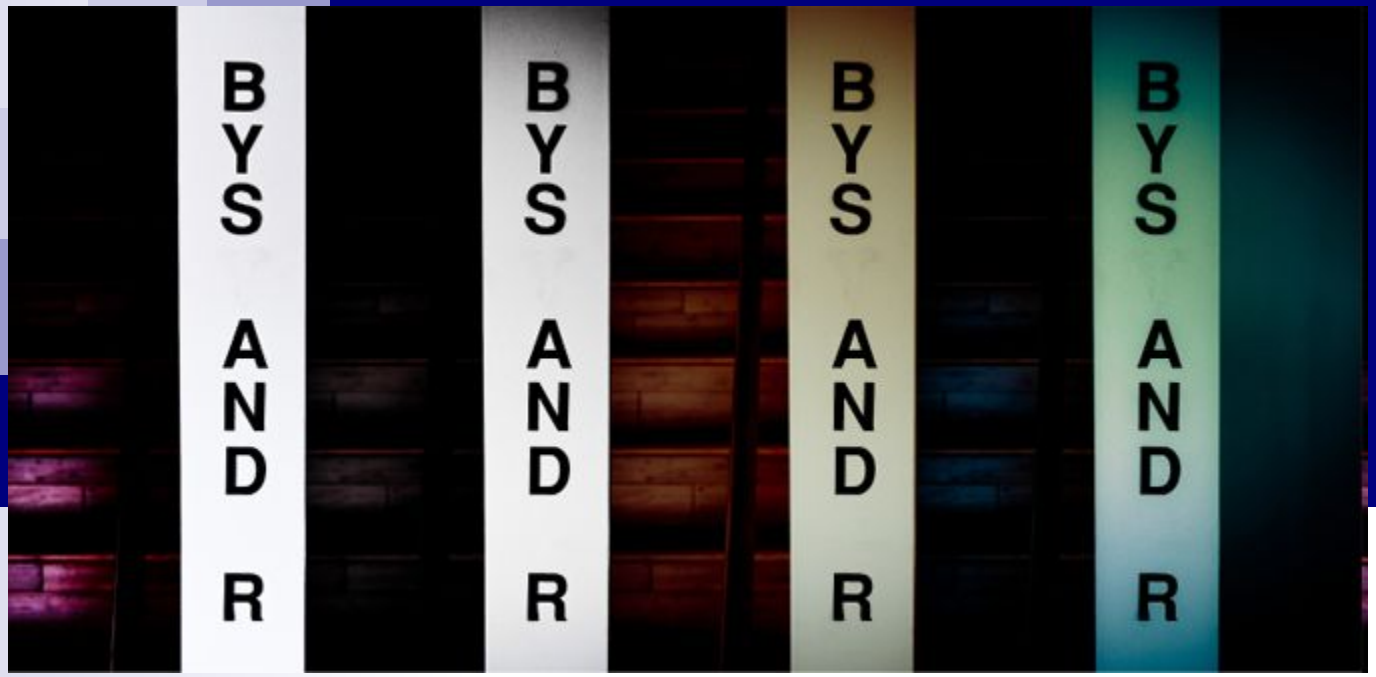
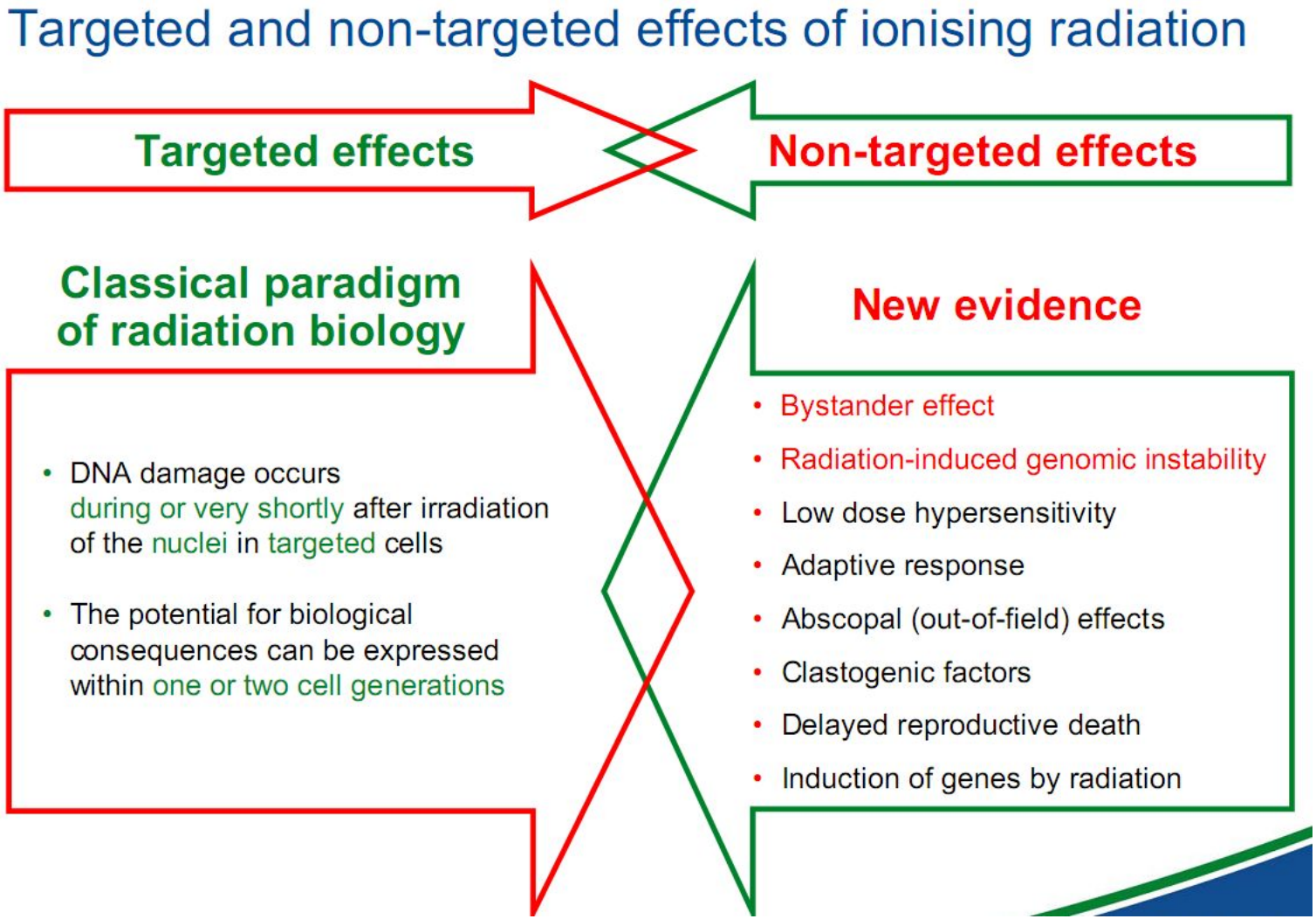
BYSTANDER EFFECT



- The bystander effect refers to the induction of biological effects in cells that are not directly traversed by a charged particle. The data available concerning the bystander effect fall into two quite separate categories, and it is not certain that the two groups of experiments are addressing the same phenomenon. First, there are experiments involving the transfer of medium from irradiated cells, which results in a biological effect in nirradiated cells. Second, there is the use of sophisticated single particle microbeams, which allow specific cells to be irradiated and biological effects studied in their neighbors; in this case communication is by gap junction. Medium transfer experiments have shown a bystander effect for cell lethality, chromosomal aberrations and cell cycle delay. The type of cell, epithelial vs. fibroblast, appears to be important.

Non-targeted biological effects of ionizing radiation



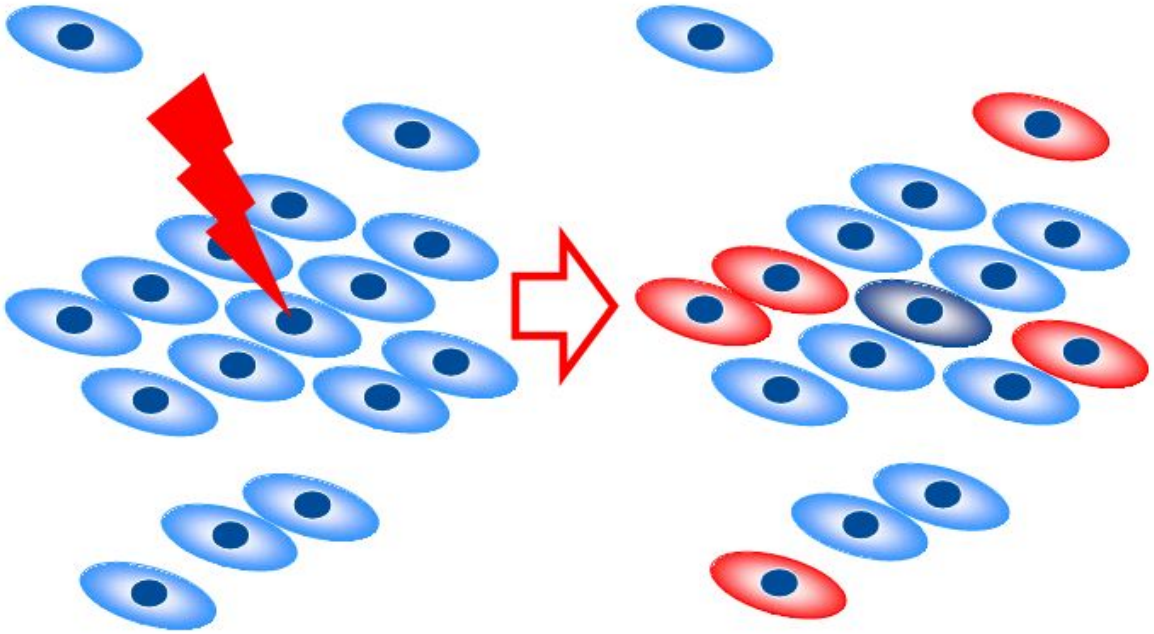
Target theory

- The *target theory* of radiation induced effects (Lea, 1946) postulates that cells contain at least one critical site or *target* that must be hit by radiation in order to kill a cell (or produce an effect).
- Therefore, radiation damage outside of the target should not cause cell death (effect).
- It is widely accepted that nuclear DNA is the critical target for radiation induced cell death (and not death related efefcts).

Non-targeted effects of ionizing radiation as a new paradigm of radiation biology

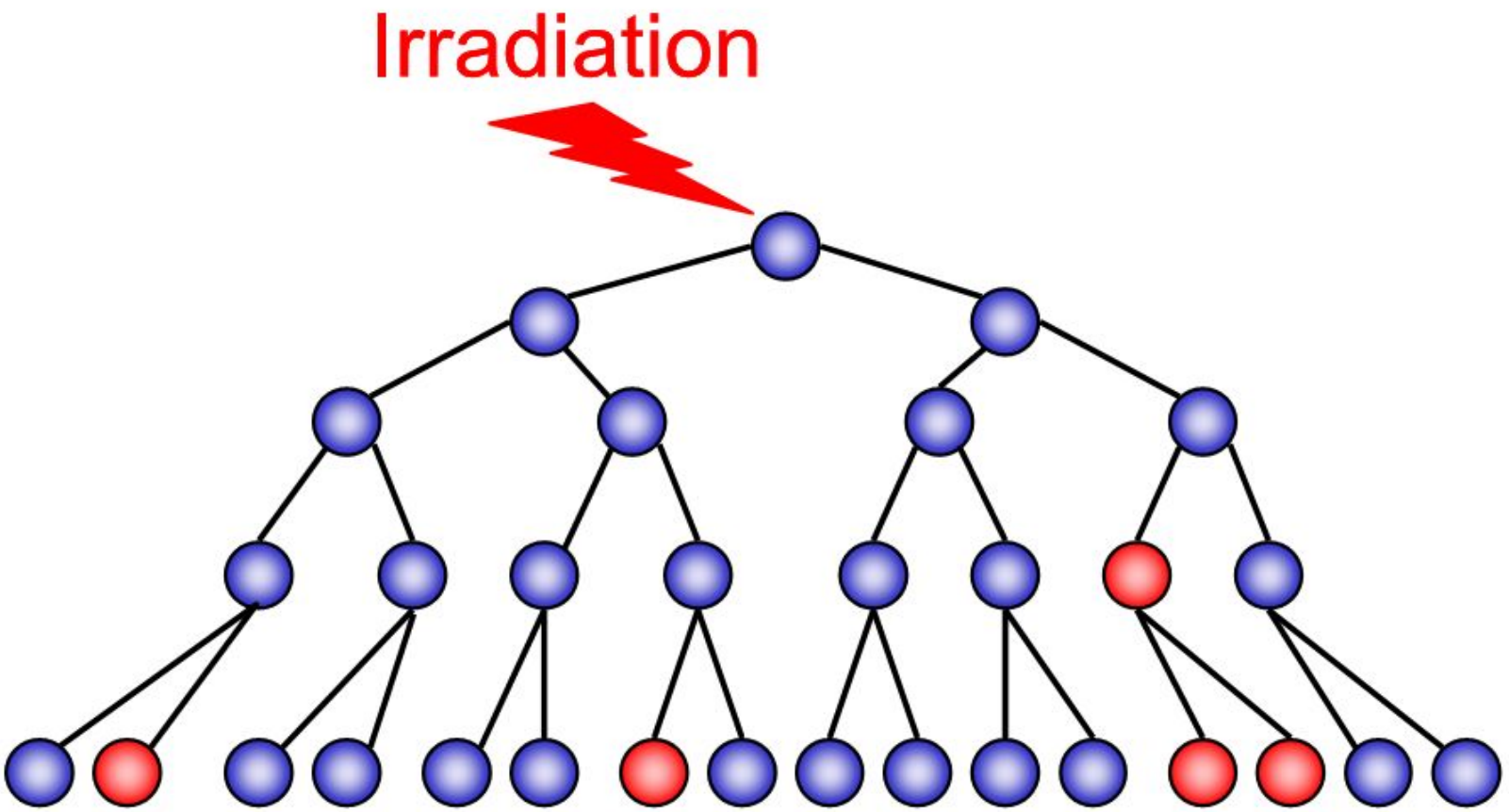
- Ward, J. (1999) New paradigms for Low-Dose Radiation Response In Proceedings of the American Statistical Association Conference on Radiation and Health. San Diego, California, USA. June 14-17, 1998. Radiat Res, 151:1, 92-117.

Radiation induced bystander effect



The radiation-induced bystander effect is a phenomenon whereby cellular damage is expressed in unirradiated neighboring cells near to an irradiated cell or cells.

Radiation-induced genomic instability



Radiation-induced genomic instability is defined as a persistent elevation in the rate of *de novo* appearance of genetic changes within a clonal population.

Non-targeted *versus* targeted effects

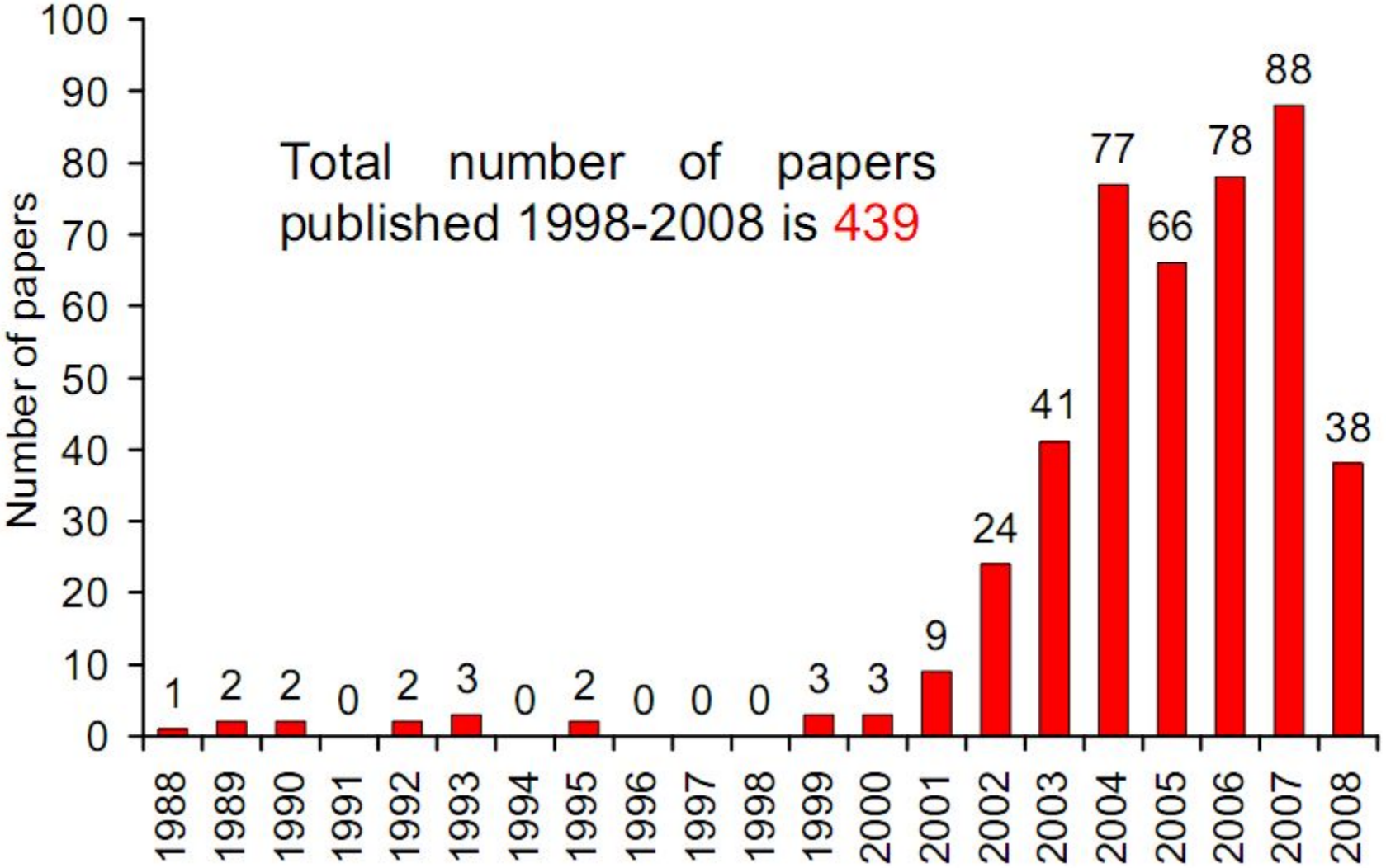
- Non-targeted effects do not contradict to "*target theory*" but increase size of the target in such extent that concept of "target" became meaningless.
- For example, bystander effect increases target *spatially* to the size of cell group, tissue or even organ.
- Genomic instability increases it *temporarily* by prolongation of damage over many cell generations or even transgenerationaly.

Need for a new paradigm of Radiation Biology

- Recent evidence for non-targeted effects suggests a new paradigm for radiation biology that challenges the universality of target theory.
- An essential feature of "non-targeted" effects is that they do not require a direct nuclear exposure by irradiation to be expressed and they are particularly significant at low doses.
- This new radiation biology paradigm should cover both targeted (direct) and non-targeted effects of ionising (and possibly non-ionising) radiation.

Baverstock, K. and Belyakov, O.V. (2005) Classical radiation biology, the bystander effect and paradigms: a reply. *Hum Exp Toxicol*, vol. 24, pp. 537-42.

Number of papers related to radiation induced non-targeted effects, bystander effect and genomic instability referred by Medline



Rationale for the current interest in non-targeted responses

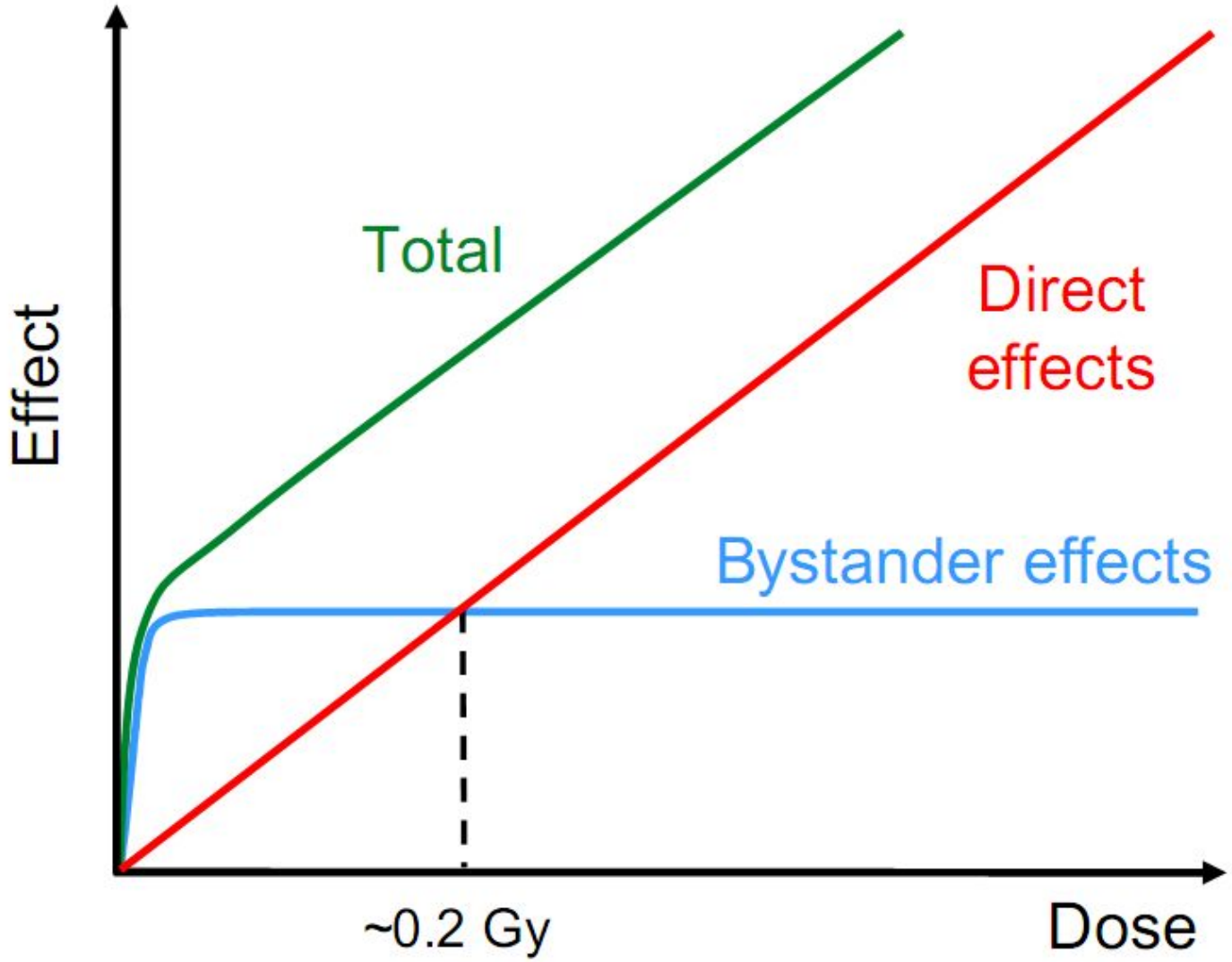
- There is a growing interest in low dose effects.
- Advances in the technical possibilities for precise low dose irradiation such as development of microbeams, imaging and computerized automation.
- Development of more specific and sensitive methods of cellular and molecular biology.
- Change of classic paradigm of radiation biology and challenging the target principle.

Bystander effect and genomic instability

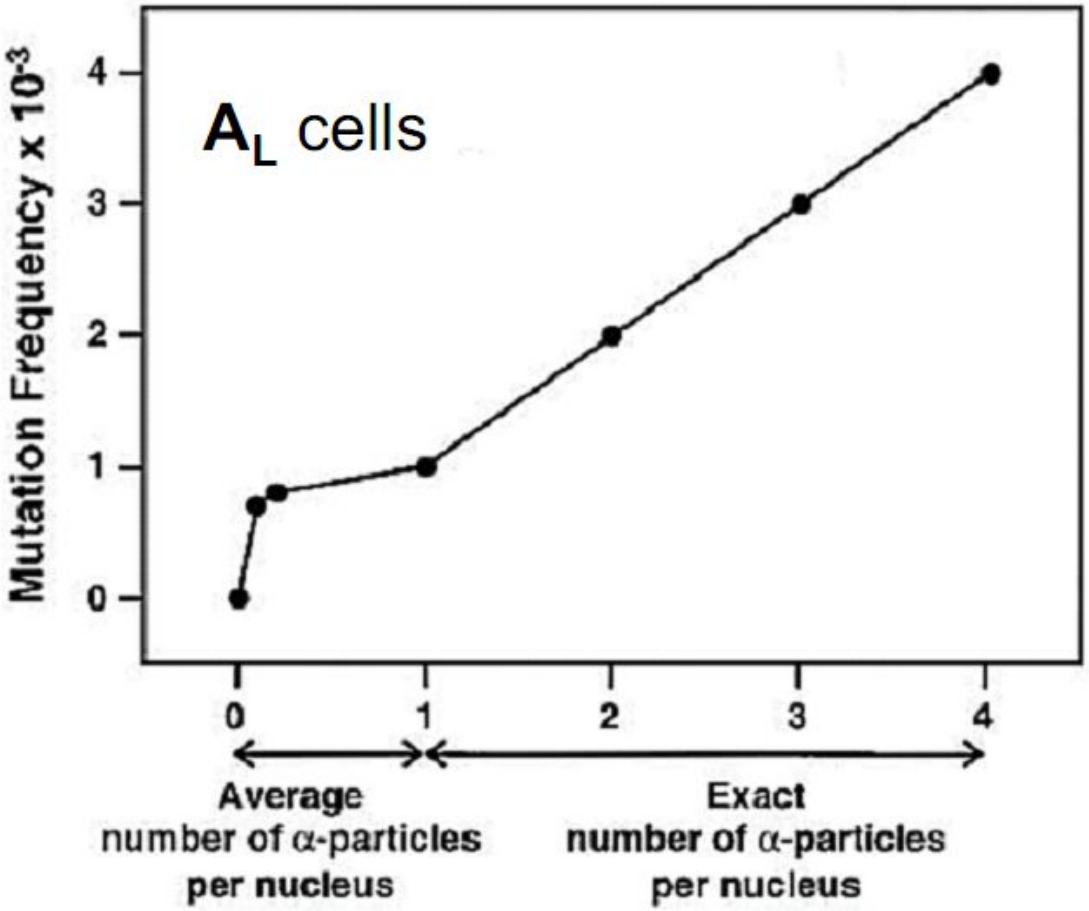
Evidence for radiation induced non targeted effect

- Increased levels of SCE in CHO cells irradiated with low doses of α-particles (Nagasawa and Little, *Cancer Res,* 1992).
- Increased p53 expression in epithelial cells exposed to α-particles (Hickman *et al., Cancer Res,* 1994).
- Extracellular factors involved in SCE following α-particle exposure (Lehnert and Goodwin, *Cancer Res,* 1997).
- Medium from γ-rays irradiated cells reduces the survival of unirradiated cells (Mothersill and Seymour, *Radiat Res,* 2001).
- Bystander effect after microbeam irradiation of a single cell (Belyakov *et al., BJC,* 2001).
- Induction of a bystander mutagenic effect after α-particle microbeam irradiation (Zhou *et al., PNAS,* 2000).
- Increased bystander neoplastic transformation after treatment with medium from irradiated cells (Lewis *et al., Radiat Res,* 2001).
- Bystander effect and genomic instability under *in vitro* (Lorimore *et al., PNAS,* 1998) and *in vivo* conditions (Watson *et al., Cancer Res,* 2000).

Contribution of bystander and direct components to the radiation induced damage



Dose response relationship for direct and bystander mutations

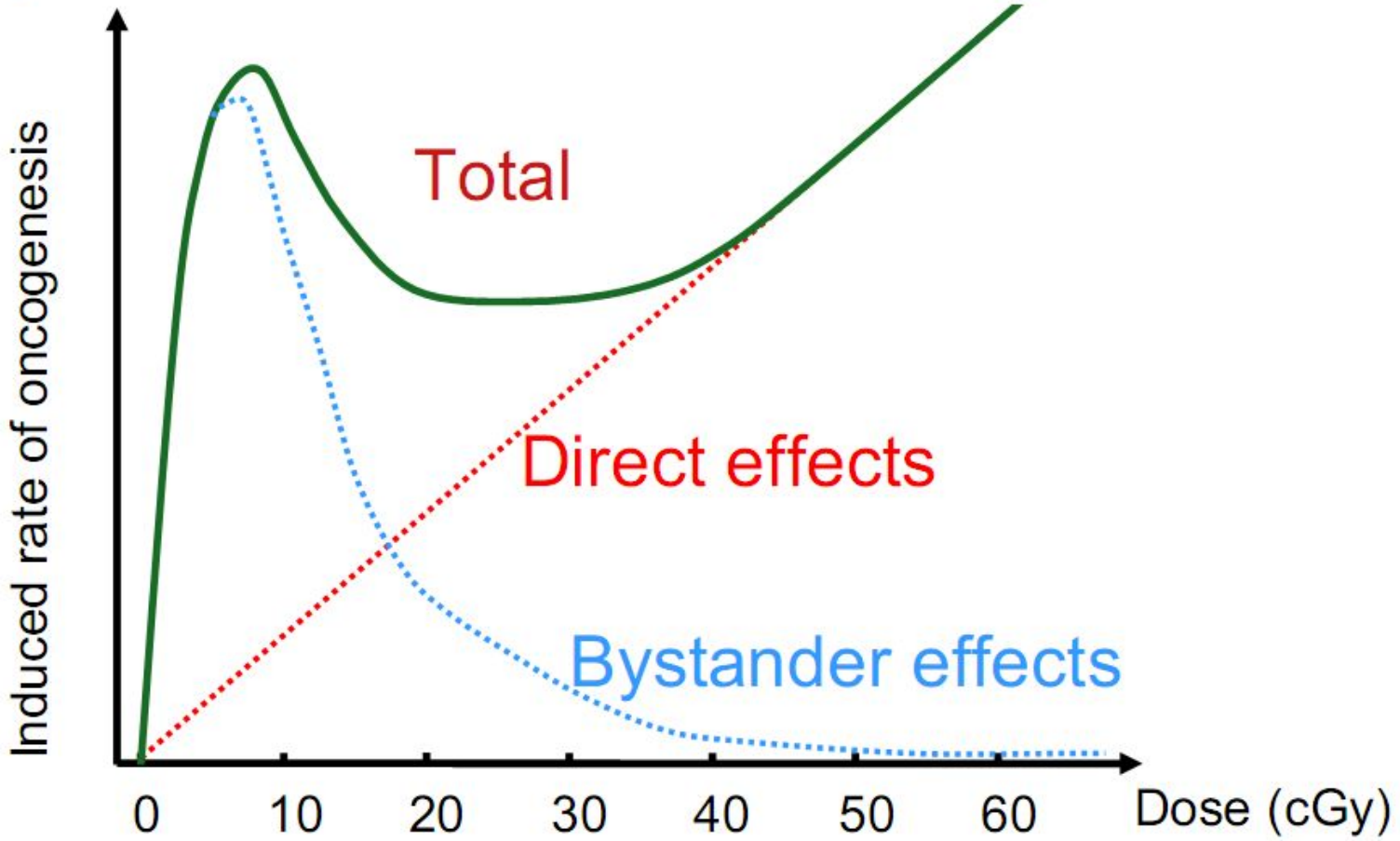


Hall, E.J. and Hei, T.K. (2003) Genomic instability and bystander effects induced by high-LET radiation. *Oncogene*, **22**:45, 7034-7042 (based on the data of Zhu *et al.*, *Radiat Res*, 1996; Hei *et al.*, *PNAS*, 1997; Zhou *et al.*, *PNAS*, 2001)

Mathematical models of bystander effects

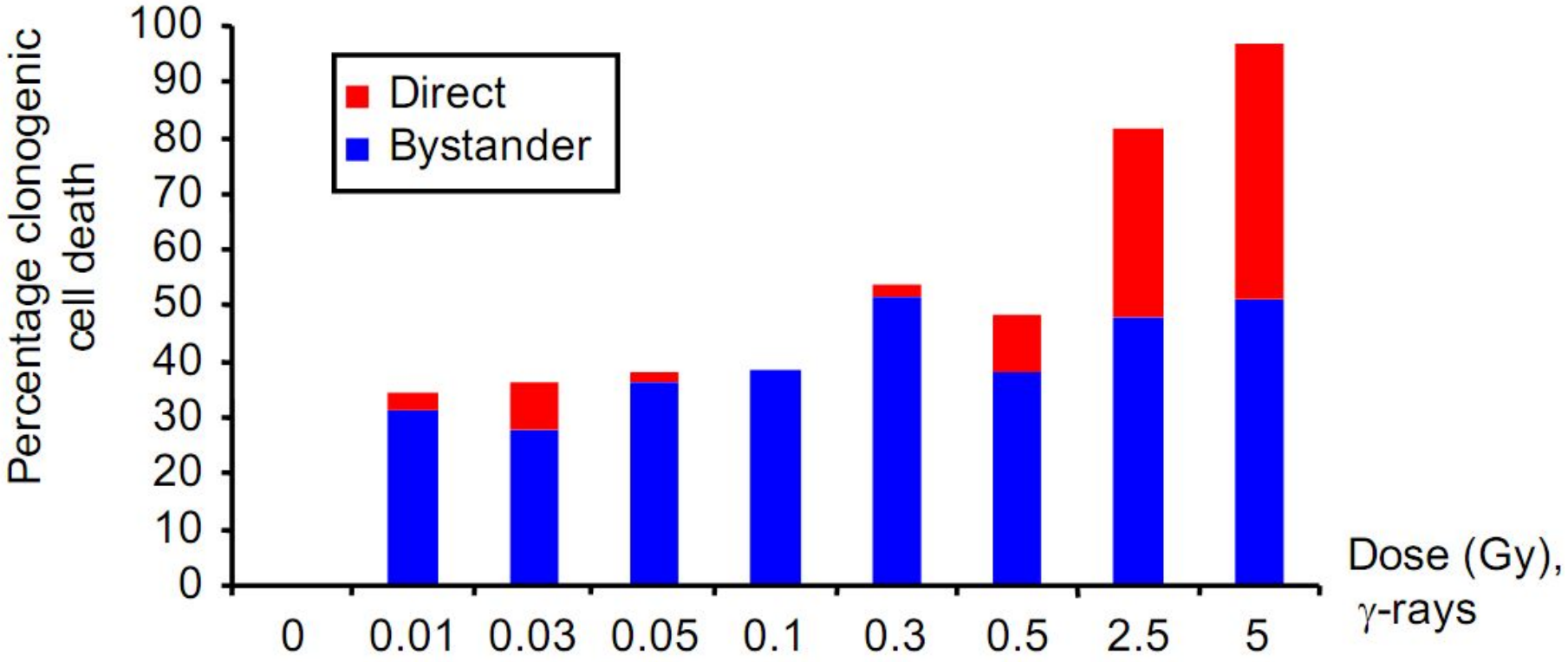
- State-vector model (SVM)
  (Schollnberger, et al., *IJRB*, 2002)
  A biomathematical neoplastic transformation model that includes radioprotective bystander mechanisms. The model successfully simulates experimental data.
- ByStander Diffusion Modell (BSDM)
  (Nikjoo and Khvostunov, *IJRB*, 2003)
  A quantitative model of the radiation-induced bystander effect based on diffusion-type spreading of bystander signal communication between the hit and non-hit cells.
- 3D lattice model
  (Little, et al., *J Theor Biol*, 2005)
  A model for bystander effects, with allowance for spatial position and the effects of cell turnover. It assumes a three-dimensional lattice of points and suitable for tissue modelling.

BaD model, contribution of bystander and direct component to the radiation induced oncogenesis



Brenner, D.J., Little, J.B. and Sachs, R.K. (2001) The bystander effect in radiation oncogenesis: II. A quantitative model. *Radiat Res*, 155:3, 402-8.

What is the relative contribution of “direct" and "bystander" effects to cell death?



Clonogenic cell death measured in human keratinocytes. The whole bar represents the total death after direct exposure. The red portion of the bar represents bystander death measured after exposure to medium from irradiated cells. The remaining death is represented by the blue portion of the bar, giving a value for death not attributable to bystander effect (Seymour and Mothersill, *Radiat Res*, 2000).

Mechanisms of the bystander effects

- Cell type dependent
- Depends on cell proliferative state
- Energy/REDOX metabolism may be involved
- Bystander effect can be induced by low and high LET irradiation
- Different underlying mechanisms
  - Gap junction (GJIC) mediated
  - Medium borne factors mediated

Hypothetical messenger(s)

At least two types of the bystander messenger might exist

Primary

- emitted by targeted cell
- short lived
- unstable
- travels through gap junctions
- water soluble
- non-protein

Long-lived organic radicals

Antioxidants (thiols)

Ca^{2+} or Ip3

cAMP

Secondary

- produced by activated cells
- long lived
- stable
- media borne
- most likely a protein

Lipid hydroperoxidases

Death ligand exfoliation

Cytokines

TNF-α, TGF-β or IL-1

Medium borne primary or secondary messengers

- Reactive oxygen species (H_2O_2/O^{-2}) have been proposed as possible signals involved in bystander responses (Narayanan, *et al.*, *Cancer Res*, 1997; Iyer and Lehnert, *Cancer Res*, 2000)
- Nitric oxide (NO) might play a central role in mediation of bystander effect (Matsumoto, *et al.*, *IJRB*, 2000; Matsumoto, *et al.*, *Radiat Res*, 2001) potentially having a protective value.

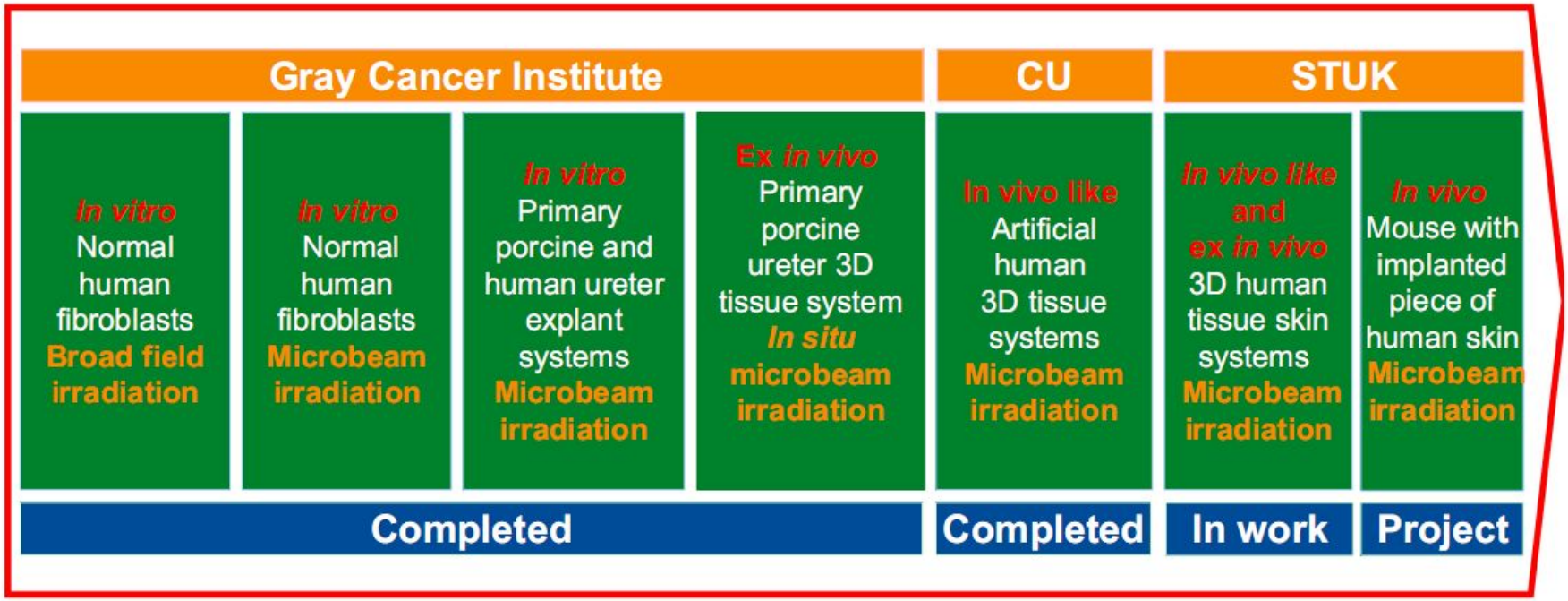
Secondary electrons cannot be involved in the bystander effect

- In our research we are using charged particles with energies of 3-4 MeV per nucleon.
- Secondary electrons produced by these particles cannot be involved in the bystander effect because of very short range.
- 7 MeV $^{4}He^{2+}$ maximal calculated energy of secondary electrons would be ≈3.8 keV, which corresponds to a few hundreds of nanometers range. This is much less than size of cell or cell nucleus. Therefore secondary electrons even would not be able to get out of nucleus after it was targeted with microbeam.
- On other hand, hypothetical bystander messenger is proven to be capable of travel for millimeters.

Bystander effect and genomic instability are closely related

- Bystander effect and genomic instability are non-targeted effects of irradiation and might have common mechanisms (Kadhim *et al*., *Mutat Res,* 2004).
- Chromosomal instability could be induced in bystander cells (Lorimore *et al*., *PNAS,* 1998).
- There is a recent evidence that the bystander effect persists for many generations (Lorimore *et al*., *Cancer Res,* 2005).
- This evidence suggests that the initial cross-section for radiation damage is increased by the bystander effect, and cells that are affected by the bystander mechanism may remain at an increased risk of genetic change for many generations.

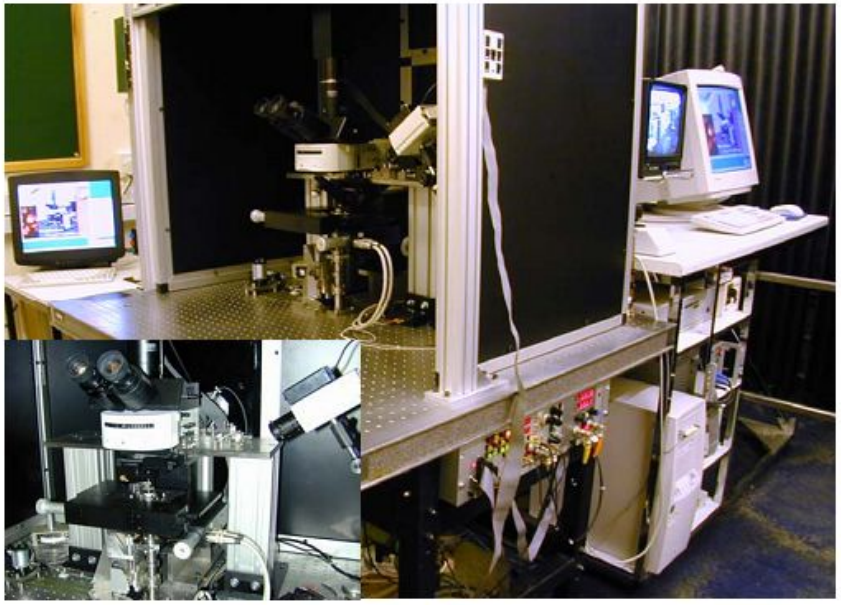
Studies of bystander effects: a *gradual* movement from *in vitro* cell culture towards *in-vivo* system



Rationale

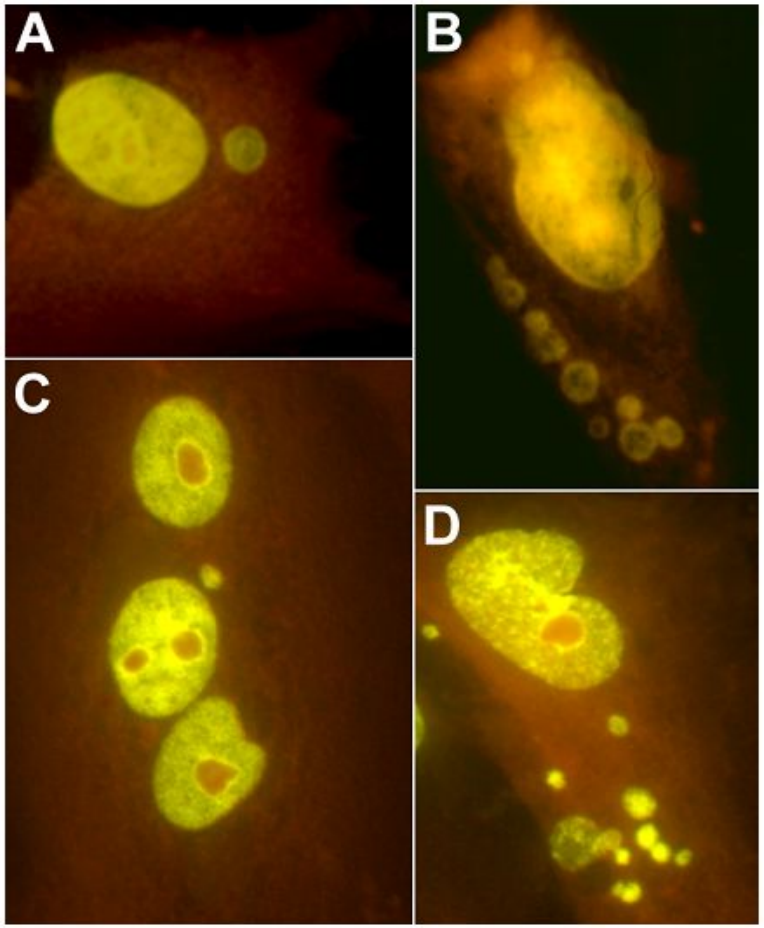
- Radiation effects at the tissue level under normal conditions prove that individual cells cannot be considered as isolated functional unit within most tissues of a multicellular organism.
- Experimental models, which maintain tissue-like intercellular cell signalling and three-dimensional (3D) structure, are essential for proper understanding of bystander effects.
- The main rationale for our research is that the bystander effect is likely to be natural phenomena which should be studied in an *in vivo* like multicellular system with preserved 3D tissue microarchitecture and microenvironment.
- This necessitates moving from *in vitro* cell culture systems to tissue-based systems.

Microbeam technology as a tool for bystander research

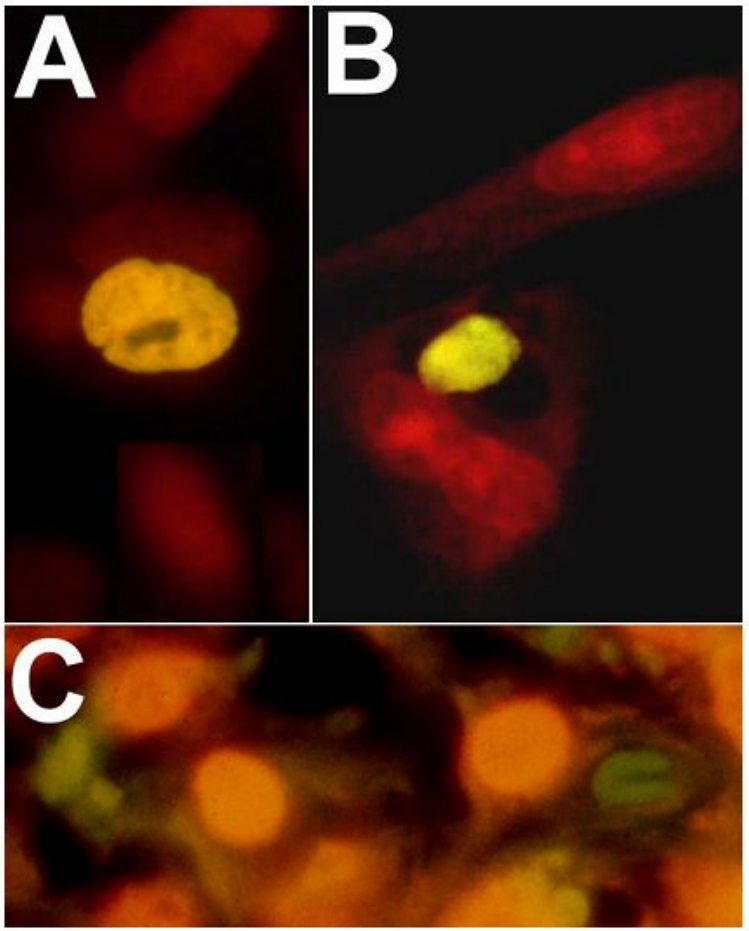


Microbeams are facilities that allow irradiation of individual cells or cell regions with precise numbers of charged particles with *micrometer* precision (see for example: Randers-Pehrson *et al*, *Radiat Res*, 2001; Folkard *et al*, *Int J Radiat Biol*, 1997).

Micronucleated and apoptotic cells



Mironucleated AG01522 fibroblasts (A, B) and urothelial cells (C, D), acridine orange staining.



AG01522 fibroblasts (A and B), porcine urothelium explant outgrowth (C).

Studies of bystander effects in AG01522 normal human fibroblasts

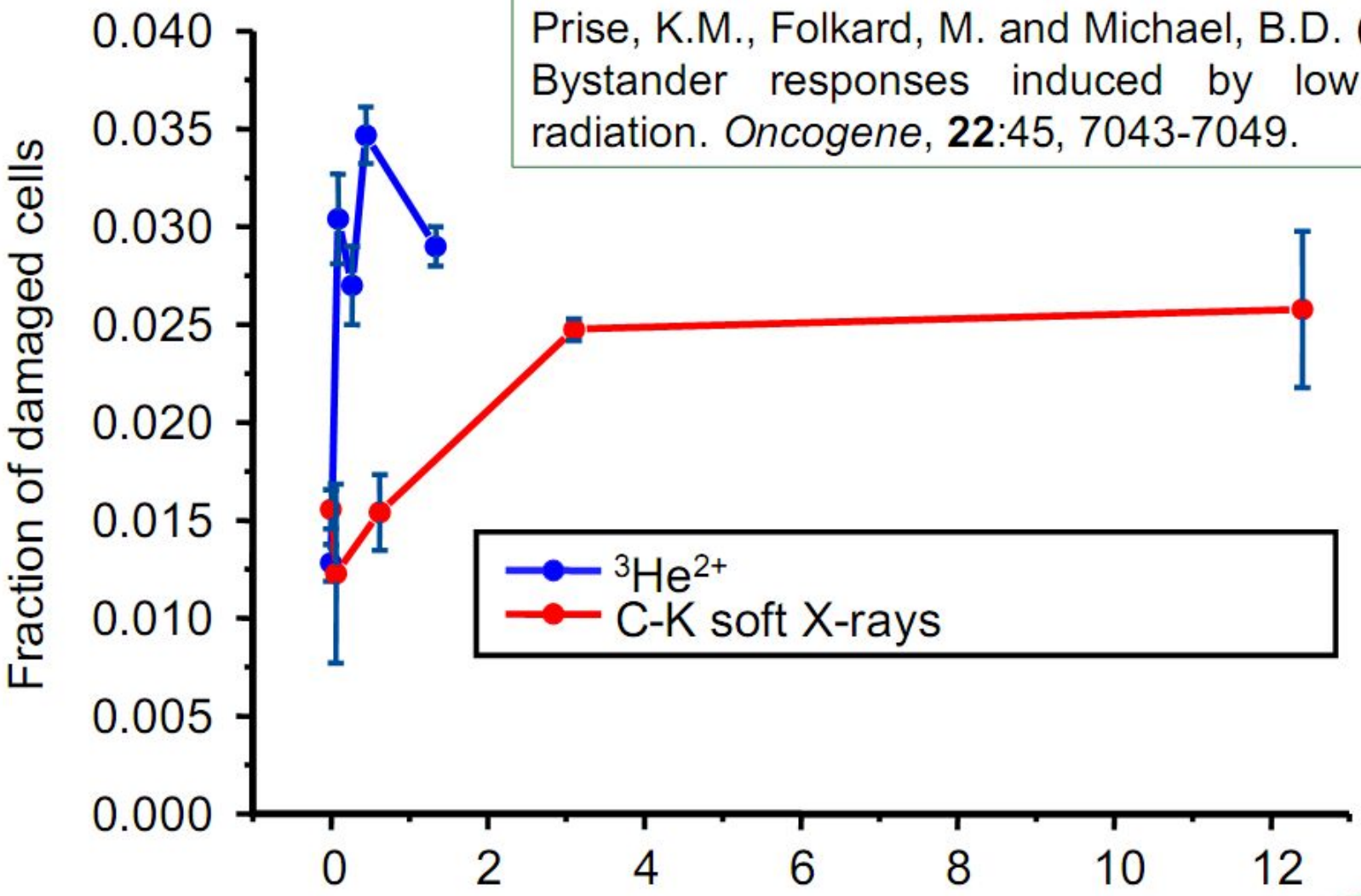
- First direct evidence for a bystander effect.
- Micronucleated and apoptotic cells were scored 3 days after irradiation in AGO1522 primary human fibroblasts.
- Irradiation of 1 fibroblast among a few hundred cells with 1 $^{3}He^{2+}$ particle produced a significant rise in damaged cells from approximately 1% to 3% in the surrounding unirradiated population.
- Further increase of dose does not change the dose response.

Belyakov, O. V., Malcolmson, A. M., Folkard, M., Prise, K. M. and Michael, B. D. (2001). Direct evidence for a bystander effect of ionizing radiation in primary human fibroblasts, *Br J Cancer* **84:5**, 674-679.

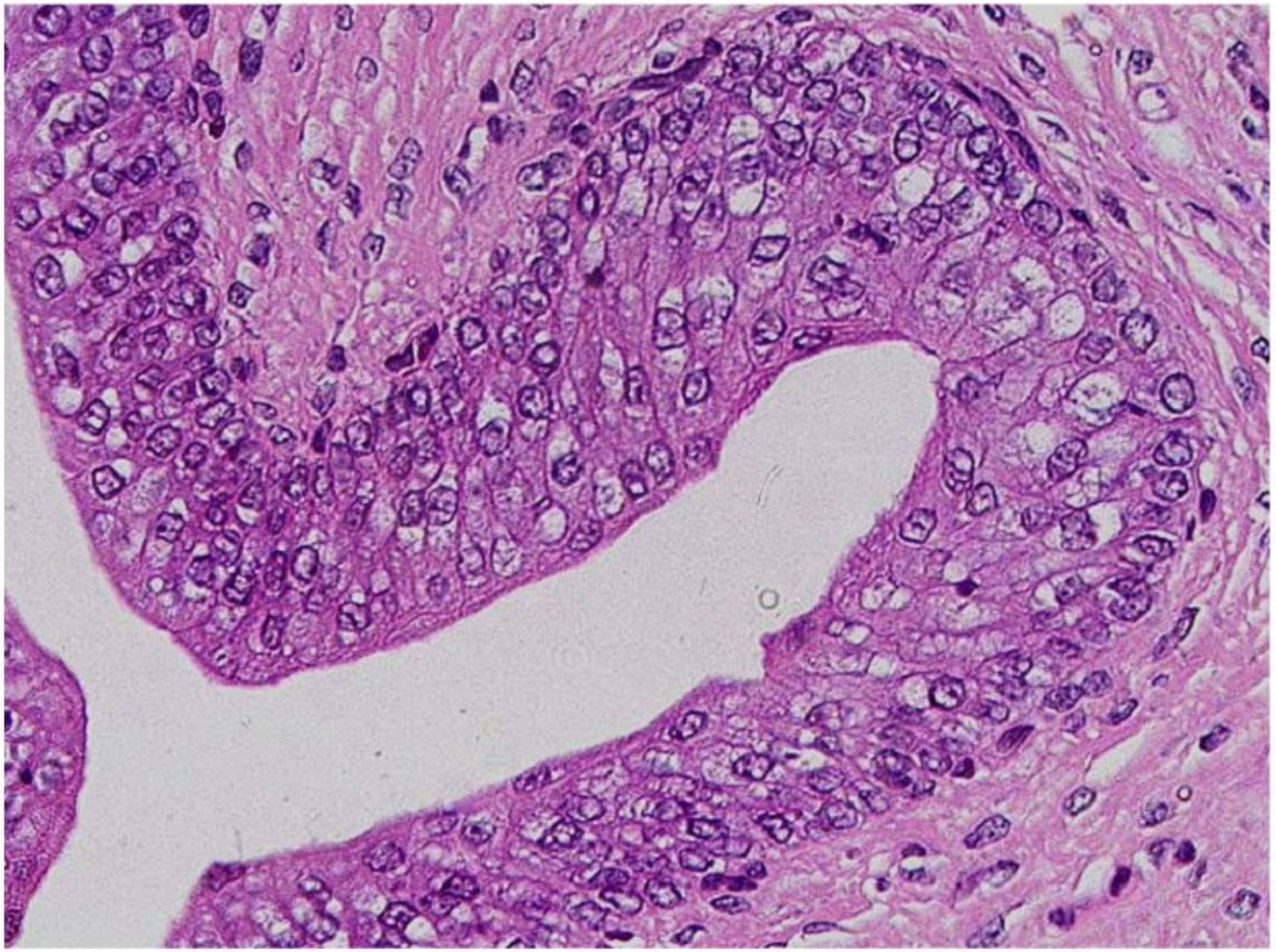
Prise, K.M., Belyakov, O.V., Folkard, M. and Michael, B.D. (1998) Studies of bystander effects in human fibroblasts using a charged particle microbeam. *Int J Radiat Biol*, **74:6,** 793-8.

Bystander effect in human fibroblasts after $^{3}He^{2+}$ microbeam and ultra soft X-ray microprobe irradiation of a single cell

Prise, K.M., Folkard, M. and Michael, B.D. (2003) Bystander responses induced by low LET radiation. *Oncogene*, **22**:45, 7043-7049.



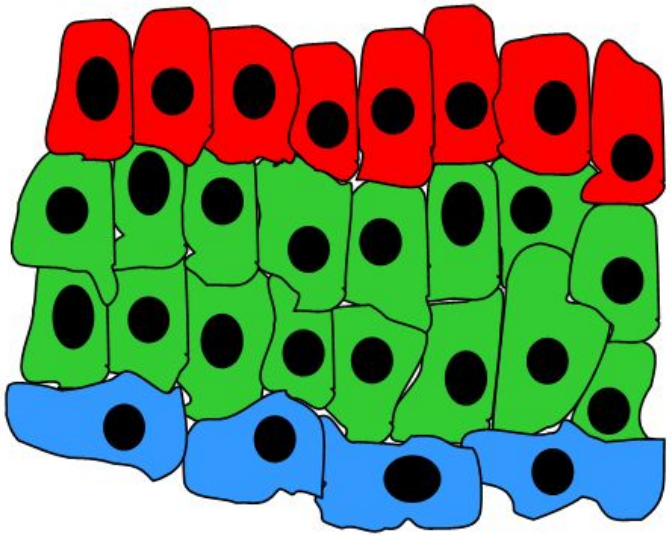
Porcine ureter section



4 µm paraffin section, Haemotoxylin-Eosin staining

Ureter tissue microarchitecture

Lamina propria



Basal cell layer, dividing

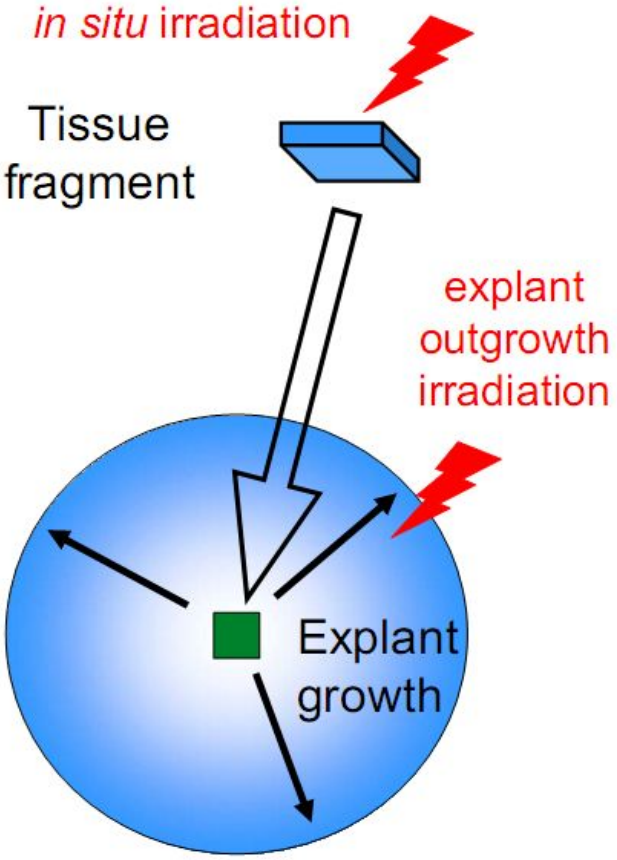
2-3 intermediate cell layers - semi-differentiated, non-dividing

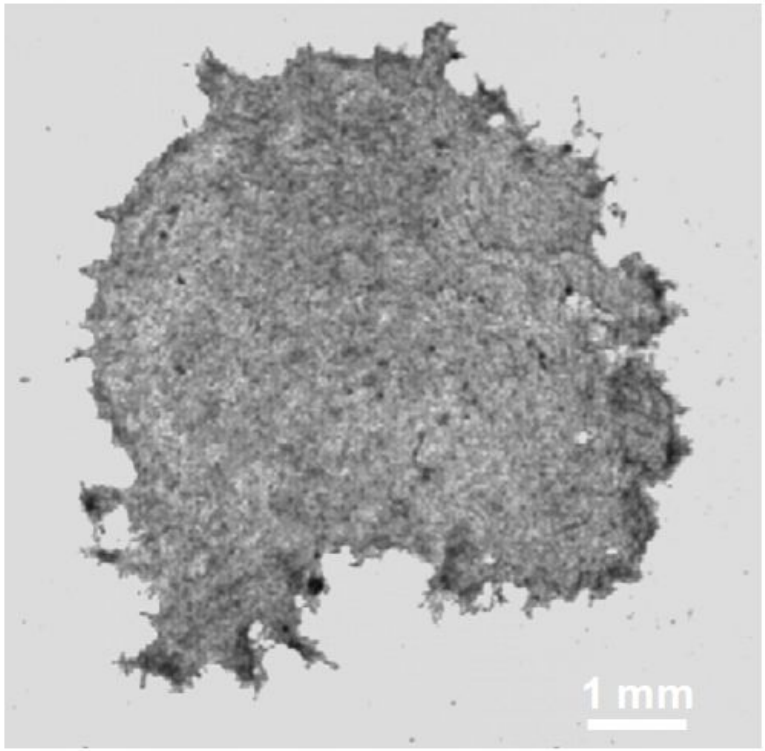
Superficial cell layer - differentiated

Cell movement

Lumen

Primary explant technique





Human urothelial explant outgrowth

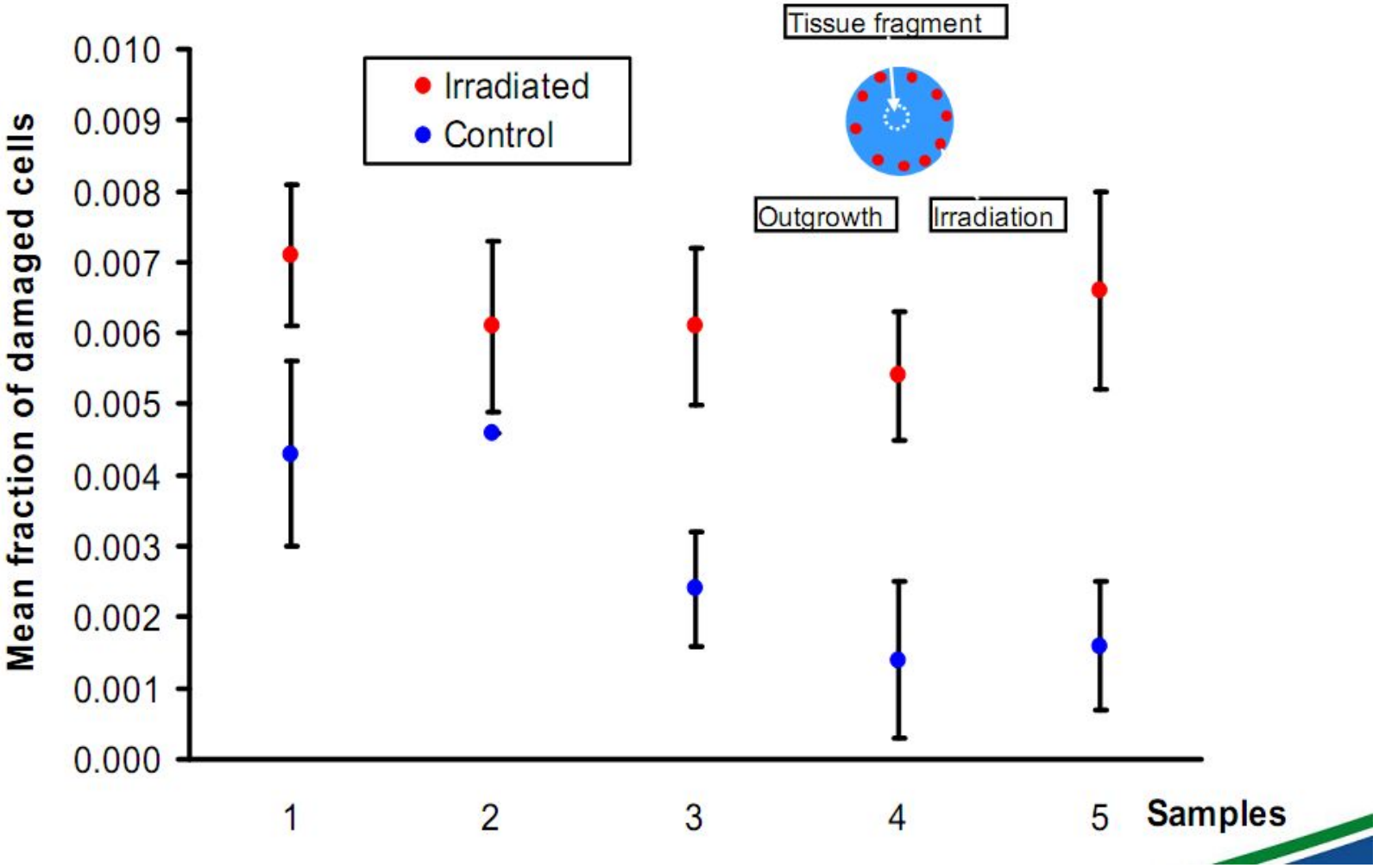
Outgrowth is a 2D representation of 3D tissue microarchitecture including *in vivo* like differentiation pattern.

A proliferation-dependent bystander effect in urothelial explants

- A significant bystander-induced effect was observed only when the periphery of the explant outgrowth (consisting of proliferating cells) was targeted.
- Approximately 2000-6000 additionally damaged cells were produced after irradiation of a few cells initially.
- This finding suggests a cascade mechanism of cell damage induction.
- The fraction of damaged cells did not exceed 1-2% of the total number of the cells within the explant outgrowth.
- The bystander-induced damage depends on the proliferation status of the cells and can be observed with this *in vivo* like explant model.

Belyakov, O.V., Folkard, M., Mothersill, C., Prise, K.M. and Michael, B.D. (2003) A proliferation-dependent bystander effect in primary porcine and human urothelial explants in response to targeted irradiation. *Br J Cancer*, **88**:5, 767-74.

Fraction of damaged cells after microbeam irradiation at the *periphery* of urothelial explant outgrowth, 10 cells have been irradiated at the edge of each explant (10 $^{3}He^{2+}$ particles/cell)

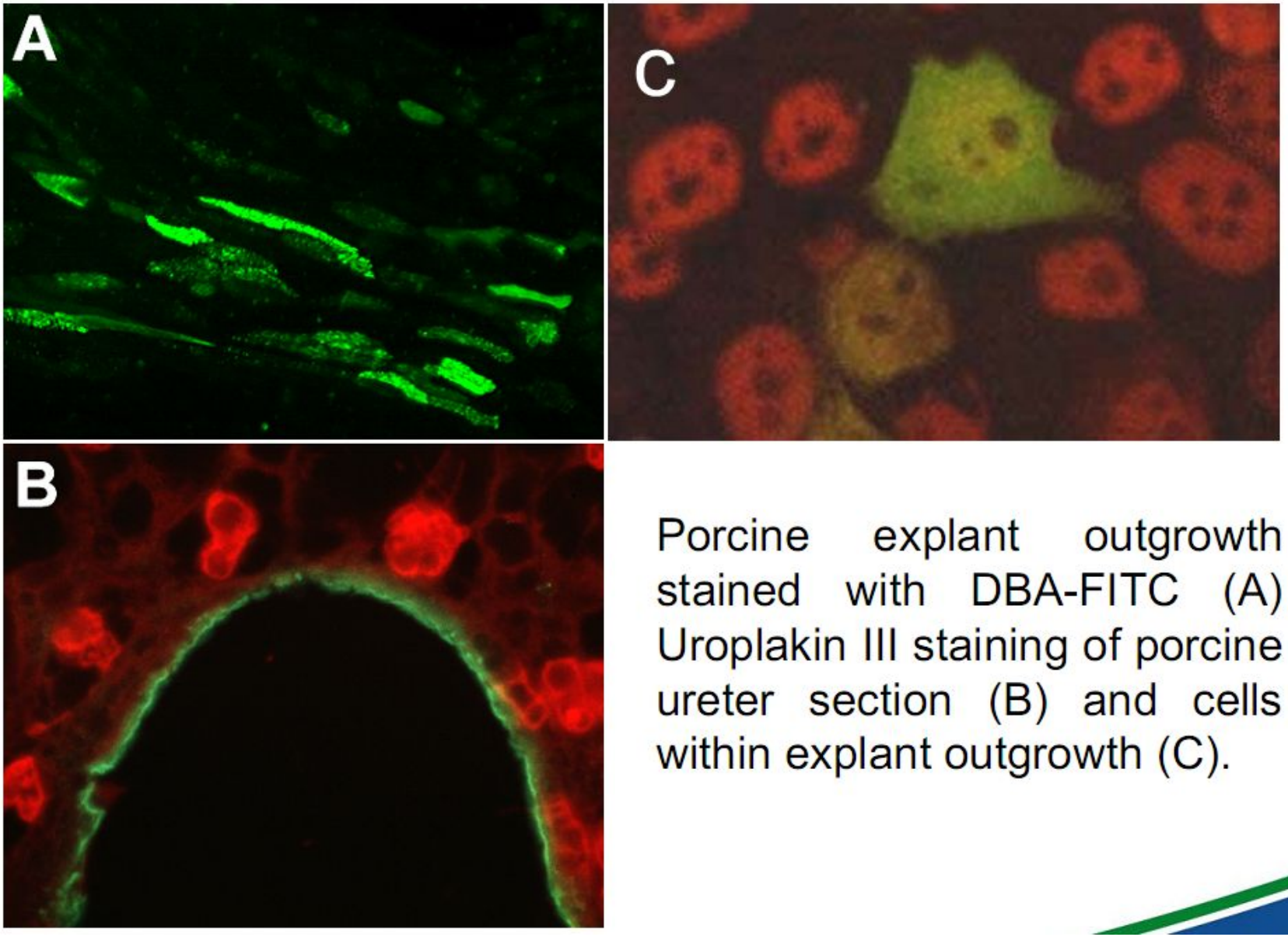


Bystander-induced differentiation in porcine ureter tissue models following *in situ* microbeam irradiation

- A single 2 μm location on ureter tissue section was pre-irradiated with 10 3He2+ particles (5 MeV; LET 75 keV/μm).
- Differentiation was estimated using antibodies to Uroplakin III, a specific marker of terminal urothelial differentiation.
- Micronucleation and apoptosis involve only a small fraction of cells (typically 1-2% of total cell number).
- Irradiated samples demonstrate about 10-15% additional differentiation in comparison to control. By far the biggest bystander response has a protective role rather than a damaging one by switching on differentiation.

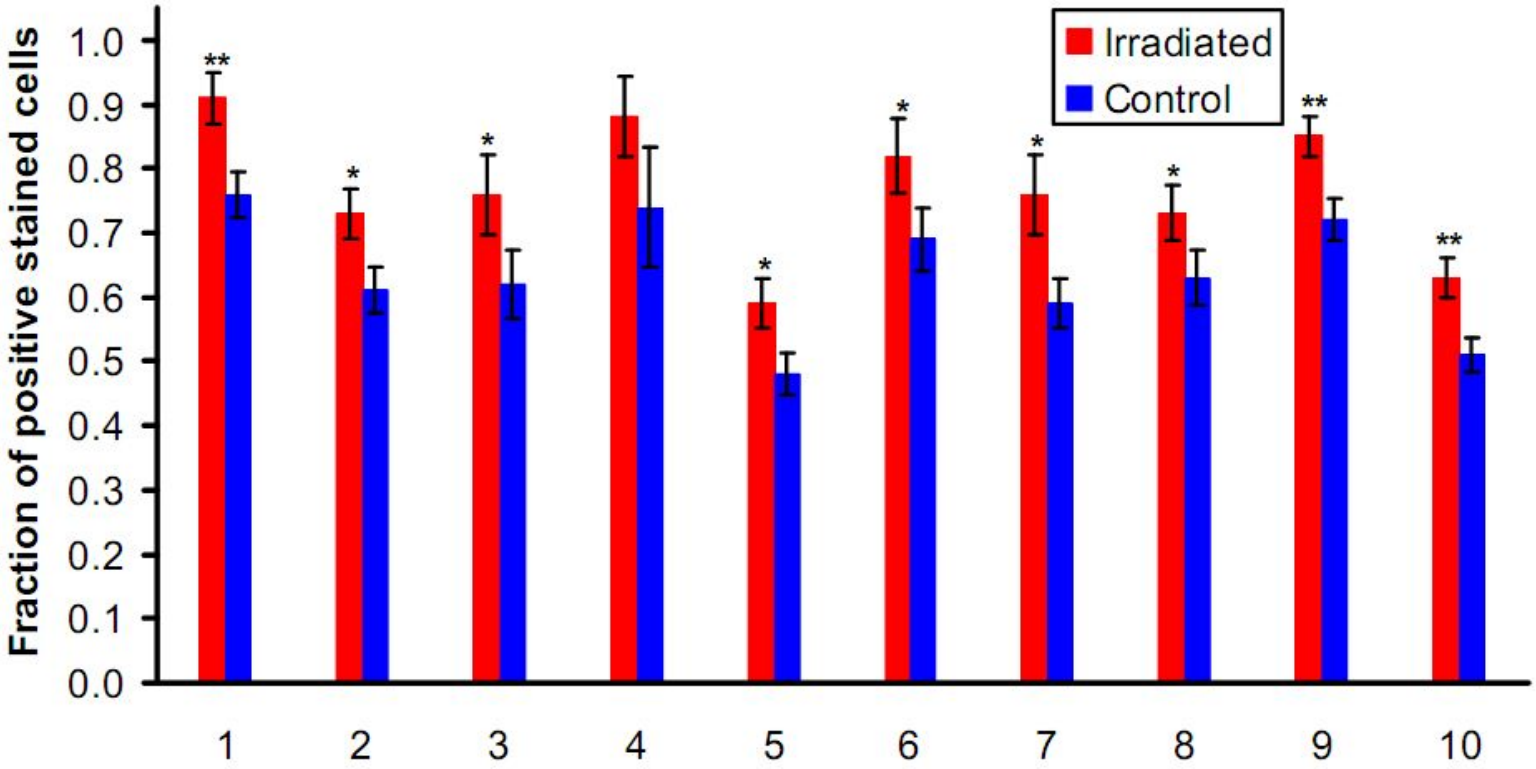
Belyakov, O.V., Folkard, M., Mothersill, C., Prise, K.M. and Michael, B.D. (2006) Bystander-induced differentiation: A major response to targeted irradiation of a urothelial explant model. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis*, **597**:1-2, 43-49.

Markers of urothelial differentiation



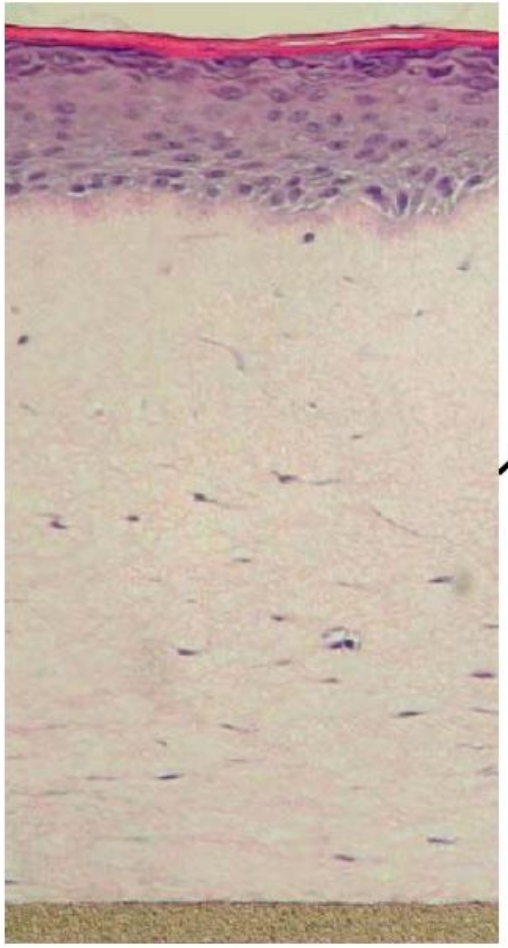
Porcine explant outgrowth stained with DBA-FITC (A) Uroplakin III staining of porcine ureter section (B) and cells within explant outgrowth (C).

Fraction of differentiated cells measured with Uroplakin III immunostaining in porcine urothelial explant outgrowths

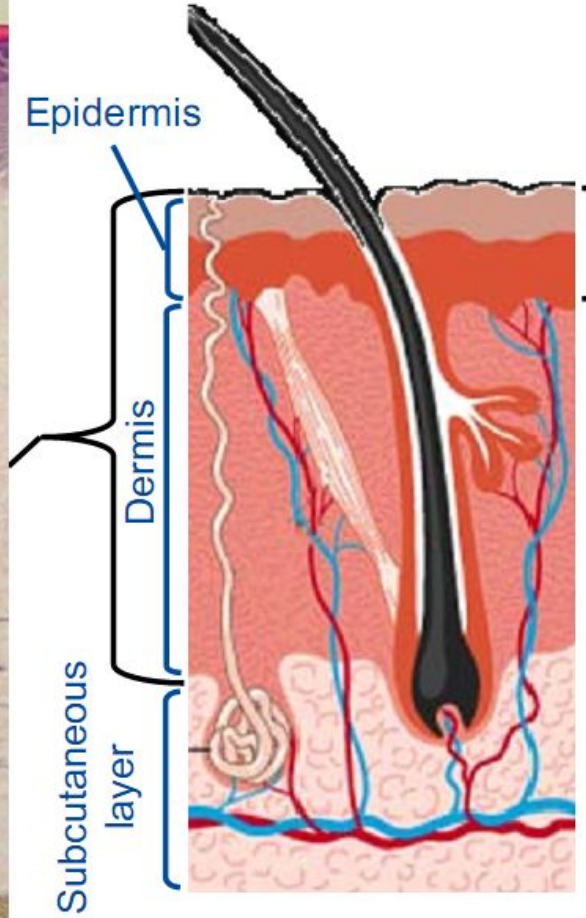


Error bars represent standard error of the means.
Significance tests were made using Student's *t*-test
($^{*}P<0.05$; $^{**}P<0.01$).

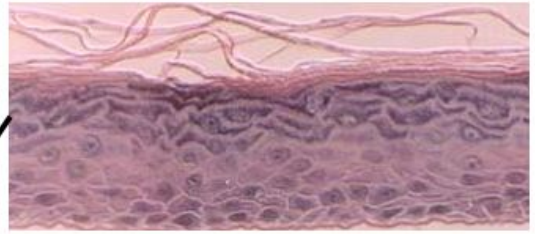
Artificial human skin tissue system



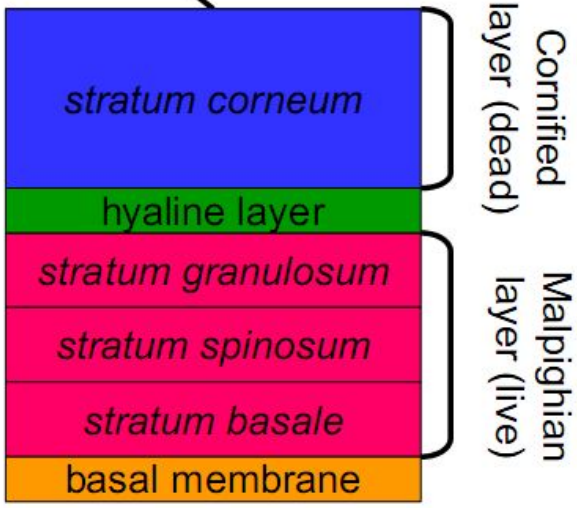
EpiDermFT



Scheme of human skin

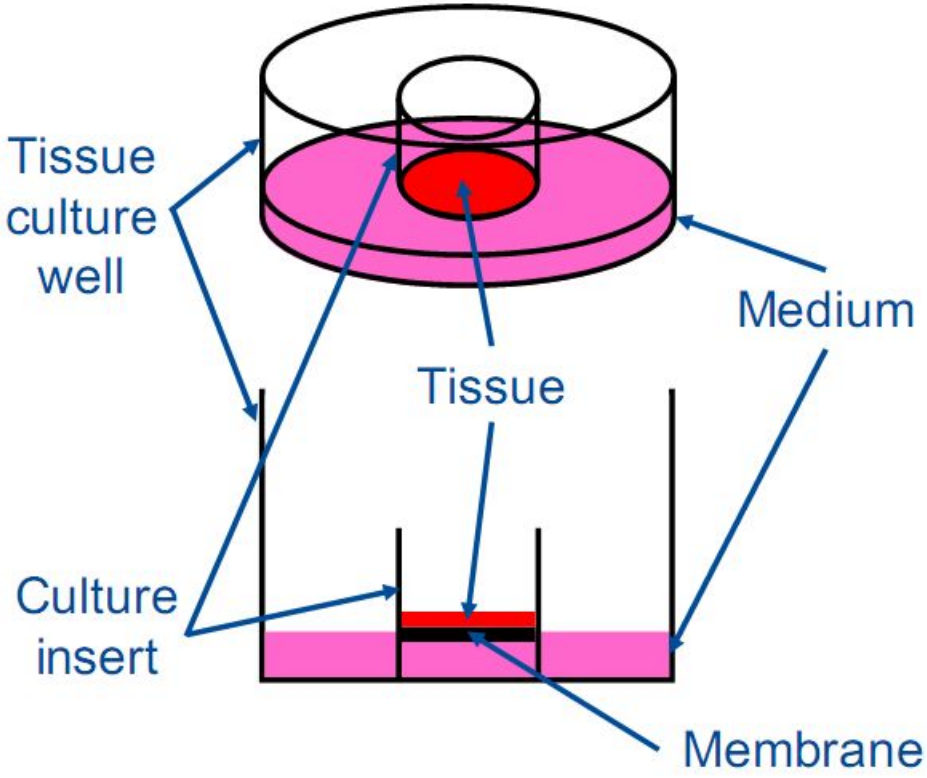


EpiDerm, EPI-200

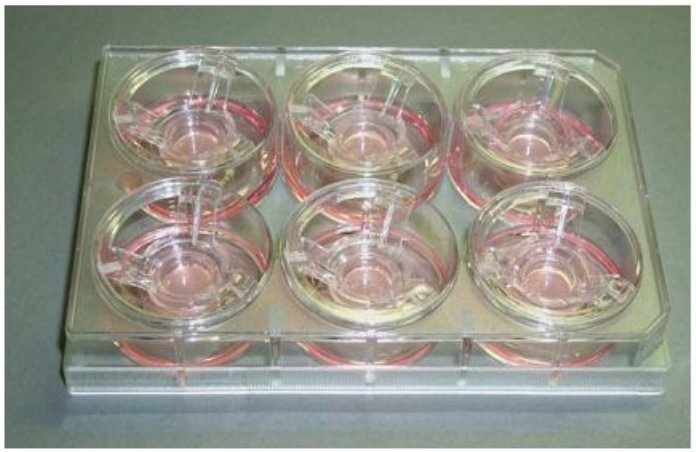


Scheme of epidermis

Cultivation



Schematic representation of the Air-Liquid Interface tissue culture technique



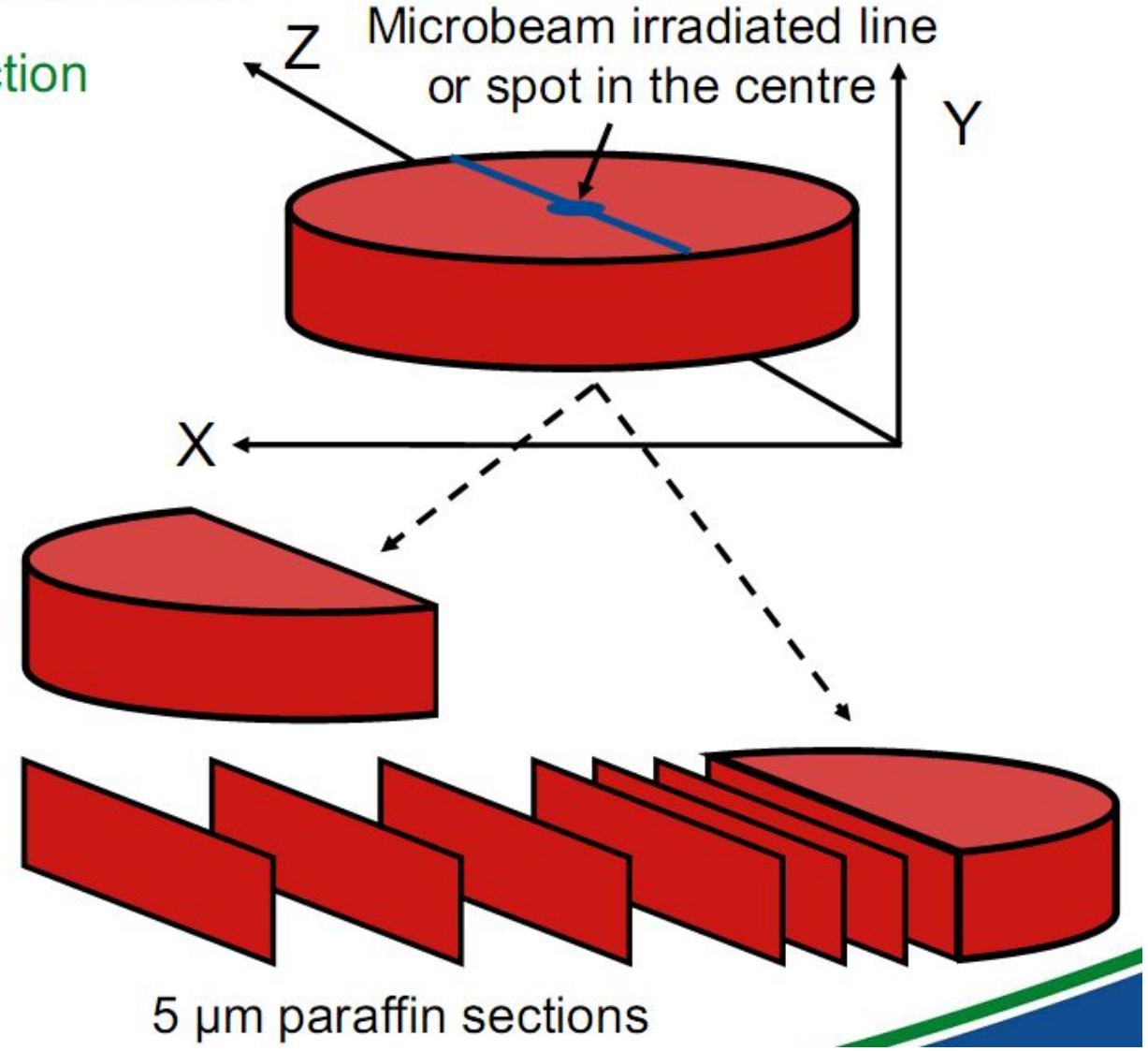
EpiAirway (AIR-100-SNP)

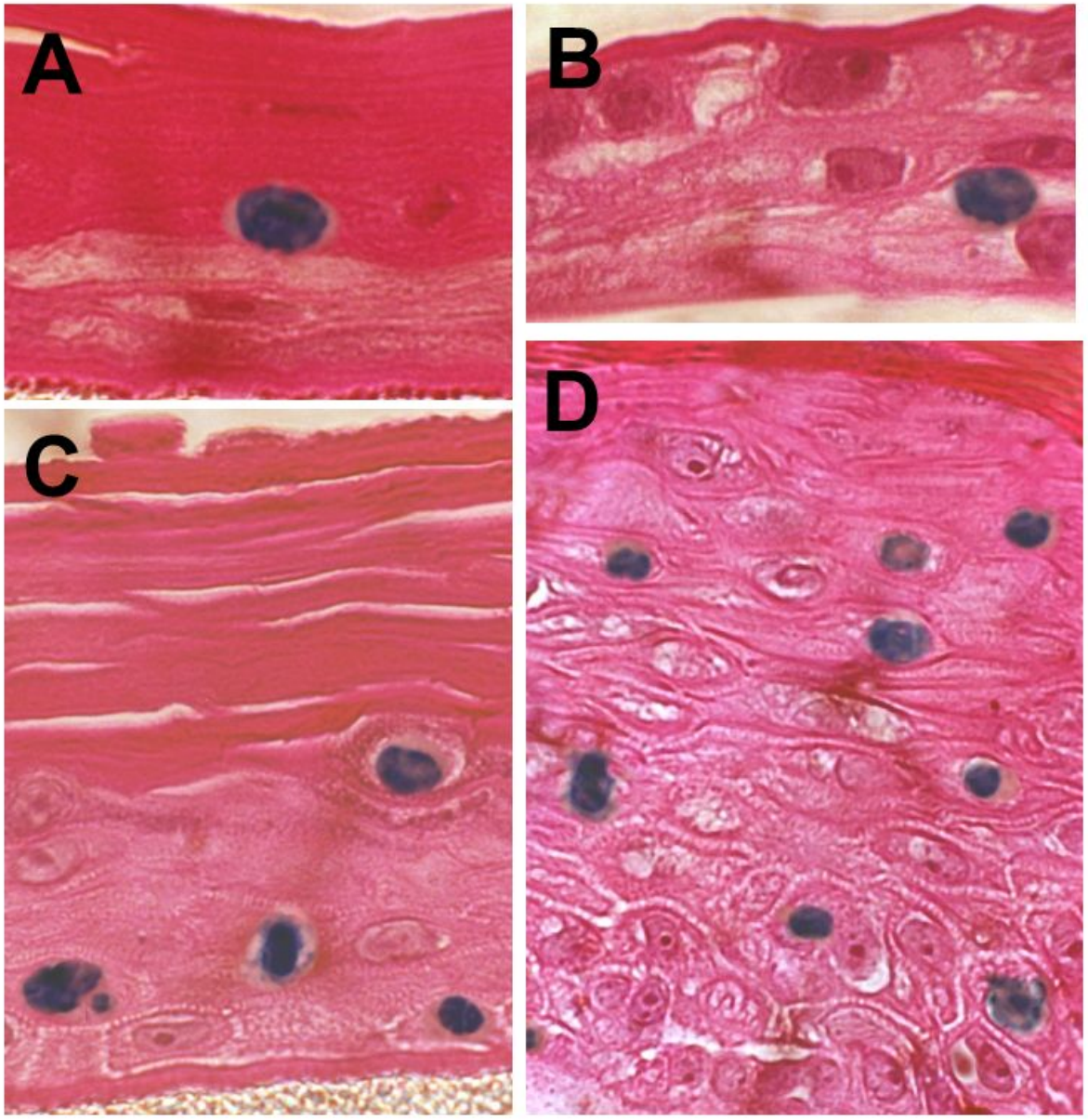
EpiDerm (EPI-212)

Distance-dependent assay after microbeam irradiation

Paraffin histological section preparation

- Incubation for 1-3 days.
- Fixation in 10% neutral buffered formalin.
- Tissue is cut in half along line of irradiation.
- Paraffin embedding.
- Sample is to be cut in series or levels along X axis.





Bystander apoptosis

Bystander induced apoptosis in artificial human skin systems stained with Derma TACS apoptosis kit. Positive apoptotic cells appear blue.

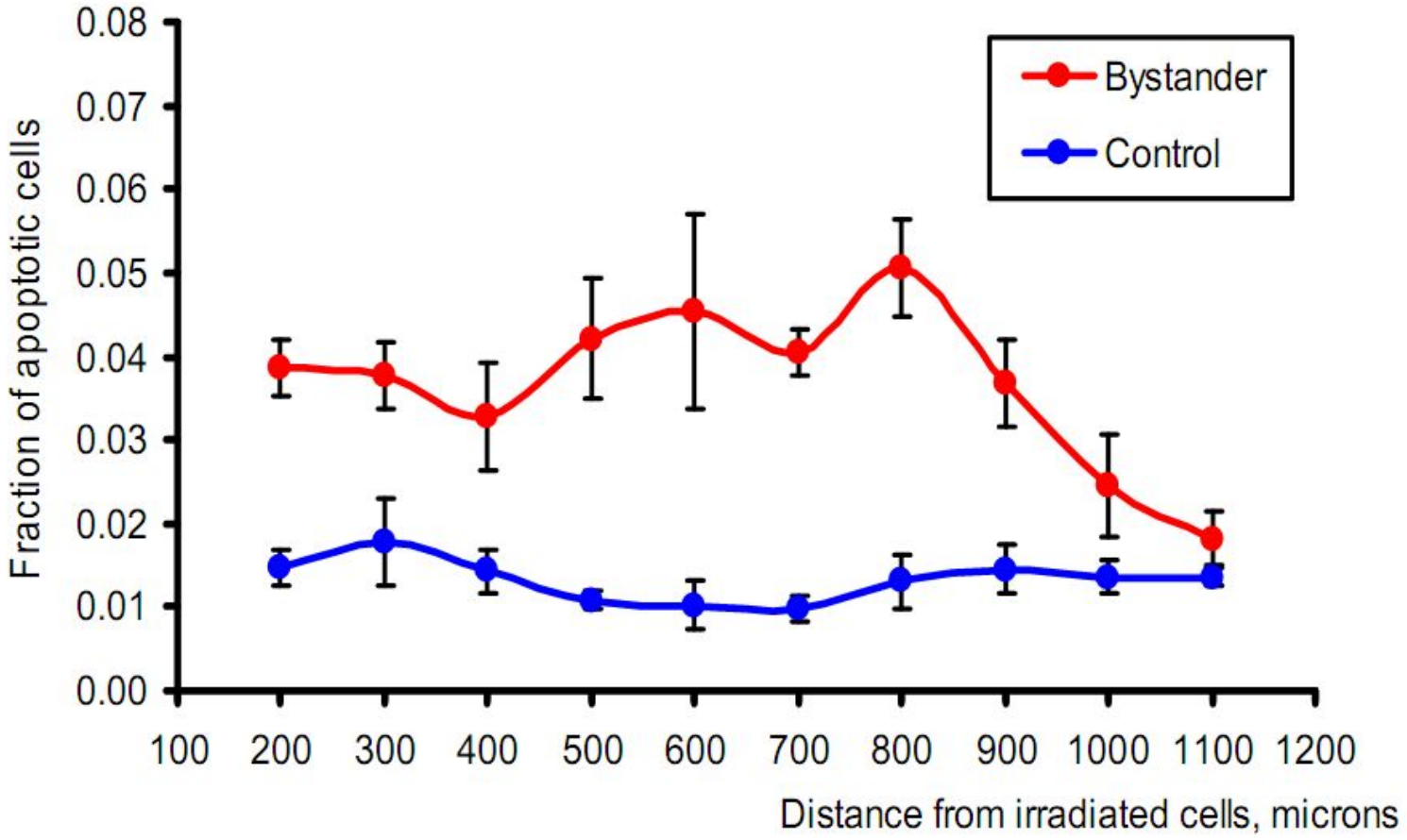
- EPI-201 (A)
- EPI-200-3s (B)
- EPI-200 (C)
- EFT-100 (D)

Bystander effect propagates up to 1 mm away from the irradiated site

- Artificial skin models were irradiated along a straight line across tissue sample (8 mm) every 100 (or 20) μм with α-particles (~7.2 MeV).
- Fractions of micronucleated and apoptotic cells were estimated.
- Mean fraction of bystander apoptotic cells was 3.7±0.6% in irradiated cells and 1.3±0.3% in control.
- Using distance-dependent assay we demonstrated for the first time that bystander effect can be propagated up to 1 mm in tissue after irradiation with α-particle microbeam.

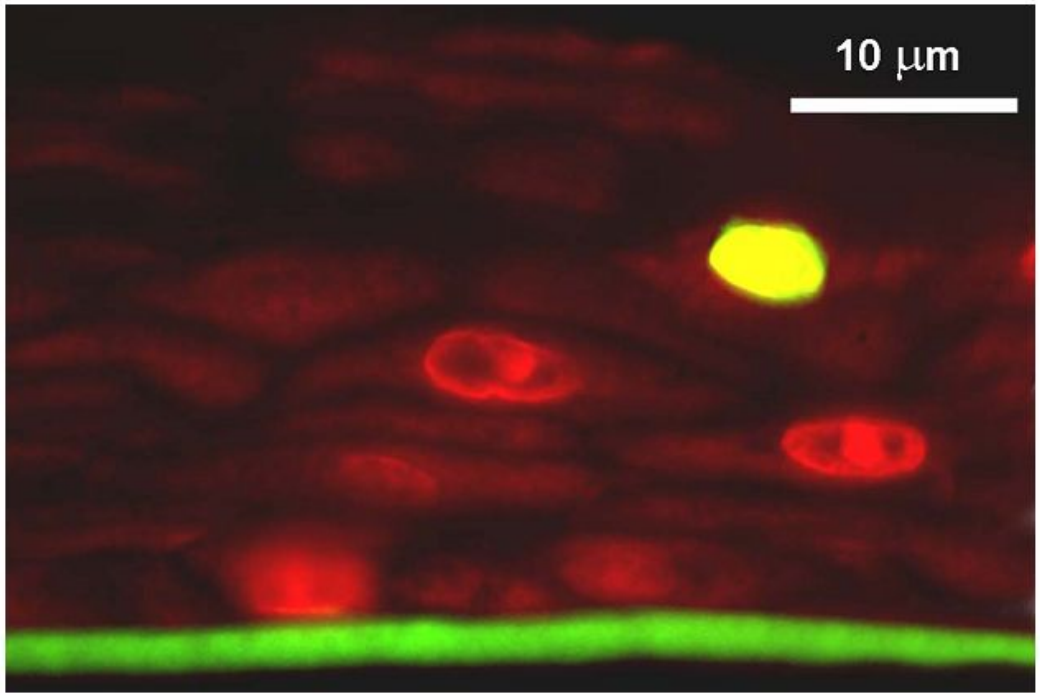
Belyakov, O.V., Mitchell, S.A., Parikh, D., Randers-Pehrson, G., Marino, S.A., Amundson, S.A., Geard, C.R. and Brenner, D.J. (2005) Biological effects in unirradiated human tissue induced by radiation damage up to 1 mm away. *Proc Natl Acad Sci U S A*, **102**:40, 14203-8.

Bystander apoptosis in EPI-200 artificial human tissue after microbeam irradiation



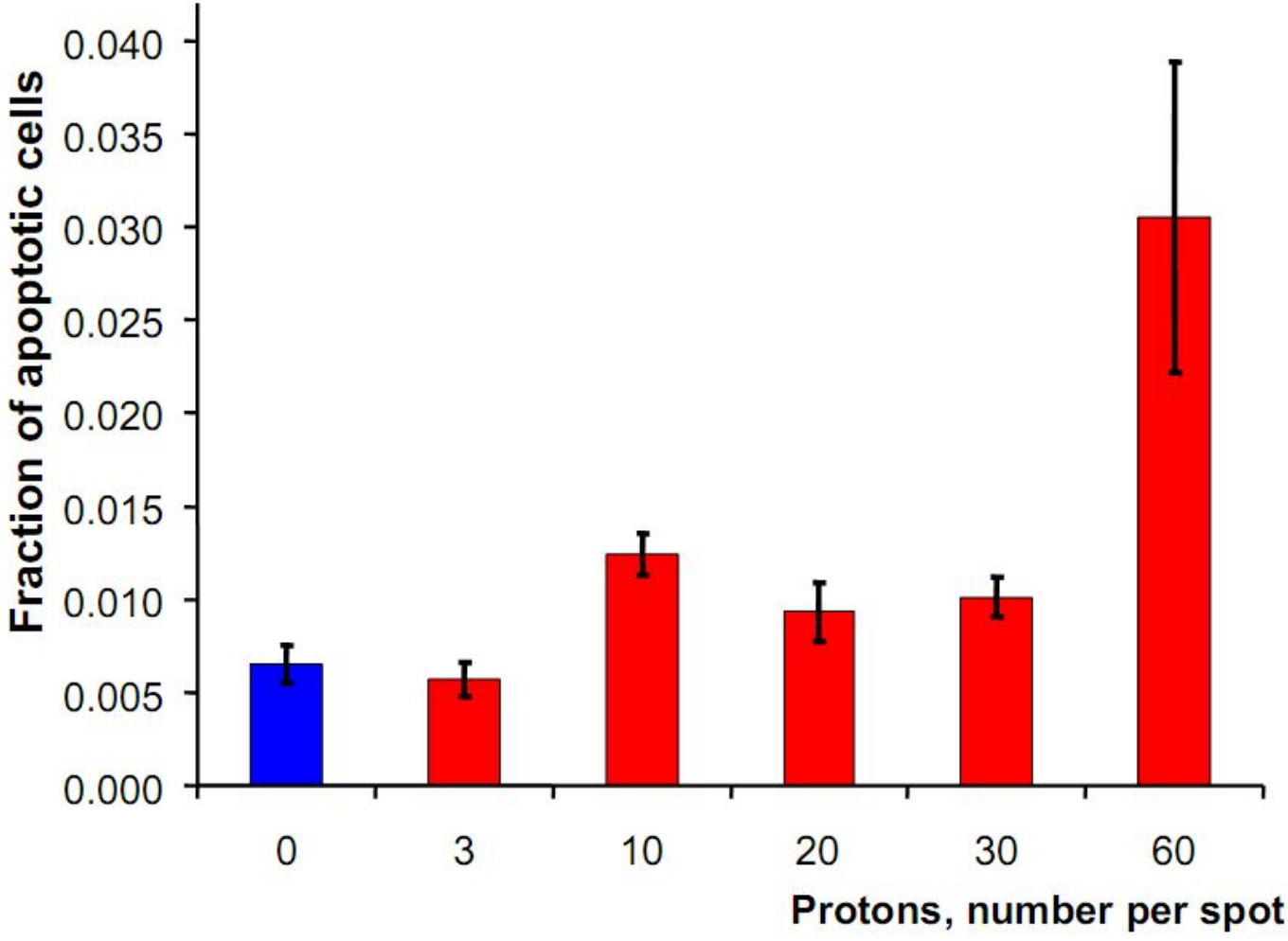
Experimental setup

- Microbeam irradiation of a single 2 μm spot with protons and $^{3}He^{2+}$ ions.
- *In situ* apoptosis assay with 3'-OH DNA end-labelling based technique.
- Studies of bystander-induced differentiation under *in situ* conditions using morphological measurements in underdeveloped EPI-201 model.

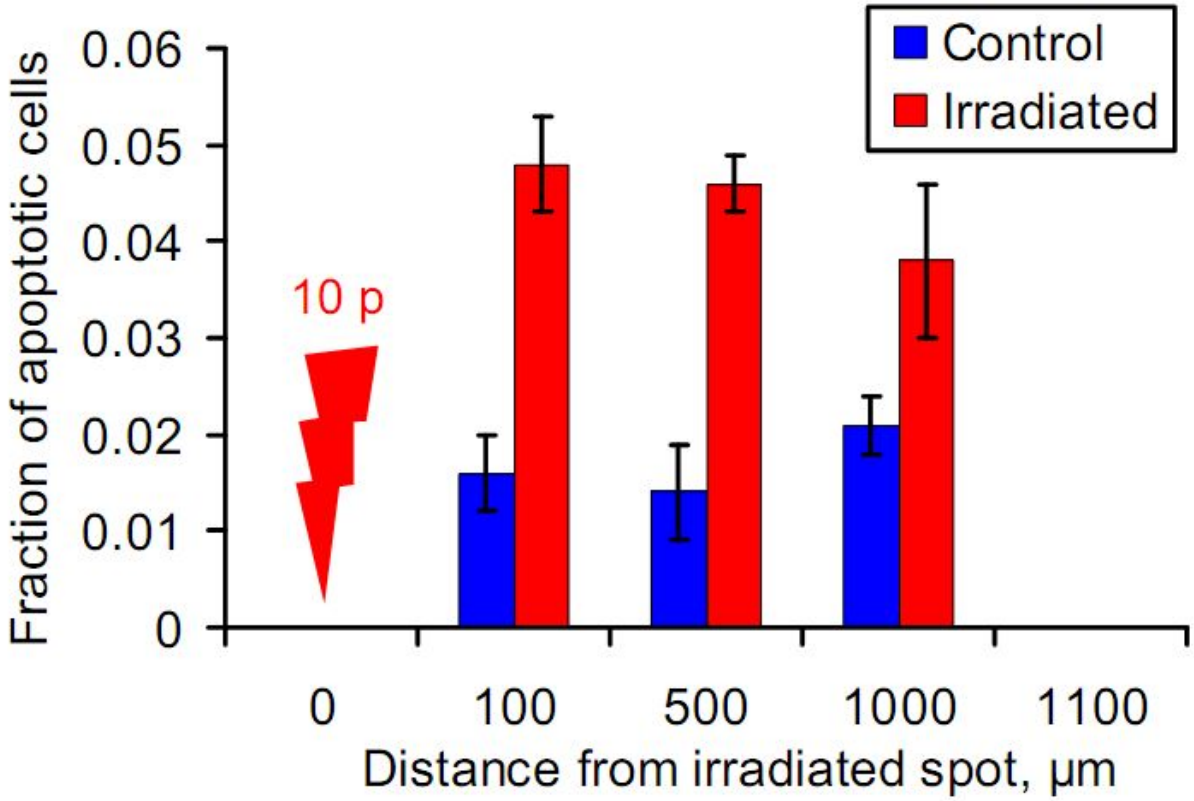


EPI-200, 4 μm paraffin section, 3' OH DNA end-labelling, positive apoptotic cell are green, fluorescent microscope.

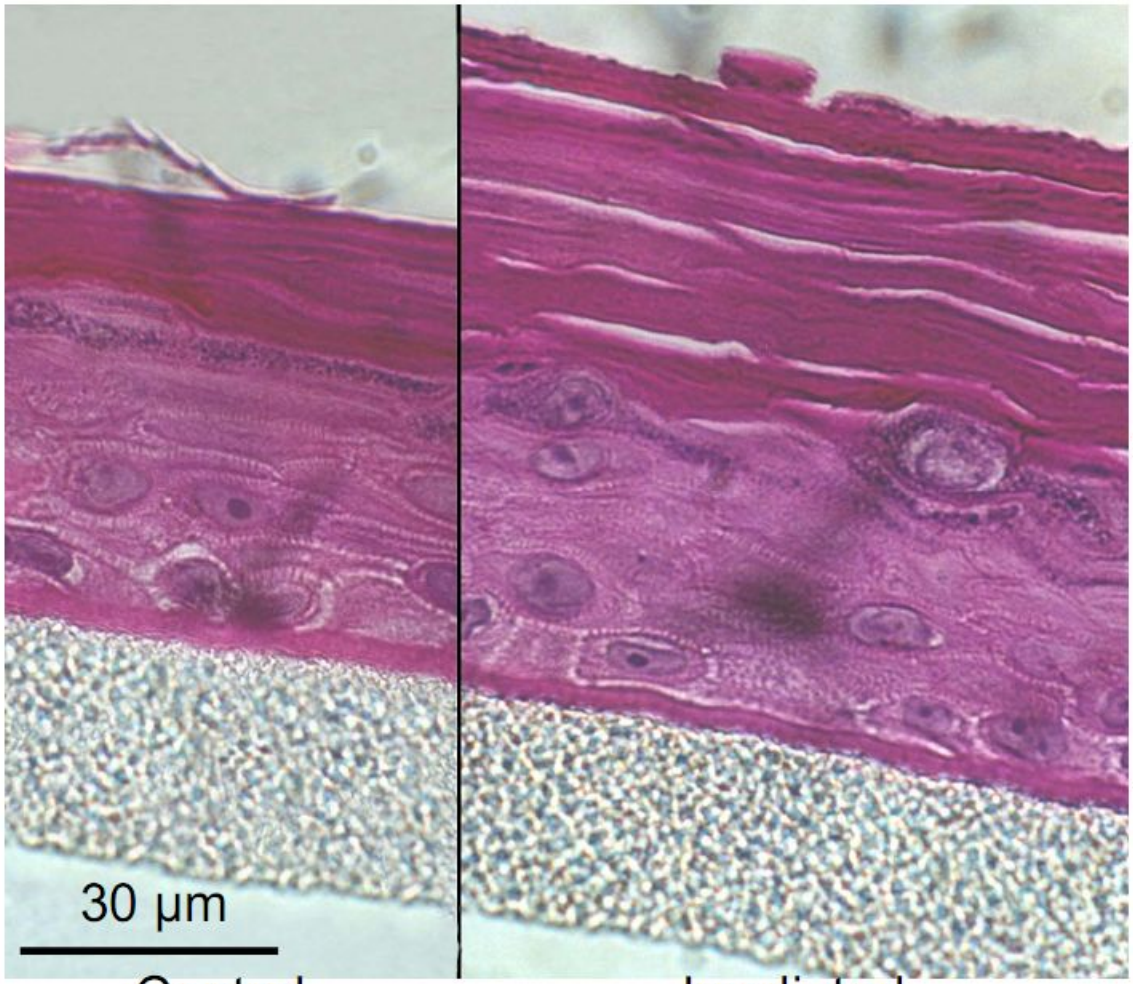
Dose-effect dependency for bystander induced apoptosis in EPI-200 artificial human skin models after microbeam irradiation with protons



Bystander apoptosis in EPI-200 artificial human skin after spot microbeam irradiation with 10 protons



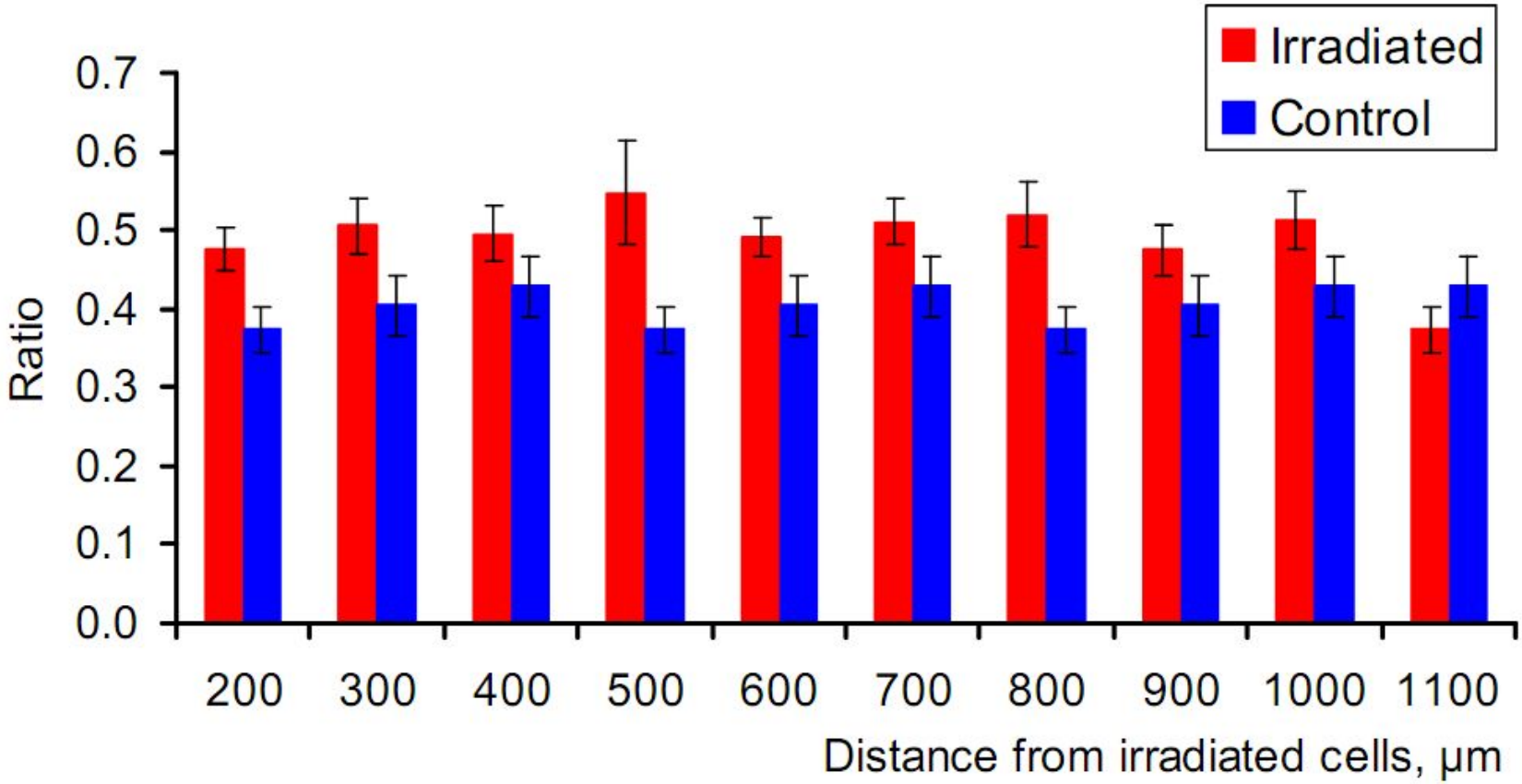
Changes in bystander differentiation pattern after microbeam irradiation EPI-201, 3 days after irradiation



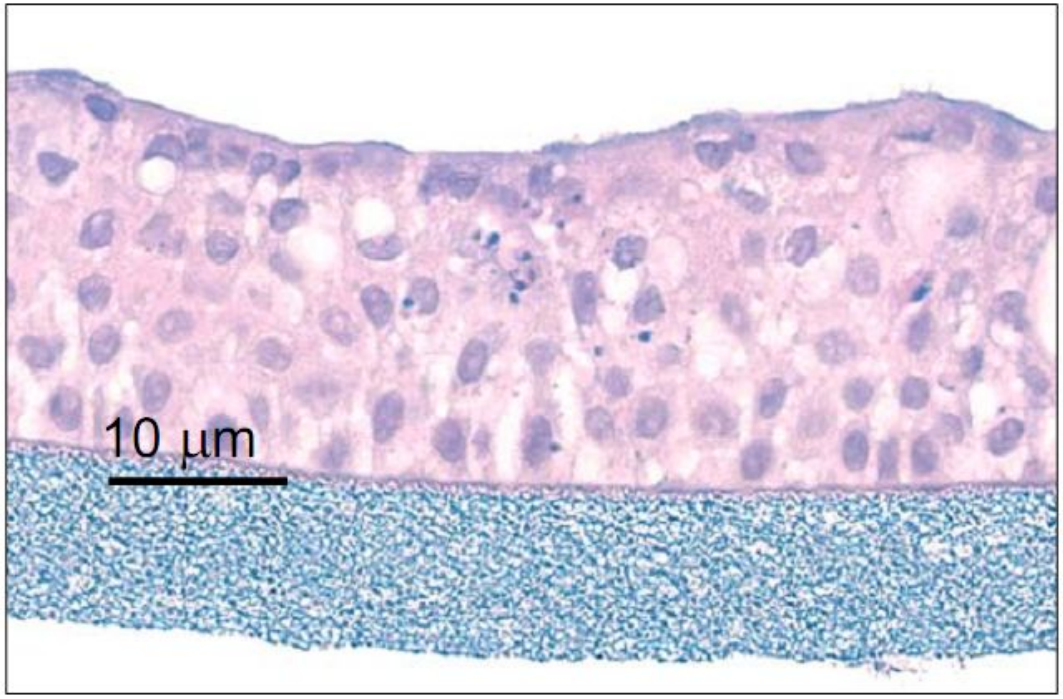
Cornified layer (terminally differentiated cells)

Malpighian layer (non-differentiated, live cells)

Microbeam irradiation increases ratio “cornified layer / total thickness”



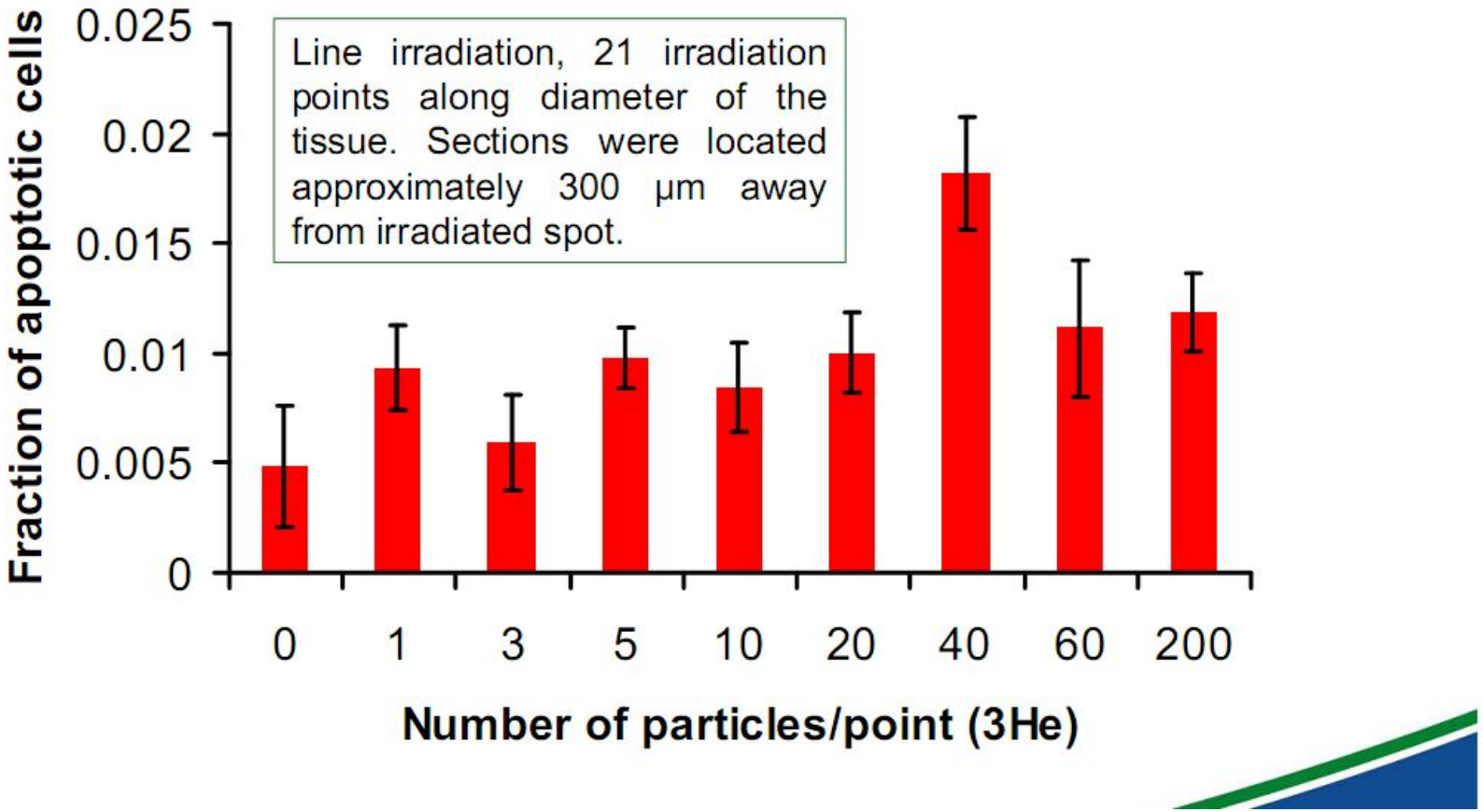
MatTek artificial tracheal/bronchial epithelial tissue system



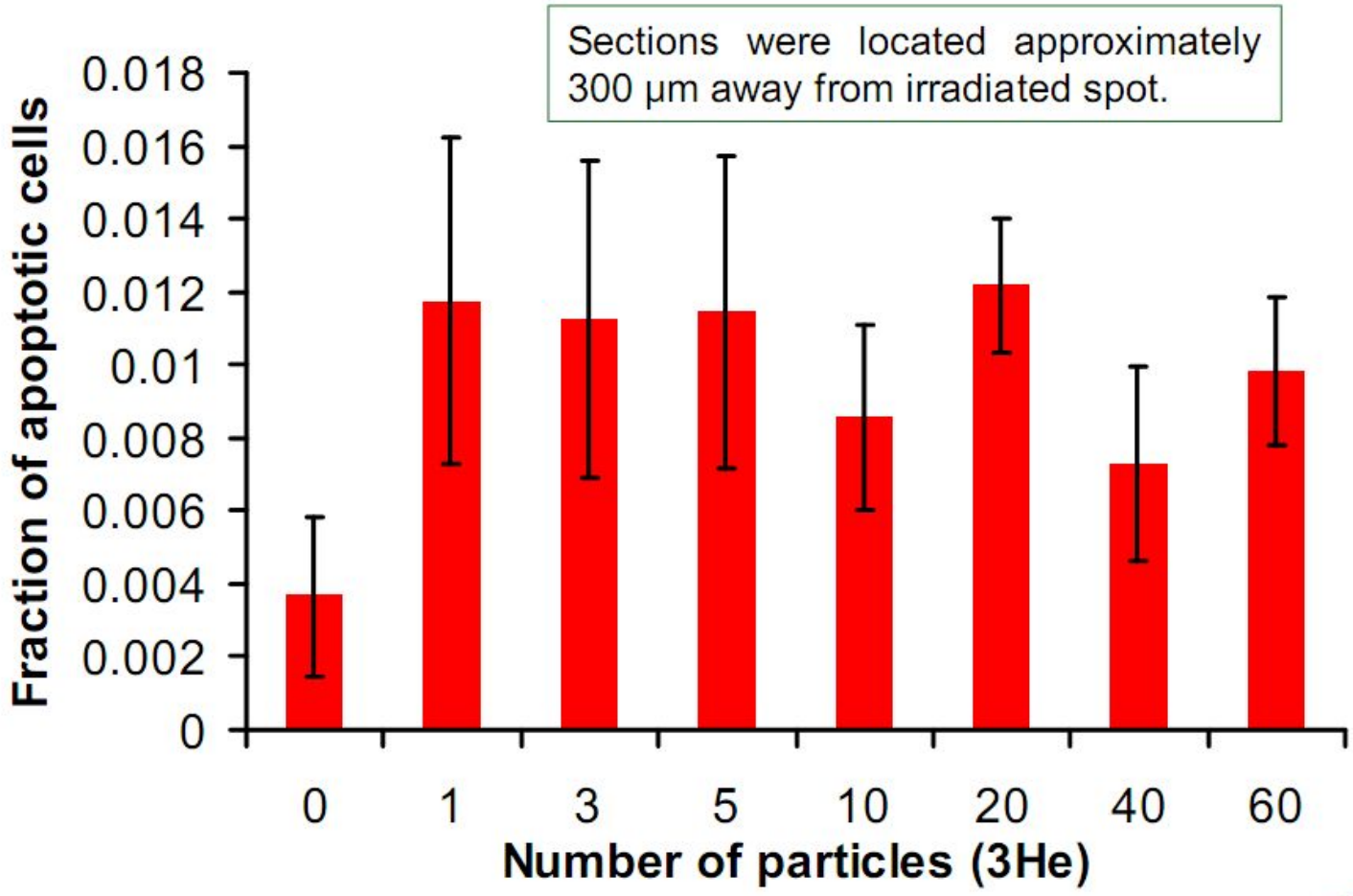
MATTEK CORPORATION

4 µm paraffin section, Haematoxylin - Eosin staining

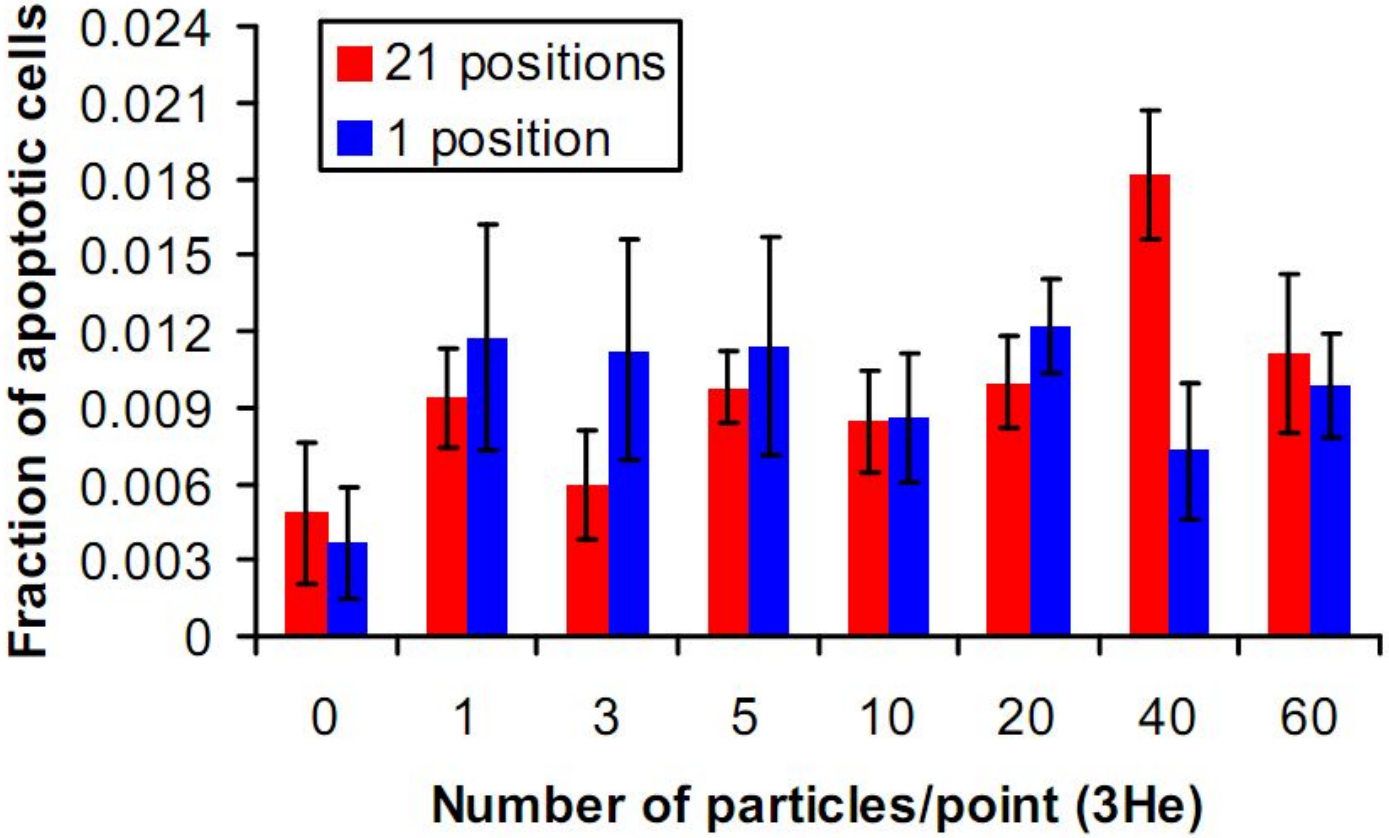
Bystander induced apoptosis following line $^{3}He^{2+}$ microbeam irradiation



Bystander induced apoptosis following single spot $^{3}He^{2+}$ microbeam irradiation



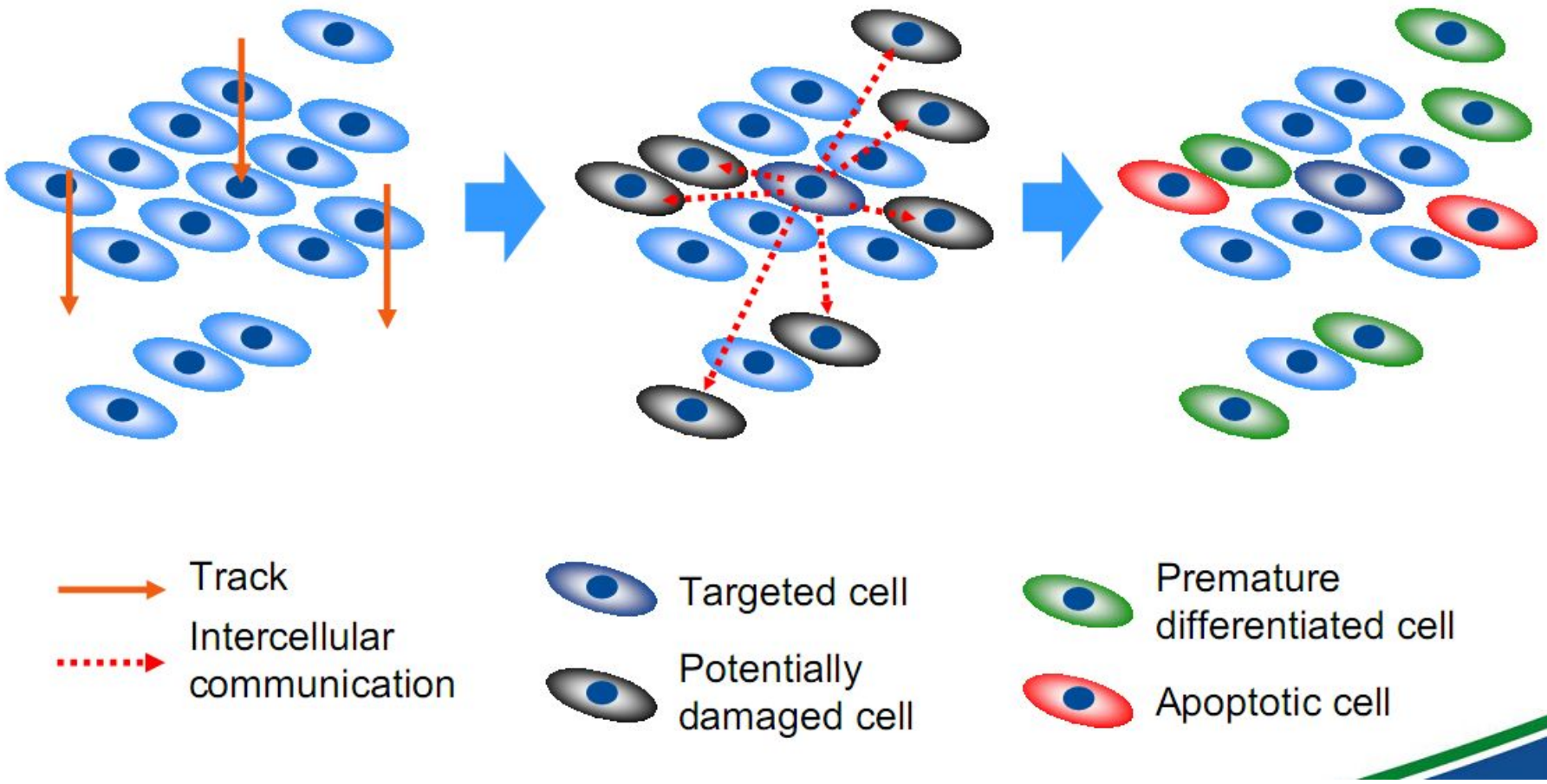
Bystander induced apoptosis following line and spot $^{3}He^{2+}$ microbeam irradiation



Hypothesis - bystander effect is a protective mechanism

- Remove potentially damaged *functional group* of cells to decrease risk of transformation.
- Maximal at low doses when a small fraction of cells is exposed.
- Normal tissue microarchitecture amplifies the response.
- Apoptosis is an important contributor.
- Irreversible differentiation is a major pathway of removing potentially damaged cells from proliferating population.

A general scheme of radiation induced bystander effect in tissue systems



Summary

- Bystander response measured as increase in apoptosis, and differentiation was observed in cell cultures, explants and 3D tissue models.
- Bystander induced apoptosis is propagated over large distances in 3D tissue.
- Tissue sample acts as a single unit in response to microbeam irradiation. A cascade mechanism of bystander effect induction might be involved.
- It is tempting to suggest that the bystander response has the function of eliminating potentially damaged cells in the vicinity of radiation induced DNA damage by apoptosis and increased differentiation.

Implications for Radiation Protection

- Non-targeted effects could be important in several radiation related areas.
- It might contribute to better estimation of cancer risk from domestic radon exposure and uranium in drinking water.
- Effects of HZE (high-charge-and-energy) particles during space missions.
- High energy radiotherapy outcome.
- Health effects of air crew and nuclear power station personnel.
- In particular, bystander effect is potentiality significant for radiation protection issues and may have implications for the applicability of the Linear-No-Threshold (LNT) model in extrapolating radiation risk data into the low-dose region.

Significance of the bystander effects for radiotherapy

- The spectrum of secondary malignancies in radiotherapy patients may suggest some contribution of the bystander effect (Hall, *Cancer J*, 2000).
- Microbeam radiation therapy (Thomlinson, *et al.*, *Cell Mol Biol (Noisy-le-grand)*, 2000) is a new technology of cancer treatment, which might utilise non-targeted effects.
- Finding of a significant bystander induced differentiation after microbeam irradiation would suggest a potential value of the bystander effect for differentiation therapy of cancer treatment; see review of (Beere and Hickman, *Anticancer Drug Des*, 1993).

Future trends in non-targeted research

Experimental systems: opportunities

Currently available

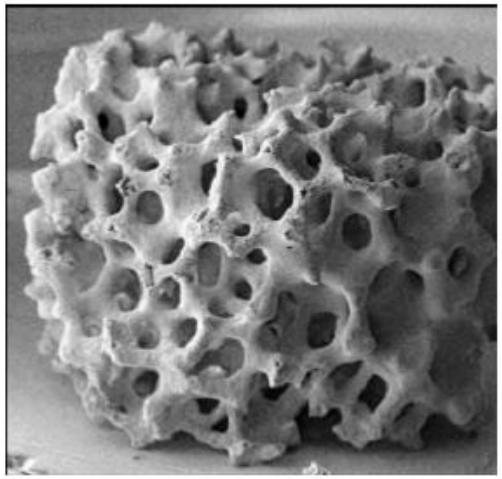
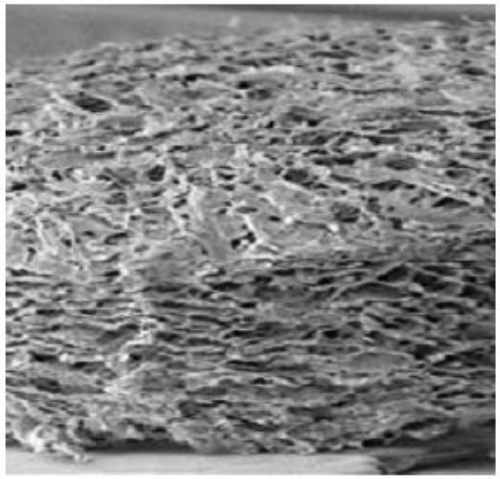
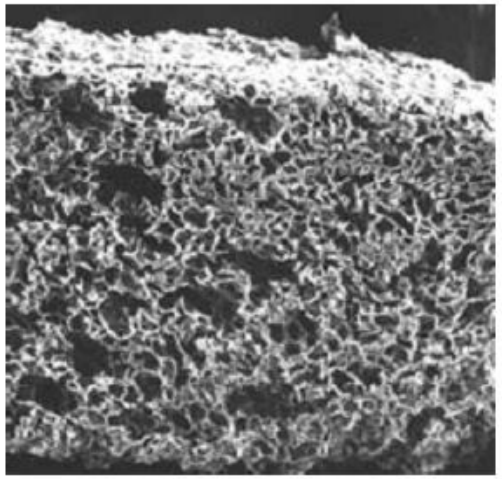
- Primary explant techniques
- Artificial human skin tissue systems
- Tissue scaffolding
- …

Future directions

- Adaptation of the "window chamber technique" for radiobiological experiments
- Tissue transplants, for example, piece of human tissue grafted on a nude mice
- …

Tissue scaffolding

- Allows to use conventional cells cultures to form tissue-like 3D microarchitecture.
- Easy to handle, cells could be easily inoculated and extracted with conventional cell culture techniques.
- Preparation of histological sections and non invasive 3D deep tissue imaging is possible.
- Stable, highly reproducible model.

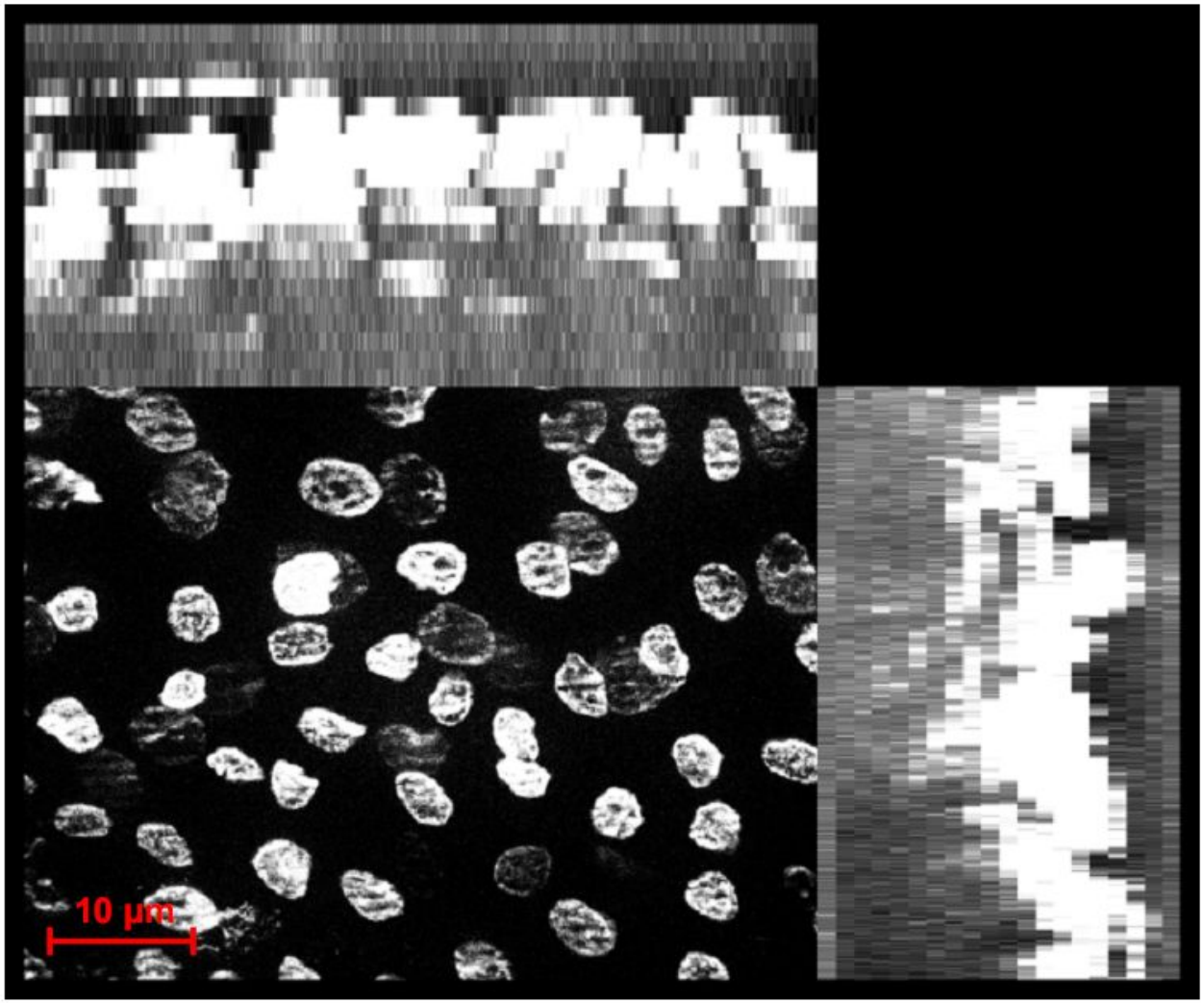




The BD Three Dimensional (3D) Scaffolds: 3D Calcium Phosphate Scaffold (left), 3D Collagen Composite (centre) and OPLA® (Open-Cell Poly-Lactic Acid [right]) scaffolds.

Endpoints

- All models are suitable for histological examination and consequent histoimmunochemistry.
- Deep tissue non-invasive imaging techniques are under development (confocal, 3-photon imaging, Zeiss ApoTome systems).
- Non-destructive life tissue examinations are possible.
- Mutations (?) and epigenetic changes.
- Genomic instability and bystander effect.
- Markers of proliferation and differentiation.
- Malignant conversion (?).
- Progression to invasive cancer (using transformed cell lines and tissue scaffolding or co-culture techniques).

Non-invasive deep tissue imaging



Non-invasive deep fixed and unfixed tissue imaging using Zeiss ApoTome system.

Priorities

- The main priority is a shift from *in vitro* cell systems towards *in vivo* (or at least 3D) tissue models.
- Possible use of human cell lines (with tissue scaffolds), tissue transplants, window chambers technique and other *in vivo* human model systems.
- Low dose irradiation can be performed with broad and microbeam charged particle and X/γ-ray facilities.

Constraints

- Significant inter-individual variability (in case of explants).
- Tissue models typically contain several types of cells, role of tissue microenvironment is significant.
- Cells in tissues are in different proliferation and differentiation states.
- 3D tissue difficult to irradiate quantitatively with existing charge-particle microbeams because of low range (typically tenths of micrometers).
- 3D tissue studies would require new methods of non-invasive deep tissue imaging to preserve 3D microarchitecture and study spatial distribution.

Non-targeted effects and radiation protection

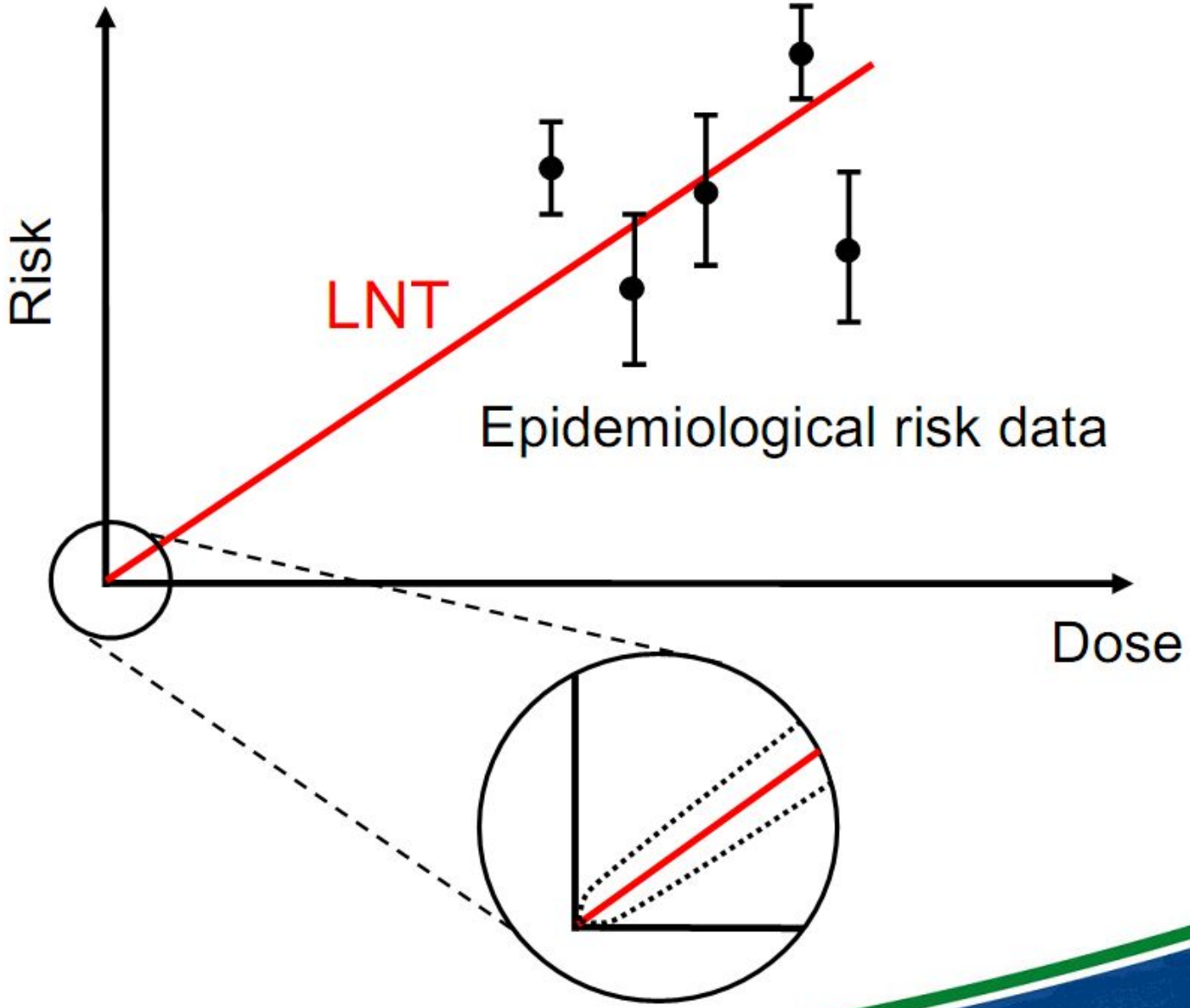
System of radiation protection

- Present estimations of radiation risk is based on direct epidemiological evidence, as well as on radiation biology research.
- The system is designed to protect against both deterministic and stochastic effects.
- Linear-Non-Threshold (LNT) model is used for estimation of long-term health effects including carcinogenesis and genetic effects.
- A dose and dose-rate correction factor is used to relate the effects of acute exposures to chronic exposures (DDREF).
- Radiation dose is used as a surrogate for risk.
- The effects produced by different types of radiation are assumed to be qualitatively the same.
- Doses can be summed to predict overall risk.

Challenges of the present radiation protection system

- The main objective of the system is to protect the individual. The protection system is generally applicable, in the same fashion, to all age groups, males and females.
- The protection system include the principles of justification, optimisation and exposure restrictions.
- There is a broad international agreement among governmental bodies that the current system of radiation protection is effective, robust and adequately protects people and the environment.
- There are, however, scientific challenges that may bring into question various aspects of the current approach, and which may have significant policy, regulatory and operational implications.
- These challenges include non-targeted effects.

LNT and uncertainties in extrapolation of radiation risk



Key question

Do non-targeted effects increase or decrease low dose risk in relation to LNT?

The bystander effect might be harmful

- The bystander-induced mutagenesis

  Nagasawa and Little, *Rad Res,* 1999

  Zhou *et al., Radiat Res*, 2000; Zhou *et al., PNAS*, 2001

- Bystander-induced transformation

  Lewis *et al., Radiat Res,* 2001

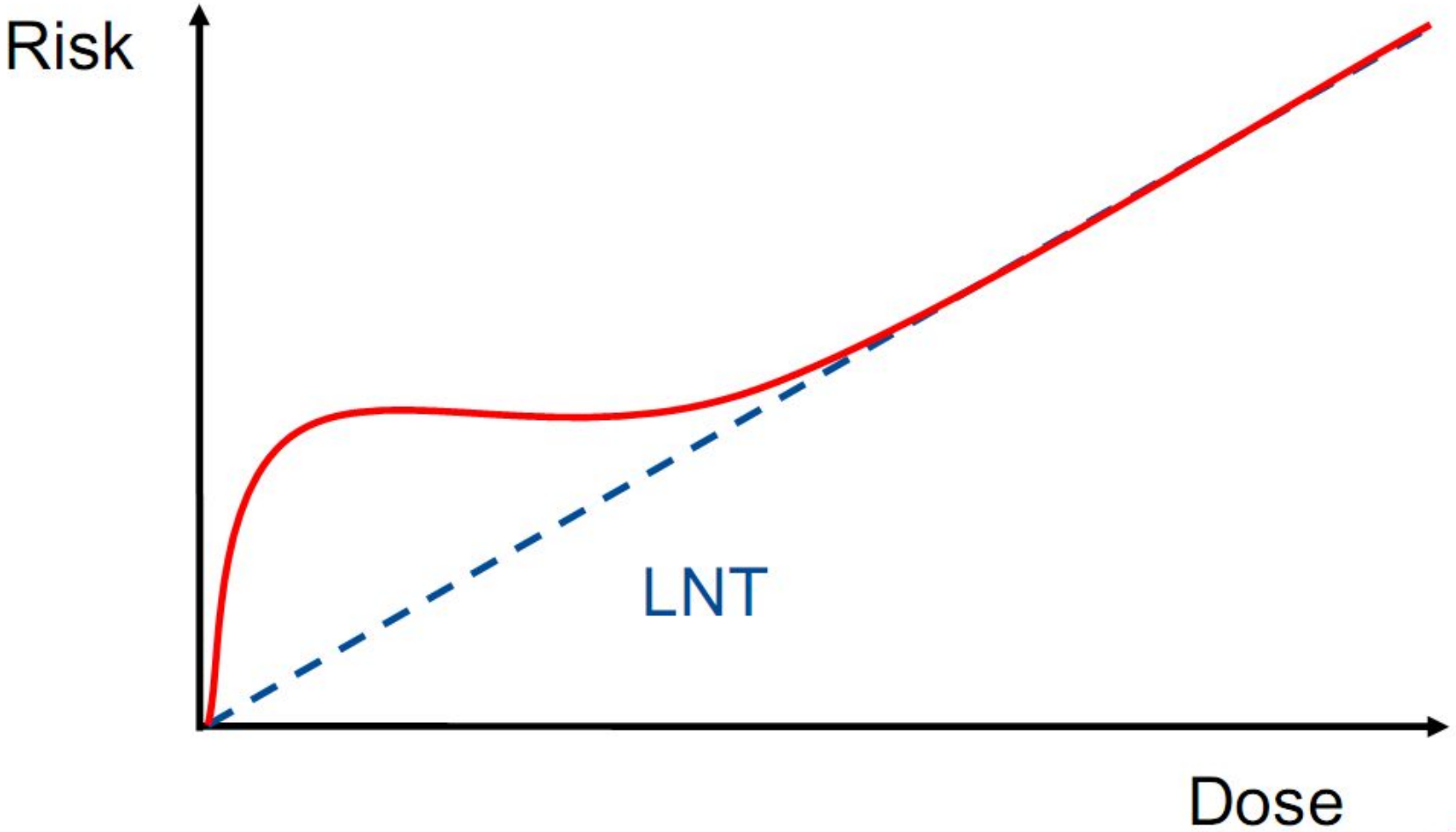
  Sawant *et al., Radiat Res*, 2001

- Chromosomal instability could be induced in bystander cells

  Lorimore *et al., PNAS,* 1998

  Watson *et al*., *Cancer Res*, 2000

The risk at low doses might be *greater* than predicted by LNT



The bystander effect might be protective

- A gross bystander induced differentiation in the urothelial explant outgrowth after microbeam irradiation

  Belyakov *et al., Mut Res,* 2006

- Cell survival is increased after treatment with medium from irradiated cells

  Matsumoto *et al.*, *Radiat Res*, 2001

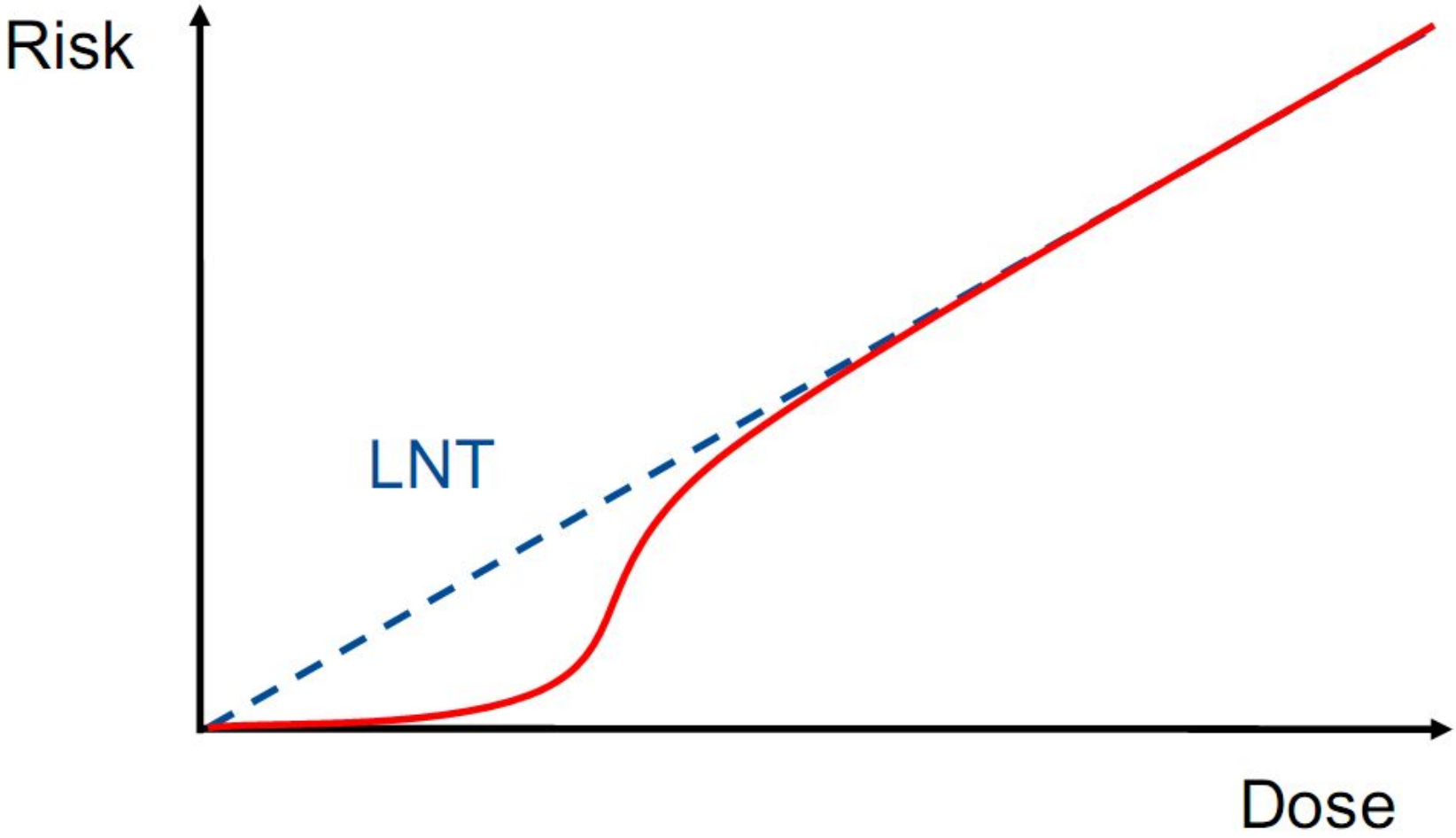
- Increase in cell proliferation after low doses of α-particle exposure

  Iyer and Lehnert, *Cancer Res*, 2000

- Bystander effect is a mechanism of tissue integrity maintenance

  Barcellos-Hoff and Brooks, *Rad Res*, 2001

The risk at low doses might be *less* than predicted by LNT



Summary

	RISK	
Bystander effects:		
cell death	-	+
mutation		+
chromosomal damage	-	+
malignant transformation		+
premature differentiation	-	
Other non-targeted effects:		+
genomic instability	-	
adaptive responses		

Implications for radiation protection

- The observation of the non-targeted effects are preliminary in nature, and the applicability of any conclusion derived from *in vitro* studies to *in vivo* situation is still uncertain.
- The risk at low doses might be *greater* or *less* than predicted by a linear extrapolation of the high dose.
- However, non-targeted effects will clearly result in an overall risk, which is a *non-linear* function of dose.
- It would be *premature* to consider revising current risk calculations on the basis of current studies of bystander phenomena.
- On other hand, the LNT model is important for radiation protection as a simple method to optimise procedures and regulations. However, it should not be mistaken as a scientific model directly derived from the present state of knowledge of the processes involved in radiation risk estimations.

- Experiments suggest that the effect is due to a molecule secreted by irradiated cells, which is capable of transferring damage to distant cells. Use of a single microbeam has allowed the demonstration of a bystander effect for chromosomal aberrations, cell lethality, mutation, and oncogenic transformation. When cells are in close contact, allowing gap junction communication, the bystander effect is a much larger magnitude than the phenomenon demonstrated in medium transfer experiments. A bystander effect has been demonstrated for both high- and low-LET radiations but it is usually larger for densely ionizing radiation such as alpha particles. Experiments have not yet been devised to demonstrate a comparable bystander effect on a three-dimensional normal tissue. Bystander studies imply that the target for the biological effects of radiation is larger than the cell and this could make a simple linear extrapolation of radiation risks from high to low doses of questionable validity.

- The radiation-induced bystander effect is defined as “the induction of biological effects in cells that are not directly traversed by a charged particle but are in close proximity to cells that are.” Although these bystander effects have been demonstrated with a variety of biological endpoints in both human and rodent cell lines (as well as in 3D tissue samples), the mechanism of the phenomenon is not known. Although gap junction communication and the presence of soluble mediator(s) are both known to play important roles in the bystander response, the precise signaling molecules have yet to be identified.

- GENERATIONS OF students in radiation biology have been taught that heritable biological effects require direct damage to DNA. In fact, evidence has been available for many years that this simple statement is not strictly true. As early as the 1940's there were reports that the inactivation of biological entities may be brought about equally by ionizations produced within the entity or by the ionization of the surrounding medium (Dale 1940, 1942, 1943; Lea et al. 1944). By 1947, Kotval and Gray had shown that alpha particles that pass close to the chromatid thread, as well as those which pass through it, have a significant probability of producing chromatid and isochromatid breaks or chromatid exchanges.
- The term used today to describe such phenomena is "The Bystander Effect," a name borrowed from the gene therapy field where it usually refers to the killing of several types of tumor cells by targeting only one type of cell within a mixed population (Cheng et al. 1999, for example).

- In the radiation field, it has come to be loosely defined as the induction of biological effects in cells that are not directly traversed by a charged particle, but are in close proximity to cells that are. Interest in this effect was sparked by the report of Nagasawa and Little (1992) that, following a low dose of alpha particles, a larger proportion of cells showed biological damage than were estimated to have been hit by an alpha particle; specifically 30% of the cells showed an increase in sister chromatid exchanges even though less than 1% were calculated to have undergone a nuclear traversal. The number of cells hit was arrived at by a calculation based on the fluence of alpha particles and the cross-sectional area of the cell nucleus. The conclusion was thus of a statistical nature since it was not possible to know on an individual basis which cells were hit and which were not.

- The plethora of data now available concerning the bystander effect fall into two quite separate categories, and it is not certain that the two groups of experiments are addressing the same phenomenon. First, there are experiments involving the transfer of medium from irradiated cells, which results in a biological effect in unirradiated cells. Second, there is the use of sophisticated single particle microbeams, which allow specific cells to be irradiated and biological effects studied in their neighbors (Randers-Pehrson et al. 2001).

Medium transfer experiments

- Experiments involving the transfer of medium from irradiated to unirradiated cells have demonstrated a highly significant reduction in the plating efficiency of both normal and malignant epithelial cells—whether or not the cells were irradiated (Mothersill and Seymour 1997).

- This bystander effect suggested that irradiated cells secreted a molecule into the culture medium that was capable of killing cells when that medium was transferred onto unirradiated cells. By contrast, medium irradiated in the absence of cells had no effect. Further experiments demonstrate that not all cells were capable of producing the toxic factor, nor were all cells capable of receiving the secreted signal (Mothersill and Seymour 1997, 2001). In later experiments using explants of human uroepithelium, Mothersill et al. (2001) show that there is considerable variation in the release of the bystander factor into the surrounding cell culture medium. The effect roduced by epithelial cell cultures is dependent on the cell number at the time of irradiation, can be observed as soon as 30 min post irradiation, and is still effective if taken from the irradiated cells up to 60 h after irradiation. This bystander effect can be induced by radiation doses as low as 0.25 mGy and is not significantly increased up to doses of 10 Gy. Forty-eight hours after receiving irradiated medium there were many apoptotic bodies present, suggesting that apoptosis may be a prominent mechanism of cell death responsible for the reduced clonogenic survival. In addition to increased levels of cell death and reduced cloning efficiency, medium transfer experiments have shown an increase in neoplastic transformation as well as genomic instability in cells that have not themselves been irradiated.

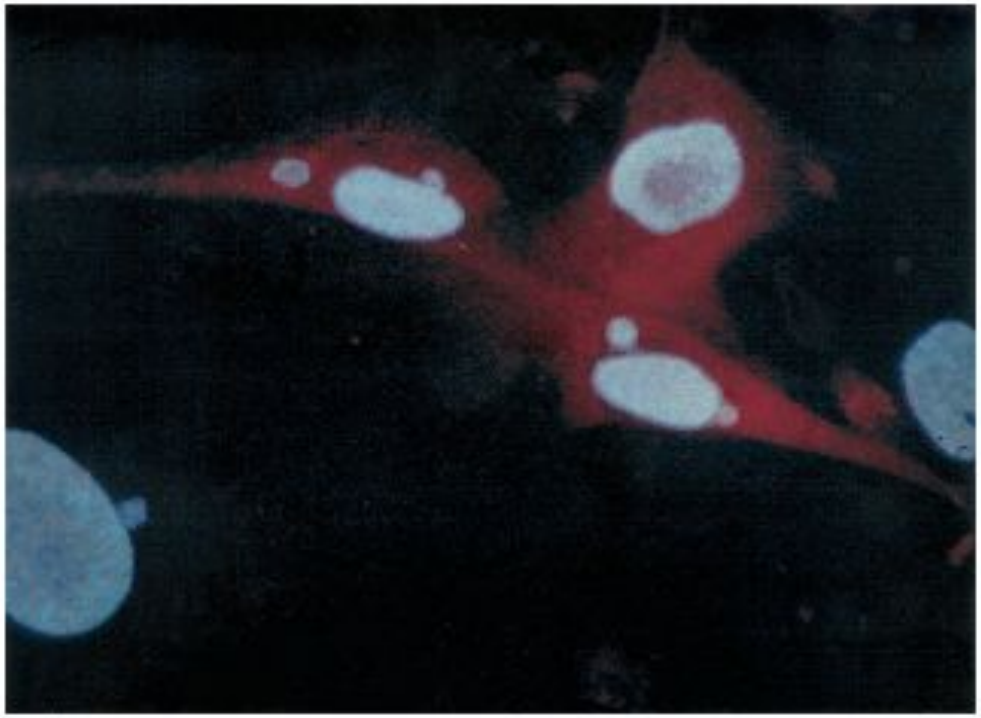
- Some limited progress has been made in the search for the mechanisms involved in this bystander effect. Following exposure to radiation, the first detectable effect of transferred medium on recipient cells was a rapid calcium pulse (1–2) followed 30–120 min later by changes in mitochondrial membrane permeability and the induction of reactive oxygen species (Lyng et al. 2002). Gap junction communication between cells was not required to induce killing of bystander cells, but medium from cell cultures irradiated at high densities induced the greatest amount of cell death. Furthermore, the use of apoptosis inhibitors or medium from lactate dehydrogenase or glucose-6-phosphate dehydrogenase mutant cells reduced or prevented the bystander effect. Treatment with the anti-oxidants L-lactate and l-deprenyl prevented bystander factor associated cell kill suggesting that energy/REDOX metabolism may be involved in the medium mediated bystander response.

The majority of bystander experiments involving medium transfer have utilized low-LET x or gamma rays, in contrast to microbeam experiments where alpha particles or protons have been the particles of choice.

THE BYSTANDER EFFECT DEMONSTRATED BY MICROBEAM EXPERIMENTS

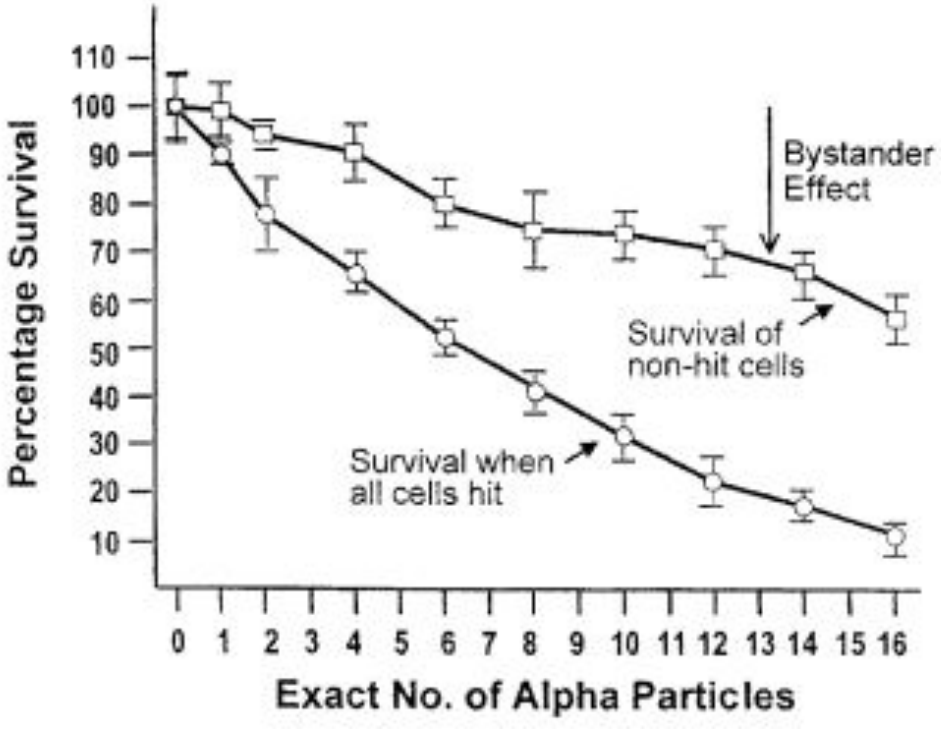
Experiments described here involve the scoring of micronuclei, cell lethality, mutation, and oncogenic transformation.

- Micronuclei in normal human fibroblasts Perhaps the most direct and most dramatic demonstration of the bystander effect involves the observation of micronuclei in irradiated human fibroblasts. Cells of one population were lightly stained with cyto-orange, a cytoplasmic vital dye, while cells of another population were lightly stained blue with a nuclear vital dye. The two cell populations were mixed and allowed to attach to the culture dish, and the computer controlling the accelerator was programmed to irradiate only blue-stained cells with 10 alpha particles directed at the centroid of the nucleus. The cells were fixed and stained 48 h later, at which time micronuclei and chromosome bridges were visible in a proportion of the nonhit (i.e., orange-stained) cells (Fig.1). This is an astonishing emonstration of the bystander effect because the development of micronuclei implies significant chromosome damage and rearrangement, which is clearly visible in nonhit cells that have been fixed in situ.



- Fig. 1. The bystander effect with human fibroblasts. Cells of one population were stained with the vital nuclear dye Hoechst 3342 (blue fluorescence), and cells of another population were stained with the vital cytoplasmic dye cell tracker orange (orange fluorescence) and mixed at a ratio of 1:1. Only blue nuclei were microbeam irradiated with alpha particles; the orange cells were thus “bystanders.” Cells were fixed and stained 44 h after exposure to radiation. A micronucleus is clearly visible in an orange (nonhit) cell (courtesy of Charles Geard).

- Cell lethality Lines of hygromycin- and neomycin-resistant V79 cells were produced. Before exposure the hygromycinresistant cells were stained with a low concentration of a vital nuclear dye. They were then plated in micro wells in the proportion nine neomycin-resistant for every one hygromycin-resistant cell. The computer was programmed to irradiate only the 10% of cells stained with a nuclear dye with various numbers of alpha particles from 1–16 aimed at the centroid of the nucleus. The cells were then removed and cultured for survival in the appropriate growth media, which made it possible to obtain survival curves for hit and nonhit cells.



- Fig. 2. The bystander effect for cell survival in V79 cells. Each data point (mean ± SE) on the line with circles refers to the survival of cells when all cell nuclei on each dish were exposed to the same exact numbers of alpha particle traversals using the microbeam system. The squares show survival for various numbers of alpha particles, from 1–16, traversing 10% of the cell population. The extent to which this falls below the 100% survival for the nonhit is an indication of the magnitude of the bystander effect. Each data point represents the mean ± SD of the clonogenic survivals from three culture plates (redrawn from Sawant et al. 2002).

- There is a considerable degree of cell killing in the nonhit cells, implying a substantial bystander effect. The magnitude of the bystander effect in these studies is much greater than that reported by The Gray Institute for Cancer Research where only 5 to 10% lethality is seen in nonhit cells, using protons or soft x rays in a microbeam. The difference is probably accounted for by the cell density. In The Gray Institute studies, only about 200 cells were seeded in an area of 10 X10 mm. The average distance between cells, therefore, was some hundreds of microns, so it is likely that communication via gap junction did not contribute to the effect observed. By contrast, in the studies reported here, 1,000 to 1,200 cells were plated in a mini-well of 6.3 mm diameter so that 50 to 60% were in contact, allowing gap junction communication that has been demonstrated to be of importance in mutation studies with the microbeam. Therefore, the current study also supports the need for gap junction communication as a mediator of bystander effects in relation to radiation-induced cell killing.

- Mutagenic effects in human-hamster hybrid cells Zhou et al. (2000) reported a study in which human-hamster hybrid (AL) cells were exposed to alpha particles by use of the Columbia microbeam. After all cells on the dish were identified and located, the computer was programmed to expose 20% of the cells, randomly selected, to 20 alpha particles directed through the centroid of the nucleus. This irradiation allows less than 1% of the cells to survive, and yet when assayed for mutations in the human chromosome 11, the mutation yield was four times that of the background (Fig. 3).

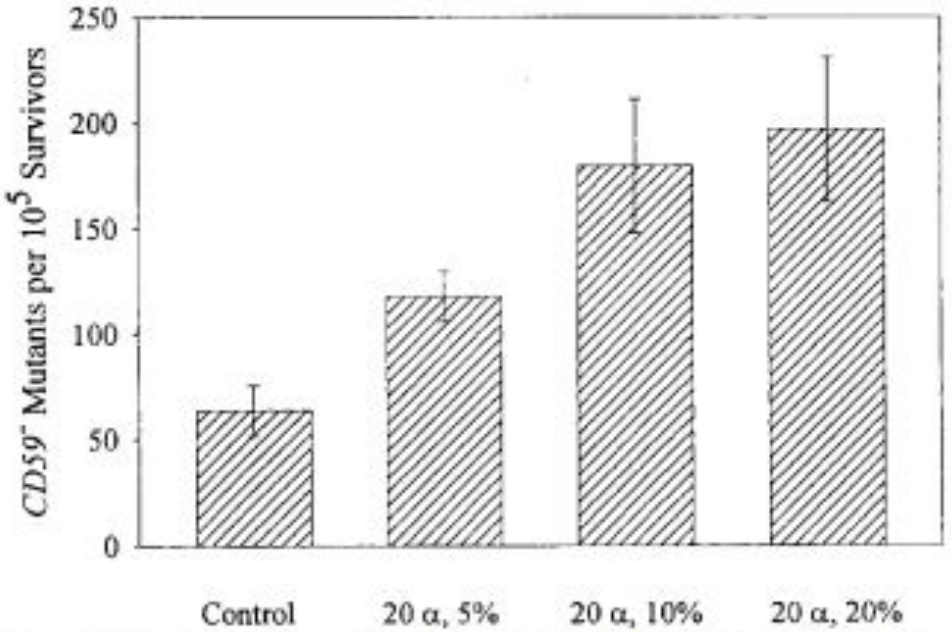


Fig. 3. The bystander effect for mutations in the human-hamster hybrid (A_L) cells when 20% of the cells receive 20 nuclear traversals by alpha particles. There is a substantial incidence of mutations over the background level, despite the fact that no irradiated cells survive (redrawn from the data of Zhou et al. 2000).

- These mutations must clearly arise from neighbor cells, not directly exposed, but in close proximity to irradiated cells. A further series of experiments identified the importance of cell-cell communication via gap junctions as a mechanism of the bystander effect. When AL cells were transfected with a dominant negative connexin 43 vector (DN6), which eliminates gap junction communication, the bystander effect essentially disappears. This is illustrated in Fig. 4.

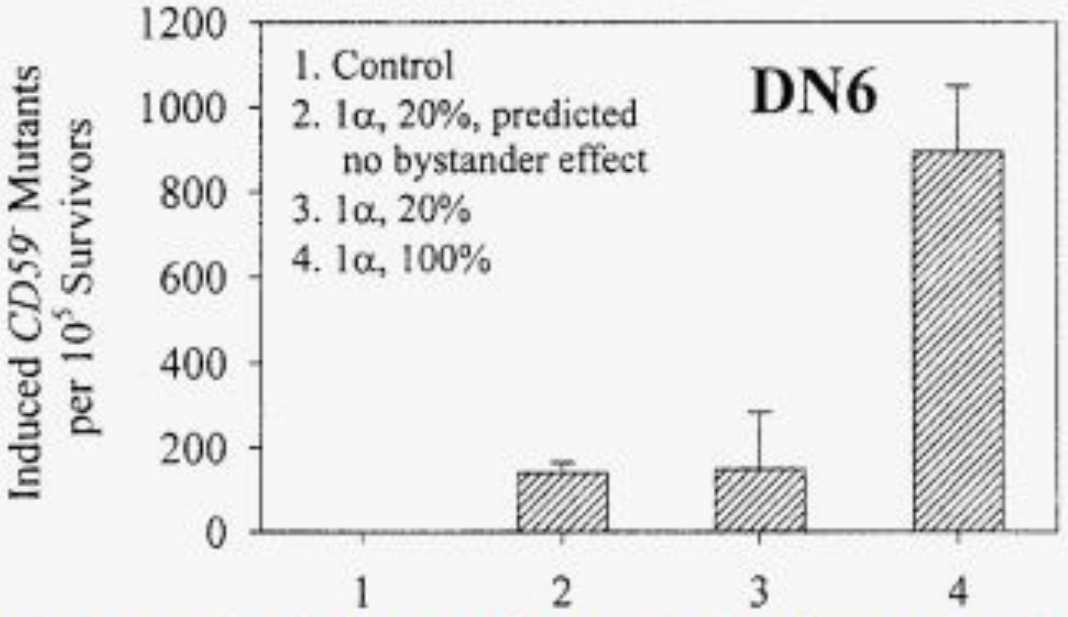


Fig. 4. Mutation fraction (M_F) from population of A_L-AH1-9 cells transfected with a dominant negative connexin 43 vector (DN6). Error bars represent ± SD. The population of AH1-9 cells used in these experiments have higher mutant induction as well as background mutant level than the parental A_L cells (redrawn from the data of Zhou et al. 2001).

- Oncogenic transformation in mouse fibroblasts Mouse fibroblast (C3H10T1⁄2) cells were plated in a monolayer, and the computer was programmed to irradiate either every cell, or every tenth cell, selected at random with 1–8 alpha particles directed at the centroid (Sawant et al. 2001) of the cell nucleus. The cells were subsequently removed by trypsinization, replated at low density, and transformed foci were identified 6 wk later by their morphologic appearance. The results are shown in Fig. 5 and illustrate that (1) more cells can be inactivated by alpha particles than were actually traversed by an alpha particle and (2) when 10% of the cells on a dish are exposed to two or more alpha particles, the resulting frequency of induced oncogenic transformation is indistinguishable from that when all the cells on the dish are exposed to the same number of alpha particles.

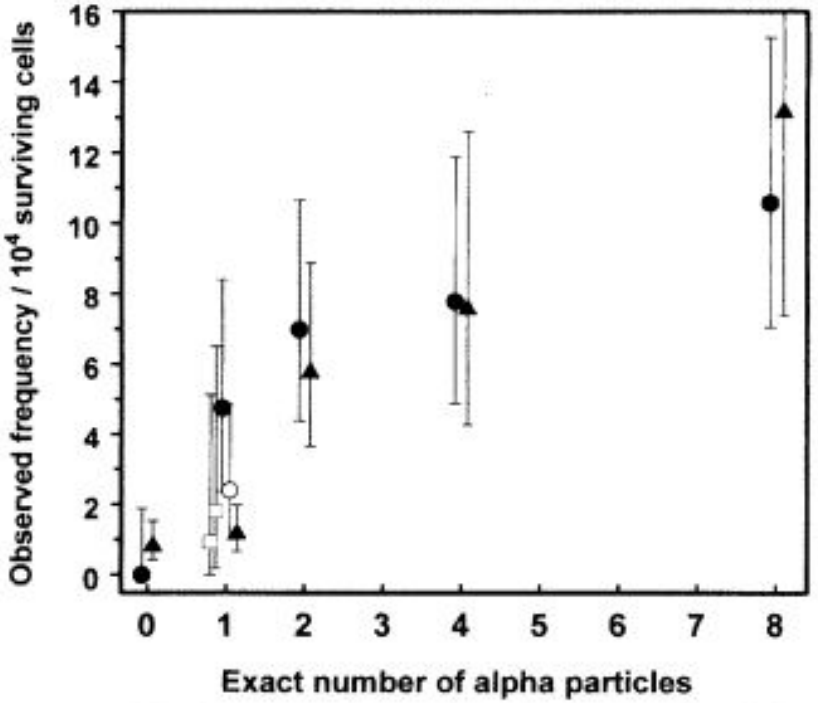


Fig. 5. Yield of oncogenically transformed cells per 10^4 surviving C3H10T½ cells produced by nuclear traversals by 5.3 MeV alpha particles. Triangles represent exposure of all cell nuclei on each dish to exact numbers of alpha particles using the microbeam system. Solid circles represent exposure of 1–10 cell nuclei on each dish to exact numbers of alpha particles. Open squares represent subsequent repeats of the experiment in which 1–10 cell nuclei were exposed to exactly one alpha particle. Open circle represents combined data for all the experiments in which 1–10 cell nuclei were exposed to one alpha particle including these repeat experiments (with caveats described in the text). Standard errors (± SD) were estimated assuming an underlying Poisson-distributed number of transformed cells (26) (redrawn from the data of Sawant et al. 2001).

- It is important to note that the experimental results discussed in this paper involve laboratory model systems, since bystander experiments with in vivo systems, particularly in the human, are clearly not possible at the present time. However, if these results were applicable in vivo, they could have significant consequences in terms of extrapolation of radiation risks from high to low doses, implying that the relevant target for radiation oncogenesis is larger than an individual cell, and that the risk of carcinogenesis would increase more slowly, if at all, at intermediate doses. Thus a simple linear extrapolation of radiation risk from intermediate doses (where they can be measured) to lower doses (where they must be inferred) would be of questionable validity, at least at high-LET.

- This is illustrated in Fig. 6 which combines the data of Zhou et al. (2001), in which only a proportion of cells are irradiated with a single particle (allowing the bystander effect to be manifest), together with a previous compilation of data by Zhou et al. (2000) where all cells were exposed to various numbers of particles from 1–4.

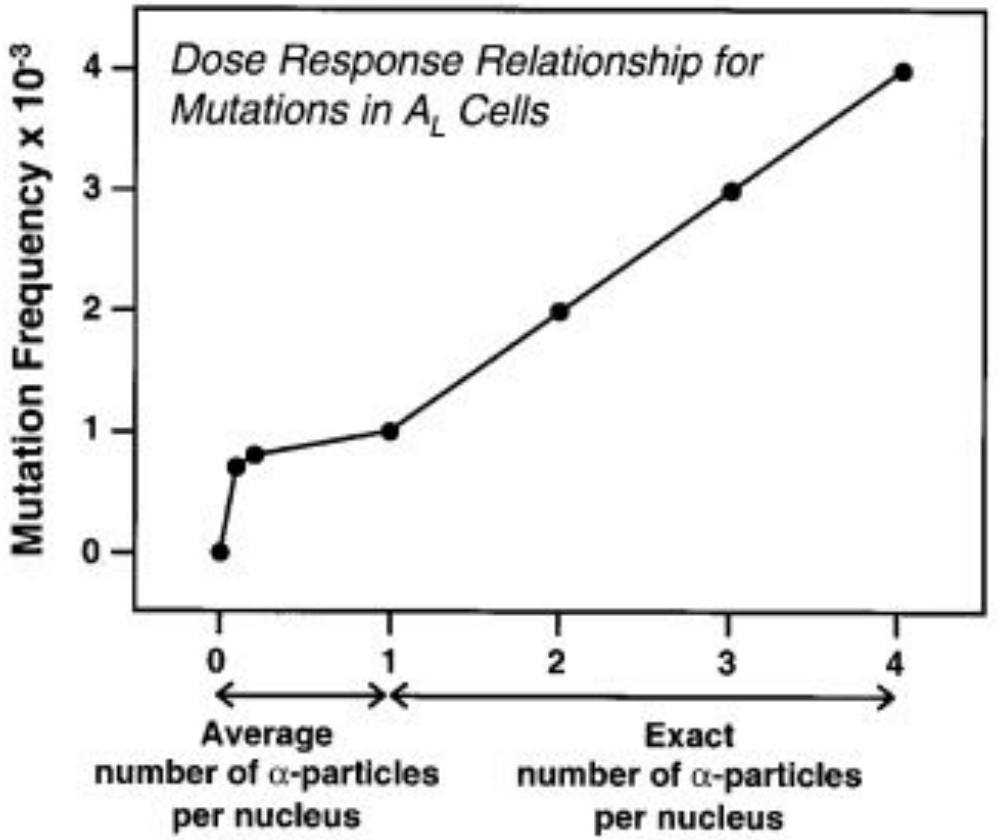
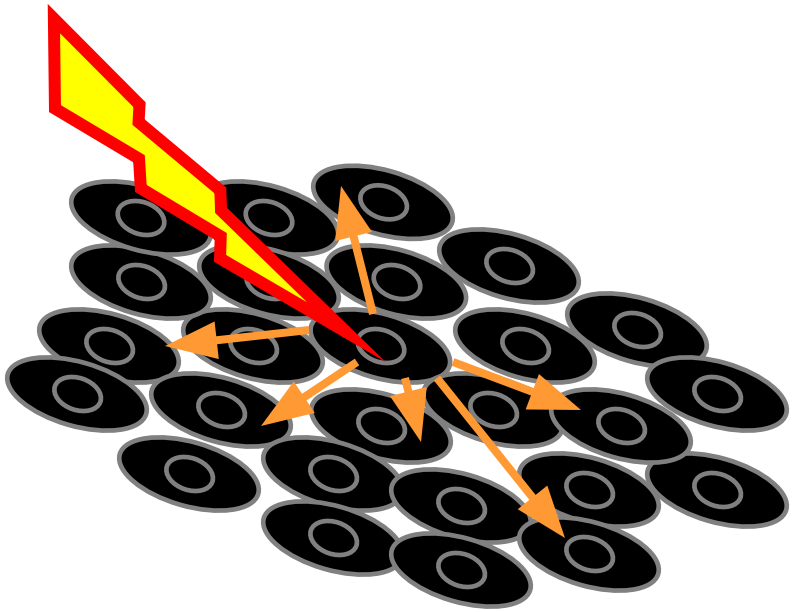


Fig. 6. Mutation frequency as a function of the number of alpha particles per nucleus (data fractions of the cell population were exposed to a single alpha particle). Due to the bystander effect which is evident when only a proportion of the population is exposed, the risk at low doses is higher than predicted by a linear extrapolation from high doses (based on the data of Zhou et al. 2000, 2001).

- Under these experimental conditions, it is evident that a linear extrapolation of risks from high doses to low doses (which average less than one particle per cell) would underestimate the risks at low doses. This applies, at this stage, strictly to alpha particles, and it is not known whether it would apply in an in vivo situation to, for example, radon exposure in homes and mines.

BYSTANDER EFFECT

Ionizing radiation

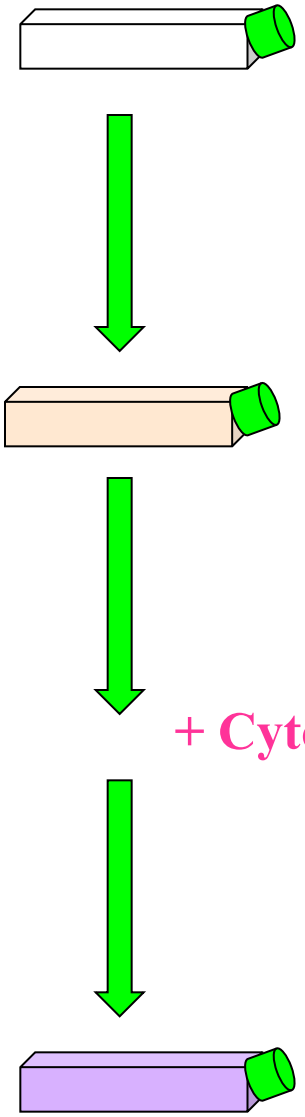


Radiated cell

BE factor migration (signal)

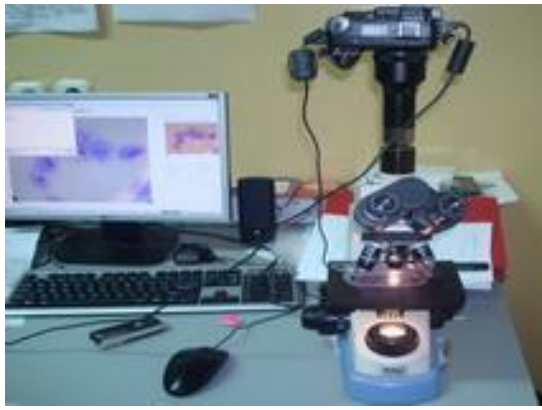
Effects in non - radiated cells

Scheme of BE induced *in vivo* (MN test)



6000 cells seeded in 24 cm^2 flasks

Flasks are incubated for 1-2 days at 37°C

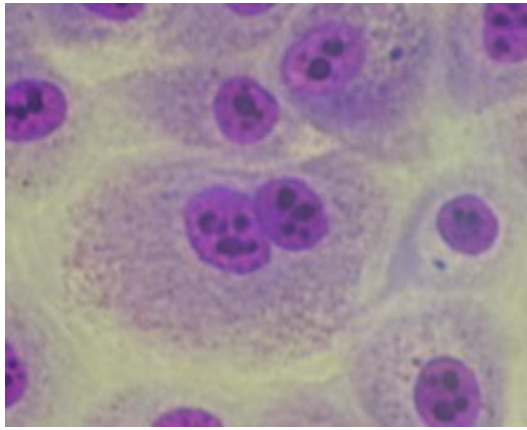


Blood serum samples from affected by the Chernobyl accident populations are added

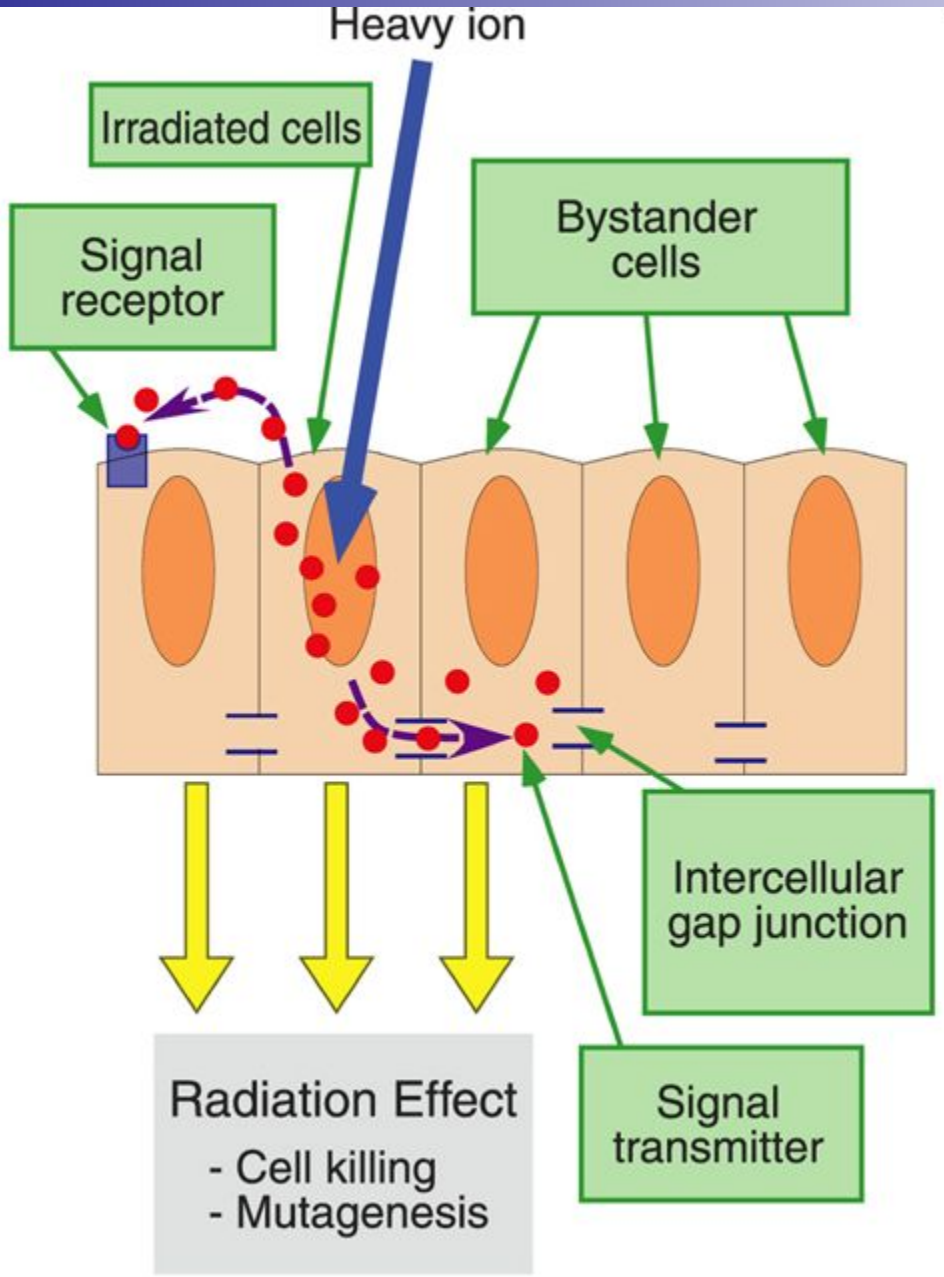
Flasks are incubated for 2 hours at 37°C

+ Cytochalasin B

Cells incubated for 24 hours at 37°C



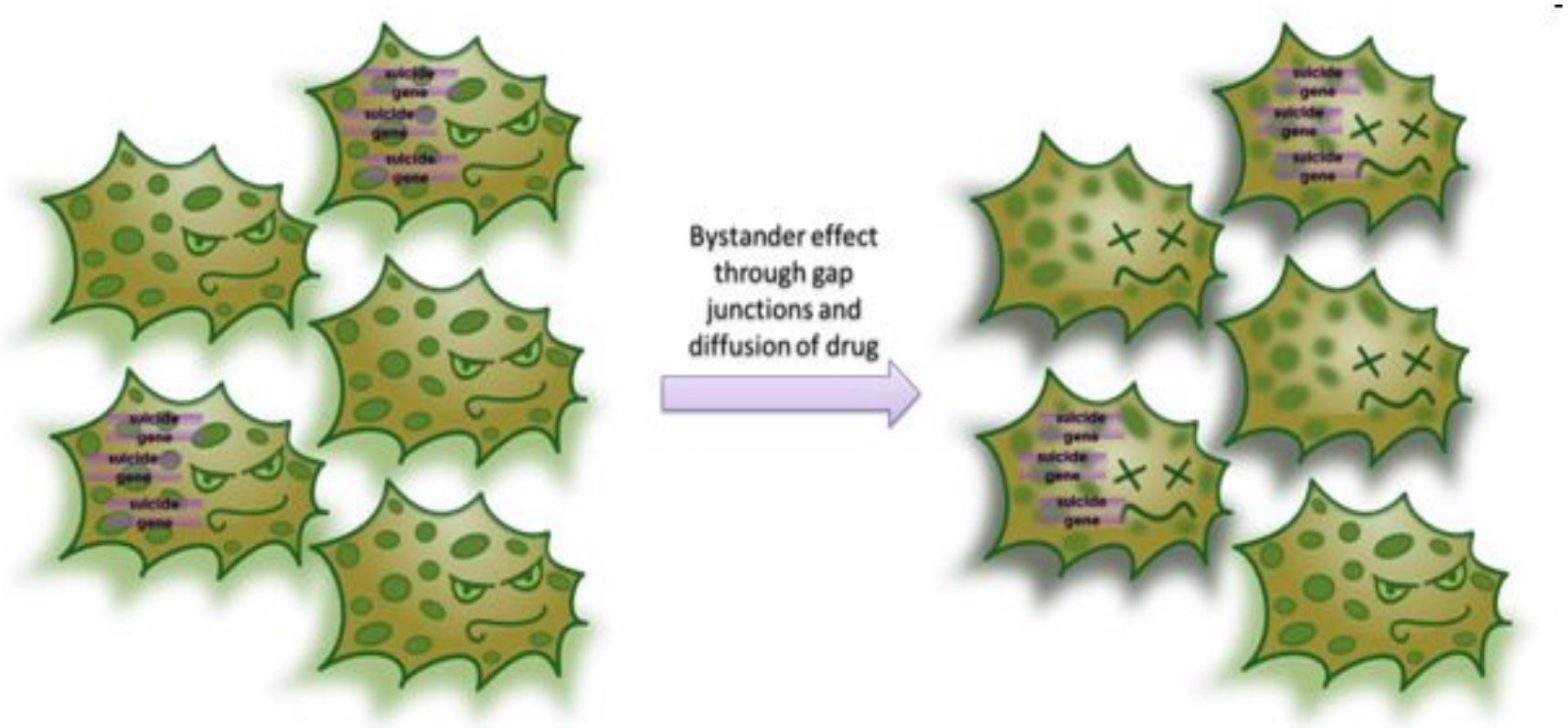
Cells washed in PBS, fixed and stained with Giemsa solution


Heavy ion
Irradiated cells
Signal receptor
Bystander cells
Intercellular gap junction
Signal transmitter
Radiation Effect
- Cell killing
- Mutagenesis

Killing Non-transduced Tumor Cells via Bystander Effect

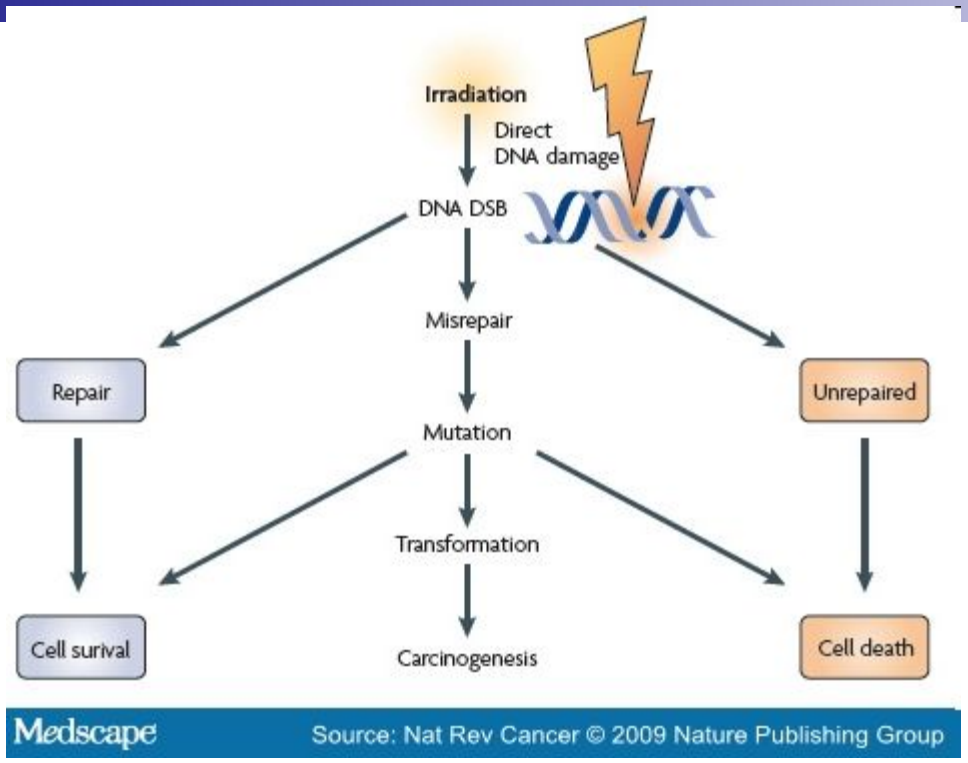
- The bystander effect was first reported by Moolten (1986) showing that prodrug convertase negative cells surrounded by suicide enzyme positive cells did not survive prodrug treatment. Besides efficient killing of targeted tumor cells, neighboring, non-transduced cells are killed as well, providing an important effect in cancer treatment. Since 5-Fluorouracil is soluble and can diffuse into adjacent cells (Huber et al. 1993) (Huber et al. 1994), the bystander effect was demonstrated using cytosine deaminase as gene of interest.

Schematic overview of the Bystander effect



- Our understanding of how radiation kills normal and tumour cells has been based on an intimate knowledge of the direct induction of DNA damage and its cellular consequences. What has become clear is that, as well as responses to direct DNA damage, cell-cell signalling — known as the bystander effect — mediated through gap junctions and inflammatory responses may have an important role in the response of cells and tissues to radiation exposure and also chemotherapy agents. This Review outlines the key aspects of radiation-induced intercellular signalling and assesses its relevance for existing and future radiation-based therapies.

- When ionizing radiation interacts with biological material, energy is deposited and chemical bonds are broken. In cells, the basic components of proteins, lipids and nucleic acids can all be damaged. However, a key consequence is that direct damage occurs to DNA within the nucleus, producing a range of lesions of which DNA double strand breaks (DSBs) have a pivotal role in determining whether cells survive radiation exposure.[1] If DNA damage is not correctly repaired two direct consequences can occur. Residual or unrepaired damage leads directly to chromosomal aberrations, loss of genetic material and cell death. Also, unrepaired or incorrectly repaired (misrepaired) damage can lead to mutations that might result in carcinogenesis or cell death (Figure 1).



Direct DNA damage radiation model. The schematic shows the standard model of DNA damage responses to radiation in biological systems, with direct DNA damage having a central role and the production of DNA double strand breaks (DSBs) leading to downstream biological consequences. Cells have complex pathways for sensing DNA damage and correctly repairing the DNA damage to survive the radiation exposure. If the DNA damage is not repaired, there is a high probability of cell death. DNA damage that is misrepaired can lead to mutations, increasing the probability of transformation and carcinogenesis.

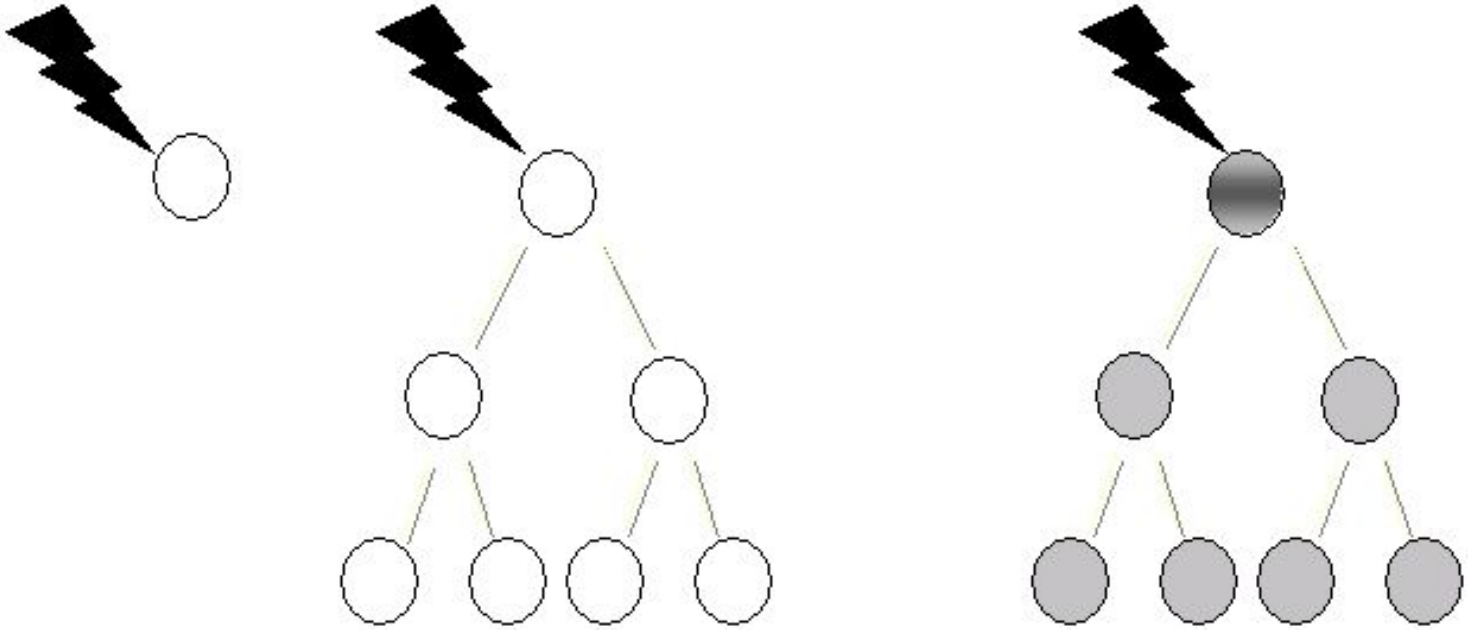
- The mechanisms underpinning DNA damage and repair processing in irradiated cells have been extensively studied since the discovery of DNA as the genetic material by Watson and Crick over 50 years ago. This has included exhaustive study of the DNA damage sensing and signalling pathways underpinning the DNA damage response that is present in cellular systems.[2] What is clear from these efforts is that cells have multiple and complex processes for sensing and repairing changes to their genomes to enable future propagation and stability. A series of sensor, transducer and effector proteins give cells important choices in response to radiation-induced DNA damage, such as DNA repair, cell cycle delay and cell death (apoptosis).[3]

- Evidence now shows that, as well as these direct DNA damage-dependent effects, irradiated cells also send signals to their neighbours. These non-irradiated cells respond to signals produced by neighbouring irradiated cells by what has been termed a bystander effect (extensively reviewed in Refs[4-7]). The term bystander effect is not new and has been observed in response to a range of other insults including ultraviolet radiation,[8] photodynamic therapy,[9] heat[10] and chemotherapy agents.[11] Its observation in response to chemotherapy agents underpins its considerable importance in gene therapy regimens, in which not all tumour cells are targeted and indirect killing of non-targeted cells is required to ensure maximal tumour cell kill.[12]

- For example, the archetypal gene therapy model is the herpes simplex virus-thymidine kinase (HSV-TK) system. In this system the HSV-TK gene is transfected into cells and these are incubated with the non-toxic agent ganciclovir, which is converted to a toxic analogue that diffuses and kills neighbouring cells.[13] This bystander effect involves direct cell-cell communication through gap junctional intercellular communication (GJIC) and requires expression and surface location of CX43 (also known as GJC1) gap junctions.[14] By contrast, the bystander effect mediated by the thymidine phosphorylase-5'-deoxy-5-fluorouridine suicide gene system involves a factor released into the medium that is independent of GJIC.[15] So, the paradigm of a bystander response after radiation treatment is not new in other fields; in essence it is a manifestation of intercellular signalling that is either specific or non-specific in its mode of action.

- Radiation-induced bystander responses have been observed in a range of cell types, tissue models and in vivo. Although the majority of the evidence for bystander effects has come from cellular studies, a range of other responses have been classified as bystander effects in the literature. In humans, in response to radiotherapy, longer-range effects occurring within or between tissues have also been reported and have been termed abscopal, out-of-field or distant bystander responses.[16] Radiation-induced bystander responses have been observed, not just from studies with external beam irradiation, but also from approaches using targeted radioisotopes. Several key questions emerge from these observations in terms of their relevance to cancer therapy. First, does an understanding of bystander mechanisms highlight new potential targets for cancer therapies and, if so, can this be modulated to either increase tumour cell killing or protect normal tissues? Second, do these bystander responses, especially after low-dose irradiation, contribute to increased carcinogenic risks associated with radiation exposure?

Targeted Effects of Ionizing Radiation



Cell death genetic

Damage repaired

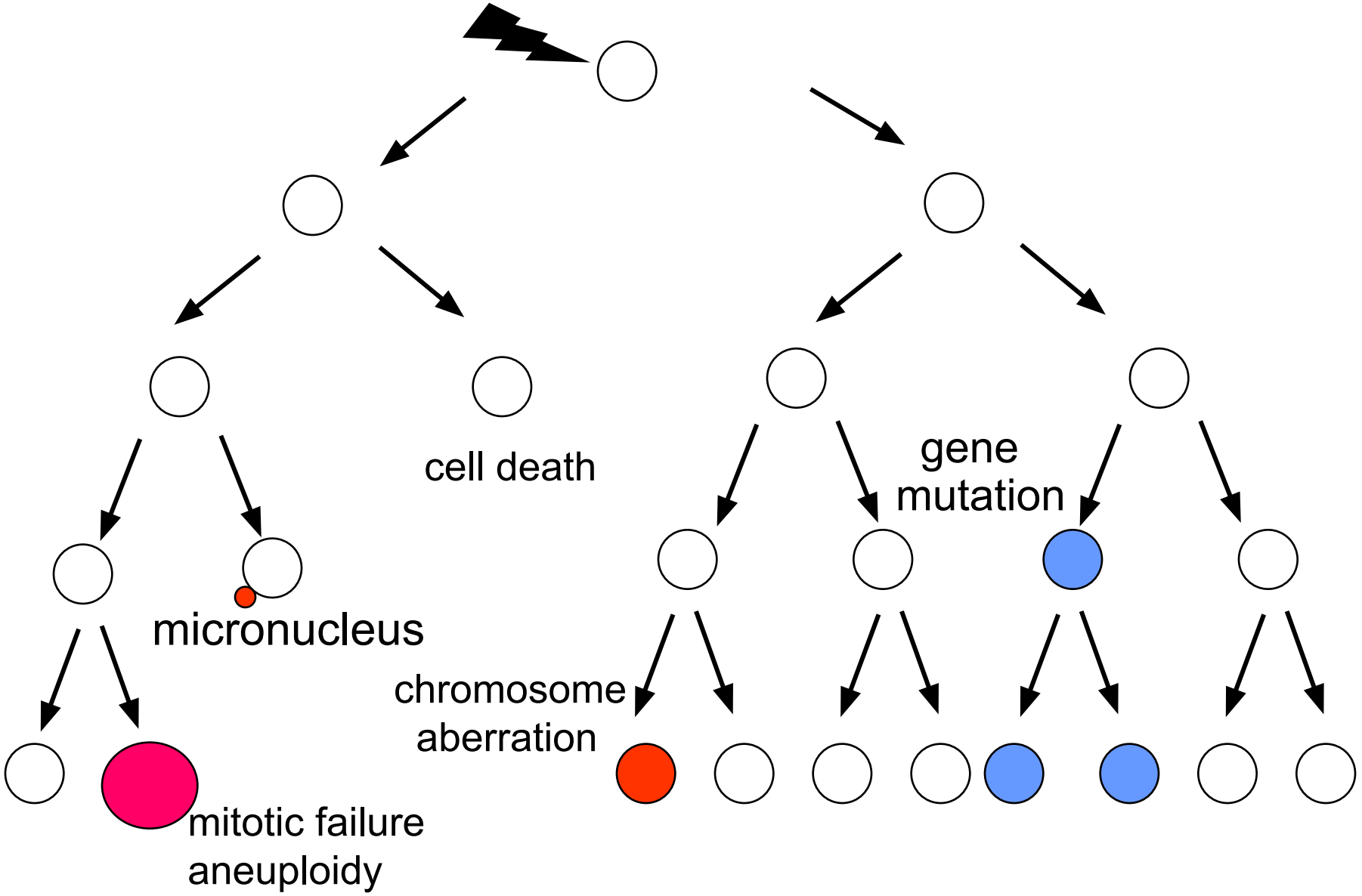
Induction of clonal alteration

Untargeted Effects of Exposure to Ionizing Radiation

Effects in unexposed cells and their progeny
i.e. in cells not directly hit.

Genomic instability **Bystander Effects**

Radiation-induced Genomic Instability



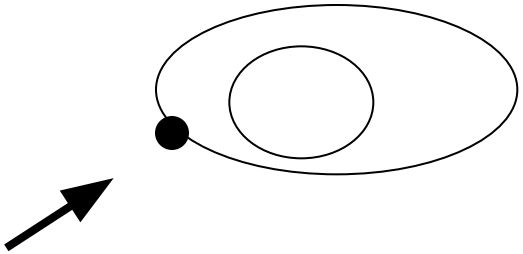
Radiation-induced Genomic Instability

- A genome-wide process induced at very high frequency
- High LET tends to be more effective inducer
- Genetic, morphological and functional abnormalities
- Persists over many cell generations (indefinitely ?)
- Not universally expressed
- Expression influenced by cell type & genetic factors
- Inter-individual variation in irradiated inbred mice
- Lesions tend to resemble “spontaneous” abnormalities
- Free radical-mediated mechanisms implicated

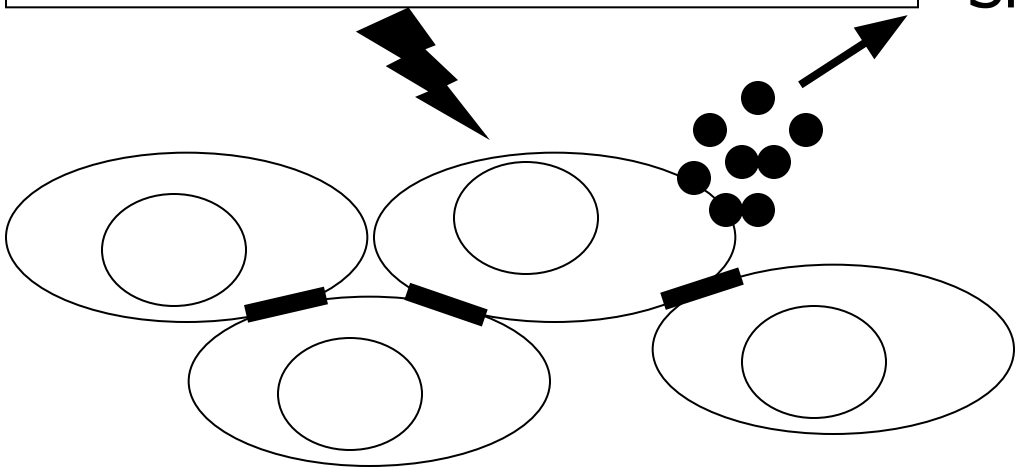
Bystander Effects of Ionizing Radiation

1990s:

- Effects in more cells than irradiated by α-particles
- Cytotoxic factor(s) after low dose low LET exposure



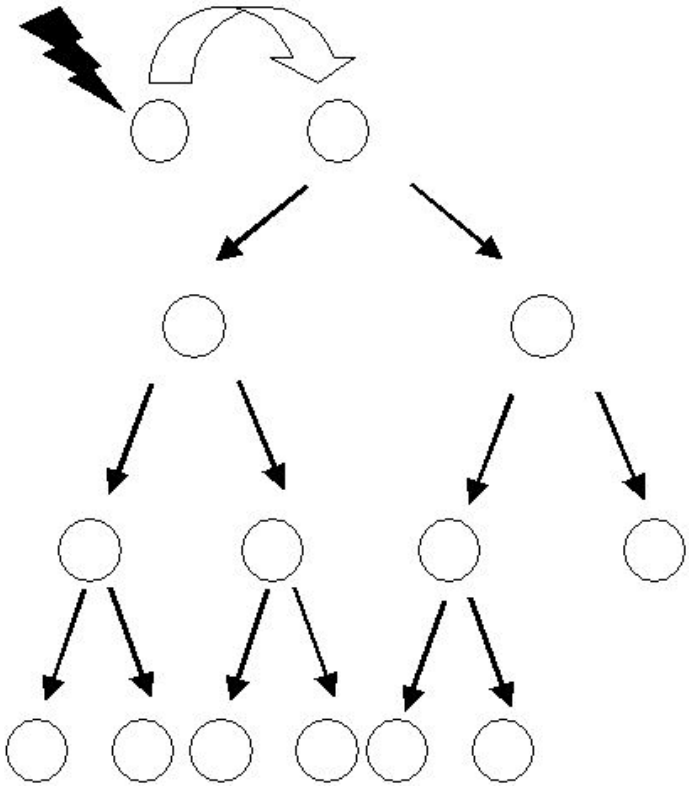
Signals via medium/plasma



Signals via gap junctions

N.B. 1950s and 60s
Reports of clastogenic factors in blood of exposed individuals

Bystander Effects of Ionizing Radiation



- Increases in damage-inducible proteins
- Decreases in damage-inducible proteins
- Increases in reactive oxygen species
- Decreases in reactive oxygen species
- Cell death
- Cell proliferation
- Mutations
- Chromosome aberrations
- 10T ½ transformation
- Chromosomal instability

Bystander Effects of Ionizing Radiation

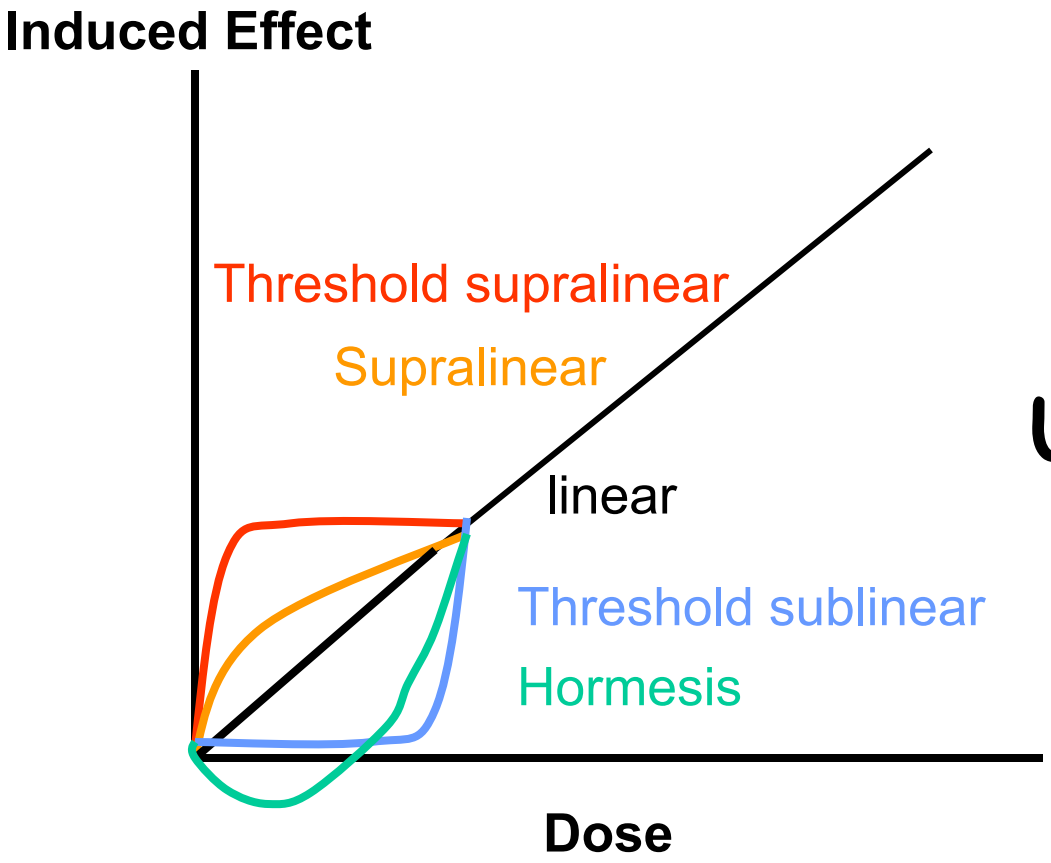
- Target for biological effects is larger than the cell
- Important implications for low dose effects

- At very low doses bystander effects may dominate overall response
- At higher doses targeted effects may dominate overall response

Potential for underestimation of low-dose risk extrapolated from intermediate/high doses?

The Linear No Threshold Problem

Non-targeted effects important at low doses



Increased risk?

⇧

Mutational changes

⇧

Untargeted Effects

⇩

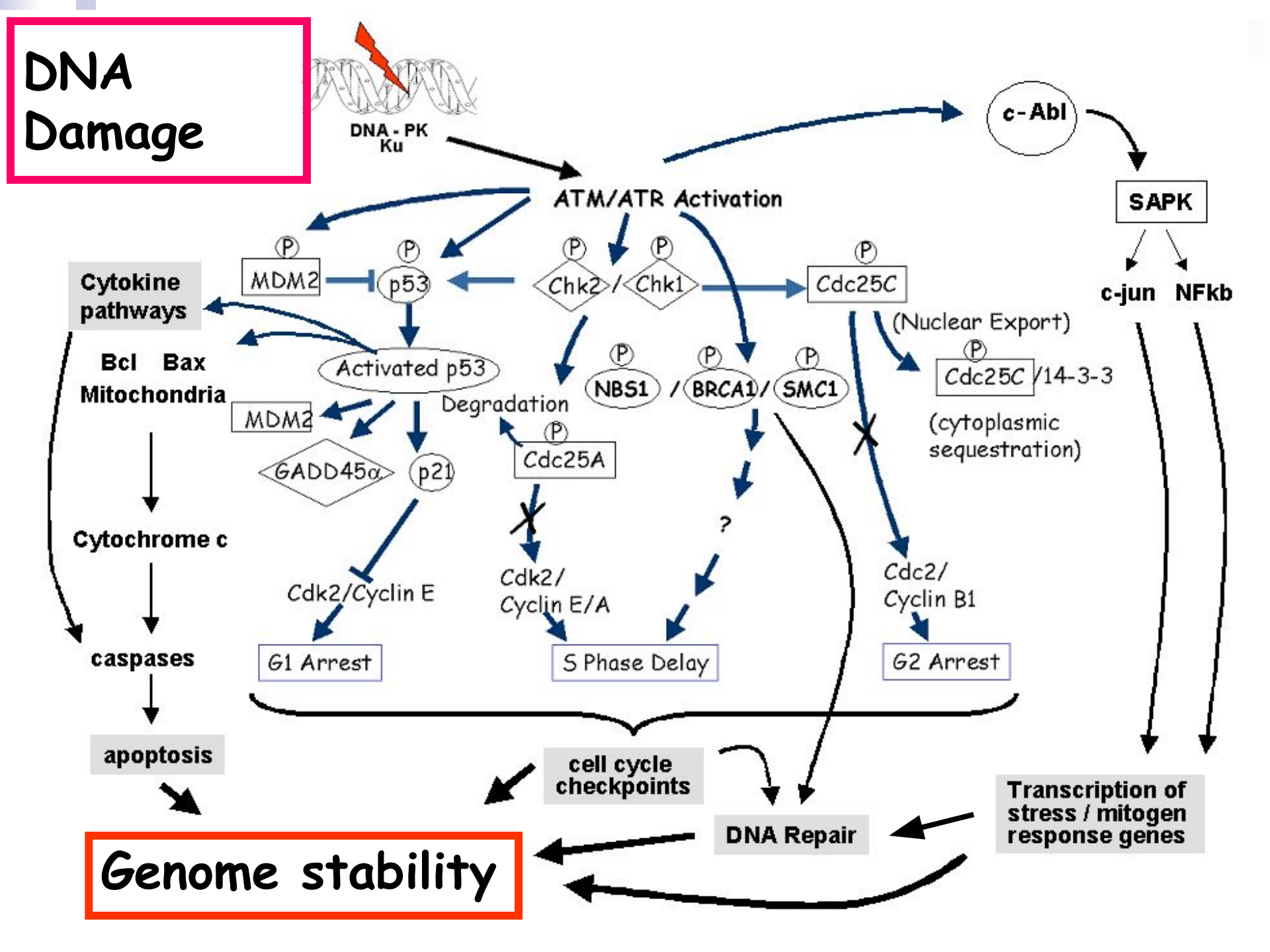
Cell death

⇩

Decreased risk?

Uncertainty at Low Doses

DNA Damage


DNA - PK
Ku
c-Abl
SAPK
ATM/ATR Activation
c-jun
NFkb
MDM2
p53
Chk2
Chk1
Cdc25C
Cytokine pathways
(Nuclear Export)
Bcl Bax
Mitochondria
Activated p53
NBS1
BRCA1
SMC1
Cdc25C /14-3-3
(cytoplasmic sequestration)
MDM2
Degradation
GADD45α
p21
Cdc25A
Cytochrome c
?
Cdk2/Cyclin E
Cdk2/
Cyclin E/A
Cdc2/
Cyclin B1
caspases
G1 Arrest
S Phase Delay
G2 Arrest
apoptosis
cell cycle checkpoints
DNA Repair
Transcription of stress / mitogen response genes
Genome stability

Untargeted Effects and Life/death Responses

Potential for increased risk

Potential for decreased risk

Cell Survival

Apoptosis

Repair/misrepair

ATM

DNA-PK

p53

Genetic factors

Growth arrest

p21

Bax