Lack of Bystander Effects From High LET Radiation For Early Cytogenetic Endpoints.

Torsten Groesser, Brian Cooper and Bjorn Rydberg

Lawrence Berkeley National Laboratory, Life Sciences Division, Department of Genome Stability, Berkeley, CA 94720, USA

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Corresponding author: Dr. T. Groesser, Lawrence Berkeley National Laboratory, Building 74-157, 1 Cyclotron Road, Berkeley, CA 94720, USA. email: tgroesser@lbl.gov

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The aim of this work was to study radiation induced bystander effects for early cytogenetic endpoints in various cell lines using the medium transfer technique after high and low LET radiation. Cells were exposed to 20 MeV/amu Nitrogen ions, 968 MeV/amu Iron ions or 575 MeV/amu Iron ions followed by transfer of the conditioned medium from the irradiated cells to unirradiated test cells. The effects studied included DNA double-strand break induction, γ-H2AX foci formation, induction of chromatid breaks in prematurely condensed chromosomes, and micronuclei formation using DNA repair proficient and deficient hamster and human cell lines (xrs6, V79, SW48, MO59K and MO59J). Cell survival was also measured in SW48 bystander cells using X-rays. Although it was possible to occasionally detect an increase in chromatid break levels using Nitrogen ions and seeing a higher number of γ-H2AX foci formation using Nitrogen and Iron ions in xrs6 bystander cells in single experiments, the results were not reproducible. After pooling all the data, we could not verify a significant bystander effect for any these endpoints. Also, we did not detect a significant bystander effect for DSB induction or micronuclei formation in these cell lines or for clonogenic survival in SW48 cells. The data suggest that DNA damage and cytogenetic changes are not induced in bystander cells. In contrast, literature data show pronounced bystander effects in a variety of cell lines, including clonogenic survival in SW48 cells and induction of chromatid breaks and micronuclei in hamster cells. To reconcile these conflicting data, it seems possible that the epigenetic status of the specific cell line or the precise culture conditions and medium supplements, such as serum, might be critical for inducing bystander effects.

INTRODUCTION

For decades it has been a central dogma in radiation biology that the energy from ionizing radiation must be deposited in the cell nucleus of the affected cell or at least in the cell's cytoplasm to induce a radiation response. However, in the last years data have provided evidence that cells that are not hit directly by ionizing radiation are also influenced by damaged neighboring cells. This now well accepted phenomenon, called the bystander effect, has been reported in various cell types and biological endpoints such as sister chromatid exchange (*1, 2*), clonogenic survival (*3, 4*), micronucleus formation (*5-10*), chromatin damage (*11*), chromosome aberrations (*12*), foci formation (*5*), apoptosis (*13*) etc (for an overview see reviews (*14-21*) and summaries (*22, 23*)). Two major mechanisms are described in the literature. First a transfer of factors through gap junctions (*6*) and second a medium mediated transport of the bystander factors independent of cell-cell contact (*3, 24*). However, the factors that are involved in the transmission of the effects are still unknown. Due to the fact that the bystander effect can be detected at very low doses (see (*20*) for a review) this phenomenon is important for radiation risk assessment at doses below 1 Gy (overview (*14, 15, 21*)).

The majority of results published on the bystander phenomenon show damage in the bystander cells that are similar to the damage in the irradiated cells, such as single-strand breaks (SSBs), base damage and point mutations (overview see (*25*)). Nevertheless there are a few publications which mention that no bystander effect was detectable in some cell lines. Kashino et al. (*26*) observed a bystander effect in repair-deficient CHO cells (xrs6 and EM9) but not in the wild type CHO cells using a X-ray microbeam and the micronucleus assay. Mothersill *et al*. measured clonogenic survival in CHO-K1 and 1Br3 cells after incubation with radiation cell conditioned medium (ICCM) using γ-rays and was not able to observe a significant reduction in cell survival of bystander cells (*3*). The same group reported that medium from irradiated epithelial cells had a toxic effect on unirradiated fibroblasts while medium from irradiated fibroblasts showed no effect on clonogenic survival (*24*).

In contrast Yang *et al*. reported a couple of years later a bystander effect in human fibroblasts after low LET radiation for various biological endpoints including cell survival (*5*). Geard *et al*. (*8*) was able to detect a bystander effect for micronuclei formation using α-particles in human fibroblast (BJ-1/h-tert) but not in human epithelial cells (RPE/h-tert).

Here we summarize our observations concerning the bystander effect for various cell lines and biological endpoints and discuss possible reasons why a significant bystander effect was not observed for these endpoints of interest.

MATERIALS AND METHODS

Cell culture:

Chinese hamster lung fibroblast V79 (with a mutated nonfunctional p53) and a DNA double-strand break repair-deficient Chinese hamster ovary cell line xrs6 (deficient in Ku80) were gifts from Dr. Priscilla Cooper. Human glioblastoma MO59K and MO59J (deficient in DNA-PKcs) are fibroblast-like cells from the same 33 year-old male patient with untreated glioblastoma (obtained from Dr. Janice Pluth). The epithelia-like p53 wild type human colon cancer cells SW48 (deficient in mismatch repair due to methylation) were purchased from ATCC.

The rodent cells (xrs6 and V79) were cultured in α-MEM medium (Gibco) with 10% FBS (Gibco), 2 mM L-glutamine (Gibco), 100 Units/ml penicillin:streptomycin solution plus 0.25 μg/ml Amphotericin B as Fungizone (Gibco), and 10 mM Hepes buffer (Sigma). The human cell lines MO59K and MO59J cells were cultured in DMEM medium (Gibco) with 10% FBS, 2 mM L-glutamine, 100 Units/ml penicillin: streptomycin plus 0.25 μg/ml Amphotericin B as Fungizone, and 10 mM Hepes buffer. The human colon cancer cell line SW48 was cultured initially in Leibovitz's L-15 medium (ATCC) with 10% FBS (Gibco; Lot No.: 1292494) and 2 mM L-

glutamine and after two weeks gradually transferred to DMEM medium. Cells in Leibovitz's L-15 medium were incubated at 37°C, 95% humidity in 100% air. Cells in α-MEM and DMEM were incubated at 37°C, 95% humidity, and 5% CO_2. All cell culture was performed in T25 and T75 flasks (Gibco) except cells for immunostaining which were seeded in 4 or 8 well glass chamber slides (NUNC). Cells were trypsinized with 0.1% trypsin (Gibco) plus 0.5 mM EDTA (Sigma) for about 5 min at 37°C after washing the cell monolayer once with 1x PBS.

All cell lines were tested for mycoplasma (Bionique Test Labs, Saranac Lake, NY) and only cells tested negative were used.

Irradiation:

Exposures with 20 MeV/amu Nitrogen ions (LET = 150 keV/μm) was performed at the 88inch cyclotron at Lawrence Berkeley National Laboratory (LBNL). Cells were irradiated through the bottom of the cell culture flask using dosimetry as previously described (*27*). Irradiation with Fe ions with energies of 968 MeV/amu (LET = 151 keV/μm) or 575 MeV/amu (LET = 176 keV/μm) were performed at the NASA Space Radiation Laboratory (NSRL) at Brookhaven National Laboratory (BNL) (*28*). The dose rate for the heavy charged particle experiments was 1 Gy/min (1.5 Gy) and 0.1 Gy/min (0.15 Gy). X-ray experiments were performed using 320 kVp X-rays filtered through 0.5 mm copper at a dose rate of 0.6 Gy/min (1.5 Gy) and 0.12 Gy/min (0.15 Gy). All exposures were performed at room temperature and cells were out of the incubator for not more than 5 minutes.

Medium transfer:

For the medium transfer experiments, the protocol of Mothersill and Seymour (*24*) was used. Near-confluent cells (about 80%) in T75 flasks with 15 ml medium were exposed to doses of 0, 0.15, or 1.5 Gy and incubated

at 37°C for 1.5 h after exposure. The cell culture medium (Irradiation Conditioned Cell-culture Medium ICCM) was then removed, filtered using a 0.22 μm filter unit (Corning) and added to the recipient cells in T25 flasks or chamber slides. The cultures were then incubated for various times (1, 3, 6, 24, 48 h) depending on the biological endpoint studied. In addition to the sham irradiated samples (0 Gy) the experiments included control samples without a medium change at all. Donor and recipient cells were always of the same cell line, for example, xrs6 recipient cells received medium from irradiated xrs6 cells.

Premature chromosome condensation and scoring of chromatid breaks:

After incubating the cells for 1, 3 or 6 h with ICCM, premature chromosome condensation was induced by adding 30 nM of Calyculin-A. Within the 30 min incubation with Calyculin A the cells rounded up and detached. The cells were collected, centrifuged, resuspended in 0.075 M potassium chloride (KCl) and incubated for 10 min at 37°C. The cells were then fixed twice in 25% acetic acid (glacial) in methanol. Cells in fresh fixative were dropped on wet slides, air dried, and stained in 4% Giemsa for 10 min. After covering the slides with mounting media, chromatid breaks in 50 – 100 G2/M-phase cells were scored blindly. Only gaps that were wider than a chromatid were counted as a chromatid break.

Immunostaining for γ-H2AX:

Cells for γ-H2AX immunostaining were cultured and stained in 4 or 8 well glass chamber slides (NUNC). After incubating the cells with ICCM for 1 or 3 h the cell culture medium was removed and the cells were washed twice with PBS. Cells were then fixed in 2% Paraformaldehyde in PBS for 10 min at room temperature, washed twice in PBS and treated with pre-cooled 100% methanol for 30 min at -20°C to open up the plasma membrane. After two additional washes with PBS at room temperature, the slides were stored at 4°C for a maximum of 2 weeks. Samples from BNL (NSRL) were transported back to LBNL filled up with PBS on ice and stained

within two weeks after fixation. Samples from the 88inch cyclotron were also stored refrigerated at least one night in 1x PBS. Before immunostaining the samples were blocked for 1 h in 1% BSA/1x PBS. After blocking, the cells were incubated for 2 h in a mouse monoclonal IgG1 anti phospho-histone H2AX (serine 139) antibody (Upstate, 1mg/ml, 1:500 dilution in 1% BSA/1x PBS). After washing the cells three time in 1% BSA/1x PBS, they were incubated 1 h with the secondary antibody Alexa Fluor 488 goat anti-mouse IgG (Molecular Probes, 2 mg/ml, 1:200 dilution in PBS). Cells were washed twice in 1x PBS, counterstained with 0.1 μg/ml DAPI/1x PBS for 5 min, and washed one more time with 1x PBS for 10 min. Slides were air dried and mounted with Vectashield (Vector Labs). γ-H2AX foci were scored manually in coded samples using a fluorescence microscope with 63x objective (Zeiss Axioskop). S-phase cells, identified by a high uniform label and/or a large number of small foci, were excluded from the analysis.

Micronuclei formation:

Micronuclei formation was measured in Cytochalasin B (3 μg/ml) induced binucleated cells 24 h (rodent cells) or 48 h (human cells) after changing the medium to ICCM. Cytochalasin B was added to the cells at the time of medium transfer. After 24 h or 48 h incubation respectively cells were trypsinized, centrifuged, and resuspended in 7 ml 0.075 M potassium chloride and incubated for 10 min at 37°C. A mild fixation was performed by adding 3 ml of 100% methanol to each centrifuge tube and the cells were incubated for at least 1 h at room temperature. The cells were then fixed twice in 25% acetic acid in methanol and spun down on glass slides. The cells were air dried and stained with Diff Quick (Dade Behring) following the manufacture's protocol. For each sample (blinded), micronuclei in 300 – 1000 binucleated cells were counted by eye following the scoring criteria described in detail in Fenech et al, 2003 (*29*).

Flow cytometry based measurement of γ-H2AX levels:

For the flow cytometry analysis, cells were immunostained for γ-H2AX using a protocol designed by Dr. J. Pluth (personal communication). Briefly, cells were cultured in T25 flasks, trypsinized at 1 h or 3 h after medium transfer to ICCM, centrifuged, and washed once in PBS. Cells were resuspended in 200 µl PBS and fixed by adding 800 µl of 70% ethanol dropwise while vortexing. Fixed cells were stored at –20°C until subsequent staining. Approximately 1 x 10^6 fixed cells were stained as follows. Cells were spun down to remove ethanol, washed once in PBS, then once in 1 ml of 2% goat serum in PBS. After centrifugation, cells were resuspended in 100 µl of PBS containing 2 % goat serum and a 1:800 dilution of γ-H2AX mouse monoclonal antibody (Upstate). Cell suspensions were incubated with gentle shaking for 1 h on ice. After incubation with the primary antibody, cells were pelleted and washed with 200 µl of 2% serum-containing PBS. After centrifugation, cells were resuspended in secondary fluorescent-labeled goat anti-mouse antibody (Alexa 488, Molecular Probes) diluted 1:400 in PBS and incubated for 1 h with gentle shaking. Cells were washed in 200 µl of PBS, the supernatant was removed and the cells resuspended in PBS containing 1 mg/ml of RNase A and incubated for an additional 20 min at room temperature. After a final spin, pellets were resuspended in PI-containing PBS (10 µg/ml), covered with foil, and refrigerated until flow cytometry analysis. Cells were analyzed on a Becton Dickinson FACScan, collecting at least 10,000 cells per data point. Medianfold increases in levels of Alexa 488 conjugated γ-H2AX fluorescence as compared to controls were determined for each sample.

Clonogenic survival of SW48 cells:

The clonogenic survival experiment was performed following the protocol of Mothersill *et al*. (*3*) with minor modifications. SW48 cells in DMEM medium were exposed to X-rays in T75 flasks followed by incubation at 37°C for 1.5 h. Cells seeded at clonogenic densities in T25 flasks were then receiving the ICCM from the irradiated flasks. After 18 days the cells were washed once in 1x PBS, fixed and stained in 0.5 g/l crystal violet

plus 10% Paraformaldehyde in PBS for 20min at room temperature. Colonies with at least 50 cells were counted (*30*)**.**

Measurements of DSBs by Pulsed-field gel electrophoresis:

In order to detect DSBs with high sensitivity we used a method that involves using mixtures of ^{3}H-thymidine and ^{14}C-thymidine labeled cells to include internal controls in pulsed-field gel experiments (full details to be published separately). Using this methodology we could measure DSB induction in mammalian cells down to a dose of about 0.2 Gy. Results are given as ΔFAR values, which represent the difference in fraction activity released (FAR) between treated and non-treated samples run in the same lane in the agarose gel. The following is a brief description of the method.

The experimental setup contained flasks with ^{3}H-thymidine labeled cells and flasks with ^{14}C-thymidine labeled cells used for treatments (direct irradiation, or exposure to medium from irradiated cells) and used as non-treated controls. After treatment, the cells were trypsinized, centrifuged, and resuspended in a small amount of PBS. Treated ^{3}H-labeled cell suspensions were then mixed with an equal volume from a ^{14}C-labeled control cell suspension, and similarly, treated ^{14}C-labeled cell suspensions were supplemented with an equal volume from a ^{3}H-labeled control cell suspension. To 100 µl ice-cold ^{3}H/^{14}C cell mixtures were then added an equal volume of 1.5 % low melting temperature agarose (Sigma, type 7) in PBS (kept at 50°C), and 200 µl plugs were cast using a BioRad plug-former. The plugs were then lysed at 50°C for at least 24 hrs in 0.4 M EDTA, 2% sarkosyl, 0.5 mg/ml proteinase-K, pH 8.

To increase the sensitivity of the following FAR assay and use a portion of the FAR induction curve that is approximately linear with dose (*31*), the plugs with mixtures of ^{3}H and ^{14}C labeled DNA were then equilibrated in 0.4 M EDTA and given an additional X-ray dose of 50 Gy to induce random breaks and bring the FAR values up to a level of around 10%. The EDTA serves as an efficient scavenger that minimizes the influence of

DNA concentration in the yield of DSBs formed. Since the same numbers of additional breaks are induced in the ^{3}H-labeled DNA and the ^{14}C-labeled DNA, the difference in FAR will still reflect the initial DSBs present in the treated cells. Fractions of plugs containing at least 10,000 cpm ^{3}H and 5,000 cpm ^{14}C were loaded onto 0.8% agarose gels in 0.5xTBE and run for 24 hrs at 14°C in a BioRad Chef II pulsed-field gel electrophoresis unit at 50 V with a constant switch time of 1 hr. This regimen produces a compression band of DNA fragments of sizes in the range of 0.1 - 5 Mbp. Each lane was cut into three gel slices of equal size using an array of razor blades. The first slice contained the original plug, and the other two slices covered the compression band. The gel slices were put in liquid scintillation vials together with 0.5 ml 1 M HCl and the capped vials were placed in a boiling water bath for 20 minutes. After cooling to room temperature, 15 ml Optifluor scintillation liquid (Packard) was added and the samples were well mixed. The samples were typically counted for 10 min to obtain sufficient statistical accuracy, and recounted for verification. Background counts were subtracted and corrections were made for spill-over of ^{14}C counts into the ^{3}H channel and ^{3}H counts into the ^{14}C channel. The corrected values were used to separately calculate FAR for the ^{3}H DNA and the ^{14}C DNA in each lane and the difference (ΔFAR) between treated and non-treated samples were calculated for each lane. Finally, the average ΔFAR for treated ^{3}H-labeled cells mixed with ^{14}C-labeled control cells and ΔFAR for equally treated ^{14}C-labeled cells mixed with ^{3}H-labeled control cells was used to cancel out a small amount of DNA breaks caused by the radioactive labels.

RESULTS

The results presented below are given either as absolute numbers or as bystander effect sample values fold over sham values (0 Gy), and all errors in the text are ± error of the mean (SEM). For general reference, Table 1 summarizes the bystander effect results with standard deviation and SEM for each cell line, biological endpoint

and radiation quality individually (fold over sham) as well as pooled results for each biological endpoint, while Table 2 shows the absolute control values for different cell lines and biological endpoints with errors of the mean and the number of independent samples (N).

Chromatid breaks in prematurely condensed chromosomes:

For the medium transfer, the protocol of Mothersill and Seymour (*24*) was used. We started to test if irradiation conditioned cell culture medium (ICCM) would induce chromatid breaks after relatively short times of incubation with non-irradiated recipient cells. Therefore xrs6 and V79 cells were exposed to Nitrogen ions and, after 1.5 h of incubation, the ICCM was transferred to unexposed recipient cells. After incubation for 1, 3 or 6 h, the chromosomes were condensed by Calyculin-A, and chromatid breaks were scored for cells in G2/M-phase. In Figure 1 the results from 3 independent experiments are summarized for xrs6 cells. In experiment A an increase of 1.6 fold (0.15 Gy) and 2.3 fold (1.5 Gy) over the individual sham levels (0 Gy) was found after 1 hr incubation with ICCM. Also after 3 h incubation with ICCM elevated levels of chromatid breaks in bystander samples were detected (1.7 fold for 0.15 Gy and 1.6 fold for 1.5 Gy). After 6 h incubation the fold values over sham were close to 1 due to a higher control level for this particular time point (1.12 fold for 0.15 Gy and 1.0 fold for 1.5 Gy). However this increase was not reproducible, and after pooling all the data for the Nitrogen experiments with xrs6 cells (experiment A – C) an average fold over the sham (0 Gy) of 1.08 ± 0.08 was calculated. Additional data for Iron ion exposure are plotted as fold over average sham (measured for each cell line) for xrs6 and V79 cells in Figure 2A. In neither of these cell lines were we able to observe an increase of chromatid breaks in the bystander samples over the average sham control after pooling the data (0.97 ± 0.07 for xrs6 and 0.80 ± 0.09 for V79). The average fold over average sham of all bystander samples (including V79 and xrs6 cell data collected in Nitrogen and Iron ion experiments) for the induction of chromatid breaks in premature condensed chromosomes is 0.92 ± 0.06 (Table 1). This contrasts with directly exposed cells, where a

20 – 37 fold increase in chromatid breaks per cell and Gy over control levels was observed in xrs6 and V79 cells depending on the radiation quality (data not shown).

Formation of γ-H2AX foci:

γ-H2AX foci formation after incubation with ICCM from Iron ion exposed cells is plotted as bystander effect sample values fold over sham in Fig. 2B. Values in xrs6 (1.23 ± 0.34), V79 (0.91 ± 0.09), and M059K (0.97 ± NA) cells were not significantly different from 1 while M059J cells showed a 1.72 fold higher average number of foci in the bystander effect samples over the sham (0 Gy). This result is only based on two data points and overall after pooling all data for this endpoint from the different cell lines it was not significantly higher than the sham (1.18 ± 0.18). In comparison, γ-H2AX foci formation per Gy in directly exposed cells was 5 – 20 fold higher than controls (data not shown).

Flow cytometry based measurement of γ-H2AX stained cells:

Measuring mean levels of Alexa 488 conjugated γ-H2AX fluorescence in bystander cells by flow cytometry showed no increase over sham level for xrs6 cells (1.03 ± 0.04) after incubated with ICCM from Iron ion exposed cells (Fig. 2C). V79 cells showed a slightly higher value of 1.10 ± 0.07 fold over sham level which is due to only one data point (1.84 fold over average sham). All the other data points are scattered closely around a value of 1. Pooled data (xrs6 and V79) showed an average fold over the sham level of 1.07 ± 0.04 (Table 1).

Micronuclei formation:

Micronuclei (MN) formation was measured in binucleated cells after medium transfer of Nitrogen and Iron ion exposed cells (Fig. 2D). In M059K and SW48 cells the fold over control in the bystander effect samples after

incubation with ICCM from Nitrogen exposed cells was 1.28 ± 0.29 and 1.28 ± 0.23 respectively. In M059K and V79 cells an average value of 1.00 was obtained after receiving medium from Iron ion exposed cells. Lower values were found in M059J cells after incubation with ICCM from Nitrogen ion (0.53 ± 0.22) and Iron ion (0.86 ± 0.08) as well as in xrs6 cells receiving ICCM from Iron ion irradiated cells (0.89 ± 0.13). If we pool all the data from the different cell lines for this endpoint we obtain a value of 1.00 ± 0.08 (Table 1). In directly exposed cells we have previously observed a 10 – 30 fold increase per Gy in MN formation over control levels for the different cells lines (for xrs6 and V79 data see (*32*)).

DSB induction in irradiated and bystander cells:

A novel dual-label pulsed-field gel electrophoresis method was used to sensitively detect DSBs in irradiated and bystander xrs6 cells. These cells are deficient in DSB repair and are therefore expected to show accumulation of DSBs during ICCM incubation. Figure 3 shows DSB induction after N-ion irradiation at low doses. The directly irradiated cells have a linear induction of DSBs, while no induction is detected in bystander cells after incubation with ICCM for 3 h. This supports the data given above showing lack of γ-H2AX induction.

Clonogenic survival in SW48 cells:

Clonogenic survival was measured in SW48 cells after 18 days of incubation with ICCM from X-ray exposed SW48 donor cell. In Fig. 4 results for 3 different cell densities are pooled which were plated in triplicates and no significant change in the bystander cells could be observed. This contrasts with data published by Mothersill *et al*. (*3, 4*), who reported a significant decrease in bystander cell survival for these cells. However, we note that our plating efficiency (44%) was very different from that reported by Mothersill *et al.* (10 - 13%), indicating a major difference in the cells or culture conditions.

To further investigate the reasons for the lack of a bystander effect, in collaboration with Dr. Mothersill, we sent our frozen SW48 cells adapted to grow in DMEM or Leibovitz's L-15 medium as well as aliquots of our serum to Dr. Mothersill's laboratory. Performing bystander effect experiments under our cell culture conditions using our cells and serum, Mothersill *et al.* did not detect a bystander effect for cell survival (personal communication), thus confirming our results. However, the SW48 cells were then adapted over a month to the cell culture conditions used in her lab (including the serum), growing the cells in DMEM:F12 (1:1) medium. Under these culture conditions the plating efficiency of the SW48 cells dropped and the clonogenic survival of the cells was 50% reduced in bystander samples compared to control levels (Dr. Mothersill, personal communication). These results underline the importance of cell culture conditions for the induction of a bystander effect.

DISCUSSION

In the present work we have been particularly careful to include sham irradiations in each individual experiment and all our samples were scored blindly. We studied several different cell lines including DNA double-strand break repair deficient hamster xrs6 and human M059J cells to allow for accumulation of DSBs during ICCM incubation without their removal by repair. As was pointed out by Nagasawa and Little (*12*) the bystander effect for chromosomal aberrations in wild-type cells is quite small compared to repair deficient cells, and Little *et al.* (*33*) reported that dsbs in repair deficient donor cells ($NHEJ^-$) produced a more pronounced bystander effect. In

addition, we have only used cells that tested negative for mycoplasma contamination, thus eliminating any effects possibly due to mycoplasma.

Under these conditions, we did not detect a consistent bystander effect using the medium transfer technique in several early endpoints including DNA double-strand breaks, cytogenetic damage and cell survival. These results are consistent with results of Fournier *et al.* (personal communication), where similar endpoints but different methodologies were used to study bystander effects. On the other hand, the results are contradictory to other published reports (*3, 5, 11, 24, 34*), where bystander effects have been reported for similar endpoints. It should be noted to reach the negative conclusion in our study we have included all our data without discrimination, i.e. we have not eliminated negative results. Our data are consistent with the interpretation that bystander effects in general do not involve generation of DNA damage or early cytogenetic changes in the bystander cells. However, to reconcile literature data with our data, a possibility to consider is that bystander effects may occur only in certain particular, but not understood circumstances. It is possible to speculate for example that agents that transmit bystander signals can be inactivated by serum components, and therefore depend on the specific serum lot used. Another factor could be the status of the cells, such as epigenetic differences. We note, for example, that our SW48 cells had a high plating efficiency and did not show a bystander effect, while Mothersill *et al.* (*3, 24*) reported a much lower plating efficiency for the same cell line and a pronounced bystander effect.

In all our experiments the donor cells were incubated for 1.5 h after exposure before transferring the ICCM to the recipient cells, following the protocol used by Mothersill et al. (*24*). This short incubation time was chosen because Mothersill's group (*24*) showed that the factors responsible for the bystander effect seemed to be secreted into the medium shortly after exposure and the bystander effect did not change when the ICCM was harvested over a time period of 0.5 h to 18 h. However, we cannot rule out the possibility that a bystander effect

would have been detected in our experiments if we had harvested the ICCM medium at later times after irradiation.

Our study involved the use of accelerated heavy ions of relatively high LET in the range of 150 –176 keV/μm. This LET is only slightly higher than the LET of typical alpha particles, a radiation that has been used extensively in bystander experiments (*2, 6, 8, 9, 11*). We therefore do not think that our negative results are due to the specific type of radiation used.

In this work we studied the bystander effect after irradiation conditioned cell medium transfer. This complements the methods used by Fournier *et al.* (personal communication), where microbeam, low fluence and co-culture methodologies were utilized to study bystander effects of early cytogenetic damage, also with negative results.

Various cell lines and biological endpoints were included in this work. The formation of chromatid breaks was measured in premature condensed chromosomes (PCC) of xrs6 and V79 cells 1, 3 and 6 h after incubation with ICCM. No significant increase in the number of chromatid breaks in bystander samples could be observed over control (Fig. 1, 2a). This contrasts with the data reported by Suzuki *et al*. in 2004 (*11*). In this bystander effect study α-particles (LET=124 KeV/μm) were used to investigate the induction of chromatid fragments (chromatid breaks, isochromatid deletions, and acentric fragments) in a human-hamster hybrid cell line (A_L) using the double-Mylar technique. Under these co-culture conditions they were able to detect a bystander effect over a dose range of 0.1 to 100 Gy at different time points (3, 24, 48 h). As pointed out above, in our experimental setup the irradiated cells had only a short time (1.5 h) to produce and secret bystander effect factors into the medium and also the incubation time of unirradiated cells with the ICCM was short (1, 3, 6 h). Nevertheless, Suzuki *et al*. (*11*) was able to detect a significant bystander effect already after 3 h of co-culturing with an increase of the bystander effect over time (max. after 48 h) but with no clear dose dependence.

Foci formation in bystander cells after X-ray (*5, 34*), γ-ray (*35*) , α-particle (*35*) and heavy charged particle (*34*) exposure have been shown by several groups. Burdak-Rothkamm *et al*. (*36*) reported that foci induced in bystander cells were restricted to S-phase cells, which in our study were excluded from analysis. Foci in S-phase cells might arise from replication errors, not from primary induced DSBs. On the other hand, foci formation in other cell cycle phases in bystander cells has been reported (*34*), and co-localization of γ-H2AX foci with other proteins involved in DNA repair such as 53BP1, ATM, and Mre11 has suggested that foci induced in bystander cells correlate with DSBs (*35*). However this contradicts our study where we could not detect DSBs in xrs6 bystander cells by direct measurements using pulsed-field gel electrophoresis (Fig. 3).

Bystander effects for micronuclei (MN) formation have previously been observed in various cells lines. Yang *et al*. (*34*) reported a two fold higher number of cells with MN in normal human fibroblasts (AG01522) after co-culturing the cells with Iron ion exposed cells. This increase was only observed when the bystander cells were co-cultured with the irradiated cells within a 3 h window after the exposure which suggested that signal transduction from irradiated cells occurred shortly after irradiation. Assuming that the bystander factors are stable at 37ºC in cell culture medium we would therefore expect to see with our protocol an increase of MN frequency in the bystander cells. This was not the case for the cell lines used in our study (Fig. 2D and Table 1) except maybe for the MO59J cells after transfer of Nitrogen ion conditioned media.

Clonogenic survival in bystander cells has been measured extensively using the medium transfer technique (*3, 24, 26*), including the SW48 cells used here (*3, 24*). However, as shown in Fig. 4, no significant change in survival of the SW48 bystander cells could be observed by us. This contrasts with data published by Mothersill *et al*. (*3, 24*), who reported a significant decrease in cell survival for these cells. It can be pointed out that the plating efficiency in our hands was much higher (44%) than reported by Mothersill *et al*. (10-13%) for the same cell line. This might be due in part to the use of different medium and medium components. The SW48 cells in our lab were adapted to grow in DMEM medium after starting them in Leibovitz's L-15 medium (ATCC) as

recommended by ATCC while in Mothersill's lab SW48 cells were cultured in DMEM:F12 medium (1:1). In addition to the medium, also differences in medium supplements such as serum can not be ignored. For example, Nagasawa and Little (*37*) showed that apparently normal human fibroblast strains show significant different numbers for chromosomal aberrations and gaps when two different lots of serum were used. The tests performed for us in Dr. Mothersill's lab (see Results) further strengthens the importance of cell culture conditions. Mothersill *et al*. (*4*) have also pointed out that the cell density of the irradiated cells can be an important factor in medium transfer experiments, with a high cell density being more efficient than a low cell density. However, that is unlikely to explain our negative results since we consistently used near-confluent cell cultures (about 80% confluence).

Bystander effects in the literature are often reported as having no dose dependence (for example (*11, 13, 35*), review (19)), meaning that all treated samples have the same level of effect, while only the control is different. This has been a cause of criticism since the whole effect then depends on the single value of the control (*38*). From this standpoint, we have in our study included separate controls in each experiment and scored the samples blindly. Our data are therefore not dependent on single control values, but on equal number of control and treated samples.

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Figure legends:

Fig. 1: Chromatid breaks in DSB repair deficient xrs6 cells after medium transfer from Nitrogen ion exposed cells. Data are shown for three independent experiments. Cells were incubated with the irradiation conditioned cell culture medium (ICCM) for 1, 3, or 6 h followed by addition of Calyculin-A to induce PCC and scoring of chromatid breaks in G2/M cells. Data are for control cells (no medium change at all), for medium recipients of sham irradiated cells (0 Gy), and for medium recipients of cells that had been exposed to 0.15 and 1.5 Gy of Nitrogen ions.

Fig. 2: Summary of bystander effect data for the different cell lines and biological endpoints after medium transfer from cells exposed to Iron (Fe) ions or Nitrogen (N) ions. Data points are fold over average sham (0 Gy). A: Chromatid breaks measured in prematurely condensed chromosomes (PCC) using Calyculin A. B: Foci formation in γ-H2AX immuno-fluorescence samples. C: Fluorescent units measured in γ-H2AX immuno-stained flow cytometry samples. D: Micronuclei formation in binucleated cells. Closed symbols represent results from recipient cells which received irradiation conditioned cell culture medium (ICCM) from cells which were exposed to 0.15 Gy while open symbols represent data points for the 1.5 Gy samples. The values with error bars on the right side of each plot show the average value ± error of the mean including all data points for the particular biological endpoint. In none of the biological endpoints the average fold value over control was significantly higher than one, indicating a lack of bystander response.

Fig. 3. DSB induction in xrs6 cells (ku80-) after N-ion irradiation. ΔFAR represents the difference between treated and control cells in dual-label pulsed-field gel electrophoresis experiments. Although an induction of DSBs is seen in the irradiated cells at these low doses, there is no indication of DSB induction in the bystander cells incubated with ICCM from cultures given 0.15 or 1.5 Gy N-ions.

Fig. 4: Bystander effect data for clonogenic survival of SW48 cells after medium transfer of X-ray exposed SW48 donor cells. Plotted are data of cells without a medium change (control), cells that received medium where only the medium was exposed to 1.5 Gy X-rays (w/o cells), and cells that received medium from cells that were irradiated with 0 Gy (sham), 0.15 Gy, or 1.5 Gy. The error bars represent the standard deviation of 8 to 9 samples. The experiment was set up in triplicates in three different cell densities (500, 1000, and 2000 cells per T25 flasks). Survival results for the different cell densities were pooled because no significant difference was observed. No decrease in cell survival was detected in the bystander samples in comparison to the sample that received medium from sham irradiated cells.

TABLE 1

Summary of Nitrogen and Iron ion bystander data shown as fold over sham (0 Gy) for different biological endpoints and cell lines separately and pooled (all)

Cell line	Type of radiation	Fold over sham (0 Gy) for the different biological endpoints (Standard deviation; error of the mean)			
		Chromatid breaks per G_2/M-phase cell	Flow cytometry γ-H2AX	MN in BNC	γ-H2AX foci
xrs6	Nitrogen	1.08 (0.29; 0.08)	-	-	-
	Iron	0.75 (0.30; 0.11)	1.03 (0.10; 0.04)	0.89 (0.36; 0.13)	1.23 (0.95; 0.34)
V79	Iron	0.80 (0.25; 0.09)	1.10 (0.24; 0.07)	1.00 (0.30; 0.13)	0.91 (0.18; 0.09)
M059K	Nitrogen	-	-	1.28 (0.57; 0.29)	-
	Iron	-	-	1.00 (0.18; 0.09)	0.97 (NA; NA)*
M059J	Nitrogen	-	-	0.53 (0.44; 0.22)	-
	Iron	-	-	0.86 (0.16; 0.08)	1.72 (NA; NA)*
SW48	Nitrogen	-	-	1.28 (0.65; 0.23)	-
All	all	0.92 (0.32; 0.06)	1.07 (0.20; 0.04)	1.00 (0.47; 0.08)	1.18 (0.71; 0.18)

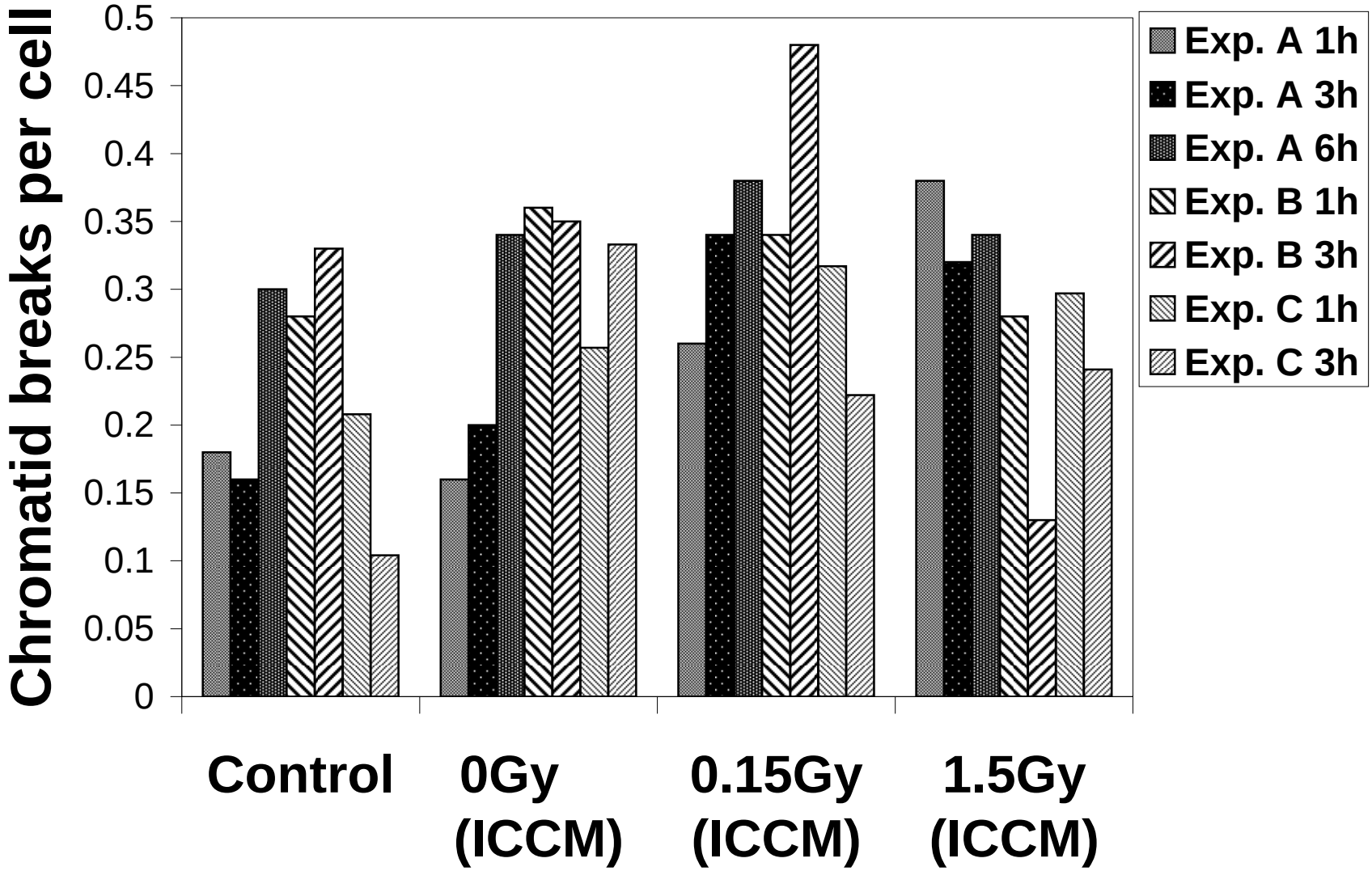
Note: MN = micronuclei; BNC = binucleated cells; * = only two data points for these endpoints; – = no data were collected.

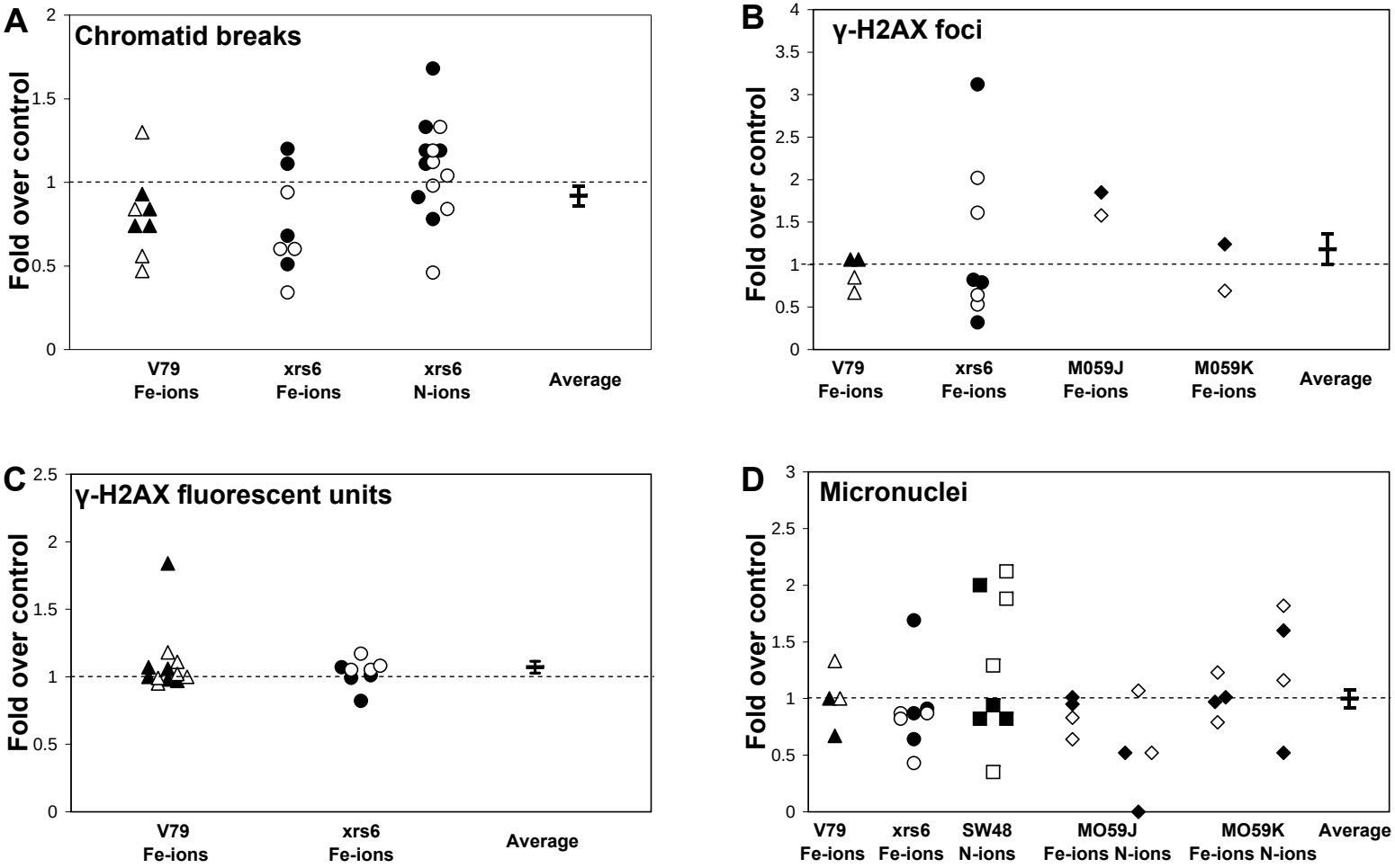
TABLE 2

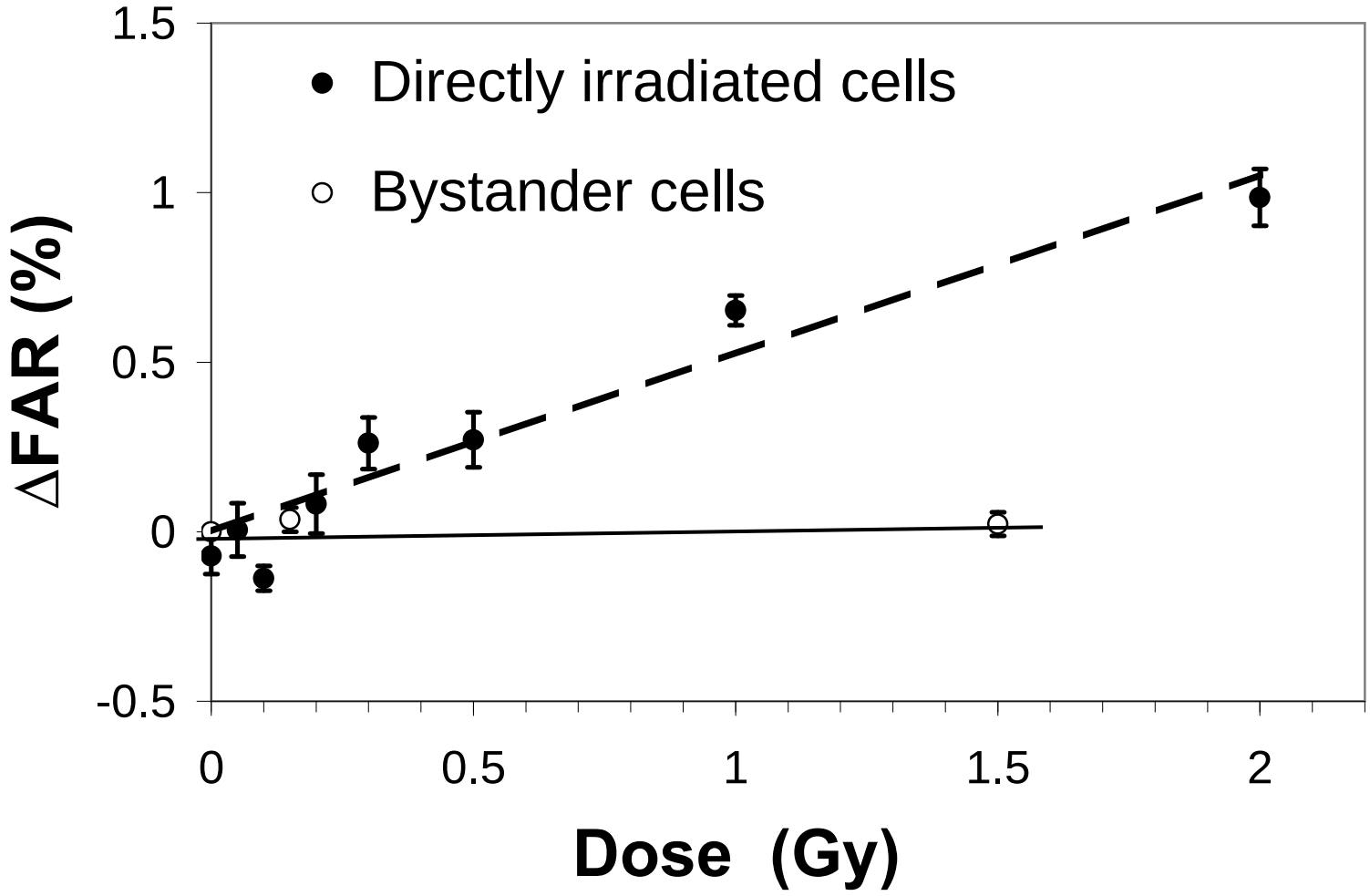
Summary of control values (0 Gy and sham) for different biological endpoints and cell lines

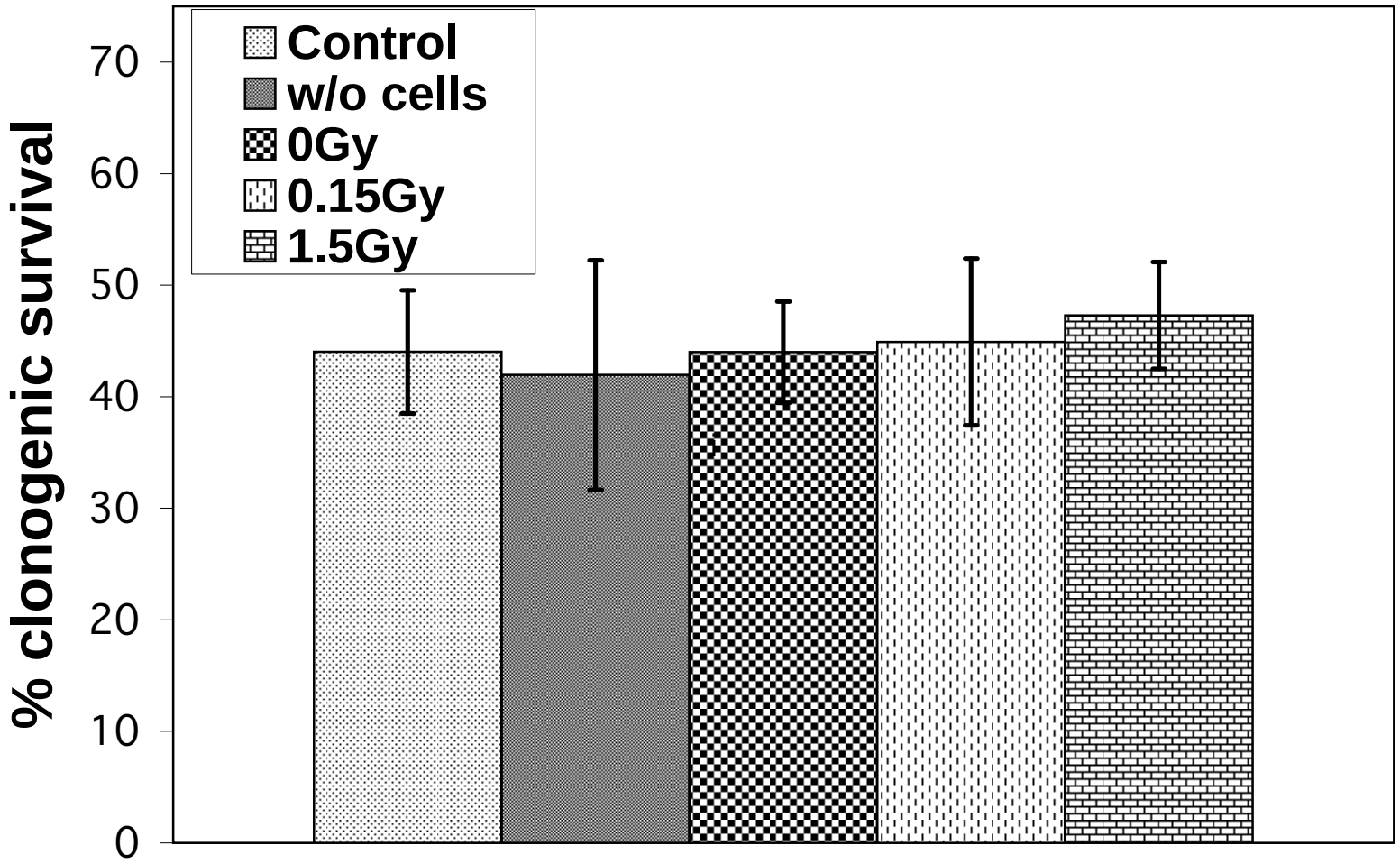
Cell lines	Control levels for the different biological endpoints ± error of the mean (N) (N= number of independent samples)		
	Chromatid breaks per G_2/M-phase cell	MN in 100 BNC	γ-H2AX foci per cell
xrs6	0.36 ± 0.04 (32)	3.30 ± 0.47 (25)	0.71 ± 0.18 (15)
V79	0.31 ± 0.06 (13)	1.02 ± 0.09 (21)	2.00 ± 0.26 (10)
M059K	-	6.88 ± 1.78 (9)	1.44 ± NA* (2)
M059J	-	3.14 ± 0.53 (11)	1.16 ± 0.17 (3)

Note: N = number of independent samples; MN = micronuclei; BNC = binucleated cells;
* = only two data points for these endpoints; – = no data were collected.


Chromatid breaks per cell
0.5
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
Exp. A 1h
Exp. A 3h
Exp. A 6h
Exp. B 1h
Exp. B 3h
Exp. C 1h
Exp. C 3h
Control
0Gy (ICCM)
0.15Gy (ICCM)
1.5Gy (ICCM)


A
Chromatid breaks
Fold over control
0
0.5
1
1.5
2
V79 Fe-ions
xrs6 Fe-ions
xrs6 N-ions
Average
B
γ-H2AX foci
Fold over control
0
0.5
1
1.5
2
2.5
3
3.5
4
V79 Fe-ions
xrs6 Fe-ions
M059J Fe-ions
M059K Fe-ions
Average
C
γ-H2AX fluorescent units
Fold over control
0
0.5
1
1.5
2
2.5
V79 Fe-ions
xrs6 Fe-ions
Average
D
Micronuclei
Fold over control
0
0.5
1
1.5
2
2.5
3
V79 Fe-ions
xrs6 Fe-ions
SW48 N-ions
MO59J Fe-ions N-ions
MO59K Fe-ions N-ions
Average


Directly irradiated cells
Bystander cells
ΔFAR (%)
1.5
1
0.5
0
-0.5
0
0.5
1
1.5
2
Dose (Gy)


Control
w/o cells
0Gy
0.15Gy
1.5Gy
% clonogenic survival
70
60
50
40
30
20
10
0