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Title Page:

An evaluation of novel real-time technology as a tool for measurement of radiobiological and radiation induced bystander effects.

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ABSTRACT:

The xCELLigence Real Time-Cell Impedance System uses a non-invasive and label free method to create a cell index that is a composite measure of cell proliferation. The aim of this study was to evaluate xCELLigence against clonogenic assay (gold standard) for measuring radiobiological effects and radiation-induced bystander effects (RIBE). A radiobiological study was conducted by irradiating EMT6.5, 4T1.2 and NMUMG cell lines with different radiation doses, while a RIBE study was done using transfer of conditioned media (CM) harvested from donor to the same type of recipient cell (EMT6.5, 4T1.2, NMUMG, HACAT and SW48). CM was harvested using two protocols which differed in the dose chosen and the exposure to the recipient cells. Results showed that xCELLigence measured a radiobiological effect which correlated with the clonogenic assay. For the RIBE study, no statistically significant differences were observed between xCELLigence or clonogenic survival in control or recipient cells incubated with CM in protocol one. However, there was a significant increase in cell index slope using CM from EMT-6.5 cells irradiated at 7.5 Gy compared to the control group under the second protocol. No other evidence of RIBE were detected by either xCELLigence or clonogenic assay. In conclusion, xCELLigence methods can measure radiobiological effects and the results correlate with clonogenic assay. We observed a lack of RIBE in all tested cell lines with the clonogenic assay, however we observed a RIBE effect in EMT6.5 cells under one particular protocol that showed RIBE is cell type dependent, not universally observed and can be detected in different assays.

KEYWORDS:

Radiotherapy, Bystander effects, clonogenic assay, xCELLigence RT-CIS

INTRODUCTION

Non-targeted effects of radiotherapy were first demonstrated over 2 decades ago (H Nagasawa and Little 1992) and have been a controversial subject since. These non-targeted effects are known as radiation-induced bystander effects (RIBE) (Prise et al. 2003) and can be defined as biological effects that occur in cells/ tissues that have not been directly traversed by ionizing radiation but are in close proximity to cells that have been traversed.

Several studies have demonstrated the non-targeted effects of radiotherapy where radiation-induced damage occurs indirectly in various experimental designs (Furlong et al. 2013; C. Mothersill and Seymour 1997; C. Mothersill and Seymour 1998; C. E. Mothersill et al. 2004b; Vines et al. 2008). The majority of outcomes on RIBE have shown that ionizing radiation elicits damage to adjacent cells, however several publications have shown an absence or a lack of a bystander effect (Belyakov et al. 2006; Groesser et al. 2008; Sowa et al. 2010; Terzoudi et al. 2010; Zyuzikov et al. 2011).

There are several possible mechanisms to explain bystander effects, usually classified into two main types; either an effect which is mediated through gap-junctions and requires cell to cell contact (Azzam et al. 2001) or, the secretion of soluble factors/ signals by irradiated cells (Belyakov et al. 2006; Butterworth et al. 2011; C. Mothersill and Seymour 1998). To study these mechanisms, two major approaches have been used *in vitro*; (a) direct targeting of individual cells within a population, or (b) media transfer method in which donor cells are irradiated and the media collected after a certain time (30 minutes – 24 hours). Unirradiated cells are then exposed to the CM. There are different biological end points for measuring bystander effects, for example increased sister chromatid exchanges (SCE), chromosomal abnormalities, micronucleus formation, genomic instability, and phosphorylated-H2AX formation (Konopacka et al. 2011; Morgan 2003a, 2003b; Morgan and Sowa 2007; H. Nagasawa et al. 2005)

Despite the evidence and proposed mechanisms in the literature regarding RIBE (Hei et al. 2008; Morgan 2003b; C. Mothersill and Seymour 2006; Prise and O'Sullivan 2009; Sowa et al. 2010), no consensus regarding a mechanism has been reached. Various studies have suggested a role for different cytokines and signalling molecules that might be responsible for RIBE, including reactive oxygen species (Lyng et al. 2011), TGF-β (Gow et al. 2010; Shao et al. 2008), cyclooxygenase -2 (Hei et al. 2008) and calcium influx (Shao et al. 2006).

There are various challenges to studying the RIBE and the mechanisms involved, particularly because of the different experimental designs that have been chosen and the parameters that have been investigated (Vines et al. 2009). In short, radiation induced bystander effects are highly variable, cell type dependent and not universally observed (Groesser et al. 2008).

The clonogenic assay is the 'gold standard' method for measuring radio-biological effects. This cell survival assay is based on the ability of a single cell to grow into a colony where a colony is defined to consist of at least 50 cells. Clonogenic assay may take up to 2 weeks to obtain the results depending on cell type. There are limitations to using clonogenic assay as a routine approach including variable assay success rates, labour costs and perhaps most significantly, long time required (2-4 weeks) to generate a result (Franken et al. 2006; Warenius and Bleehen 1982; West and Sutherland 1986).

The xCELLigence RT-CIS technology (Roche Applied Science) offers a real-time, non-invasive and label free method to access cellular proliferation, cell viability, and migration. This method uses impedance changes to measure rate of cell proliferation (Atienza et al. 2006; Limame et al. 2012). Several studies have shown the xCELLigence RT-CIS can be used to measure radiobiological effects and the results of the impedance assay agreed with clonogenic data, providing sensitive measurements within 72 hours compared to 14 days for the clonogenic assay (Limame et al. 2012; Roa et al. 2011).

The main goal of this study was to evaluate the xCELLigence RT-CIS technology as a tool for measurement of radiation dose effects and radiation induced bystander effects in an *in vitro* model using different cell types. The first aim was to validate and optimize the xCELLigence assay with clonogenic and cell proliferation assays using unirradiated and directly-irradiated cells. The second aim was to use the xCELLigence assay to investigate bystander effects in different cell lines and compare these with clonogenic assay. We hypothesized the xCELLigence assay would be suitable for measuring dose-dependent effects of radiation on cells, and that we could use these assays to detect the RIBE in conditioned media taken from *in vitro* irradiated cells.

The results of our work are divided into two parts; the first part describes how we optimised and validated our assays i.e. the cell seeding densities, serum concentration and comparison of xCELLigence with clonogenic and traditional cell proliferation assays and the second part describes our scientific findings on RIBE by *in vitro* conditioned medium.

MATERIALS AND METHODS

Cell Culture

Various normal and cancer cell lines from animal and human sources were tested to establish the assays to study RIBE *in vitro*. These cell lines included the mouse tumour cell lines EMT 6.5 and 4T1.2, wild type macrophages (WTMacs), a mouse mammary epithelial cell line (NMUMG), a human keratinocyte cell line (HACAT) and a human adenocarcinoma cell line (SW48). The EMT6.5 and 4T1.2 cell lines were kindly provided by Prof. Robin Anderson, Peter MacCallum Cancer Centre, East Melbourne, Victoria, Australia. The NMUMG and WTMacs were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). The SW48 and HACAT cell lines, (ATCC, Manassas, VA, USA) were provided by Dr Nuzhat Ahmed from the Women's Cancer Research Centre, Royal Women's Hospital, Victoria, Australia. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Life Technologies, Mulgrave, Australia) supplemented with 10% Fetal Bovine Serum (FBS), 100 IU mL−1 penicillin G, and 100 µg mL−1 streptomycin (Gibco, Life technologies, Mulgrave, Australia) in a humidified incubator with 5% CO_2 at 37 °C.

Cell irradiation and conditioned media

In this study, we investigated the bystander effect using conditioned media (CM) that was harvested and then exposed to the same cell line. We employed two protocols to prepare the CM, which the protocols differed in the concentrations of serum supplement used to prepare the CM, how the CM was used to treat the recipient cells, whether it had been stored and thawed before use or freshly added to recipient cells after harvesting, and finally the dose chosen to irradiate the donor cells.

Protocol 1

Donor cells for providing the conditioned media were grown in T12.5 flasks in 5 ml DMEM media supplemented with 10 % FBS until 70 – 80% confluent. One hour before irradiation, the media was replaced

with DMEM containing 2% serum. Briefly, flasks were sealed and transferred in heat pack at 37^{o}C. Cells were irradiated using a Co-60 teletherapy unit at the Walter Eliza Hall Institute (WEHI), Melbourne, Australia at room temperature operating at a dose rate of approximately 2 Gy/min. For this protocol, the doses chosen were 5 Gy and 50 Gy. Control flasks were sham-irradiated. After irradiation, cells were returned to the incubator for 4 hours at 37^{o}C to allow cell communication or secretion of soluble factors into the media before the media were collected. Media were collected from the donor flasks and filtered using a 2 µm syringe filter to remove any debris or cells but retain soluble proteins and factors (Asur et al. 2012). From this step onwards, the media are referred to as conditioned media (CM). CM was aliquoted in eppendorf tubes (2 ml volume) for xCELLigence and 5 ml tubes for clonogenic assay and CM was stored in -80^{o}C freezer. Each eppendorf tube was thawed once for each experiment and the leftover was discarded.

Protocol 2

The second protocol for harvesting CM was similar to the first, with the following modifications: (i) The 10% serum was maintained for irradiation and CM collection instead of being replaced with 2% serum 1 hour prior to irradiation as in protocol 1, (ii) The radiation doses used were 1.5, 3.8 Gy or 7.5 Gy and (iii) The CM was used immediately after harvesting. CM was added to unirradiated recipient cells in 6 cm culture dishes for clonogenic assay and E-plates for xCELLigence RT-CIS assay.

xCELLigence Real time Cell Impedance Sensing (RT-CIS)

We determined cell proliferation using xCELLigence (RT-CIS). The impedance-based measurement is detected through gold electrodes in the bottom of the E-plate and is proportional to a combination of cell attachment, spreading, morphology, proliferation and metabolism. Integrated RTCA software expresses the cell impedance changes as the cell index value. Briefly, growth curves for each cell lines were established by culturing 100 µl of cell stock at various seeding densities (1 – 10 x 10^3 cells/ ml) that were harvested from 70-80% confluent cultures. Cell attachment and growth were monitored and impedance measurements were recorded every hour. Cell proliferation/ growth were analysed by calculating cell index changes over a period of time and expressed as cell index slope. For the RIBE study, the optimum seeding densities for each cell line were established and the cells seeded in DMEM media with 2% FBS and media were replaced with CM between 4 - 24 hours after cells were seeded in the E-plate.

Clonogenic assay

We used a modified clonogenic assay technique (Franken et al. 2006; Puck and Marcus 1956). Plating efficiency (PE) for each cell line was established by culturing various seeding densities (1 – 10 x 10^3 cells/ ml) harvested from 70-80% confluent cultures for 1 – 2 weeks. Colonies were fixed by staining with toluidine blue/70% ethanol and the plates were scanned using a gel image scanner (Image Scanner III, Labscan™ 6, GE Healthcare, Rydalmere, New South Wales, Australia). Colonies were counted using macros created in either ImageJ or Metamorph software v7 (Molecular Devices, LLC, Sunnyvale, CA, USA). PE was calculated by dividing the number of colonies formed by the number of seeded cells. The seeding density with the highest PE percentage was chosen for RIBE studies. Clonogenic assay for RIBE study was accomplished by seeding the recipient cells in the CM and results were analysed by survival curve. Radiation survival curves were constructed by normalizing the number of surviving colonies (irradiated cells) against the number of colonies in control plates (unirradiated cells).

Classic end point cell proliferation assays – Cell Titre Blue

Classic cell proliferation was measured using the CellTitre-Blue® cell viability assays (CTB) (Promega, Madison, WI, USA) according to manufacturer's instructions. This proliferation assay was used to validate with xCELLigence RT-CIS assay only. Briefly, cells were seeded in 96 well plates and incubated at 37°C for 24 h, 48 h, 72 h, 96 h, 120 h and 144 h to match the xCELLigence RT-CIS data. The CTB reagent was added into each well and incubated for two hours at 37°C before experiment termination each day until day 6. The fluorescent signal was measured at $560_{ex}/590_{em}$ nm using the FLUOstar OPTIMA plate reader (BMG LabTech, Mornington, Victoria, Australia). All assays were performed in triplicate. Data were expressed as absorbance (arbitrary units).

Optimization of cell number and serum concentration for xCELLigence and clonogenic assays

Initial experiments were conducted for each cell line to optimize the concentration of serum added to the conditioned media as well as the optimum cell number to seed for both assays. The goal was to use the lowest serum concentration to eliminate the effect of the intrinsic protein from the serum and establish the optimum seeding densities for each cell line for each assay. Optimization was conducted by seeding various cell numbers of each cell line in DMEM supplemented with 2%, 5% and 10% serum. The seeding density and serum

concentration that gave a lag phase within 3-5 days before plateauing was chosen for the xCELLigence RT-CIS assay and the highest PE percentage for the clonogenic assay.

Validation of xCELLigence with clonogenic assay and classic end point proliferation assay

We validated xCELLigence RT-CIS with a clonogenic assay and a classic cell proliferation assay (CTB) in unirradiated and irradiated EMT6.5, 4T1.2 and NMUMG cells. Similar cell seeding densities were used for all three assays. Results were correlated between the 3 different assays.

Statistical Analysis.

All data are expressed as mean ± SEM. Statistical analysis was conducted on raw data by one-way ANOVA followed by Tukey's post-hoc test ($p<0.05$ taken as significant) after testing for normal distribution using SPSS software version 20 (SPSS Inc, Chicago, IL). Spearman correlation coefficient was used to analyse correlation between absorbance and colony number/ survival rate with cell index.

RESULTS

Optimisation of clonogenic and xCELLigence RT-CIS assays in unirradiated cells

NMUMG, EMT6.5, WTMacs and 4T1.2 cell lines were grown in media supplemented with 2%, 5 % and 10% serum (Supp. Fig. 1). Using xCELLigence RT-CIS assay, cell index showed that each cell line had a specific growth pattern and the values were different between cell lines i.e.: EMT6.5> NMUMG> 4T1.2> WTMacs reflecting the proliferation of the cell, size of the cell and biological activity. The cell index was proportional to the concentration of serum (2%, 5% and 10%) in the media (Supp. Fig. 1). The 2% serum concentration was selected as the concentration to be added to the CM for all subsequent xCELLigence RT-CIS experiments as a compromise between reducing growth factor signaling by the serum but at the same time permitting a steady but low rate of cell growth within 3-5 days.

For the clonogenic assay, the results show that all cell lines formed colonies in media supplemented with 10% serum but showed very low Plating Efficiency (PE) percentages in 2% and 5% serum (Supplementary Fig. 2). Different cell lines exhibited different colony formation capabilities in different serum concentrations. Therefore, 10% serum concentration was used in subsequent clonogenic assay. Different cell numbers per plate were required for each cell line to produce good colony formation, with different incubation lengths resulting in different PE percentages.

Validation of xCELLigence RT-CIS, clonogenic and classic end point assays using unirradiated and irradiated cells

We measured the dynamic proliferation of different cell lines of EMT6.5, 4T1.2 and NMUMG using xCELLigence RT-CIS and classic cell proliferation end point assays by the Cell Titre Blue (CTB) assay at similar seeding densities for each well between two assays for 6 days and compared the proliferation pattern (Supp. Fig. 3). In unirradiated cells, results show that the proliferation profile measured by xCELLigence RT-CIS and CTB assays produced a similar growth pattern for all cell lines. Using the cell index and absorbance value, we plotted the correlation curve for each cell line (Fig. 1 a-c). There was a strong correlation coefficient between cell proliferation measured by the CTB and xCELLigence RT-CIS assays for each cell line: EMT6.5 (r^2=0.94, r=0.99, p<0.01)), 4T1.2 (r^2=0.93, r=0.96, p<0.01) and NMUMG (r^2=0.91, r=0.96, p<0.01). We also assessed the correlation between cell index value and colony number formation in the clonogenic assay (Fig. 2

a-c). Results indicate that there were strong correlations between the measured cell index and colony count at similar densities for each cell line: EMT6.5 (r^2=0.95, r=0.99, p<0.01), 4T1.2 (r^2=0.97 r=0.99, p<0.01) and NMUMG (r^2=0.98, r=0.99, p<0.01).

Using irradiated cells, similar results were observed between the proliferation profile measured by the CTB assay and xCELLigence RT-CIS assay for EMT 6.5 and 4T1.2, however the pattern not similar for NMUMG particularly at the highest dose (7.5) (Fig. 3). Furthermore there were strong correlations between cell index and absorbance for EMT6.5 (r^2=0.91, r=0.91, p<0.01), 4T1.2 (r^2=0.90, r=0.89, p<0.01) and NMUMG (r^2=0.80, r=0.97, p<0.01) (Fig. 4 a-c). For NMUMG, although the proliferation profiles measured by the CTB assay and xCELLigence were not similar, the correlation between these assays was significant. Fig. 5 a-c shows plots of correlation between cell index slope and cell cell survival fraction for EMT6.5, 4T1.2 and NMUMG in irradiated cells. There was a strong correlation between cell index and cell survival fraction using clonogenic assay for each cell line: 4T1.2 (r^2=0.95, r=0.99, p<0.01) and NMUMG (r^2=0.84, r=0.99, p<0.01). However, low correlation was observed in EMT6.5 (r^2=0.75, r=0.95) and the correlation level did not reach significance.

The effect of CM on recipient cell proliferation and survival fraction

Fig. 6 is a plot which demonstrates the lack of effect of CM supplemented with 2% FBS from recipient cells on cell proliferation of donor cells as measured via xCELLigence RT-CIS assay (protocol 1). Results are shown for EMT 6.5, 4T1.2, WT Macs and NMUMG cells. We did not observe any significant differences in cell index between sham controls and recipient cells incubated with 5 Gy and 50 Gy CM from donor cells irradiated in all tested cell lines, however there was a trend for a reduction in 4T1.2 cells.

Fig. 7 depicts the effect of CM supplemented with 10% FBS from recipient cells on cell proliferation of donor cells as measured via xCELLigence RT-CIS assay (protocol 2). Results are shown for EMT 6.5, 4T1.2, HACAT and SW48. There was a statistically significant increase in cell index slope in the EMT 6.5 cell line between sham control and recipient cells incubated with 7.5 Gy CM (p<0.05). However no significant differences in normalized cell index slope were observed in the other cell lines.

Fig. 8 shows the lack of effect of CM on cell colony formation by clonogenic assay for protocol 1. Results are shown for EMT 6.5, 4T1.2 and NMUMG cell lines. There were no significant differences in cell survival

fraction between sham controls and recipient cells incubated with CM 5 Gy and 50 Gy from donor cells irradiated in all tested cell lines. Fig. 9 is a plot which demonstrates the lack of effect of CM on cell colony formation by clonogenic assay for protocol 2. Results are shown for EMT 6.5, 4T1.2, HACAT and SW48 cell lines. There were no statistically significant differences in cell survival fraction between sham controls and recipient cells incubated with CM from donor cells irradiated with 1.5 Gy, 3.8 Gy and 7.5 Gy in all tested cell lines.

DISCUSSION

In the present work, we undertook a study to evaluate the potential of xCELLigence assays as a tool to measure radiobiological effects and the radiation-induced bystander effects (RIBE). We used a cell culture medium transfer method (referred to as conditioned media, CM), from irradiated donor cells to unirradiated recipient cells and compared results with those obtained using the clonogenic assay, an assay that is widely used to study RIBE (C. Mothersill et al. 2014).

The xCELLigence RT-CIS assay is a new technology that monitors cell growth in real-time but the basic principle is similar to that of other endpoint assays. Previous studies have showed that xCELLigence assays provided quick, rapid and repeatable outcomes (Atienza et al. 2006; Limame et al. 2012; Roa et al. 2011). In the first part of the study, we optimized the serum concentrations and seeding densities of different cell lines that we used in our laboratory for xCELLigence and clonogenic assays and we further validated the xCELLigence method with classic cell proliferation and clonogenic assays on both treated and untreated cells to investigate the capability of xCELLigence assay to measure the radiation dose effect. The rationale for using the low serum concentration was to reduce the effects of growth factor in the serum that might influence the results of the assays. It is important to choose the optimum seeding density for xCELLigence since the cells have different size and doubling times. Seeding at too higher cell numbers results in rapid cell growth in the limited growth factor environment of the culture chamber. As a consequence, cell growth passes through the exponential phase and plateaus within 24 to 48 hours, and the assay is then unable to show differential cell growth in response to different radiation exposures.

The results show that the xCELLigence assay can discriminate the effect of different radiation doses in a similar manner to clonogenic and CTB assays. A linear relationship between xCELLigence RT-CIS data and CTB in all cell lines and clonogenic assay in 4T1.2 and NMUMG cell lines confirmed that the xCELLigence RT-CIS system provides a robust approach to measure cell growth for the chosen cell line as reported by previous studies. Rao et al. (2011) demonstrated a strong correlation between cell index value and colony formation in the MCF-7 breast cancer cell line. Limame et al. (2012) showed xCELLigence RT-CIS was an accurate platform for non-invasive detection of cell viability using the MDA-MB-231 breast cancer cell line and reported strong correlation with sulforhodamine B (SRB) cell proliferation assay. Thus, xCELLigence RT-CIS can be used as a useful alternative to clonogenic assay. Our results clearly show that xCELLigence RT-CIS assay can

be used to determine radiation dose effect in irradiated cells in a short period of time and the ability to conduct high throughput screening with multiple radiation doses.

The key novel aspect of this study comes from our second aim, determination of RIBE from CM by xCELLigence assays in several cell lines, which has not been reported earlier. We tested 6 mammalian cell lines; 2 human cell lines and 4 mouse cell lines. We used EMT6.5, 4T1.2, NMUMG and WTMACs and cell lines for protocol 1 and EMT6.5, 4T1.2, SW48 and HACAT for protocol 2.

Initially in protocol 1, we used very high (50 Gy) and medium (5 Gy) radiation doses in low serum concentration (2% FBS) in order to find the range of experimental dose for RIBE, however no significant RIBE effects were detected. We observed no significant change in cell index slope or clonogenic survival in recipient cells receiving CM for protocol 1.

Some studies have reported that a RIBE occurs predominantly at lower doses of radiation (less than 1 Gy) but equally, other studies show RIBE in doses ranging from 5 – 15 Gy (Gow et al. 2010; Veldwijk et al. 2014). Mothersill et al. (2014) reported using high dose homogenous field radiation (35 Gy and 350 Gy) to investigate bystander effects in cage mates as compared to high dose microbeam radiotherapy. Mothersill et al. noted that high radiation doses may cause immediate cell death, evoke an inflammatory response and may lead to abscopal effects in the same animal. Thus, the inter-animal study enables the study of a bystander effect mechanism.

Since we observed no significant change with protocol 1, we therefore altered the protocol and used 10% supplemented media during irradiation for CM harvesting and fresh CM after harvesting to expose to the recipient cells for both assays. The doses chosen were between 1.5 to 7.5 Gy which are in medium to high categories (Kadhim et al. 2013). For protocol 2, we did observe a statistically significant bystander effect on the cell index slope for the EMT6.5 cell line for a dose of 7.5 Gy (Fig. 7). We did not observe any significant effects for the other doses nor in the cell survival fraction. We observed an increasing trend in the cell index slope for the SW48 human cell lines, but the trend was not statistically significant (Fig. 7).

Results from another study that employed doses ranging from 0- 9 Gy CM suggested a RIBE effect of reduced survival fraction in the dose range from 0 to 2 Gy, but clonogenic kill plateaued at doses greater than 2 Gy

(Boyd et al. 2006). In our study we measured increased cell index slope due to RIBE using xCELLigence RT-CIS with high dose CM from our EMT6.5 cell line.

Increased cell index can be caused by increased cell number, motility, metabolism or altered cell morphology. Decreased survival fraction is the most common RIBE reported however, some studies have also shown increased cell proliferation (Baskar 2010). Surprisingly our experiment showed a significant increase in cell index in EMT6.5 and a trend towards an increase in SW48. To confirm our radiation-induced cell proliferation in the EMT6.5 cell line, we monitored EMT6.5 cells that were directly irradiated with 7.5 Gy from a Co-60 source in 6 well plates for 4 days. Control cells proliferated well and the numbers increased, however the numbers of EMT6.5 cells in treated group were reduced after 3 days. Morphologically, the treated EMT6.5 cell were larger compared to controls cells at day 1 post-radiation and some of the cells were qualitatively, very large by day 3 before rupturing on day 4 (Supp. Fig. 4) Cell enlargement occurs in a short period of time after irradiation and is not measured in clonogenic assay. We were unable to replicate the finding (cell enlargement in the EMT 6.5 irradiated cells) with the EMT 6.5 cells treated with CM due to the limited amount of the CM. It appears that cell enlargement rather than cell proliferation may be giving the significant RIBE effect that caused an increased cell index slope.

To our knowledge, no study has reported a RIBE using EMT6.5, 4T1.2, NMUMG and WT MAC cell lines *in vitro* with a CM protocol. There are conflicting reports about the RIBE on the SW48 cell line. Using clonogenic survival, the research group led by Prof Carmel Mothersill reported a significant decrease in survival fraction (C. Mothersill and Seymour 1997; C. Mothersill et al. 2004a) while Groesser and his group did not observe significant change in survival of SW48 cells (Groesser et al. 2008). In our study, no significant changes were observed in SW48 survival fraction. Various RIBE studies have shown opposite or conflicting outcomes. For example, one study showed T98G and HT29 cells did not exhibit a bystander effect as measured using the clonogenic survival assay (C. Mothersill and Seymour 1997) (C. Mothersill et al. 2002) but another study by Shao et al (Shao et al. 2006) with the T98G cell line showed an increased calcium flux and micronucleus formation. It is possible to hypothesize that the RIBE outcomes depend on the end point assay chosen for the study (Morgan 2003a, 2003b). In this current study, the xCELLigence RT-CIS assay was able to measure an acute RIBE effect while clonogenic assay determined long-term survival of the cell. The diversity of the RIBE

results reported could also be due to the different cellular pathway activation that can be detected by the different assays (Kadhim et al. 2013).

In conclusion, we showed that the xCELLigence RT-CIS system can be used to determine radiobiological effects in a short period of time and the results correlated with clonogenic assay. Furthermore, we observed the RIBE in EMT6.5 using the high dose CM (7.5 Gy) by xCELLigence assay but not with clonogenic assay. It is reasonable to conclude that RIBE is cell type dependent, not universally observed and can be detected using different assays.

COMPLIANCE WITH ETHICAL STANDARDS:

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Conflict of Interest:

All authors declare no conflicts of interest. Premila Paiva and Jeffrey C. Crosbie are recipients of Early Career Researcher Fellowships from the National Health & Medical Research Council (NHMRC), Australia. Mohammad Johari Ibahim receives PhD scholarship from The Ministry of Higher Education, Malaysia.

Ethical approval:

This article does not contain any studies with human participants or animals performed by any of the authors.

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FIGURES

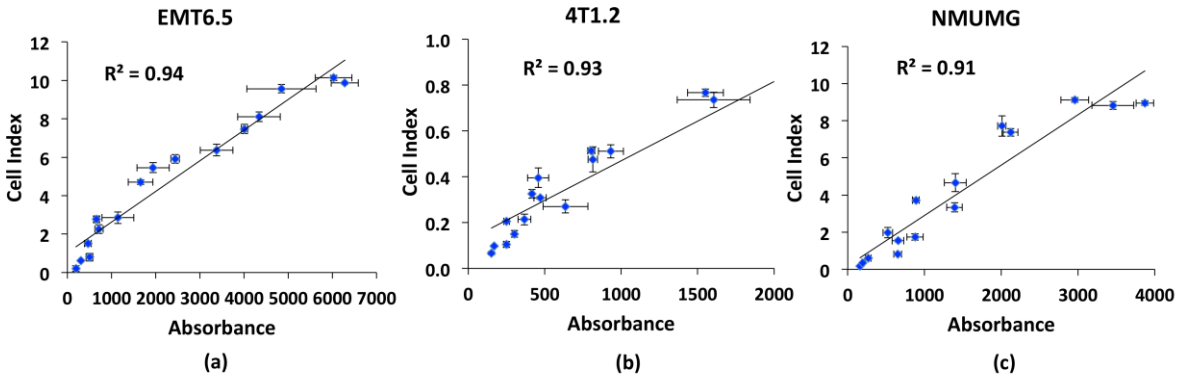

Fig. 1 a-c There was a strong correlation between cell proliferation measured by CTB assay and cell index from xCELLigence RT-CIS assay in unirradiated cells at similar seeding densities for each cell line: EMT6.5 (r^2=0.94, r=0.99, p<0.01) 4T1.2 (r^2=0.93, r=0.96, p<0.01) and NMUMG (r^2=0.91, r=0.96, p<0.01) (Data: Mean, n=3 ± S.E.M). ** There are some error bars that are smaller than the symbol.

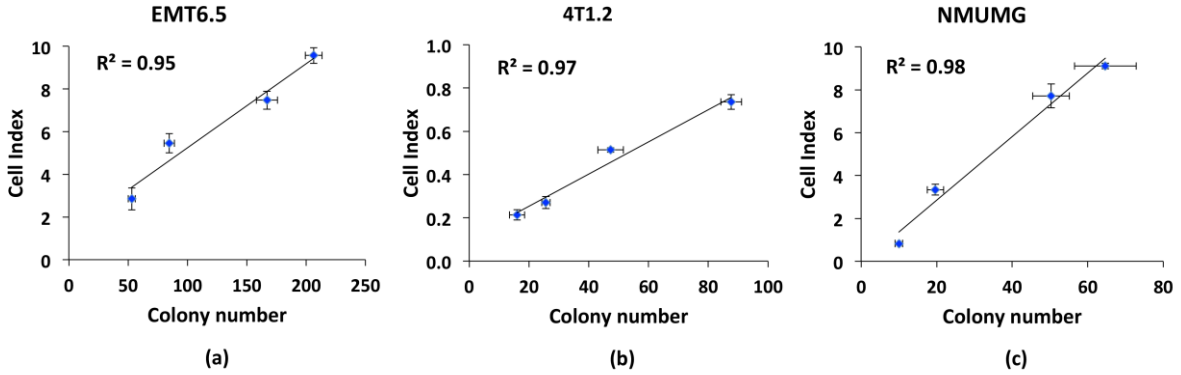

Fig. 2 a-c There was a strong correlation between colony number using clonogenic assay and cell index from xCELLigence RT-CIS in unirradiated cells at similar seeding densities for each cell line: EMT6.5 (r^2=0.95, r=0.99, p<0.01), 4T1.2 (r^2=0.97, r=0.99, p<0.01) and NMUMG (r^2=0.98, r=0.99, p<0.01) (Data: Mean, n=3 ± S.E.M). ** There are some error bars that are smaller than the symbol.

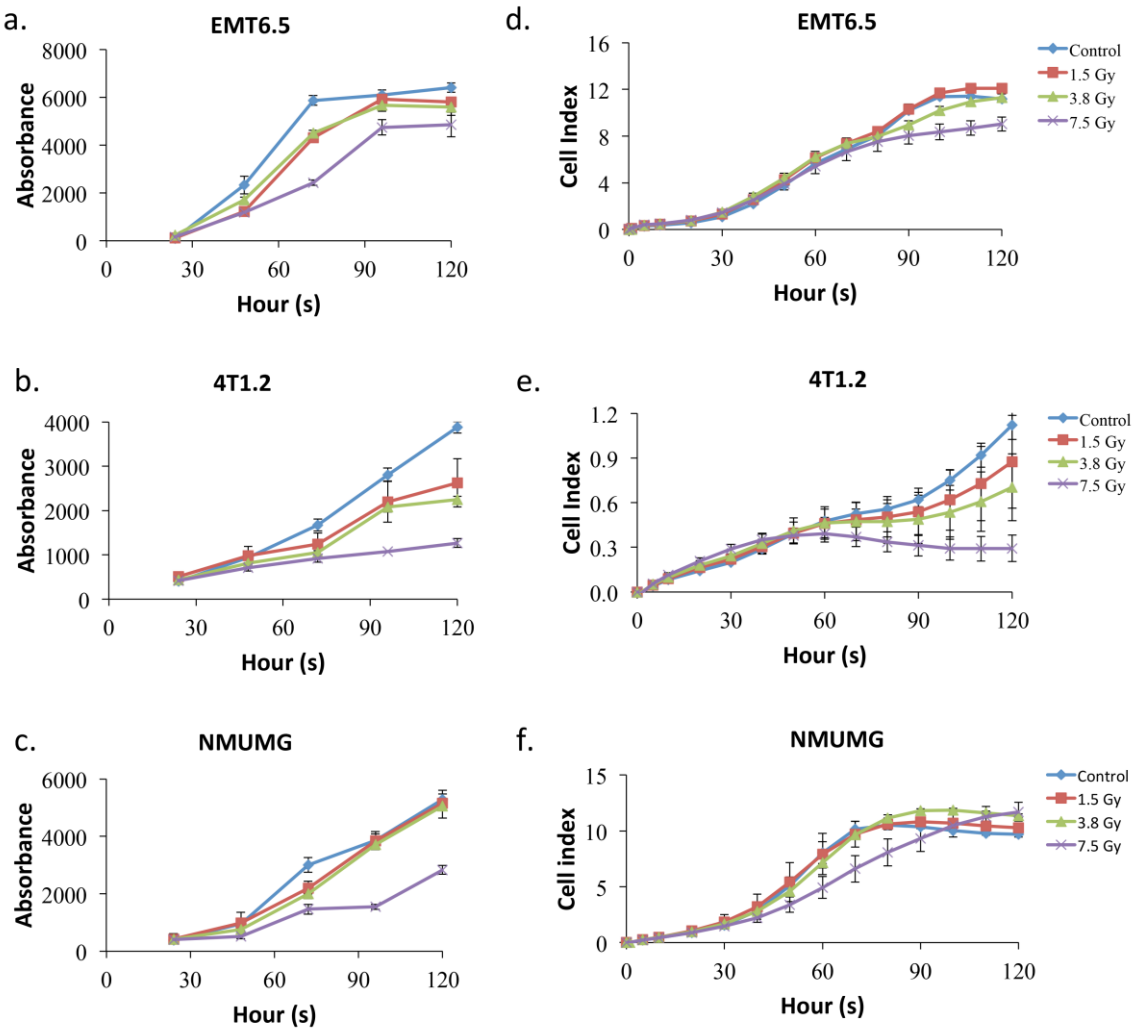

Fig. 3 Similar results were observed between the proliferation profile measured by the CTB assay and xCELLigence RT-CIS assay for EMT 6.5 and 4T1.2 cell lines, however a similar pattern was not seen for NMUMG cells, particularly at the highest dose (7.5 Gy). Dynamic proliferation of irradiated EMT6.5, 4T1.2 and NMUMG cell lines measured as cell index using xCELLigence RT-CIS (left panel) and as absorbance by the CTB assays (right panel) in irradiated cells (1.5 Gy, 3.8 Gy and 7.5 Gy) using Co-60 (Data: Mean ± S.E.M, n=3). ** There are some error bars that are smaller than the symbol

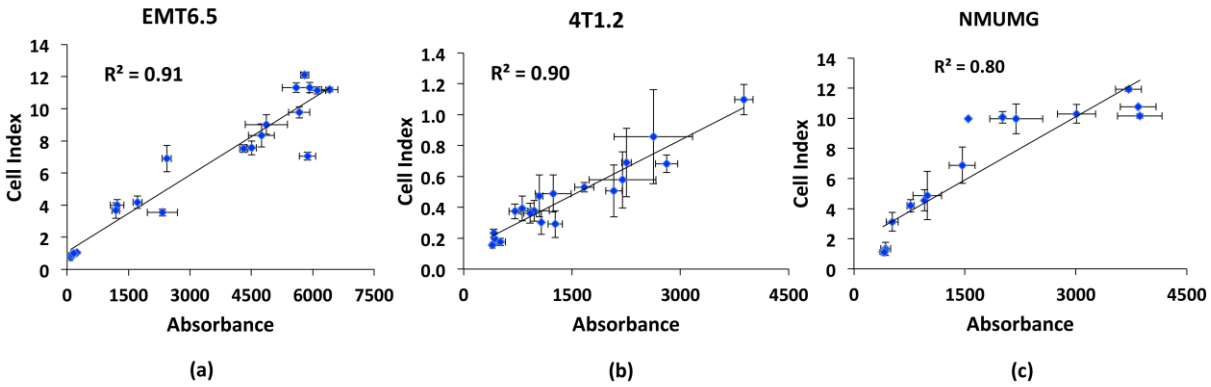

Fig. 4 a-c There was a strong correlation between cell proliferation measured by CTB and cell index from xCELLigence RT-CIS in Co-60 irradiated cells (1.5 Gy, 3.8 Gy and 7.5 Gy) for each cell line: EMT6.5 (r^2=0.91, r=0.91, p<0.01), 4T1.2 (r^2=0.90, r=0.89, p<0.01) and NMUMG (r^2=0.80, r=0.97, p<0.01) (Data: Mean, n=3 ± S.E.M). ** There are some error bars that are smaller than the symbol.

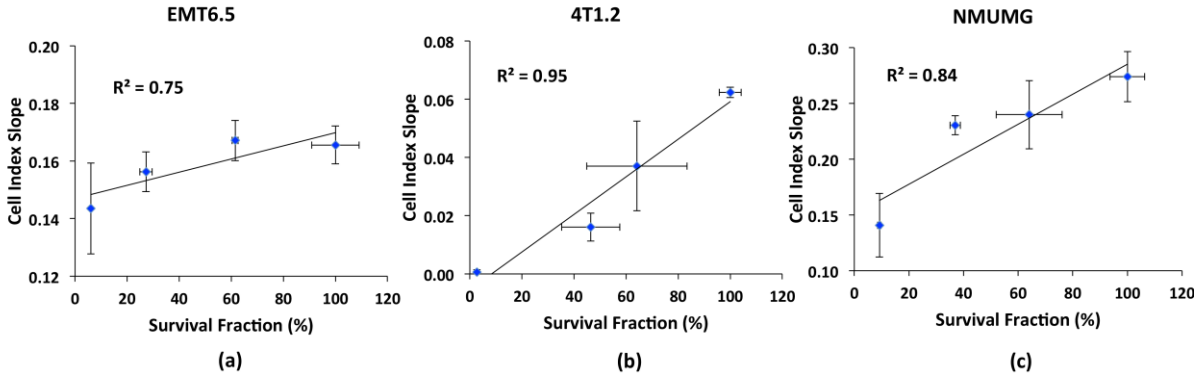

Fig. 5 a-c There was a strong correlation between cell survival fraction measured by clonogenic assay and cell index from xCELLigence RT-CIS in Co-60 irradiated cells for each cell line: 4T1.2 (r^2=0.95, R=0.99, p<0.01) and NMUMG (r^2=0.84, R=0.96, p<0.05). However, a lack of correlation was observed in EMT6.5 (r^2=0.75, R=0.85) and this did not reach significance. (Data: Mean, n = 3 ± SE.M). ** There are some error bars that are smaller than the symbol.

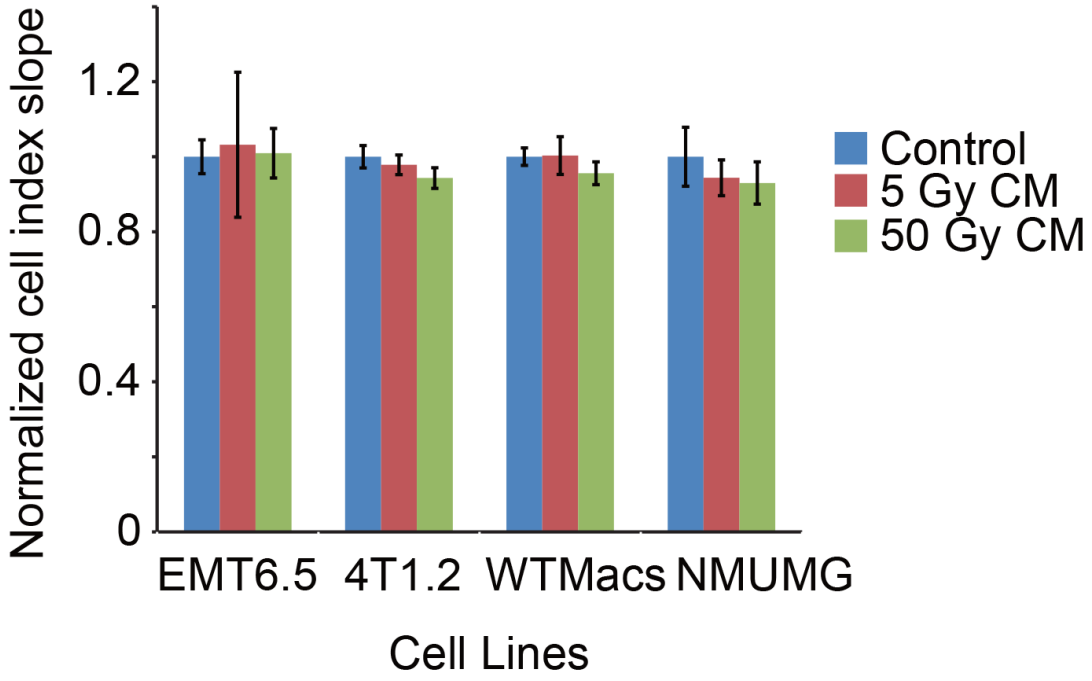

Fig. 6 No significant differences in normalized cell index slope in all cell lines were observed between control, 5 Gy and 50 Gy CM harvested in 2% serum condition. Rate of cell growth measured as cell index by xCELLigence RT-CIS assay of EMT6.5, 4T1.2, WTMacs and NMUMG cells treated with CM harvested in 2% serum from donor cells irradiated at 0, 5 Gy and 50 Gy (Data: Mean ± S.E.M, n= 4).

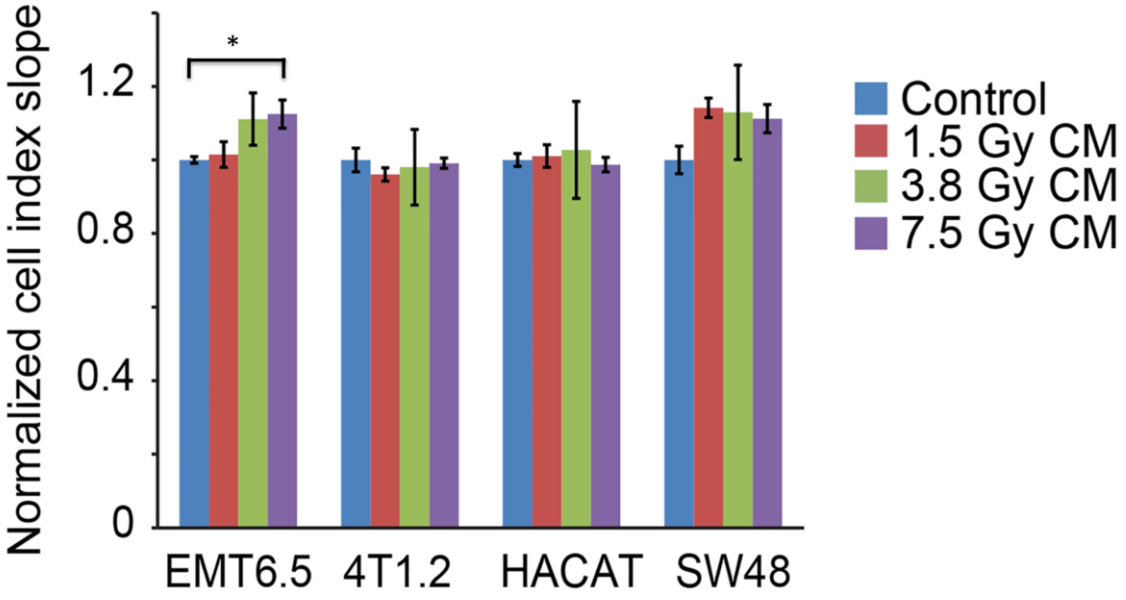

Fig. 7 Increased cell index slope in 7.5 Gy CM harvested in 10% serum in EMT6.5 compared to control. Rate of cell growth measured as cell index by xCELLigence RT-CIS of EMT6.5, 4T1.2, SW 48 and HACAT cells treated with CM harvested in 10% serum from donor cells irradiated at 0, 1.5 Gy, 3.8 Gy and 7.5 Gy (Data: Mean ± S.E.M, n= 3) (* $p<0.05$).

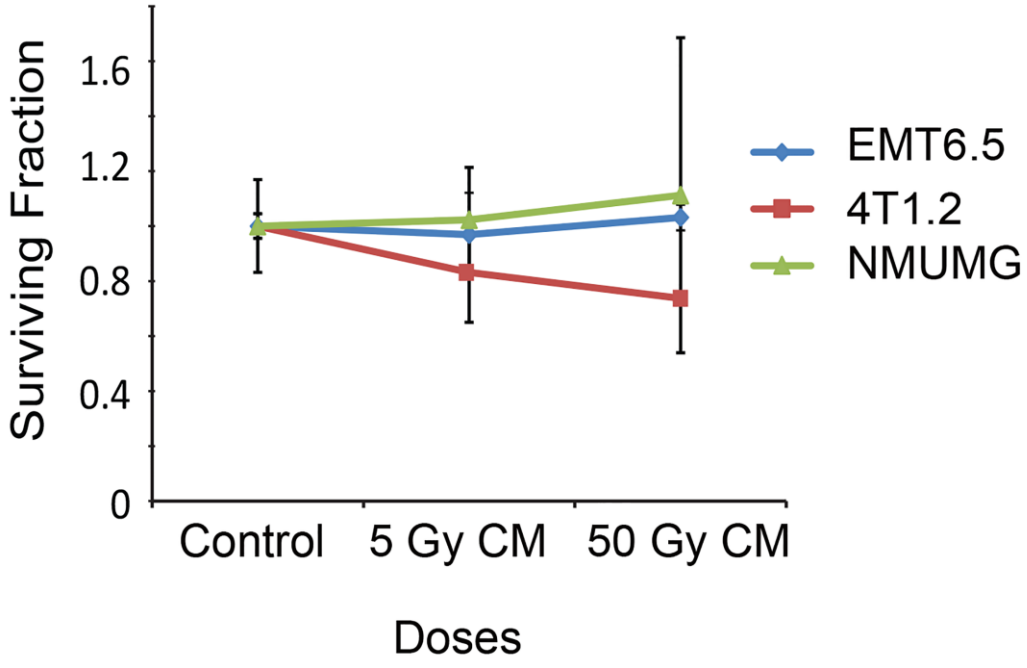

Fig. 8 No significant differences in the surviving fraction in all cell lines between control, 5 Gy and 50 Gy CM harvested in 2% serum. Surviving fraction obtained from clonogenic assay of EMT 6.5, 4T1.2 and NMUMG cells assays treated with CM harvested in 2% serum from donor cells irradiated at 0, 5 Gy and 50 Gy (Data: Mean ± S.E.M, n= 4 (EMT6.5 and 4T1.2), n=2 (NMUMG)).

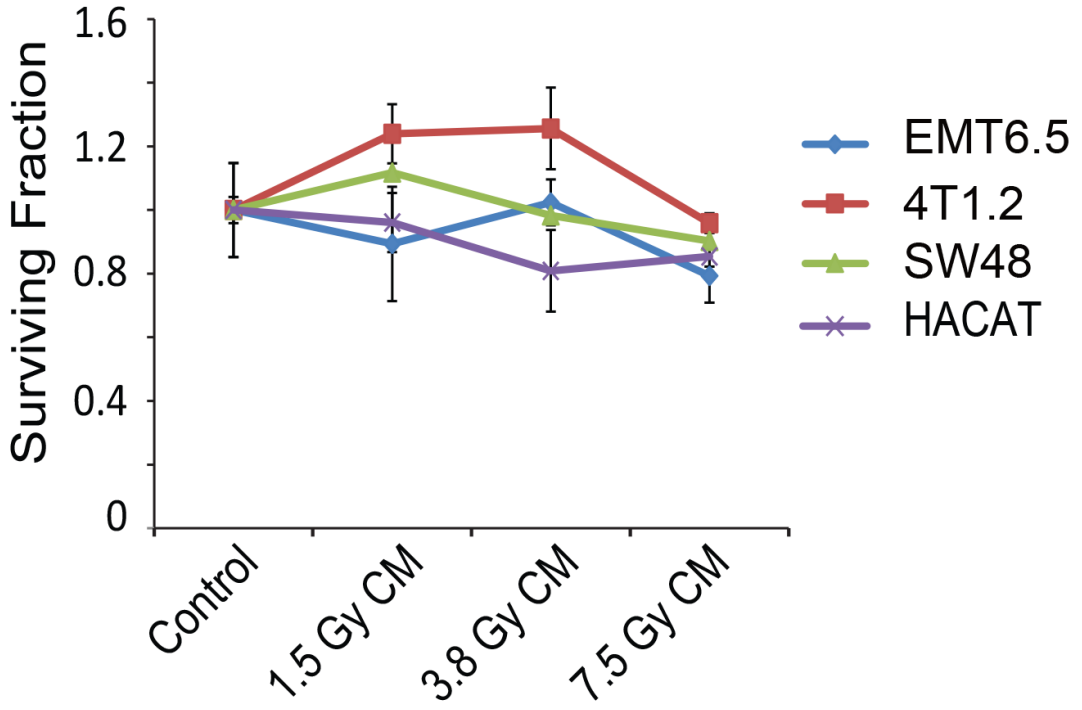

Fig. 9 No significant differences in the surviving fraction in all cell lines between control, 1.5 Gy, 3.8 Gy and 50 Gy CM harvested in 10% serum. Surviving fraction obtained from clonogenic assay of EMT6.5, 4T1.2, SW48 and HACAT cells treated with conditioned media from donor cells irradiated at 0, 1.5 Gy, 3.8 Gy and 7.5 Gy (Data: Mean ± S.E.M, n= 3 for all cell lines except SW48 (n=1))