., Protopopov, A., Tang, J., Seth, S., Koch, M., Zhang, J., Weitz, J., Chin, L., Futreal, A., Kalluri, R. (2014). Identification of double-stranded genomic DNA spanning all chromosomes with mutated KRAS and p53 DNA in the serum exosomes of patients with pancreatic cancer. *The Journal of Biological Chemistry*, 289(7), pp.3869–3875.

Kalluri, R. (2016). The biology and function of exosomes in cancer. *The Journal of Clinical Investigation*, 126(4), pp.1208–1215.

Kalluri, R., LeBleu, V. (2016). Discovery of Double-Stranded Genomic DNA in Circulating Exosomes. *Cold Spring Harbor Symposia on Quantitative Biology*, 81, pp.275-280.

Kamerkar, S., LeBleu, V., Sugimoto, H., Yang, S., Ruivo, C., Melo, S., Lee, J., Kalluri, R. (2017). Exosomes facilitate therapeutic targeting of oncogenic KRAS in pancreatic cancer. *Nature*, 546(7659), pp.498-503.

Kanemoto, S., Nitani, R., Murakami, T., Kaneko, M., Asada, R., Matsuhisa, K., Saito, A., Imaizumi, K. (2016). Multivesicular body formation enhancement and exosome release during endoplasmic reticulum stress. *Biochemical and Biophysical Research Communications*, 480(2), pp.166–172.

Kang, J., Lienhard, M., Pastor, W., Chawla, A., Novotny, M., Tsagaratou, A., Lasken, R., Thompson, E., Surani, M., Koralov, S., Kalantry, S., Chavez, L., Rao, A., (2015). Simultaneous deletion of the methylcytosine oxidases Tet1 and Tet3 increases transcriptome variability in early embryogenesis. *Proceedings of the National Academy of Sciences of the United States of America*, 112(31), pp.E4236-45.

Kawamura, Y., Sanchez Calle, A., Yamamoto, Y., Sato, T., Ochiya, T., (2019). Extracellular vesicles mediate the horizontal transfer of an active LINE-1 retrotransposon. *Journal of Extracellular Vesicles*, 8(1), pp. 1643214.

Kawamura, Y., Yamamoto, Y., Sato, T., Ochiya, T. (2017). Extracellular vesicles as trans-genomic agents: Emerging roles in disease and evolution. *Cancer Science*, 108(5), pp.824–830.

Keszenman, D., Kolodiuk, L., Baulch, J. (2015). DNA Damage in cells exhibiting radiation-induced genomic instability. *Mutagenesis*, 30(3), pp.451-8.

Kim, G., Chandrasekaran, K., Morgan W. (2006). Mitochondrial dysfunction, persistently elevated

levels of reactive oxygen species and radiation-induced genomic instability: a review. *Mutagenesis*, 21(6), pp.361-367.

Kim, K., Kim, H., Lee, H., Lee, K., Hong, D., Lim, H., Cho, K., Jung, N., Yi, Y. (2016). Application of a non-hazardous vital dye for cell counting with automated cell counters. *Analytical Biochemistry*, 492, pp.8-12.

King, H., Michael, M., Gleadle, J. (2012). Hypoxic enhancement of exosome release by breast cancer cells. *BMC Cancer*, 12, pp.421.

Kitai, Y., Kawasaki, T., Sueyoshi, T., Kobiyama, K., Ishii, K., Zou, J., Akira, S., Matsuda, T., Kawai, T. (2017). DNA-Containing Exosomes Derived from Cancer Cells Treated with Topotecan Activate a STING-Dependent Pathway and Reinforce Antitumor Immunity. *Journal of Immunology*, 198(4), pp.1649-1659.

Klein-Scory, S., Tehrani, M., Eilert-Micus, C., Adamczyk, K., Wojtalewicz, N., Schnölzer, M., Hahn, S., Schmiegel, W., Schwarte-Waldhoff, I. (2014). New insights in the composition of extracellular vesicles from pancreatic cancer cells: implications for biomarkers and functions. *Proteome Science*, 12, 50.

Kozak, M. (1989). The scanning model for translation: an update. *J Cell Biol.*, 108(2), pp.229-241.

Kurywchak, P., Tavormina, J., Kalluri, R. (2018). The emerging roles of exosomes in the modulation of immune responses in cancer. *Genome Medicine*, 10(1), 23.

Kuhmann, C., Weichenhan, D., Rehli, M., Plass, C., Schmezar, P., Popanda, O., (2011). DNA methylation changes in cells regrowing after fractionated ionizing radiation. *Radiotherapy Oncology*, 101(1), pp.116-121.

Kurywchak, P 2018, 'Identification and utility of DNA in exosomes', PhD thesis, The University of Texas M D Anderson Cancer Center, Houston.

Lan, Y., Londoño, D., Bouley, R., Rooney, M., Hacohen, N. (2014). Dnase2a deficiency uncovers lysosomal clearance of damaged nuclear DNA via autophagy. *Cell Reports*, 9(1), pp.180–192.

Lans, H., Marteijn, J. A., Vermeulen, W., (2012). ATP-dependent chromatin remodeling in the DNA-damage response. *Epigenetics & Chromatin*, 5, pp.4.

Lázaro-Ibáñez, E., Sanz-Garcia, A., Visakorpi, T., Escobedo-Lucea, C., Siljander, P., Ayuso-Sacido, A., Yliperttula, M. (2014). Different gDNA content in the subpopulations of prostate cancer extracellular vesicles: apoptotic bodies, microvesicles, and exosomes. *The Prostate*, 74(14), pp.1379–1390.

Lee, E., Iskow, R., Yang, L., Gokcumen, O., Haseley, P., Luquette, L., Lohr, J., Harris, C., Ding, L., Wilson, R., Wheeler, D., Gibbs, R., Kucherlapati, R., Lee, C., Kharchenko, P., Park, P. (2012). Landscape of somatic retrotransposition in human cancers. *Science*, 337(6097), pp.967-71.

Lee, T., Chennakrishnaiah, S., Meehan, B., Montermini, L., Garnier, D., D'Asti, E., Hou, W., Magnus, N., Gayden, T., Jabado, N., Eppert, K., Majewska, L., Rak, J. (2016). Barriers to horizontal cell

transformation by extracellular vesicles containing oncogenic H-ras. *Oncotarget*, 7(32), pp.51991-52002.

Lehmann, B., Paine, M., Brooks, A., McCubrey, J., Renegar, R., Wang, R., Terrian, D. (2008). Senescence-associated exosome release from human prostate cancer cells. *Cancer Research*, 68(19), pp.7864-7871.

Li, P., Kaslan, M., Lee, S. H., Yao, J., Gao, Z. (2017). Progress in Exosome Isolation Techniques. *Theranostics*, 7(3), pp.789–804.

Li, Z., Yanfang, W., Li, J., Jiang, P., Peng, T., Chen, K., Zhao, X., Zhang, Y., Zhen, P., Zhu, J., Li, X. (2018). Tumor-released exosomal circular RNA PDE8A promotes invasive growth via the miR-338/MACC1/MET pathway in pancreatic cancer. *Cancer Letters*, 432, pp.237-250.

Liang, D., Peng, Y., Lv, W., Deng, L., Zhang, Y., Li, H., Yang, P., Zhang, J., Song, Z., Xu, G., Cram, D., Wu, L. (2014). Copy number variation sequencing for comprehensive diagnosis of chromosome disease syndromes. *Journal of Molecular Diagnostics*, 16(5), pp.510-526.

Liao, H., Xiao, Y., Hu, Y., Xiao, Y., Yin, Z., Liu, L. (2015). microRNA-32 induces radioresistance by targeting DAB2IP and regulating autophagy in prostate cancer cells. *Oncology Letters*, 10(4), pp.2055-62.

Litvinchuk, A., Vachelova, J., Michaelidesova, A., Wagner, R., Davidkova, M. (2015). Dose-dependent micronuclei formation in normal human fibroblasts exposed to proton radiation. *Radiation and Environmental Biophysics*, 54(3), pp.327-334.

Liu, M., Williams, K., Werth, V. (2016). Microvesicles in autoimmune diseases. *Advances in Clinical Chemistry*, 77, pp.125-175.

Liu, S., Song, L., Cram, D., Xiong, L., Wang, K., Wu, R., Liu, J., Deng, K., Jia, B., Zhong, M., Yang, F. (2015). Traditional karyotyping vs copy number variation sequencing for detection of chromosomal abnormalities associated with spontaneous miscarriage. *Ultrasound in Obstetrics & Gynecology*, 46(4), pp.472-7.

Lodish, H., Berk, A., Zipursky, S. L., Matsudaira, P., Baltimore, D., Darnell, J., (2000). *Molecular Cell Biology*. 4th edn. New York: W. H. Freeman

Luzhna, L., Ilnytskyy, Y., Kovalchuk, O. (2015). Mobilization of LINE-1 in irradiated mammary gland tissue may potentially contribute to low dose radiation-induced genomic instability. *Genes & Cancer*, 6(1-2), pp.71–81.

Ma, J., Kim, E., Haber, J., Lee, S. (2003). Yeast Mre11 and Rad1 proteins define a Ku-independent mechanism to repair double-strand breaks lacking overlapping end sequences. *Molecular and Cellular Biology*, 23(23), pp.8820-8828.

Mackenzie, K., Carroll, P., Martin, C., Murina, O., Fluteau, A., Simpson, D., Olova, N., Sutcliffe, H., Rainger, J., Leitch, A., Osborn, R., Wheeler, A., Nowotny, M., Gilbert, N., Chandra, T., Reijns, M., Jackson, A. (2017), cGAS surveillance of micronuclei links genome instability to innate immunity. *Nature*, 548(7668), pp.461-465.

- Maia, J., Caja, S., Strano Moraes, M., Couto, N., Costa-Silva, B. (2018). Exosome-Based Cell-Cell Communication in the Tumor Microenvironment. *Frontiers in cell and developmental biology*, 6, 18.
- Maierhofer, A., Flunkert, J., Dittrich, M., Müller, T., Schindler, D., Nanda, I., Haaf, T. (2017). Analysis of global DNA methylation changes in primary human fibroblasts in the early phase following X-ray irradiation. *PLOS One*, 12(5), e0177442.
- Marcu, L. (2009). Cellular bystander effects and radiation hormesis. *Analele Universitatii din Oradea, Fascicula Biologie*, pp.66-70.
- Matsumoto, H., Hayashi, S., Hatashita, M., Ohnishi, K., Shioura, H., Ohtsubo, T., Kitai, R., Ohnishi, T., Kano, E. (2001). Induction of radioresistance by a nitric oxide-mediated bystander effect. *Radiation Research*, 155, pp.387-396.
- Meehan, K., Vella, L. (2016). The contribution of tumour-derived exosomes to the hallmarks of cancer. *Critical Reviews in Clinical Laboratory Sciences*, 53(2), pp.121-31.
- Meldolesi, J. (2018). Exosomes and ectosomes in intercellular communication. *Current Biology*, 28(8), pp.R435-R444.
- Melo, S., Sugimoto, H., O'Connell, J., Kato, N., Villanueva, A., Vidal, A., Qiu, L., Vitkin, E., Perelman, L., Melo, C., Lucci, A., Ivan, C., Calin, G., Kalluri, R. (2014). Cancer exosomes perform cell-independent microRNA biogenesis and promote tumorigenesis. *Cancer Cell*, 26(5), pp.707-21.
- Millipore (2013) Muse™ Cell Cycle Kit User's Guide. Available at: '[http://www.icms.qmul.ac.uk/flowcytometry/uses/musekits/protocols/MCH1001064600-3387MAN \[B\] MUSE CELL CYCLE KIT USER'S GUIDE.pdf](http://www.icms.qmul.ac.uk/flowcytometry/uses/musekits/protocols/MCH1001064600-3387MAN[B]MUSE%20CELL%20CYCLE%20KIT%20USER'S%20GUIDE.pdf)'. (Accessed: 25th July 2019).
- Mills, R., Bennett, E., Iskow, R., Devine, S. (2007). Which transposable elements are active in the human genome? *Trends in Genetics*, 23(4), pp.183-191.
- Miousse, I., Chang, J., Shao, L., Pathak, R., Nzabarushimana, É., Kutanzi, K., Landes, R., Tackett, A., Hauer-Jensen, M., Zhou, D., Koturbash, I. (2017). Inter-Strain Differences in LINE-1 DNA Methylation in the Mouse Hematopoietic System in Response to Exposure to Ionizing Radiation. *International Journal of Molecular Sciences*, 18(7), 1430.
- Mladenov, E., Magin, S., Soni, A., Iliakis, G. (2016). DNA double-strand-break repair in higher eukaryotes and its role in genomic instability and cancer: Cell cycle and proliferation-dependent regulation. *Semin. Cancer Biology*, 37-38, pp.51-64.
- Morales, J., Snow, E., Munane, J. (2003). Environmental factors affecting transcription of the human L1 retrotransposon. II. Stressors. *Mutagenesis*, 18(2), pp.151-158.
- Morawetz, C. (1987). Effect of irradiation and mutagenic chemicals on the generation of ADH2-constitutive mutants in yeast significance for the inducibility of Ty transposition. *Mutation Research*, 177, pp53–60.

Morgan, W. (2003). Non-targeted and Delayed Effect of Exposure to Ionizing Radiation: I. Radiation-Induced Genomic Instability and Bystander Effects *In Vitro. Radiation Research*, 159, pp.567-580.

Morgan, W., Sowa, M. (2007). Non-targeted bystander effects induced by ionizing radiation. *Mutation Research*, 616(1-2), pp.159-64.

Morini, J., Babini, G., Barbieri, S., Baiocco, G., Ottolenghi, A. (2017). The Interplay between Radioresistant Caco-2 Cells and the Immune System Increases Epithelial Layer Permeability and Alters Signaling Protein Spectrum. *Frontiers in Immunology*, 8, 223.

Morse, B., Rotherg, P., South, V., Spandorfer, J., Astrin, S. (1988). Insertional mutagenesis of the myc locus by a LINE-1 sequence in a human breast carcinoma. *Nature*, 333(6168), pp.87–90.

Mrowczynski, O., Madhankumar, A., Sundstrom, J., Zhao, Y., Kawasawa, Y., Slagle-Webb, B., Mau, C., Payne, R., Rizk, E., Zacharia, B., Connor, J. (2018). Exosomes impact survival to radiation exposure in cell line models of nervous system cancer. *Oncotarget*, 9(90), pp.36083-36101.

Müller, W., Nüsse, M., Miller, B., Slavotinek, A., Viaggi, S., Streffer, C. (1996). Micronuclei: a biological indicator of radiation damage. *Mutation Research/Reviews in Genetic Toxicology*, 366(2), pp.163–169.

Muradyan, A., Gilbertz, K., Stabentheiner, S., Klause, S., Madle, H., Meineke, V., Ullmann, R., Scherthan, H. (2011). Acute High-Dose X-Radiation-Induced Genomic Changes in A549 Cells. *Radiation Research*, 175(6), pp.700-707.

Mutschelknaus, L., Azimzadeh, O., Heider, T., Winkler, K., Vetter, M., Kell, R., Tapio, S., Merl-Pham, J., Huber, S., Edalat, L., Radulović, V., Anastasov, N., Atkinson, M., Moertl, S. (2017). Radiation alters the cargo of exosomes released from squamous head and neck cancer cells to promote migration of recipient cells. *Scientific Reports*, 7(1), 12423.

Nagasawa, H., Little, J. (1992). Induction of Sister Chromatid Exchanges by Extremely Low Doses of α -Particles Advances in Brief Induction of Sister Chromatid Exchanges by Extremely Low Doses of α -Particles. *Cancer Research*, 52(22), pp.6394–6396.

O'Driscoll, M., Jeggo, P. A., (2006). The role of double-strand break repair – insights from human genetics. *Nature Reviews Genetics*, 7, pp.45-54.

Olshen, A., Venkatraman, E., Lucito, R., Wigler, M. (2004). Circular binary segmentation for the analysis of array-based DNA copy number data. *Biostatistics*, 5(4), pp.557-72.

Ono, R., Yasuhiko, Y., Aisaki, K., Kitajima, S., Kanno, J., Hirabayashi, Y., (2019). Exosome-mediated horizontal gene transfer occurs in double-strand break repair during genome editing. *Communications Biology – Nature*, 57(2).

Ostrowski, M., Carmo, N., Krumeich, S., Fanget, I., Raposo, G., Savina, A., Moita, C., Schauer, K., Hume, A., Freitas, R., Goud, B., Benaroch, P., Hacohen, N., Fukuda, M., Desnos, C., Seabra, M., Darchen, F., Amigorena, S., Moita, L., Thery, C. (2010). Rab27a and Rab27b control different steps of the exosome secretion pathway. *Nature Cell Biology*, 12(1), pp.19-30.

Parolini, I., Federici, C., Raggi, C., Lugini, L., Palleschi, S., De Milito, A., Coscia, C., Iessi, E., Logozzi, M., Molinari, A., Colone, M., Tatti, M., Sargiacomo, M., Fais, S. (2009). Microenvironmental pH Is a Key Factor for Exosome Traffic in Tumor Cells. *The Journal of Biological Chemistry*, 284(49), pp.34211–34222.

Peinado, H., Alečković, M., Lavotshkin, S., Matei, I., Costa-Silva, B., Moreno-Bueno, G., Hergueta-Redondo, M., Williams, C., García-Santos, G., Ghajar, C., Nitadori-Hoshino, A., Hoffman, C., Badal, K., Garcia, B., Callahan, M., Yuan, J., Martins, V., Skog, J., Kaplan, R., Brady, M., Wolchok, J., Chapman, P., Kang, Y., Bromberg, J., Lyden, D. (2012). Melanoma exosomes educate bone marrow progenitor cells toward a pro-metastatic phenotype through MET. *Nature Medicine*, 18(6), pp.883–891.

Petit-Frère, C., Capulas, E., Lyon, D., Norbury, C., Lowe, J., Clingen, P., Riballo, E., Green, M., Arlett, C. (2000). Apoptosis and cytokine release induced by ionizing or ultraviolet B radiation in primary and immortalized human keratinocytes. *Carcinogenesis*, 21(6), pp.1087-95.

Pigati, L., Yaddanapudi, S., Iyengar, R., Kim, D., Hearn, S., Danforth, D., Hastings, M., Duelli, D. (2010). Selective release of microRNA species from normal and malignant mammary epithelial cells. *PloS One*, 5(10), e13515.

Pikor, L., Thu, K., Vucic, E., Lam, W. (2013). The detection and implication of genome instability in cancer. *Cancer and Metastasis Reviews*, 32(3-4), pp.341-352.

Prise, K. M., Folkard, M., Michael, B. D. (2003). A review of the bystander effect and its implications for low-dose exposure. *Radiation Protection Dosimetry*, 104, pp.347-355.

Qiao, Y., Zhang, P., Wang, C., Ma, L., Su, M. (2014). Reducing X-Ray Induced Oxidative Damages in Fibroblasts with Graphene Oxide. *Nanomaterials*, 4(2), pp.522–534.

Ramirez, M., Amorim, M., Gadelha, C., Milic, I., Welsh, J., Freitas, V., Nawaz, M., Akbar, N., Couch, Y., Makin, L., Cooke, F., Vettore, A., Batista, P., Freezor, R., Pezuk, J., Rosa-Fernandez, L., Carreira, A., Devitt, A., Jacobs, L., Silva, I., Coakley, G., Nunes, D., Carter, D., Palmisano, G., Dias-Neto, E. (2018). Technical challenges of working with extracellular vesicles. *Nanoscale*, 10(3), pp.881-906.

Rashed, M., Bayraktar, E., Helal, G., Abd-Allah, M. F., Amero, P., Chavez-Reyes, A., Rodriguez-Aguayo, C. (2017). Exosomes: From Garbage Bins to Promising Therapeutic Targets. *International Journal of Molecular Sciences*, 18(3), pp.538.

Riley, P. A. (1994). Free radical in biology: oxidative stress and the effects of ionizing radiation. *International Journal of Radiation Biology*, 65 (1), pp.27-33.

Robbins, P. Morelli, A. (2014). Regulation of immune responses by extracellular vesicles. *Nature Reviews Immunology*, 14(3), pp.195–208.

- Rodgers, K., McVey, M. (2016). Error-Prone Repair of DNA Double-Strand Breaks. *Journal of Cellular Physiology*, 231(1), pp.15-24.
- Rossi, M., Battafarano, G., D'Agostini, M., Del Fattore, A. (2018). The Role of Extracellular Vesicles in Bone Metastasis. *International Journal of Molecular Sciences*, 19(4), 1136.
- Russell, R. 2008. *Can living cells repair damage from radiation?* [Online]. [Accessed 10 April 2019]. Available from: http://www.windows2universe.org/earth/Life/images/radiation_dna_damage_big_jpg_image.html
- Saadeldin, I., Oh, H., Lee, B. (2015). Embryonic–maternal cross-talk via exosomes: potential implications. *Stem Cells and Cloning*, 8, pp.103-107.
- San Lucas, F., Allenson, K., Bernard, V., Castillo, J., Kim, D., Ellis, K., Ehli, E., Davies, G., Petersen, J., Li, D., Wolff, R., Katz, M., Varadhachary, G., Wistuba, I., Maitra, A., Alvarez, H. (2016). Minimally invasive genomic and transcriptomic profiling of visceral cancers by next-generation sequencing of circulating exosomes. *Annals of Oncology*, (4), pp.635-41.
- Savina, A., Furlan, M., Vidal, M., Colombo, M. (2003). Exosome release is regulated by a calcium-dependent mechanism in K562 cells. *Journal of Biological Chemistry*, 278(22), pp.20083-20090.
- Sazgarnia, A., Montazerabadi A., Bahreyni-Toosi, M., Ahmadi, A., Aledavood, A. (2013). In vitro survival of MCF-7 breast cancer cells following combined treatment with ionizing radiation and mitoxantrone-mediated photodynamic therapy. *Photodiagnosis and Photodynamic Therapy*, 10(1), pp.72-78.
- Schneck, H., Blassl, C., Meier-Stiegen, F., Neves, R., Janni, W., Fehm, T., Neubauer, H. (2013). Analysing the mutational status of PIK3CA in circulating tumor cells from metastatic breast cancer patients. *Molecular Oncology*, 7(5), pp.976–986.
- Shadeo, A., Lam, W. L. (2006). Comprehensive copy number profiles of breast cancer cell model genomes. *Breast Cancer Research*, 8(1), R9.
- Shao, C., Stewart, V., Folkard, M., Michael, B. D., Prise, K. (2003). Nitric oxide-mediated signaling in the bystander response of individually targeted glioma cells. *Cancer Research*, 63, pp.8437-8442.
- Shelke, G., Jang, S., Yin, Y., Lässer, C., Lötvall, J. (2016). Human mast cells release extracellular vesicle-associated DNA. *Matters*, 2(2), pp.1
- Shen, Y., Le Bert, N., Chitre, A., Koo, C., Nga, X., Ho, S., Khatoor, M., Tan, N., Ishii, K., Gasser, S. (2015). Genome-derived cytosolic DNA mediates type I interferon-dependent rejection of B cell lymphoma cells. *Cell Reports*, 11(3), pp.460–473.
- Shu, H., Wang, Y. (2014). Adding to the STING. *Immunity*, 41(6), pp.871-3.
- Simi, L., Pratesi, N., Vignoli, M., Sestini, R., Cianchi, F., Valanzano, R., Nobili, S., Mini, E., Pazzagli, M., Orlando, C. (2008). High-resolution melting analysis for rapid detection of KRAS, BRAF, and PIK3CA gene mutations in colorectal cancer. *American Journal of Clinical Pathology*, 130(2), pp.247-53.

- Sinibaldi-Vallebona, P., Matteucci, C., Spadafora, C. (2011). Retrotransposon-encoded reverse transcriptase in the genesis, progression and cellular plasticity of human cancer. *Cancers*, 3(1), pp.1141–1157.
- Sisirak, V., Sally, B., D'Agati, V., Martinez-Ortiz, W., Özçakar, Z. B., David, J., Rashidfarrokhi, A., Yeste, A., Panea, C., Chida, A., Bogunovic, M., Ivanov, I., Quintana, F., Sanz, I., Elkon, K., Tekin, M., Yalçinkaya, F., Cardozo, T., Clancy, R., Buyon, J., Reizis, B. (2016). Digestion of Chromatin in Apoptotic Cell Microparticles Prevents Autoimmunity. *Cell*, 166(1), pp.88–101.
- Skog, J., Würdinger, T., van Rijn, S., Meijer, D., Gainche, L., Sena-Esteves, M., Curry, W., Carter, B., Krichevsky, A., Breakefield, X. (2008). Glioblastoma microvesicles transport RNA and proteins that promote tumour growth and provide diagnostic biomarkers. *Nature Cell Biology*, 10(12), pp.1470–1476.
- Snijders, A., Mannion, B., Leung, S., Moon, S., Kronenberg, A., Wiese, C. (2015). Micronucleus formation in human keratinocytes is dependent on radiation quality and tissue architecture. *Environmental and Molecular Mutagenesis*, 56, pp.22–31.
- Snyder, A., Morgan, W. (2005). Lack of consensus gene expression changes associated with radiation-induced chromosomal instability. *DNA Repair*, 4(9), pp.958-970.
- Suzuki, K., Ojima, M., Kodama, S., Watanabe, M. (2003). Radiation-induced DNA damage and delayed genomic instability. *Oncogene*, 22, pp.6988-6993.
- Symer, D., Connelly, C., Szak, S., Caputo, E., Cost, G., Parmigiani, G., Boeke, J. (2002). Human I1 retrotransposition is associated with genetic instability in vivo. *Cell*, 110(3), pp.327-338.
- Szatanek, R., Baj-Krzyworzeka, M., Zimoch, J., Lekka, M., Siedlar, M., & Baran, J. (2017). The Methods of Choice for Extracellular Vesicles (EVs) Characterization. *International Journal of Molecular Sciences*, 18(6), pp.1153.
- Tai, Y., Chen, K., Hsieh, J., Shen, T. (2018). Exosomes in cancer development and clinical applications. *Cancer Science*, 109(8), pp.2364–2374.
- Takahashi, A., Okada, R., Nagao, K., Kawamata, Y., Hanyu, A., Yoshimoto, S., Takasugi, M., Watanabe, S., Kanemaki, M., Obuse, C., Hara, E. (2017). Exosomes maintain cellular homeostasis by excreting harmful DNA from cells. *Nature Communications*, 8, 15287.
- Takei, Y., Takigahira, M., Mihara, K., Tarumi, Y., Yanagihara, K. (2011). The Metastasis-Associated microRNA miR-516a-3p Is a Novel Therapeutic Target for Inhibiting Peritoneal Dissemination of Human Scirrhus Gastric Cancer. *Cancer Research*, 71(4), pp.1442-53.
- Tanaka, A., Nakatani, Y., Hamada, N., Jinno-Oue, A., Shimizu, N., Wada, S., Funayama, T., Mori, T., Islam, S., Hogue, S., Shinagawa, M., Ohtskui, T., Kobayashi, Y., Hoshino, H. (2012). Ionising irradiation alters the dynamics of human long interspersed nuclear elements 1 (LINE1) retrotransposon. *Mutagenesis*, 27(5), pp.599-607.
- Thakur, B., Zhang, H., Becker, A., Matei, I., Huang, Y., Costa-Silva, B., Zheng, Y., Hoshino, A., Brazier, H., Xiang, J., Williams, C., Rodriguez-Barrueco, R., Silva, J., Zhang, W., Hearn, S., Elemento, O.,

Paknejad, N., Manova-Todorova, K., Welte, K., Bromberg, J., Peinado, H., Lyden, D. (2014). Double-stranded DNA in exosomes: a novel biomarker in cancer detection. *Cell Research*, 24(6), pp.766–769.

Théry, C., Witwer, K. W., Aikawa, E., Alcaraz, M. J., Anderson, J. D., Andriantsitohaina, R., ... Zuba-Surma, E. K. (2018). Minimal information for studies of extracellular vesicles 2018 (MISEV2018): a position statement of the International Society for Extracellular Vesicles and update of the MISEV2014 guidelines. *Journal of Extracellular Vesicles*, 7(1), pp.1535750.

Todorova, D., Simoncini, S., Lacroix, R., Sabatier, F., Dignat-George, F. (2017). Extracellular Vesicles in Angiogenesis. *Circulation Research*, 120(10), pp.1658–1673.

Vignard, J., Mirey, G., Salles, B. (2013). Ionizing-radiation induced DNA double-strand breaks: a direct and indirect lighting up. *Radiotherapy Oncology*, 108(3), pp.326-329.

Vlassov, A., Magdaleno, S., Setterquist, R., Conrad, R. (2012). Exosomes: current knowledge of their composition, biological functions, and diagnostic and therapeutic potentials. *Biochimica et Biophysica Acta*, 1820, pp.940-8.

Waddell, N., Pajic, M., Patch, A., Chang, D., Kassahn, K., Bailey, P., Johns, A., Miller, D., Nones, K., Quek, K., Quinn, M., Robertson, A., Fadlullah, M., Bruxner, T., Christ, A., Harliwong, I., Idrisoglu, S., Manning, S., Nourse, C., Nourbakhsh, E., Wani, S., Wilson, P., Markham, E., Cloonan, N., Anderson, M., Fink, J., Holmes, O., Kazakoff, S., Leonard, C., Newell, F., Poudel, B., Song, S., Taylor, D., Waddell, N., Wood, S., Xu, Q., Wu, J., Pinese, M., Cowley, M., Lee, H., Jones, M., Nagrial, A., Humphris, J., Chantrill, L., Chin, V., Steinmann, A., Mawson, A., Humphrey, E., Colvin, E., Chou, A., Scarlett, C., Pinho, A., Giry-Laterriere, M., Rooman, I., Samra, J., Kench, J., Pettitt, J., Merrett, N., Toon, C., Epari, K., Nguyen, N., Barbour, A., Zeps, N., Jamieson, N., Graham, J., Niclou, S., Bjerkvig, R., Grützmann, R., Aust, D., Hruban, R., Maitra, A., Iacobuzio-Donahue, C., Wolfgang, C., Morgan, R., Lawlor, R., Corbo, V., Bassi, C., Falconi, M., Zamboni, G., Tortora, G., Tempero, M., Gill, A., Eshleman, J., Pilarsky, C., Scarpa, A., Musgrove, E., Pearson, J., Biankin, A., Grimmond, S. (2015). Whole genomes redefine the mutational landscape of pancreatic cancer. *Nature*, 518(7540), pp.495-501.

Wang, J., Wang, W., Li, R., Li, Y., Tian, G., Goodman, L., Fan, W., Zhang, J., Li, J., Zhang, J., Guo, Y., Feng, B., Li, H., Lu, Y., Fang, X., Liang, H., Du, Z., Li, D., Zhao, Y., Hu, Y., Yang, Z., Zheng, H., Hellmann, I., Inouye, M., Pool, J., Yi, X., Zhao, J., Duan, J., Zhou, Y., Qin, J., Ma, L., Li, G., Yang, Z., Zhang, G., Yang, B., Yu, C., Liang, F., Li, W., Li, S., Li, D., Ni, P., Ruan, J., Li, Q., Zhu, H., Liu, D., Lu, Z., Li, N., Guo, G., Zhang, J., Ye, J., Fang, L., Hao, Q., Chen, Q., Liang, Y., Su, Y., San, A., Ping, C., Yang, S., Chen, F., Li, L., Zhou, K., Zheng, H., Ren, Y., Yang, L., Gao, Y., Yang, G., Li, Z., Feng, X., Kristiansen, K., Wong, G., Nielsen, R., Durbin, R., Bolund, L., Zhang, X., Li, S., Yang, H., Wang, J. (2008). The diploid genome sequence of an Asian individual. *Nature*, 456(7218), pp.60–65.

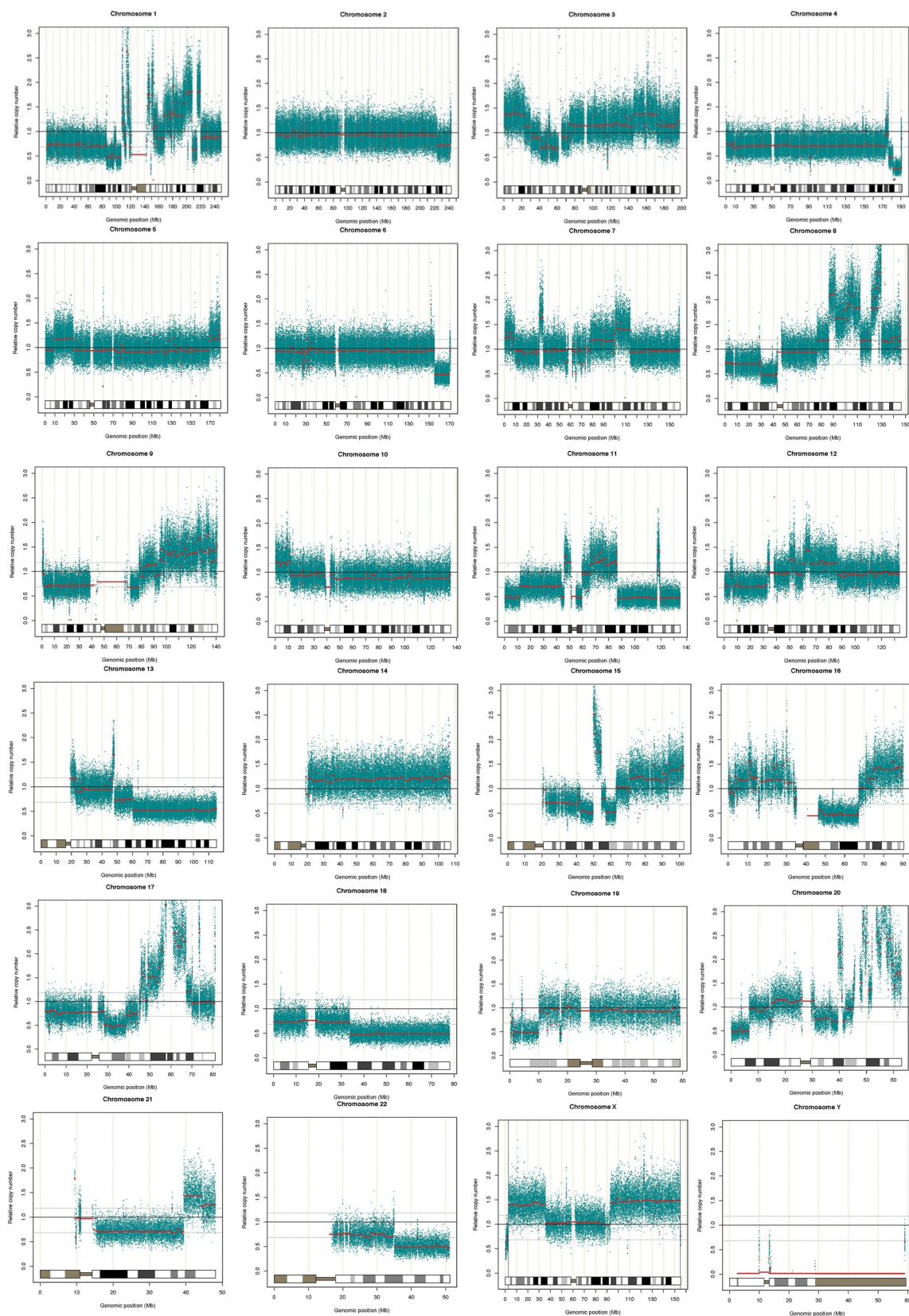
Wang, L., Li, Y., Guan, X., Zhao, J., Shen, L., Liu, J. (2018). Exosomal double-stranded DNA as a biomarker for the diagnosis and preoperative assessment of pheochromocytoma and paraganglioma. *Molecular Cancer*, 17(1), 128.

Weigert, A., Johann, A., von Knethen, A., Schmidt, H., Geisslinger, G., Brune, B. (2006). Apoptotic cells promote macrophage survival by releasing the antiapoptotic mediator sphingosine-1-phosphate. *Blood*, 108(5), pp1635-42.

- Wen, S., Sceneay, J., Lima, L., Wong, C., Becker, M., Krumeich, S., Lobb, R., Castillo, V., Wong, K., Ellis, S., Parker, B., Möller, A. (2016). The Biodistribution and Immune Suppressive Effects of Breast Cancer-Derived Exosomes. *Cancer Research*, 76(23), pp.6816-6827.
- Wickman, G., Julian, L., & Olson, M. F. (2012). How apoptotic cells aid in the removal of their own cold dead bodies. *Cell Death and Differentiation*, 19(5), pp.735–742.
- Wilkins, A. S. (2010). The enemy within: An epigenetic role of retrotransposons in cancer initiation. *Bioessays*, 32, pp.856-865.
- Wolff, E., Byun, H., Han, H., Sharma, S., Nichols, P., Siegmund, K., Yang, A., Jones, P., Liang, G., (2010). Hypomethylation of a LINE-1 promoter activates an alternate transcript of the MET oncogene in bladders with cancer. *PLoS Genetics*, 6(4), pp.e1000917.
- Wright, E. (2000). Inducible genomic instability: new insights into the biological effects of ionizing radiation. *Medicine Conflict and Survival*, 16(1), pp.117-130.
- Wu, J., Sun, L., Chen, X., Du, F., Shi, H., Chen, C., & Chen, Z. J. (2013). Cyclic GMP-AMP is an endogenous second messenger in innate immune signaling by cytosolic DNA. *Science*, 339(6121), pp.826–830.
- Wu, X., Zhang, Y., (2017). TET-mediated active DNA demethylation: mechanism, function and beyond. *Nature Reviews Genetics*, 18(9), pp.517–534.
- Xiao, X., Yu, S., Li, S., Wu, J., Ma, R., Cao, H., Zhu, Y., Feng, J. (2014). Exosomes: decreased sensitivity of lung cancer A549 cells to cisplatin. *PloS One*, 9(2), pp.e89534.
- Xing, J., Witherspoon, D., Jorde, L. (2013). Mobile element biology: new possibilities with high-throughput sequencing. *Trends in Genetics*, 29(5), pp.280–289.
- Xiong, J., Zhang, Z., Chen, J., Huang, H., Xu, Y., Ding, X., Zheng, Y., Nishinakamura, R., Xu, G., Wang, H., Chen, S., Gao, S., Zhu, B., (2016). Cooperative action between SALL4A and TET proteins in stepwise oxidation of 5-methylcytosine. *Molecular Cell*, 64(5), pp. 913–925.
- Xu, Y., He, J., Wang, Y., Zhu, X., Pan, Q., Xie, Q., Sun, F. (2015). miR-889 promotes proliferation of esophageal squamous cell carcinomas through DAB2IP. *FEBS Letters*, 589(10), pp.1127-35.
- Yang, C., He, H., Zhang, T., Chen, Y., Kong, Z. (2016). Decreased DAB2IP gene expression, which could be induced by fractionated irradiation, is associated with resistance to γ -rays and α -particles in prostate cancer cells. *Molecular Medicine Reports*, 14(1), pp.567-73.
- Yang, H., Wang, H., Ren, J., Chen, Q., Chen, Z. J. (2017). cGAS is essential for cellular senescence. *Proceedings of the National Academy of Sciences of the United States of America*, 114(23), pp.E4612–E4620.
- Yu, Q., Zhang, W., Zhang, X., Zeng, Y., Wang, Y., Wang, Y., Xu, L., Huang, X., Li, N., Zhou, X., Lu, J., Guo, X., Li, G., Hou, Y., Liu, S., Li, B. (2017). Population-wide sampling of retrotransposon insertion polymorphisms using deep sequencing and efficient detection. *GigaScience*, 6(9), pp.1–11.

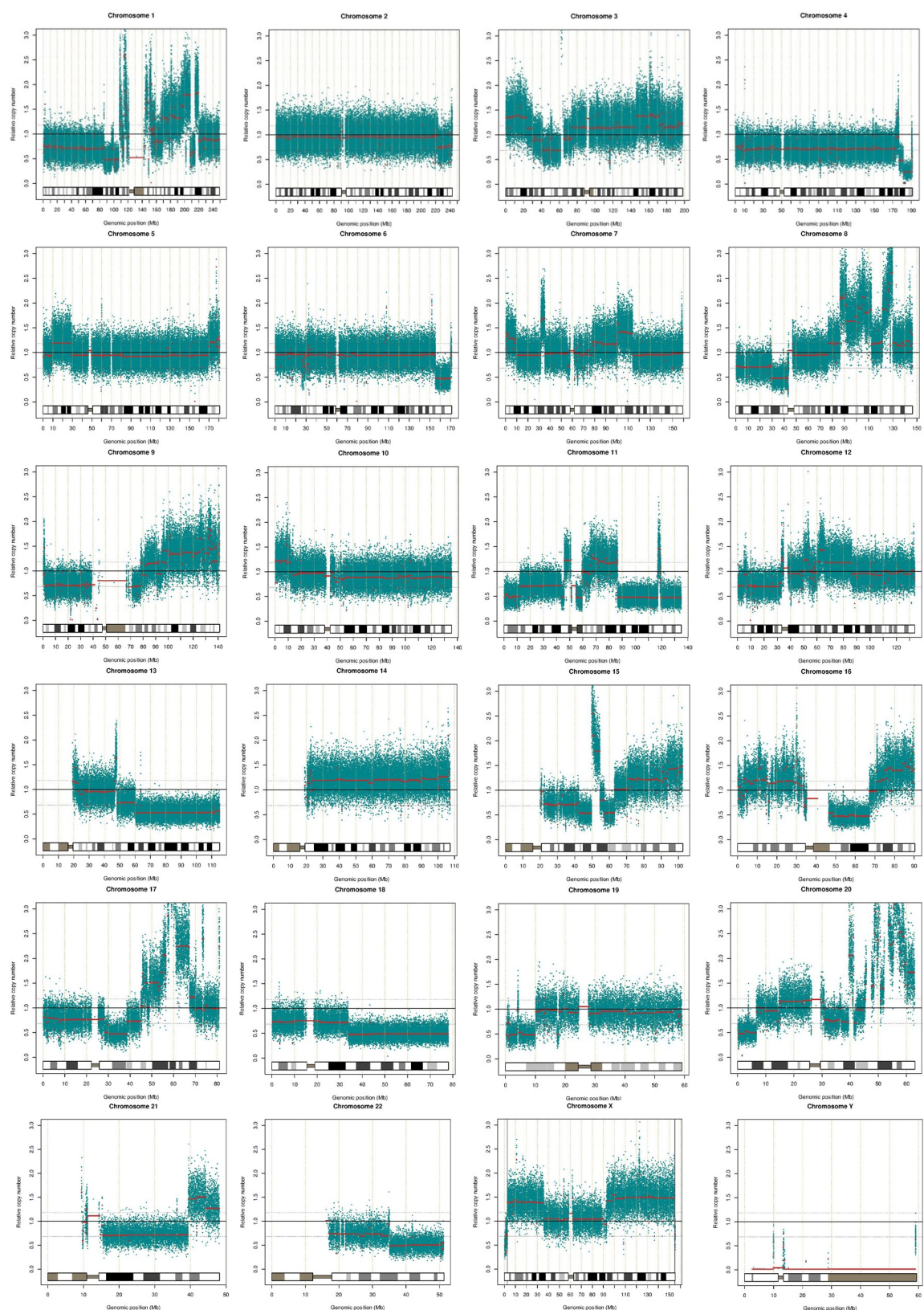
- Yu, S., Cao, H., Shen, B., Feng, J. (2015). Tumor-derived exosomes in cancer progression and treatment failure. *Oncotarget*, 6(35), pp.37151–37168.
- Yu, X., Harris, S., Levine, A. (2006). The Regulation of Exosome Secretion: a Novel Function of the p53 Protein. *Cancer Research*, 66(9), pp.4795-4801.
- Zaborowski, M., Balaj, L., Breakefield, X., Lai, C. (2015). Extracellular Vesicles: Composition, Biological Relevance, and Methods of Study. *BioScience*, 65(8), pp.783-797.
- Zappulli, V., Friis, K., Fitzpatrick, Z., Maguire, C., Breakefield, X. (2016). Extracellular vesicles and intercellular communication within the nervous system. *The Journal of Clinical Investigation*, 126(4), pp.1198–1207.
- Zarovni, N., Corrado, A., Guazzi, P., Zocco, D., Lari, E., Radano, G., Muhhina, J., Fondelli, C., Gavrilova, J., Chiesi, A. (2015). Integrated isolation and quantitative analysis of exosome shuttled proteins and nucleic acids using immunocapture approaches. *Methods*, 87, pp.46-58.
- Zerlinger, E., Barta, T., Li, M., Vlassov, A. (2015). Strategies for isolation of exosomes. *Cold Spring Harbor Protocols*, (4), pp. 319-23.
- Zhang, C., Ji, Q., Yang, Y., Li, Q., Wang, Z. (2018). Exosome: Function and Role in Cancer Metastasis and Drug Resistance. *Technology in Cancer Research & Treatment*, 17, 1533033818763450.
- Zhang, H., Deng, T., Liu, R., Bai, M., Zhou, L., Wang, X., Li, S., Wang, X., Yang, H., Li, J., Ning, T., Huang, D., Li, H., Zhang, L., Ying, G., Ba, Y. (2017). Exosome-delivered EGFR regulates liver microenvironment to promote gastric cancer liver metastasis. *Nature Communications*, 8, 15016.
- Zhang, M., Jin, K., Gao, L., Zhang, Z., Li, F., Zhou, F., Zhang, L. (2018). Methods and Technologies for Exosome Isolation and Characterization, *Small Methods*, 2, pp.1800021.
- Zhang, P., Ludwig, A., Hastert, F., Rausch, C., Lehmkuhl, A., Hellmann, I., Smets, M., Leonhardt, H., Cardoso, M., (2017). L1 retrotransposition is activated by Ten-eleven-translocation protein 1 and repressed by methyl-CpG binding proteins. *Nucleus*, 8(5), pp.548-562.
- Zhang, Y., Hu, Y., Zheng, L., Wang, Q. (2017). Characteristics and Roles of Exosomes in Cardiovascular Disease. *DNA and Cell Biology*, 36(3), pp.202-211.
- Zhang, Y., Liu, Y., Liu, H., Tang, W. (2019). Exosomes: biogenesis, biologic function and clinical potential. *Cell & Bioscience*, 9, 19.
- Zhao, H., Yang, L., Baddour, J., Achreja, A., Bernard, V., Moss, T., Marini, J., Tudawe, T., Seviour, E., San Lucas, F., Alvarez, H., Gupta, S., Maiti, S., Cooper, L., Peehl, D., Ram, P., Maitra, A., Nagrath, D. (2016). Tumor microenvironment derived exosomes pleiotropically modulate cancer cell metabolism. *eLife*, 5, pp.e10250.

Appendix



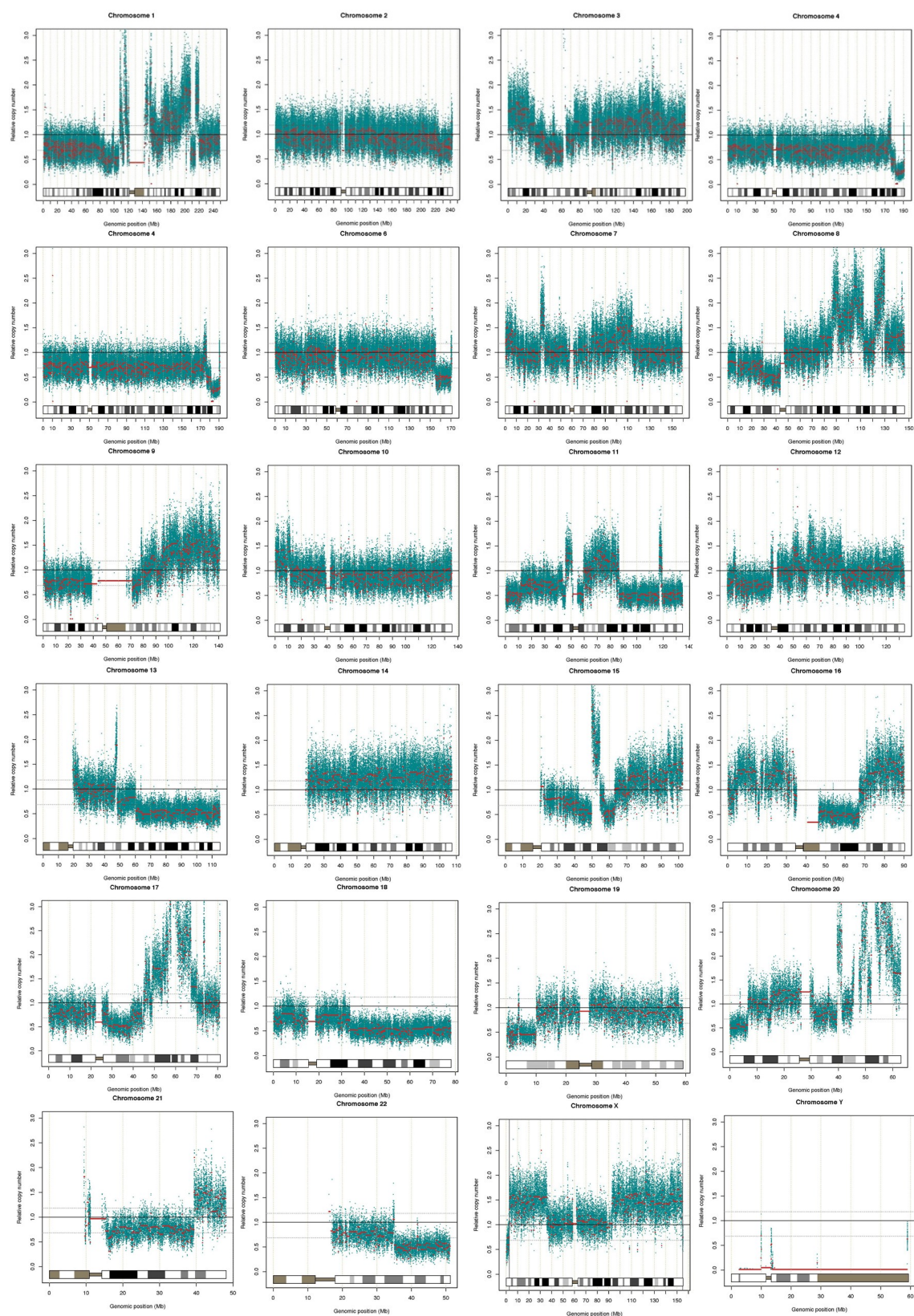
Appendix A. CNV Analysis, Sample 1 (as per section Figure 5.5).

CNV Sequencing analysis of 0 Gy exoDNA originating from MCF7 cell line. Complete set of chromosome profiles showing chromosomal gains/amplifications (above the baseline) and losses (below the baseline). The y-axis represents the log ratio of patient to control read counts (green) and the copy number call of each segment (red). The corresponding chromosome position (Mb) is shown beneath on the x-axis.



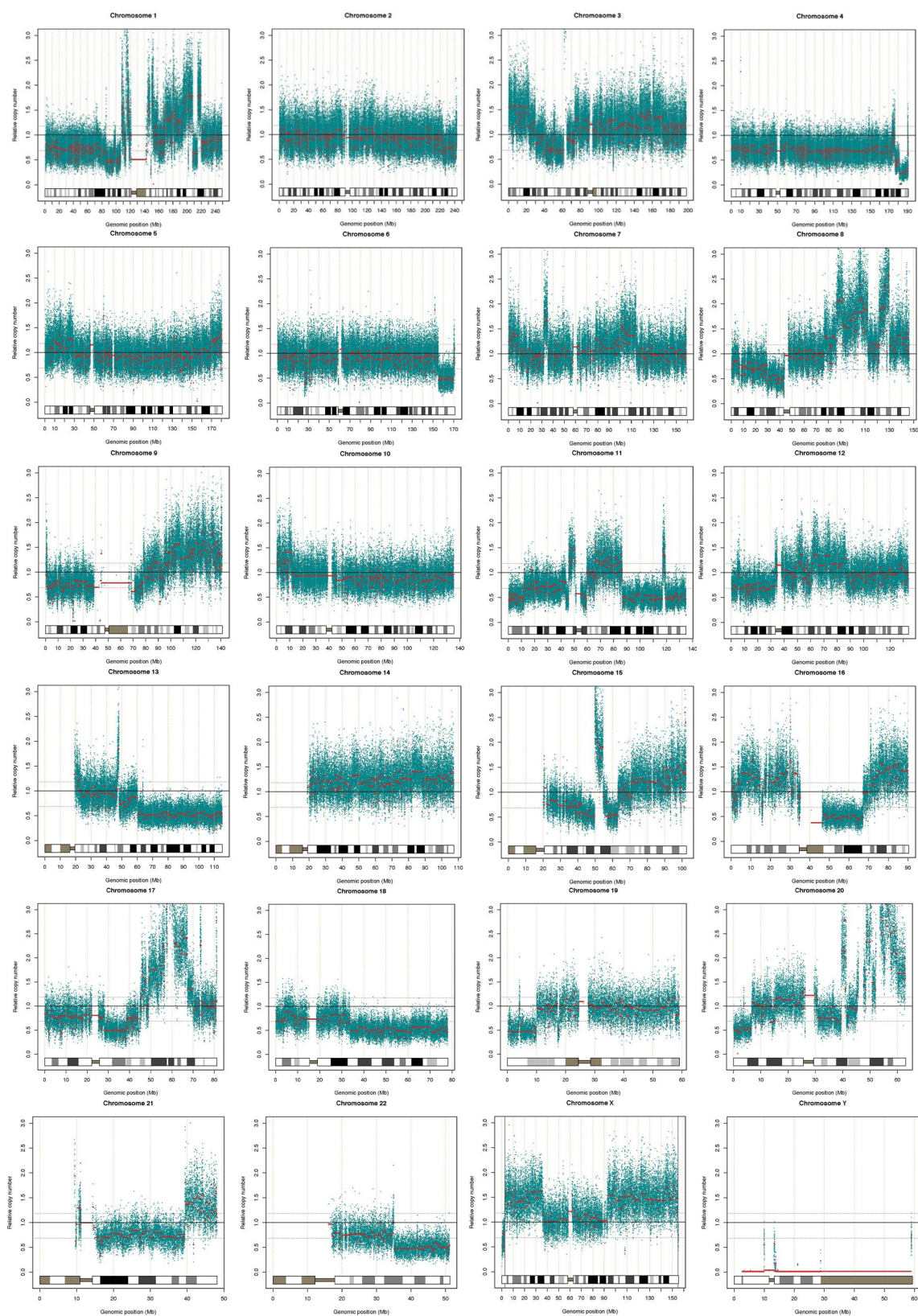
Appendix B. CNV Analysis, Sample 3 (as per section Figure 5.5).

CNV Sequencing analysis of 0 Gy exoDNA originating from MCF7 cell line. Complete set of chromosome profiles showing chromosomal gains/amplifications (above the baseline) and losses (below the baseline). The y-axis represents the log ratio of patient to control read counts (green) and the copy number call of each segment (red). The corresponding chromosome position (Mb) is shown beneath on the x-axis.



Appendix C. CNV Analysis, Sample 4 (as per section Figure 5.5).

CNV Sequencing analysis of 2 Gy exoDNA originating from MCF7 cell line. Complete set of chromosome profiles showing chromosomal gains/amplifications (above the baseline) and losses (below the baseline). The y-axis represents the log ratio of patient to control read counts (green) and the copy number call of each segment (red). The corresponding chromosome position (Mb) is shown beneath on the x-axis.



Appendix D. CNV Analysis, Sample 6 (as per section Figure 5.5).

CNV Sequencing analysis of 2 Gy exoDNA originating from MCF7 cell line. Complete set of chromosome profiles showing chromosomal gains/amplifications (above the baseline) and losses (below the baseline). The y-axis represents the log ratio of patient to control read counts (green) and the copy number call of each segment (red). The corresponding chromosome position (Mb) is shown beneath on the x-axis.