DISSERTATION

INVESTIGATIONS OF TELOMERES AND TELOMERASE FOLLOWING IONIZING RADIATION EXPOSURE

Submitted by

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ABSTRACT

INVESTIGATIONS OF TELOMERES AND TELOMERASE FOLLOWING IONIZING RADIATION EXPOSURE

Telomeres are critical structures located at the termini of eukaryotic chromosomes that regulate the replicative lifespan of human cells. Telomeres shorten with cell division, a process that eventually leads to telomere based growth arrest and cellular senescence. Telomere length is maintained through the activity of the reverse transcriptase, telomerase that functions in embryonic and adult stem cells to elongate telomeres and prolong replicative lifespan. Telomerase is repressed in the vast majority of human somatic cells, and its reactivation is a critical early step in carcinogenesis. Thus, telomerase and telomere maintenance are critical factors in the processes of carcinogenesis, tumor maintenance, and tumor recurrence following interventional therapy.

Ionizing radiation (IR) has long been acknowledged as both a potent carcinogen and an effective agent in the treatment of cancer. To investigate the role telomeres and telomerase play in the cellular response to IR exposure, we tracked telomerase activity and telomere length in a panel of cancer and immortalized non-cancer cell lines following both acute and low dose rate (LDR) exposures to γ-rays. We observed elevations of telomerase activity in cancer, but not non-cancer, cell lines following acute exposures to IR. Further, telomere length was significantly reduced in both cancer and non-cancer cells post-acute IR exposure. Taken together, these studies suggest telomerase activity is playing a role in accelerated tumor repopulation following

radiation therapy and that the associated telomere loss may be contributing to genomic instability.

As IR induced enrichment of cancer stem cells (CSC) in established cancer cell lines was recently suggested to play a role in accelerated tumor repopulation following radiation therapy, we investigated a potential role for telomerase in the IR induced enrichment of CSCs. Consistent with previous reports, we detected a significant enrichment of putative breast CSCs in MCF-7 mammary carcinoma cells at 5 days post exposure, and demonstrate significant enrichment of putative CSCs in the non-tumorigenic MCF-10a, WTK1, and LCL15044 cell lines. Further small molecule inhibition of telomerase activity was able to effectively block CSC enrichment in both MCF-7 and MCF-10a cells. Together, these results suggest that telomerase inhibition is a significant player in the IR induced enrichment of putative CSCs in both cancer and non-cancer mammary epithelial cells. Further, this process seems to be driven by non-canonical roles of telomerase.

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CHAPTER 1

INTRODUCTION

Telomeres and Telomerase

Telomere Structure and Function

Telomeres are nucleoprotein complexes located at the termini of linear, eukaryotic chromosomes. The human telomere sequence, conserved throughout vertebrate biology, consists of the tandem repeat DNA sequence 5'-TTAGGG-3' (Meyne, Ratliff, & Moyzis, 1989), which is directly bound by Telomere Repeat Factor 1 (TRF1), Telomere Repeat Factor 2 (TRF2), and Protection of Telomeres 1 protein (POT1), and together with TRF1 Interacting Nuclear Factor 1 (TIN2), Tripeptidyl Peptidase 1 (TPP1), and Replication Associated Protein 1 (Rap1) form a tightly regulated chromatin structure termed Shelterin (de Lange, 2005). The telomeric DNA sequence ends in a 3' single stranded overhang, which with the help of the shelterin complex, folds back onto itself and invades the double stranded telomeric DNA duplex forming a lariat structure known as the T-loop (Griffith et al., 1999). A schematic of the T-loop structure can be found in Figure 1.1. It is believed that this T-loop structure serves to protect the end of the chromosome from nucleolytic degradation, in addition to preventing the telomere (essentially a naturally occurring double strand DNA break) from being recognized as a break in need of repair (Bailey, 2008).

Due to the semi-conservative nature of DNA replication and the requirement for an RNA primer in lagging strand DNA synthesis, conventional DNA polymerases are unable to replicate to the absolute end of the chromosome (Olovnikov, 1973; Watson, 1972). This phenomenon has been termed the "End Replication Problem" and results in the progressive loss of telomere

sequence with each round of cell division (Counter et al., 1992; Prowse & Greider, 1995; Vaziri et al., 1993). Telomere loss accompanies cell division and aging (Cherif, Tarry, Ozanne, & Hales, 2003; Harley, Futcher, & Greider, 1990), and is the molecular mechanism underlying the finite replicative lifespan of human somatic cells known as the Hayflick Limit (Hayflick & Moorhead, 1961). As telomeres shorten, they eventually reach a state of critical shortness, which triggers a persistent DNA damage response resulting in cellular senescence or apoptosis, thus signaling the end of the cell's replicative lifespan (Bodnar et al., 1998; Harley & Villeponteau, 1995). Telomere shortening and defects in telomere length maintenance have been linked to diseases of premature aging including Werner syndrome, as well as degenerative diseases such as idiopathic pulmonary fibrosis (IPF) and dyskeratosis congenital (DKC) (H. Zhu, Belcher, & van der Harst, 2011). Alternatively, cancer cells which require a mechanism to maintain telomere length in order to avoid a state of crisis, utilize the activity of the reverse transcriptase telomerase to synthesize telomeric DNA de novo; thereby overcoming the end replication problem and acquiring replicative immortality. We now recognize telomeres and telomere biology as critical players situated at the crossroads between normal human aging, degenerative disease, and cancer.

Telomeres and Telomerase History and Significance

Telomeres were first functionally described circa 1931 when Barbara McClintock and Herman Muller, working in maize and drosophila respectively, utilized x-rays to fragment chromosomes and noticed that the natural ends of chromosomes are functionally different than those existing at the site of an induced chromosomal break (McClintock, 1938; Muller, 1938). While McClintock and Muller lacked the necessary tools to understand the structure of telomeres, the description of the DNA by James Watson and Francis Crick occurring in the

1950's (Watson & Crick, 1953), and the discovery of DNA polymerases by Arthur Kornberg in the late 1950's (Kornberg, 1957) lead to the realization of end replication problem, and further, how was it solved at telomeres? Elizabeth Blackburn sought to answer this question, and working in *Tetrahymena thermophilia*, demonstrated that telomeres were composed of a tandem repeat DNA sequence varying between 20 to 70 repeats (Blackburn & Gall, 1978). The conserved, repeat nature of telomeric DNA was later confirmed by Blackburn and colleagues in *Saccharomyces cerevisae*, indicating that telomere structure transcends the boundaries of kingdoms in the evolutionary spectrum (Szostak & Blackburn, 1982). We now appreciate that the structure and function of telomeric DNA, while varying in sequence and associated proteins, is conserved throughout domain Eukarya (Blackburn, Greider, & Szostak, 2006). While the structural nature of telomeric DNA had been described in model organisms, a fundamental solution to the end replication problem remained unknown. Working as a graduate student under Blackburn, Carol Greider began a series of experiments to identify the mystery enzyme capable of synthesizing telomeric DNA. In December of 1984, Greider confirmed the existence of such an enzyme, which was capable of adding 6-base pair repeats to a specified oligonucleotide consistent with the *Tetrahymena* telomere sequence (Greider & Blackburn, 1985). Blackburn and Greider later termed this enzyme "telomerase." Telomerase is now appreciated as a reverse transcriptase complex consisting of a catalytic protein component, the telomerase reverse transcriptase (TERT), and a telomerase RNA template component (TERC) both of which are essential for the elongation activity of telomerase to occur (Autexier & Lue, 2006). Thus telomerase is an enzyme capable of circumventing the end replication problem by preventing critical telomere shortening and extending the telomere sequence *de novo*.

Soon thereafter, the race to identify the human telomere sequence ended with the identification of the repetitive TTAGGG sequence via fluorescence in situ hybridization (FISH) (Moyzis et al., 1988). Together with the discovery of telomerase, an explosion of research was ignited that sought to characterize telomeres and telomerase in a variety of organisms and settings. A critical role for telomerase in cancer was confirmed in cultured cancer lines and telomerase knockout mouse models, in which loss of telomerase activity limited cell division and tumor production (Blackburn et al., 2006). These and numerous other studies have led to the development of telomere based therapies in the clinic, most specifically telomerase inhibitors which are now undergoing Phase III clinical trials for human use (Thompson et al., 2013). Additionally, DNA repair inhibitors such as phosphatidylinositol 3 kindase (PIK3) and poly ADP-ribose polymerase (PARP) inhibitors also show promise.

Blackburn, Greider, and their collaborator Jack Szostak were awarded the Nobel Prize for Medicine and Physiology in 2009 for the discovery of telomeres and telomerase; highlighting the important role that these discoveries have played not only in our understanding of the critical biological processes surrounding the nature of chromosome ends, but also the role they have played in the study and treatment of human disease.

Stem Cell Biology

Stem cells or progenitor cells are defined as having the potential to mitotically divide and develop into many different cell types (Health, 2009). An early example of stem cell theory was conducted in 1961 when it was determined that only a small percentage of bone marrow cells were able to form colonies in the spleen on nude mice (Till & Mc, 1961). In many tissues, stem cells serve as an internal repair system dividing with few limits to replenish lost or damaged cells (Chopra, Hans, & Shetty, 2013). When stem cells divide, the daughter cells have the potential to

remain a stem cell or differentiate into another type of cell with a more specialized function, a process known as asymmetric cell division (Morrison & Kimble, 2006). Stem cells are also capable of self-renewal undergoing symmetric cell division to produce equivalent daughter stem cells (Q. Z. Wang, Lu, Jiang, Diao, & Xu, 2010). Experimentally, stem cells can be coaxed to differentiate into various specialized cell types under different microenvironmental or experimental conditions (Sokolov & Neumann, 2013). These properties of self-renewal and the ability to differentiate along multiple lineages (potency) are defining characteristics of stem cells (Mitalipov & Wolf, 2009). Historically, stem cells have been divided into two categories, embryonic stem cells (ESCs) and adult (somatic) stem cells (Health, 2009).

ESCs are defined as primitive (undifferentiated) cells derived from pre-implantation embryos capable of dividing for prolonged periods of time without differentiation in culture (Health, 2009). These are considered pluripotent stem cells, in that they have the potential to develop into cells of any of the three germ layers; ectoderm, mesoderm, or endoderm and can give rise to any fetal or adult cell type (Mitalipov & Wolf, 2009). ESCs were first isolated from the blastocysts of murine embryos in 1981 (Martin, 1981), and human ESCs (hESCs) were derived using an analogous method in 1998 (Thomson et al., 1998). Early embryonic stem cells and cell lines were derived for the purpose of *in vitro* fertilization techniques and later donated with informed consent for research purposes (Health, 2009). ESCs were the first type of stem cell line to be derived and cultured from humans, however ethical issues associated with the production of these cell lines has limited their use as a research tool.

Adult or somatic stem cells are rare undifferentiated cells found among specialized cell types in many organs and differentiated tissues with a limited capacity for self-renewal and differentiation (Health, 2009). Adult stem cells are considered multipotent or able to differentiate

into a limited number of cell types (Mitalipov & Wolf, 2009). For example a blood or hematopoietic stem cell is able to differentiate into lymphocytes and monocytes but not muscle or brain cells. Differentiation of these cells is generally limited to the specialized cells within the organ of origin and their role is generally limited to maintaining and repairing the tissue in which they reside (Mitalipov & Wolf, 2009).

Stem cells, self-renewal, differentiation, and potency are concepts central to developmental biology and organismal structure. Gaining a deeper understanding and broadening the clinical applications of these concepts has potential implications for regenerative medicine, treatment of degenerative disease, and cancer therapy.

Telomeres and Telomerase in Aging, Degenerative Disease, and Cancer

In the absence of a mechanism to elongate telomeres, the end replication problem leads to a progressive shortening of telomeres eventually triggering senescence (Blasco, 2005). During embryonic development ESCs express the human telomerase holoenzyme, which contains both the human TERT component (hTERT) and the human telomeric RNA component (hTERC), in addition to numerous other proteins which aid in telomerase function (Choudhary, Karande, & Raghavan, 2012). As ESCs divide and begin to differentiate, telomerase expression is reduced and eventually suppressed around the time of birth, as evidenced by the fact that telomerase transcription is repressed in the vast majority of human somatic cells (Flores & Blasco, 2010). The exceptions to this rule include cells of the germ-line which express telomerase to a high degree and adult stem cells which express lower levels of telomerase necessary for their elongated replicative lifespan (Shay & Wright, 2011). Figure 1.2 details a schematic of telomere length and maintenance in normal cell aging and cancer. Telomerase is a key regulator of the

replicative lifespan of human cells and lies at the crux between aging, degenerative disease, stem cell maintenance, and tissue self-renewal.

Loss of telomerase function has been associated with multiple degenerative diseases and diseases of tissue failure including dyskeratosis congenita (DKC) and idiopathic pulmonary fibrosis (IPF) (Garcia, Wright, & Shay, 2007). DKC is a rare multisystem disorder characterized by skin pigmentation, nail dystrophy, and leukoplakia in the mouth (Drachtman & Alter, 1995). Patients appear normal at birth but develop somatic disorders of the bone marrow, gastrointestinal tract, endocrine system, and neurological systems within the first two decades, leading to death. About 40% of DKC cases involved a mutation in genes related to telomere function (Vulliamy et al., 2006) and positional cloning in patients diagnosed with DKC identified a common mutation in the DKC1 gene encoding dyskerin which is associated with telomerase (Heiss et al., 1998). Dyskerin aids in the processing of hTERC into mature RNA and loss of function results in decreased hTERC levels and shortened telomeres (Mitchell, Wood, & Collins, 1999; J. M. Wong & Collins, 2006). Thus mutations in DKC1 caused decreased levels of hTERC lead to a defect in telomerase that result in progressive DKC, eventually triggering fatal bone marrow failure. IPF is a progressive, fatal lung disease characterized by abnormal lung scarring and inefficient gas exchange (Gross & Hunninghake, 2001). A small subset of patients have a familial form of the disease, and these familial cases tend to have nucleotide deletions in the hTERT gene leading to a truncated protein missing large portions of the reverse transcriptase domain (Tsakiri et al., 2007). In both DKC and IPF, loss of functional telomerase (presumably vital to adult stem cell compartments) results in progressive, genetic disease leading to tissue failure and premature aging phenotypes.

In contrast to differentiated human somatic cells and the degenerative diseases of telomerase failure highlighted above, cancer cells maintain their telomere length to prevent cellular senescence and crisis. The vast majority of cancers overcome this tumor suppressive barrier through the re-activation of telomerase (N. W. Kim et al., 1994; Shay, 2005; Shay & Wright, 2011). Ninety percent of human cancers express high levels of telomerase which maintains telomeres above a critically shortened length. The other ten percent of cancers maintain telomere length through a telomerase independent Alternative Lengthening of Telomeres (ALT) pathway (Heaphy et al., 2011). Therefore, telomere length maintenance is crucial to the survival of cancer cells, and all cancers must have a mechanism to maintain telomere length to sustain growth. However, the mechanism of telomerase activation and the stage at which it becomes necessary for carcinogenesis to progress, remains in question and lies at the heart of one of the greatest controversies in cancer research: the multi-step or cell of origin models of carcinogenesis.

The Armitage-Doll multistage model of carcinogenesis states that accumulated, heritable mutations in multiple genes over time provides a selective growth advantage leading to cancer (Armitage & Doll, 1954; Boyd & Barrett, 1990). As somatic cells divide, telomere shortening eventually triggers telomere based growth arrest and cellular senescence leading to an aging phenotype. However, in the presence of growth signals such as oncogene activation, cells with critically short telomeres will continue to divide until they reach a stage known as crisis, which is marked by critically short telomeres and telomere-telomere end fusions resulting in chromosome aberrations and mitotic cell death (Greenberg, 2005). This crisis phase is overcome via the reactivation of telomerase, which maintains telomeres at a length above the crisis levels, thereby contributing to immortalization of the cell population and resulting in tumor formation. In the

multi-step model of carcinogenesis, telomerase reactivation prevents mitotic cell death resulting from telomere-telomere fusions in crisis endowing the cell with an essentially infinite replicative lifespan, providing a selective growth advantage, and facilitating tumor formation.

Alternatively, the cell of origin or cancer stem cell (CSC) model of carcinogenesis states that cancer arises from mutations in the small population of adult stem cells that have a much greater replicative lifespan and are naturally more "cancer like" than differentiated somatic cells (Armanios & Greider, 2005). CSC's are defined as possessing properties of indefinite self-renewal, slow replication, intrinsic resistance to chemo- and radiotherapy and the ability to give rise to differentiated progeny (O'Flaherty et al., 2012). In this case, adult stem cells that already express telomerase can become deregulated through mutations in various genes, which are then propagated to the progeny of the adult stem cell that naturally has a large propensity for cell division. This eventually results in the generation of a bulk tumor containing both therapy resistant CSC's, which function to renew the tumor following treatment, as well as differentiated non-stem cancer cells (NSCC's). Pre-existing telomerase activity in adult stem cell populations give great replicative potential, which, through oncogenic mutation, transition to a CSC and drives tumor progression.

Regardless of the model in which cancer arises, telomeres and telomerase are key players in regulating cellular lifespan. Therefore, a more comprehensive understanding of what processes and pathways telomeres and telomerase influence during carcinogenesis and cancer therapy will better inform both the treatment and prevention of cancer.

Human Telomerase Function, Regulation, and Roles in Cancer

The telomerase holoenzyme is a ribonucleoprotein composed of two primary components; the catalytic reverse transcriptase unit (TERT) and the telomerase RNA component

(TERC) that acts as a template for the synthesis of telomeric repeats (Harrington et al., 1997; Weinrich et al., 1997). hTERT is encoded by a single gene located on the short arm of chromosome 5 at 5p15.33 and is roughly 40 kb in length containing 15 introns and 16 exons (NCBI website,(Cong, Wen, & Bacchetti, 1999). hTERC is located on the long arm of chromosome 3 at 3p26 (NCBI website) and the mature transcript contains 451 nucleotides (NCBI rna database) and is believed to be non-polyadenylated (Collins & Mitchell, 2002; D. Fu & Collins, 2003; Jady, Richard, Bertrand, & Kiss, 2006; Li & Blackburn, 2006). While hTERC expression is ubiquitous among different cell types, the expression of hTERT is tightly regulated. hTERT is expressed in adult stem cell compartments and embryonic stem cells, while its expression is repressed in somatic cells (Blasco, 2005). Additionally, cancer cells reactivate hTERT as a means of overcoming replicative senescence during carcinogenesis (Shay & Wright, 2011). Transfection of hTERT into somatic cells extends the replicative lifespan and overcomes senescence (N. W. Kim et al., 1994), as well as increasing the invasive capacity of U2OS ALT, osteosarcoma cells (Yu et al., 2009).

The protein encoded by hTERT is comprised of 1132 amino acid residues and is generally considered to have four distinct, functional domains (Autexier & Lue, 2006), which include the telomerase essential N-terminal (TEN) domain necessary for appropriate function of telomerase at the telomere (Autexier & Lue, 2006), the TERT RNA-binding (TRB) domain that selectively binds TERC, the reverse transcriptase (RT) domain that contains the motifs necessary for hTERT's reverse transcriptase activity, and the TERT C-terminal extension domain that participates in numerous protein-protein interactions and regulates enzyme localization and processivity (Zvereva, Shcherbakova, & Dontsova, 2010).

The mechanisms that regulate hTERT expression are poorly understood, however evidence for a cell signaling feedback network in which hTERT promotes its own transcription is beginning to emerge, that has implications for carcinogenesis and cancer treatment. Xiang et al. demonstrated that when hTERT was overexpressed in human lens epithelial cells, it induced an enhanced growth phenotype accompanied by a downregulation of the p53 and p21 tumor suppressor genes, which induced hyper-phosphorylation of the retinoblastoma protein (Rb) and upregulation of E2F transcriptional activity, thus promoting advancement of the cell cycle through the G1/M checkpoint (Xiang et al., 2002). Furthermore depletion of hTERT via RNA interference (RNAi) in human ovarian and breast cancer cells resulted in increased expression of p53 and p21 (Lai, Cunningham, Huynh, Andrews, & Tollefsbol, 2007). It has also been demonstrated that overexpression of p53 represses telomerase activity (Kanaya et al., 2000), and that abrogation of p53 function induced cellular immortalization and the reactivation of telomerase (Yang et al., 2007). Taken together, these findings suggest a negative feedback loop between telomerase and the Rb/p53/E2F/p21 G1/M checkpoint machinery that may regulate hTERT expression throughout the cell cycle.

hTERT expression has also been linked to the Wnt/β-catenin signaling network, which plays a significant role in the development of cancer (Anastas JN, 2013). Canonical Wnt signaling involves the accumulation of β-catenin in the cytoplasm, which then migrates into the nucleus where it acts as a transcriptional activator promoting the expression of genes that affect cell proliferation and differentiation including c-myc, oct4, sox2 and other oncogenes (Wu et al., 2013). In the absence of Wnt ligand signaling, two proteins in the destruction complex, axin and adenomatous polyposis coli (APC), bind newly translated β-catenin and target it for phosphorylation by glycogen synthase kinase 3-β (GSK3β). This phosphorylation event targets

β-catenin for ubiquitination and degradation by the proteasome, effectively preventing the transcription of its target genes. However, in the presence of Wnt ligand bound to frizzled receptors (FZD) on the cell surface, GSK3β kinase activity is repressed leading to a cytoplasmic and eventually a nuclear accumulation of β-catenin and therefore the transcription of β-catenin target genes (Wu et al., 2013).

A link between TERT and the Wnt pathway was first uncovered in mouse skin cells expressing a catalytically inactive TERT, where it was demonstrated that stem cells expressing TERT protein exhibited transcriptional activation of genes regulated by Wnt and Myc (Choi et al., 2008). TERT was also shown to act as a cofactor for the β-catenin transcriptional complex in mouse embryonic stem cells (mES) (Park et al., 2009). In this study, TERT was shown to form a complex with the Wnt transcription factor BRG1/SMARCA4 that is a Switch/Sucrose Non Fermentable (SWI/SNF) related chromatin remodeling protein. This allowed TERT to physically occupy the promoters of Wnt target genes including cyclin D1 and c-myc and activate these reporter genes in culture and *in vivo.* It has also been demonstrated that β-catenin can directly regulate the expression of TERT, as β-catenin occupies the TERT promoter in human colorectal cancer cells (Hoffmeyer et al., 2012). hTERT was also shown to interplay with β-catenin to facilitate cancer progression by inducing epithelial to mesenchymal transition (EMT) and CSC phenotype (Liu et al., 2013). Additionally, c-myc which is also under the control of Wnt/ β-catenin signaling; is a well-known regulator of TERT transcription (Greider, 2012; Stower, 2012). These studies provide evidence for a positive feedback loop between hTERT and Wnt/ β-catenin signaling, however, a recent report by Elizabeth Blackburn's group has generated controversy by questioning the direct interaction of β-catenin and the hTERT promoter (Listerman, Gazzaniga, & Blackburn, 2014).

Additionally, the telomere associated protein tankyrase 1 (TNKS) is a well-known regulator of the Wnt/ β-catenin signaling pathway. TNKS is an enzyme containing poly(ADP-ribosylation) (PARP) activity that regulates the amount of TRF1 localized at telomeres (Hsiao & Smith, 2008; Smith, Giriat, Schmitt, & de Lange, 1998). TNKS adds PARP groups to TRF1, a modification that releases TRF1 from the telomere, thereby controlling access to and telomere elongation by telomerase (Smith & de Lange, 2000). Additionally, TNKS regulates Wnt/ β-catenin signaling via modifications of axin; TNKS PARsylates the axin protein, which simultaneously targets it for degradation via the proteasome. Loss of axin results in an accumulation of β-catenin in the cytoplasm therefore activating the Wnt/ β-catenin signaling network even in the absence Wnt ligand being bound to Fzd receptors. Therefore, inhibition of TNKS PARP activity result the stabilization of axin, degradation of β-catenin, inhibits Wnt signaling (S. M. Huang et al., 2009), and potentially contributes to the feedback loop described above.

Telomerase Biogenesis

hTERT and hTERC are brought together and assembled into the telomerase holoenzyme at small nuclear sub-organelles known as Cajal bodies (Y. Zhao et al., 2011; Y. Zhu, Tomlinson, Lukowiak, Terns, & Terns, 2004). Cajal bodies are small and spherical, located in the nucleus and primarily identified by the presence of the protein coilin (Andrade et al., 1991). Cajal bodies are common in cells with high metabolism such as neurons and cancer and are thought to facilitate RNA biogenesis and maturation (Cremer & Cremer, 2001).

hTERC is transcribed by RNA polymerase II and is believed to be non-polyadenylated (Gallardo & Chartrand, 2008). hTERC expression occurs in normal cells and its localization appears to be evenly distributed across the nucleus, indicating that it does not associate with

telomeres, Cajal bodies, or other nuclear structures (Y. Zhu et al., 2004). It was demonstrated that the localization of hTERC to Cajal bodies and telomeres is dependent on hTERT, whereas hTERT knockdown did not affect the overall level of hTERC expression, it did affect hTERC localization to hTERT, Cajal bodies, and telomeres (Tomlinson et al., 2008; Y. Zhu et al., 2004). Therefore it appears that the primary factor controlling localization of hTERC is the hTERT protein. Elevation of hTERC levels is an early step in the development of cancer and hTERC expression correlates better with cancer malignancy than the levels of hTERT expression or telomerase activity (Blasco et al., 1997). Additionally, numerous studies have shown that TERC polymorphisms correlate with breast cancer (Pellatt et al., 2013) and colorectal cancer risk (Jones et al., 2012). Single nucleotide polymorphisms (SNPs) (Chubb et al., 2013) and hTERC gene amplification (Eid et al., 2013; Nowak et al., 2006; Xu, Gruber, Peterson, & Pisa, 1998) have also been associated with risk of acute myelogenous leukemia (AML). Like hTERT, these findings suggest that hTERC functions not only as a part of canonical telomerase activity, but that it may also possess some additional functions important for carcinogenesis.

Taken together, these findings suggest that the traditional role of telomerase in elongating telomeres is not the only important role it plays in carcinogenesis (Low & Tergaonkar, 2013). Throughout this project we will seek to assess the roles hTERT, hTERC, and telomeres play in the process of radiation induced carcinogenesis, as well as tumor repopulation following radiation therapy.

Ionizing Radiation

Introduction and Overview

Ionizing radiation (IR) is a unique agent for which exposure is known to both drive carcinogenesis and treat existing tumors. Ionizing radiation is defined as radiation with sufficient

energy to eject one or more orbital electrons from an atom or molecule (Hall, 2006). This is in contrast to non-ionizing or excitation radiation, which is able to raise orbital electrons to a higher energy state without actual ejection from the atom (Hall, 2006). Each ionization event results in the absorption of around 33 electron volts (eV), which is more than enough energy to break a strong (covalent) chemical bond (Hall, 2006). When occurring in living tissue, this breaking of chemical bonds results in damage to cellular structures including DNA, RNA, lipids, and proteins which can result in tissue injury, mutation, loss of function, and cell death. This is the fundamental process driving both radiation carcinogenesis and therapeutic gains resulting from clinical radiation therapy in the treatment of cancer.

IR is generally classified as being either electromagnetic or particulate. Electromagnetic IR includes γ-rays and X-rays, which do not differ in their nature or properties, only in their atomic location of origin (Hall, 2006; Turner, 2007). γ-rays are produced intranuclearly (in the nucleus) and generally have higher energy whereas X-rays are produced extranuclearly (from changes in state of orbital electrons) and generally have lower energies. Particulate IR includes α-particles, electrons or β-particles, protons, neutrons, or larger heavy charged particles (HZE), which arise from the decay of unstable radio-isotopes or exist almost exclusively in the deep space environment (Turner, 2007). Most terrestrial exposures to IR result from galactic cosmic rays that penetrate the earth's atmosphere, inhalation of radon generated from the natural decay of uranium in the earth, and medical diagnostic modalities (Hall, 2006). Radiation therapy on the other hand utilizes a combination of high energy photons and charged particles including electrons, protons, neutrons, and heavy charged particles such as carbon ions and neon ions (Hamada et al., 2010).

The biological effects of IR exposure occur as a result of damage to critical targets. When radiation is absorbed inside a biological material, there is a stochastic probability that it will interact directly with critical molecular targets in the cell, which results in ionization events within the atoms of the critical target that can lead to chemical modification and result in a biological change. This is known as direct damage. Additionally IR can ionize the water surrounding critical targets, and generate free radicals and reactive oxygen species (ROS). ROS can diffuse throughout the aqueous solution surrounding critical targets and induce chemical alterations in critical targets. This is known as indirect damage. Both direct and indirect damage resulting from IR exposure can lead to cell death and other biological effects resulting from IR exposure (Hall, 2006).

DNA is considered to be the principle target for the biological effects resulting from IR. IR induces base damage, single strand breaks (SSB), and most consequentially double strand breaks (DSB) to DNA, which when unrepaired or repaired incorrectly can lead to the formation of permanent changes such as chromosome aberrations, mutations, large scale deletions, and genomic instability. These changes all correlate with increasing IR dose and contribute to radiation induced cell death. Radiation induced cell death is defined as loss of function in differentiated, non-dividing cells, and as loss of reproductive integrity in proliferating cell populations. Loss of reproductive integrity is known as reproductive or mitotic cell death and is the primary end-point measured in vitro (Hall, 2006).

The amount of damage induced by IR is generally proportional to its ionization density, which is traditionally measured by Linear Energy Transfer (LET). LET is defined as the rate of energy transferred per unit length along the radiation track and is generally denoted in units of keV/um. LET is primarily dependent on the mass, charge, and acceleration of the particle (Hall,

2006). Photons, electrons, and high energy protons are generally considered low LET radiations whereas heavy ions, low energy protons, neutrons, and alpha particles are considered high LET radiations (Turner, 2007).

While LET is a useful physical measurement for comparing radiation quality, it may not adequately reflect the variation of energy deposition along an ionization track. The relative biological effectiveness (RBE) is a more relevant tool to compare the differing biological effects of different radiation types, as RBE is defined as the ratio of the amount of a test radiation necessary to achieve the same quantifiable biological endpoint as a set amount of a reference radiation (usually 250 kV X-rays) (Hall, 2006). RBE depends on radiation quality, radiation dose, the fractionation or dose rate, and the biological end point examined.

The effect of physical properties on RBE highlights the fact that all radiation, and therefore all radiation exposures, are not created equal. Specifically factors such as LET, dose-rate, and dose fractionation significantly alter the response of a biological system to radiation. For example, while a dose response is generally observed for endpoints like survival, mutation, and chromosome aberrations, exposures to high acute doses/dose rates often exhibit lower incidences of oncogenic transformation, suggesting that low dose and low dose rate exposures may present a greater risk for carcinogenesis, the prevailing explanation is that dead cells cannot make tumors.

Ionizing Radiation Induced Carcinogenesis

Much of our understanding of the cancer risks associated with IR exposure arise from epidemiological studies that have tracked exposed individuals throughout their lifespans, the most notable of which is the Hiroshima and Nagasaki atomic bomb (A-bomb) survivors lifetime cohort study (Gilbert, 2009). In addition there are now numerous published studies reporting the

risk associated with IR exposure in medical, occupational, and environmental settings (Gilbert, 2009).

Leukemia was the first cancer to be linked with low LET radiation exposure in A-bomb surviors (Folley, Borges, & Yamawaki, 1952; Gilbert, 2009) and has the highest relative risk of any cancer. The risk of leukemia incidence decreases with age at time of exposure and with the number of years since that exposure occurred (Council, 2006; Preston et al., 2004). Essentially this highlights a complex relationship between age of IR exposure and cancer risk; exposure at younger ages carries an elevated risk of developing cancer later in life, a phenomenon that is near universal to IR induced cancers. Leukemia incidence in the A-bomb surviors peaked within the first 5-10 years following exposure and declined after that point, in contrast to the incidence of solid cancers, which had a latency period following exposure and increase in incidence with time since exposure (Gilbert, 2009). Preston et al. reported that significant dose response relationships for cancers of the oral cavity, esophagus, stomach, colon, liver, lung, non-melanoma skin, female breast, ovary, bladder, central nervous system, thyroid, and "all other solid cancers" among A-bomb survivors (Pawel, Preston, Pierce, & Cologne, 2008; Preston et al., 2008). Additional case control studies have confirmed a direct link between radiation dose and breast cancer risk (Ronckers, Erdmann, & Land, 2005), lung cancer risk (Pierce, Sharp, & Mabuchi, 2003), salivary gland tumors (Land et al., 1996), liver cancer(Cologne, Tokuoka, Beebe, Fukuhara, & Mabuchi, 1999), basal cell carcinoma (Ron et al., 1998), and tumors of the nervous system and pituitary gland (Preston, Ron, et al., 2002) among this cohort.

In addition to environmental exposures such as those described in the A-bomb survivors study, medical exposures resulting from diagnostic imaging modalities and radiation therapy have also provided a unique opportunity for studying IR induced cancer risk resulting from both

low and high LET exposures. An association between leukemia and medical treatment with radiation was first acknowledged in 1957 (Court-Brown & Doll, 2007). Since then numerous studies have found links between leukemia occurrence and radiation therapy (Boice et al., 1987; Weiss, Darby, Fearn, & Doll, 1995). Interestingly, the risk of leukemia first increases with dose then decreases as dose is escalated, suggesting cell killing in doses exceeding the 2 to 4 Gy range (Little et al., 1999). This is consistent with findings in patients treated for uterine cancer with brachytherapy in which relatively low dose and low dose rate exposures generated the same risk as those exposed to higher dose rate external beams, indicating that the low dose rate exposure is more carcinogenic than equivalent large, acute doses (Curtis et al., 1994; Travis et al., 1994).

A dose-response relationship for breast cancer has also been demonstrated in numerous medical radiation studies. Preston et al. conducted a pooled analysis of breast cancer incidence from seven cohort studies of patients treated for benign disease and demonstrated a significant dose response with evidence of a downturn at high doses (Preston, Mattsson, et al., 2002). Case control studies of breast cancer risk in patients treated for malignant disease have revealed a significant dose response relationship for patients exposed under the age of 45 (Boice, Harvey, Blettner, Stovall, & Flannery, 1992; Hooning et al., 2008; Stovall et al., 2008). Elevated incidence of breast cancer has also been observed in Hodgkin Lymphoma (Travis et al., 2003; van Leeuwen et al., 2003) and childhood cancer survivors (Guibout et al., 2005) treated with radiation therapy.

Case control studies have also been conducted demonstrating dose-response relationships between lung cancer and patients treated previously with IR for ankylosing spondylitis (Weiss, Darby, & Doll, 1994), peptic ulcers (Carr et al., 2002), female breast cancer (Inskip, Stovall, & Flannery, 1994), and Hodgkin lymphoma (van Leeuwen et al., 1995). Occupational and

environmental exposures to radon in a Colorado cohort of uranium miners was also shown to significantly elevate lung cancer risk, however the absolute risk increase was greater among smokers (Council, 1999; Lubin et al., 1995).

All of these studies highlight the role of IR as a carcinogen in humans, as most solid cancers and leukemia have been found to be associated with radiation exposure, however the most reliable risk estimations exists for leukemia, breast, lung, and thyroid (Gilbert, 2009). They also highlight the inherent risks associated with using radiation to treat cancer and non-cancer diseases as well as the need for a greater understanding of the biological and molecular processes involved in radiation carcinogenesis.

Radiobiology in Radiation Therapy

About half of those diagnosed with cancer in the United States receive radiation therapy as part of their treatment (Ballas, Elkin, Schrag, Minsky, & Bach, 2006). Radiation therapy is defined as the use of high energy photon or particle radiation to shrink tumors and kill cancer cells (Institute; Lawrence TS, 2008). Radiation therapy can be divided into external beam which includes the use of photons and charged particles and internal or brachytherapy which utilizes internal emitters or radioisotopes located near or absorbed by the tumor to produce therapeutic gains (Institute). In the United States, radiation therapy is dominated by the use of photon based modalities including external beam radiation therapy (EBRT), intensity modulated radiation therapy (IMRT), image guided radiation therapy (IMRT), tomotherapy, stereotactic radiosurgery (SRS), and stereotactic body radiation therapy (SBRT) which all utilize traditional radiobiological methodology to treat tumors with curative or palliative intent (Institute).

Additionally, charged particle regimens including proton, electron, and carbon ion therapy are commonly used in the treatment of rare, radioresistant tumors (Institute).

The overarching goal of radiation therapy is to generate tumor killing with either curative or palliative intent while also sparing normal, non-tumor tissues the detrimental effects of radiation exposure (Hall, 2006). Traditional radiation therapy is based in the principle of fractionation where the delivery of total radiation dose is divided into smaller (usually 2 Gy) fractions separated by time (usually one day). Fractionation maximizes the therapeutic gains from radiation therapy by killing the quickly dividing tumor cells and sparing the slowly dividing normal tissues thereby preventing unwanted side effects which limit the amount of radiation delivered to the tumor. It is now understood that the therapeutic gains from radiation fractionation arise due to the four R's of radiation therapy which include reassortment, repair, reoxygenation, and repopulation (Hall, 2006).

The first of these principles, reassortment, arises from differences in radiation sensitivity of cells at different points in the mitotic cell cycle. Specifically, cells in G1 and late S-phase are more radio resistant than those in other phases of the cell cycle (Hall, 2006). Conversely, cells in G2/M and early S-phase tend to be more radiosensitive. When a dividing cell population is exposed to radiation, those cells in more radiosensitive phases of the cell cycle are preferentially killed. This is an advantage to fractionated radiation therapy because in the time following a single dose, those cells which survive will progress (reassort) into less radio resistant phases of the cell cycle and the population will be more sensitive at the time the next radiation fraction is delivered. This is also beneficial to sparing of normal tissues and cells because they divide more slowly than tumors, and are more likely to be in the radio resistant G0/G1 phase.

Both normal and cancer cells have numerous pathways that repair the damage to DNA caused by IR. Due to this fact, much of the damage caused by IR can be referred to as sub-lethal damage (SLD), as it is unable to kill a cell assuming it gets repaired quickly and efficiently. SLD damage is the operational term for the increase in cell survival that is observed if a given radiation dose is split into two fractions separated by a time interval (Hall, 2006). SLD repair provides radio resistance to both tumor and normal cell populations under fractionated radiotherapy as the time between fractions allows for the SLD to be repaired (Hall, 2006). In the case of low dose rate radiation exposure, which can be thought of as the ultimate hyper-fractionation scheme, repair can actually occur while IR exposure is taking place and result in decreased cell death (K. K. Fu, Phillips, Kane, & Smith, 1975). Work done by Elkind et al demonstrated SLD repair in a two fraction experiment in which two equal fractions of radiation were delivered to chinese hamster ovary cells (CHO) separated by time and maintained at room temperature to prevent movement through the cell cycle. Dividing the two fractions and waiting for 30 minutes between dose deliveries increased the overall survival of cells relative to a single acute dose. As the time interval between the two doses was increased, the surviving fraction increased and plateaued at around 2 hours between doses (Elkind, Sutton-Gilbert, Moses, Alescio, & Swain, 1965). In a parallel experiment where cells were maintained at their normal growing temperature, the pattern of survival was drastically different. If the second fraction was delivered in the first few hours after the first fraction, SLD repair was evident and mirrored the room temperature experiment, however survival fell dramatically at five hours between the two fractions, and increased dramatically thereafter (Elkind et al., 1965). This can be explained by reassortment, and cells entering more sensitive phases of the cell cycle following the first fraction when the second fraction is delivered 2-6 hours after the first fraction (Hall, 2006). Thus

by dividing the total dose delivered into fractions, and allowing time between those fractions, cells are able to repair SLD and display increased cell survival. While this increases the survival of tumor cells, it also helps to minimize the effects of IR exposure on normal cells that may be in the radiation field.

Oxygen has long been recognized as a potent radiation sensitizer as cells grown in the presence of oxygen are more sensitive to the effects of IR exposure than those under hypoxic conditions (Palcic & Skarsgard, 1984). The ratio of the difference in dose necessary to achieve the same biological effect with and without oxygen is known as the oxygen enhancement ratio (OER) (Hall, 2006). The OER is high for photons and lower for high LET particle radiation (Barendsen et al., 1966). In order for the OER to be observed, molecular oxygen must be present at the time of radiation exposure. The molecular basis for this phenomenon is described by the oxygen fixation hypothesis in which absorbed radiation generates free radicals that interact with DNA producing DNA radicals. In the presence of molecular oxygen, these DNA radicals can react with oxygen producing chemically un-restorable organic peroxides in the DNA (Barilla & Lokajicek, 2000; Ewing, 1998; Spiro, Ling, Stickler, & Gaskill, 1985). The OER is important to radiation therapy because solid tumors often have areas of poorly vascularized and therefore hypoxic, necrotic cores (Brown & Giaccia, 1998). The cancer cells residing in these necrotic regions are relatively radio resistant due to a decreased partial pressure of oxygen (Brown, 1979), thus large acute doses of radiation will selectively kill the well oxygenated tumor cells leaving the hypoxic tumor cells to divide. Fractionation serves to alleviate this problem by allowing the necrotic region of the tumor to reoxygenate in the time between radiation fractions as the tumor progressively shrinks.

Treatment with cytotoxic agents such as IR or chemotherapy can trigger surviving cells (clonogens) in a tumor to divide more rapidly than before, a phenomenon known as accelerated repopulation (Hall, 2006; Withers, 1977; Withers, Taylor, & Maciejewski, 1988). A transplanted tumor model in rats first demonstrated accelerated repopulation when a single acute dose of 20 Gy x-rays resulted in an initial decrease in tumor volume indicating regression; however the surviving clones divided more rapidly than prior to radiation treatment, effectively repopulating the tumor more quickly than it was growing prior to IR (Hermens & Barendsen, 1969). This is important because as the tumor was overtly shrinking, the surviving clones were dividing more rapidly than ever (Hall, 2006). A similar phenomenon was also reported in human head and neck cancers (Withers et al., 1988). In this study, Withers et al reported accelerated repopulation at 28 days after the initiation of radiotherapy in a fractionated regiment. Furthermore they reported that an additional dose increment of about 0.6 Gy/day was necessary in order to account for this accelerated repopulation (Hall, 2006; Withers et al., 1988). Thus in the treatment of human tumors, the regimen should be completed as quickly as possible in order to avoid complications imposed by accelerated repopulation. While there is ample experimental evidence supporting the concept of accelerated tumor repopulation following radiotherapy, the molecular aspects and pathways involved remain poorly understood, although recent reports suggest an intricate balance in which signals from dead or apoptotic cells induce a wound healing response (Q. Huang et al., 2011).

The goal of traditional, fractionated radiation therapy is to utilize the fundamental radiobiological principles described above in order to increase tumor killing while at the same time sparing normal tissues the detrimental effects of IR exposure, the definition of a therapeutic advantage. Recent advances in the physics of radiation delivery and diagnostic imaging have

allowed for the creation of hypo-fractionated regimens in which much larger acute doses of IR are delivered specifically to the tumor while preferentially sparing normal tissues due to more accurate delivery of radiation dose. While effective, the underlying radiobiology and signaling pathways, in particular, those involving telomeres and telomerase associated with these new modalities including IMRT, SRS, SBRT, and heavy ion therapies remain poorly understood and represent research opportunities for improving radiotherapy.

Telomeres and Ionizing Radiation

Telomeres were first identified as functional structures in experiments using X-rays to fragment chromosomes in maize and drosophila (McClintock, 1938; Muller, 1938). In these experiments Mueller and McClintock noted that telomeres behave quite differently than the DSB's induced by ionizing radiation. Telomeres are essential features for preventing chromosome ends from being recognized as damaged DNA, therefore telomere function is closely related to maintaining genomic stability (Silvestre & Londono-Vallejo, 2012). Telomeres can become dysfunctional either through excessive shortening (loss of sequence) or loss of end capping function (loss of structure) due to defects in the proteins that for or associated with the shelterin complex (Frias, Pampalona, Genesca, & Tusell, 2012). Thus, multiple studies have been undertaken to investigate the link between telomere function and radiation sensitivity.

Telomere shortening was shown to play a role in radiation sensitivity when mouse embryonic fibroblasts (MEFs) deficient in TERC and expressing critically short telomeres, exhibited symptoms reminiscent of radiation sensitivity syndromes, including decreased survival of MEF's (K. K. Wong et al., 2000). Additionally, this radiosensitivity correlated with delayed repair of DSB's, persistent chromosomal breaks, complex chromosomal rearrangements, and

widespread chromosomal fragmentation (Bailey & Cornforth, 2007). It has also been shown that short telomeres in telomerase knockout mice are also capable of fusing to the sites of radiation induced double strand breaks (Genesca et al., 2006; Latre et al., 2003). In humans, it has been noted that short telomeres were predictive of increase chromosomal instability following treatment of patients with Hodgkin's lymphoma (M'Kacher et al., 2007). Such findings suggest a strong association between shortened telomeres and radiation induced genomic instability and provide a potential explanation for why radiosensitivity increases with age.

A second manner by which telomeres can become dysfunctional results from loss of telomere end-capping function (Bailey & Cornforth, 2007), which can occur due to loss of function of various proteins known to play a critical role at telomeres. In addition to proteins of the shelterin complex, this list also includes proteins associated with the repair of IR induced double strand breaks via non-homologous end joining (NHEJ) and includes the DNA dependent protein kinase (DNA-PK) holoenzyme comprised of the Ku70/Ku80 heterodimer and the DNA-PK catalytic subunit (DNA-PKcs) (Iliakis et al., 2004; Jeggo, 1998). Cells deficient in DNA-PKcs demonstrate hyper radiosensitivity and cancer susceptibility (Auckley et al., 2001; Ponnaiya, Cornforth, & Ullrich, 1997; Someya et al., 2006). Bailey et al demonstrated that loss of function of DNA-PKcs resulted in reduced telomeric end-capping function and the formation of telomere-telomere and telomere-DSB fusion events in the absence critical telomere shortening (Bailey, Brenneman, et al., 2004; Bailey, Cornforth, Ullrich, & Goodwin, 2004; Bailey et al., 1999). Since DNA-PKcs mutations are known to occur in humans, this is of particular importance to radioprotection and carcinogenesis.

In addition to the roles mentioned above, IR and oxidative stress have also been shown to induce telomere shortening (Z. Wang, D. B. Rhee, et al., 2010; Zongaro, Verri, Giulotto, &

Mondello, 2008) and loss of telomere function has been implicated as a major driver of carcinogenesis (Ayouaz, Raynaud, Heride, Revaud, & Sabatier, 2008). Thus it becomes abundantly clear that telomeres are not only contributors to radiation sensitivity and survival, but with their intimate link to DSB repair, telomeres are also important drivers of carcinogenesis.

T*elomerase and Ionizing Radiation*

Human telomerase plays a vital role in embryonic development and the maintenance of the adult stem cell niche (Flores & Blasco, 2010). hTERT is repressed in the majority of adult somatic cells, being reactivated during the development of most cancers and so classification as an oncogene (Daniel, Peek, & Tollefsbol, 2012). Recent findings, including connections to the Wnt/β-catenin and NF-κB (Chung, Aroh, & Vadgama, 2013) signaling pathways and links to angiogenesis (Falchetti et al., 2008), have led to the hypothesis that telomerase not only regulates cellular lifespan, but is central to all the hallmarks of cancer (Low & Tergaonkar, 2013). This body of work has led us to speculate that telomerase plays critical roles in radiation carcinogenesis and accelerated tumor repopulation following radiation therapy.

In the late 1990's, it was demonstrated by multiple groups that telomerase activity is elevated by ionizing radiation in cancer and can be used to assess the radio curability of tumor cells (Finnon, Silver, & Bouffler, 2000; Hyeon Joo, Hande, Lansdorp, & Natarajan, 1998; Karimi-Busheri, Rasouli-Nia, Mackey, & Weinfeld, 2010; Leteurtre, Li, Gluckman, & Carosella, 1997; Neuhof, Ruess, Wenz, & Weber, 2001; Ogawa et al., 1998; Pandita & Roti Roti, 2003; Perez Mdel et al., 2002; Sawant et al., 1999; Terashima et al., 1998; X. Wang et al., 2000; P. Zhao, Li, Yang, & Wang, 1999). In one study, HeLa cells exposed to therapeutically relevant, acute doses of ionizing radiation experienced a significant increase in telomerase activity that

persisted up to 5 days post IR exposure (Sawant et al., 1999). The traditional explanation for this phenomenon was that telomerase is played a role in DNA repair (Leteurtre et al., 1997). However, Elkind et al (Elkind & Sutton, 2012) demonstrated that most of the damage induced by IR was repaired within the first 4-12 hours following exposure, leading to the question of why telomerase remains activated. Additionally, in-vivo assessment of relapsed tumors also showed elevated telomerase activity relative to cured tumor controls, providing further evidence that telomerase may be playing a role in accelerated repopulation of tumors (Sawant et al., 1999).

Furthermore, findings of increased telomerase activity have been observed in other model systems including MCF-7 mammary carcinoma cells (Karimi-Busheri et al., 2010), KG1a AML cells (Perez Mdel et al., 2002), colon carcinoma (Hyeon Joo et al., 1998), squamous cell carcinomas of the oral cavity (Abe et al., 2008), lymphoma and myeloma (Terashima et al., 1998), chronic human skin ulcers related to skin cancer (P. Zhao et al., 1999), murine leukemia (Finnon et al., 2000), nasopharyngeal carcinoma (X. Wang et al., 2000), and immortalized human lymphoblast cells (Neuhof et al., 2001). While each of these studies suggested that telomerase activity was elevated following IR exposure, the doses necessary to induce the elevation, the time telomerase remained elevated post exposure to IR, and the degree of elevation was wildly inconsistent. Furthermore, little work was done to assess the response of telomerase activity in normal human cells, following low dose rate IR exposure, or for periods longer than 3 days, or to explain what was causing the changes in telomerase activity. Therefore, the studies reported here seek to assess the mechanisms of telomerase activation following IR exposure in both normal and cancer cell lines exposed to varying doses of both high and low dose rate IR.

Radiation induced Stem Cell Enrichment in Cancer and Normal Tissues

Cancer Stem Cells in the Treatment of Cancer

Like ESCs, cancer stem cells can self-renew and have pluripotent capacity. CSCs are thought to be the basis for tumor initiation, development, metastasis, and recurrence of cancer following therapy (Chen, Huang, & Chen, 2013). The concept was first acknowledged in 1963 when it was determined that only 1-4% of lymphoma cells were able to form colonies *in vitro* or initiate carcinoma in mouse spleen (Bruce & Van Der Gaag, 1963). However it was not until 1997 when reports that only a $CD34^{+}/CD38^{-}$ subpopulation of human AML cells could initiate malignancy in immunodeficient mice that strong evidence for the theory emerged (Bonnet & Dick, 1997). The first report of a CSC population in solid tumors came in 2003 when a sub-population of $CD44^{+}/CD24^{-}$ breast tumor cells contained the CSC characteristics similar to those found in AML (Al-Hajj, Wicha, Benito-Hernandez, Morrison, & Clarke, 2003). Currently, CSC populations have been identified in nearly all solid tumors including lung, colon, prostate, ovarian, brain, melanoma, and osteosarcoma (Chen et al., 2013). A list of the markers used to identify CSCs in various tumors can be found in table 1.

Cancer stem cells are thought to be one of the predominant factors leading to failure of current therapeutic strategies, as they are thought to be the drivers of resistance to both radiation and chemotherapy (LaBarge, 2010; Lacerda, Pusztai, & Woodward, 2010; Z. Wang, Y. Li, et al., 2010). Due to this fact it is necessary to target and eliminate the CSC population in order to improve treatment outcome, however most conventional therapies do not adequately eliminate CSC populations and may even increase them (Chen et al., 2013).

Cellular Plasticity and Somatic Cell Reprogramming

Potency and self-renewal are the defining characteristics of stem cells, however experiments conducted within the last decade have given rise to the question of whether "stemness" refers to a specific cell population or a cell property (Antoniou, Hebrant, Dom, Dumont, & Maenhaut, 2013). Traditionally, cell fate was thought to be irreversible; meaning that once a cell has committed to a lineage it cannot be reversed. However, the induction of pluripotency was demonstrated in differentiated mouse (Yamanaka & Takahashi, 2006) and later human (Takahashi et al., 2007; Zaehres & Scholer, 2007) fibroblasts through the introduction of transcriptional reprogramming factors, effectively reversing the committed lineage and reverting these cells to more primitive ESC-like states of pluripotency. This feature of cell fate is now referred to as "plasticity" and the process by which it occurs is termed "reprogramming" (Elshamy & Duhe, 2013).

Reprogramming has been demonstrated in several experimental models. Direct reprogramming to pluripotency can occur through introduction of transcription factors octamer binding transcription factor 4 (Oct4), krupple like factor 4 (Klf4), sex determining region Y box 2 (Sox2), and cMyc (Yamanaka & Takahashi, 2006). Such induced pluripotent stem cells (iPSCs) can then be cultured under appropriate conditions to differentiate into any specialized cell type. Additionally, it was recently demonstrated that differentiated cells of one type can be directly reprogrammed into cells of another differentiated cell type without first proceeding through a pluripotent state through the forced expression of two or three transcription factors (Efe et al., 2011; Ieda et al., 2010; J. Kim et al., 2011).

Reprogramming can also occur through the process of somatic cell nuclear transfer (SCNT). First demonstrated using frog embryos in 1958, the nuclei of a differentiated, somatic

cell was transferred to the cytoplasm of an enucleated egg, resulting in the development of a genetically identical adult frog (Gurdon, Elsdale, & Fischberg, 1958). SCNT proved that the nucleus of a somatic cell contains all the information necessary and sufficient to make another organism, and that it can be reprogrammed by factors present in the egg cytoplasm. This same process was used to clone the famous sheep "Dolly" in 1997 (Wilmut, Schnieke, McWhir, Kind, & Campbell, 1997), and has been demonstrated in human cells as recently as 2013 (Tachibana et al., 2013).

Finally, reprogramming can occur due to changes in environmental signaling, first demonstrated in 1998 when the bone marrow of normal adult mice was transferred into the skeletal muscle of different adult mice. The transplanted bone marrow was able to form new muscle fiber nuclei indicating that the microenvironment of the skeletal muscle exerted a reprogramming effect on the bone marrow cells, effectively turning them into skeletal muscle (Ferrari 1999). Thus, transplanting adult cells from one tissue type into another may give rise to one or more cell types that are typical of the recipient environment. The predominant thought is that extracellular signaling molecules in the cellular microenvironment help to dictate cell fate and changing that microenvironment induces reprogramming or conversion (not necessarily through a pluripotent state). Thus, plasticity can be thought of as the ability of a cell to change its phenotype in response to extra-cellular signals, however, this ignores the "trans-differential" aspects presented by PSC induction and SCNT.

The concept of cellular plasticity has led several groups to propose rethinking of the rigid, unidirectional, hierarchal model of cellular differentiation in normal epithelial and mesenchymal tissues and cancer in favor of a bidirectional, phenotypic equilibrium model in which stochastic processes allow for the interconversion of differentiated and stem cells

(Elshamy & Duhe, 2013; Gupta et al., 2011). It also provides potential new (and perhaps universal) targets in the effort to treat cancer as this interconversion between cell types has been linked to carcinogenesis, tumor progression, metastasis, and recurrence following therapy.

The epithelial to mesenchymal transition (EMT) and the reverse process of mesenchymal to epithelial transition (MET) are common phenomenon occurring during embryogenesis that aid in the differentiation of specific gestational layers to form the organs of the human body (Nakaya & Sheng, 2013). Both of these transitions are intimately connected to cancer progression and may provide the driving force for metastasis (Elshamy & Duhe, 2013), as they are processes of cellular reprogramming and play important roles in cancer. Cancer cells undergoing EMT are capable of surviving genotoxic stress and are resistant to both chemo- and radiation therapy (Fillmore & Kuperwasser, 2008). Additionally, it has been demonstrated on numerous occasions that invasive, metastatic cells also undergo EMT (Du et al., 2011). While undergoing EMT, breast carcinoma cells often acquire stem like properties including increases in CD44 levels as well as decreases in CD24 levels (Louie et al., 2010; Mani et al., 2008) indicating an increase in the number of CSCs.

Together, these findings suggest that phenotypically, cancer stem cells, reprogrammed non-stem cancer cells, those cells undergoing EMT, and metastatic cells are all essentially the same, indicating that cellular plasticity not only drives tumor development and metastasis, but may also prevent therapies targeting CSC's from proving effective for the treatment of cancer.

Radiation Induced Enrichment of Cancer Stem Cells

The qualities of CSCs concerning to cancer treatment include resistance to radiation and chemotherapy, the ability to repopulate the tumor following therapy, and the fact that the

processes involved in the maintenance or generation of previously non-existing (reprogrammed) CSCs contribute to cancer development, therefore therapy induced second cancers, and tumor recurrence. This assumption is confirmed by the fact that biomarkers for CSCs are indicative of poor treatment outcome and the inactivation of CSCs within a tumor determines permanent local control (Butof, Dubrovska, & Baumann, 2013). Therefore it can be assumed that any process that increases the number of CSCs within a tumor could potentially lead to a poor clinical outcome.

CSC enrichment is pertinent to radiation therapy, as it was demonstrated in 2006 (Lagadec, Vlashi, Della Donna, Dekmezian, & Pajonk, 2012; Lagadec et al., 2010; Phillips, McBride, & Pajonk, 2006) that exposure to therapeutic doses of X-rays significantly increased the proportion of $CD44^{+}/CD24^{-}$ breast cancer stem cells in culture. Furthermore other groups have demonstrated a similar stem cell enrichment of colorectal and breast CSCs following exposure to therapeutically relevant doses of γ-rays (Cui et al., 2011; Yang et al., 2012). Interestingly, significant increases in CSCs populations were not observed following equivalent doses of carbon ion therapy, indicating that radiation quality may play a role (Cui et al., 2011). Together, these findings suggest that one reason for tumor relapse and secondary cancers following radiation therapy may be due to an enrichment of CSCs that are inherently more radio resistant, have a larger capacity for cell division, and are more invasive than NSCCs therefore contributing to accelerated repopulation.

The original explanation for the enrichment of CSC following radiation therapy was that existing CSCs within the tumor at the time of dose delivery were more radiation resistant than their NSCC counterparts and therefore were more likely to survive fractionated radiation therapy. Such a scenario implies that CSC enrichment is due to the selection, mobilization, and propagation of surviving CSCs. However, it is now recognized that CSCs can arise from the

interconversion (reprogramming) of NSCC's into CSCs and that radiation encourages this process (Lagadec et al., 2012; Yang et al., 2012). Even more intriguingly, Yang et al. demonstrated that CSCs spontaneously convert to NSCC and vice versa even without radiation exposure to maintain an equilibrium population of CSCs, and that exposure to radiation accelerated the rate of interconversion back to equilibrium (Yang et al., 2012). Therefore, tumors not only rely on CSCs to propagate, but they also have a mechanism to maintain a stochastic equilibrium percentage of CSCs.

While selection and reprogramming are two different paths leading to enrichment of CSCs, it is likely that within a tumor, both processes play a role in CSC enrichment and therefore tumor repopulation. These findings suggest that in order to improve radiation therapy, a combination of cell killing achieved through maximizing the delivered dose, targeting and eliminating existing CSCs, and preventing the conversion of NSCCs into CSCs via reprogramming must be achieved.

Conclusions

Our goal in the present studies was to characterize the response of telomeres and telomerase to ionizing radiation in a panel of normal, non-cancer immortalized, and cancer cell lines. We first sought to verify elevations of telomerase activity in cancer cell lines following IR exposure and to determine whether those elevations correlated with changes in hTERT and hTERC expression and protein levels. We then expanded these studies to examine changes in telomerase activity in primary and non-tumorigenic immortalized cell lines of mammary and hematopoietic origin, as well as to prolonged low dose rate (LDR) exposures and telomere length analyses. Our findings suggest that telomerase activity and transcription levels are elevated for

24-48 hours post exposure in mammary carcinoma and AML cell lines, but not in non-tumorigenic and primary cell lines. These findings suggest that the deregulation of telomerase that occurs during carcinogenesis plays a distinct role in the survival and repopulation of human cancer cell lines exposed to IR, however checkpoint activation may prevent telomerase from acting in the same way in non-tumorigenic and primary cell lines. Furthermore, as changes in telomerase activity did not track with increases in telomere length, we can conclude that telomerase is acting outside of its canonical role in telomere elongation.

We next sought to elucidate the mechanisms leading to increases in telomerase activity. We hypothesized that since stem cells (and CSCs) have a higher background telomerase activity relative to their non-stem (and NSCC) counterparts that stem cell enrichment following IR exposure may correlate with increases in telomerase activity. Our results however suggest that elevation in telomerase activity precedes stem cell enrichment and that there is no association between the percentage of stem cells in a culture and telomerase activity. To determine if telomerase is necessary for the radiation induced enrichment of stem cells, we applied a combined small molecule inhibitor and RNA interference (RNAi) approach. Our results suggest that the reverse transcriptase activity of telomerase is necessary for radiation induced stem cell enrichment.

Taken together, these findings demonstrate a critical role of telomerase in the survival and repopulation of tumors following radiation therapy which may be independent of its canonical reverse transcriptase function. Our results also suggest a potential role for combined radiation and anti-telomerase therapy to produce higher rates of recurrence free survival as well as preventing potential secondary cancers which may arise from the irradiation of non-cancer

tissues. Future studies will seek to discover additional telomere based therapies which could be used to increase the efficacy of traditional cancer treatment.

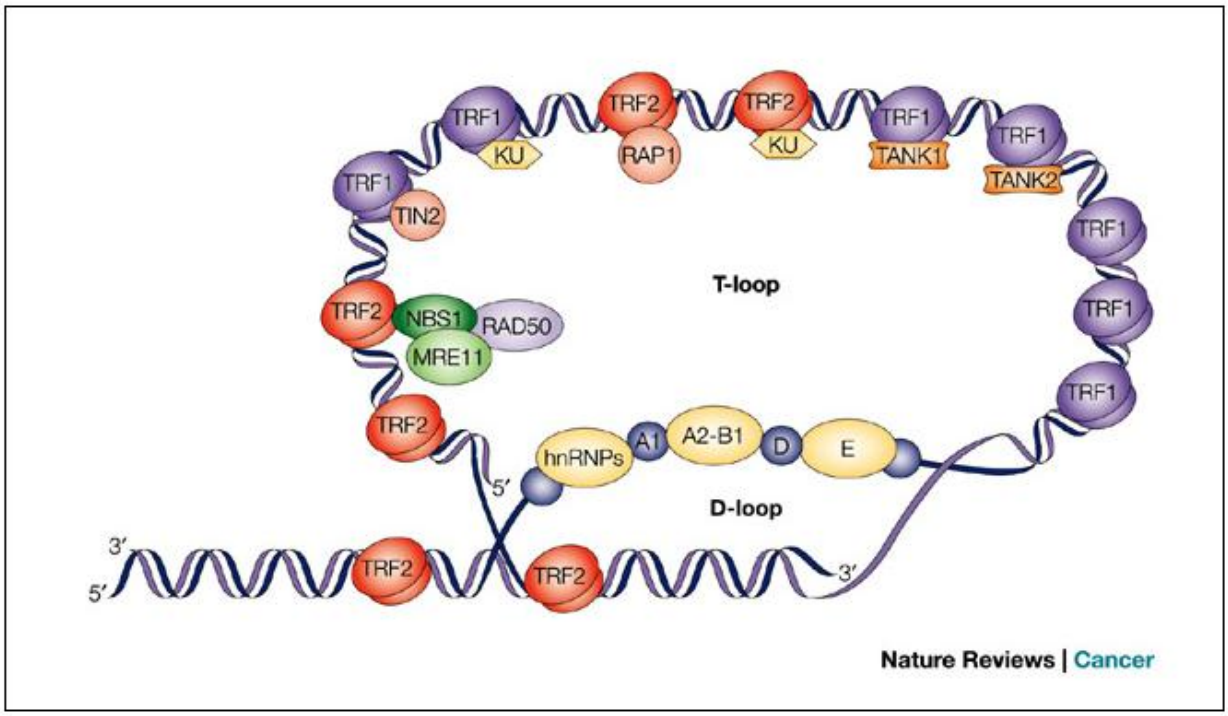

Figure 1.1: Schematic of the T-loop structure. TRF1, TRF2, and POT1 (not shown) bind directly to the telomere sequence. Proteins associated with double strand break repair pathways also interact with the telomere through protein-protein interactions with shelterin. The 3' single stranded overhang binds the DNA duplex displacing the upstream DNA duplex to form a "capping structure" or D-loop. (Neumann & Reddel, 2002)

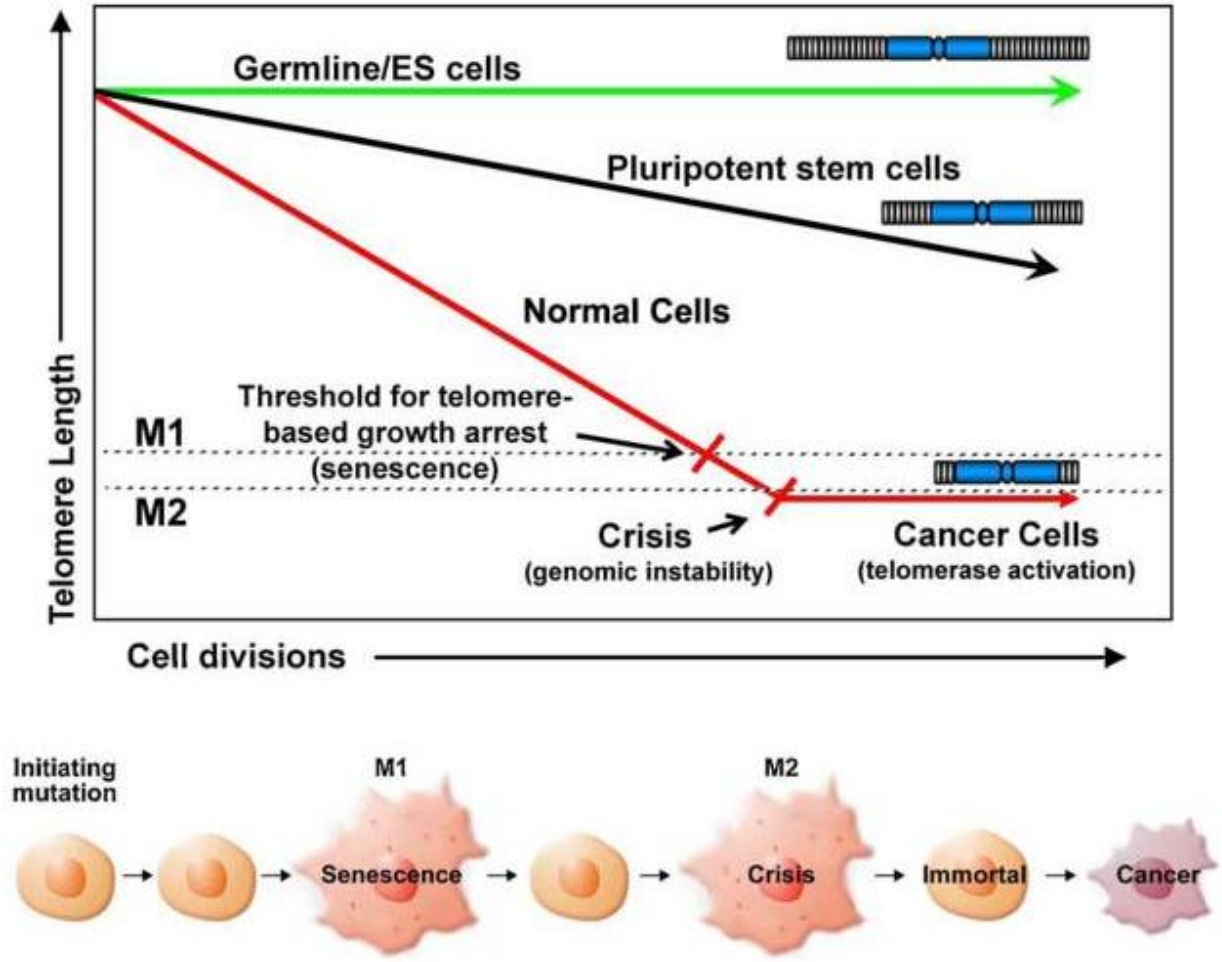

Figure 1.2: Telomere shortening in normal tissue aging and carcinogenesis. In normal human somatic tissues, telomeres shorten by 30-100 base pairs per cell division, a process that eventually leads to telomere based growth arrest/senescence. Adult stem cells and germ line cells maintain telomere length through the activity of telomerase. Human tumors must overcome telomere based growth arrest and accomplish this through reactivation of telomerase or the alternative lengthening of telomeres pathway (Shay & Wright, 2011).

Table 1: List of cancer stem cell markers by tumor type (Chen et al., 2013).

Tumor type	Phenotype of CSCs markers
Leukemia	$CD34^{+}CD38^{-}$HLA-DR-$CD71^{-}CD90^{-}CD117^{-}CD123^{+}$
Breast cancer	$ESA^{+}CD44^{+}CD24^{-/low}Lineage^{-}$, $ALDH\text{-}1^{high}$
Liver cancer	$CD133^{+}$, $CD49f^{+}$, $CD90^{+}$
Brain cancer	$CD133^{+}$, $BCRP1^{+}$, $A2B5^{+}$, $SSEA\text{-}1^{+}$
Lung cancer	$CD133^{+}$, $ABCG2^{high}$
Colon cancer	$CD133^{+}$, $CD44^{+}$, $CD166^{+}$, $EpCAM^{+}$, $CD24^{+}$
Multiple myeloma	$CD138^{-}$
Prostate cancer	$CD44^{+}$, $\alpha2\beta1^{high}$, $CD133^{+}$
Pancreatic	$CD133^{+}$, $CD44^{+}$, $EpCAM^{+}$, $CD24^{+}$
Melanoma	$CD20^{+}$
Head and neck cancer	$CD44^{+}$

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CHAPTER 2

CHARACTERIZING CHANGES IN TELOMERASE ACTIVITY FOLLOWING IONIZING RADIATION EXPOSURE

Summary

Telomerase, the reverse transcriptase that serves to maintain telomere length, is a critical player regulating the proliferative lifespan of human cells. Reactivation of telomerase is a critical rate limiting step in the process of carcinogenesis as well. Interestingly, increased telomerase activity has also been linked to accelerated tumor repopulation following radiation therapy and so may provide a critical target for improving therapeutic intervention strategies. We hypothesized that telomerase activity would be elevated in normal and non-cancer cell lines as well as cancer cell lines following IR exposure. To more clearly elucidate the effects of ionizing radiation (IR) exposure on telomerase activity, we tracked changes in telomerase, expression of telomerase components hTERT and hTERC, and telomere length in a panel of human cancer and non-cancer cell lines following acute and low dose rate (LDR) IR exposures. Elevation of telomerase activity following acute IR exposure was observed specifically in cancer cells, while LDR exposure decreased rather than increased telomerase activity. Furthermore, and to the best of our knowledge, we are the first group to report significant telomere shortening across all the individual telomeres examined in dividing cell populations as a result of acute IR exposure. These findings indicate that the interplay between telomeres, telomerase, carcinogenesis, and IR exposure are complex and highlight the need for studies that increase our understanding of the factors involved.

Introduction

Telomerase is expressed at high levels in the majority of human tumors, facilitating cellular immortalization and evasion of cellular senescence (Shay & Wright, 2011). Normal adult somatic cells do not have telomerase activity with the exception of rare adult, multipotent stem cell (ASC) populations which repopulate and heal tissues following injury (Flores & Blasco, 2010). Telomerase activity in ASCs conveys an extended replicative lifespan and the ability to self-renew through symmetric and asymmetric cell division (Armanios & Greider, 2005). Activation of telomerase is a critical, early step in the process of carcinogenesis leading to its classification as an oncogene (Low & Tergaonkar, 2013).

Ionizing radiation (IR) is a potent carcinogen linked to the development of a variety of solid and blood cancers, most notably breast cancer and leukemia (Gilbert, 2009). Interestingly, it has been demonstrated that acute γ- and x-ray exposures induce increased telomerase activity in human tumors and cell lines including mammary carcinoma (Karimi-Busheri et al., 2010), acute myeloid leukemia (AML) (Leteurtre et al., 1997; Perez Mdel et al., 2002), colon carcinoma (Hyeon Joo et al., 1998), squamous cell carcinoma of the oral cavity (Y. Ogawa et al., 1998), and lymphoma (Terashima et al., 1998) for up to 5 days post IR exposure. This increase in telomerase activity has been linked to accelerated repopulation of tumors following radiation therapy, and so may represent a potent target to increase the clinical efficacy of radiation therapy (Pandita & Roti Roti, 2003; Sawant et al., 1999). Increased telomerase activity following IR has generally been regarded as evidence of telomerase activity in DNA repair. However, considering that most of the damage resulting from IR exposure is repaired within the first 2-12 hours following exposure (Elkind et al., 1965), we thought it unlikely that telomerase is functioning directly in a repair capacity for up to 5 days post exposure (Hall, 2006).

While the effects of IR exposure on telomerase activity are well studied in tumors and cancer cell lines, little has been done to evaluate the effects of IR on telomerase in normal (telomerase negative) and non-tumorigenic (telomerase positive, but not deregulated) cell lines. IR may well induce changes in telomerase activity in these cells potentially increasing their cellular lifespan. Because telomerase activity was elevated in cancer cell lines, we hypothesized that acute IR exposure would activate telomerase in normal and non-tumorigenic immortalized cell lines, and thereby contribute to cellular immortalization and carcinogenesis.

To test this hypothesis we selected a panel of normal, non-tumor immortalized, and cancer cell lines of mammary epithelial and hematopoietic origin, exposed them to acute doses of ionizing radiation, and tracked changes in telomerase activity utilizing the quantitative real time polymerase chain reaction (q-RT PCR) based telomere repeat amplification protocol (TRAP) (Herbert, Hochreiter, Wright, & Shay, 2006). In our mammary epithelial cell panel, we further examined hTERT and hTERC expression levels in response to IR. We also tracked changes in telomerase activity following equivalent doses of chronic low dose rate (LDR) IR exposure to determine if changes in telomerase activity exhibit a dose rate effect. Additionally, to assess whether changes in telomerase activity had an associated effect on telomere length, we tracked changes in telomere length using quantitative fluorescence in situ hybridization (Q-FISH) (Poon & Lansdorp, 2001).

Our results, in agreement with the published literature, indicate that telomerase activity is elevated in cancer cell lines 24-48 hours post exposure to various doses of IR, and corresponds to changes in hTERT mRNA expression levels. No noticeable increase in telomerase activity in normal or non-cancer cell lines was observed. Additionally, telomeres shortened (~20-30%) in the mammary epithelial cells 5 days post IR.

Materials and Methods

Cell Culture

The human mammary epithelial non-tumorigenic cell line MCF-10a was purchased from ATCC and was cultured as described previously (Le, Maranon, Altina, Battaglia, & Bailey, 2013) in 1:1 Dulbecco's Modified Essential Medium (D-MEM)/Ham's F12 growth medium (Hyclone) supplemented with 5% fetal bovine serum (FBS), 10 µg/mL insulin (Sigma), 20 ng/mL epidermal growth factor (EGF; Sigma), 0.5 µg/mL hydrocortisone (Sigma), 0.1 µg/mL cholera toxin (Sigma), and 1% glutamax (Life Technologies). The human mammary carcinoma cell line MCF-7 (kind gift from L. Chubb, CSU Animal Cancer Center) was grown in D-MEM supplemented with 10% FBS and 1% glutamax. The primary mammary epithelial cell line AG11137 (Coriell) was grown in MCDB 170 complete growth medium (US biological) supplemented with 5 µg/mL insulin, 10 ng/mL EGF, 0.5 µg/mL hydrocortisone, 56 µg/mL bovine pituitary extract (Life Technologies), and 1% glutamax.

A human primary immortalized, low passage lymphoblast cell line (LCL15044 kind gift from A. Sigurdsson, National Institute of Health) was grown in RPMI medium supplemented with 15% FBS and 1% glutamax. The WTK1 immortalized lymphoblast cell line (kind gift from H. Liber, Colorado State University) was grown in RPMI medium supplemented with 10% fetal horse serum and 1% glutamax. Human acute myeloid leukemia cells KG1a (Kind gift from Michelle LeBeau, University of Chicago) were grown in RPMI media supplemented 20% FBS and 1% Glutamax. The human osteosarcoma ALT cell lines U2OS and SAOS2 (kind gift from D. Gustafson Colorado State University Animal Cancer Center) were grown in McCoy's 5A growth medium (Life Technologies) supplemented with 10% FBS and 1% glutamax. Primary human foreskin fibroblasts BJ1 (kind gift from J. Shay, University of Texas Southwestern

Medical School) and hTERT immortalized primary human fibroblasts (BJ1 hTERT) (kind gift from J. Bedford, Colorado State University) were grown in a 4:1 mixture of D-MEM high glucose medium (Hyclone) / M-199 (Hyclone) supplemented with 10% FBS and 1% glutamax. All cells were grown at in a humidified incubator at 37^{O}C in 5% CO_2 and passaged 1-2 times per week.

Irradiations

Cells were exposed to various, acute doses of ^{137}Cs γ-rays in a Mark I irradiator (J.L. Shepherd) located at Colorado State University. Cells were exposed at a dose rate of 2.5 Gy/min with rotation. For LDR exposure, cells were incubated under a ^{137}Cs source for total doses of 1 or 4 Gy of γ-rays at dose rates of 4.9 and 3.12cGy/hour. Un-irradiated controls were kept in a separate incubator under identical conditions.

Quantitative Real Time Polymerase Chain Reaction (qRT-PCR) Telomere Repeat Amplification Protocol (TRAP)

Telomerase activity was detected via the telomere repeat amplification protocol (TRAP) assay originally described by Herbert (Herbert et al., 2006) et al and adapted for real time PCR by Hou et al (Hou, Xu, Bjorkholm, & Gruber, 2001). Briefly, whole cell lysates were prepared from cultured cell pellets, lysed in cold MPER mammalian protein extraction buffer (Thermo Fischer) containing a protease inhibitor cocktail (Roche) and RNasin ribonuclease inhibitor (Promega) at a ratio of 100 µl of buffer per 1,000,000 cells. Lysates were cleared by centrifugation at 14,000 RPM for 10 minutes at 4^{o}C, aloiquotted, and stored at -80^{o}C. Protein concentration was determined using the Bradford Assay (Biorad).

The SYBR green master mix (Promega) included all necessary components to complete the RTQ-PCR reaction. Each well contained between 0.1 and 0.25µg of protein lysate, 50%

volume of SYBR green master mix, 0.2µg T4 gene32 protein (New England Biolabs, Ipswitch, MA), 0.1µg of each primer TS (5'-AATCCGTCGAGCAGAGTT- 3') and ACX (5'-GCGCGG(CTTACC)3CTAACC-3') (Integrated DNA Technologies) and RNase/DNase free water to achieve a final well volume of 25ul. The PCR and detection were performed on a CFX 96 (Biorad). In addition to the treatment samples, a series of controls were also included on each plate: (1) no template control with TS primer only, (2) no template control with ACX primer only, (3) no template control with TS and ACX primers (used in normalization of samples), (4) heat inactivated control with template (protein lysate) and TS and ACX primers, (5) HeLa cell lysate with TS and ACX primers (a positive control with robust telomerase activity).

The RTQ-PCR program includes the following steps: Step 1- 1cycle 25℃ 20 min (telomerase elongates the TS primer by adding TTAGGG repeat sequences); Step 2- 1cycle 95℃ 3 min (heat activation of the enzyme in the SYBR master mix); Step 3- 40 cycles of 95℃ for 20 sec, 50℃ for 30 sec and 72℃ for 1min 30 sec (PCR amplification allows for detection by real time instrument); Step 4- 80 cycles 0.10 sec per cycle (melt curve to ensures no primer dimer formation). Each sample is run in triplicate on a 96 well plate format allowing for an average Ct to be obtained per sample. Utilizing the average Ct value, the relative percent telomerase activity in each sample is calculated using the Delta Delta Ct method ($2^{-\Delta\Delta CT}$) (Livak & Schmittgen, 2001). Briefly, to calculate the percent relative activity for each sample first normalize the average sample Ct to the no template control with TS and ACX primers. This is referred to as the delta Ct value. The delta Ct value of each sample is subtracted from the delta Ct value of a chosen comparative sample, in this case a normal feline mucous membrane cell lysate, yielding a delta delta Ct value ($\Delta\Delta$Ct). Using the $2^{-\Delta\Delta CT}$, a relative value is generated for each sample comparison and when multiplied by 100 is the relative percent of telomerase activity (RTA) of

the sample compared to the normal feline mucous membrane. The RTA can be compared between samples assayed across different plates. Results from two runs were averaged.

Reverse Transcriptase PCR to Assess Changes in hTERT and hTERC Expression Levels

Total RNA was harvested from irradiated and un-irradiated samples using the Qiagen RNeasy kit (Qiagen). RNA was quantified using a Nanodrop 1000 spectrophotometer and reverse transcribed using the Verso cDNA kit (Thermo Scienfitic). Real-time PCR was performed using SYBR green master mix (Promega) according to the manufactures protocol and performed using a CFX 96 system (Biorad). The real time cycle was as follows: Cycle 1 at 95^{O}C for 15 minutes, cycle 2 (50X) step 1 at 95^{O}C for 15 seconds, step 2 at 58^{O}C for 30 seconds, step 3 at 72^{O}C for 30 seconds. A melt curve was included to assess for primer dimers and non-specific amplification as follows: cycle 3 at 95^{O}C for 30 seconds, cycle 4 at 55^{O}C for 30 seconds and cycle 5 (80X) at 55^{O}C for 10 seconds. Primers were designed using the Primer3 program (Rozen, 1998) using a published cDNA library for hTERT ((NCBI)) and hTERC ((NCBI)). hTERT primers were added at a final concentration of 300 nM including a forward sequence: CCATCAGAGCCAGCTTCACCT and reverse sequence: TCACCTGCAAATCCAGAAACA. hTERC primer were added at a final concentration of 300 nM including a forward sequence: AAGAGTTGGGCTCTGTCAGC and reverse sequence: TCCCACAGCTCAGGGAATC. Primers for transferrin receptor (TFRC) at a final concentration of 100 nM were included as a housekeeping gene with the forward sequence: CGCTGGTCAGTTCGTGATTA and the reverse sequence: GCATTCCCGAAATCTGTTGT. Relative hTERT and hTERC RNA expression were analyzed using the $2^{-\Delta\Delta CT}$ method.

Telomere Length Analysis via Interphase Quantitative Fluorescence in situ Hybridization (FISH)

Samples were prepared for interphase telomere length analysis using standard cytogenetic techniques as described previously with slight modifications (Le et al., 2013; Ourliac-Garnier & Londono-Vallejo, 2011; Poon & Lansdorp, 2001). Briefly, cultured cell pellets were resuspended in 8 mL of 75 mM potassium chloride (KCl; hypotonic) and incubated for 30 min at 37^{O}C. Following incubation, 1 mL of fixative (3:1 methanol acetic acid) was added, cells were pelleted at 1000 RPM for 5 minutes, resuspended in 6 mL fixative, and stored at -20^{o}C. Fixed cell pellets were then washed and dropped onto glass slides for telomere FISH analysis. Telomere FISH was performed as described previously with modifications (Dregalla et al., 2010).

For telomere fluorescence *in situ* hybridization (FISH), slides were treated with 100ug/ml RNASE A in 150mM NaCl, 15mM Sodium Citrate buffer for 30 minutes at 37^{O}C, dehydrated through an ethanol series (75%, 85%, and 100%), and denatured in a 70% formamide/2X saline sodium citrate (SSC) solution at 70^{O}C for 2 minutes. A G-rich telomere peptide nucleic acid (PNA) probe $(TTAGGG)_3$ labeled with Cy-3 was subsequently hybridized onto the slides at 37^{O}C overnight. Slides were washed twice each in 50% formamide/2X SSC, 2X SSC, and 0.1% NP-40 in 2X SSC for 2.5 minutes each at 43^{O}C. Finally slides were mounted in Prolong Gold Antifade reagent (Invitrogen) with 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI).

Image Z stacks were taken using a Zeiss Axio Imager.Z2 microscope, with a Coolsnap ES2 camera running Metamorph 7.7 (Molecular Devices). For each slide between 30 and 50 images were obtained, each consisting of 22, 0.2um stacks. Metamorph nearest neighbor deconvolution and stack compression functions were then applied followed by image thresholding (upper and lower threshold values were held consistent across experiment). Finally

a region of interest was created for each nucleus and the intensities of individual telomeres were obtained in metamorph.

Flourescence values obtained in each batch of FISH were standardized to the fluorescence intensity of an LY-R mouse lymphoma cell pellet as an internal control. LY-R cells have long brightly staining telomeres and their use for standardization, which was adapted from Q-FISH (H. P. Wong & Slijepcevic, 2004), represents a way to accurately compare relative telomere lengths from run to run.

Statistical Analyses

Statistical differences were detected using a Student's t-test against relevant controls. Statistical analysis was conducted using the Graph Pad Prism 5 software for windows XP.

Results

Telomerase Activity in Normal and Cancer Human Cell Lines

To evaluate the sensitivity of the real time PCR based TRAP assay, we first compared telomerase activity across a panel of normal, non-tumor immortalized, and cancer human cell lines (Figure 2.1) that had been previously reported to express varying levels of telomerase activity (Karimi-Busheri et al., 2010; Kim et al., 1994; Sawant et al., 1999). HeLa cell lysates were utilized as a positive control and all values are reported as fold activity changes relative to HeLa cells. Consistent with published reports, cancer cell lines KG1a and MCF-7 had telomerase activity levels at 3.09 and 0.615 fold differences of HeLa cells respectively, indicating relatively high levels of telomerase activity (Figure 2.1 left panel). WTK1 cells, an immortalized p53

heterozygous cell line (Neuhof, Ruess, Wenz, & Weber, 2001), had telomerase activity levels at a very high 12.83 fold increase compared HeLa cells (Figure 2.1 left panel).

For comparison, the non-tumor immortalized cell lines BJ1 hTERT cells, which were transfected to express the telomerase hTERT component, the spontaneously immortalized mammary epithelial cell line MCF-10a, and the EBV immortalized low passage lymphoblast cell line LCL15044 displayed telomerase activity levels of 0.278, 0.0424, 0.0412 the activity of HeLa cells, indicating telomerase activity expression but at lower levels than those of comparable cancer cell lines (Figure 2.1 left panel). The normal human fibroblast cell line BJ1, primary mammary epithelial cells AG11137, and osteosarcoma cell lines U2OS and SAOS2 that maintain telomere length through the telomerase independent ALT pathway, all displayed telomerase levels at less than 0.035 fold of HeLa cells. Data on telomerase low and negative cell lines can be observed in the right panel of figure 2.1, which omits the inclusion of WTK1 and KG1a cell line telomerase activity to better accentuate the differences in telomerase activity in non-tumorigenic immortalized and normal cell lines (Figure 2.1 right panel). Based on these findings, it can be concluded that any cell line with less than about 0.04 fold the activity of HeLa cells is functionally telomerase negative using this assay. In addition, differences in telomerase activity were confirmed across numerous orders of magnitude, indicating extreme sensitivity over very large range of detection.

Telomerase Activity in Cell Lines Exposed to Acute IR

Numerous reports have demonstrated that IR induces elevation of telomerase activity in cancer cell lines for up to several days post exposure (Falchetti et al., 2008; Finnon, Silver, & Bouffler, 2000; Hyeon Joo et al., 1998; Karimi-Busheri et al., 2010; Leteurtre et al., 1997;

Ogawa et al., 1998; Pandita & Roti Roti, 2003; Perez Mdel et al., 2002; Sawant et al., 1999; Terashima et al., 1998; Wang et al., 2000; Zhao, Li, Yang, & Wang, 1999). These reports have suggested that elevation of telomerase activity may play a role in accelerated tumor repopulation following radiation therapy, as well as contribute to the overall survival of cancer cells exposed to IR (Pandita & Roti Roti, 2003; Sawant et al., 1999). Despite numerous reports of telomerase activity being induced by IR in cancer, none have been conducted examining telomerase activity following IR exposure in non-cancer cell lines. We hypothesized that IR exposure would also elevate telomerase activity in non-tumor cell lines, and thereby contribute to IR induced carcinogenesis by prolonging the proliferative lifespan of cells carrying oncogenic mutations resulting from IR exposure (Shay & Wright, 2011).

To verify these findings and to expand upon previous reports by examining changes of telomerase activity following IR exposure in non-tumor immortalized and normal cell lines, we tracked changes in telomerase activity as a factor of time at 24 hour intervals for up to 240 hours post-acute exposure to 10 Gy γ-rays in mammary epithelial cells MCF-7, MCF-10a, and AG11137. We also tracked changes in telomerase activity in hematopoietic cell lines KG1a, WTK1, and LCL15044 post-acute exposure to 1 and 4 Gy γ-rays up to 72 hours post exposure. All data are reported as values relative to un-irradiated (0 Gy) control samples within the same cell line.

Our results demonstrate significant elevation of telomerase activity in MCF-7 adenocarcinoma cells for up to 48 hours post IR exposure. This significant elevation returns to background levels by 72 hours and decreases steadily thereafter until telomerase activity is significantly decreased in irradiated cells at 120 and 240 hours post exposure (Figure 2.2 left panel), consistent with previous reports. In contradiction to our hypothesis, we did not observe

significant elevation in telomerase activity in either the non-tumorigenic MCF-10a or the primary mammary epithelial AG11137 cell lines following IR exposure (Figure 2.2 middle and right panels). A significant decrease in telomerase activity in AG11137 cells was observed at 48 hours post exposure was observed, however, it is difficult to draw meaningful conclusions from this finding, as AG11137 cells are essentially telomerase negative to begin with.

We conducted a similar study in hematopoietic cell lines following IR exposure and observed similar trends in cancer vs. non-cancer cell lines. Specifically, telomerase activity was significantly elevated in KG1a AML cells following exposures to both 1 and 4 Gy γ-rays, which persisted for up to 72 hours (Figure 2.3 left panel). A significant increase was detected in the non-tumor immortalized WTK1 cell line 24 hours post exposure to 4 Gy, which had disappeared by 48 hours post exposure and remained at background levels thereafter (Figure 2.3 middle panel). No significant changes in telomerase activity in the low passage normal LCL15044 cells were detected post exposure to either 1 or 4 Gy γ-rays (Figure 2.3 right panel).

Our results suggest that elevation of telomerase activity following exposure to IR only occurs in cells possessing high levels of telomerase, like cancer cells, as the only cell lines to display a consistent increase telomerase activity were MCF-7 and KG1a. No significant elevation in telomerase activity was detected in the low telomerase positive MCF-10a cell line post exposure, nor in the normal AG11137 and LCL15044 cell lines. Thus, counter to our original hypothesis, telomerase is not significantly elevated post IR exposure in non-tumor cell lines, thus it is unlikely that telomerase is contributing to an extended replicative lifespan in these cells following exposure. To highlight this trend, Figure 2.4 illustrates a direct comparison of mammary epithelial cells (left panel) and hematopoietic cells (right panel), all relative to HeLa cell lysates.

Telomerase Activity in Cell Lines Exposed to Low Dose Rate IR

Epidemiological studies indicate that low dose rate exposures may be more carcinogenic than equivalent acute exposures (Gilbert, 2009). To determine whether telomerase activity is elevated in telomerase positive cell lines following chronic LDR exposures, we incubated the telomerase positive cell lines MCF-7, MCF-10a, KG1a, and WTK1 under LDR γ-ray exposures at dose rates of 4.9 or 3.12 cGy/hour for total accumulated doses of 1 and 4 Gy (Figure 2.5). Results suggest a dose rate dependent decrease in telomerase activity in all four cell lines examined that appears to be accentuated at cumulative doses of 1 Gy as opposed to 4 Gy. One possible explanation for this finding is that LDR exposure could be modifying the cell cycle distribution or potentially slowing overall cell growth enough to effect telomerase activity.

Changes in hTERC and hTERT mRNA levels in mammary epithelial cells following Acute IR exposure

To assess whether changes in telomerase activity were associated with changes in the expression of hTERT mRNA or hTERC RNA in MCF-7 and MCF-10a cells, and both of these genes are transcriptionally regulated, we utilized quantitative reverse transcriptase PCR (qRT-PCR) to evaluate hTERT and hTERC levels post exposure to 10 Gy of γ-rays (Figure 2.6). Consistent with the observed elevation of telomerase activity in MCF-7 cells following IR exposure, both hTERT and hTERC levels were significantly elevated. hTERT levels, like telomerase activity, were significantly elevated at 24 hours post IR exposure and decreased thereafter, returning to background levels by 72 hour post IR. hTERC levels were significantly increased at 24 hours post IR exposure and increased from that point forward, remaining that way until at least 120 hours post exposure. These findings suggest that the increases in

telomerase activity observed post exposure observed in MCF-7 cells is associated with elevation in hTERT and hTERC RNA levels, but do not account for the significant decrease in telomerase activity detected at 120 hours post exposure. In contrast, MCF-10a cells experienced a significant decrease in hTERT and hTERC levels following exposure to 10 Gy γ-rays with the prior starting at 24 hours and the later beginning at 72 hours post exposure. While no overall decrease in telomerase activity associated with IR exposure was detected in MCF-10a cells, the lack of increased expression in these cells following IR exposure could explain why telomerase activity levels did not change. These findings are consistent with the idea that elevation of telomerase activity following IR exposure is a trait unique to cancer cells.

Telomere Length Analysis of MCF-7 and MCF-10a Cells Exposed to Acute IR

To assess whether changes in telomerase activity and hTERT or hTERC RNA levels led to an appreciable change in telomere length, we utilized interphase quantitative fluorescence *in situ* hybridization (Q-FISH) to assess changes in telomere length in MCF-7 and MCF-10a cells at 120 hours post exposure to 10 Gy of γ-rays (Figure 2.7). Surprisingly, and despite an elevated telomerase activity in MCF-7 cells in the first several days post IR exposure, telomere length was reduced by ~20% at both the cell population level (Figure 2.7 top left panel) and the individual telomere level (Figure 2.7 lower left panel) relative to un-irradiated controls 5 days post IR exposure. A similar trend was observed in MCF-10a cells, with the only difference being that the observed decrease appeared to be greater at roughly 30% (Figure 2.7). The difference in the degree of telomere loss observed between MCF-7 and MCF-10a cells may be explained by the difference in elevations of telomerase activity during the first 48 hours post exposure (Figure 2.2 left panel).

It has been demonstrated that cells with shorter telomeres tend to be more radiosensitive than cells with longer telomeres (Bailey & Cornforth, 2007; Genesca et al., 2006; Latre et al., 2003; M'Kacher et al., 2007; K. K. Wong et al., 2000). However, this would suggest that cells containing shorter telomeres at the time of irradiation would be less likely to survive to 5 days, therefore truncating the histogram of individual telomere length on the left end (elimination of cells with short telomeres). This is not what we observed; rather it appears that all telomeres were shortened as a result of IR exposure shifting the normal distribution of individual telomere lengths to the left in both MCF-7 and MCF-10a cells (Figure 2.7 lower panel). These results suggest that an additional mechanism of telomere loss resulting from IR exposure is causing all telomeres to shorten disproportionately faster than un-irradiated cells, as opposed to selectively killing those cells with shorter telomeres at the time of irradiation.

Discussion:

Telomerase reactivation is a critical rate limiting step in the process of carcinogenesis and is required for cellular immortalization and tumor formation the vast majority of cancers (Shay & Wright, 2011). Consistent with previous reports, we found telomerase activity significantly increased in both MCF-7 mammary adenocarcinoma cells (Karimi-Busheri et al., 2010) and KG1a acute myeloid leukemia cells (Perez Mdel et al., 2002) for several days post-acute IR exposure. A possible explanation for this trend includes the idea that telomerase is playing a role in DNA repair (Bailey, 2008). However, Elkind *et al* previously demonstrated that most of the damage caused by low LET radiation is repaired within the first 2-12 hours following exposure (Elkind et al., 1965). Therefore, it is unlikely that telomerase is acting in a DNA repair capacity up to 48-72 hours post exposure. An alternative explanation involves telomerase as promoting

tumor repopulation by increasing the replicative potential of surviving clonogens. This concept is supported by a report by Padita *et al* that demonstrated telomerase activity was significantly elevated in relapsed, irradiated tumors in a mouse xenograft model (Pandita & Roti Roti, 2003; Sawant et al., 1999). Pandita et al. speculated that telomerase activity may be used as a marker to predict the radio curability of solid tumors.

We reasoned that telomerase activity may play a similar role in the process of carcinogenesis in normal, telomerase negative cells, and/or telomerase positive adult stem cells exposed to IR may utilize telomerase activity to increase their replicative lifespan and inadvertently propagate oncogenic mutations to their progeny, promoting tumor formation. However, our results tracking changes in telomerase activity in telomerase positive immortalized, non-tumorigenic mammary epithelial (MCF-10a) and lymphoblastoid (WTK1) cells yielded no significant increase in telomerase activity. Nor did we observe increases in telomerase activity in telomerase negative primary mammary epithelial cells (AG11137) or in telomerase positive low passage lymphoblastoid cells (LCL15044). One possible explanation for this finding could be activation of the G1/S phase checkpoint that several reports suggest acts in a negative feedback loop suppressing the transcription of the hTERT gene (Kanaya et al., 2000; Lai, Cunningham, Huynh, Andrews, & Tollefsbol, 2007; Xiang et al., 2002; Yang et al., 2007). Specifically, overexpression of p53 was shown to suppress telomerase activity in a variety of cancer cell lines due to increased levels of p21 protein (Xiang et al., 2002; Yang et al., 2007). Consistently, expression of hTERT in telomerase negative cell lines induced hyper phosphorylation of the retinoblastoma protein (Rb) promoting progression past the G1 checkpoint into S-phase (Lai et al., 2007). Therefore, activation of the G1 checkpoint following IR exposure via the p53 mediated DNA damage response, could be suppressing telomerase

activity in non-tumor cells, explaining the lack of an observed increase when regulation of these pathways is normal/functional. Conversely, in MCF-7 and KG1a cancer cells that lack a functional G1 checkpoint, regulation is lost and telomerase activity increases following IR.

To further characterize the response of telomerase components hTERT and hTERC in MCF-7 and MCF-10a cells, we also examined changes in the levels of hTERT mRNA and hTERC in response to an acute 10 Gy exposure (Figure 2.6). Consistent with the increase in telomerase activity observed at 24 hours post exposure in MCF-7 cells, hTERT mRNA levels were significantly elevated starting at 24 hours post exposure and returned to background levels at 72 hours. hTERC RNA levels were also significantly elevated at 24 hours and steadily increased peaking at the 120 hour time point (latest time point examined). In contrast, both hTERT mRNA and hTERC were significantly decreased in MCF-10a cells following exposure, again highlighting that with regards to telomerase, the responses of tumor vs non-tumor is very different and is consistent with the checkpoint activation response described earlier.

Epidemiologic studies have demonstrated that LDR exposure to IR is more carcinogenic than equivalent acute exposures (Gilbert, 2009). To our knowledge, we are the first group to examine the response of telomerase activity to LDR exposure. To determine whether LDR had a differential effect on telomerase activity compared to acute exposures, we incubated telomerase positive cells under standard conditions at dose rates of 4.9 and 3.12 cGy/hour for a time necessary to accumulate doses of 1 and 4 Gy. Surprisingly, LDR incubation appeared to have no significant effect on telomerase activity relative to un-irradiated controls in all of the cell lines examined (Figure 2.5). A statistically insignificant dose rate effect was observed as a dose rate of 4.9 cGy/hour, which appeared to be more effective at reducing telomerase activity than 3.12 cGy/hour; an effect that was more pronounced at total doses of 1 Gy. This lack of a change

could be due to an increased cycling time resulting from LDR exposure effectively decreasing the need for cells to elongate telomeres and mitigate telomere loss because they are cycling more slowly.

As the traditional role of telomerase is to elongate telomeres, a discussion of its effect on telomere length is crucial to any discussion of telomerase activity. Very little information exists on the effect of acute IR exposure on telomere length in dividing cells, however the work that has been done suggests simply that cells with shorter telomeres are more radiosensitive than cells with longer telomeres (Bailey & Cornforth, 2007; Genesca et al., 2006; Latre et al., 2003; K. K. Wong et al., 2000). To assess the effect of acute IR exposure, we examined telomere length in MCF-7 and MCF-10a cells at 120 hours post exposure to 10 Gy of γ-rays using interphase Q-FISH (Figure 2.7).

Counter to the expected findings, we observed a significant shortening of telomeres in both cell lines at the population level of approximately 20% in MCF-7, and a 30% decrease in MCF-10a. To further understand this change, we generated a histogram of the individual telomeres from the cells examined at the population level and superimposed the 0 and 10 Gy populations on top of each other. Rather than observing a selective elimination of specific telomeres, in both MCF-7 and MCF-10a cells, the length of all telomeres in the population were decreased effectively shifting the normal distribution of telomere lengths to the left (Figure 2.7 lower panel). This finding suggests an alternative mechanism that results in a general shortening of all telomeres, as opposed to a mechanism that selectively acts on shorter telomeres. Additionally, significant shortening of telomeres was observed in both tumor and non-tumor cell lines, however the degree of shortening was greater in MCF-10a cells presumably due to low

levels of telomerase both before and after exposure, whereas MCF-7 had elevated levels after exposure, which likely helped to mitigate telomere shortening to some extent.

This finding also raises the issues of inducing genomic instability, one of Weinberg's hallmarks of cancer (Hanahan & Weinberg, 2011). The significance of genomic instability and ties to cancer were first suggested by Nowell in 1976 and manifest in the form of random, ongoing mutation, gene amplification, and chromosomal changes (Nowell, 1976). Shortened telomeres have also been implicated as playing a role in promoting genomic instability as well as carcinogenesis (Kong, Lee, & Wang, 2013). The fact that IR shortens the entire population of telomeres implies that it will also induce instability; therefore surviving clonogens may have increased levels of instability leading to increased risk of tumor progression in non-cancer cells. Further, elevation in telomerase activity in cancer cells that appears to reduce overall telomere shortening may increase the survival of cancer cells, while also masking genomic instability and promoting metastasis or tumor recurrence following radiation therapy.

Based on these findings, we conclude that the response of telomerase to IR exposure varies significantly between cancer and non-tumor cells. This is likely explained by the deregulation of telomerase that occurs during carcinogenesis and potentially results from the abrogation of functional cell cycle checkpoint machinery. LDR exposure also appears to effectively reduce telomerase activity indicating that dose rate is a critical factor to examine in future experiments. Furthermore, acute IR exposure causes telomere shortening in both normal and cancer cells, which appears to be mitigated by telomerase in cancer. This finding suggests that genomic instability can result from acute IR exposure generating critically short telomeres. Therefore the issue of telomeres and telomerase in the treatment and prevention of cancer is much more complex than the simple long and short of telomeres and the positives and negatives

of telomerase. Future studies will seek to more clearly define this elaborate molecular interplay to provide insights and suggestions for the improvement of radiation therapy.

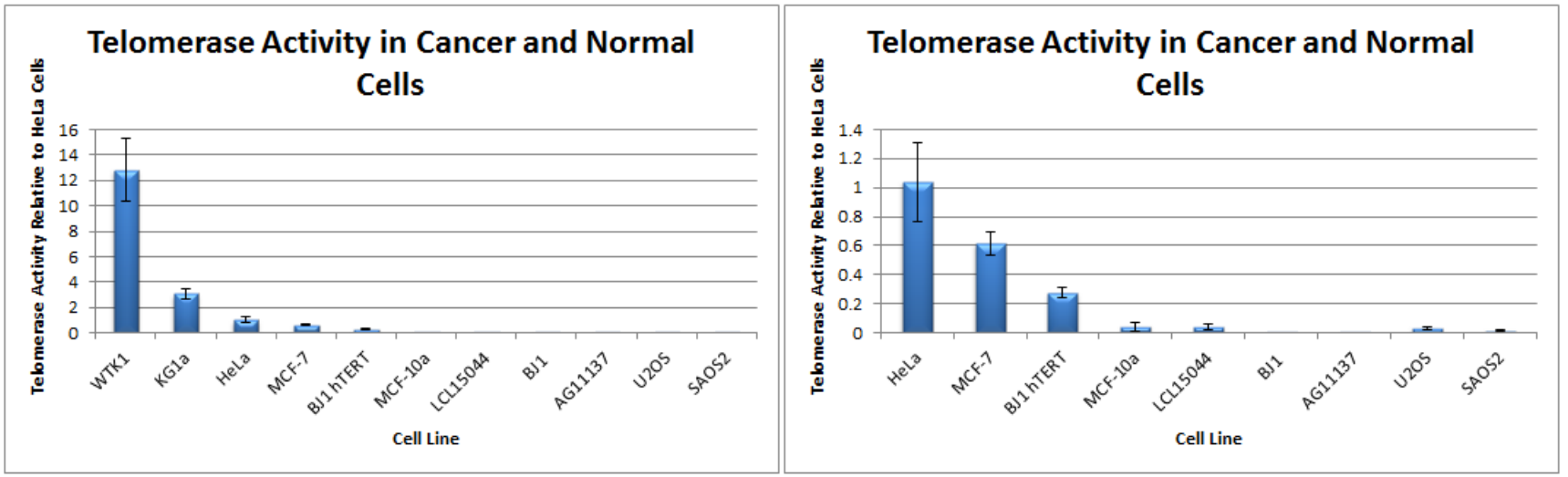

Figure 2.1: Telomerase activity relative to HeLa Cell Lysates in a panel of human cell lines. Left panel illustrates all cell lines examined and right panel demonstrates the same data, but with cell lines WTK1 and KG1a omitted to highlight differences in telomerase activity at low levels in normal and ALT cell lines. Based on these results, further analysis will consider cells with a telomerase activity at less than 3.5% of HeLa cells as functionally telomerase negative.

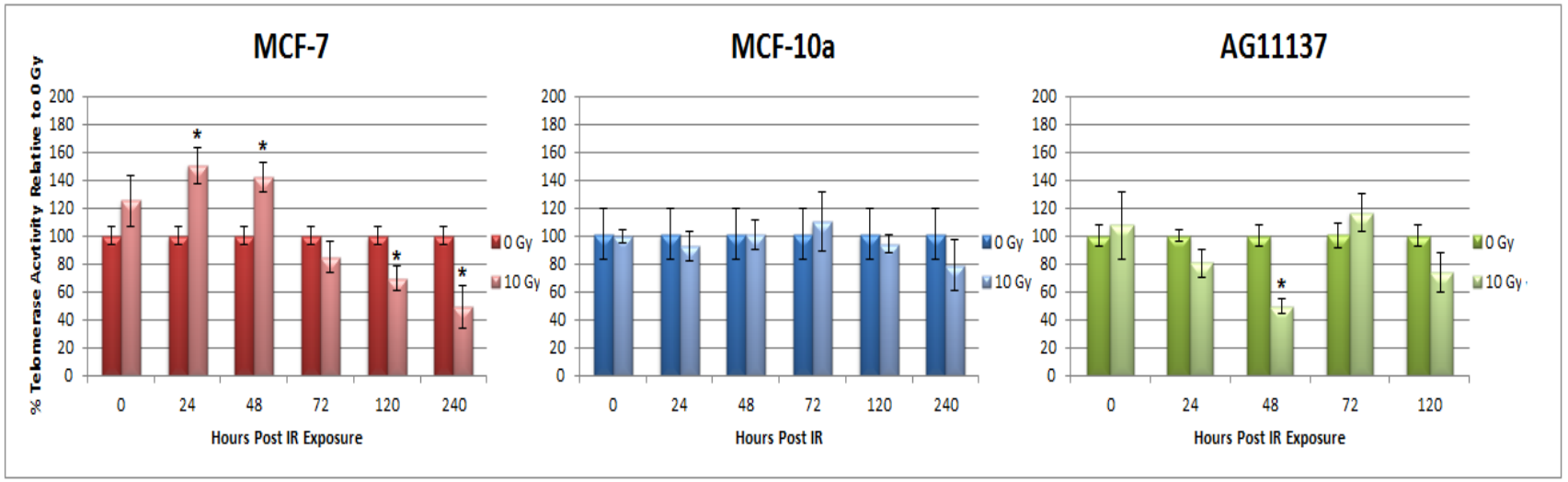

Figure 2.2: Telomerase activity in mammary epithelial cells exposed to an acute dose of 10 Gy of γ-rays at various time points post exposure. Values are expressed as the percentage relative telomerase activity compared to an unirradiated control in MCF-7 adenocarcinoma cells (left panel), MCF-10a non-tumor immortalized mammary epithelial cells (middle panel), and AG11137 primary mammary epithelial cells (right panel). Stars (*) represent data points which are significantly different from unirradiated controls as determined by Student's t-test. Error bars express standard deviation of the mean.

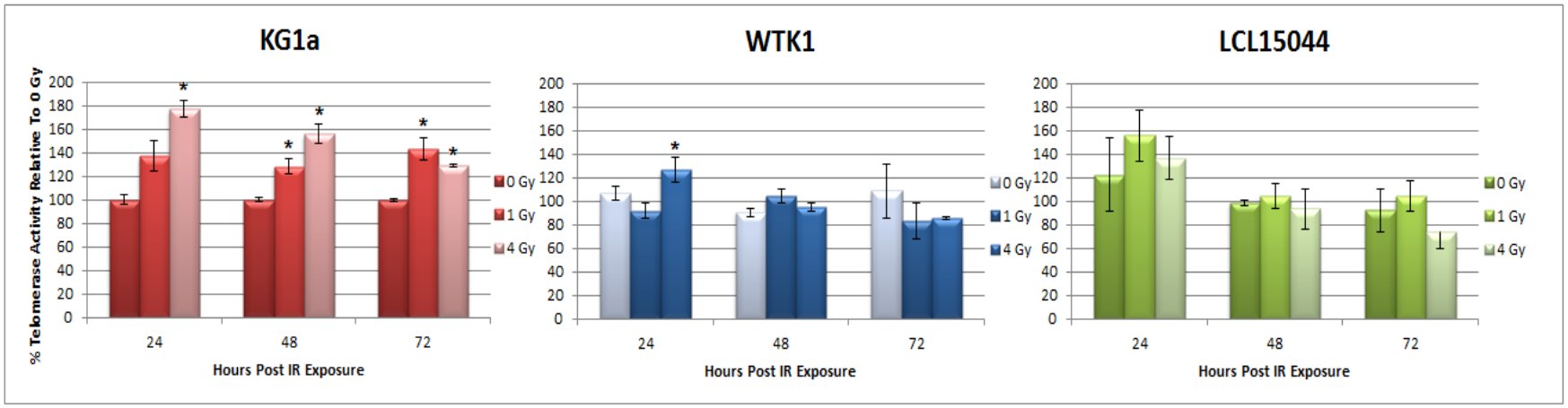

Figure 2.3: Telomerase activity in in hematopoietic cells exposed to an acute dose of 1 and 4 Gy γ-rays at various time points post exposure. Values are expressed as the percentage relative telomerase activity compared to an unirradiated control in KG1a acute myeloid leukemia cells (left panel), WTK1 non-tumor immortalized lymphoblasts (middle panel), and LCL15044 primary immortalized low passage lymphoblast cells (right panel). Stars (*) represent data points which are significantly different from unirradiated controls as determined by student's t-test. Error bars express standard deviation of the mean.

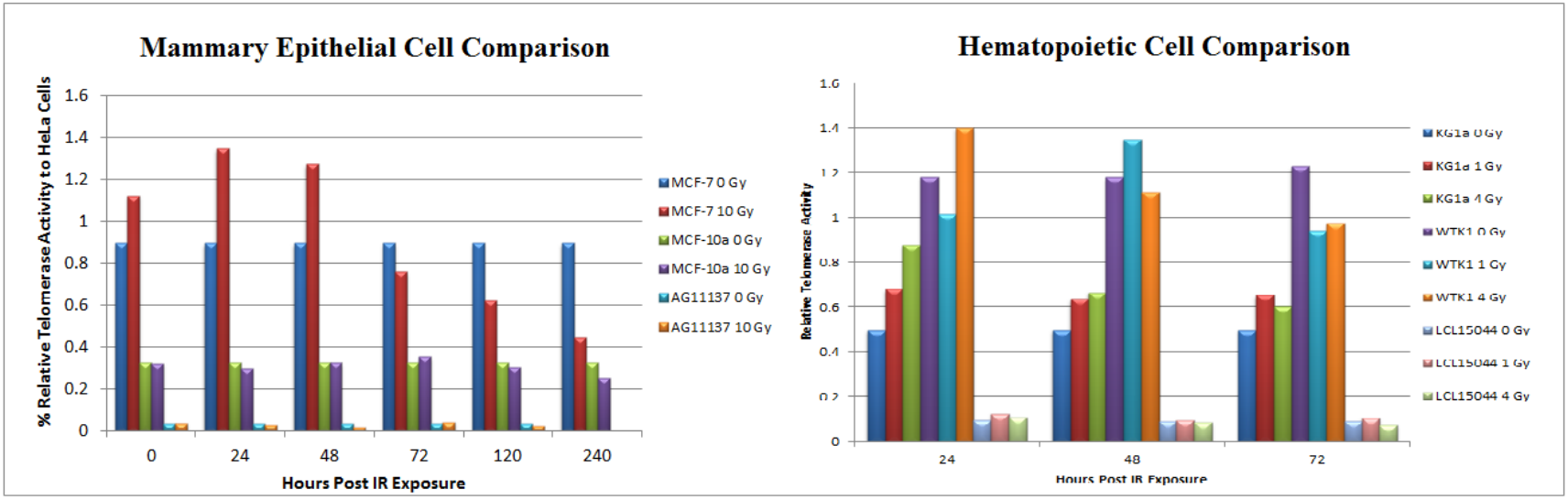

Figure 2.4: Telomerase activity in mammary epithelial cell lines exposed to acute doses of 10 Gy (left panel) and hematopoietic cell lines exposed to 1 or 4 Gy γ-rays (right panel). All data is scaled relative to unirradiated HeLa cell lysates (positive control) to provide an accurate comparison between cell lines and highlight the overall different response of various cell lines exposed to IR.

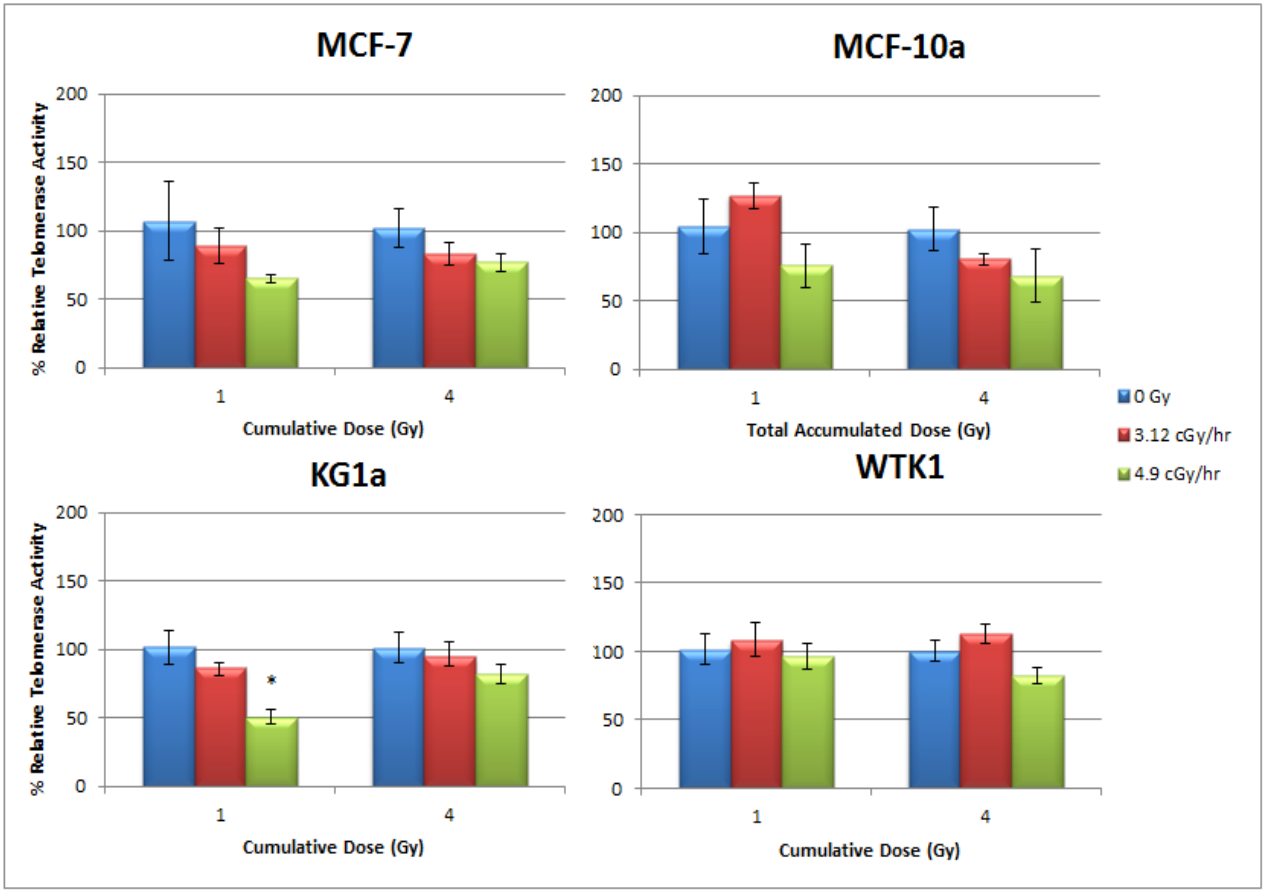

Figure 2.5: Telomerase activity in telomerase positive cell lines exposed to cumulative doses of 1 or 4 Gy γ-rays at dose rates of 0, 3.12, or 4.9 cGy/hr in MCF-7 (top left panel), MCF-10a (top right panel), KG1a (bottom left panel), and WTK1 (bottom right panel). Stars (*) represent data points which are significantly different from un-irradiated controls as determined by student's t-test. Error bars express standard error of the mean.

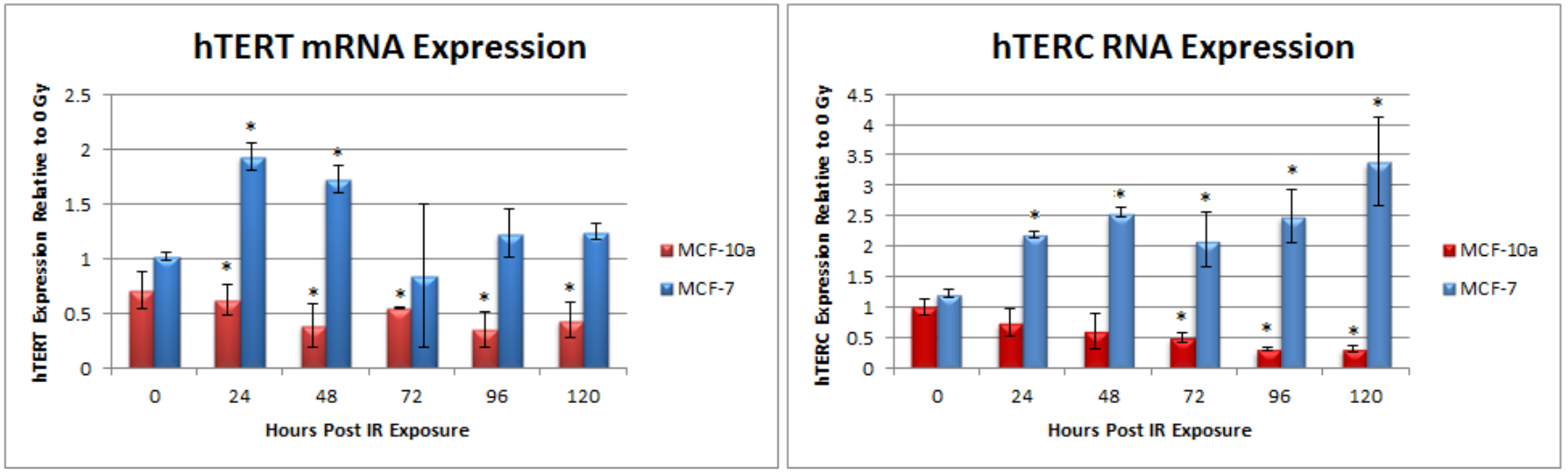

Figure 2.6: Relative changes in hTERT (left panel) and hTERC (right panel) expression in MCF-10a (red bars) and MCF-7 (blue bars) in a five day timecourse a single acute exposure to 10 Gy of γ-rays at a dose rate of 2.5 Gy/min. Values represent expression changes relative to an un-irradiated control samples. Error bars represent standard deviation of the mean and stars (*) represent values which are significantly different than zero hours.

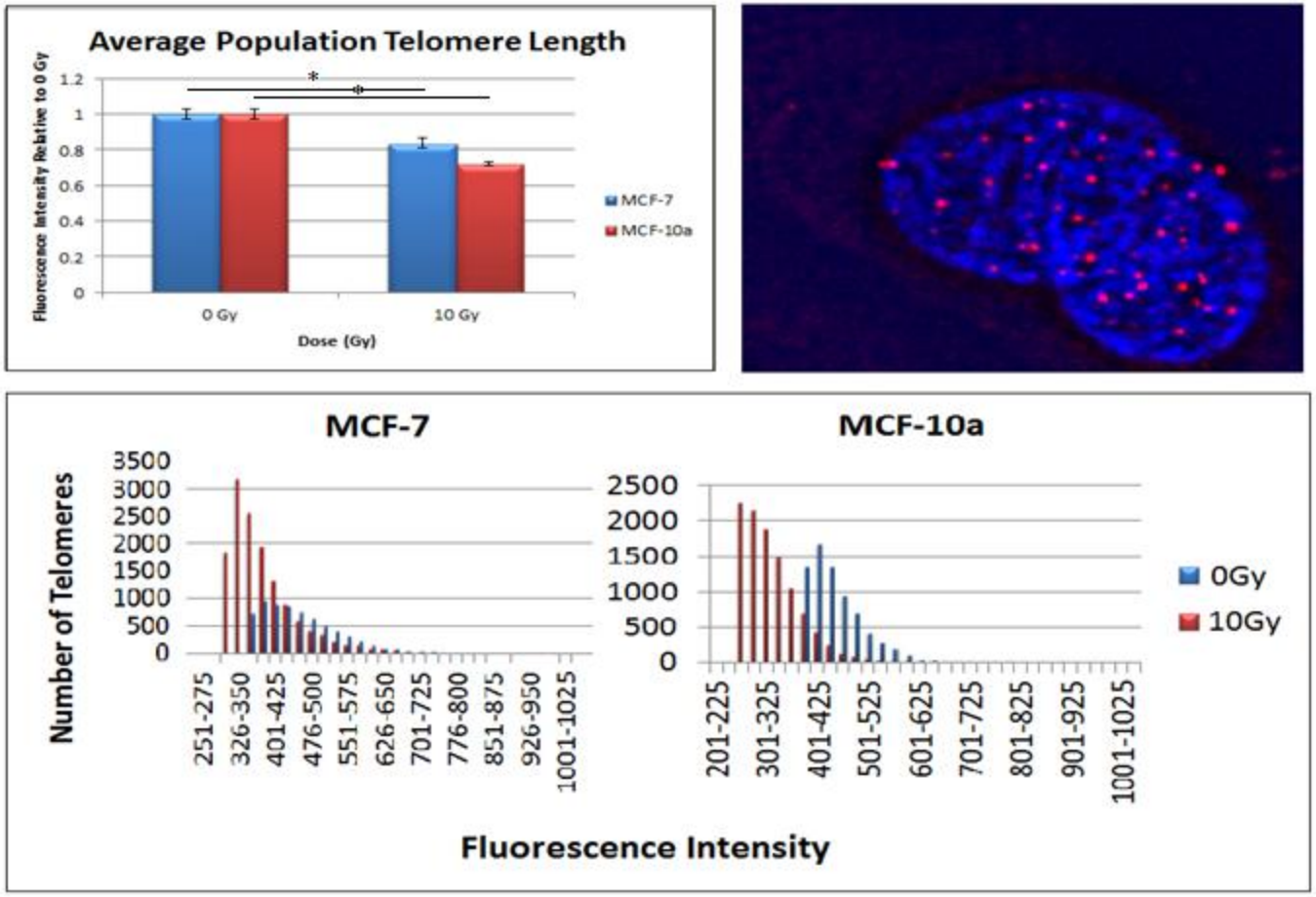

Figure 2.7: Telomere length in MCF-7 and MCF-10a cells following 5 day incubation after a dose of 10 Gy γ-rays. Top left panel represents the average cell population telomere length in both un-irradiated controls and 10 Gy samples. Top right panel is a representative image of interphase nuclei hybridized with a Cy-3 telomere PNA probe utilized to generate telomere fluorescence intensities. Bottom panel is histograms displaying the length of individual telomeres in MCF-7 (bottom left) and MCF-10a (bottom right) cells following a 5 day incubation after 0 Gy (blue bars) or 10 Gy (red bars) exposures. Stars (*) represent values significantly different than unirradiated controls as determined by Student's t-test.

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CHAPTER 3

TELOMERASE REVERSE TRANSCRIPTASE ACTIVITY PROMOTES RADIATION INDUCED CANCER STEM CELL ENRICHMENT

Summary

Cancer stem cells (CSCs) are a sub-population of highly clongoenic cells in human tumors that possess the embryonic stem cell (ES) like properties of self-renewal and the ability to differentiate along many lineages. CSCs are believed to be more radio resistant than their non-stem cancer cell counter parts (NSCCs) and contribute to accelerated tumor repopulation following radiation therapy. Recent reports have shown that ionizing radiation (IR) exposure enriches putative CSCs in established human cancer cell lines. As telomerase plays a critical role in the maintenance of adult stem cells (ASCs), and we previously demonstrated that telomerase activity was elevated in human cancer cell lines following IR exposure, we reasoned that telomerase may promote radiation induced putative stem cell enrichment. In the studies presented here, we confirm previous reports of putative CSC enrichment in mammary carcinoma cells, and demonstrate CSC enrichment in non-cancer cell lines MCF-10a, WTK1, and LCL15044. Utilizing MST-312, a small molecule inhibitor of telomerase activity, we were also able to confirm that telomerase activity is critical for the enrichment of CSCs following IR exposure in telomerase positive breast cancer and non-cancer cell lines. Telomerase was not necessary for IR induced CSC enrichment in telomerase negative cancers. Taken together these findings imply that telomerase isn't just for elongating telomeres anymore, and may play a more substantial role in carcinogenesis and tumor repopulation following radiation therapy than previously acknowledged.

Introduction

It has been previously demonstrated that almost all solid tumors and leukemias contain a subpopulation of primitive, highly clonogenic cells with self-renewing capability and the ability to differentiate along multiple lineages (Ailles & Weissman, 2007). When transplanted into immunodeficient mice, these cells were able to form tumors at much lower limiting dilutions than their more differentiated counterparts indicating a much higher invasive capacity (Bonfanti, Barrandon, & Cossu, 2012; Chen et al., 2013). This sub population, regarded as cancer stem cells (CSCs), is thought be the driver of tumor progression, metastasis, tumor recurrence, and resistance to radiation and chemotherapy (K. Ogawa et al., 2013); thus, much effort has been allocated to understanding CSCs and identifying pathways that can be utilized to therapeutically target them.

Ionizing radiation (IR) is commonly used to treat solid tumors due to its ability to selectively and effectively kill dividing cells (Hall, 2006). CSCs have long been regarded as more radio resistant than their more differentiated non-stem cancer cell (NSCC) counterparts. Proposed reasons for CSC increased radio resistance include: slower cycling times, enhanced DNA repair kinetics, elevated defenses against reactive oxygen species (ROS), and occupation of hypoxic tumor regions (K. Ogawa et al., 2013). Recently, experiments demonstrating the plasticity of cellular differentiation status with somatic cell nuclear transfer (SCNT) (Tachibana et al., 2013) and transcription factor mediated nuclear reprogramming (Takahashi et al., 2007; Zaehres & Scholer, 2007) indicate an ability to interconvert between cellular niches, specifically CSCs and NSCCs. Because of this, it can be inferred that CSC radio resistance is a transient cellular property governed by reprogramming factors and differentiation status rather than intrinsic to a specific cell population.

It has been demonstrated that therapeutically relevant doses of IR induce the enrichment of CSCs in established human tumor cell lines including mammary carcinoma (Phillips et al., 2006), colorectal cancer (Cui et al., 2011), and lung cancer (O'Flaherty et al., 2012). It was originally believed that such stem cell enrichment was the result of selection of the radio resistant CSC population surviving and dividing to repopulate the tumor. However, it has recently been shown by Lagadeck et. al. that IR can induce reprogramming (inter-conversion) of NSCCs into CSCs in mammary carcinoma cell lines (Lagadec et al., 2012). Furthermore, Hei et al. demonstrated that reprogramming occurs at background levels to maintain an equilibrium percentage of CSC's and that IR accelerated the inter-conversion kinetics (Yang et al., 2012). Thus, both selection of radio resistant CSCs and reprogramming of NSCCs into CSCs are likely contributing to stem cell enrichment post IR exposure. Importantly, CSC enrichment has been linked to accelerated tumor repopulation following radiation therapy (K. Ogawa et al., 2013; Trott, 1994). Gaining a deeper understanding of the molecular mechanisms involved in CSC enrichment following IR could therefore provide novel therapeutic targets with the potential to improve the efficacy of radiation therapy.

Telomerase activity is required for maintaining telomere length and prolonging cellular lifespan in embryonic stem (ES) and adult stem cell (ASC) populations, and is also high in NSCCs (Shay & Wright, 2011). Interestingly, cancer cell lines that have been enriched for CSC populations by way of addition of defined growth factors (sphere forming assay) have been shown to have higher telomerase activity than cultures with much lower percentages of CSCs (Karimi-Busheri et al., 2010). This has led us to hypothesize that previously reported elevations in telomerase activity following acute IR exposure in cancer cell lines may be the result of CSC enrichment. To test this hypothesis we analyzed putative cancer stem cell populations in

mammary epithelial cell lines ($CD44^+/CD24^{low/-}$), in hematopoietic cell lines ($CD34^+$ or $CD34^+/CD38^-$), and osteosarcoma cell lines ($CD44^+/CD133^+$) following IR exposure. Further, we employed a small molecule inhibitor of telomerase activity to evaluate a potential role for telomerase in radiation induced stem cell enrichment.

Results suggest that radiation induced stem cell enrichment is a general phenomenon applying to mammary epithelial, hematopoietic, and osteosarcoma cell lines. Additionally, and to the best of our knowledge, we are the first to report $CD44^+/CD24^{low/-}$ CSC cell enrichment in the non-tumorigenic MCF-10a cell line, cells that upon reprogramming have been shown to lead to tumor formation and metastasis when injected into immunodeficient mice. Furthermore, elevation of telomerase activity appears to precede cancer stem cell enrichment in MCF-7 adenocarcinoma cells. It was inversely associated with stem cell populations in KG1a acute myeloid leukemia (AML) cells. Enrichment occurred in non-tumorigenic cell lines (MCF-10a, LCL15044, and WTK1) despite no significant changes in telomerase activity with IR, as well as in osteosarcoma cell lines U20S and SAOS2 which maintain telomeres using the telomerase independent alternative lengthening of telomeres (ALT) pathway. Additionally, small molecule inhibition of telomerase activity blocked radiation induced stem cell enrichment in both MCF-7 and MCF-10a cells despite the later having no detectable increases in telomerase activity associated with IR exposure. Additionally, small molecule inhibition of Tankyrase 1 (TNKS1) and DNA dependent protein kinase catalytic subunit (DNA-PKcs), important players in regulation of telomere structure and function, had no discernable effect on stem cell enrichment following IR.

We found that elevated telomerase activity post IR exposure temporally preceded CSC enrichment and so did not simply reflect mobilization of stem cells following insult. However,

small molecule inhibition of telomerase activity demonstrated that it is necessary for radiation induced stem cell enrichment following IR exposure in telomerase positive backgrounds. Thus, our results support telomerase inhibition as a potentially effective strategy to prevent radiotherapy induced CSC enrichment and reduce tumor recurrence.

Materials and Methods

Cell Culture

The human mammary epithelial non-tumorigenic cell line MCF-10a was purchased from ATCC and was cultured as described previously (Le et al., 2013) in 1:1 Dulbecco's Modified Essential Medium (D-MEM)/Ham's F12 growth medium (Hyclone) supplemented with 5% fetal bovine serum (FBS), 10 µg/mL insulin (Sigma), 20 ng/mL epidermal growth factor (EGF; Sigma), 0.5 µg/mL hydrocortisone (Sigma), 0.1 µg/mL cholera toxin (Sigma), and 1% glutamax (Life Technologies). The human mammary carcinoma cell line MCF-7 (kind gift from L. Chubb, CSU Animal Cancer Center) was grown in D-MEM supplemented with 10% FBS and 1% glutamax. The primary mammary epithelial cell line AG11137 (Coriell) was grown in MCDB 170 complete growth medium (US biological) supplemented with 5 µg/mL insulin, 10 ng/mL EGF, 0.5 µg/mL hydrocortisone, 56 µg/mL bovine pituitary extract (Life Technologies), and 1% glutamax. A human primary immortalized, low passage lymphoblast cell line (LCL15044 kind gift from A. Sigurdsson, National Institute of Health) was grown in RPMI medium supplemented with 15% FBS and 1% glutamax. The WTK1 immortalized lymphoblast cell line (kind gift from H. Liber, Colorado State University) was grown in RPMI medium supplemented with 10% fetal horse serum and 1% glutamax. Human acute myeloid leukemia cells KG1a (Kind gift from Michelle LeBeau, University of Chicago) were grown in RPMI media supplemented 20% FBS

and 1% Glutamax. The human osteosarcoma ALT cell lines U2OS and SAOS2 (kind gift from D. Gustafson Colorado State University Animal Cancer Center) were grown in McCoy's 5A growth medium (Life Technologies) supplemented with 10% FBS and 1% glutamax. All cells were grown at in a humidified incubator at $37^{O}C$ in 5% CO_2 and passaged 1-2 times per week. Cells were grown at $37^{o}C$ in a humidified incubator at 5% CO_2 passaged 1-2 times per week.

Irradiation

Cells were exposed to various, acute doses of ^{137}Cs γ-rays in a Mark I irradiator (J.L. Shepherd) located at Colorado State University. Cells were exposed at a dose rate of 2.5 Gy/min with rotation.

Clonogenic Cell Survival

Cells were seeded at ~50% density in T-25 culture flasks 48 hrs prior to irradiation. Following irradiation at doses of 0, 1, 2, 4, 8, and 10 Gy of γ-rays, cells were trypsinized, resuspended at low density, and plated to allow for 30-50 colonies in 30 mm cell culture dishes (TPP Cell Culture). Cells were grown for ~10 population doublings (10-14 days), fixed using 100% ethanol, and stained using 5% crystal violet (Sigma). Colonies were scored based on the presence of 50 or more cells/colony and scored independently by two individuals.

Flow Cytometry

Human mammary epithelial CSCs were identified based upon expression of $CD44^{+}/CD24^{low/-}$ surface markers (Phillips et al., 2006; Sheridan et al., 2006; Sun et al., 2012). CSCs from human hematopoietic cell lines were identified based upon expression of $CD34^{+}/CD38^{low/-}$ or $CD34^{+}$ expression alone (Bonnet & Dick, 1997). CSCs in human

osteosarcoma cell lines were identified based on expression of $CD133^{+}/CD44^{+}$ (L. Yu et al., 2013). All analyses were performed on a CyAn ADP Analyzer with 9-color capability (Beckman Coulter CY20130) located at the Colorado State University Veterinary Teaching Hospital.

Staining for cancer stem cell markers

Monolayers MCF-7 and MCF-10a mammary epithelial cells were trypsinized and stained for CD44 and CD24 expression as described previously (Lagadec et al., 2012; Lagadec et al., 2010; Sheridan et al., 2006). Briefly, ~300,000 cells were dissociated from cell culture surface using 0.25% Trypsin-EDTA, pelleted, washed, and resuspended in 30 uL of Flow Cytometry wash buffer (1X PBS, 1% FBS, and 1% Penicillin/Streptomyocin). 6 µL of direct FITC conjugated mouse monoclonal anti-human CD44 antibody (BD Pharmingen #555478) and 6 µL of direct PE conjugated mouse monoclonal anti-human CD24 antibody (BD Pharmingen #555428). Cells were then incubated for 30-60 min in the dark at $4^{O}C$. Following incubation cells were pelleted and resuspended in 500 µl of cold 1X PBS and kept on ice until analysis. Analysis gates were established using cells from unstained controls and anti-mouse Ig,κ antibody capture beads (BD Pharmingen #552843). For lymphoblastoid suspension cultures, cells were stained as above with 6 µL of direct PE conjugated mouse monoclonal anti-human CD34 antibody (BD Pharmingen #555822) and 6 µL of direct FITC conjugated mouse monoclonal anti-human CD38 antibody (BD Pharmingen #560982). Assessment of CSCs in Osteosarcoma cell lines was done as described above using anti-human CD44 antibody and anti-human CD133/2 antibody (Milteny Biotect #293C3-APC).

Quantitative Real Time PCR Based Telomere Repeat Amplification Protocol (TRAP)

Telomerase activity was detected via the telomere repeat amplification protocol (TRAP) assay originally described by Herbert (Herbert et al., 2006) et al and adapted for real time PCR by Hou et al (Hou et al., 2001). Briefly, whole cell lysates were prepared from cultured cell pellets, lysed in cold MPER mammalian protein extraction buffer (Thermo Fischer) containing a protease inhibitor cocktail (Roche) and RNasin ribonuclease inhibitor (Promega) at a ratio of 100 µl of buffer per 1,000,000 cells. Lysates were cleared by centrifugation at 14,000 RPM for 10 minutes at 4^{o}C, aloiquotted, and stored at -80^{o}C. Protein concentration was determined using the Bradford Assay (Biorad).

The SYBR green master mix (Promega) included all necessary components to complete the RTQ-PCR reaction. Each well contained between 0.1 and 0.25µg of protein lysate, 50% volume of SYBR green master mix, 0.2µg T4 gene32 protein (New England Biolabs, Ipswitch, MA), 0.1µg of each primer TS (5'-AATCCGTCGAGCAGAGTT- 3') and ACX (5'-GCGCGG(CTTACC)3CTAACC-3') (Integrated DNA Technologies) and RNase/DNase free water to achieve a final well volume of 25ul. The PCR and detection were performed on a CFX 96 (Biorad). In addition to the treatment samples, a series of controls were also included on each plate: (1) no template control with TS primer only, (2) no template control with ACX primer only, (3) no template control with TS and ACX primers (used in normalization of samples), (4) heat inactivated control with template (protein lysate) and TS and ACX primers, (5) HeLa cell lysate with TS and ACX primers (a positive control with robust telomerase activity).

The RTQ-PCR program includes the following steps: Step 1- 1cycle 25℃ 20 min (telomerase elongates the TS primer by adding TTAGGG repeat sequences); Step 2- 1cycle 95℃ 3 min (heat activation of the enzyme in the SYBR master mix); Step 3- 40 cycles of 95℃ for 20 sec, 50℃ for

30 sec and 72℃ for 1min 30 sec (PCR amplification allows for detection by real time instrument); Step 4- 80 cycles 0.10 sec per cycle (melt curve to ensures no primer dimer formation). Each sample is run in triplicate on a 96 well plate format allowing for an average Ct to be obtained per sample. Utilizing the average Ct value, the relative percent telomerase activity in each sample is calculated using the Delta Delta Ct method ($2^{-\Delta\Delta CT}$) (Livak & Schmittgen, 2001). Briefly, to calculate the percent relative activity for each sample first normalize the average sample Ct to the no template control with TS and ACX primers. This is referred to as the delta Ct value. The delta Ct value of each sample is subtracted from the delta Ct value of a chosen comparative sample, in this case a normal feline mucous membrane cell lysate, yielding a delta delta Ct value ($\Delta\Delta$Ct). Using the $2^{-\Delta\Delta CT}$, a relative value is generated for each sample comparison and when multiplied by 100 is the relative percent of telomerase activity (RTA) of the sample compared to the normal feline mucous membrane. The RTA can be compared between samples assayed across different plates. Results from two runs were averaged.

Small Molecule Inhibiton

Inhibition of telomerase activity was accomplished through the use of MST-312 (Sigma) also known as telomerase inhibitor IX (Seimiya et al., 2002). Inhibition of tankyrase 1 (TNKS1) was accomplished through the use of inhibitors XAV-939 (ToCris) and IWR-1 (Sigma) (Bao et al., 2012; Narwal, Venkannagari, & Lehtio, 2012). Nu7026 (Sigma) was used to inhibit DNA-PKcs kinase activity (Le et al., 2013). Briefly, all inhibitors were solubilized at concentrations recommended by the manufacturer in sterile DMSO, aloiquotted, and stored at -20^{O}C for no more than 1 month prior to use. MST-312 was added to cultures 6 hours prior to experimentation whereas XAV-939 and IWR-1 were added to culture 24 hours prior to experimentation.

Thiazolyl Blue Tetrazolium Bromide (MTT) Assay

Potential cytotoxic effects of the telomerase inhibitor MST-312 were determined through the use of the MTT assay as described previously (Bes et al., 2004). Briefly 2000 cells per well of a 96-well plate were seeded 24 hours prior to addition of inhibitor. Media was removed from wells and fresh media containing varying concentrations of MST-312 or an equivalent DMSO control were added to all wells. Cells were incubated in the presence of inhibitor for 48 and 72 hours. At the time of analysis, media was once again removed from wells, and cells were resuspended in fresh media containing 0.5 mg/ml MTT reagent and incubated at $37^{O}C$ for 3.5 hours. Following incubation, media was removed and 150 µl of MTT solvent (4mM HCl, 0.1% NP-40, all in isopropanol) was added to each well and set to agitate on a shaker at room temperature for 15 min. After agitation, plates were read on a Modulus Microplate reader (Turner Biosystems) at 600 nm absorbance.

Statistical Analyses

Statistical differences were detected using a Student's t-test against relevant controls. Statistical analysis was conducted using the Graph Pad Prism 5 software for windows XP.

Results

Ionizing Radiation Induced Breast Cancer Stem Cell Enrichment

Breast cancer cell populations grown on low adherence plates containing selective media form structures known as mammospheres (Joseph et al., 2010; Karimi-Busheri et al., 2010; Xie et al., 2012). Mammospheres are enriched for $CD44^{+}/CD24^{low/-}$ breast CSCs and have been shown to have greater telomerase activity than the same cells grown in monolayer (Phillips et al.,

2006) supporting breast CSCs having higher telomerase activity than NSCCs. Further, ionizing radiation has been shown to enrich CSC population in monolayer cultures of established cancer cell lines for breast (Al-Hajj et al., 2003), lung (O'Flaherty et al., 2012), colon (Yang et al., 2012), and glioblastoma (Hale, Sinyuk, Rich, & Lathia, 2013). Such findings led us to originally hypothesize that the elevated telomerase activity following exposure to acute doses of ionizing radiation, might result from CSC enrichment following IR exposure.

Consistent with previous reports, breast cancer stem cells were enriched in MCF-7 cell populations at 5 days post exposure (Figure 3.1). Unexpectedly, we also observed a significant increase in CSCs in MCF-10a cells at 5 days post IR exposure. To our knowledge, we are the first to report this finding and it suggests that IR induced CSC enrichment may play a role in the transformation of non-tumor tissues. In earlier studies, we demonstrated that telomerase activity was significantly elevated within MCF-7 cells in the first 48 hours post IR exposure, and that by 120 hrs, telomerase activity had dropped below levels observed in un-irradiated cells. Therefore, at least in MCF-7 cells, stem cell enrichment (occurring at 120 hours post IR exposure) does not seem to temporally correlate with changes in telomerase activity. Furthermore, CSC enrichment was also observed in MCF-10a cells, which did not exhibit a significant change in telomerase activity following IR exposure. Thus, it appears as though our original hypothesis was incorrect, in that fluctuations in telomerase activity post IR (occurs 24-48 hr) do not appear to be temporally associated with enrichment of breast cancer stem cell populations (occurring at 120 hr).

To further characterize breast cancer stem cell enrichment in MCF-10a cells, we tracked stem cell enrichment relative to un-irradiated controls for 10 days post exposure to 10 Gy of γ-rays (Figure 3.3 top panel). These results indicated that enrichment peaked at 120 hours and

steadily declined thereafter, returning to background levels 168 hr after exposure. We also examined potential dose dependence of CSC enrichment in MCF-10a cells, as a threshold dose had been previously demonstrated in breast cancer cell lines (Lagadec et al., 2010). Stem cell enrichment did not occur at doses of 5 Gy and below, but a dose response was detected at 10 and 20 Gy (Figure 3.3 bottom panel). Therefore, a threshold dose necessary to trigger CSC enrichment is between 5 and 10 Gy (γ-rays) and beyond that threshold, increased dose appears to enrich CSC populations in a dose dependent manner.

Potential Role of IR Induced CSC Enrichment in Tumor Repopulation Following Radiation Therapy

It has been suggested that CSCs play a crucial role in the accelerated repopulation of tumors following radiation therapy (Trott, 1994). Originally, it was believed that populations of radio-resistant CSCs existing in a tumor were better to able to survive radiation therapy, and once mobilized through accelerated asymmetric cell division repopulated the NSCCs and the bulk tumor (Butof et al., 2013; K. Ogawa et al., 2013). However, recent experimental evidence suggesting plasticity of the stem cell niche, including successful nuclear reprogramming (Takahashi et al., 2007), somatic cell nuclear transfer (Tachibana et al., 2013) of human cells, and more recently radiation induced reprogramming of NSCC into CSCs (Lagadec et al., 2012; Yang et al., 2012), have raised a critical question: is cancer stem cell enrichment following radiation therapy a result of selecting for existing CSCs or a result of the NSCCs being converted into CSCs?

Experimentally this is a very difficult question to answer, however we examined growth kinetics of both MCF-7 and MCF-10a cells following exposure to 10 Gy of γ-rays to tease out an answer. We first performed a clonogenic survival assay to determine the fraction of cells

surviving radiation at an acute dose of 10 Gy (Figure 3.2 top panel). In the case of both the MCF-7 and MCF-10a cells, survival was below 1% suggesting that 10 Gy reproductively killed most of the cells in the culture. However, despite few surviving cells, the cell populations continued to divide for at least 5 days, although at a much slower rate in both cases (Figure 3.2 middle panel). From this, it could be assumed that surviving cells (presumably the existing CSCs) are repopulating the tumor in a process akin to selection. However, cell cycle analysis using propidium iodide (PI) staining and flow cytometry in MCF-10a cells, revealed very few cycling cells at later time points, suggesting CSC enrichment is resulting from inter-conversion or reprogramming rather than selection (Figure 3.2 bottom panel). Further experimentation is necessary to understand this complex relationship, however contrasting results suggest that both selection and reprogramming play a role in IR induced stem cell enrichment.

IR Induced CSC Enrichment in Hematopoietic Cell Lines

To determine if IR induced stem cell enrichment was a phenomenon unique to solid tumor cell lines, we tracked the proportion of $CD34^+$ cells in immortalized primary (LCL15044) and p53 mutated (WTK1) lymphoblastoid cell lines. CD34 is a classical marker of primitive hematopoietic stem cells and is commonly associated with CSCs in leukemia and lymphoma cell lines and exists at low levels in differentiated lymphocytes (Bonnet & Dick, 1997; Calloni, Cordero, Henriques, & Bonatto, 2013). We exposed suspension lymphoblast cultures to acute doses of 1 or 4 Gy γ-rays and tracked $CD34^+$ cell populations for up to 120 hours post IR exposure (Figure 3.4). Both the WTK1 and LCL15044 cell lines displayed a dose and time dependent increase in the percentage of $CD34^+$ cells (Figure 3.4 top and middle panels). This increase in CD34+ cells is important because, like is observed with MCF-10a cells, it suggests an

IR induced enrichment of more primitive cell populations in non-cancer cell lines; a finding with important implications for carcinogenesis and in particular secondary cancers following radiation therapy.

We also examined the dynamics of a $CD34^{+}/CD38^{-}$ CSC population in KG1a acute myeloid leukemia (AML) cells following acute exposures to 1 and 4 Gy up to 72 hours post IR (Figure 3.4 bottom panel). Interestingly, CSC percentages in this cell line were decreased in a dose dependent manner. This was the only observed exception to IR induced stem cell enrichment observed in all other cell lines. One possible explanation is that KG1a is therapy induced leukemia, and therefore may respond very differently to IR (Mrozek, Tanner, Heinonen, & Bloomfield, 2003). Additionally, as KG1a has a relatively high background population of CSCs, it is possible that the CSC population is more heterogeneous in its radiation sensitivity. Taken together with the studies conducted using breast cancer stem cell markers, we can conclude that changes in telomerase activity do not correspond with increases in CSC populations.

Telomerase Reverse Transcriptase Activity in IR Induced CSC Enrichment

As significant elevation of telomerase activity preceded enrichment of CSC populations in irradiated MCF-7 cultures, we hypothesized that telomerase activity is necessary for enrichment to occur following IR. To test this hypothesis we utilized the small molecule inhibitor MST-312, which non-covalently binds to the reverse transcriptase domain of the telomerase catalytic subunit effectively blocking its activity (Seimiya et al., 2002). MST-312 effectively reduced telomerase activity as verified by TRAP assay at 1 μM doses in MCF-10a cells (Figure 3.5 top right panel) and at 5 μM doses in MCF-7 cells (Figure 3.5 top left panel).

Cytotoxicity of MST-312 was determined via MTT assay. It was determined that future experiments would be conducted with both 1 and 3 μM doses in MCF-7 cells, and at a 1 μM dose in MCF-10a cells to avoid complications from cytotoxicity (Figure 3.5 middle panel). Essentially doses of MST-312 were chosen that gave minimal cytotoxicity while still achieving a reasonable level of telomerase inhibition. MST-312 was added to cultures 6 hours prior to irradiation to allow for binding and inhibition to occur. Cultures were then irradiated with 10 Gy of γ-rays, incubated for 120 hr and analyzed for the presence of $CD44^{+}/CD24^{low/-}$ breast CSC populations (Figure 3.5 bottom panel). Doses of MST-312 (1 μM) effectively blocked radiation induced CSC enrichment in MCF-10a and MCF-7 (3 μM) cells. This is consistent with the much higher background levels of telomerase activity in MCF-7 cells as compared to MCF-10a. MST-312 did not appear to effect background levels of CSCs in either cell line examined. These findings suggest that telomerase activity is necessary for CSC enrichment following IR.

To further evaluate the role of telomerase in IR induced stem cell enrichment, we determined whether IR induced ($CD44^{+}/CD133^{+}$) putative CSC enrichment in the U2OS and SAOS2 osteosarcoma cell lines which maintain their telomere length via the ALT pathway (L. Yu et al., 2013). To our surprise, both osteosarcoma cell lines displayed a significant increase in CSC percentages at 5 days post exposure to 15 Gy (Figure 3.6 top panel). A dose of 15 Gy was chosen in this case, as previous reports suggested that osteosarcoma cells are more radio-resistant than carcinomas. These findings suggest that more than telomerase is at play in this finding and other pathways may be acting in accordance or substituting for the lack of telomerase activity in ALT cell lines. Thus, despite the fact that telomerase inhibition prevented IR induced CSC enrichment in telomerase positive cancers, it does not appear to be required for that same enrichment in a telomerase negative background, indicating that a more complex role is in play.

Inhibition of Telomerase, Not Other Telomere Related Pathways Prevents IR Induced Stem Cell Enrichment

To determine if blocking telomerase activity prevented IR induced stem cell enrichment through direct loss of telomerase activity or general effects on telomere structure, we repeated previous experiments using inhibitors known to affect telomere structure. TNKS1 is a poly ADP-ribose polymerase which post-translationally modifies proteins (PARsylation) by adding chains of NAD^+ groups that affect binding properties (S. M. Huang et al., 2009; Smith et al., 1998). TNKS1 PARsylates the telomere repeat binding factor 1 (TRF1), leading to TRF1 dissociation from the telomere chromatin structure and facilitating access by telomerase (Hsiao & Smith, 2008). Loss of TNKS1 leads to telomere shortening and instability (Smith & de Lange, 2000). DNA-PKcs kinase activity is also critical for appropriate telomere end capping function, as its loss results in telomere telomere end fusions and telomere-double strand break fusions (Bailey, Brenneman, et al., 2004; Fabre et al., 2011).

We utilized small molecule inhibitors directed against TNKS1 (XAV-939 and IWR-1) and DNA-PKcs (Nu7026) to induce telomere dysfunction in MCF-10a cells. Cells were treated with inhibitors for 24 hr (XAV-939 and IWR-1) and 6 hours (Nu7026) prior to irradiation and evaulated for $CD44^+/CD24^{low/-}$ stem cell populations 5 days post IR (Figure 3.7). None of these reagents were effective at blocking radiation induced CSC enrichment. Interestingly, XAV-939 and IWR-1 treatment appeared to significantly elevate background levels of CSCs in the cell population, while treatment with Nu7026 significantly decreased background levels. These findings present new avenues to pursue in ongoing future research.

Discussion

Cancer stem cells are thought to drive carcinogenesis and underlie solid tumor recurrence following treatment (Butof et al., 2013). This concept has promoted a great deal of research to better understand and target existing CSCs in order to improve clinical outcomes (Chen et al., 2013). Foremost among such examples would be the successful conversion of non-stem human cells into pluripotent stem cells through the processes of transcription factor mediated nuclear reprogramming or somatic cell nuclear transfer. These studies highlighted cellular plasticity and fluidity of the stem cell niche in both cancer and normal tissues, and supported the potential to contribute to carcinogenesis and tumor repopulation following radio therapy.

Here, we sought to establish a causative link between telomerase, the reverse transcriptase critical in maintaining the human ASC niche, and cellular plasticity-specifically the enrichment of CSCs following radiation therapy. We examined a population of $CD44^{+}/CD24^{low/-}$ cells that have been identified as putative breast CSCs in human tumors, and exhibit the ability to self-renew and differentiate along multiple lineages (Al-Hajj et al., 2003; Fillmore & Kuperwasser, 2008). It has been shown that a population of breast CSCs exists in established cultured tumor cell lines (Fillmore & Kuperwasser, 2008; Ponti et al., 2005), and further that exposure to IR significantly enriched this population (Lagadec et al., 2012; Lagadec et al., 2010; Phillips et al., 2006). Consistent with previous reports, we observed a significant elevation of $CD44^{+}/CD24^{low/-}$ breast CSCs in the MCF-7 mammary epithelial line at 5 days post exposure to IR. As $CD44^{+}/CD24^{low/-}$ cells require much lower limiting dilution to form tumors in mice (Ponti et al., 2005), and exposure to IR enriches this population, it is highly likely that clongoenic capacity is increased and tumor repopulation accelerated following therapy.

To our knowledge, we are the first to report an enrichment of $CD44^{+}/CD24^{low/-}$ cells in the non-tumorigenic MCF-10a cell line (Figure 3.1). A population of $CD44^{+}/CD24^{low/-}$ cells had previously been documented in this cell line, however it was uncertain whether these breast CSCs possessed the same properties as CSCs from metastatic tumor cultures (Sheridan et al., 2006). MCF-10a cells have been reprogrammed to pluripotency through the introduction of nuclear transcription factors, leading to a homogenous population of $CD44^{+}/CD24^{low/-}$ cells (Nishi et al., 2014). Reprogrammed MCF-10a cells were able to form tumors in nude mice, whereas their more differentiated/non-reprogrammed counterparts were not, indicating that this reprogrammed population did indeed exhibit properties of CSCs. These results have important implications for carcinogenesis, because IR exposure appears to be enriching MCF-10a cells for a population with tumor promoting properties. Further, characterization of MCF-10a stem cell enrichment following IR exposure revealed a peak in $CD44^{+}/CD24^{low/-}$ cells at 120 hours post IR exposure that appeared to have dose dependence above a threshold around 10 Gy.

The lymphoblastoid cell lines WTK1 and LCL15044 also displayed a significant increase in cells expressing the hematopoietic stem cell (HSC) marker CD34 following IR exposure (Figure 3.4). CD34 is a cell surface glycoprotein believed to mediate the attachment of HSCs to the bone marrow extracellular matrix (Simmons, Satterthwaite, Tenen, & Seed, 1992) that is used as a marker for CSCs in various types of leukemia (Calloni et al., 2013). Radiation doses of both 1 and 4 Gy induced stem cell enrichment evidenced by the increased percentage of cells expressing CD34. This finding suggests that radiation induced stem cell enrichment is not restricted to cells of epithelial origin, but that it also presents a carcinogenic risk to other cell types, as it occurs in hematopoietic cells. The only exception to stem cell enrichment following IR was seen in the KG1a cell line, which expresses an about ~20 percent background level of a

previously described $CD34^{+}/CD38^{-}$ population of leukemic CSCs (Hoang, Zepeda-Moreno, & Ho, 2012). Following exposures to both 1 and 4 Gy, KG1a cells displayed a significant decrease in the number of $CD34^{+}/CD38^{-}$ CSCs indicating that radiation selectively eliminated the CSC population.

It has long been assumed that enrichment of CSCs following radiation therapy in human tumors and established cancer cell lines resulted from the more radio resistant CSCs surviving to repopulate the tumor following injury (K. Ogawa et al., 2013). However, it was recently demonstrated that sorted populations of near-pure breast NSCCs exposed to therapeutically relevant doses of IR were reprogrammed into a more stem like state (Lagadec et al., 2012; Yang et al., 2012). This important observation that IR triggers the inter conversion of NSCCs into CSCs, suggests IR-induced CSC enrichment results from more than simply selecting for existing radiation resistant stem cells, and further that the radio-resistant properties CSC exhibit may be a transient cellular property rather than specific cell state. Indeed, when pure populations of sorted breast and colon NSCCs and CSCs were maintained in long term culture, both eventually returned to the background percentages of CSCs identified in unsorted culture (Gupta et al., 2011). Return to this background equilibrium state was accelerated in both directions through exposure to IR, suggesting the activation of pathways that maintain a sort of kinetic homeostasis (Yang et al., 2012). Thus, to effectively eliminate CSCs over the course of radiation therapy, not only must existing CSC populations be targeted, but the inter conversion of NSCC into CSC must also be blocked to most effectively prevent IR induced stem cell enrichment and accelerated tumor repopulation.

Our previous findings (Chapter 2) demonstrated that telomerase activity was elevated in established cancer cell lines following exposure to IR. As telomerase activity was elevated in

breast cancer mammosphere cultures that have elevated levels of $CD44^{+}/CD24^{low/-}$ CSCs (Karimi-Busheri et al., 2010), we originally hypothesized that telomerase activity was elevated due to enrichment of CSC populations. However, in the case of MCF-7 cells, telomerase activity was elevated from 24 to 48 hours, whereas CSC enrichment did not occur until 120 hours post exposure. KG1a cells, which had significantly elevated levels of telomerase activity post IR, displayed decreased levels of CSCs at the same time. Furthermore, the non-cancer cells MCF-10a, WTK1, and LCL15044 had significantly elevated levels of CSC markers in cells exposed to IR, despite a lack of change in telomerase activity. Therefore, CSC enrichment is not associated with increased telomerase activity.

Recent reports linking telomerase to pathways that promote "stemness" including Wnt/β-catenin and NF-κB signaling, and recent reports of hTERT transfection in ALT cells inducing stem cell enrichment (L. Yu et al., 2013) caused us to rethink our hypothesis (Choi et al., 2008; Hoffmeyer et al., 2012; Low & Tergaonkar, 2013; Stower, 2012). Because telomerase activity was elevated prior to the enrichment of breast CSCs, perhaps it was acting to promote "stemness" upstream of CSC enrichment. To test this hypothesis we treated MCF-7 and MCF-10a cells with the telomerase inhibitor MST-312 6 hours prior to irradiation. MST-312 is a small molecule inhibitor of telomerase activity that, blocks its reverse transcriptase activity by non-covalently binding to the elongation cleft of hTERT (Seimiya et al., 2002). Telomerase inhibition effectively blocked CSC enrichment resulting from IR, but did not appear to effect background levels of CSC populations in MCF-7 cells. Interestingly, telomerase inhibition had the same effect in MCF-10a cells despite a lack of telomerase activity increase following IR exposure. Thus, while changes in telomerase activity following IR exposure does not temporally associate with CSC enrichment, telomerase activity is required for IR induced CSC enrichment. These

results suggest that telomerase inhibition can block stem cell enrichment regardless of whether it occurs via selection of surviving CSCs or through reprogramming.

Since telomerase activity was necessary for IR induced CSC enrichment in mammary epithelial cells, we next asked whether CSCs in osteosarcoma cell lines that maintain telomere length through the telomerase independent, ALT pathway were able to be enriched following IR exposure. A population of $CD44^{+}/CD133^{+}$ CSCs were recently described in osteosarcoma cells (L. Yu et al., 2013). Following exposure of the osteosarcoma cell lines U20S and SAOS2 to an acute dose (15 Gy γ-rays), the number of $CD44^{+}/CD133^{+}$ cells in the population 5 days post IR exposure was determined. Interestingly, these cell lines were also enriched for CSCs despite the lack of telomerase activity. Therefore, while telomerase activity may be required for the efficient CSC enrichment in telomerase positive breast cancer cell lines, it is not required for CSC enrichment in telomerase negative, ALT cancer cell lines.

This finding led us to ponder whether the absence of breast CSC enrichedment following IR exposure upon telomerase inhibition was due to lack of telomerase activity or a disruption of telomere structure. Telomerase positive cell lines display accelerated telomere shortening and instability under telomerase inhibition (Bechter, Zou, Walker, Wright, & Shay, 2004). By this reasoning, if enrichment was blocked due to telomere dysfunction rather than lack of telomerase activity in telomerase positive cancer, inhibiting telomerase activity would not induce telomere dysfunction in ALT. To test this hypothesis, we induced telomere dysfunction utilizing small molecule inhibitors directed against TNKS1 and DNA-PKcs, proteins which are important for appropriate telomere maintenance, but not directly related to telomerase. TNKS1 is a PARP that covalently modifies TRF1, a component of shelterin which directly binds telomere double stranded DNA (Hsiao & Smith, 2008). Loss of TNKS1 PARP activity has been shown to induce

telomere shortening independent of telomerase activity (Smith & de Lange, 2000). DNA-PKcs is a protein required for classical DNA double strand break repair via non-homologous end joining (NHEJ) that is also necessary for the appropriate capping function of telomeres (Bailey, Brenneman, et al., 2004). Loss of DNA-PKcs kinase activity leads to telomere-telomere fusions and other markers of telomere instability (Bailey & Goodwin, 2004). Neither inhibition of TNKS1 nor DNA-PKcs prevented IR induced CSC enrichment in MCF-10a cells, supporting the need for telomerase in IR induced enrichment. Interestingly, while TNKS1 inhibition was unable to prevent IR induced CSC enrichment, it significantly elevated background levels of CSCs in MCF-10a cells without irradiation. It is possible that the connection between TNKS1 and TRF1 explains this finding, as TRF1 was recently demonstrated to be necessary for maintenance of the adult stem cell niche (Schneider et al., 2013), however further experimentation must be conducted to establish such a link. Further experimentation is necessary to determine what cells are actively dividing and potentially contributing to IR induced stem cell enrichment. Agent based mathematical modeling as well as future experimentation assessing the fraction of S-phase, apoptotic, and senescent cells will help to determine if IR induced putative stem cell enrichment is a result of the mobilization of existing, and inherently radio-resistant stem cell populations, or resulting from reprogramming.

Throughout the course of this study we have demonstrated IR induced CSC enrichment in various cancer and normal cell lines. These findings highlight a potential opportunity to enhance clinical radiation therapy through the targeting of processes that influence CSC enrichment, thereby slowing or preventing accelerated tumor repopulation. Furthermore, the demonstration of IR induced enrichment of CSC populations in non-tumor cell lines suggests that this same process may also be relevant in the generation of second cancers following

radiation therapy. We have identified telomerase inhibition as an effective candidate in accomplishing such goals. Future studies will seek to more clearly define the role of telomeres and telomerase in IR induced CSC enrichment, as well as identify other pathways that may be effective in eliminating CSCs throughout the course of radiation therapy.

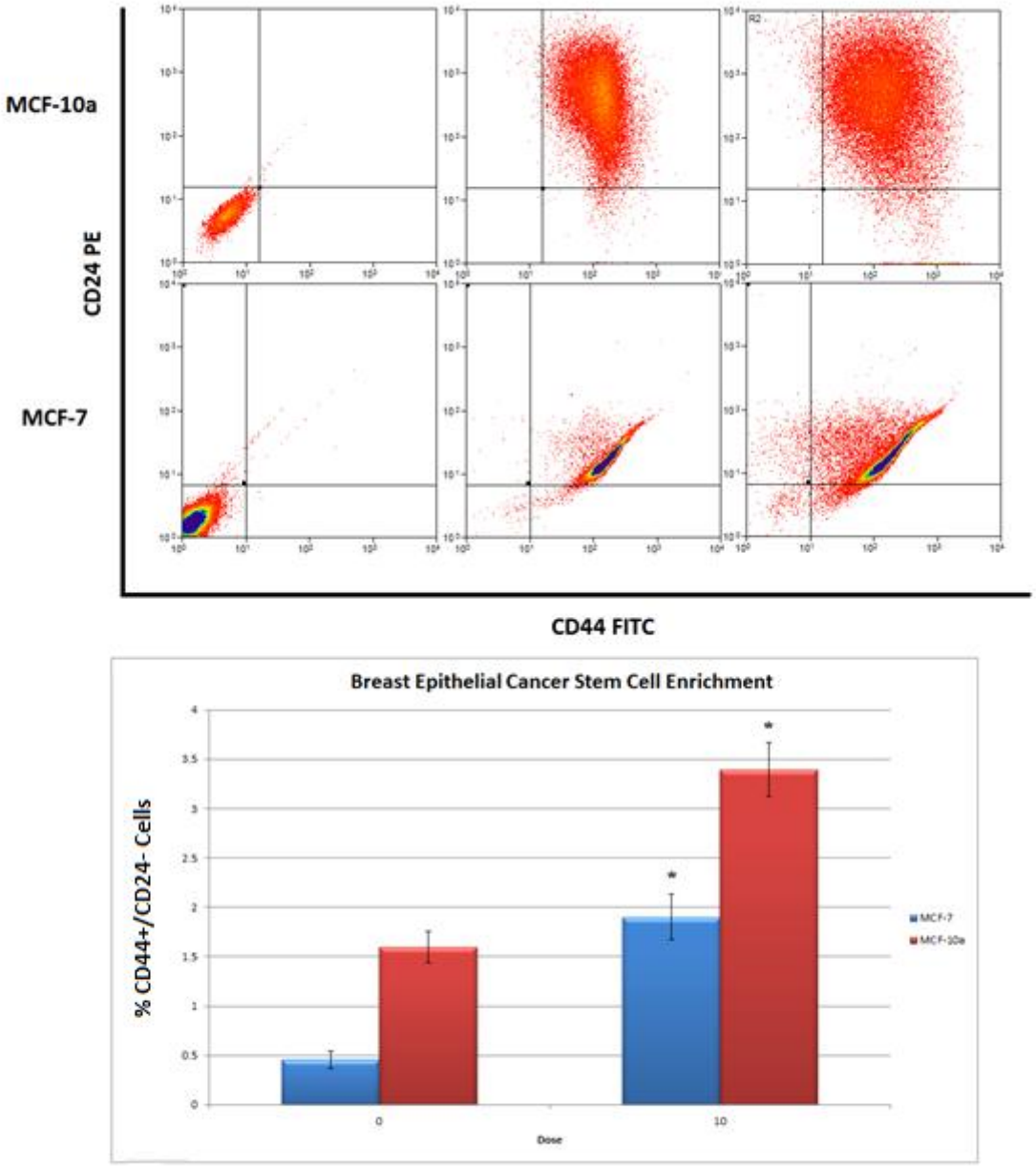

Figure 3.1: Enrichment of $CD44^{+}/CD24^{low/-}$ breast cancer stem cells in MCF-7 and MCF-10a cell lines 5 days post exposure to an acute dose of 10 Gy of γ-rays. Top panel illustrates representative scatter plots generated using flow Cytometry and bottom panel illustrates the absolute change in frequency of the percentage of $CD44^{+}/CD24^{low/-}$ cancer stem cells. Error bars represent the standard deviation of the mean and stars (*) represent values which are significantly different from unirradiated controls within each cell line as determined by a Student's t-test.

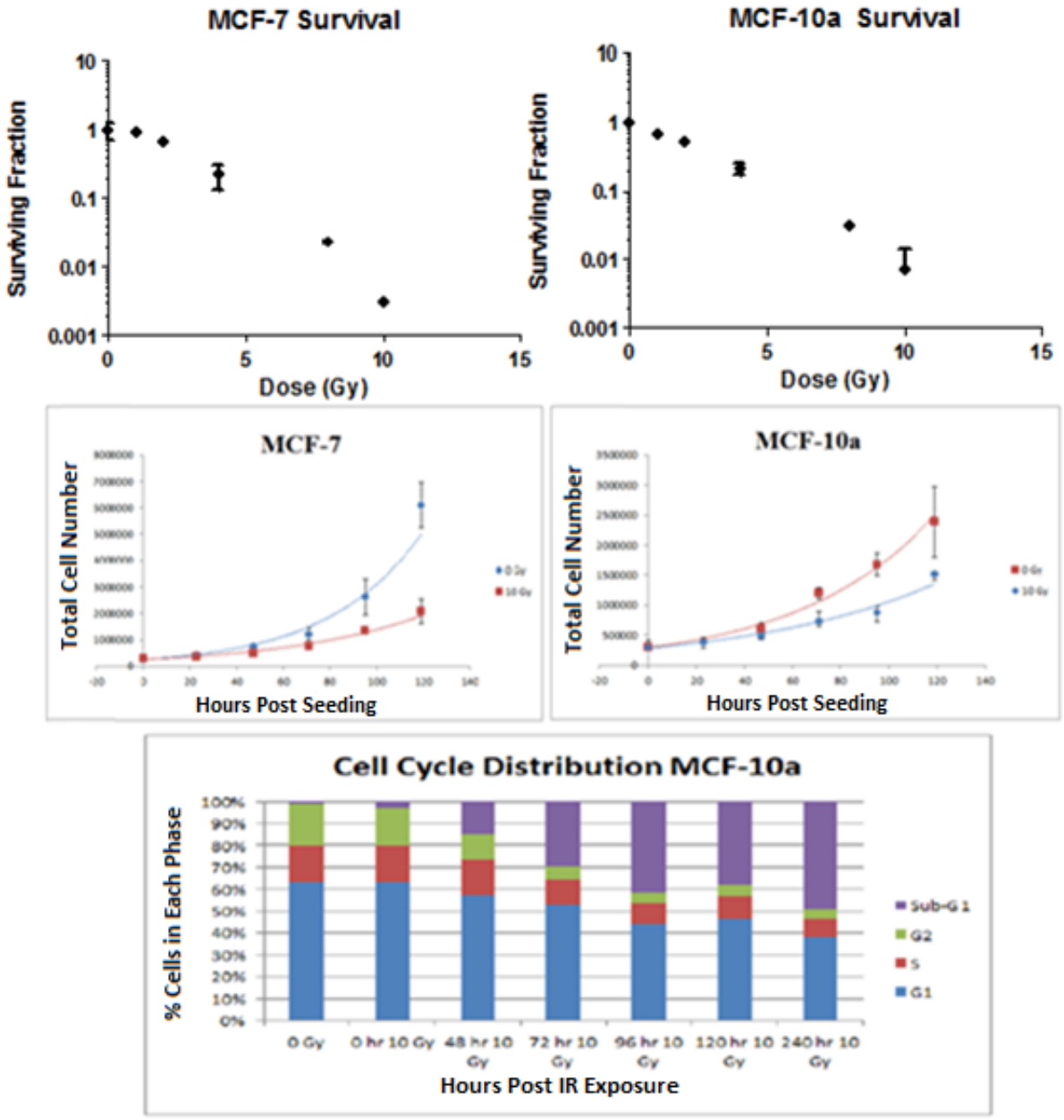

Figure 3.2: Cell growth dynamics of MCF-7 and MCF-10a cells following an acute dose of 10 Gy γ-rays. Top panel: Clonogenic survival curves of MCF-7 (left) and MCF-10a cells (right). Middle panel: changes in cell growth kinetics in MCF-7 (left) and MCF-10a (right) resulting from 10 Gy γ-ray exposures. Cell cycle distribution of MCF-10a cells for up to 240 hours post IR in MCF-10a cells.

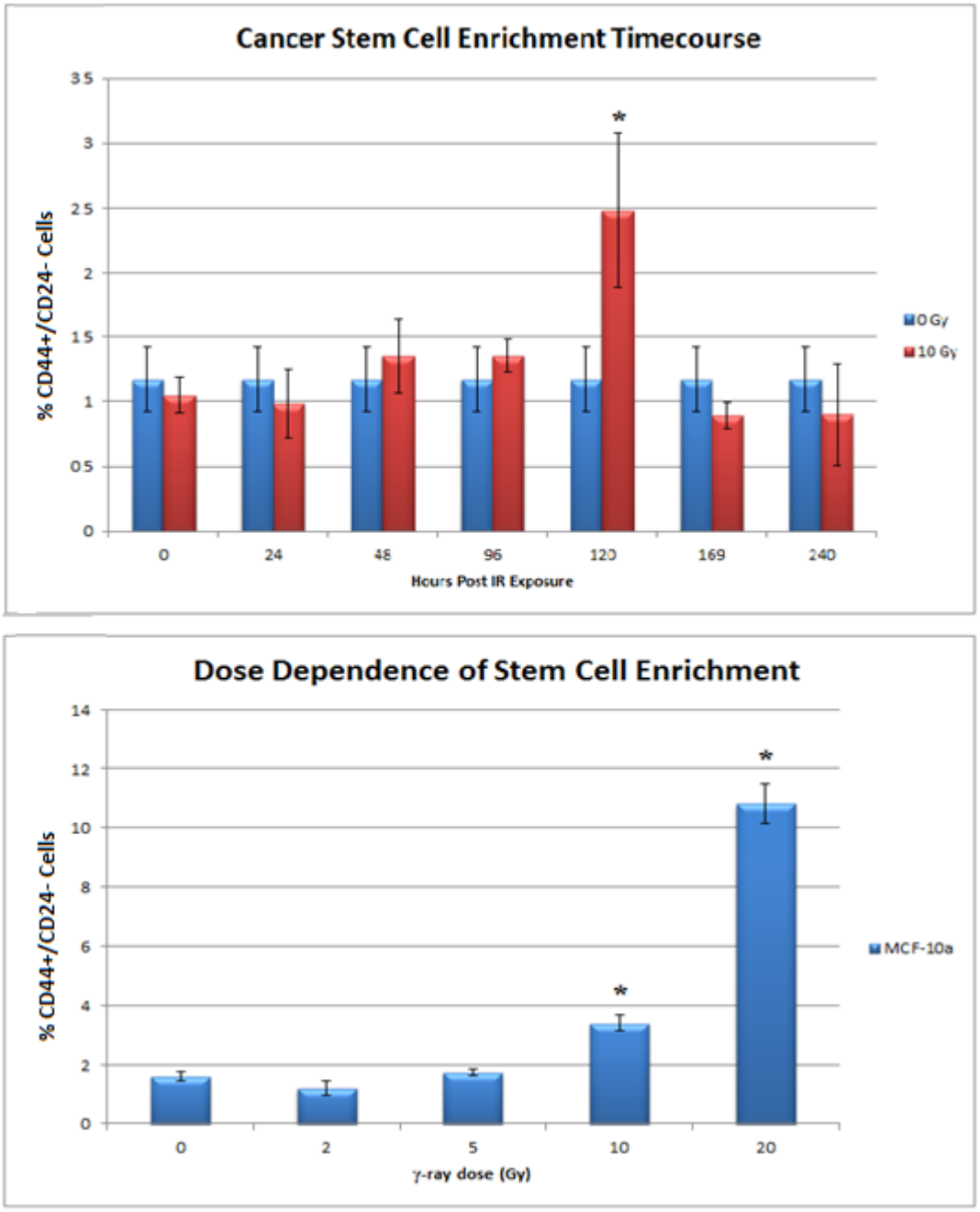

Figure 3.3: Enrichment of $CD44^{+}/CD24^{low/-}$ breast cancer stem cells in MCF-10a cells peaks at 5 days post IR exposure and declines to background levels thereafter (top panel). CSC enrichment at 5 days post exposure is dose dependent with a threshold dose of between 5 and 10 Gy (bottom panel) in MCF-10a cells and increases significantly between 10 and 20 Gy. Error bars represent the standard deviation of the mean and stars (*) represent values which are significantly different from the unirradiated control at each specific timepoint as determined by a Student's t-test.

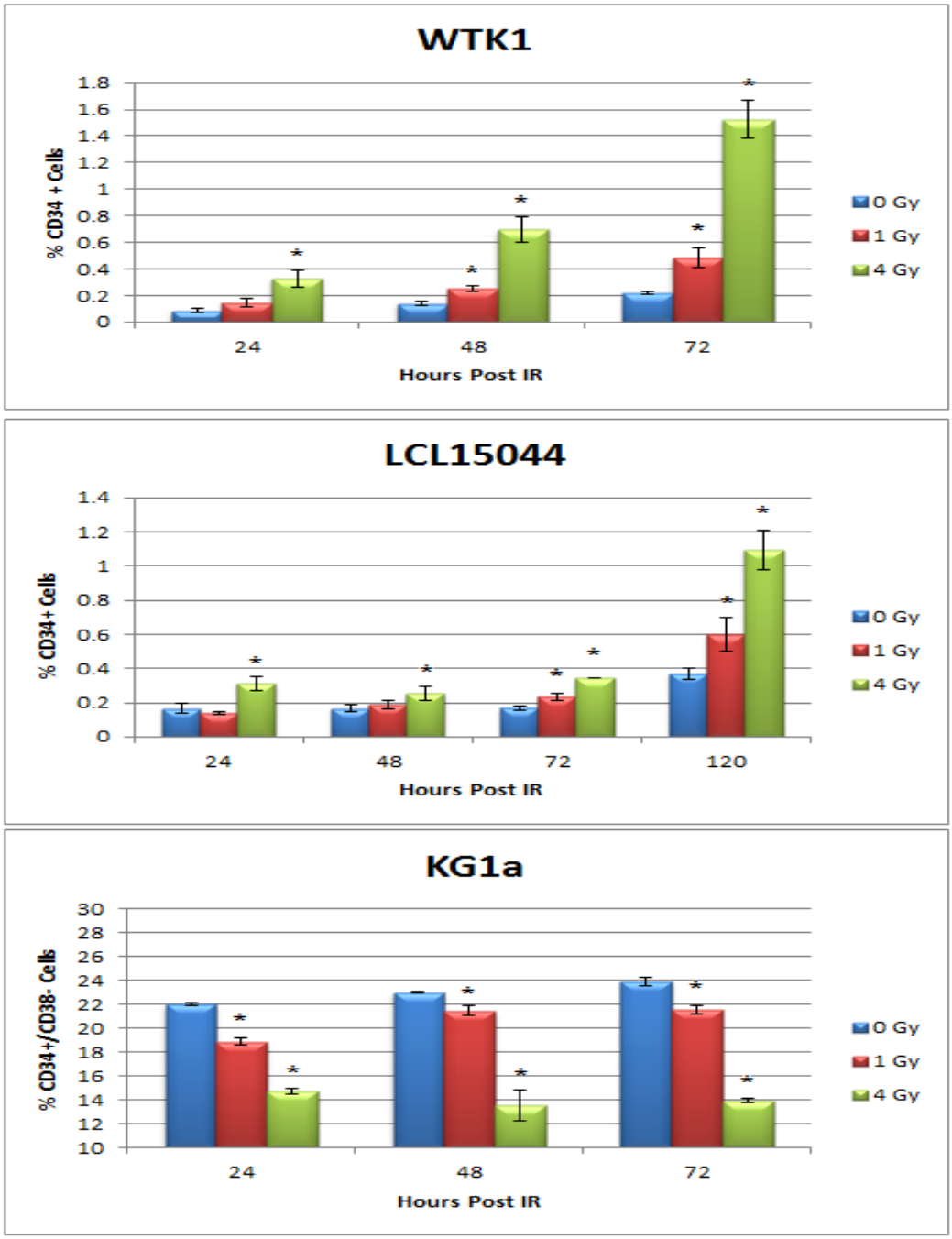

Figure 3.4: Time course of cancer stem cell enrichment of hematopoietic cell lines exposed to acute doses of 1 and 4 Gy γ-rays. Percentages of $CD34^+$ cells were examined in human lymphoblast cell lines WTK1 (top panel) and LCL15044 (middle panel) for up to 120 hours post IR. Percentages of $CD34^+/CD38^-$ leukemic stem cells were evaluated following IR in KG1a AML cells for 72 hours post exposure (bottom panel). Error bars represent standard error of the mean and stars (*) represent values significantly different than unirradiated controls.

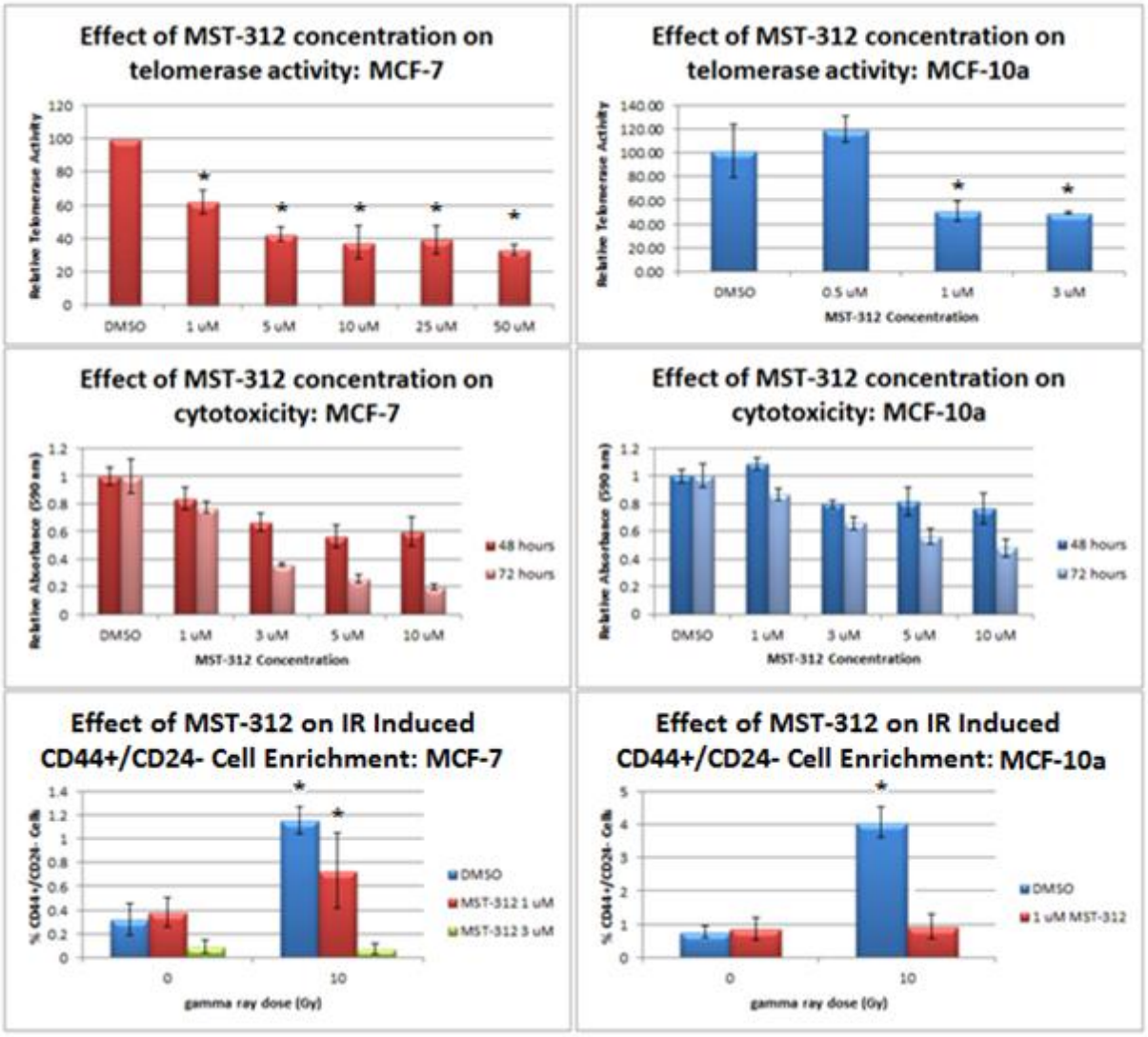

Figure 3.5: Telomerase inhibition blocks breast cancer stem cell enrichment following IR. Top panel: effect of MST-312 telomerase inhibitor on telomerase activity in MCF-7 (left) and MCF-10a cells (right). Middle panel: cytotoxicity of MST-312 at 48 and 72 hours post exposure as determined by MTT assay in MCF-7 (left) and MCF-10a (right) cells. Bottom panel: telomerase inhibition blocks radiation induced stem cell enrichment in both MCF-7 (left) and MCF-10a cells (right). Error bars represent standard error of the mean and stars (*) represent values significantly different than unirradiated, untreated, or vehicle controls.

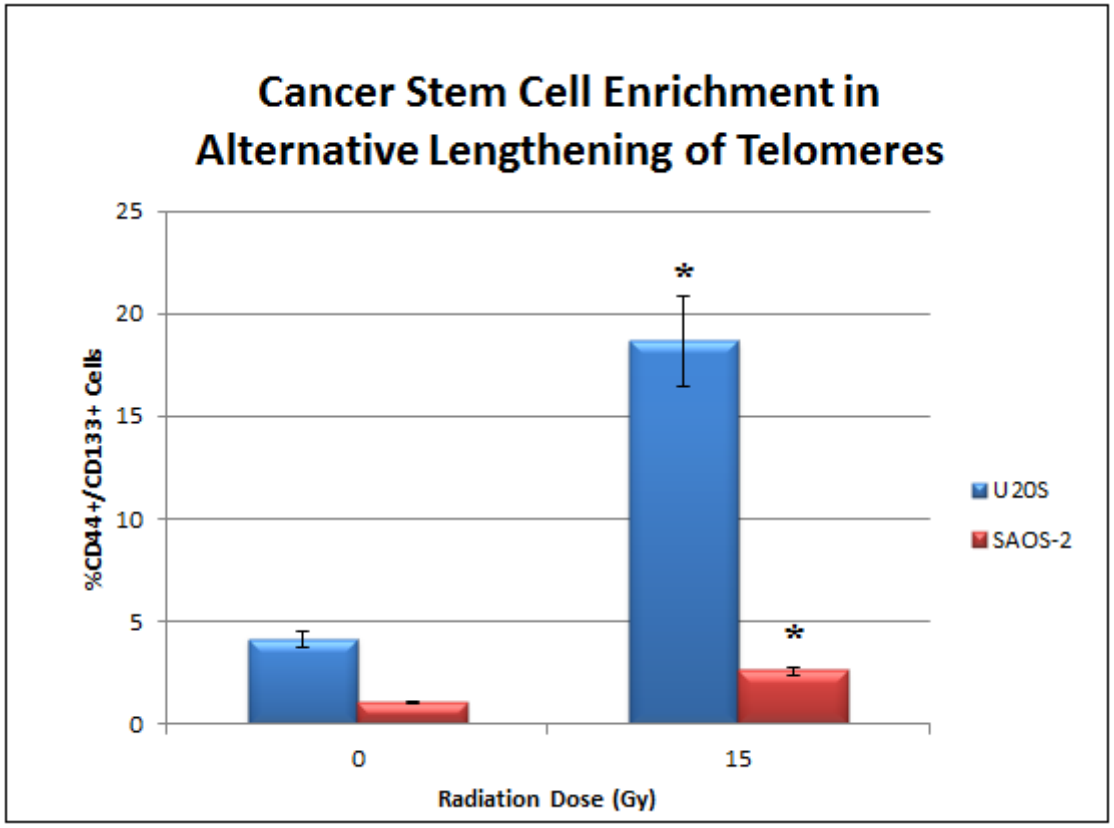

Figure 3.6: Enrichment of $CD44^{+}/CD133^{+}$ osteosarcoma cancer stem cells in the telomerase negative, ALT cell lines U20S and SAOS2 at 5 days post an acute dose of 15 Gy γ-rays. Error bars represent standard error of the mean and stars (*) represent values significantly different than unirradiated controls within each cell line examined.

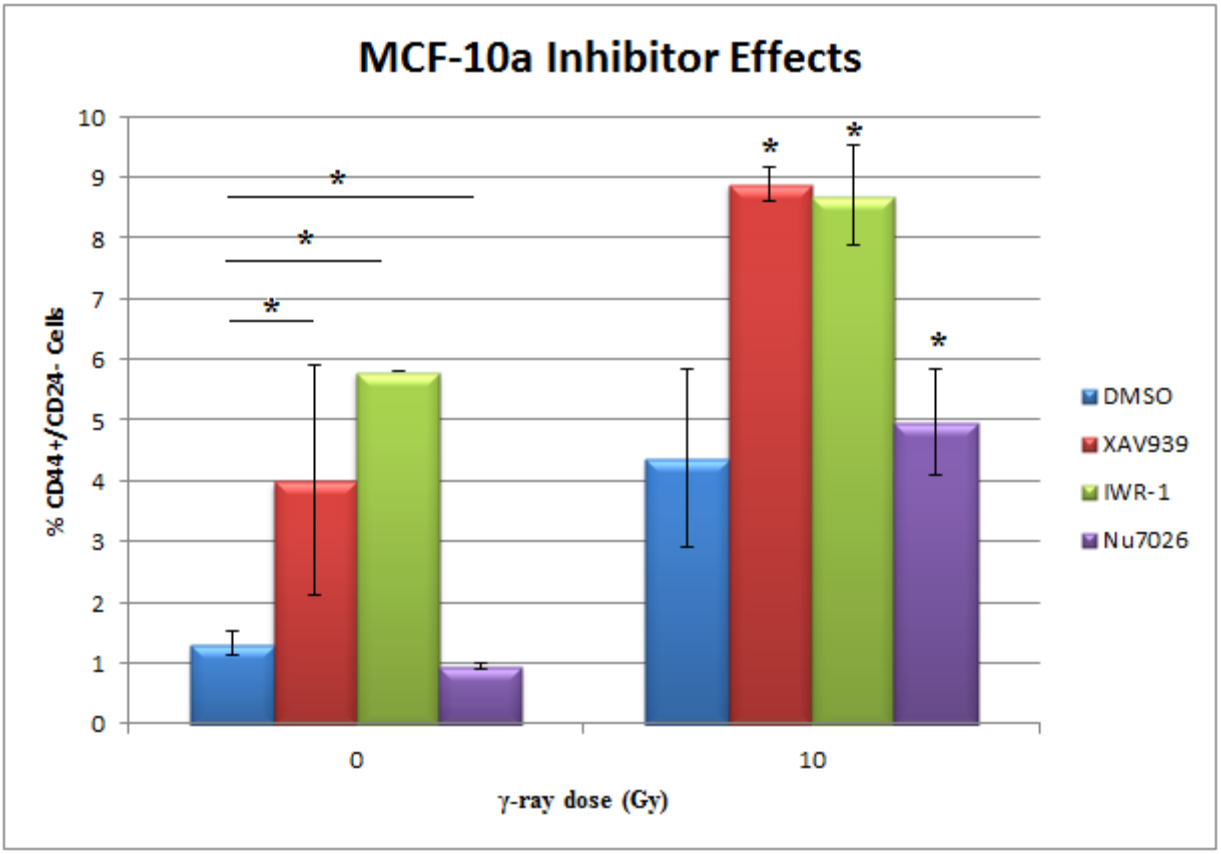

Figure 3.7: Effect of various telomere related inhibitors on $CD44^{+}/CD24^{low/-}$ breast cancer stem cell enrichment in MCF-10a cells 5 days post exposure to IR. Neither TNKS1/Wnt signaling inhibitors IWR-1 or XAV-939 were effective at blocking radiation induced stem cell enrichment; however, they did significantly elevate background breast cancer stem cell levels. DNA-PKcs inhibitor Nu7026 significantly decreased background CSC levels, but did not impact overall stem cell enrichment as a result of irradiation. Error bars represent standard error of the mean and stars (*) represent values significantly different than unirradiated control and each inhibitor. Bars with stars represent values which are significantly different than DMSO (vehicle treated) controls in unirradiated samples.

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CHAPTER 4

CONCLUSIONS AND DISCUSSION

Overview

Since Barbara McClintock and Herman Muller first identified telomeres, researchers have sought to understand the role these deceptively simple structures play in a myriad of biological processes (McClintock, 1938; Muller, 1938). With the identification of telomerase, the reverse transcriptase that elongates telomeres (Blackburn et al., 1989), and recognition of its up-regulation in the vast majority of human cancers (N. W. Kim et al., 1994), the field of telomere biology quickly grew and expanded to encompass numerous fields ranging from aging and degenerative disease, to stem cell biology and cancer. We now appreciate that telomeres and telomerase lie at the intricate crossroads between normal tissue aging and cancer, and so may be critical targets for slowing human aging and winning the war on cancer (Blasco, 2005). Telomerase is repressed in the vast majority of human somatic cells, but serves to endow replicative immortality to adult stem cells (ASCs) and embryonic/pluripotent stem cells (ESCs) (Flores & Blasco, 2010). Additionally, telomerase is reactivated or alternatively cells which already contain telomerase activity such as ASC's are mutated to become cancer stem cells, therefore driving carcinogenesis (Shay & Wright, 2011). Thus, as telomerase and telomeres are crucial players in carcinogenesis and tumor maintenance, understanding their role in response to known carcinogens and cancer therapies will help to identify potential mechanisms with potential to aid both the prevention and treatment of cancer.

Telomeres are critical players in the biological response to radiation as short telomeres have been associated with increased radiation sensitivity of human cells (Shim, Ricoul, Hempel,

Azzam, & Sabatier, 2014). Further, dysfunctional telomeres can join incorrectly to DNA double strand breaks following radiation exposure thereby contributing to genomic instability (Latre et al., 2003). As roughly two in three patients diagnosed with cancer will undergo radiation therapy as part of their treatment (Oncology, 2008), gaining a deeper understanding of the interplay between telomere biology and radiation exposure will help to identify key processes that can be targeted and exploited to improve clinical outcomes. In the studies reported here, we investigated the response of telomeres and telomerase to ionizing radiation in both normal and cancer cells.

Our goal was to 1) characterize the effect of both high and low dose rate (LDR) ionizing radiation (IR) on telomerase activity and telomere length in both normal and cancer cell lines, 2) more thoroughly define the enrichment of cancer stem cells (CSCs) following IR exposure, and 3) investigate the potential role of telomeres and telomerase in the radiation induced enrichment of cancer stem cells.

Changes in Telomerase Activity Following IR Exposure

Telomerase, a critical regulator of cellular lifespan (Blasco, 2005; Flores & Blasco, 2010; Shay & Wright, 2011), has been shown to be upregulated in a wide variety of primary a0nd recurrent tumors following radiation therapy, presenting a critical question; is the upregulation of telomerase activity following IR exposure aiding in the accelerated repopulation of tumors following radiation therapy? To address this question, changes in telomerase activity in a panel of normal and cancer cell lines of both mammary and hematopoietic origin were analyzed. Results demonstrated that telomerase activity was elevated in cancer cell lines (high telomerase) following exposure to IR, but not in normal (telomerase negative) or immortalized non-tumorigenic cell lines (telomerase positive) that have a functional checkpoint response. These observations trend were supported by examining expression levels of telomerase components

hTERT and hTERC, both of which were upregulated in MCF-7 breast adenocarcinoma cells following acute exposures, but downregulated in the non-tumorigenic MCF-10a immortalized mammary epithelial cell line. LDR IR exposure yielded slight, but not significant decreases in telomerase activity in all cell lines examined, suggesting that the response of telomerase to IR is dependent on dose delivery regimen and highlighting the need to address changes in telomerase activity in dosing regimens similar to those that occur in fractionated radiation therapy.

Telomere length in MCF-7 and MCF-10a cells was also evaluated following acute exposures to 10 Gy γ-rays. As it was previously reported that cells with short telomeres are radio sensitive (Bailey, 2008; Gisselsson et al., 2001), and we observed increased telomerase activity in cancer cells, we expected to see a loss of short telomeres due to apoptotic removal of cells with short telomeres, and an increase in the population's overall telomere length. However in both MCF-7 and MCF-10a we observed significant shortening of all telomeres within the cell population, rather than selective loss of shorter telomeres at the time of irradiation or elongation resulting from telomerase activity increases. The overall loss in average telomere length in the MCF-7 cells was around 20 percent while the overall loss in MCF-10a cells was closer to 30 percent. This difference may be explained by the elevation in telomerase activity observed in MCF-7 cells that was absent in MCF-10a cells. Thus, while telomerase activity is elevated in cancer cells following IR, and this activity appears to help mitigate telomere loss, it did not result in an overall elongation of telomeres. This suggests that the response of telomerase following IR is may function to mitigate loss of telomere length via its canonical role in telomere elongation; however, further investigation is necessary to elucidate its role in tumor repopulation following radiation therapy. What is difficult to explain is the overall loss of telomere length suggesting that an IR induced mechanism causing telomere erosion is activated. Furthermore, short

telomeres have also been linked to genomic instability, one of Weinberg's hallmarks of cancer. If acute exposure to IR is inducing telomere shortening in both cancer and non-tumor cells, the role of such shortening in inducing genomic instability must be examined, as irradiation of non-tumor cells during radiation therapy could lead to therapy induced second cancers.

Implications and Future Directions

As telomerase activity was elevated in cancer cells following exposure to therapeutically relevant, acute doses of IR, it is possible that such an elevation is contributing to accelerated tumor repopulation (Pandita & Roti Roti, 2003; Sawant et al., 1999), and therefore may represent a viable target in preventing tumor recurrence following radiation therapy. Additionally, IR exposure led to an overall shortening of telomeres in both normal and cancer cells, regardless of telomerase activity. Therefore, understanding the fundamental consequences of IR-induced telomere shortening may be vital to preventing genomic instability in non-tumor tissues exposed during the course of radiation therapy. Future studies will seek to characterize the response of telomerase and telomere length to radiations of different qualities including heavy charged particles found in the deep space environment and those used in carbon ion and proton radiation therapy. Such studies will help to define and mitigate the risk these exposures may pose to human pioneers in space, as well as to those who receive cancer treatment through carbon ion therapy.

Telomerase in the Radiation Induced Enrichment of Cancer Stem Cells

IR Induced Elevation of Telomerase Activity Precedes Stem Cell Enrichment

In chapter 3 described experiments in which confirmed reports of $CD44^{+}/CD24^{-}$ breast CSC stem cell enrichment following exposure to therapeutically relevant, acute doses of IR in the mammary carcinoma cell line MCF-7 (Ailles & Weissman, 2007; Fillmore & Kuperwasser,

2008; Lagadec et al., 2012; Lagadec et al., 2010; Phillips et al., 2006). To our knowledge, we are the first to report CSC enrichment in the non-tumorigenic MCF-10a cell line following IR exposure. We also report an increase in the percentage of lymphoblastoid cells expressing the hematopoietic stem cell (HSC) marker CD34 in response to radiation exposure (Calloni et al., 2013). The consequences of such stem cell enrichment are largely unknown, however as CSCs are more resistant to radiation therapy and possess increased replicative lifespan, it can be inferred that IR induced CSC enrichment may well contribute to accelerated tumor repopulation following radiation therapy, as well as drive tumor progression in non-tumor tissues (Butof et al., 2013; Lacerda et al., 2010; K. Ogawa et al., 2013; Z. Yu, Pestell, Lisanti, & Pestell, 2012).

Our initial goal was to determine whether the enrichment of CSCs in irradiated MCF-7 cells correlated with elevations in telomerase activity. Our results revealed that telomerase activity was elevated within the first 48 hours post IR exposure in cancer cell lines (expressing high levels of telomerase), but not in non-tumor cell lines (telomerase low or negative). Additionally, breast CSC enrichment did not occur until 120 hours post IR exposure. Therefore, elevations in telomerase activity temporally preceded the enrichment of CSCs and so was clearly not a result of stem cell mobilization following IR injury. This conclusion was supported by examining $CD34^{+}/CD38^{-}$ CSC populations in the AML cell line KG1a (Bonnet & Dick, 1997; Hoang et al., 2012; Perez Mdel et al., 2002), which displayed increased telomerase activity post exposure to both 1 and 4 Gy of γ-rays that coincided with decreased CSCs at these same time points. Further the non-tumor cells MCF-10a, LCL15044, and WTK1 displayed significant increases in the proportion of CSCs despite no change in telomerase activity. This suggests that while telomerase might promote IR induced CSC enrichment, it's increase is independent of stem cell enrichment.

Telomerase Reverse Transcriptase is required for IR Induced Breast Cancer Stem Cell Enrichment

Due to telomerase activity being elevated prior to the enrichment of breast CSCs populations following exposure to IR, we reasoned that telomerase may be acting upstream in a pathway that promotes CSC enrichment. To test this hypothesis we utilized the small molecule inhibitor MST-312 which blocks the reverse transcriptase activity of telomerase (Seimiya et al., 2002). Indeed, inhibiting telomerase activity blocked the enrichment of CSCs following IR exposure in MCF-7 cells. Telomerase inhibition also blocked enrichment of CSCs in MCF-10a, despite a lack of elevated telomerase activity following IR, supportive of a causative role. Interestingly, in neither MCF-7 nor MCF-10a did telomerase inhibition affect the background percentage of CSCs, indicating that inhibition is acting specifically to block a radiation induced phenomenon, in this case stem cell enrichment.

Since telomerase appeared to be playing a critical role, we sought to determine whether IR induced CSC enrichment occurred in cancer cells that maintain telomere length through the telomerase independent alternative lengthening of telomeres (ALT) pathway (Cesare & Reddel, 2010; Heaphy et al., 2011; Ulaner et al., 2004). A population of $CD44^{+}/CD133^{+}$ CSCs was recently identified in the U2OS and SAOS2 cell lines (Tirino et al., 2008; Tirino et al., 2011; L. Yu et al., 2013), so we exposed these cells to acute exposures of 15 Gy γ-rays and found that CSC enrichment also occurred in the absence of telomerase activity. This gave further confirmation that inhibition of telomerase in telomerase positive cancers may be affecting upstream pathways in telomerase positive cancers or that loss of telomere integrity through the inhibition of telomerase (in telomerase positive cancers) may be preventing IR induced CSC enrichment.

Several candidate pathways may provide insight. It was recently demonstrated that telomerase interacts with the Wnt/β-catenin and NF-κB signaling networks, which are known to promote "stemness" and block differentiation in colon carcinoma (Choi et al., 2008; Greider, 2012; Hoffmeyer et al., 2012; Low & Tergaonkar, 2013; Stower, 2012). Several recent reports have demonstrated that hTERT is transcriptionally regulated by Wnt/β-catenin signaling; however this finding has come under scrutiny, as Blackburn et. al. disputed this claim (Listerman et al., 2014). Telomerase has also been implicated as a direct regulator of the NF-κB inflammation response, as hTERT binds directly to NF-κB to promote transcription of cytokines including IL-6 (Ghosh et al., 2012; Wu et al., 2013). Furthermore, a mode of action through NF-κB may help explain why CSC enrichment was observed in MCF-10a cells despite an elevation of telomerase activity as overexpression of the NF-κB protein was shown to substitute for loss of telomerase activity (Ghosh et al., 2012). Future studies will seek to define the link between IR, telomerase, and CSC enrichment following IR exposure (Dreesen & Brivanlou, 2007).

Furthermore, neither inhibition of tankyrase 1 (TNKS1) poly-ADP ribose polymerase activity that has been previously demonstrated to induce telomere shortening (Hsiao & Smith, 2008; Smith & de Lange, 2000; Smith et al., 1998), nor DNA dependent protein kinase c (DNA-PKcs) kinase activity that is required for efficient end-capping function of telomeres (Bailey, 2008; Bailey, Brenneman, et al., 2004; Dregalla et al., 2010; Le et al., 2013) were able to prevent IR induced enrichment of CSCs. These results suggest that the inability of CSCs to be enriched following IR exposure upon telomerase inhibition was specifically due to telomerase activity, and potentially telomerase related pathways, rather than disruption of the telomere structure. Potential explanations could include increases in the percentage of senescent cells resulting from telomerase inhibition.

Implications and Future Directions:

CSC enrichment following IR exposure likely plays a critical role in tumor repopulation following radiation therapy. With the finding that stem cell enrichment also occurs in non-tumorigenic cell lines, it may also be a contributing factor to carcinogenesis and tumor progression in non-tumor tissues exposed to IR. We identified telomerase reverse transcriptase activity as a critical player in this process; the evidence reported here suggests that telomerase is acting through pathways that promote "stemness" rather than due to direct effects at telomeres. Future studies will seek to expand on this knowledge and explore potential links to the Wnt/β-catenin and NF-κB signaling networks.

Implications and Future Directions

We explored both canonical and potential non-canonical roles for telomeres and telomerase in the response of various cultured cell lines to IR exposure. We determined that acute IR (γ-ray) exposure elevated telomerase activity in established cancer cell lines. Further we suggested that checkpoint activation may be the mechanism responsible for keeping telomerase activity under control in IR damaged, non-tumor cells. Telomeres were significantly shortened as a result of IR exposure in both normal and cancer mammary epithelial cells, indicating potential induction of telomere driven genomic instability that may promote tumor recurrence and carcinogenesis following radiation exposure. We also demonstrated that telomerase activity is required for the enrichment of CSCs following IR exposure in telomerase positive breast carcinoma cells, and suggest that pathways such as Wnt/β-catenin and NF-κB signaling may be involved upstream of telomerase to promote stem cell enrichment following IR exposure.

Taken together, the findings we report here imply that telomerase isn't just for telomeres anymore, perhaps particularly with regard to radiation exposure. Moving forward, we have

developed an admittedly telo-centric working model (Figure 1), where telomeres, telomerase, and "stemness" are merged with canonical modes of cancer progression including mutation, chromosome aberrations, and genomic instability, to highlight the critical role of these fascinating structures in the process of carcinogenesis and tumor repopulation following radiation therapy.

The proposed model (Figure 1) builds upon the accepted premise that IR induces carcinogenic mutation of normal tissues (telomerase negative), induce telomere shortening, and leading to genomic instability as supported by evidence in Chapter 2. IR induced instability can lead to further mutation and cancer progression. Additionally, when adult stem cells (telomerase positive) undergo oncogenic transformation and/or an adult somatic cells are reprogrammed from IR exposure (reactivating telomerase), they can become or evolve into CSCs and propagate mutations to progeny leading to bulk tumor formation. In both of these scenarios, IR leads to the generation of a bulk tumor (telomerase positive) that must be treated with radiation therapy. That treatment can then induce CSC enrichment via selection of existing CSCs and/or through the reprogramming of NSCCs into CSCs resulting in tumor regrowth, accelerated repopulation, and potential recurrence. Further, radiotherapy poses another risk in that it can spark the carcinogenic processes described above in normal tissues irradiated over the course of treatment, leading to treatment induced secondary cancers.

The model proposes that telomerase and the genomic instability arising from the generation of short telomeres through IR exposure, are critical drivers in the process of carcinogenesis. Reactivation and subsequent deregulation of telomerase via mutation of checkpoints for example lead to telomerase activity elevation promoting growth and CSC enrichment in tumors and non-tumor tissues irradiated over the course of cancer treatment,

factors that contribute to tumor recurrence and potentially second cancers. Thus, when it comes to carcinogenesis and cancer treatment, the reality is much more complex than the long and short of telomeres and positives and negatives of telomerase activation. The studies we described here highlight and propose new avenues of research building on the appreciation for telomerase and telomeres underlying all the hallmarks of cancer.

Future studies will seek to expand upon those described previously and highlight the effects of high LET radiation exposure such as that experienced in the deep space environment and as a result of proton and carbon ion based radiation therapy on telomeres and telomerase. Additionally we intend to expand our studies of telomerase and telomere length in response to IR in apparently healthy stimulated human blood samples and look for evidence of lymphocyte de-differentiation. Results will have immediate implications for potential health risk assessment in astronauts living aboard the International Space Station and beyond. Furthermore, identification of pathways and processes outside of telomerase inhibition that that could be manipulated to improve cancer treatment and prevent carcinogenesis will be a vital contribution to the field.

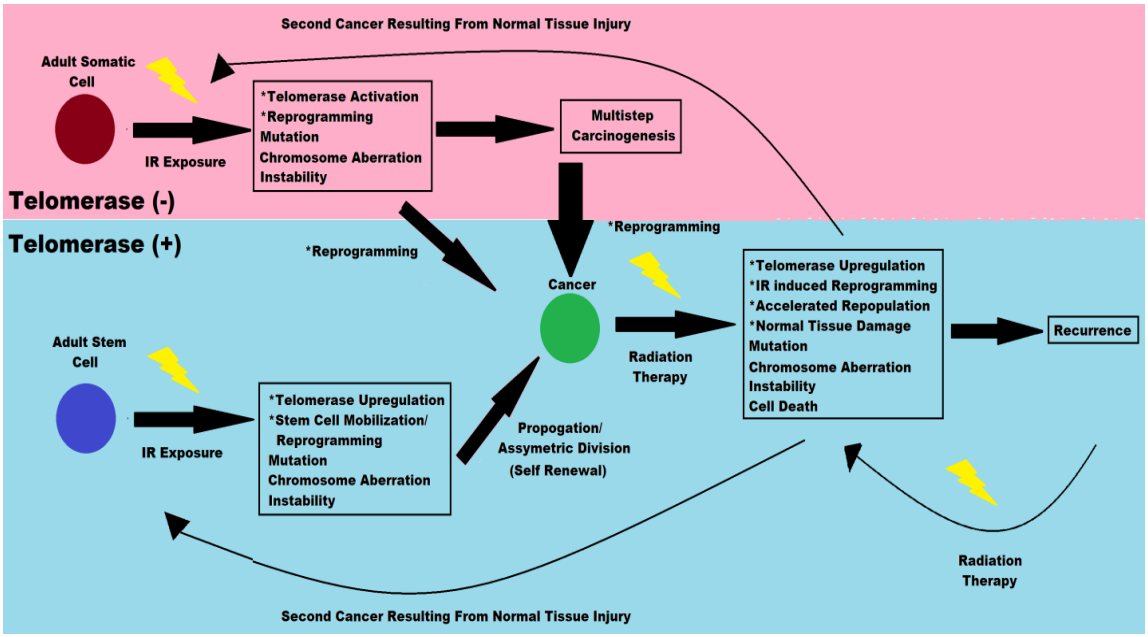

Figure 4.1: Working model depicting the potential role of telomeres and telomerase in ionizing radiation induced carcinogenesis starting in telomerase negative adult somatic cells (top portion) and telomerase positive adult stem cells (bottom portion). Model depicts the process of ionizing radiation induced carcinogenesis effecting telomere length and differentiation state which may lead to the development of cancer cells via the multistage model of carcinogenesis or through the conversion of an ASC into a CSC. Further treatment of cancer via radiation therapy induces similar elevation of telomerase activity and differentiation status, potentially leading to accelerated tumor repopulation and tumor recurrence or radiation induced carcinogenesis of normal tissues exposed as a result of treatment.

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APPENDIX

List of Abbreviations

(Listed in alphabetical order)

Symbols

α	Alpha
β	Beta
γ	Gamma
Δ	Delta
μ	Micro
κ	Kappa

Abbreviations

A-bomb	Atomic Bomb
ADP	Adenosine diphosphate
ALT	Alternative Lengthening of Telomeres
AML	Acute Myleoid Leukemia
APC	Adenomatous Polyposis Coli
ASC	Adult Stem Cell
BLM	Bloom
BRG1/SMARCA4	Transcription Activator BRG1
BSA	Bovine Serum Albumin

cDNA	Complementary DNA
CHO	Chinese Hampster Ovary
CO_2	Carbon Dioxide
Cs	Cesium
CSC	Cancer Stem Cells
DAPI	4',6-diamidino-2-phenylindole, dihydrochloride
DKC	Dyskeratosis Congenita
DKC1	Dyskeratosis Congenita Gene 1
D-loop	Displacement Loop
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribose Nucleic Acid
DNA-PK	DNA Dependent Protein Kinase
DNA-PKcs	DNA Dependent Protein Kinase Catalytic Subunit
DSB	Double Strand Break
E2F	E2F Transcription Factor
EBRT	External Beam Radiation Therapy
EDTA	Ethylenediaminetetraacetic Acid
EGF	Epidermal Growth Factor
EMT	Epithelial to Mesynchymal Transition
ESC	Embryonic Stem Cell

eV	Electron Volt
FBS	Fetal Bovine Serum
FISH	Fluorescent *in situ* Hybridization
Fzd	Frizzled Receptor
g	gram
GSK3-β	Glycogen Syntase Kinase 3 β
Gy	Gray
hTERC	Human Telomerase RNA Component
hTERT	Human Telomerase Reverse Transcriptase
HZE	High Charge and Energy Particle
IMRT	Intensity Modulated Radiation Therapy
IPF	Interstitial Pulminary Fibrosis
iPSC	Induced Pluripotent Stem Cell
IR	Ionizng Radiation
KCl	Potassium Chloride
keV	Kilo Electron Volt
LDR	Low Dose Rate
LET	Linear Energy Transfer
MEF	Mouse Embryonic Fibroblast
MEM	Minimum Essential Media
mES	Mouse Embryonic Stem Cell
MET	Mesynchymal to Epithelial Transition
mL	Milli Liter

mM	Milli Molar
mRNA	Messenger Ribose Nucleic Acid
NF-κB	Nuclear Factor Kappa Light Chain Enhancer of Activated B Cells
NHEJ	Non-Homologous End Joining
nM	Nano Molar
Oct-4	Octamer Binding Transcription Factor 4
OER	Oxygen Enhancement Ratio
p21	p21/Cyclin Dependant Kinase Inhibitor 1 /CDK Interacting Protein 1
p53	Cellular Tumor Antigen p53
PARP	Poly-ADP Ribose Polymerase
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
PNA	Peptide Nucleic Acid
POT1	Protection of Telomeres Protein 1
PSC	Pluripotent Stem Cell
Q-FISH	Quantitative Fluorescent *in situ* Hybridization
qRT-PCR	Quantitative Reverse Transcriptase Polymerase Chain Reaction
Rap1	Replication Associated Protein 1

Rb	Retinoblastoma Protein
RBE	Relative Biological Effectiveness
RNA	Ribose Nucleic Acid
RNAi	RNA interference
ROS	Reactive Oxygen Species
RPMI	Roswell Park Memorial Institute
RT	Reverse Transcriptase
SCNT	Somatic Cell Nuclear Transfer
siRNA	Small Interfering Ribose Nucleic Acid
SLD	Sublethal Damage
SNP	Single Nucleotide Polymorphism
SOX2	Sex Determining Region Y-Box 2
SRS	Stereotactic Radiosurgery
SSB	Single Stranded Break
SSC	Saline Sodium Citrate
SWI/SNF	Switch/Sucrose Non-Fermentable
TEN	Telomerase Essential N-Terminal Domain
TERC	Telomerase RNA Component
TERT	Telomerase Reverse Transctiptase
TIN2	TRF1 Interacting Nuclear Factor 1
T-loop	Telomere Loop
TNKS	Tankyrase 1
TPP1	Tripeptydl Peptidase 1

TRB	TERT RNA Binding Domain
TRF1	Telomere Repeat Binding Factor 1
TRF2	Telomere Repeat Binding Factor 2