Neoplastic Human Embryonic Stem Cells as a Model of Radiation Resistance of Human Cancer Stem Cells

By

Steve Dingwall, B.Sc. (Hons)

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AUTHOR: Steve Dingwall

SUPERVISORS: Drs. D. R. Boreham and M. Bhatia

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Abstract

Recent studies have implicated that a small sub population of cells within a tumour, termed cancer stem cells (CSCs), have an enhanced capacity for tumour formation in multiple cancers and may be responsible for recurrence of the disease after treatment. Further work has suggest that CSCs are radioresistant relative to other cell types composing tumours, in several solid cancers. The genetic and phenotypic heterogeneity of malignant CSCs, as well as the difficulty associated with culturing these cells *in vitro*, limits the capacity to study the response of CSCs to ionizing radiation. Further, the absence of normal known counterparts for many CSCs has made it difficult to compare the radiation responses of CSCs with the normal stem cells required for post radiotherapy tissue regeneration. Here we have shown that transformed human embryonic stem cells (t-hESCs), showing features of neoplastic progression, produce tumours resistant to radiation relative to their normal counterpart. We further show that t-hESCs have a reduced capacity for radiation induced cell death via apoptosis and exhibit altered cell cycle arrest *in vitro,* relative to hESCs. We found that decreased levels of p53ser15, following DNA double strand break induction, is associated with this radiation resistance.

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1. Introduction

1.1 General

Radiation therapy is one of the most important and effective modalities for the treatment of cancer patients with solid tumors. It is often used in conjunction with surgery and/or chemotherapy, and is utilized in approximately 40-50% of all cancer therapies (Delaney et al, 2005). Radiation therapy aims to eliminate primary tumors, as well as regional lymph node metastases (Yaromina, 2012). Successful elimination of tumours is dependent on the radiation dose that can be delivered throughout the course of radiotherapy. This dose is limited by the tolerance of normal tissue surrounding the tumour (Barnett et al., 2009). Technological advances in medical imaging (Dawson et al., 2006) and intensity modulated radiation therapy (Veldeman et al., 2008) have greatly improved the capacity to target tumours while limiting normal tissue toxicity, however many patients still suffer from locally recurrent disease after therapy. Such recurrence can occur as late as ten or more years post treatment for some cancers, such as breast (Zielske et al., 2011) and prostate (Desgrandchamps, 2008). Further, it has been estimated that up to 20% of patients treated with radiotherapy develop second primary malignancies (Tubiana, 2009), potentially resulting from radiation exposure to normal tissues during treatment. Factors associated with tumour recurrence include the capacity of tumour cells to repopulate during radiotherapy (Kim and Tannock, 2005), the degree of

hypoxia in the tumour (Nordsmark et al., 2005), as well as the intrinsic radiation sensitivity of the cells composing the tumour (West et al., 1993).

.1.2 Cancer Stem Cells

The cancer stem cell hypothesis is founded on the observation that not all of the cells composing a tumor have the same capacity to initiate and maintain tumorigenesis. It postulates that, similar to normal tissues, tumors are derived and maintained from a subpopulation of cells capable of self-renewal as well as differentiation into the multiple cell types within the tumor (Reya et al., 2001). The capacity of a sub-fraction of tumor cells to recapitulate the heterogeneity of the parent tumor was observed as early as 1964, in a series of experiments by Kleinsmith and Pierce. They observed that less than 3% of single cells derived from murine teratocarcinomas were able to form secondary teratocarcinomas, and that the cellular composition recapitulated that observed in the primary tumors (Kleinsmith and Pierce, 1964). A subpopulation of cells with an enhanced capacity for recapitulating the cancer from which they originated, in non-obese diabetic mice with severe combined immunodefieciency disease (NOD/SCID mice), were later identified in human acute myeloid leukemia (AML) (Bonnet and Dick., 1997). These cells were distinguished by the presence of the cell surface protein CD34 and the absence of the cell surface protein CD38, a phenotype shared with normal human hematopoietic cells capable of repopulation in NOD/SCID mice. Xenotransplantation is an important and powerful, albeit restricted, research tool in the cancer stem cell field. Markers for cancer stem cells can be used to identify cells capable of recapitulating the heterogeneity of the original tumor when injected in immune compromised mice.

However, for ethical reasons, it is not possible to elucidate whether these markers identify cells with the same capacity in humans (Kelly et al., 2007). However, the presence of these CSC markers has been correlated with poor prognosis in some cancers including oligodendroglial tumors (Beier, 2008) and breast cancer (Zhou et al., 2010). Further, murine mammary stem cell markers have been used to identify CSCs in a mouse model of breast cancer, and were shown to enrich for tumorigenic potential in mouse-mouse transplants (Zhang et al., 2008).

Cell surface markers for solid tumor CSCs were first identified in human breast cancer. Cells expressing the cell surface markers $CD^{44+}CD^{24/low}$ were able to recapitulate the morphological and phenotypic heterogeneity of the tumor of origin, when as few as 100 cells were injected into immune deficient NOD/SCID mice. However, when tens of thousands of tumor cells with alternate phenotypes were injected into NOD/SCID mice, tumors failed to form (Al-Hajj et al., 2003). This population was later refined by the addition of another cell surface marker, aldehyde dehydrogenase (ALDH) and tumor formation was observed in NOD/SCID mice injected with as few as 20 $CD^{44+}CD^{24/low}$ Lin^{-} $ALDH^{+}$ cells (Ginestier, 2007). While the use of cell surface markers is required for live sorting of cells, the mechanistic role that many of these markers play in the CSC phenotype is often unclear. It is possible that some of the proteins used to identify CSC do not play a direct role in facilitating enhanced tumor formation capacity; however, they do provide an important target for identification of the sub-populations that contain the cells responsible for tumorigenesis.

After the identification of CSCs in breast cancer, cell surface markers indicative of CSCs were identified in multiple other solid tumors including gliobastoma multiforme (GBM) (Signh et al., 2004), lung (Kim et al., 2005; Eramo et al., 2008), prostate (Collins et al., 2005), head and neck (Prince et al., 2007), colon (O'Brien et al., 2007; Ricci-Vitiani et al., 2007; Dalerba et al., 2007) and Liver (Yang, 2009) cancers. A synopsis of cell surface markers which correlates with enhanced tumor initiating capacity in these human cancers is presented in Figure 1.1.

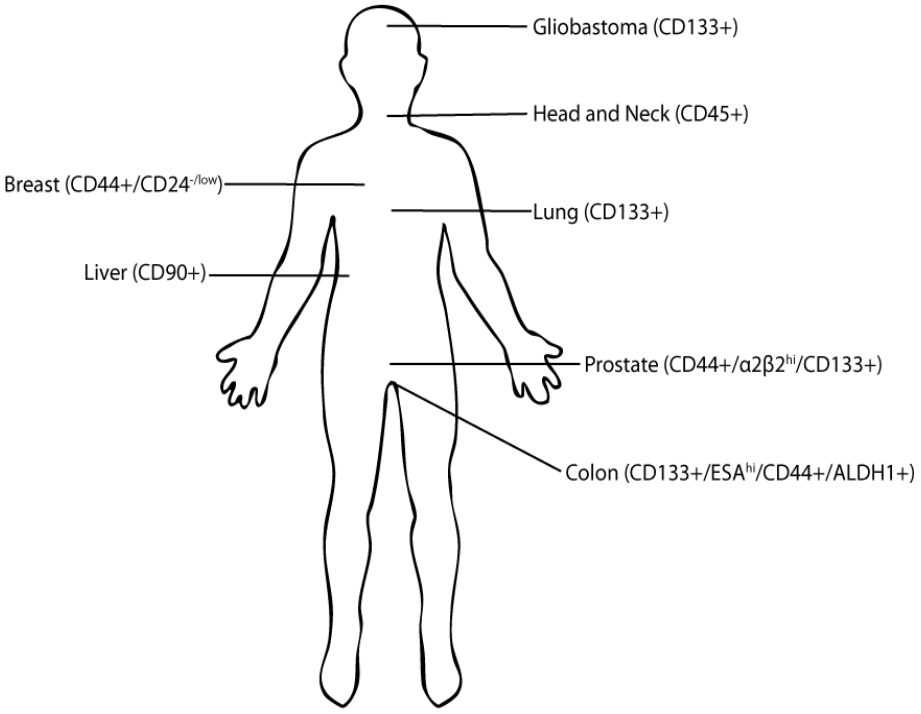


Figure 1.1: Examples of solid tumors where prospective CSC cell surface markers have been identified. Prospective markers for cancer stem cells have been identified in multiple solid cancers including Glioblastoma (CD133+), Head and Neck (CD45+), Breast (CD44+/CD24low), Lung (CD133+), Liver (CD90+), Prostate (CD44+/α2β2hi/CD133+), and Colon (CD133+/ESAhi/CD44+/ALDH1+).

1.3 Radiation Resistance in Cancer Stem Cells

Recent studies have found that CSCs are resistant to radiation relative to the other cells composing a tumor (Bao et al., 2006; Phillips et al., 2006; Chiou et al., 2008; Blazek et al., 2009; Charafe-Jauffret et al., 2009; Diehn et al., 2009; Zhang et al., 2010). These cells represent an important target for radiotherapy, and failure to eliminate these cells could result in the eventual recurrence of the cancer. The initial success of radiotherapy is generally measured by reduction in tumor volume. However, if a small sub-population, capable of repopulating the tumor is relatively radiation resistant, reduction in tumor volume may not be indicative of successful elimination of the disease. Targeting CSCs during radiation therapy has therefore become a priority among many within the field of radiation oncology. Several mechanisms for radiation resistance in cancer stem cells, relative to the non-CSC population in tumours, have been proposed including enhanced capacity for DNA repair, enhanced reduction of reactive oxygen species, and alterations in molecular pathways associated with apoptosis.

1.3.1 Direct and Indirect Interactions and DNA Double Strand Breaks

Ionizing radiation induced cell death is closely correlated with the induction of DNA double strand breaks (DSBs) (Radford, 1968), as well as the cell's ability to repair these DSBs (Kemp et al., 1984; Dikomey et al., 1998). A single DSB can be sufficient to kill a cell if it is located in a critical region of the DNA and induces apoptosis (Rich et al., 2000). As such, induction of DNA DSBs in tumor cells is generally considered the primary objective of radiotherapy. DSBs can result from direct interactions of energized particles or photons with DNA, as well as from indirect reactions with free radicals

produced by the ionization of water molecules in the cell (Johansen and Howard-Flanders, 1965). While, the ratio of DSBs arising from direct versus indirect interactions varies with the type and quality of radiation used, the majority of DSBs induced in cells during common radiotherapy (photon and x-rays) result from indirect reactions (Ward, 1985).

1.3.2 Enhanced DNA Repair in Cancer Stem Cells

The relative radiation resistance of CSCs was first reported in the CD133+ fraction of glioma cells (Bao et al., 2006). In this study, CD133+ human primary glioma cells were found to survive irradiation (both *in vitro* and in the brain of immune compromised mice) in increased proportions, relative to cells not expressing CD133. This radiation resistance was correlated with increased activation of proteins involved in the DNA damage checkpoint response (ATM, Rad17, Chk1, and Chk2) and enhanced DNA repair capacity in the CD133+ fraction. Radiation resistance in CSC populations associated with an enhanced DNA damage response has since been observed in Daoy medulloblastoma cell lines (Blazek et al., 2009), primary atypical teratoid/rhabdoid tumor (AT/RT) cells (Chiou et al., 2008), a murine breast cancer model (Zhang et al., 2010) and human breast cancer cell lines (Charafe-Jauffret et al., 2009). While an enhanced response to DNA damage may facilitate radiation resistance in some CSC population, it is not likely responsible for radiation resistance in all CSC populations. For example, while enhanced DNA repair was observed in both murine and human breast cancer cells by some groups, other groups did not observe this phenomenon (Phillips et al., 2006; Diehn et al., 2009). This may be a

result of different genetic mutations initiating transformation in the murine models used, and differences in breast cancer cell lines used in the human experiments.

1.3.3 Enhanced Free Radical Scavenging in Cancer Stem Cells

Another mechanism of radiation resistance in CSCs is an enhanced capacity to reduce free radicals. At a cellular level, this may be achieved by increasing the production of free radical scavengers such as glutathione and various superoxide dismutases. The scavenging of free radicals produced by radiation-induced hydrolysis of water molecules within the cell protects DNA and other vital cellular components from damage (Dikomey et al., 1998). It has been recently demonstrated that CSC populations in the human breast cancer cell line MCF-7 maintain lower reactive oxygen species (ROS) levels, relative to non CSCs, in response to ionizing radiation (Phillips et al., 2006). In this study, decreased levels of ROS correlated with decreased initial levels of γH2AX (an indicator of DSB damage) and increased cell survival in the CSC population after irradiation. Decreased basal levels of ROS have also been found in human and murine tumors, as well as in normal mammary epithelial stem cells (Diehn et al, 2009).

1.3.4 Induction of Apoptosis in Response to Ionizing Radiation

One of the hallmarks of a cancer cell is its ability to evade the induction of apoptosis (Hanahan and Weinberg, 2000). Apoptosis was first described by Kerr et al. in 1972, as a process of programmed cell death in multicellular organisms utilized during development and for the maintenance of homeostasis of cell populations in tissues. Apoptosis also acts as a defensive mechanism and can be induced in response to multiple types of cellular

stress including heat, radiation and hypoxia (Elmore, 2007). An important feature of apoptosis is the movement of normally inward facing phosphatidylserine to the outer layers of the plasma membrane, for early phagocytic recognition (Bratton et al, 1997). The presence of phosphatidylserine on the cell membrane allows for the recognition of early apoptotic cells using the recombinant protein Annexin V, in combination with a cell membrane impermeable nuclear stain such as 7-Aminoactinomycin D (7-AAD) (Arur, 2003). Cells in the early stages of apoptosis will positive for Annexin V as phosphatidylserine will have moved to the surface of the plasma membrane, but will stain negative for 7AAD as the nuclear membrane will not yet be permeabilized. As the cells progress through apoptosis they will stain positive for both 7AAD and Annexin V. Detection of apoptotic cells by flow cytometry, using 7-AAD and Annexin V is illustrated in Figure 1.2.

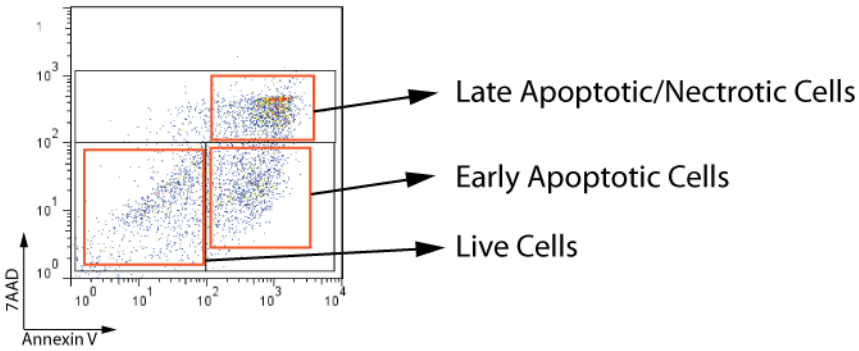


Figure 1.2: Apoptotic populations detectable by flow cytometric analysis of cells with Annexin V and 7AAD staining.

Induction of apoptosis is generally mediated through two main pathways; the extrinsic/death receptor pathway and the intrinsic/mitochondrial pathway. Of primary concern for radiation induced apoptosis is p53-mediated induction of the intrinsic pathway, in response to DNA DSBs. However, these two pathways are closely linked and molecules from both pathways have been shown to influence the others (Igney and Kramer, 2002). In response to DSBs generated by ionizing radiation the protein ataxia-telangiectasia mutated (ATM) is activated and phosphorylates p53 directly at serine-15 (Banin et al, 1998; Canman et al., 1998) and indirectly at serine-20, via phosphorylation of CHK1 and CHK2 (Shieh et al., 2000). Phosphorylation of these regions results in the interruption of p53 binding by MDM2, resulting in increased stability as MDM2 binding marks p53 for ubiquitination (Fei and El-Deiry, 2003). Upon activation, p53 regulates apoptosis, cell cycle arrest, autophagy and senescence through activation and repression of multiple downstream targets (Vousden and Prives, 2006). Of particular importance to p53 mediated apoptosis are members of the B-cell lymphoma 2 (Bcl-2) family consisting of 25 pro and anti-apoptotic members (Swanton et al, 1999) and members of the Inhibitors of Apoptosis (IAP) family (Hunter et al., 2007). Both of these protein families operate downstream from p53 and modulate p53 induced apoptosis.

1.3.5 p53 and Cancer Stem Cells

Defects in p53 may play a role in the maintenance of CSC populations within tumours. p53 has been shown to bind the promoter of CD44 and repress its expression in breast cancer cell lines, following exposure to the DNA intercalating agent doxorubicin (Godar et al., 2007). Here, decreased CD44 expression correlated with decreased tumor initiating

capacity and conversely, ectopic expression of CD44 blocked p53 induction of apoptosis. In a mouse model of breast cancer symmetric divisions were found to be more common in CSCs than their normal counterpart (Cicalese et al., 2009). In this study, targeted mutation of p53 in normal mammary stem cells resulted in the same self-renewal properties as mammary CSCs, indicating a role for p53 in controlling self-renewal of CSCs as well as apoptosis. Alterations in p53, which have been found to be present in approximately 50% of cancers (Hollstein et al., 1991), are likely to influence the frequency of CSC within a tumor and proteins associated with the CSC phenotype may play a role in altering the p53 response. p53 seems to play a similar role in embryonic stem cells where it has been shown to bind the pluripotency factor Nanog in mouse ES cells and induce differentiation (Lin et al., 2005). Further, the efficiency of induced pluripotent stem (iPS) cell reprogramming is improved by p53 interference using the SV40 large T antigen (Mali et al., 2008), or p53 small interfering RNAs (siRNAs) (Zhao et al., 2008).

1.3.6 The Bcl-2Family, Inhibitors of Apoptosis (IAPs), and Cancer Stem Cell Radiation Resistance

Alterations in molecular pathways downstream of p53 have been associated with a decreased apoptotic response in CSC populations. Radiation resistance in the CSC population of the pediatric brain tumor AT/RT has been correlated with increased activation of the anti-apoptotic protein Bcl-2 (Chiou et al., 2008). However, increased expression of Bcl-2 is also utilized in normal hematopoietic stem cells, and has been shown to be key for their survival in response to genotoxic stress (Domen et al., 2008). As a result, direct targeting of Bcl-2, to radiosensitize CSCs in AT/RT patients could have

serious side effects related to the maintenance of hematopoiesis. High expression of another Bcl-2 family member Mcl-1, in the CSC population of human primary gioblastomas, has also been associated with decreased induction of apoptosis (Tagscherer, 2008) and elevated expression levels of the anti-apoptotic proteins X-linked inhibitor of apoptosis (XIAP), cellular IAP1 (cIAP1), Survivin, Bcl-2 and Bcl-Xl have all been reported in the CD133+ of glioblastomas, relative to the CD133- fraction (Liu et al., 2006). Among these, it is interesting to note that Survivin has been correlated with the teratoma forming capacity of human ES cells, and pharmacological ablation of survivin induces apoptosis in hES cells, as well as teratomas (Blum et al., 2009).

1.4 Embryonic Stem Cells, Pluripotency, and Cancer

Targeting mechanisms of radiation resistance during radiation therapy to improve the elimination of CSCs is complicated by the potential overlap of some of these characteristics between CSCs and normal stem cells residing near the site of tumor formation. While targeting the CSC population, normal stem cells need to be spared from damage and cell death. Loss of or damage to the normal stem cell population can result in an inability to effectively repair tissue post radiotherapy, or could potentially lead to the induction of a secondary cancer. It is therefore necessary to study a normal stem cell counterpart, while devising methods for the successful elimination of cancer stem cells with ionizing radiation. Difficulties in identifying normal counterparts for CSCs, as well as in maintaining both cell types *in vitro*, have limited progress in studying the responses of normal and cancerous stem cells side by side. As a result, a human embryonic stem cell (hESC) line with features of neoplastic progression (herein referred to as t-hESCs)

has been characterized (Werbowetski-Ogilvie et al., 2009). These cells have been shown to be effective for identifying biological phenomena unique to cancer stem cells when used in conjunction with a normal hESC counterpart, and for devising therapeutics specific to CSC populations (Sachlos et al., 2012).

CSCs are thought to share some molecular characteristics with normal stem cell populations. It has been proposed that a key step in cancer initiation may include the inappropriate activation of molecular machinery normally restricted to stem cells. For example, both leukemic and normal hematopoietic stem cells require the Polycomb group gene Bmi-1 for the regulation of proliferative activity (Lessard and Sauvageau, 2003). As a result of this, a normal counterpart needs to be considered when devising mechanisms for the elimination of CSCs. Further, CSCs may not only utilize molecular programs similar to those found in normal adult stem cells, but may also re-initiate molecular programs found in embryonic stem cells. For example, leukemic stem cells in MLL initiated AML have been shown to maintain a self-renewing state using a transcriptional program more similar to that of hESCs than of normal myeloid progenitors (Somervaille et al., 2009).

Oct-4 is a transcription factor belonging to the POU family, and is normally expressed in the pluripotent cells of the developing embryo, as well as in their *in vitro* counterparts' hESCs and embryonic germ (EG) cells (Pesce and Scholer, 2001). Reactivation of Oct-4, as well as other members of regulatory networks associated with pluripotency, may play a role in tumorigenesis and the maintenance of CSC populations in some cancers. Oct-4 staining has been observed in various types of testicular germ cell tumors (Jones et al.,

2004; Gidekel et al., 2003) and expression of the activation targets of Oct4 and other pluripotency factors (Nanog, Sox2, and c-Myc), have been associated with poorly differentiated and highly aggressive breast cancers (Ben-Porath et al., 2008), increased risk of metastasis and worse outcome in adenocarcinoma patients (Wong et al., 2008), as well as with greater disease progression, increased metastasis and decreased patient survival in human bladder cancers (Chang et al., 2008).

In one study, expression of Oct-4 was modified in mouse ES cell lines and increased Oct-4 expression was correlated with the incidence of tumor formation upon injection into recipient mice (Gidekel et al., 2003). In this study, when Oct-4 expression was blocked after tumor formation, tumor regression was observed, indicating that Oct-4 expression may play a role in maintenance as well as initiation in some tumors. In other murine models induced overexpression of Oct-4 in somatic tissues resulted in dysplastic growth in epithelial tissues of the gastrointestinal tract and skin (Hochendlinger et al., 2005) and increased the metastatic capacity of bladder cancer cell lines to the lungs (Chang et al., 2008). The mechanism by which Oct-4 and other proteins associated with pluripotency influence the induction and maintenance of tumors has not yet been well characterized. However, a recent publication on the response of patient samples to radiotherapy has indicated a potential role for some of these factors in transforming non-CSC cells within a tumor towards a CSC phenotype. In this study non-CSCs from breast cancer patients and multiple breast cancer cell lines were irradiated, and a dose dependent increase in the number of cells bearing CSC markers was observed (Lagadec et al., 2012). These breast CSCs, termed induced breast cancer stem cells (iBCSCs) were shown to have increased

tumorigenic potential and express the same stemness related genes found in CSCs not induced by irradiation. This reprogramming of non-CSCs was correlated with increased expression of four pluripotency factors (Oct-4, Sox-2, Klf-4, and Nanog) also associated with the reprogramming of somatic cells to a pluripotent state (Okita et al., 2007).

1.5 Properties of Transformed Human Embryonic Stem Cells

The genetic and phenotypic heterogeneity of malignant cancer stem cells, as well as the difficulty associated with culturing these cells *in vitro*, limits the capacity to study the response of CSCs to ionizing radiation. Further a direct comparison of the radiation response of CSCs, with that of a normal counterpart has been limited by difficulty in identifying and maintaining these cells *in vitro*. However, these limitations can be addressed by the utilization of a model system consisting of normal (hESCs) and transformed (t-hESCs) human embryonic stem cells. t-hESCs have previously been characterized and shown to have features of neoplastic progression including enhanced self-renewal, growth factor independence, aberrant differentiation, and enhanced tumor initiation capacity (Werbowetski-Oglivie et al., 2009). These t-hESCs have lost the morphological and phenotypic heterogeneity characteristic of normal hESCSs (described by Morag et al., 2006), and shows elevated expression of the pluripotency markers Oct-4 (Junfeng et al., 2009), SSEA3, and SSEA4 (Werbowetski-Oglivie et al., 2009). Co-culture of normal hESCs with t- hESCs leads to the transmission of some of these neoplastic properties, including enhanced pluripotent marker expression and a differentiation blockade, to the normal hESCs (Werbowetski-Oglivie et al., 2011).

These t-hESCs have been shown to produce neural tumours which model early human paediatric brain tumours (Werbowetski-Ogilivie, 2012). As the blocked differentiation of these cells still permits some differentiation towards a neural fate *in vitro*, neural precursors (NP) derivation is possible. These NPs show similar features of neoplastic progression, including enhanced cell proliferation and an inability to mature towards the astrocyte lineage relative to NPs derived from normal hESCs. Further, intracranial injection of t-hESC derived NPs into NOD-SCID mice results in the formation of tumors with morphological, phenotypic and molecular features of a medulloblastoma (Werbowetski-Oglivie, 2012). This study shows the potential for using t-hESCs to potentially model the early stages of various cancers.

t-hESCs have also been used in conjunction with normal hESCs for chemical compound screening, resulting in the identification of a chemical compound capable of inducing differentiation specific to AML CSCs and not normal hematopoietic stem cells (Sachlos et al., 2012). This study exemplifies the usefulness of t-hESCs, in conjunction with normal hESCs, as a potential *in vitro* surrogate for CSC which can result in the identification of molecular mechanisms specific to the CSC therapeutic response.

1.6 Hypothesis and Objectives

The primary objective of this study was to test whether t-hESCs exhibited a radiation resistant phenotype similar to that observed in various CSCs. This was tested by the formation of tumours with both normal hESCs and t-hESCs, followed by subsequent localized irradiation. We showed that t-hESCs were similar to CSCs in their ability to

produce a heterogeneous tumour that is relatively resistant to ionizing radiation.We further showed that this radiation resistant phenotype correlated with the pluripotent cell fraction's ability to continue to form teratomas following irradiation. Next, the novel capacity to stably culture purified populations of cells which are capable of forming either radio-resistant (t-hESC) or relatively radio-sensitive (hESC) tumours allowed for the *in vitro* comparison of these cell types. These direct *in vitro* comparisons showed a decreased capacity for radiation induced apoptosis and an altered cell cycle response in the transformed stem cell population, relative to their normal stem cell counterpart. These altered apoptotic and cell cycle responses correlated with decreased activation of the tumour suppressor p53, which is found to be altered in approximately 50% of cancers (Hollstein et al., 1991). The results from this research implicate a potential role for aberrant p53 activation in mediating radiation resistance in CSC populations.

2. Materials and Methods

2.1 Ethics Statement

Animal experiments were approved by the Animal Care Committee (Animal Research Ethics Board), and Veterinary Services of McMaster University. Approval from our local ethics board was obtained for use of established human embryonic stem cell lines and SCOC.

2.2 Human Embryonic Stem Cell Culture

Human Embryonic Stem Cell Culture was performed as previously published (Chadwick et al, 2003; Stewart et al., 2006; Werbowetski-Ogilvie et al., 2009). Briefly, normal (H9) and transformed (v-H9-1) hESC cell lines were cultured on Matrigel (BD Biosciences) coated plates in Mouse Embryonic Fibroblast Conditioned Media (MEF-CM) supplemented with 8ng/ml of basic fibroblast growth factor (bFGF) (Invitrogen). MEF-CM was produced in house by daily collection of media used to feed irradiated mouse embryonic fibroblasts (MEFs) over a 7 day period. Media used to feed MEFs consisted of 80% knockout Dulbecco modified eagle medium, 20% knockout serum replacement, 1% non-essential amino acids, 1uM L-glutamamine (all Invitrogen), 0.1mM β-mercaptoethanol (Sigma Aldrich), and 4ng/mL bFGF. After collection MEF-CM was filtered through a 0.22-μM and stored at -30°C. Cells were dissociated for 2-5min with

collagenase IV (Gibco) and passaged at a 1:6 ratio every 4 days (t-hESCs) or at a 1:2 ratio every 7 days (hESCs). Cells were maintained in a 37°C incubator with 5% CO_2.

2.3 In Vitro Irradiation

7 (hESCs) or 4(t-hESCs) days following cell passaging, media was aspirated until just covering cells. Cells were irradiated at room temperature using a ^{137}Cs (662Kev) γ ray source (McMaster Taylor Source), at a distance of 30cm, resulting in a dose rate of 0.344Gy/min. Following irradiation, fresh media was added to cultures and cells were returned to incubator.

2.4 Flow Cytometry

2.4.1 Cell Harvest and SSEA3 Staining

hESCs and t-hESCs were treated with collagenase IV and then dissociated in cell dissociation buffer for 10min at 37°C. Cells were then resuspended in 4mL of PBS/3%FBS and centrifuged at 1500rpm for 5min. Supernatant was aspirated and cells were again resuspended in of PBS/3%FBS and filtered through a 40um filter. Cell counts were measured using an automated cell counter (Countess, Invitrogen) and diluted to a concentration of 1 x 10^6 cells/mL. Cell suspensions were then conjugated with PE-SSEA3(1:100) (BD Bioscience) for 30min at room temperature, followed by two washes with PBS/3%FBS as above .

2.4.2 Annexin V Staining

Cells were harvested 8 hrs post irradiation and stained for SSEA3, as above, then resuspended in 1x Binding Buffer (BD Bioscience). Cell suspensions were then conjugated with FITC-AnnexinV (1:20) for 15 min. The DNA stain 7-Amino-actinomycin D (*7-AAD*) (BD Bioscience) was added (1:100) and cells were incubated at room temperature for 15min. Analysis was performed using a FACSCalibur (BD Bioscience) and FlowJo software (Tree Star).

2.4.3 Cell Cycle Analysis

Day 7 hESCS and day 4 t-hESCs were exposed to 10μM BrdU for 1 hour, prior to cell harvest. Cells were harvested either 12 or 72hrs post irradiation and stained for SSEA3, as above. Cells were then washed with 1ml of Perm/Wash buffer (BD Bioscience) and centrifuged at 1500rpm for 5min and then fixed in Cytofix/Cytoperm Plus (BD Bioscience) for 10min at room temperature. Cells were again washed with Perm/Wash buffer then fixed with Cytofix/Cytoperm buffer (BD Bioscience) for 5min at room temperature. Perm/Wash buffer was repeated then cell pellets wer resuspended in DNAase (300ug/mL) (BD Bioscience) and incubated at 37°C for 70min. Cells were then washed with Perm/Wash buffer and conjugated with APC-antiBrdU (1:50) (BD Bioscience), for 20min at room temperature. Cells were then stained for DNA content with 7AAD (1:10) (BD Bioscience) at room temperature for 10min prior to analysis. Analysis was performed using a FACSCalibur (BD Bioscience) and FlowJo software (Tree Star).

2.5 Immunocytochemisty Staining, Automated Imaging, and Analysis

Cells were fixed in 2% paraformaldehyde and stained with 10µg/mL Hoechst 33342 (Invitrogen) with a Combi Multidrop Dispenser (Thermo). Standard fluorescence immunocytochemical techniques were used to stain the cells with a monoclonal antibodies for p53 (Cell Signaling Technologies) and for Oct4 (BD), and a Alexa-Fluor-488 and Alexa-Fluor-546 secondary antibodies (Invitrogen). Immunocytochemical staining was performed by a Janus automated liquid handler (Perkin Elmer). Images were acquired at 10x with an Arrayscan HCS VTI Reader (Cellomics) by means of epi-fluorescence illumination and standard filter sets. Images were acquired at 5x for hESCs, and 10x for t-hESCs, with an Arrayscan HCS VTI Reader (Cellomics) by means of epi-fluorescence illumination and standard filter sets.

Image analysis was performed using custom scripts in Acapella software (Perkin Elmer). Nuclear objects were segmented from the Hoechst signal. The fraction of nuclear-localised Alexa-Fluor-488- and Alexa-Fluor-546-positive cells was quantified. Images and well-level data were stored and analysed in a Columbus Database (Perkin Elmer).

2.6 Teratoma Injections, Irradiation, and Histology

hESCs (200K) and t-hESCs (50K) were treated with collagenase IV for 3-5 min, as described above, and resuspended in 25ul of PBS/3%FBS. Cell suspensions were injected intratesticularly into NOD.Rag1-/-.IL-2rγc-/- (NRG) *mice (Jax Laboratories).* Injected and control testicles were palpated weekly and measured using callipers, beginning 3 weeks post injection and continuing until tissue collection. Irradiations were

performed when the palpated volume of the injected testicle reached a 0.25-0.45 cm^3. A schematic representation is provided in Figure 2.1.

In Vivo Irradiations were performed using a Cs-137 γ irradiation source (Gammacell 40 Exactor, MDS Nordion) at a dose rate to the skin of 0.97Gy/min. Mice were anesthetised using 2.5% Avertin (Sigma Alrich) (0.018 ml/g body mass) and placed in lead shielding to localize the dose to the testicles (Fig. 2.2). Measurements of direct radiation dose to the inside of the testicles and indirect dose received in the small intestines, while mice were housed in lead shielding, were obtained by surgical implantation of thermo-luminescence dosimeter (TLD) chips (Harshaw TLD-100 LiF Chips). TLD chip analyses were performed by a third party specializing in clinical diagnostic radiation measurements (K&S Associates Inc., Nashville, TN).

Mice were euthanized 4 weeks following irradiation. Teratomas were extracted and volume was measured by displacement of 10% formalin in a graduated cylinder and with callipers. The weight of all teratomas was also measured and recorded. Teratomas were fixed in 10% formalin, imbedded in paraffin and sectioned in 5um intervals. This was followed by depraffinization in xylene and dehydration in a graded series of ethanol concentrations. Samples were stained with H&E or Oct4 (1:200). Histological samples were scanned at 200x and 400x magnification with an automated slide scanner (Scanscope CS, Aprio).

2.7 Statistical Analysis

All tests were performed using Prism 5 software (GraphPad Software, La Jolla, CA, USA). Descriptive statistics were used to determine significant differences including mean and s.e.m. along with one-way ANOVAs and independent sample two-tailed *t*-tests. *P*-values <0.05 were considered significant.

3. Results

3.1 t-hESCs Generate Radiation Resistant Tumours Upon Injection into Immune Compromised Mice with Normal Radiation Sensitivity

In order to test whether t-hESCs sustain tumour growth following ionizing radiation, similar to CSC population, intratesticular (IT) injections of both normal and transformed hESCs were performed and resulting tumours were irradiated as outlined in Figure 3.1a. To minimize the effects of non-targeted irradiation, and maximize the dose that could be delivered to the tumours, lead shielding was constructed to localize radiation dose (Figure 3.1b). To ensure the shielding was effective in reducing non-targeted radiation, and quantify the internal dose received in the scrotum, thermo-luminescence dosimeter (TLD) chips were surgically implanted into the scrotum and small intestine of mice. 59-61.5% of the external dose was transmitted to the scrotum and 3.5-6.5% of the external dose was transmitted to the small intestine of mice, while in the shielding apparatus (Fig. 3.1c). As the SCID mutation has previously been shown to cause general defects in DNA repair (Fulop and Phillips, 1990), it was hypothesized that immune-compromised mice containing the SCID mutation might be hypersensitive to radiation, thus limiting their utility for the assessment of the radiation sensitivity of transplanted cells. As expected, mice harboring the SCID mutation had decreased survival after exposure to significantly lower doses of irradiation, when compared with the immune compromised NOD $Rag1^{-/-}$ $Il2r\gamma^{-/-}$ (NRG) mouse strain (Fig 3.1d). After IT injection mice were palpated weekly and

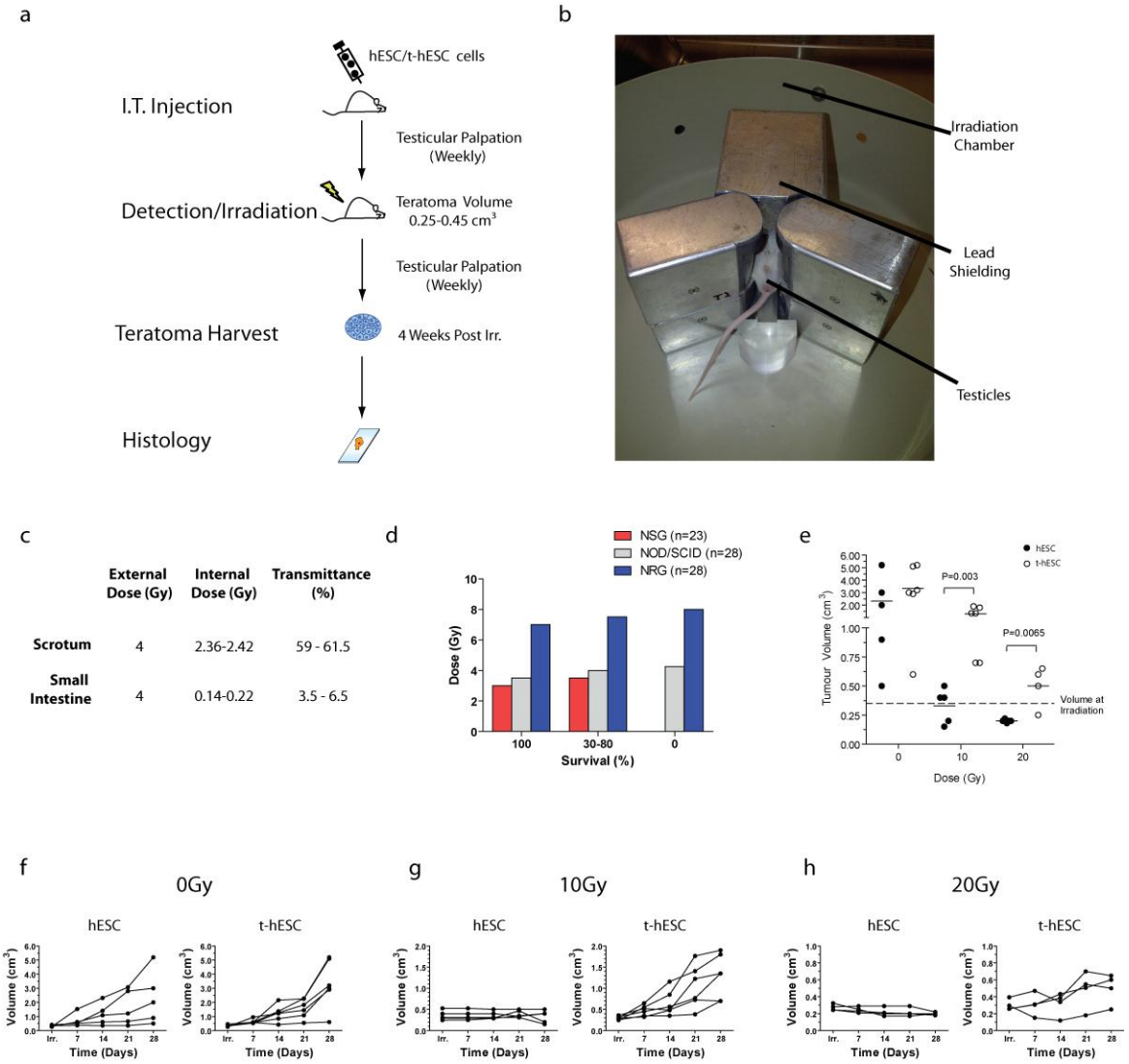


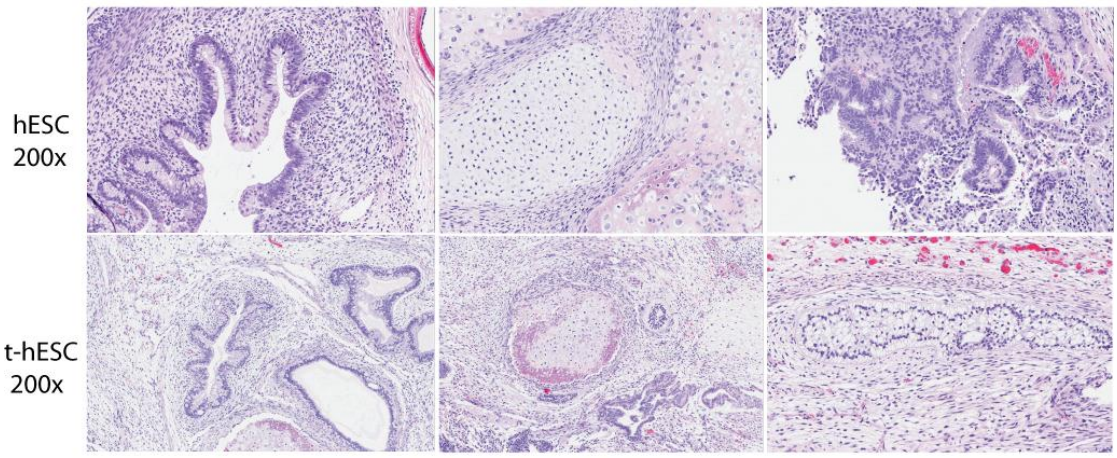
Figure 1. t-hESC derived tumours are radiation resistant relative to their hESC counterpart. (a) Schematic representation of experimental design. (b) Lead shielding apparatus. (c) Internal dose mice received while in lead shielding measured using of thermo-luminescence dosimeter (TLD) chips. (d) Survival of NOD/SCID, NOD/SCID Il2$\gamma^{-/-}$ (NSG) and NOD Rag1$^{-/-}$ Il2r$\gamma^{-/-}$ (NRG) mice 8 weeks post irradiation. (e) Final teratoma volume measured by displacement 4 weeks post irradiation show significantly larger teratomas derived from t-hESCs, relative to hESCs. (f-h) Teratoma volumes measured via weekly testicular palpations show no increased growth in teratomas derived from hESCs following either 10 or 20 Gy doses of Cs-137 γ irradiation and continued growth in multiple t-hESC derived teratomas following the same doses.

irradiated when teratoma volume reached 0.25-0.45cm^3. Four weeks later tertomas were harvested and measured by displacement. t-hESC derived teratomas were significantly larger than hESC derived teratomas following both 10Gy (P=0.003) and 20Gy (P=0.0065) doses of Cs-137 γ radiation (Figure 3.1e). Teratoma palpation volume shows that while hESC derived teratomas ceased to continue growing following irradiaion the majority of t-hESC derived teratomas continued to grow after exposure to both 10Gy (Fig 3.1g) and 20Gy doses (Fig 3.1h).

3.2 t-hESC Derived Tumours Retain Differentiation Capacity and Oct-4 Expression Following Irradiation

To test whether there was a co-relation between radiation response and the maintenance of pluripotent cells following irradiation, tumours were embedded in paraffin and analyzed via immunohistochemistry for their capacity to form tumours containing all 3 germ layers (teratomas), as well as for the presence of the pluripotency marker Oct-4. Both hESCs and t-hESCs were capable of forming teratomas when not irradiated (Fig. 3.2a) and both cell types were capable of forming all 3 germ layer even after irradiation (Fig. S1). However, the capacity for teratoma formation decreased in hESCs, but not t-hESCs, in a dose dependent fashion following exposure to radiation and differentiation was directed towards ectodermal fate in these tumours (Fig. 3.2b). The maintenance of differentiation capacity in t-hESCs, following irradiation, correlated with the detection of Oct-4+ cells in t-hESC tumours in 10Gy irradiated tumours (Fig. 3.2c).

a



b

		Teratomas	Endoderm	Ectoderm	Mesoderm	Oct-4+
hESC	**0 Gy**	5/5	5/5	5/5	5/5	0/5
	10 Gy	1/5	5/5	5/5	2/5	0/5
	20 Gy	0/5	4/5	4/5	1/5	0/5

c

		Teratomas	Endoderm	Ectoderm	Mesoderm	Oct-4+
t-hESC	**0 Gy**	4/6	4/6	6/6	6/6	5/6
	10 Gy	5/5	5/5	5/5	5/5	2/5
	20 Gy	5/5	5/5	5/5	5/5	0/5

d

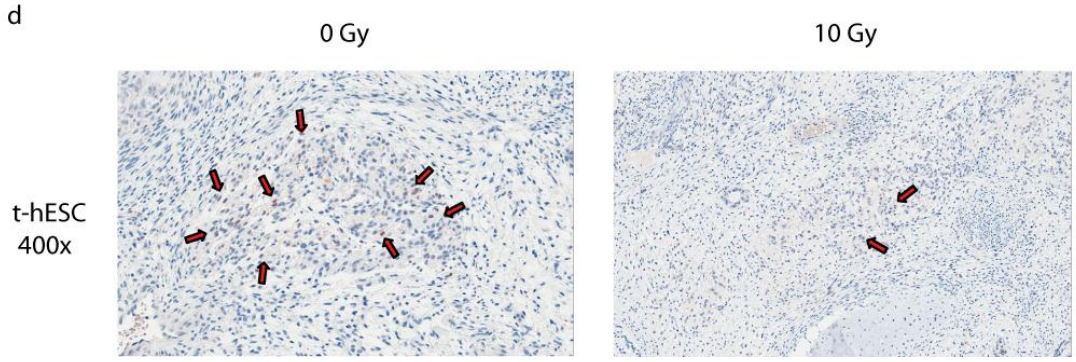


Figure 3.2. Tumours derived from t-hESCs retain teratoma formation capacity following irradiation. (a) Immunohistochemical analysis of the three germ layers present in teratomas. (b) Teratoma formation capacity is lost in a radiation dose dependent fashion and no Oct-4 positive cells were observed in hESC derived tumours. (c) t-hESC derived tumours do not lose teratoma formation capacity in response to radiation and Oct-4 positive cells were present in un-irradiated, as well as 10Gy irradiated tumours. (d) Immunohistochemical analysis show Oct-4 expression (brown staining nuclei).

3.3 t-hESCs are Resistant to Radiation Induced Apoptosis Relative to Normal hESCs

Unlike primary CSC samples, our model system affords the unique capacity for long term *in vitro* culture of an enriched human cancer stem cell population. This allowed for analysis of the mechanisms of radiation resistance and direct comparison with a normal stem cell counterpart. For *in vitro* cell studies the cell-surface pluripotency marker SSEA3 was utilized, as it did not require fixation and nuclear permeabilization of cells, thus allowing analysis of the apoptotic response of pluripotent cells. A significantly greater decrease in the # of SSEA3+ cells in hESC cultures relative to t-hESC cultures was observed 72 hours following all radiation doses tested (Fig 3.3a). To minimize the effects of cellular necrosis on apoptosis induction, the lowest dose (4 Gy) was used while quantifying the apoptotic response. Morphological changes were observable in hESC, but not t-hESC cultures by 8hrs post irradiation (Fig 3.3b). 4 hours following exposure to a 4Gy dose of Cs-137 γ rays an approximately three-fold increase in the proportion of normal hESCs undergoing apoptosis (Annexin V+, 7AAD-) was observed, however a significant increase was not observed in t-hESCs (Fig. 3.3d). A slightly decreased frequency of apoptosis was observed in the SSEA3- population of cells in normal hESC cultures relative to SSEA3+ cells in the same cultures (Figure S2), however too few SSEA3- cells were present in t-hESC cultures for analysis. The proportion of cells undergoing apoptosis correlated with a decreased number of viable cells (as measured by trypan blue exclusion) in hESCs, but not t-hESCs (Fig. 3.3e). This decreased induction of apoptosis in t-hESCs also correlates with a decreased number of Oct-4 positive cells in hESC, but not t-hESCs 6 hours following irradiation (Fig. S3).

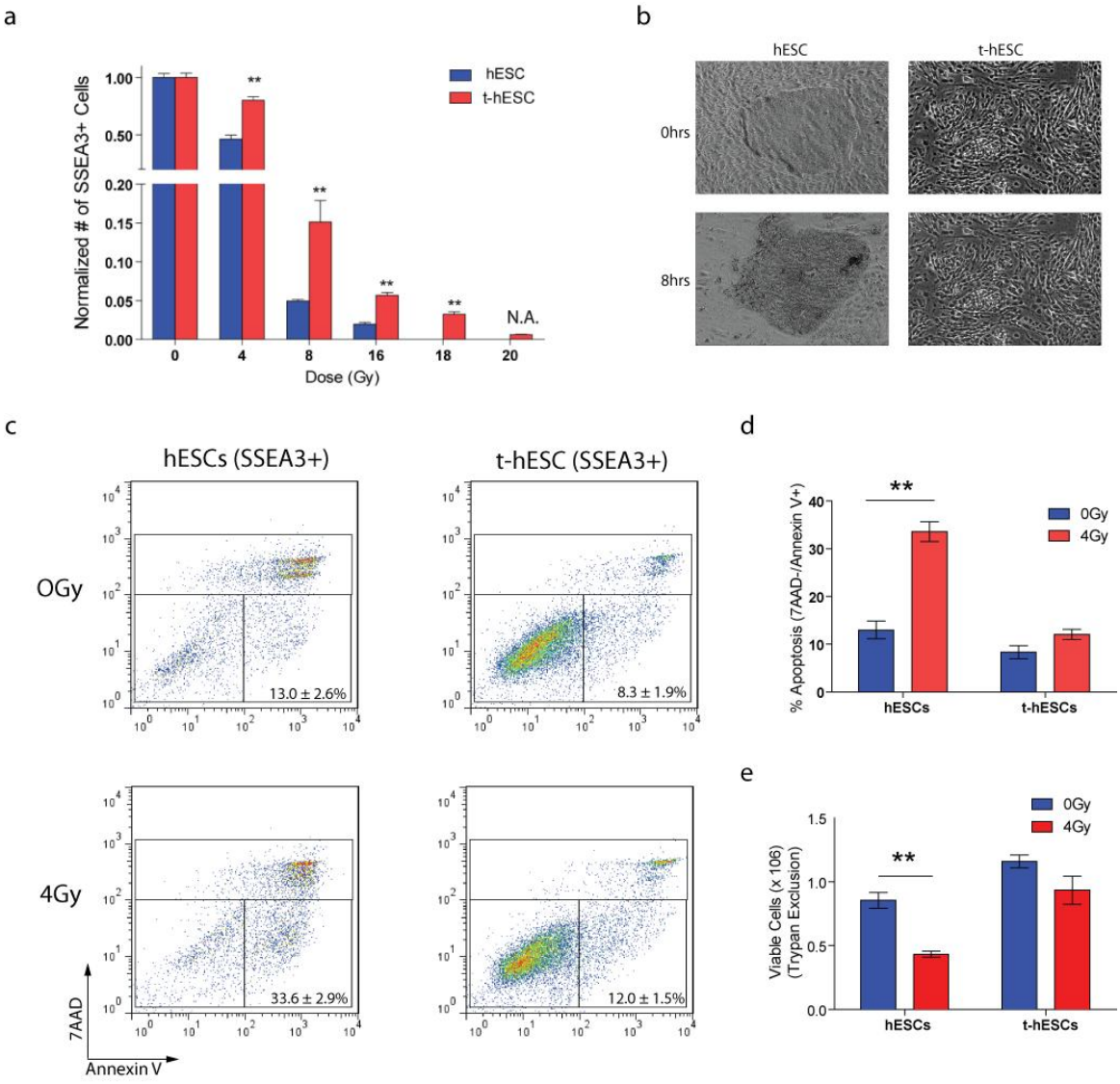


Figure 3.3 t-hESCs have a decreased propensity for radiation induced cell death via apoptosis relative to hESCs. (a) Total cell count and SSEA3 analysis by FACS reveal a greater decrease in the number of SSEA3+ hESCs relative to t-hESCs at all doses of γ radiation 72 hours post irradiation (**,$P < 0.01$, ***.,no cells observed in hESC plate, n = 6). (b) Phase contrast images of hESCs and thESCs 0 or 8 hours after exposure to a 4Gy dose of γ irradiation. (c) Representative Flow-Cytometry patterns. X-axis, externalization of phosphatidylserine shown by Annexin V binding, y-axis, DNA content shown by 7AAD binding.(d) Relative percentages of cells undergoing apoptosis (7AAD-, Annexin V +) show an increase in the induction of apoptosis in hESCs (SSEA3+) relative to t-hESCs (SSEA3+) 8 hours after exposure to a 4 Gy dose of γ irradiation (**,$P < 0.01$, n = 4). (e) Relative number of viable hESCs and t-hESCs measured via trypan blue, 8 hours after exposure to a 4 Gy dose of γ irradiation, shows a significant decrease in the number of viable hESCs, but not t-hESCs (**, $P < 0.01$, n = 4)

3.4 t-hESCs Show Altered Cell Cycle Arrest Relative to Normal hESCs

Following exposure to ionizing radiation somatic cells undergo cell cycle arrest at both the G1 and G2 checkpoints (Fournier and Taucher-Scholz, 2004), however the G1 checkpoint has been shown to be absent in hESCs (Momcilović et al., 2009). hESCs and t-hESCs were incubated for 1 hour with the thymidine analog bromodeoxyuridine BrdU and stained for DNA content with 7AAD, for analysis of the cell cycle response following ionizing radiation. Normal hESC cultures exhibited a characteristic ES cell cycle arrest in the G2 phase of the cell cycle in both the SSEA3+ and SSEA3- fractions, 12 hours following irradiation. However, t-hESCs exhibited a greater frequency of cells in G1 (Fig. 3.4g) and a lower frequency of cells in G2 (Fig. 3.4i), relative to their hESCs, a cell cycle response intermediate to the immortalized somatic fibroblast cell line AGO1522 and hESCs (Fig. S3).

3.5 t-hESCS Exhibit Decreased Levels of ser-15 Phosporylated p53 Follwing Irradiation

As p53 has been shown to be involved in the activation of multiple cellular responses to ionizing radiation, including both cell cycle response and apoptosis (reviewed by Vousden and Prives, 2006) we next assessed levels of the activated form of p53 (p53 phosphorylated at serine 15). Significantly lower levels of p53 (phospo-ser15) were detected in t-hESCs, relative to hESCs, 6 hours following all doses of irradiation tested (Fig. 3.5b). To assess whether the lack of p53 activation was a result of decreased levels of initial DNA DSB induction in t-hESCs, which could potentially result from

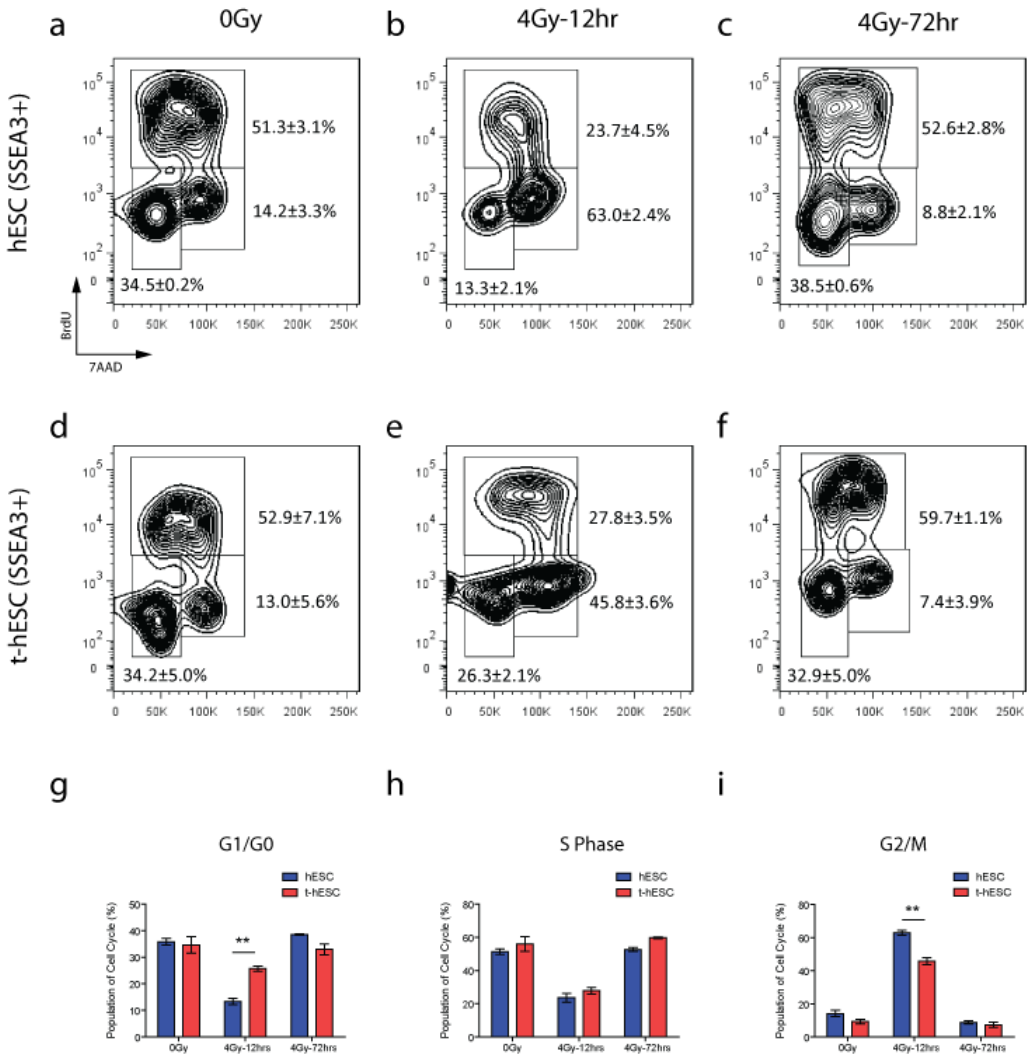


Figure 4 t-hESCs show a decreased propensity for G2 cell cycle and an increased frequency of cells in G0-G1 relative to hESCs in response to ionizing radiation. (a-f) Flow-cytometry patterns. X-axis, DNA content shown by 7-AAD binding; y-axis, Brd U uptake after a 1-h exposure. (g-f)Relative percentages of phase-specific populations show that while both hESCs and t-hESCs show an increase in the proportion of cells in G2/M, 12 hours following an exposure to 4Gy of γ irradiation, a greater percentage of hESCS are observed relative to t-hESCs (**, $P < 0.01$, $n = 4$). The relative percentages of phase-specific populations returned to those of un-irradiated cells by 72 hours post irradiation in both cell types.

increased free radical scavenging capacity in t-hESCs, we next assessed p53 activation in response to the DNA DSB inducing agent etoposide. As etoposide induces DNA DSBs through inhibition of topoisomerase II, etoposide DSB induction is not influenced by a cells free radical scavenging capacity. 24 hrs following the addition of topoisomerase, p53 (phospo-ser15) levels were significantly lower in t- hESC cells at both 1 and 10 μM concentrations, indicating defective activation of p53 following the induction of DNA DSBs (Fig 3.5c).

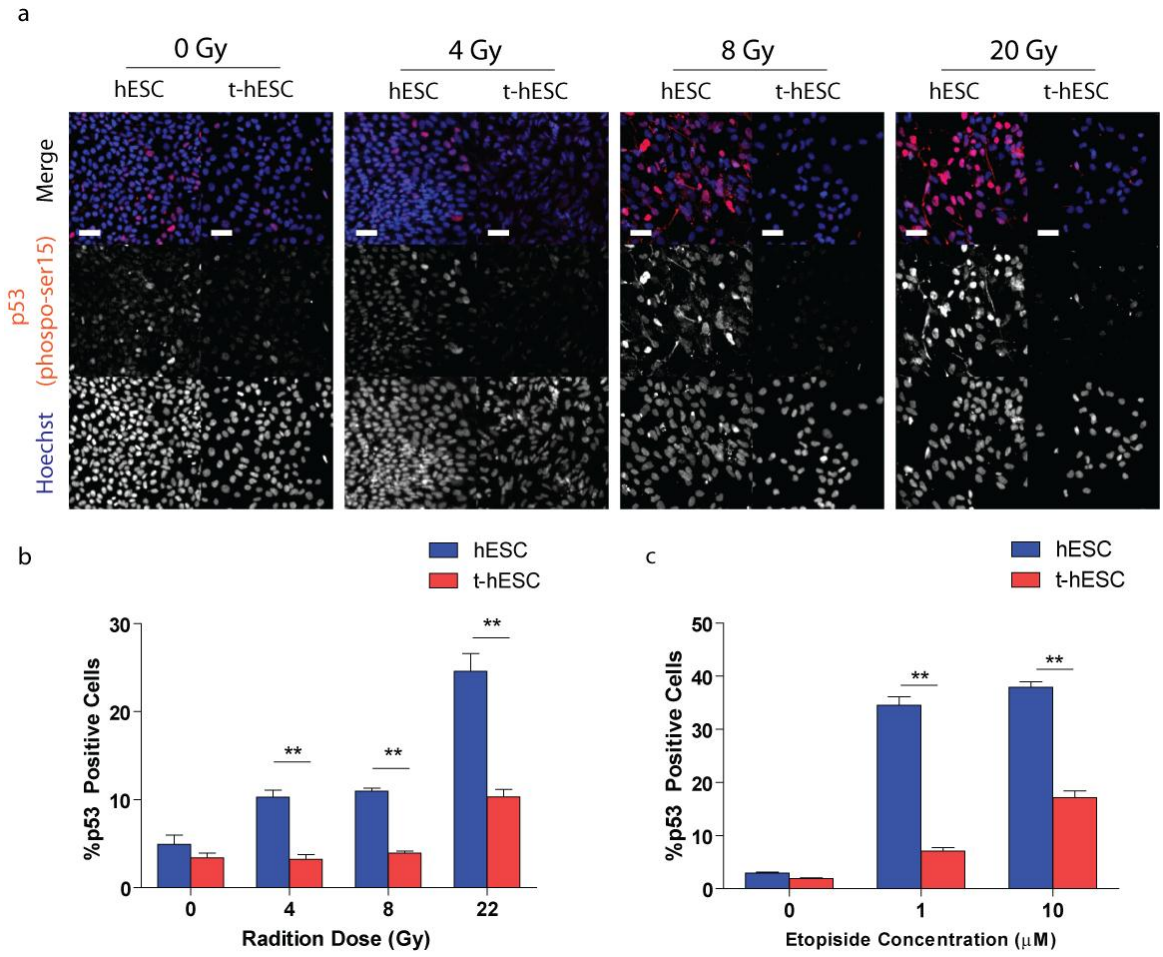


Figure 5 t-hESCs show decreased levels of p53 (phosphor-ser15) in response to γ irradiation. (a) Immunocytochemical staining of hESCs and thESC 6 hours after exposure to 0, 4, 8, or 22Gy of γ irradiation. (b) Immuno-cytochemical analysis shows a greater increase in the percentage of p53 (phosphor-ser15) positive cells in hESCs relative to t-hESCs, 6 hours following all doses of γ irradiation (**,$P < 0.01$, $n = 6$).

4. Discussion

4.1 Summary

It has been hypothesized that recurrence of tumorigenesis post radiotherapy is a result of an inability to eliminate radiation resistant CSC population within a tumour (Rich, 2007). Enhanced radiation resistance has been observed in multiple CSC populations, relative to non-CSCs residing in the same tumour (Bao et al., 2006; Blazek et al., 2009; Charafe-Jauffret, 2009; Chiou et al., 2008; Diehn et al., 2009; Phillips et al., 2006; Zhang et al., 2010). However, due to difficulty maintaining CSCs and difficulty identifying normal counterparts for analysis *in vitro,* no comparison with a normal stem cell counterpart has been made to date. Here for the first time we have shown that the transformation of stem cells towards a neoplastic state is associated with enhanced radiation resistance. We have shown that a decreased capacity for p53 activation (Fig. 5b) in transformed stem cells is associated with aberrant apoptotic (Fig. 3.3d) and cell cycle responses (Fig. 3.4g-i) following irradiation *in vitro*. Further we have shown that transformed stem cells form tumours, upon injection into immune-compromised mice which are more difficult to eliminate with radiotherapy (Fig. 1e) and retain their pluripotency potential following irradiation (Fig. 2b). The ability to easily culture t-hESCs *in vitro,* provides a unique opportunity for mechanistic studies of radiation resistance, as well as an opportunity for the screening of potential radiosensitizing agents.

4.2 Radiation Resistant Tumours derived from t-hESCs

Radiation resistant tumours comprise a serious obstacle for the clinical treatment of multiple types of cancers including breast (Hayward and Caleffi., 1987), head and neck (Lindber and Fletcher., 1978), and prostate (Fuks., 1991). Here we have produced tumours comprised of multiple tissue types, derived from either normal or transformed hESCs. We have shown that while treatment of tumours derived from normal hESCs with irradiation was effective at stopping tumour growth (Fig. 1g-h), tumours derived from t-hESCs continued to grow following irradiation. The ability of t-hESCs to continue to produce teratomas following irradiation, as well as their prolonged expression of Oct-4 suggests the maintenance of multi-potent population within the tumour following radiation. This is analogous to the proposed role of CSCs in failed radiotherapy attempts and the subsequent recurrence of tumorigenesis (Rich, 2007; Pajonk et al., 2010). However, unlike CSC populations exhibiting radiation resistance isolated from primary tumours and cell lines, we are able to isolate these cells and compare them directly with a counterpart which is capable of producing heterogeneous tumours that are not resistant to radiation. This implicates that the capacity for both self-renew, as well as the production of multiple cell types within a tumour may not be inherently linked to radiation resistance, and that further alterations to CSC populations could result in the radiation resistant phenotype observed in multiple cancers.

4.3 Decreased Induction of Apoptosis, Altered Cell Cycle Response, and Abberant p53 Response in t-hESCs Following Ionizing Radiation

Successful elimination of all the cells comprising a tumour during radiotherapy is dependent on the capacity to induce apoptosis in these cells (Dewey et al., 1995). It has been shown that multiple CSC populations are resistant to the induction of apoptosis (Bao et al., 2006; Diehn et al., 2009; Phillips et al., 2006), therefore these cells are thought to be responsible for recurrent tumorigenesis following radiotherapy. Activation of p53 has been shown to be required for the induction of apoptosis in hESCs in response to DNA DSBs (Grandela et al., 2008). Here, we have shown that the decreased capacity for the induction of apoptosis in t-hESCs is associated with an inability to activate p53 (Fig. 3.5b). This establishes a role for p53 in potentially enhancing radiation resistance in CSC populations within a tumour. We have shown that the capacity to activate p53 is decreased in t-hESCs relative to hESCs in response to etoposide treatment (Fig. 3.5c), as well as radiation, indicating that aberrant activation is the result of a process downstream of DNA DSB induction. It is possible that the lack of activation results from enhanced DNA repair such as has been observed in gliobastoma CSCs (Bao et al, 2007) or from abberant DNA damage recognition and signalling, involving proteins such as ATM and the MRN complex (Mre11, Rad50, Nbs) (Lavin, 2007).

Ionizing radiation induced DNA damage results in cell cycle arrest at both the G1 and G2 phases of the cell cycle in proliferating somatic cells (Kastan et al., 1992). However, G2 cell cycle arrest, following irradiation has been shown to be largely absent in hESCs (Momcilović et al., 2009). The absence of G1 cell cycle arrest in response to genotoxic

stress has been shown to be mediated by inhibition of p21 translation by the micro RNA family miR-302 (Card et al., 2008; Dolezalova et al., 2012). This microRNA family has also been shown to inhibit the tumorgenicity of human pluripotent stem cells via modification of CDK2 and CDK4/6 cell cycle pathways (Lin et al, 2010). The presence of increased G1 cell cycle arrest in t-hESCs relative to hESCs (Fig. 4g), as well the enhanced tumour initiating capacity of t-hESCs, relative to hESCs (Werbowetski et al., 2009) implicate a potential role for miR-302 and the cell cycle response in enhanced tumorogenecity and radiation resistance observed in t-hESCs.

4.4 Conclusion

The primary goal of this work was to evaluate the radiation response of normal and transformed human embryonic stem cells to potentially identify characteristics of transformation in stem cell population that correlates with radiation resistance observed in multiple CSC populations. We observed that, t-hESCs were radiation resistant relative to normal hESCs both *in vivo* and *in vitro*, and that this radiation resistance correlated with aberrant activation of p53. This work has resulted in a unique mode of quantification of radiation sensitivity of human tissues *in vivo*, as our group is the first to identify a severely immune deficient mouse capable sustaining complex human tumour growth as well as surviving clinically relevant doses of irradiation. This mode of quantification of the *in vivo* radiation sensitivity has great potential utility for studying the radiation response of multiple types of cancers. Further, we have for the first time directly compared the radiation response of a transformed stem cell to its normal counterpart and found that the normal counterpart is more sensitive to radiation. This result implicates the

need for molecules/techniques that will help to either enhance the ability to eliminate transformed stem cells relative to normal stem cells, or to protect only normal stem cells from damage. The capacity to isolate a purified population of radiation resistant human transformed stem cells *in vitro*, and directly compare them to a normal counterpart, provide an ideal platform for the identification of such molecules/techniques.

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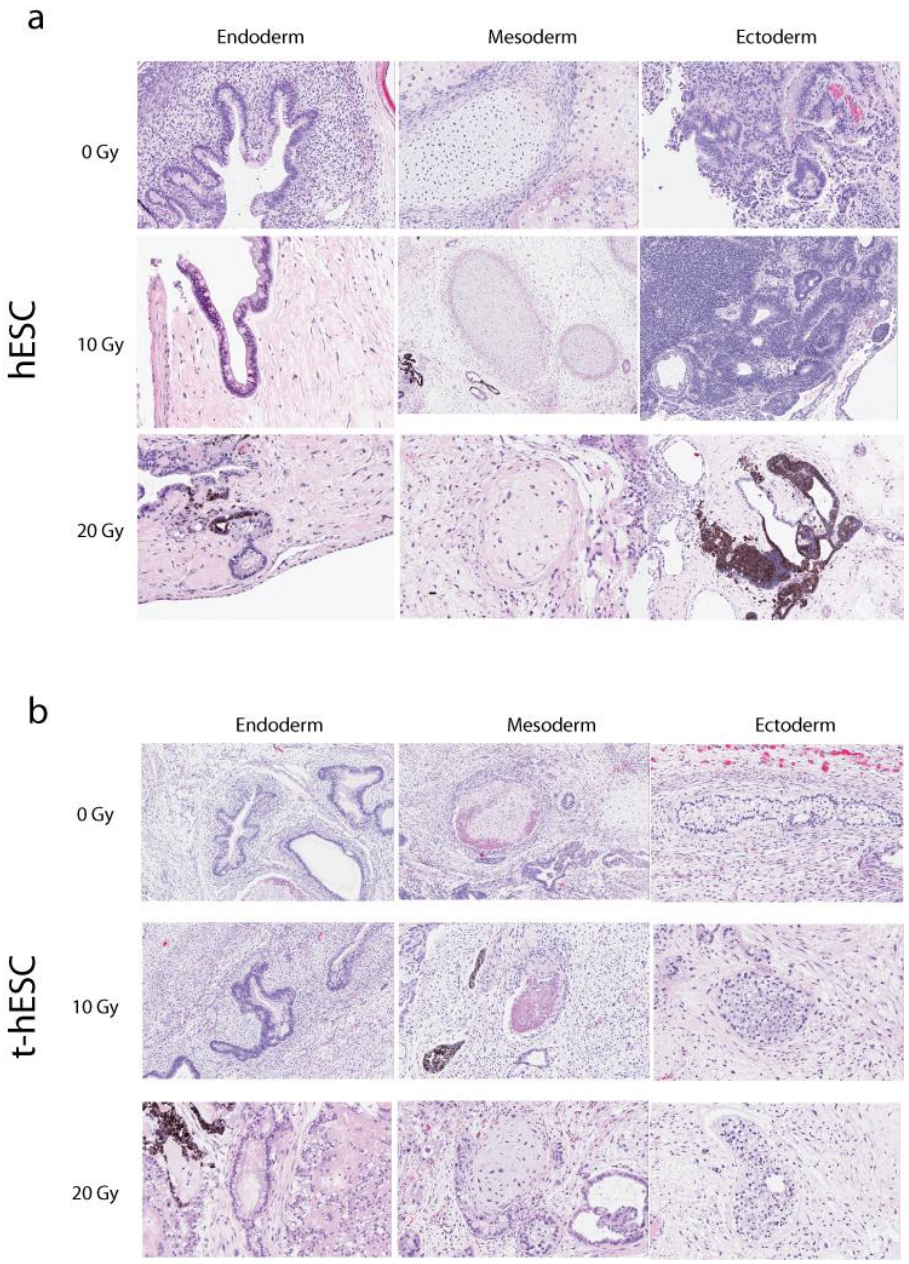
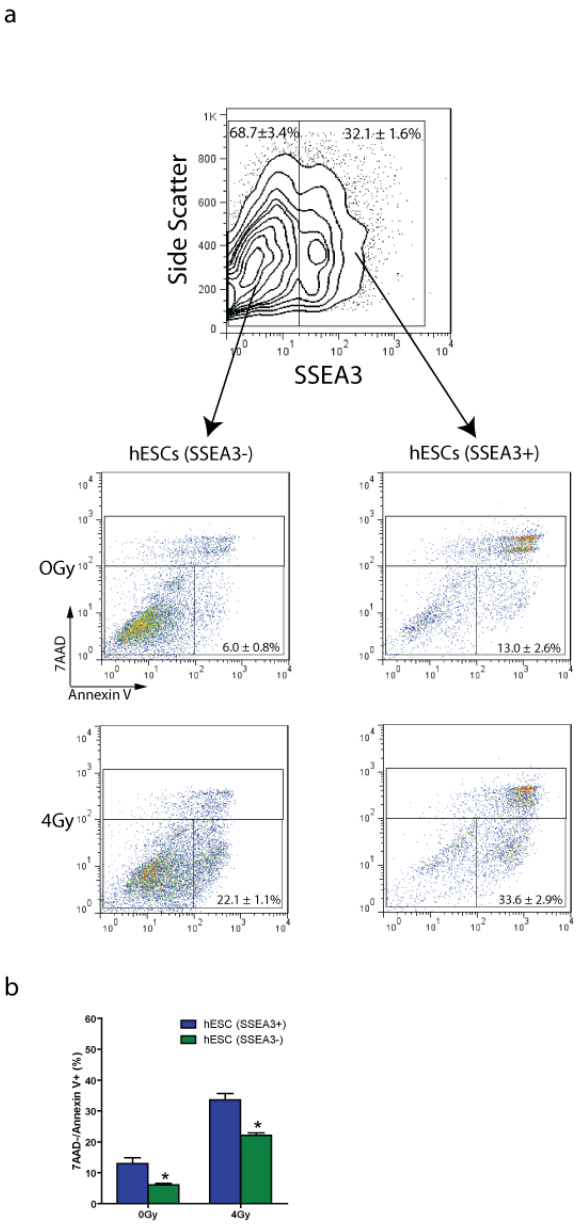
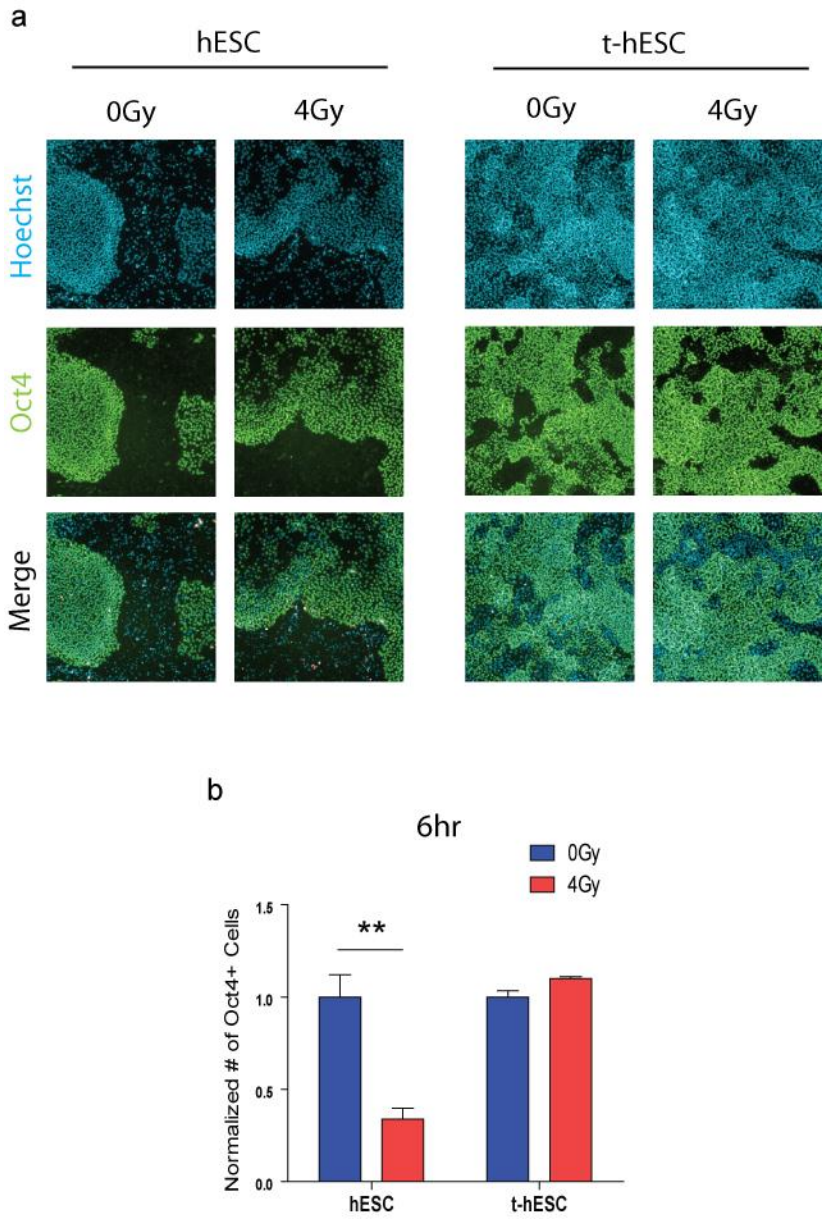
6. Supplemental Data



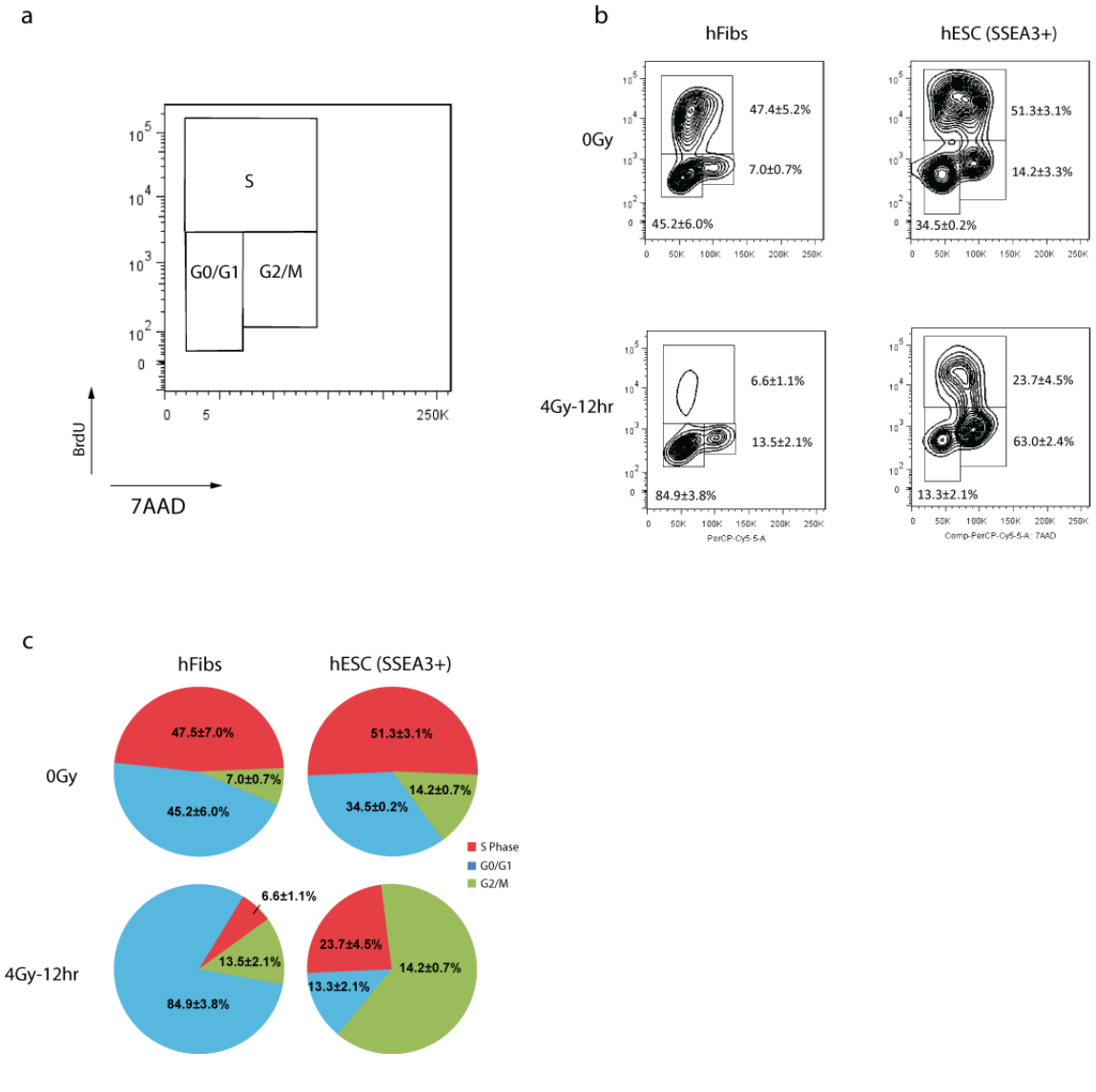
Supplemental Figure 1: hESCs and t-hESCS have some potential for formation of all three germ layers following irradiation. At least one tumour from both normal hESC (a) and t-hESC (b) injections was capable of differentiating towards endodermal, mesodermal or an ectodermal fate.



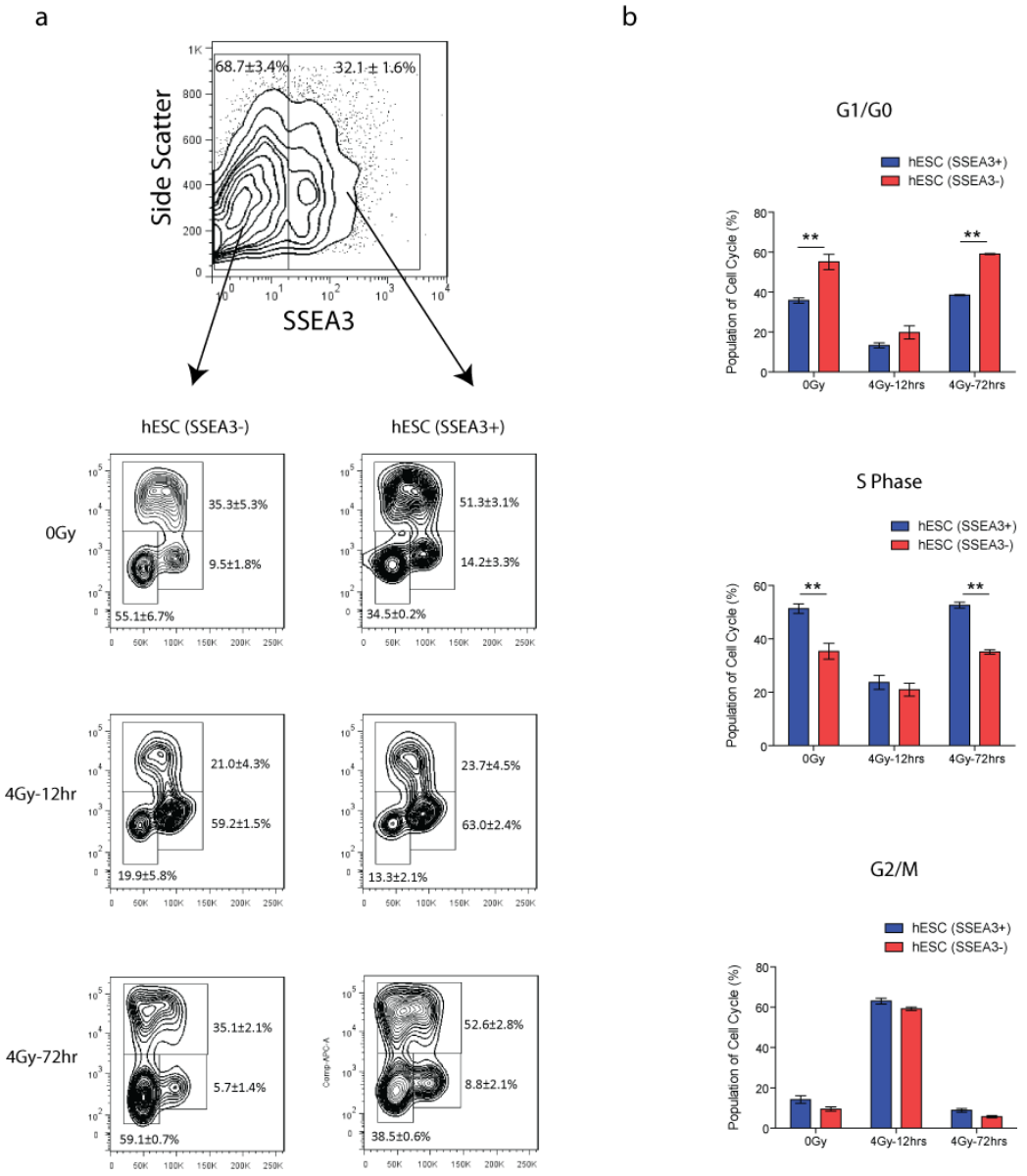
Supplemental Figure .2 SSEA3- hESCs show decreased spontaneous and radiation induced apoptosis relative to SSEA3+ hESCs. (a) Flow-cytometry patterns. X-axis, DNA content shown by 7-AAD binding; y-axis, Brd U uptake after a 1-h exposure. (b) Relative percentages of cells undergoing apoptosis (7AAD-, Annexin V +) show higher levels of both spontaneous and radiation induced apoptosis in SSEA3+ hESCS relative to SSEA3- hESCs 8 hours after exposure to a 4 Gy dose of γ irradiation (**,$P < 0.01$, n = 4).



Supplemental Figure 3. t-hESCs show an increased proportion of Oct4 positive cells present 6 hours post irradiation. (a) Immunocytochemical staining of hESCs (left panel) and t-hESCs (right panel) 6 hours after sham irradiation (0 Gy) or exposure to a 4 Gy dose of γ irradiation (dose rate = 0.344 Gy/min). (b) Immunocytochemical analysis shows a significant decrease in the number of Oct4 + hESCs while no significant decrease was observed in the t-hESCs (**,$P < 0.01$, $n = 3$).



Supplemental Figure 4. hESCs Predominantley reside in G2/M 12 hrs post irradiation while firbroblasts predominately reside in G1. (a) Schematic representation indicating cell cycle state of cells withing flow cytometry plot. (b) Flow-cytometry patterns. X-axis, DNA content shown by 7-AAD binding; y-axis, Brd U uptake after a 1-h exposure. (c) Relative percentages of phase-specific populations show hESCs accumulate in G2/M, while normal human fibroblasts predominately accumulate in G0/G1 12hours after receiving a 4Gy dose of γ irradiation (**, $P < 0.01$, $n = 4$).



Supplemental Fig. 5 A greater portion of SSEA3- hESCs reside in G0/G1 relative to SSEA3+ hESCS, however similar cell cycle distribution is observed in response to irradiation. (a) Flow-cytometry patterns. X-axis, DNA content shown by 7-AAD binding; y-axis, Brd U uptake after a 1-h exposure. **(b)** Relative percentages of phase-specific populations show a greater proportion of SSEA3- hESCs reside in G0/G1 and less in S phase under normal conditions, however similar cell cycle distribution is observed 12 hours following an exposure to 4Gy of γ irradiation (**, $P < 0.01$, $n = 4$).