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## Apoptosis is Signalled Early by Low Doses of Ionizing Radiation in a Radiation-Induced Bystander Effect

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# **Apoptosis is Signalled Early by Low Doses of Ionizing Radiation in a Radiation-Induced Bystander Effect**

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## **Abstract**

It is known that ionising radiation (IR) induces a complex signalling apoptotic cascade post-exposure to low doses ultimately to remove damaged cells from a population, specifically via the intrinsic pathway. Therefore, it was hypothesised that bystander reporter cells may initiate a similar apoptotic response if exposed to low doses of IR (0.05 Gy and 0.5 Gy) and compared to directly irradiated cells. Key apoptotic genes were selected according to their role in the apoptotic cascade; tumour suppressor gene TP53, pro-apoptotic Bax and anti-apoptotic Bcl2, pro-apoptotic JNK and anti-apoptotic ERK, initiator caspase 2 and 9 and effector caspase 3, 6 and 7. The data generated consolidated the role of apoptosis following direct IR exposure for all doses and time points as pro-apoptotic genes such as Bax and JNK as well as initiator caspase 7 and effector caspase 3 and 9 were up-regulated. However, the gene expression profile for the bystander response was quite different and more complex in comparison to the direct response. The 0.05 Gy dose point had a more significant apoptosis gene expression profile compared to the 0.5 Gy dose point and genes were not always expressed within 1 hour but were sometimes expressed 24 hrs later. The bystander data clearly demonstrates initiation of the apoptotic cascade by the up-regulation of TP53, Bax, Bcl-2, initiator caspase 2 and effector caspase 6. The effector caspases 3 and 7 of the bystander samples demonstrated down-regulation in their gene expression levels at 0.05 Gy and 0.5 Gy at both time points therefore not fully executing the apoptotic pathway. Extensive analysis of the mean-fold gene expression changes of bystander data demonstrated that the apoptosis is initiated in the up-regulation of pro-apoptotic and initiator genes but may not very well be executed to final stages of cell death due to down-regulation of effector genes.

## **1. Introduction**

A 'bystander factor' can be produced in cells exposed to ionising radiation (IR) and can subsequently affect the function and survival of surrounding un-irradiated cells due to cellular communication of the bystander signals. This communication is either through gap junctions or by secreted factors in the surrounding medium that transmits a signal [1]. The importance of understanding altered gene expression in radiation induced bystander effects (RIBE) is apparent in the literature but has yet to be fully characterised. This study investigates gene expression changes in both directly irradiated and bystander human keratinocyte HaCaT cells.

A broad range of information regarding gene expression changes in directly irradiated cells is available, but less so for bystander cells. Human epithelial keratinocytes exposed to direct and indirect irradiation has been shown to induce initiating apoptotic events specifically on the mitochondrial related intrinsic pathway, with the increase of expression of anti-apoptotic Bcl-2 [2–5]. Mothersill et al found that a threshold of approximately 1Gy exists to induce Bcl-2 in directly irradiated cells [6] but it is thought that there may be a different threshold for bystander irradiated cells [7]. It is important therefore to understand the signalling mechanisms involved on a molecular level. Expression levels of bystander factor induced-apoptosis related genes, in particular Bcl-2 and cytochrome c have been determined using fluorescent probes [5].

An attempt to establish cellular regulatory mechanisms in bystander cells was made in 2008, with measurement of global gene expression of alpha particle direct irradiated normal human lung fibroblasts four hours after exposure compared to parallel bystander effects. Both direct and bystander effects were compared with the discovery that two major transcriptional centres, P53 and NfκB which regulate the direct response also have a role in the bystander

cells but to a different extent [8]. P53 functions as a transcription factor in response to the stress of ionising radiation. Kuang et al investigated the genes that are targeted directly by P53 and this paper examined its role more thoroughly in response to indirect (bystander) radiation at very low doses [9]. The application of genome wide microarrays has been beneficial to determine changes in transcript profiles in human melanoma cells grown in conditioned medium from irradiated cells where they made the observation that factors transmitted from IR cells can affect transcript levels in non-IR cells [10]. The effect and involvement of chemicals in specific pathways in bystander responses has also been investigated with measurement of gene expression changes, in particular the MAPK downstream targets. These results showed bystander induced changes in MAPK proteins and downstream targets [11].

Elmore [12] describes two distinct pathways of apoptosis in detail in her review of programmed cell death. These pathways are the extrinsic pathway which is associated with transmembrane receptor-mediated interactions involving death receptors which form multiprotein complexes. The second, intrinsic pathway involves a diverse array of non-receptor mediated stimuli which produce intracellular signals involving the mitochondria. The two described pathways have unique events initiating the pathway and unique signalling events, but there is evidence that the two pathways are linked due to molecules in one pathway influencing the other [13]. However, studies have shown that the intrinsic pathway is a more sensitive indicator of apoptotic signals when exposed to IR [14]. Subsequently only the intrinsic apoptotic cascade was considered for this bystander study to compare the gene expression response with directly irradiated cells at the same doses and time points. A group of 'intrinsic pathway' apoptotic genes were chosen for this study, dependent on their function, location and role in apoptosis.

It is known that ionising radiation in cells initiate changes in the intrinsic apoptotic pathway directly involving the mitochondria and the activation of a group of proteases known as caspases. Two groups exist according to function, initiator Caspases (Caspase 2, 8, 9, 10) and effector caspases (Caspase 3, 6, 7). Initiator caspases cleave inactive pro-forms of effector caspases, thereby activating them. Effector caspases in turn cleave other protein substrates within the cell, to trigger the apoptotic process [15]. Caspases are the main effectors of apoptosis through cleavage of cellular substrates. Procaspases 8, 9 and 10 are the main initiators although the main function of some do not relate to apoptosis. In this study, only selected initiator caspases related to the intrinsic pathway were studied (Caspases 2 and 9) to capture their role in the cascade. The role of caspase 2 was included in the study because its precise role in apoptosis is still unclear, however it is thought to induce apoptosis in response to intrinsic and extrinsic signals [16]. All of the known effector caspases (Caspase 3, 6 and 7) were selected to capture all mechanisms which result in the execution of apoptosis.

Different modes of cell killing such as apoptosis, necrosis, mitotic catastrophe, senescence and autophagy do exist. Cells that are exposed to IR may experience rapid or delayed cell death, such as mitotic cell death which has been investigated by Howe et al, [17]. Oncosis is the term more commonly used to describe the process of necrosis, as it is a mode of cell death that refers to the degradative processes that occur after cell death [12]. One particular paper showed a significant increase of necrosis in indirectly irradiated cells (bystander cells) in comparison to directly irradiated cells and also suppressed proliferation activity [18, 19]. Autophagy is thought to arise in damaged cells that cannot be removed by engulfment cells and is thought to lead to cell death through the process of cytoplasm destruction [20]. There may very well be other significant mechanisms responsible for the response of the bystander cells such as cross-talk to other cell death pathways. In a recent publication [19] our

laboratory compared cell death pathways (apoptosis, necrosis and mitotic cell death) in directly irradiated and bystander HaCaT cells.

The bystander effect may be also be mediated by signalling pathways responsive to oxidative stress thus activating stress related kinases and their down-stream transcription factors, such as JNK and ERK. JNK is a member of the Mitogen-activated protein kinases (MAPK's), in which their pathways play an important role in transportation of stress signals such as ionising radiation from the surface of the cell to the nucleus and responses are dependent on stimulus type [21]. Zhou et al, [22] showed that inhibition of ERK can lead to an increase in number of bystander cells.

Gene expression levels of these selected apoptotic genes were measured in directly irradiated and bystander HaCaT cells exposed to low doses of IR or ICCM respectively 1 hr and 24 hrs after exposure. To our knowledge, this is the first report demonstrating the apoptotic gene expression levels in the intrinsic apoptotic pathway related to a radiation-induced bystander response and compared in parallel to the direct IR response *in-vitro*.

## **2. Materials and Methods**

### *2.1 Direct and bystander irradiation experiments in vitro*

An immortal human keratinocyte cell line, HaCaT, which was kindly received from Dr Petra Boukamp's laboratory [23] were used in these studies as they have previously been shown to be good reporters of the radiation induced bystander response [22, 23]. They were routinely cultured in DMEM: F12 (Dulbeccos Modified Eagles Medium) medium (Sigma) supplemented with 10% fetal bovine serum (FBS, Gibco) and 2mM L-Glutamine (Gibco) in DMEM medium. Cells were maintained in an atmosphere of 37°C and 5% CO<sub>2</sub> and grown to



approximately 70-80% confluency to ensure they were in the logarithmic phase of growth. Cells were removed from stock flasks using a 1:1 mix of EDTA:Trypsin, EDTA: 0.1 g of EDTA in 500 ml PBS:Trypsin 2.5% 10X) and a 1:10 dilution of Trypsin then neutralised in EDTA. 200,000 cells were counted using a Coulter Counter and were plated into T25 flasks. A set of flasks were set up at 1 hr and 24 hr direct irradiation experiment and for harvesting of bystander media for each of the dose points (0 Gy, 0.05 Gy 0.5 Gy) in triplicate. Another set of flasks were set up as bystander recipients at 1 hr and 24 hr for bystander irradiations for each of the dose points (0 Gy, 0.05 Gy 0.5 Gy) in triplicate. All flasks were incubated with a fresh media change after 1 day. The growth was monitored for 2-3 days so that the cells were allowed to reach 70-80% confluency, to ensure the phase of growth of the cells and to grow as many cells as possible per dose and time point. After 72 hours of culturing, cells (direct irradiation flasks only) were irradiated at room temperature using the cobalt 60 teletherapy unit in St. Luke's hospital (Rathgar, Dublin) with a distance from radiation source to flask of 80cm, and a field size of 25 x 25cm. All irradiated flasks were placed back into the incubator directly after irradiation until 1 hr post exposure. Our group has shown that the bystander factor is produced from 30 seconds onwards from the rapid formation of calcium fluxes in similar HPV-G keratinocyte cells exposed to low doses of irradiated cell conditioned media (ICCM) [2]. The Media (ICCM) was harvested and pooled per triplicate flask for the directly irradiated flasks at 1 hr and 24 hr time points and each of the three dose points (0, 0.05 and 0.5 Gy). This was labelled ICCM. 5ml of fresh DMEM F12 media was replaced on the 24hr direct irradiation flasks and they were re-incubated for 24 hr while an RNA extraction was conducted immediately on the 1hr direct irradiation flasks. The ICCM was filtered through a 0.22µm filter (Nalgene) to remove any dead cells or debris. The ICCM was then immediately transferred to the bystander parallel cultures of 1 hr and 24 hr time points at the three dose points (0, 0.05 and 0.5 Gy). For both the direct and bystander cell cultures, RNA was

extracted at the relevant time points of 1 hr and 24 hrs of each dose point. The Tri-Reagent (Sigma Aldrich) extraction technique [26] was used which briefly involves cell lysis, phase separation (with chloroform), Isopropanol precipitation and ethanol washing of the extracted RNA. The re-dissolved RNA in DEPC water (Sigma Aldrich 1X) was stored at -80°C for subsequent studies. The bystander ICCM experiment was set up according to the medium transfer technique developed by Mothersill & Seymour [1].

## *2.2 Gene expression studies*

### *2.2.1 Optimisation of apoptosis gene primers*

Apoptosis gene primers were first synthesised using the PRIMER3 program. Caspase 3 and caspase 7 primer sequences were sourced from (Jooyeon Hwang) [27]. Bax and Bcl-2 primer sequences were sourced from (Hans-Peter Gerber) [28]. See Table 1 for individual gene primer sequences. All primer sets were obtained from Sigma Genosys, UK and were of Homosapien in origin, desalted and scaled to 0.05µmol. They were sent lyophilised and re-suspended to a concentration of 100µM, and further diluted depending on individual primer performances. Each primer set was optimised before experimentation for gene expression by assessing different primer annealing temperatures. A conventional PCR protocol using the enzyme TAQ Polymerase (Red TAQ, Sigma) was used. Each individual reaction consisted of 1µl H<sub>2</sub>O, 2µl template DNA (HaCaT), 10µl Red Taq polymerase (Sigma), and 2µl of each primer (Forward and Reverse) to a total volume of 20µl. The PCR program incorporated the following conditions; PCR initiation activation step for 2 minutes @ 94°C, Denaturation for 40seconds @ 94°C, Annealing for 1 minute @ 63°C, Extension for 40 seconds @ 72°C, Final extension for 2 minutes @ 72°C and then holding the samples at 8°C. The denaturing/melting temperatures ( $T_m$ ) and annealing temperature ( $T_a$ ) were calculated. The  $T_m$  of the primers is calculated by the following equation

$$T_m = 2 [A+T] + 4 [G+C]$$

Ranges of annealing temperatures ( $T_a$ ) were tried for each primer set until a pure PCR product/band was obtained with 1% agarose gel electrophoresis. The clearest largest band on the gel image indicated the optimum temperature for that specific primer set.

### *2.2.2 Expression of apoptosis genes in direct and bystander irradiation samples using real-time PCR and SYBR green technology*

RNA samples were quantified on a Helios  $\gamma$  spectrophotometer which measured the absorbance of the extracted RNA at 260nm and protein concentration at 280nm. A ratio of absorbance at different wavelengths (Absorbance 260:280) was calculated and samples were selected based on whether they fell in between the permitted ratio range of 1.8 – 2.0 which indicated high purity samples. All RNA samples were then reverse transcribed into a complementary DNA copy (cDNA) using the Quantitect reverse transcription kit (Qiagen, UK). According to the manufacturer's instructions, 1 $\mu$ g/ $\mu$ l of pure RNA was used in a final volume of 20 $\mu$ l. gDNA wipeout buffer was added to RNA template and RNase-free water, and incubated for 2 minutes on ice. In a separate tube Quantiscript Reverse Transcriptase, Quantiscript RT Buffer and RT primer Mix were mixed and placed on ice. The Reverse transcription mix was added to the Genomic DNA elimination mix and incubated for 15-30 minutes at 42°C, and then incubated for 3 minutes at 95°C to inactivate Quantiscript Reverse Transcriptase. The newly synthesised cDNA was stored at -20°C until subsequent gene expression studies were carried out by real time PCR.

Real time PCR experiments were carried out using Lightcycler® 480 (Roche Diagnostics) and SYBR Green technology (Roche). The formation of PCR products was detected by

measurement of SYBR Green fluorescence signals from each experiment. SYBR Green intercalates into the dsDNA helix and the increase in SYBR Green fluorescence is directly proportional to the amount of dsDNA generated.

The real-time PCR protocol used to calculate gene efficiency involved the addition of 2µl of cDNA product, 10µl SYBR Green (Roche), 6µl H<sub>2</sub>O and 2µl of each primer (forward and reverse) to a total volume of 20µl. This protocol was used to calculate the efficiency of each gene in the study, with an expected gene efficiency value of 2. The PCR programme consisted of the following steps; PCR initial activation step for 10 minutes @ 95°C, Denaturation for 10 seconds @ 95 °C, Annealing for 1 minute at 57, 60, 64 °C (depending on optimised temperature of primer involved) and extension for 1 minute @72°C. Each sample analysed were set up in triplicate, n = 3. And mean values were calculated.

### *2.2.3 Analysis of gene expression data*

Relative quantification analysis directly from the LC480 program (from the real-time PCR experiments using SYBR green fluorescence) was used to determine the apoptosis gene expression levels in target and reference genes. The reference gene (Tubulin) normalises sample to sample differences and was important to determine the changes in expression of different genes, according to dose and time. Two ratios were compared in each experiment, the ratio of target (gene of interest) to a reference (housekeeping gene) sequence, and the ratio of sequences within a calibrator (positive sample) sample. The result was expressed as a normalised ratio which is  $(\text{conc.target}):(\text{conc.target}) / (\text{conc.reference}):(\text{conc.reference})$

The quantitative endpoint for real-time PCR is the threshold cycle (CT). Schmittgen and Livak best define this value as ‘‘*The PCR cycle at which the fluorescent signal of the reporter dye crosses an arbitrarily placed threshold*’’[29]. A mathematical model widely used in the analysis of Real-time PCR data, the *delta delta CT method* [30] was subsequently applied to

the raw CT data to give a ratio of target gene: reference genes to show the gene expression changes.

Mean-fold changes are calculated from mean normalised values of raw CT data between samples, dose/time. The *deltadelta CT* mathematical model normalises sample to sample differences. The aim was to determine the effect of both direct and indirect irradiation on the expression of the chosen target genes. This method enabled the measurement of gene expression changes of target genes normalised to Tubulin (housekeeper gene) monitored at 0.05 Gy and 0.5 Gy at 1 hr and 24 hrs exposures and relative to the expression at 0 Gy (control). The value of the mean-fold change at 0 Gy (control) was 1. The mean-fold changes in gene expression of each target gene were plotted by Microsoft Excel, and the graphs present either up-regulation or down-regulation of samples (target genes) determined whether they reach above or below the control sample value of 1.

#### *2.2.4 Statistical data analysis*

Significance of variances between doses and time points were determined, for each specific gene targeted, by the statistical one-way ANOVA test with the aid of Microsoft excel and variances were considered significant if  $p < 0.05$ . The overall outcome on the statistical analysis recorded was a 'NO' for significance in table 2 compared to those deemed significant with a 'YES'. Samples not considered statistically significant were still plotted.

### **3. Results**

#### *3.1 Optimised annealing temperatures for primers*

The set of primers which were designed with a PCR primer design tool program online, PRIMER3, were analysed by conventional PCR to optimise the melting temperature prior to the gene expression experiments. Agarose gel electrophoresis provided strong bands which correlated to a temperature gradient used in the PCR program, from this the best suited temperature was chosen for further RT-PCR experiments. Primer sequences for Bax, Bcl-2 and caspase 3, 7 were sourced from literature [23,24]. A list of forward and reverse sequences is shown in Table 1 and the optimum temperatures ( $T_m$  °C) for each primer set are also shown.

### *3.2 RT-PCR Gene expression study*

The expression levels of each gene will be discussed separately to unveil any emerging expression patterns of apoptosis in these HaCaT cells. Figures 1 to 5 display the direct in comparison to the bystander data of each target gene and highlight emerging changes in gene expression levels, be they up-regulated or down-regulated with respect to the control samples that have been set to one for each gene investigated.

#### *TP53*

Figure 1A-B displays a comparison of relative mean-fold changes in gene expression levels of tumour suppressor gene TP53 in HaCaT cells, following 1 hr and 24 hrs exposures to 0.05 Gy and 0.5 Gy in direct and indirectly irradiated cells. TP53 is up-regulated in direct cells after 1 hour exposure to 0.5 Gy and down-regulated in all other direct samples. The bystander data shows that TP53 is up-regulated after 1 hr in the lower dose of 0.05 Gy and down-regulated at other doses/time points. All TP53 data is statistically significant, direct  $p < 0.05$  and bystander  $p < 0.05$  and presented in Table 2.

The tumour suppressor gene responds to a range of stresses by inducing cell cycle arrest and/or apoptosis in damaged cells from IR. TP53 is a well described transcription factor that can induce the expression of multiple pro-apoptotic gene products such as caspase activators and pro-apoptotic members of the Bcl2- family such as Bax. It is known that TP53 has a critical role in regulating the bcl-2 family of proteins but the exact mechanisms have not yet been determined [31]. The direct and bystander data shows a role in the early initiation steps of apoptosis. As it is an initiator of further downstream apoptotic genes, the low dose may have implicated a response for the activation of TP53. The data illustrates a possible functional role for the initiation of the apoptotic process, at very low doses.

#### *Bax and Bcl2*

Pro-apoptotic Bax mean-fold gene expression changes are displayed in figure 2A-B. In the direct cells Bax is up-regulated in both doses which increases after 24 hrs and more so in the higher dose of 0.5 Gy. In the bystander samples, Bax is up-regulated at the lower dose of 0.05 Gy after 24 hrs. This instigated a somewhat delayed response.

Figure 2C-D shows that anti-apoptotic Bcl-2 in direct cells is down-regulated with 0.05 Gy after 1 hr and 24 hrs. It is then up-regulated with 0.5 Gy after 1 hr and down-regulated after a further 24 hrs. The bystander cells displayed up-regulation after 1 hr and then down-regulation after 24 hrs exposure to 0.5 Gy. However, one striking difference in Bcl-2 expression levels was for the 0.05 Gy bystander cells, where up-regulation was seen compared to direct cells. Table 2 revealed all gene expression changes of Bax and Bcl-2 to be statistically significant. Bax direct  $p < 0.05$  and Bax bystander  $p < 0.05$  and Bcl-2 direct  $p < 0.05$  and Bcl-2 bystander  $p < 0.05$

Bax and Bcl-2 are antagonistic to one another, in that pro-apoptotic Bax promotes the release of cytochrome c and other apoptotic factors whereas anti-apoptotic Bcl2 blocks the release of

these factors [32]. The up-regulation of Bax shows that mitochondria have a role to play in the intrinsic apoptotic cascade of both direct and bystander cells at low doses. Comparing the Bax and Bcl-2 direct and bystander data it is clear that Bax up-regulation is greater so it possibly overrides the anti-apoptotic effects of Bcl-2, allowing apoptosis to continue. This is expected as it is usually in favour of inducing the apoptotic pathway.

### *JNK and ERK*

Pro-apoptotic JNK expression is displayed in figure 3A-B and is up-regulated in direct samples with 0.05 Gy after 24 hrs. JNK is up-regulated but not much greater than the control sample with 0.5 Gy after 1 hr. All other samples are down-regulated. The bystander samples showed very little expression of JNK with only down-regulation observed. All changes were statistically significant as seen in Table 2. Direct  $p < 0.05$  and bystander  $p < 0.05$

Anti-apoptotic ERK expression changes can be seen in figure 3C-D ERK is up-regulated in direct samples at 0.05 Gy at both time points but to a much greater extent after 1 hr. The 0.5 Gy samples showed minor up-regulation of ERK after 1 hr and down-regulation after 24 hrs. The bystander samples displayed only down-regulation, so slight that expression cannot visually be observed in the graphical display of data. The bystander data showed no statistically significant changes in gene expression across the ERK samples displayed in Table 2, direct  $p < 0.05$  and bystander  $p > 0.05$

Multiple stress-inducible molecules such as c-jun N-terminal kinase (JNK) and mitogen-activated protein kinase MAPK/extracellular signal-regulated protein kinase (ERK) have been implied in transmitting the apoptotic signal [33–35]. JNK and ERK direct data had expected up-regulation, this is expected as JNK is pro-apoptotic and should respond to



damaging signals. An unexpected response was detected in bystander samples, in that JNK and ERK were down-regulated in bystander conditions. This was remarkable as both genes were anticipated to have roles in the initiation process. This could possibly be due to the low doses administered, suggesting that the signal from direct cells is not strong enough to induce the pro-apoptotic function of JNK in bystander cells. Consequently there could be a threshold in existence for a response of both genes under these low dose circumstances

### *Initiator Caspases (2, 9)*

Initiator caspase 2 data is presented in figure 4A-B. The direct samples demonstrated down-regulation of caspase 2 with 0.05 Gy after 24 hrs and 0.5 Gy after 1 hr. The bystander data varied to the direct in that caspase 2 was up-regulated with 0.05 Gy after 1 hr and other bystander samples were down-regulated. Table 2 reveals clear statistical significance in both the direct and bystander data, direct  $p < 0.05$  and bystander  $p < 0.05$

Caspase 9 (Figure 4C-D) expression in direct samples was comparable to the bystander, in that caspase 9 was up-regulated with a low dose of 0.05 Gy after 1 hr in both types of exposure, more so in the direct samples. Caspase 9 was down-regulated in all other direct and bystander samples. Statistical analysis as seen in Table 2 determined that caspase 9 direct data is statistically significant  $p < 0.05$  whereas bystander data is not statistically significant  $p > 0.05$  so may not be applicable to the overall response.

Caspase 9 seems to be switched on and up-regulated in response to direct irradiation which is expected as it is the key protein recruited in the intrinsic apoptotic pathway after cytochrome c has been released from the mitochondria. They bind along with apaf-1 to generate the apoptosome or otherwise called 'the wheel of death' which ultimately leads to downstream

apoptotic events its established role associated with the formation of the apoptosome (wheel of death) in the apoptotic process. It was unusual therefore to see that both initiator Caspases 2 and 9 up-regulated in response to indirect irradiation, suggesting a dual-role in both genes in a low dose bystander response and validates their role as initiators in this bystander response.

### *Effector Caspases (3, 6, 7)*

Figure 5A-F displays changes in gene expression of the effector caspases. Caspase 3 direct data was not statistically significant ( $p = 0.194$ ) specified in Table 2, but inspection of the data shows expression of the gene across all samples with an increase in up-regulation with 0.05 Gy and an increase of up-regulation in the 0.5 Gy samples. Caspase 3 was down-regulated in the bystander samples and statistically significant as  $p < 0.05$  for all doses and times and is presented in Table 2.

Caspase 6 is down-regulated in direct samples but the bystander data had a very different response in that caspase 6 was up-regulated after 1 hr exposure to 0.05 Gy, and all other expression of the gene was down-regulated in the other doses and time points. Caspase 6 data was statistically significant in both direct and bystander data in Table 2, direct  $p < 0.05$  and bystander  $p < 0.05$ .

In the direct samples caspase 7 is up-regulated at 0.05 Gy after 1 hr which decreased over the 24 hr exposure. The 0.5 Gy dose induced up-regulation and a very slight increase after 24 hrs. The bystander data showed only down-regulation of the gene, and both direct and bystander data is statistically significant in Table 2, direct  $p < 0.05$  and bystander  $p < 0.05$ .

Caspase 3 is considered to be the most important of the effector caspases and is activated by any of the initiator caspases. Caspase 3 specifically activates the endonuclease CAD which subsequently degrades chromosomal DNA within the nuclei and causes chromatin condensation, one of the prominent cellular features of apoptosis that is detected microscopically [36] . In comparison to that, caspase 6 does not present up-regulation in direct data, so it has no role to play in this direct response. Although, caspase 7 displays up-regulation in the direct samples across all doses/time points. This is quite interesting when in comparison to the bystander data. Uncharacteristically, caspase 3 and caspase 7 were not expressed in bystander data as up-regulators, but caspase 6 was. The expression of caspase 3 was expected to be up-regulated in the samples for all doses and time points since pro-apoptotic Bax and initiator caspase 2 and 9 were all consistently expressed. It's likely that caspase 6 has dual function (not yet elucidated) and it also may not be compelled to execute the final stages of the apoptotic pathway in the bystander cells, and so cell death be completed

#### **4. Discussion**

This study consolidates the role of apoptosis in directly irradiated cells *in vitro* with low doses of ionising radiation at 1 hr and 24 hr after exposure and also provides evidence for the role of the apoptotic cascade in a radiation induced bystander response at low doses of ionising radiation. As previously mentioned our laboratory [19] has compared cell death pathways (apoptosis, necrosis and mitotic cell death) in directly irradiated and bystander HaCaT cells and has been documented. Apoptosis, necrosis and mitotic cell death was observed at 24, 48 and 72 hours with higher levels in the directly irradiated cells. The induction of apoptosis in bystander cells has been shown previously by our laboratory [2, 3, 5].

Although the gene expression data was consistent and consolidated the role of apoptosis in the direct and bystander IR response, the picture for the role of apoptosis in the bystander response that emerged was more complex. It implies a different role for TP53 for the direct compared to the bystander response, which may be due to the multiple functions of TP53 and perhaps even beyond cell cycle arrest and apoptosis. It was selected in this study because of its key importance in apoptosis and for interacting with several of the specific apoptosis inducers of the intrinsic pathway. Our data indicates a definite role for mitochondria in the bystander response. This is suggested from an immediate and prolonged role of Bax in the apoptotic response for both direct and bystander samples. So evidently the pathway has been initiated in the bystander cells. Possibly Bax is working in tandem with P53 in the bystander response.

JNK has pro-apoptotic properties and due to the high expression levels consistent with Bax in these samples consolidates the hypothesis that apoptosis is a key event in the direct IR response but perhaps not in the bystander response.

The role of caspase 2 is still under much investigation but it has been suggested to be an initiator caspase of both the intrinsic and extrinsic apoptotic pathways. For this reason, both caspase 2 and 9 were included in this study (and extrinsic pathway associated initiators excluded). The direct samples demonstrated that caspase 9 permitted the progression of the apoptotic initiation events. Bystander data reported caspase 2 and as the leading initiator of further down-stream caspases in the bystander response.

The role of effector caspase 3 and 7 are still unclear but their expression levels in this study was consistent for directly irradiated cells (at the same doses and time points) indicating a possible dual role in the execution of apoptosis. It is known that caspase 3 causes chromatin condensation in the execution of apoptosis, but considering that other processes such as cell

shrinkage, membrane blebbing and apoptotic body formation, perhaps caspase 6 has a role to play in these apoptotic execution processes. For the bystander cells, it appeared that caspase 6 was the only one to be up-regulated as opposed to effector caspase 3 and 7 displaying down-regulation signifying a late response. Maybe caspase 7 does not have a huge role to play in the overall apoptotic bystander response.

The data clearly indicated differences between the apoptotic events in the direct versus the bystander response for dose and time. The direct samples demonstrated up-regulation in gene expression in both doses and time points. However bystander data displayed a common trend in that evidence for up-regulation was more notably apparent at the lower dose of 0.05 Gy. It was suggested that perhaps this was due to the extent of the damage by the low doses analysed (0.05 Gy and 0.5 Gy) or the exposure time analysed in the study 1 and 24 hrs.

Perhaps the full apoptotic cascade needs more than 24 hours to complete the process. To obtain a clearer pattern of the apoptotic gene expression profile bystander response, lower doses (0.005 Gy) would be required and additional time points at intervals between 1 to 24 hrs (6 hrs, 12 hrs ) would also be useful to indicate the changes in expression levels at specific times. The consideration of apoptosis genes from the other two known apoptosis pathways (extrinsic and Granzyme perforin pathway) would be beneficial to detect if the bystander response is a unique pathway with cross talk between genes of the apoptosis pathways. It is well known that there is cross-talk between all of the three types of apoptosis pathways and it is possible that the bystander response may be involved in this cross-talk.

Further to this study our group are currently analysing the post-translational effects of these genes, because protein expression doesn't always go hand in hand with gene up-regulation. It is possible to get post-translational modification of existing proteins, hence in their active form. Thus an RNA result showing no change does not necessarily mean no change in activity. It could suggest an alternative cell death mechanisms are in operation. From this

study we can delineate that human keratinocyte bystander cells exposed to low doses of ionising radiation have a different role of cell death than directly irradiated cells.

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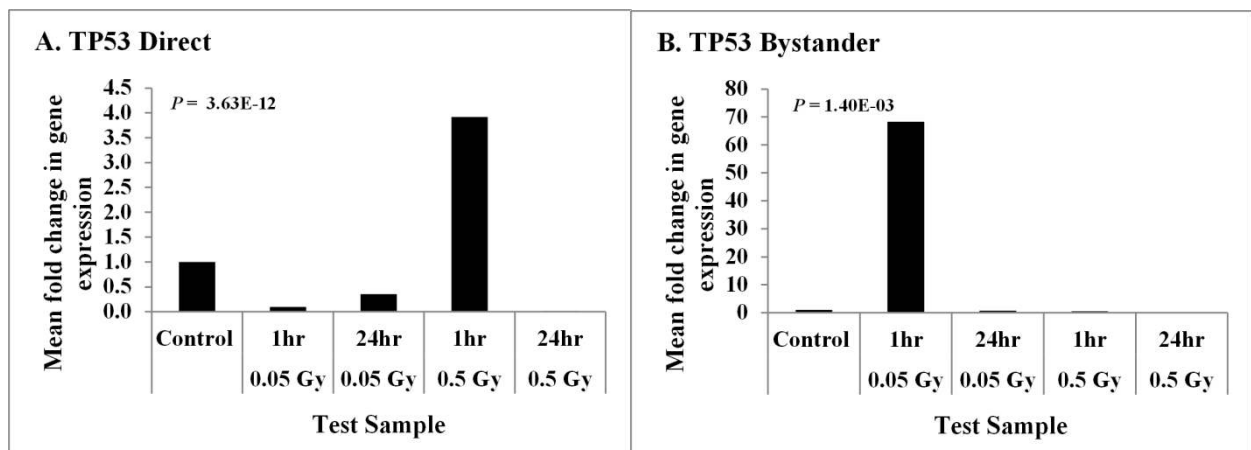
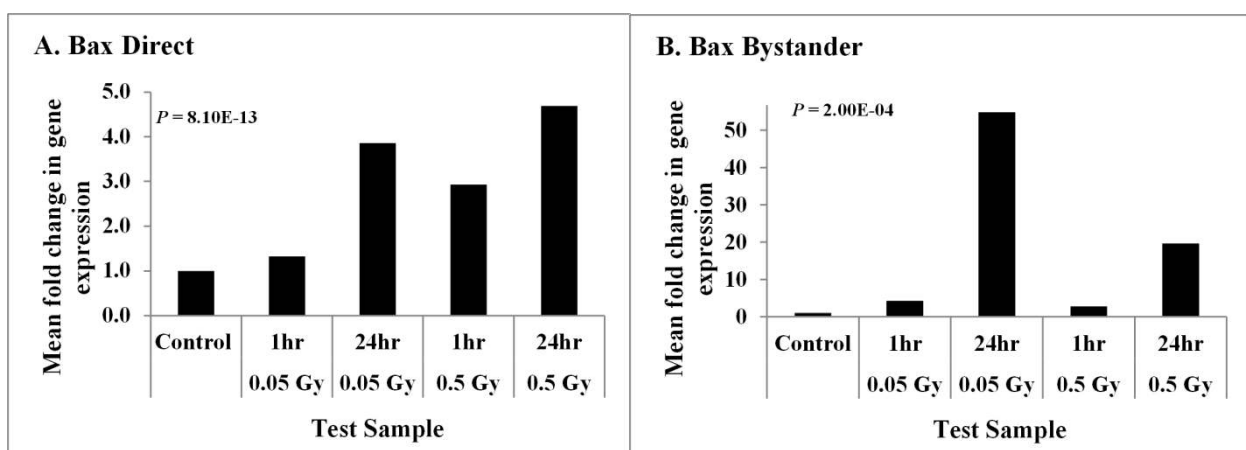


Figure 1 A-B: Comparison of relative fold-changes in gene expression levels of tumour suppressor gene TP53 in HaCaT cells, following 1 hr and 24 hrs exposures to 0.05 Gy and 0.5 Gy direct and indirect gamma irradiation.



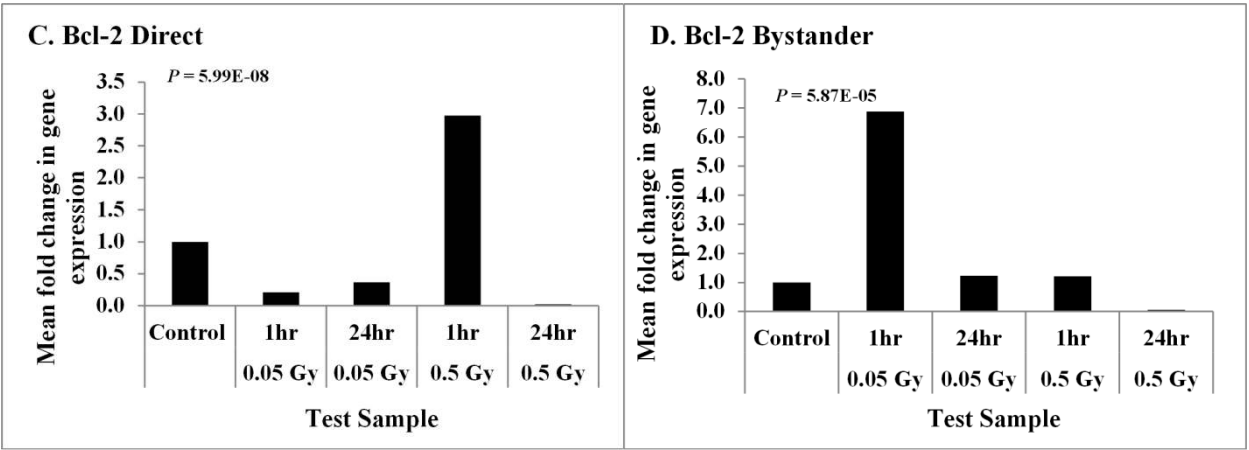


Figure 2 A-D: Comparison of relative fold-changes in gene expression levels of Pro-apoptotic Bax and anti-apoptotic Bcl-2 in HaCaT cells following 1 hr and 24 hrs exposures to 0.05 Gy and 0.5 Gy direct and indirect gamma irradiation.

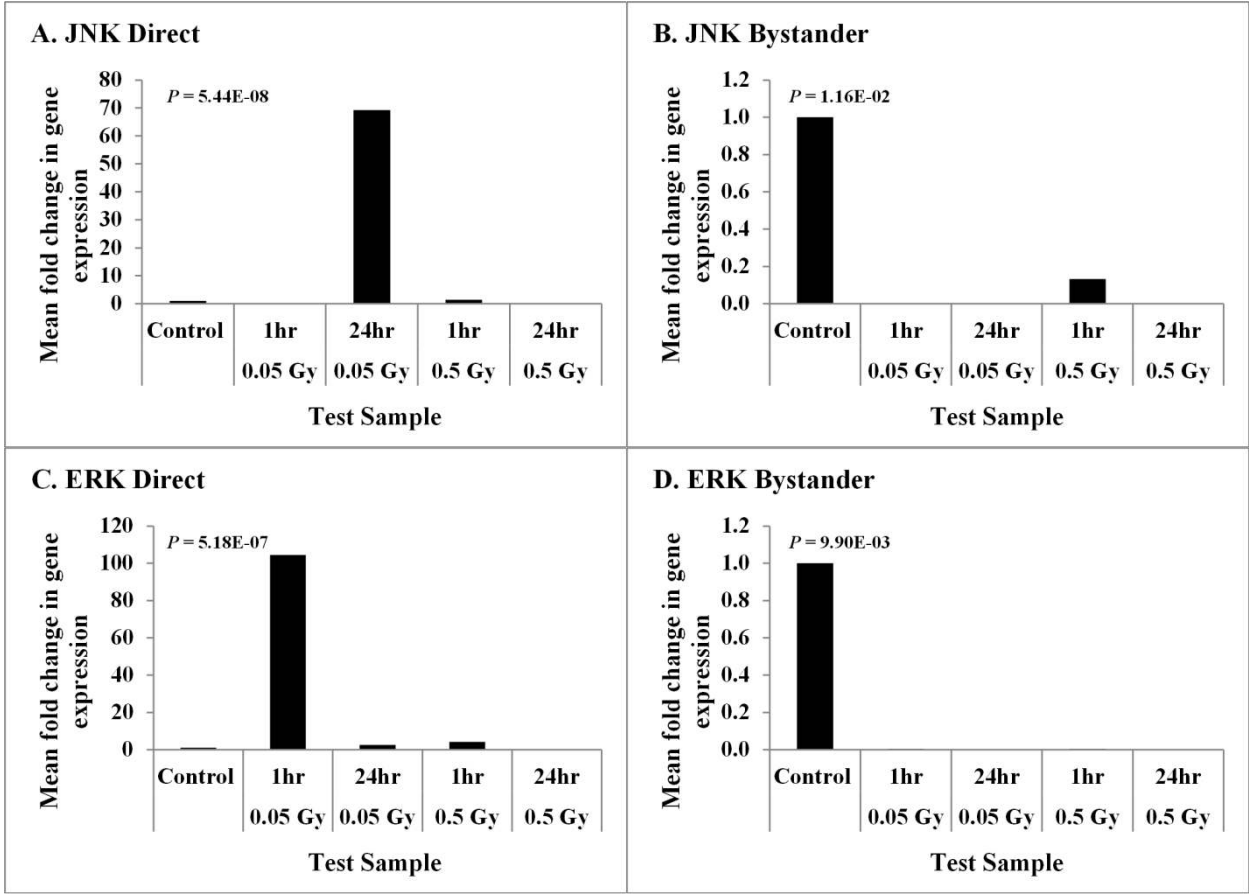


Figure 3 A-D: Comparison of relative fold-changes in gene expression levels of synergistic JNK and ERK in HaCaT cells following 1 hr and 24 hrs exposures to 0.05 Gy and 0.5 Gy direct and indirect gamma irradiation.

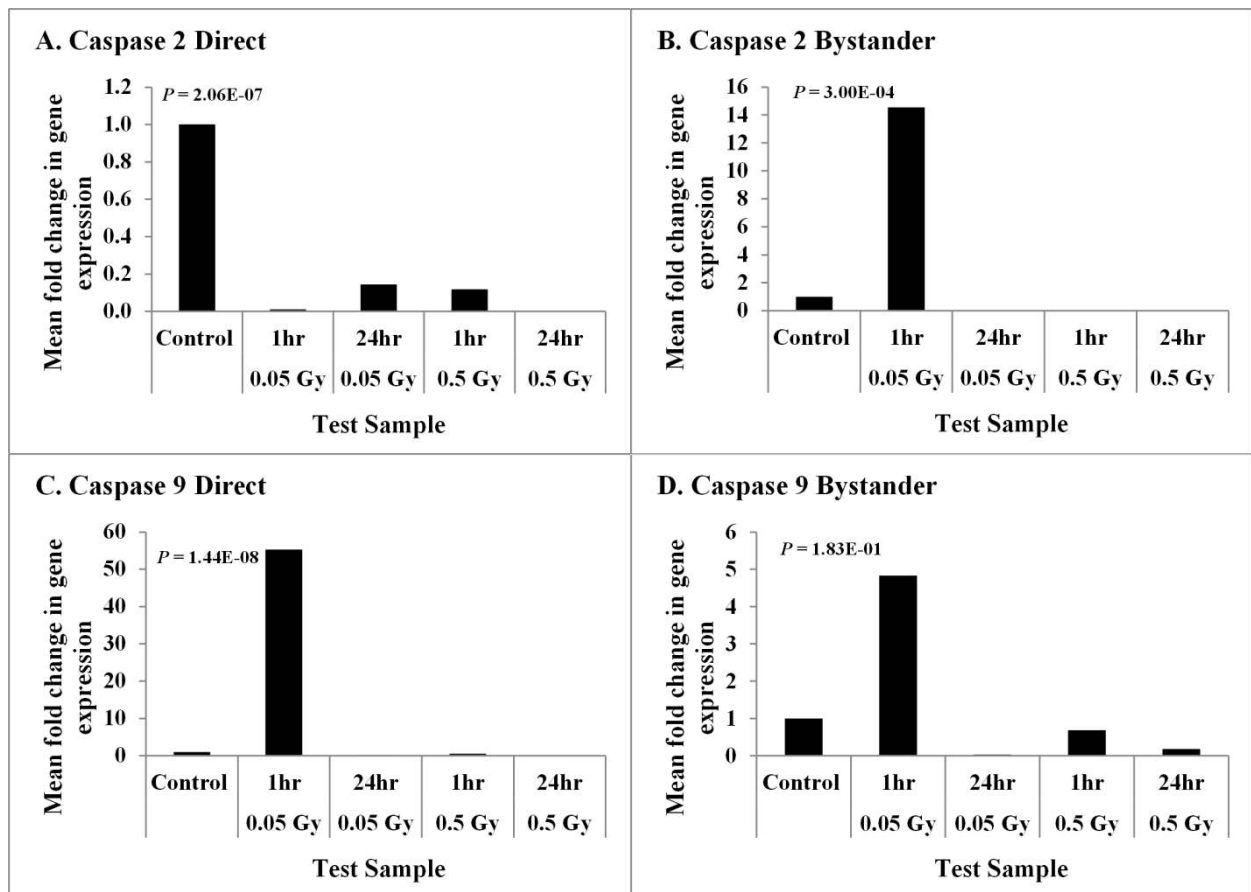
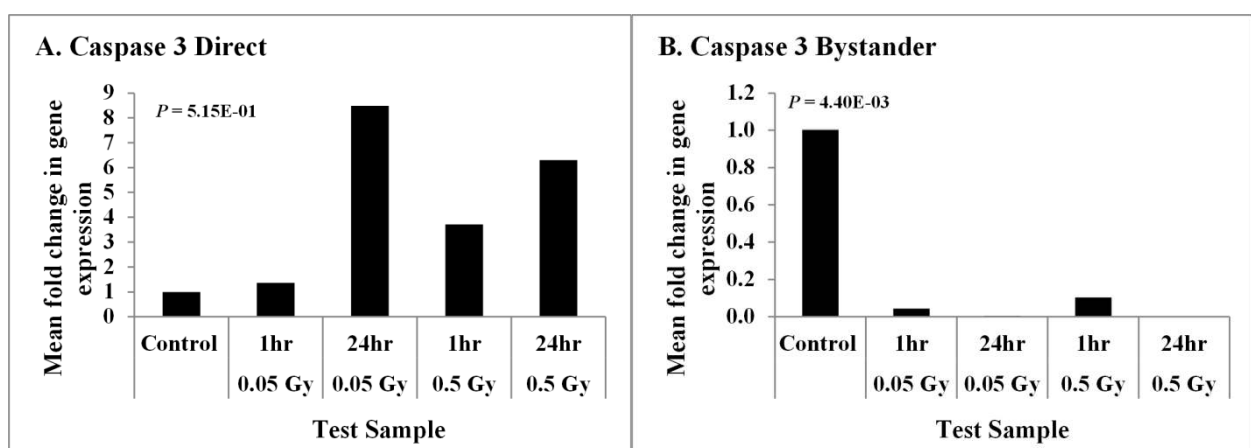


Figure 4 A-D: Comparison of relative fold-changes in gene expression levels of Initiator Caspases 2 and 9 in HaCaT cells following 1 hr and 24 hrs exposures to 0.05 Gy and 0.5 Gy direct and indirect gamma irradiation.



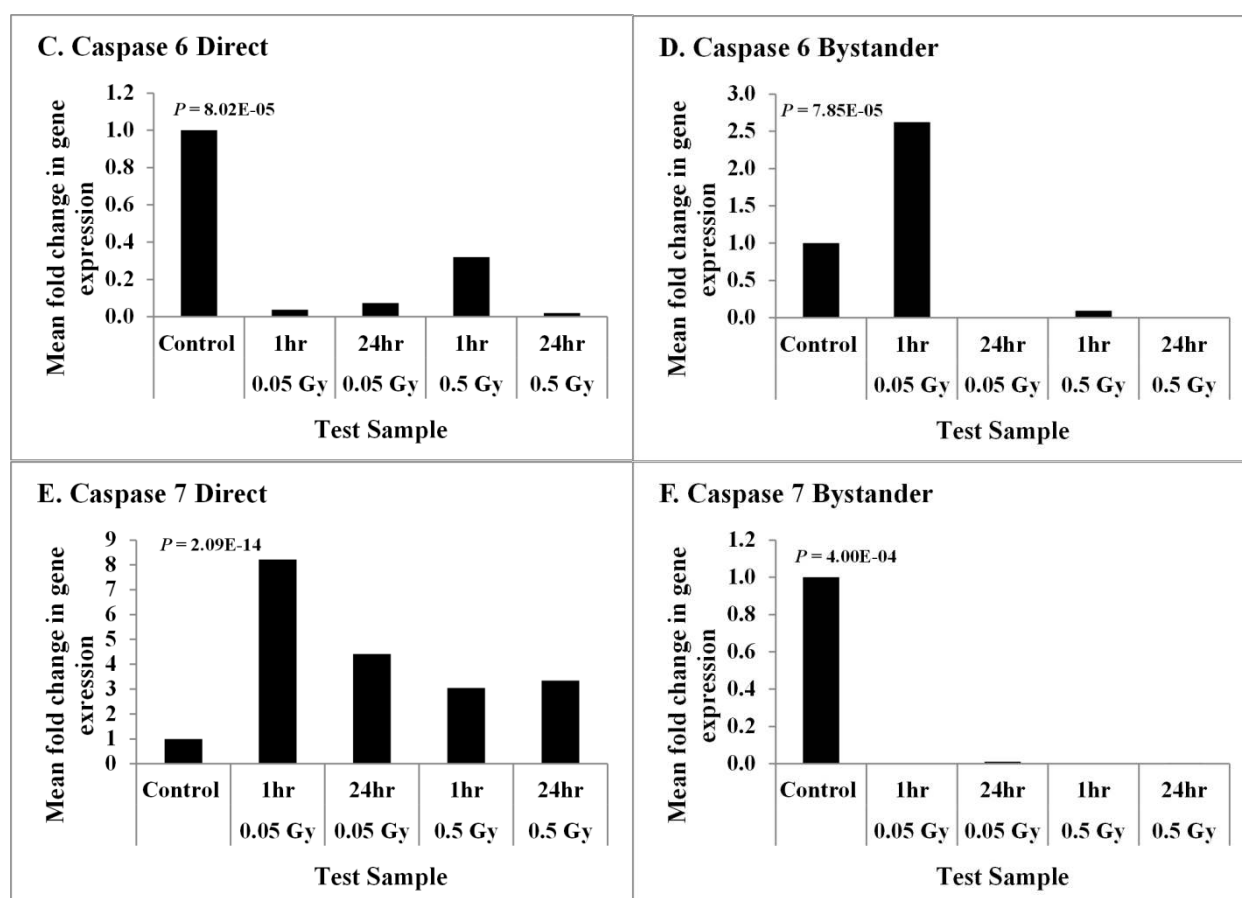


Figure 5 A-F: Comparison of relative fold-changes in gene expression levels of executioner Caspases 3, 6 and 7 HaCaT cells following 1 hr and 24 hrs exposures to direct and indirect 0.05 Gy and 0.5 Gy gamma irradiation.

Gene	Forward oligo sequence	Reverse oligo Sequence
TP53	5'GTCTTTGAACCCCTTGCTTGC'3	3'CCACAACAAAACACCAGTGC'5
Bax	5'AGGATGCGTCCACCAAGAAG'3	3'CCAGTTGAAGTTGCCGTCAGA'5
Bcl-2	5'AAGCGGTCCCGTGGATAGA'3	3'TCCGGTATTCGCAGAAAGTCC'5
ERK	5'TTAGGGCTGTGAGCTGTTCC'3	3'TCAGGAGGATGAGGACATGG'5
JNK	5'TTAAAGCCAGTCAGGCAAGG'3	3'CATTGATGTACGGGTGTTGG'5
Caspase 2	5'CTACCTGTTCCCAGGACACC'3	3'AGAACAGAAACCGTGCATCC'5
Caspase 9	5'TCCAGATTGACGACAAGTGC'3	3'AGGGACAGTGCTGAACATCC'5
Caspase 3	5'TTTGTTTGTGTGCTTCTGAG'3	3'TGAATTTTCGCCAAGAATAT'5
Caspase 6	5'CCTGACCAACATGGAGAAGC'3	3'AGTGATTCTCCTGCCTCAGC'5
Caspase 7	5'AAGATCCCAGTGGAAGCTGA'3	3'TCTCATGGAAGTGTGGGTCA'5
Tubulin	5'GCTTCTTGTTTCCACAGC'3	3'CTCCAGCTTGGAATTCTTGC'5

**Table 1** List of Forward and reverse oligo sequences of genes used in this study

**Table 2** Statistical values for Real-Time PCR gene expression analysis data presented in Figures 1.1 to 1.5. Significant differences in gene expression changes were determined with One-Way Anova, whereby the mean values of target genes were compared for significant variability in data. A gene expression change was deemed statistically significant (denoted by a 'YES' in the table below) only if  $p < 0.05$  and values are displayed below.

Gene	Dose Gy	One Way Anova (Direct) $p =$	Significance Direct	One way Anova (Bystander) $p =$	Significance Bystander
TP53	Control	3.63E-12	YES	1.40E-03	YES
Bcl-2 Family Pro and Anti-apoptotic					
Bax	Control	8.10E-13	YES	2.00E-04	YES
Bcl-2	Control	5.99E-08	YES	5.87E-05	YES
Stress Activated Protein Kinases					
JNK	Control	5.44E-08	YES	1.16E-02	YES
ERK	Control	5.18E-07	YES	9.90E-03	NO
Initiator Caspases					
Casp 2	Control	2.06E-07	YES	3.00E-04	YES
Casp 9	Control	1.44E-08	YES	1.83E-01	NO
Effector Caspases					
Casp 3	Control	5.15E-01	NO	4.40E-03	YES
Casp 6	Control	8.02E-05	YES	7.85E-05	YES
Casp 7	Control	2.09E-14	YES	4.00E-04	YES