Introduction to epigenetic effects and ionising radiation

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Overview

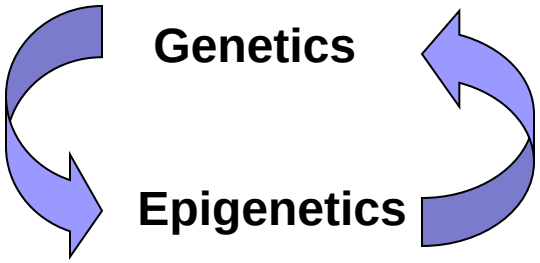
- Genetics & Epigenetics: definitions and interlink
- Radiation Response: Targeted & Non Targeted Effects (NTE)
- Main focus on NTE of Ionizing Radiation exposure
- Mechanisms: Epigenetics & NTE
- The role of Microvesicles /Exosomes in NTE
- Summary & Comments

Genetics *vs* Epigenetics:

- A big **difference** between **genetic** and **epigenetic** regulation is that **epigenetic** mechanisms **do** not involve a change to the DNA sequence, whereas **genetic** mechanisms involve the primary DNA sequence and changes or mutations to this sequence.

- "**Genetics**", conceptually, deals with **genes and gene function**, while "**epigenetics"** deals with **gene regulation**. More specifically, genetics focuses on how DNA *sequences* lead to changes in the cell/host, while "epigenetics" focuses on how DNA is *regulated* to achieve those changes.

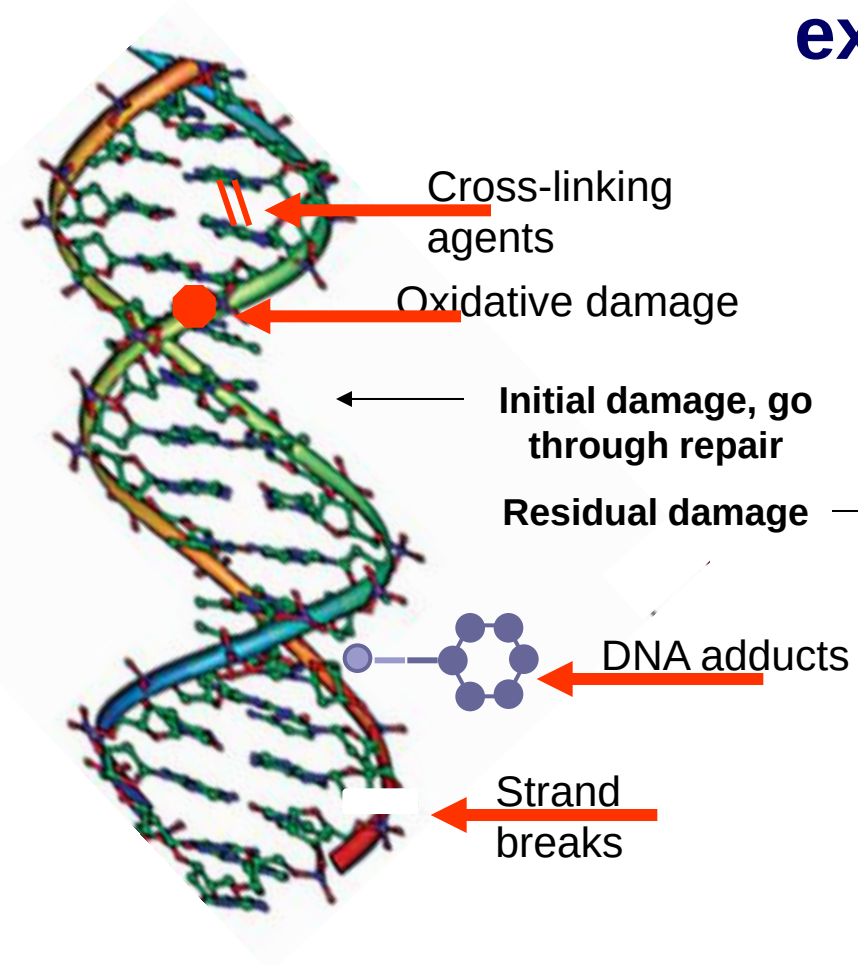
Genetics and Epigenetics



- DNA repair
  - Genetics – DNA repair enzymes
  - Epigenetics – Chromatin modifications that promote repair
- Cellular responses
  - Genetic damage, *e.g.* mutations, chromosomal change, etc..
  - Epigenetics – altered gene expression

Interactions between genetics and epigenetics (always present, but is it the same for radiation response ?)

Radiation response: consequences of radiation exposure



Mutations,
Chromosome Aberrations
Cell death

The focus is on genes and genetic damage, but what about epigenetics?

Radiation Response:

- **Targeted effects of Radiation:** it is a postulation that cells contain at least one critical site or target (mainly the DNA) that must be hit by radiation in order to kill a cell or produce an effect.

- **Non Targeted Effects of Radiation:** cell /tissue responses that does not require direct ionising radiation deposition in nuclear DNA to be expressed. These include:
  - ♦ Genomic Instability **(GI):** *de novo* genetic alterations in the progeny of irradiated cell
  - ♦ Bystander Effects **(BE) &** Abscopal Effects **(AE)** : radiation like effects in non irradiated cells/ tissue

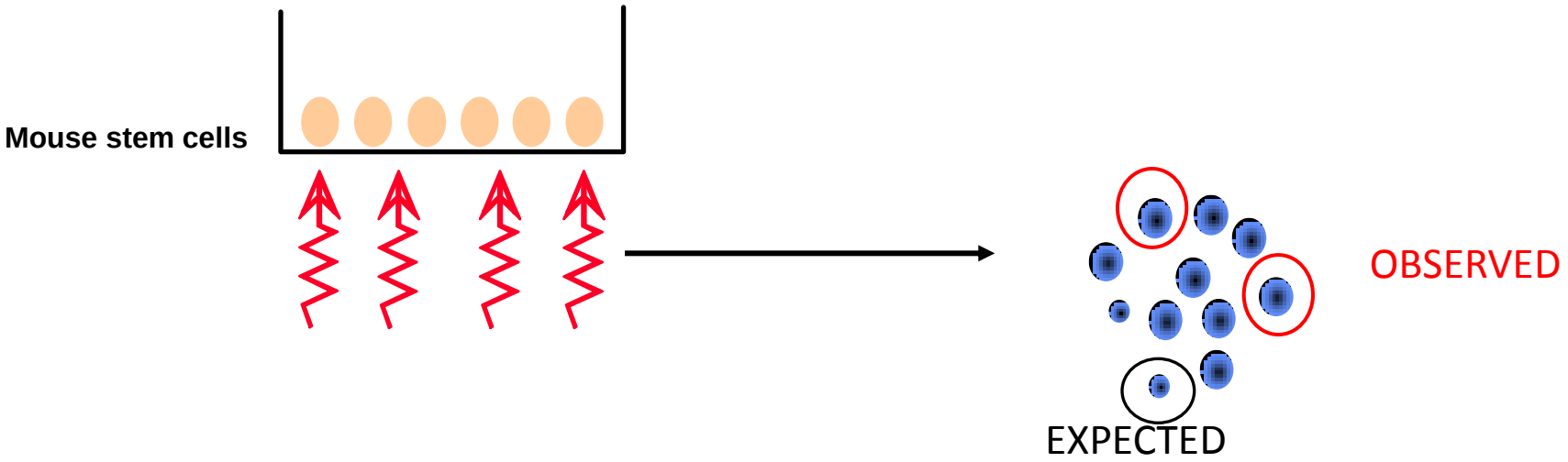
Genetics and epigenetics of cellular responses to targeted and NTE of radiation exposure

- Targeted effects involve both genetics and epigenetics

- NTE (progeny of irradiated cells & bystander cells / tissues) receive no direct radiation dose, so no DNA damage from radiation
  - Response is initiated through epigenetic mechanisms

Examples and Evidences

Chromosomal instability induced at high frequency which is inconsistent with mutation

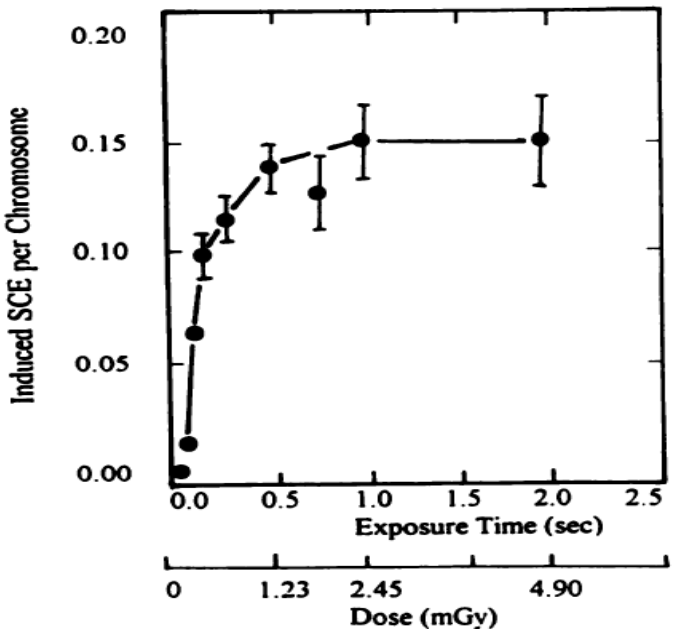


Dose (Gy)	Expected Chromosomal Aberrations/cell	Observed Chromosomal Aberrations / cell
0.25	0.055	0.400
0.5	0.105	0.579
1	0.200	0.608

Kadhim *et al*, Nature 355 (6362): 738-40

Sister chromatid exchange in non irradiated bystander cells

Sister chromatid exchange **frequency increases in 30% of cells even though 0.1% cells traversed**



Nagasawa & Little 1992

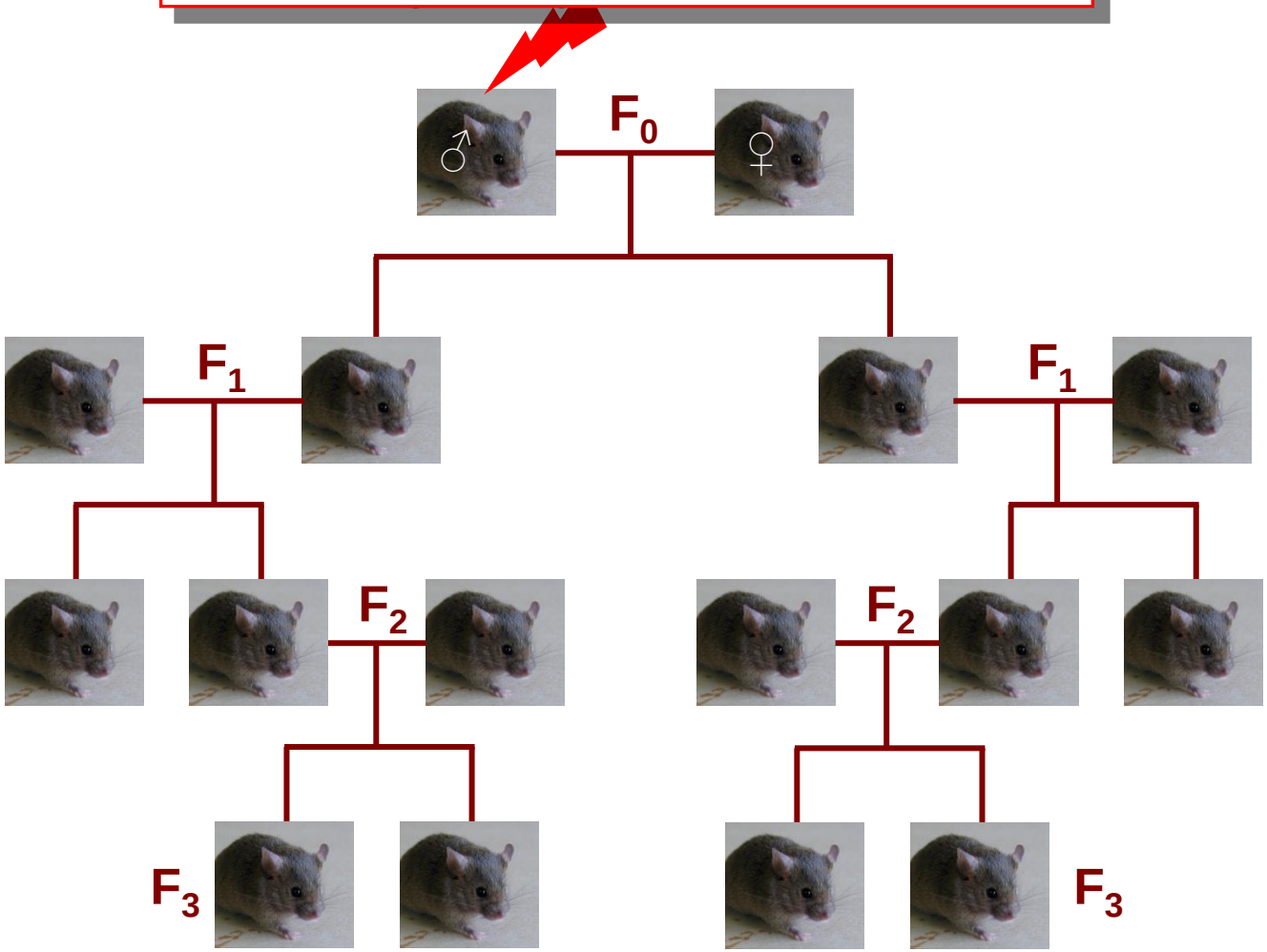
Transgenerational instability

CBA/H

BALB/c

C57BL/6J

Fission neutrons, 0.4 Gy: CBA/H; C57BL/6
Acute X-rays, 2 Gy: CBA/H
Acute X-rays, 1 Gy: BALB/c

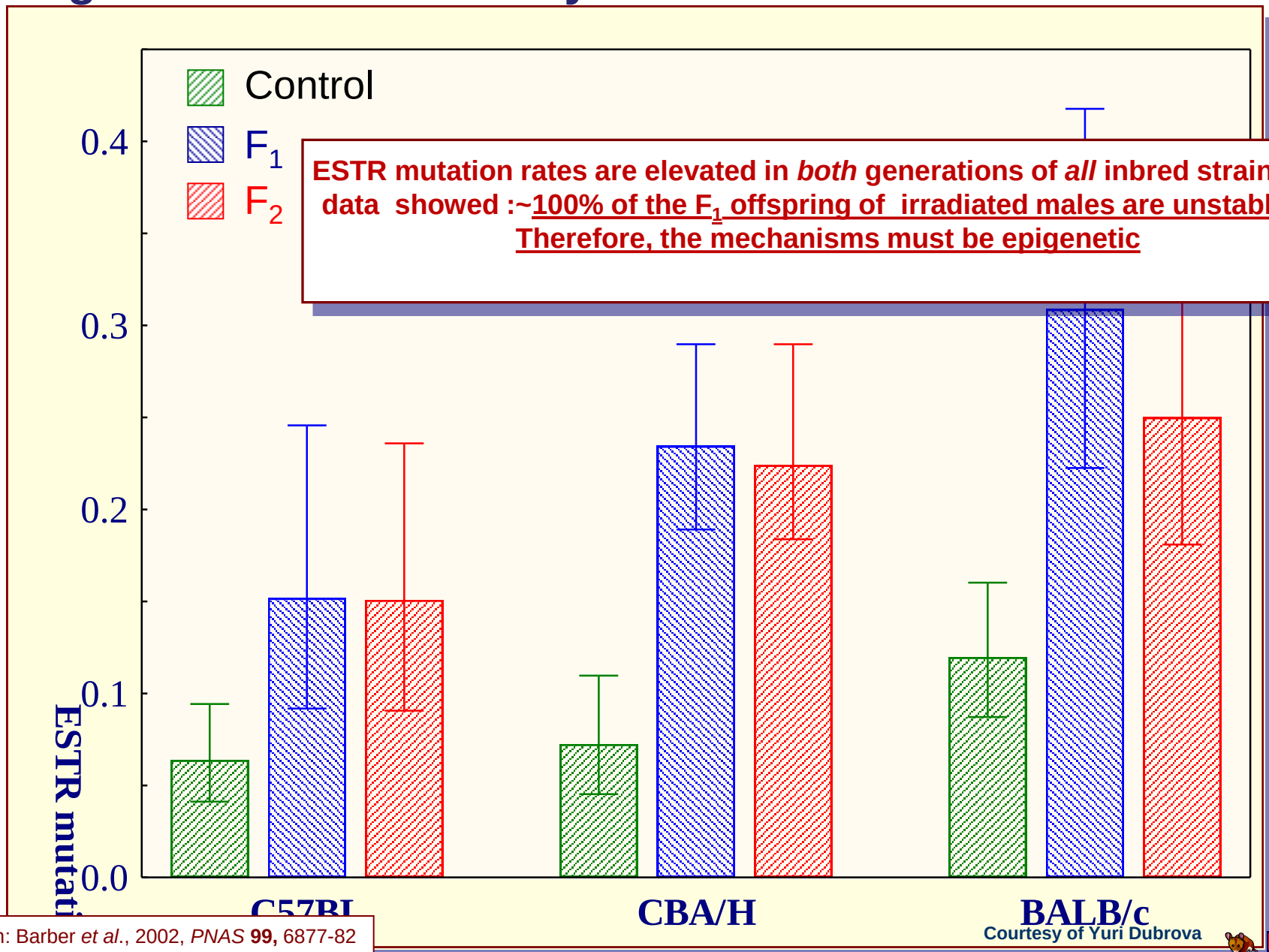


From: Barber *et al*., 2002, *PNAS* **99,** 6877-82

Courtesy of Yuri Dubrova

University of Leicester

Transgenerational instability in three inbred mouse strains



ESTR mutation rates are elevated in *both* generations of *all* inbred strains : data showed :~100% of the F_1 offspring of irradiated males are unstable. Therefore, the mechanisms must be epigenetic

From: Barber *et al*., 2002, *PNAS* **99,** 6877-82

Courtesy of Yuri Dubrova

Non- targeted effects of exposure to ionizing radiation (NTE): some features / principales

Recent Reviews : Kadhim et al, 2013; Morgan,2012; *Mothersill & Seymour 2012* ;Little et al, 2013; Butterworth et al, 2013, Kadhim& Hill *2015;Burtt et al, 2016*

1- NTE does not require direct ionizing radiation deposition in nuclear DNA to be expressed.

2- NTEs are predominantly low dose effects (≤ 0.1 Sv) and typically have non-linear dose-response relationships.

3- NTE is not universally expressed due to influencing factors (e.g. genetic predisposition, cell / tissue type, radiation dose & quality).

4- NTE response is Non-clonal aberrations & heterogeneity within populations and clones.

5- **NTE induced at higher frequency than expected for mutation in a single gene : Epigenetic mechanism.**

6-NTEs do not contradict ‘‘target theory’’ but contribute to a concept of an ‘‘expanding target’’ related to underlying biological signalling triggered by physical dose deposition, for example:

- ***GI*** *increases the target size* ***temporally*** *by prolongation of effect over many cell generations or transgenerationally*
- ***BE*** *increases the target size* ***spatially*** *to a group of cells, the tissue, or whole organism*

7- Transmission of information is NOT one-way and biological functionality is multi-level.

NTE : Epigenetic Mechanisms

Chromatin-associated changes

DNA methylation	Chromatin remodelling	Histone post-translational modification	Non-coding RNA (ncRNA) modulatio
Genome-wide hypomethylation post-irradiation	**ATP-dependent chromatin remodelling?**	**Methylation Acetylation Phosphorylation Ubiquitination**	**miRNA up and down-regulation**

Radiation

Hypomethylation:
Whole body irradiation
Pogribny *et al.* 2005;
Koturbash *et al.* 2006, 2007, 2008; Ilnytskyy *et al.* 2009
Bystander populations
Koturbash *et al.* 2006, 2007, 2008; Ilnytskyy *et al.* 2009

Radio-sensitivity:
Roy *et al.* 2006
Jin-Han Bae,et al,2015
Transgenerational:
Dubrova *et al.* 2000, 2003;
Barber *et al.* 2006

Reviews:
Ma *et al.* 2010;
Cedric R. Clapier, et al, 2017

Methylation
Pogribny *et al.* 2005
Koturbash *et al.* 2007
Ilnytskyy *et al.* 2009

Acetylation
J. Ren &, B. Li, 2017

Phosphorylation
Li L, et al, 2014

Ubiquitination
UV – monoubiquitination
(Bergnick *et al.* 2006)

miRNA
Koturbash *et al.* 2007
Koturbash *et al.* 2008
Chaudhry *et al.* 2010
Babenko et al. 2012

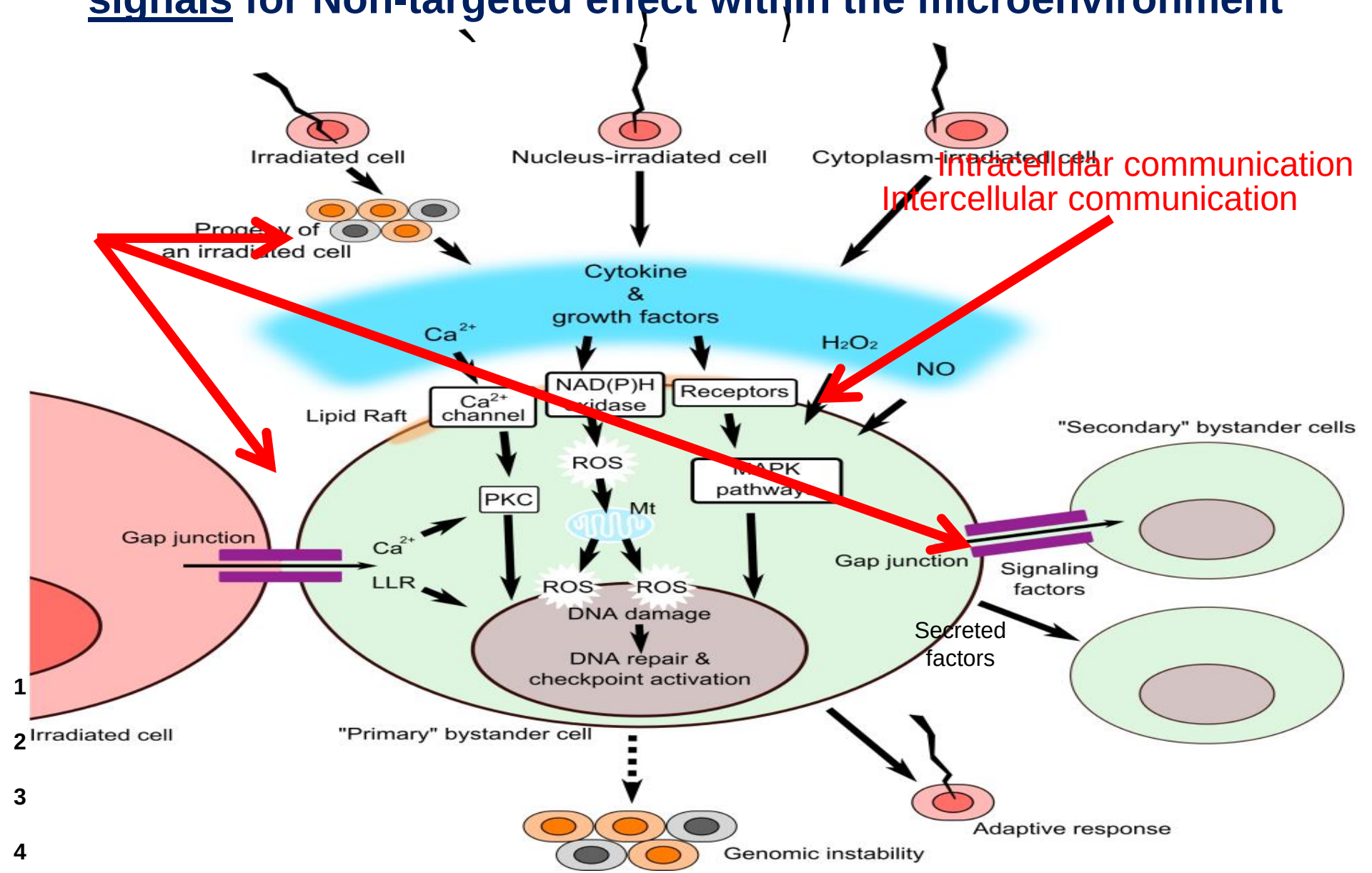
Epigenetics Mechanisms of NTE

- Our understanding of epigenetics of NTE is rapidly expanding but far from complete.

- A relevant example is the role of Microvesicles / exosomes in NTE through communicating the radiation bystander effect to naïve unirradiated cells & their progeny *(Al- Mayah et al, 2012,2015,2017 ; Jella, et al.2014;* Michelle Le, et al, 2017*).*

Role of Exosomes/MVs as secreted diffusible factors in Radiation Induced NTE (GI&BE)

Proposed model for the spatiotemporal propagation of radiation signals for Non-targeted effect within the microenvironment



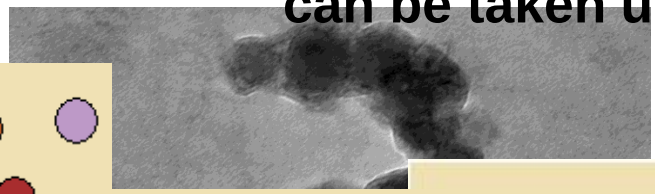
Hamada *et al*., Curr Mol Pharmacol (2011)

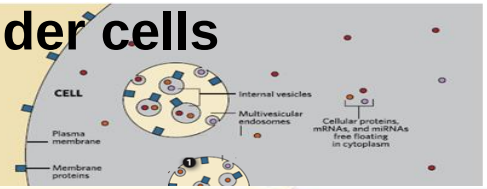
NTE Mediated Signals Molecules: Signaling within the microenvironment

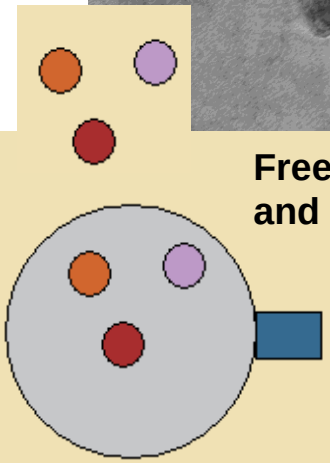
Bystander mediator	Inhibitor	Effect upon BE induction	Reference
ROS	N-acetylcysteine (NAC)	Prevention of growth arrest	(Macip et al. 2002)
Cytokines i.e. TNF-α	Anti-sense oligonucleotides	Reduction in radiation-induced apoptosis	(M. Zhang et al. 2008)
Mitochondria	DNA depletion	Reduced γ-H2AX induction	(Chen et al. 2008)
Gap-junctions	Lindane/Octanol	Reduced p53 modulation/reduced mutagenesis	(Zhou et al. 2001; Azzam et al. 1998)
COX-2	NS-398	Reduced DNA damage	(Zhou et al. 2005)
Calcium	Calcicludine	Prevention of micronuclei induction	(Shao et al. 2006b)
Extracellular vesicles/ Exosomes	RNase A & heat (protein)	Abrogation of DNA damage mediation via an RNA/ Protein dependent mechanism	(Al-Mayah et al. 2012,2015; Jella et al, 2014, O'Leary et al. 2015)

EXOSOMES

Secreted from irradiated cells to the extra cellular environment & can be taken up by recipient bystander cells



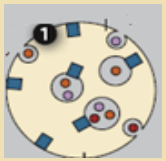


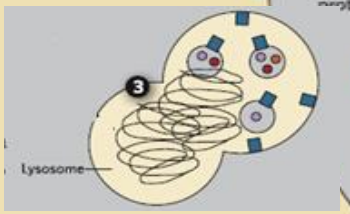


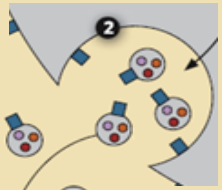
Free floating proteins and m/miRNA

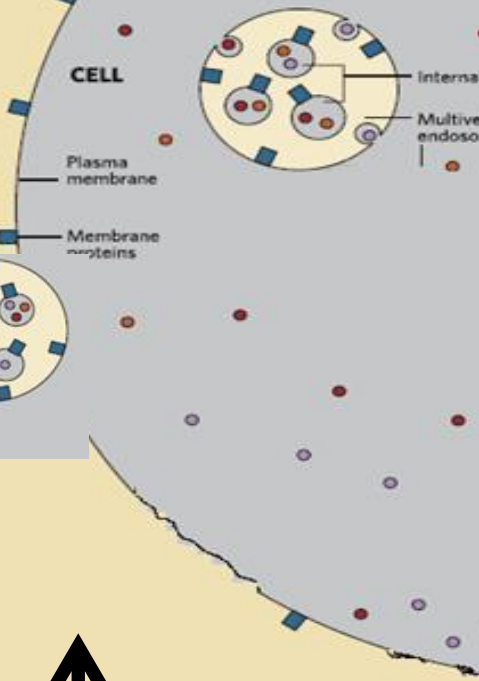
Exosome

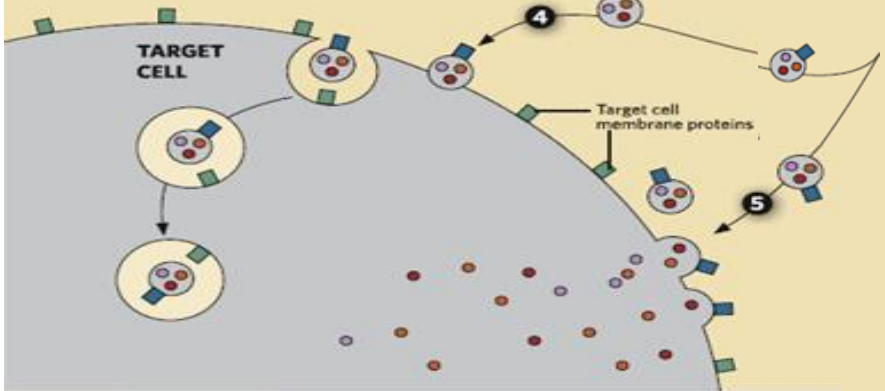
Bystander cell









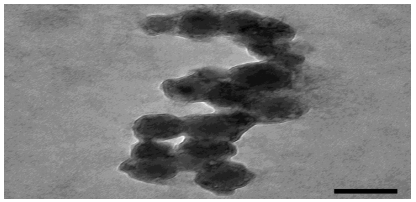


Exosome

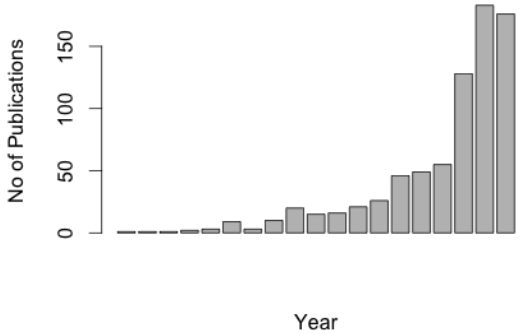
Irradiated cell

EV / Exosome : a fast growing field

Exponential Growth in scientific output **specially in cancer relevant studies** & highlighted the exosomes implication in both physiological and pathological processes.

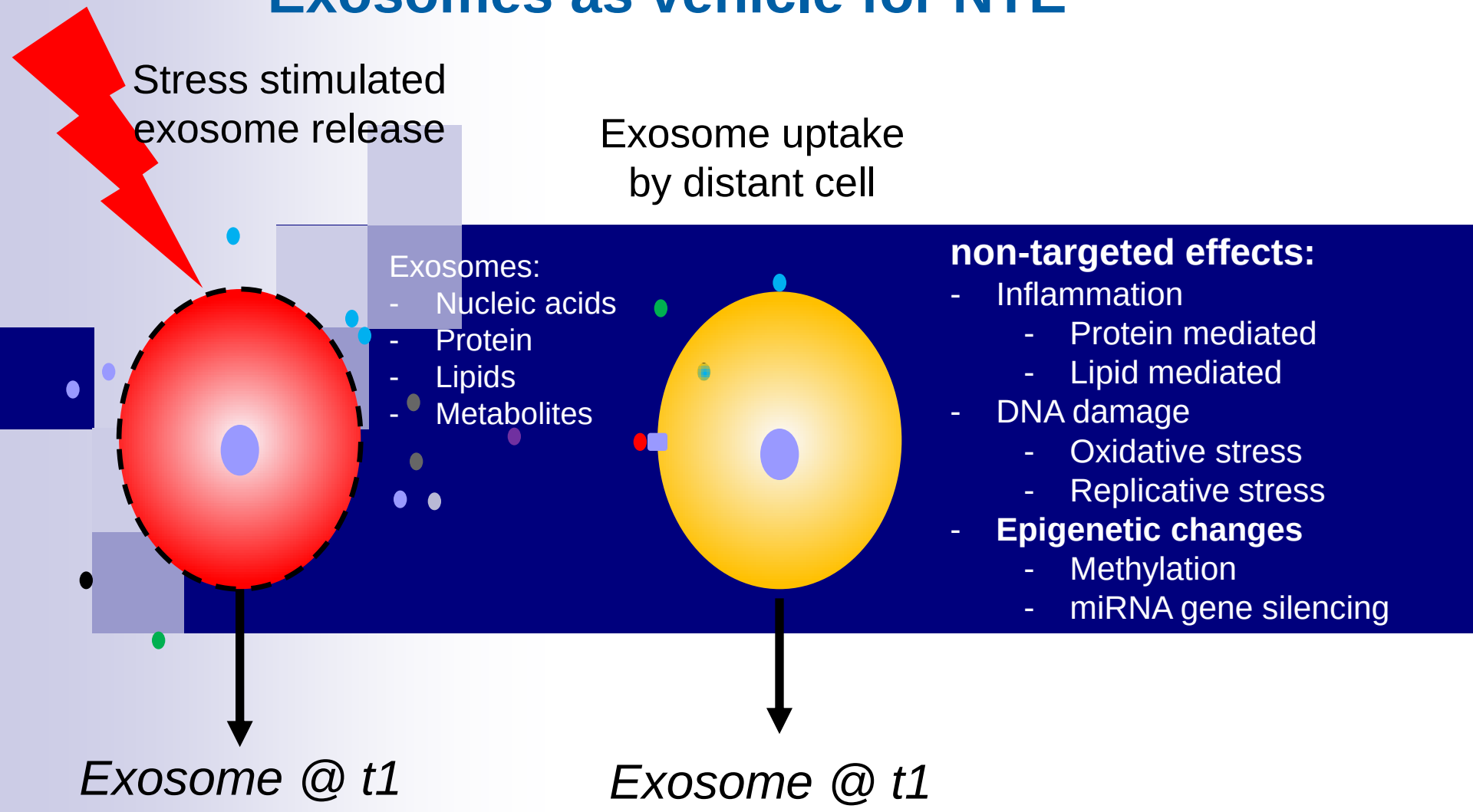


Over 500 Research groups worldwide



However,
far fewer studies pertain to the effects of radiation on cellular release and uptake mechanisms of exosomes and their **role in radiation exposure especially in targeted and non targeted effects (NTE) of ionizing radiation.**

Exosomes as vehicle for NTE



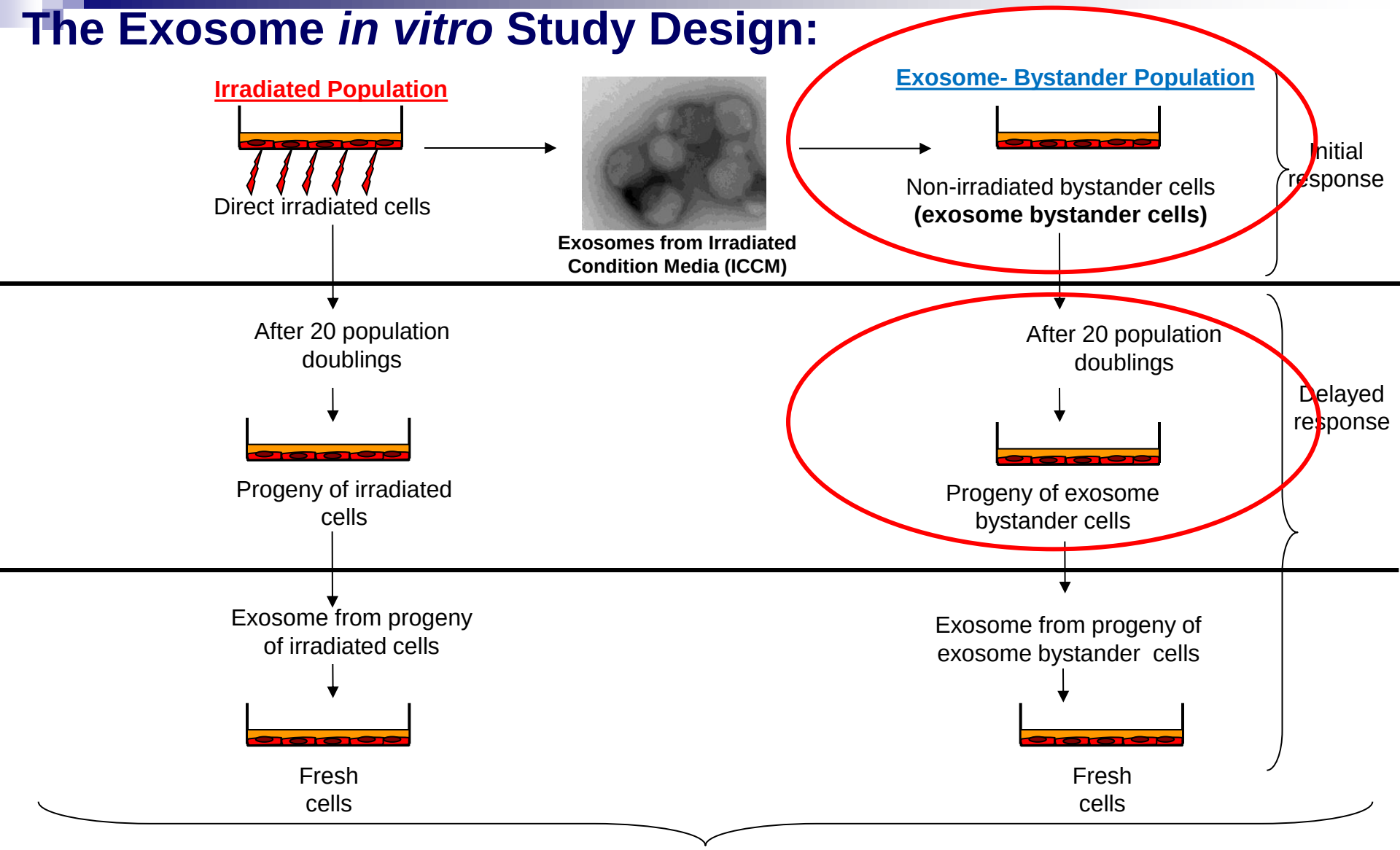
Of particular interest in relation to NTE is how exosome profile responds over time post exposure

Bright *et al,* in preparation

In Vitro Experimental design

We used Breast cancer cells in the following experiments

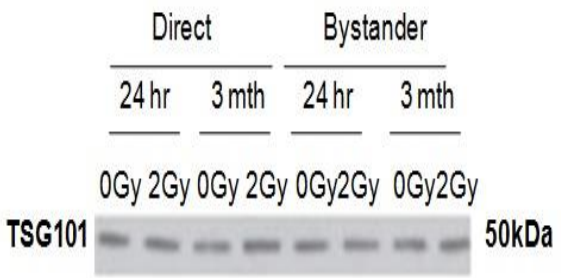
The Exosome *in vitro* Study Design:

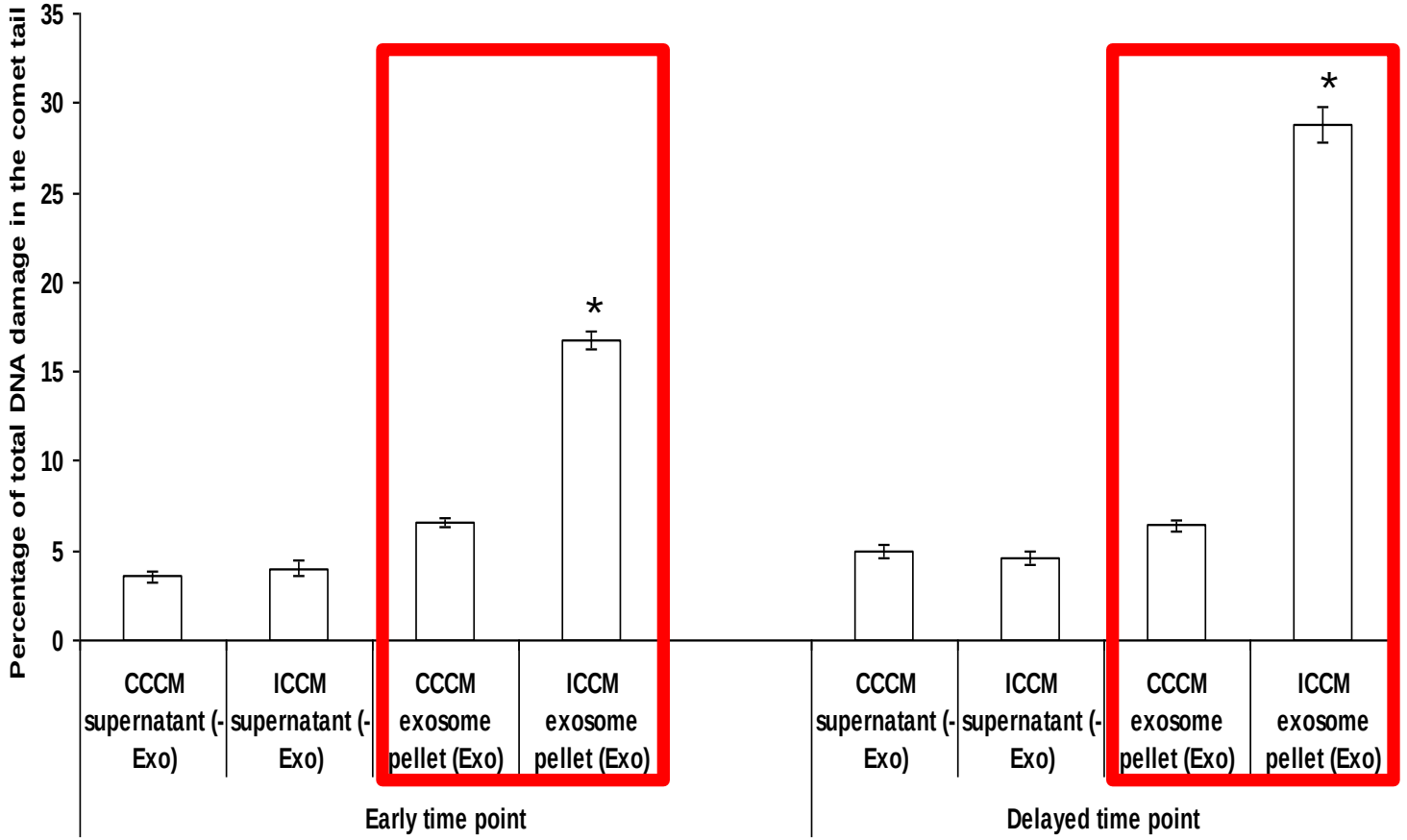


Relevant biological end points analysis including DNA damage, Chromosomal and Telomere instability

Al-Mayah et al., 2012, 2015

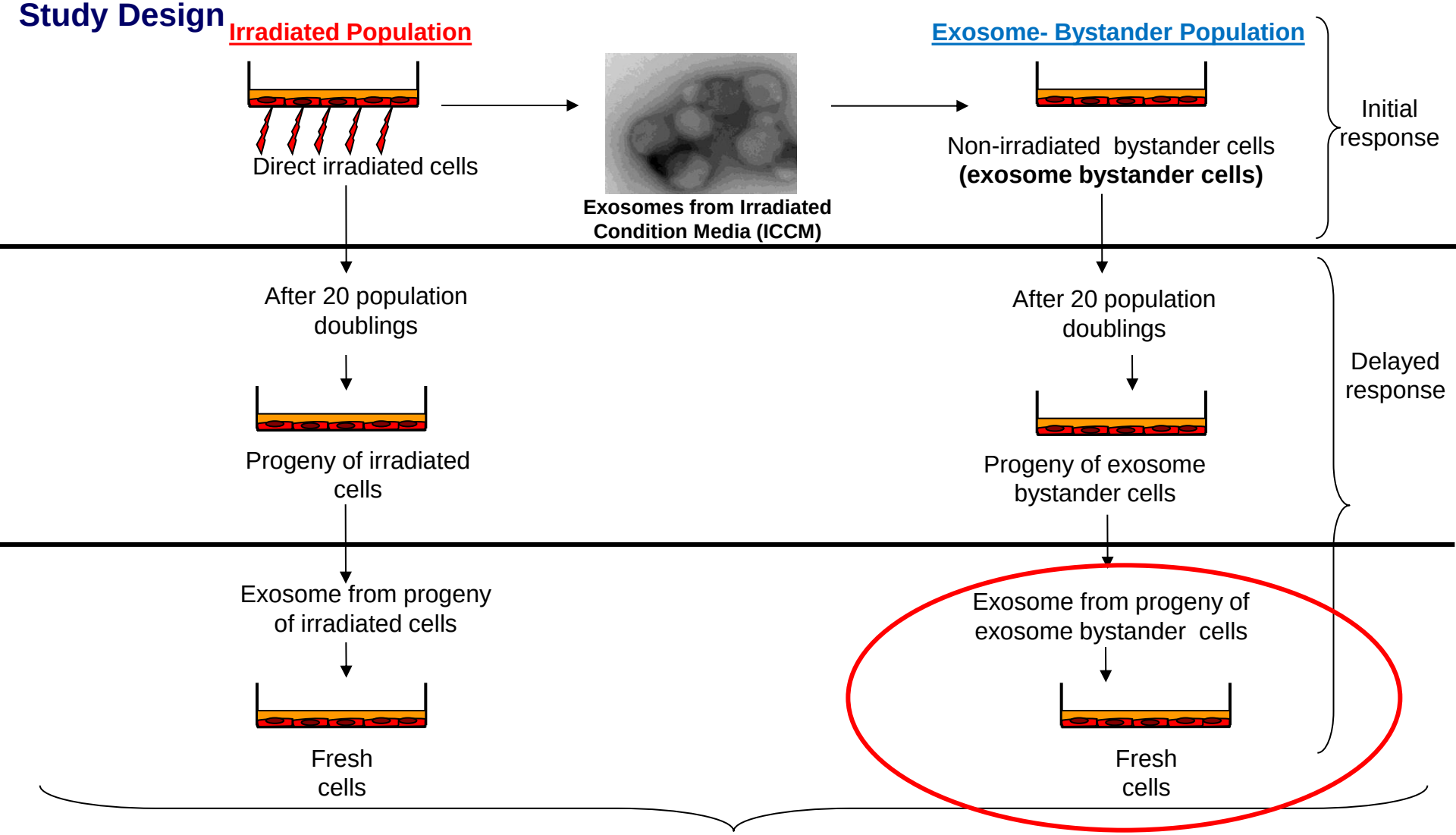
Exosomes induced DNA damage in MCF7 cells





Al-Mayah et al,. Rad Res, 2012 545(5): 539–545.

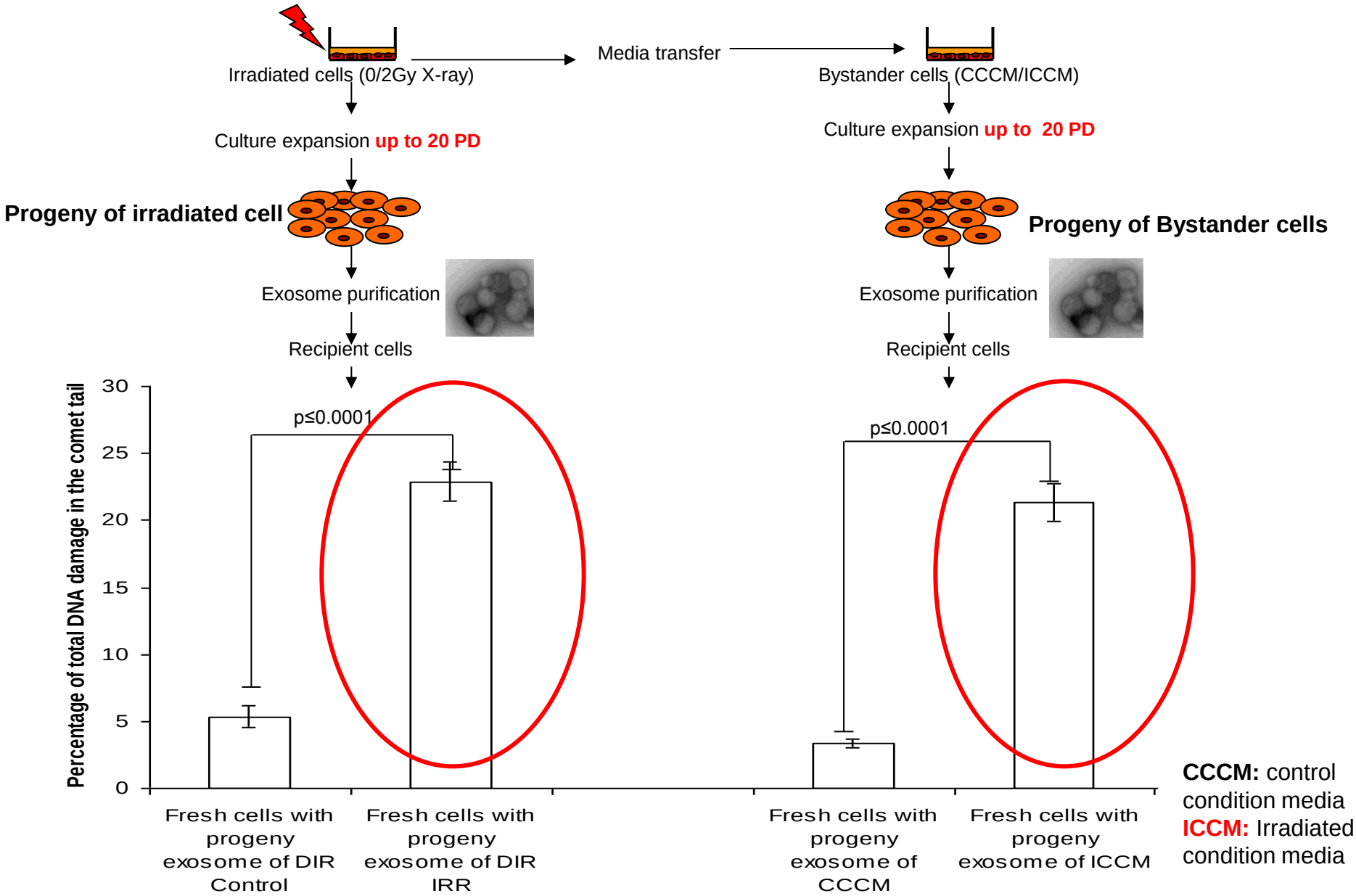
The longevity of exosome-induced activity in the progeny of irradiated and bystander cells : Study Design



Relevant biological end points analysis including DNA damage, Chromosomal and Telomere instability

Al-Mayah et al., Mut. Res. 772 (2015) 38–45

Exosomes damaging signals are persist in the progeny of irradiated and Bystander cell populations



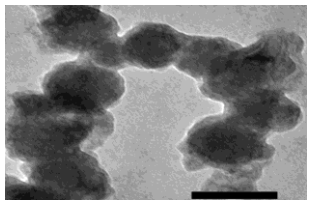
CCCM: control condition media
ICCM: Irradiated condition media

Al-Mayah et al., Mut. Res. 772 (2015) 38–45

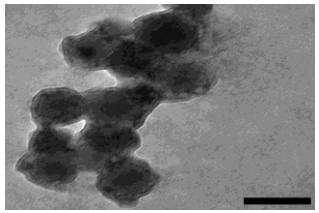
Exosome release profile

Exosomal characterisation - Electron microscopy & concentration

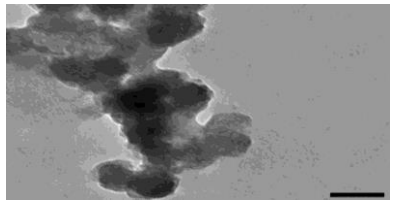
	Size (nm)
	54.8
	51.5
	58.6
	68.9
	60.5
	63.3
	72.9
	74.4
	61.4
	71.7
	55.1
	77.5
	63.4
	75
	45.8
	70.5
	72.1
Average	64.55
Stdev	8.99



Exosomes-Control

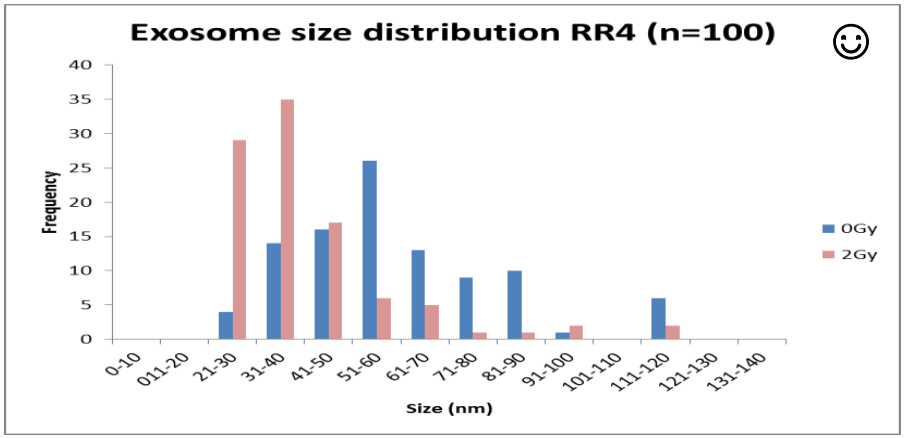


Exosomes- Irradiated cells



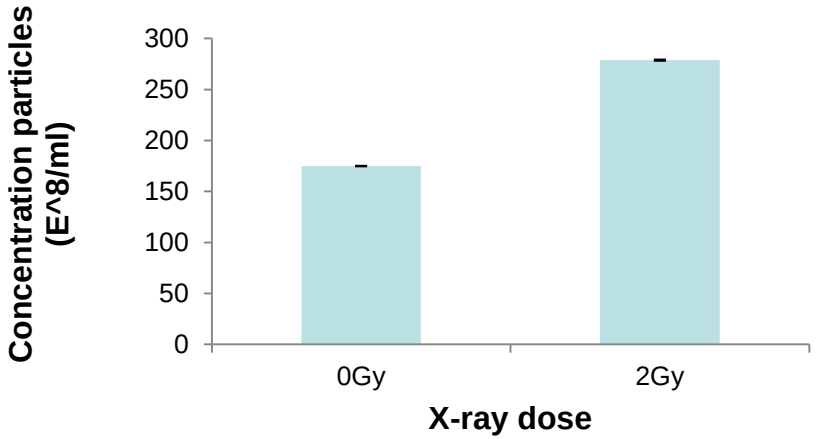
Exosomes- Bystandered cells

Al-Mayah et al, 2015

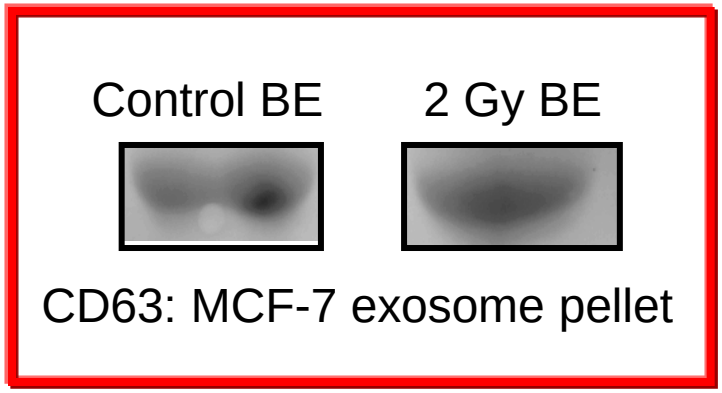


☺ **Laura Jacobs-PhD project**

Exosome release following irradiation



Western blotting confirmed their endosomal origin



Summary 1:

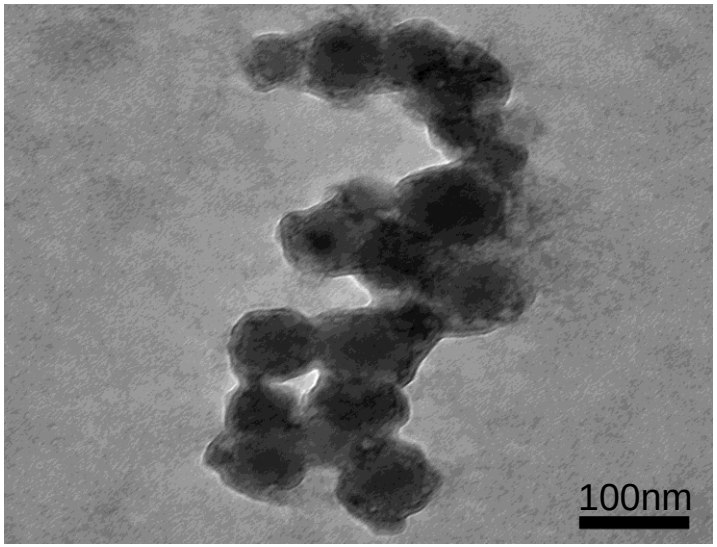
- Exosomes are transmitted factors, involved significantly in the Non Targeted Effects (GI & BE) of radiation exposure.

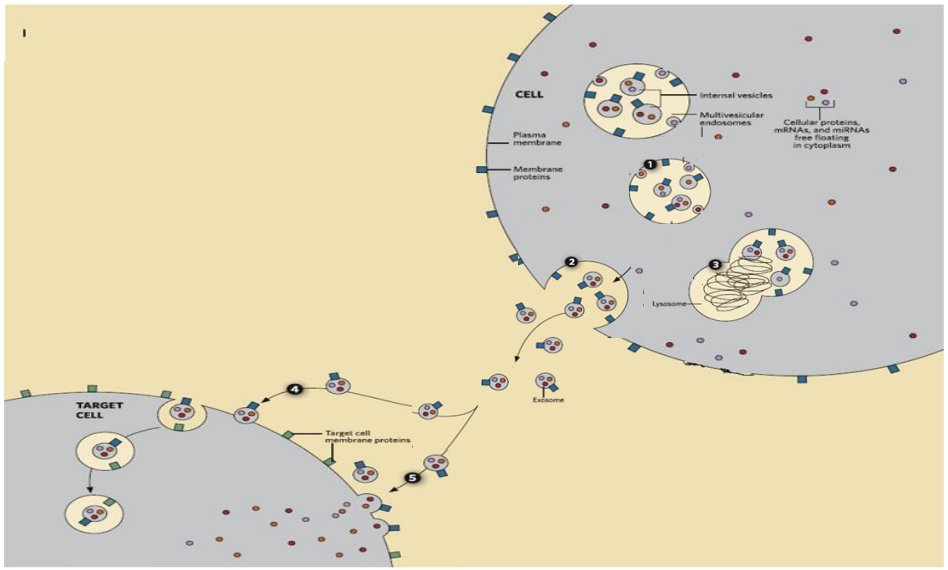
- This effect showed longevity, observed >20 doublings post-irradiation in progeny of irradiated & bystander cells

- Removal of exosomes from irradiated supernatant has shown significant reduction of Chromosomal instability & total DNA damage.

So how this might occur?

EXOSOMES





http://icn.postech.ac.kr/icn_intro_new

- Exosomes are small heterogeneous membrane vesicles (50-150 nm).
- Present in all body fluids (Blood, Urine, Saliva, Milk etc.)
- Cell-cell mediators with physiological & pathological significance
- Specific surface proteins
- **Contain both protein and RNA molecules.**
- Secreted by cells to the extra cellular environment
- Exosomes can be taken up by recipient cells in the delivery of their protein and RNA cargo.
- Cancer cells exosomes can induce oncogenic properties in the recipient cells (increase in cell division or metastatic behaviour) :*Lee et al, 2011, Semin Immunopathol DOI 10.1007/s00281-011-0250-3*

EXOSOME FUNCTIONAL CONTENTS:
RNA & Protein Cargo

Radiat Res. 2012 May;177(5):539-45.

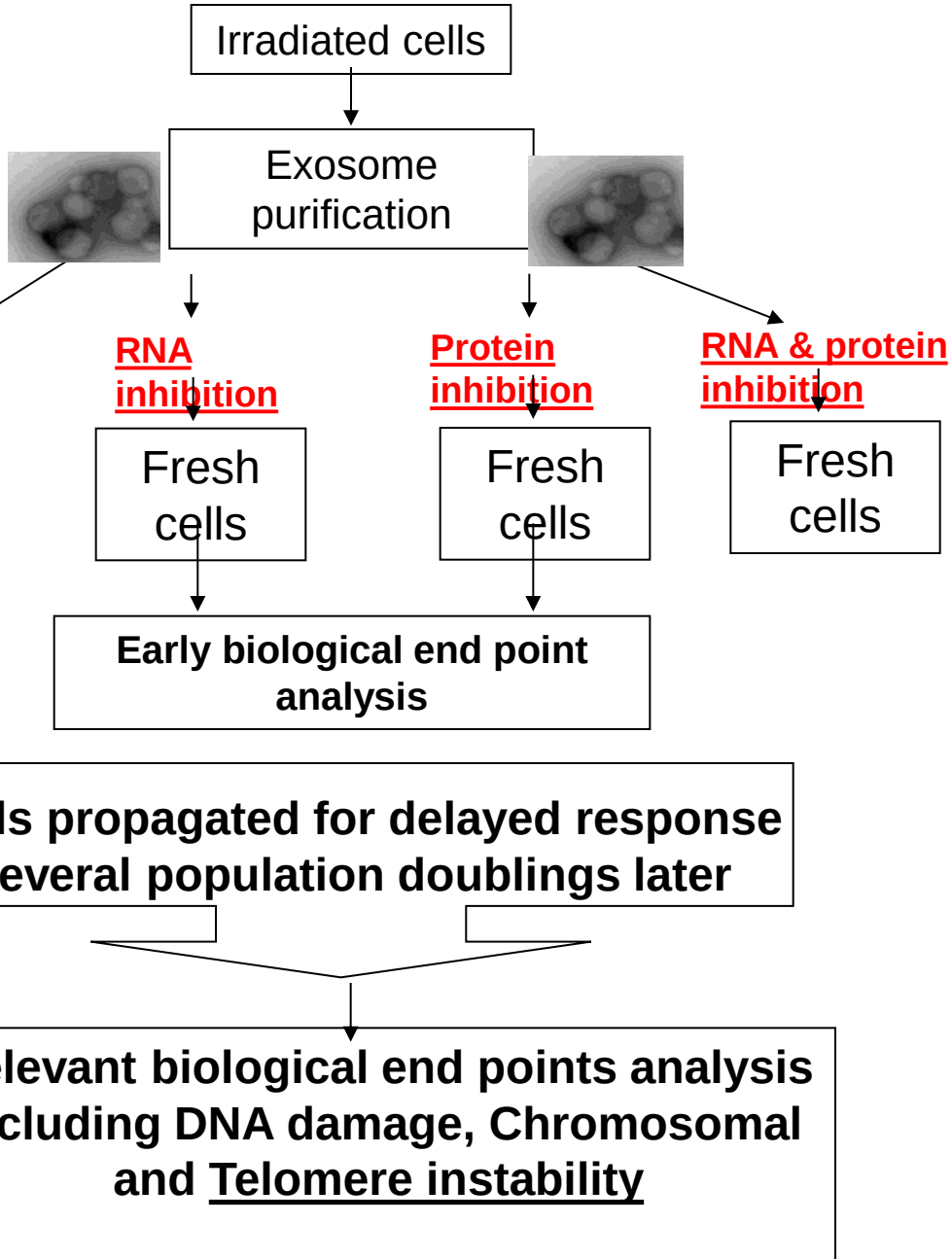
Possible role of exosomes containing RNA in mediating nontargeted effect of ionizing radiation.

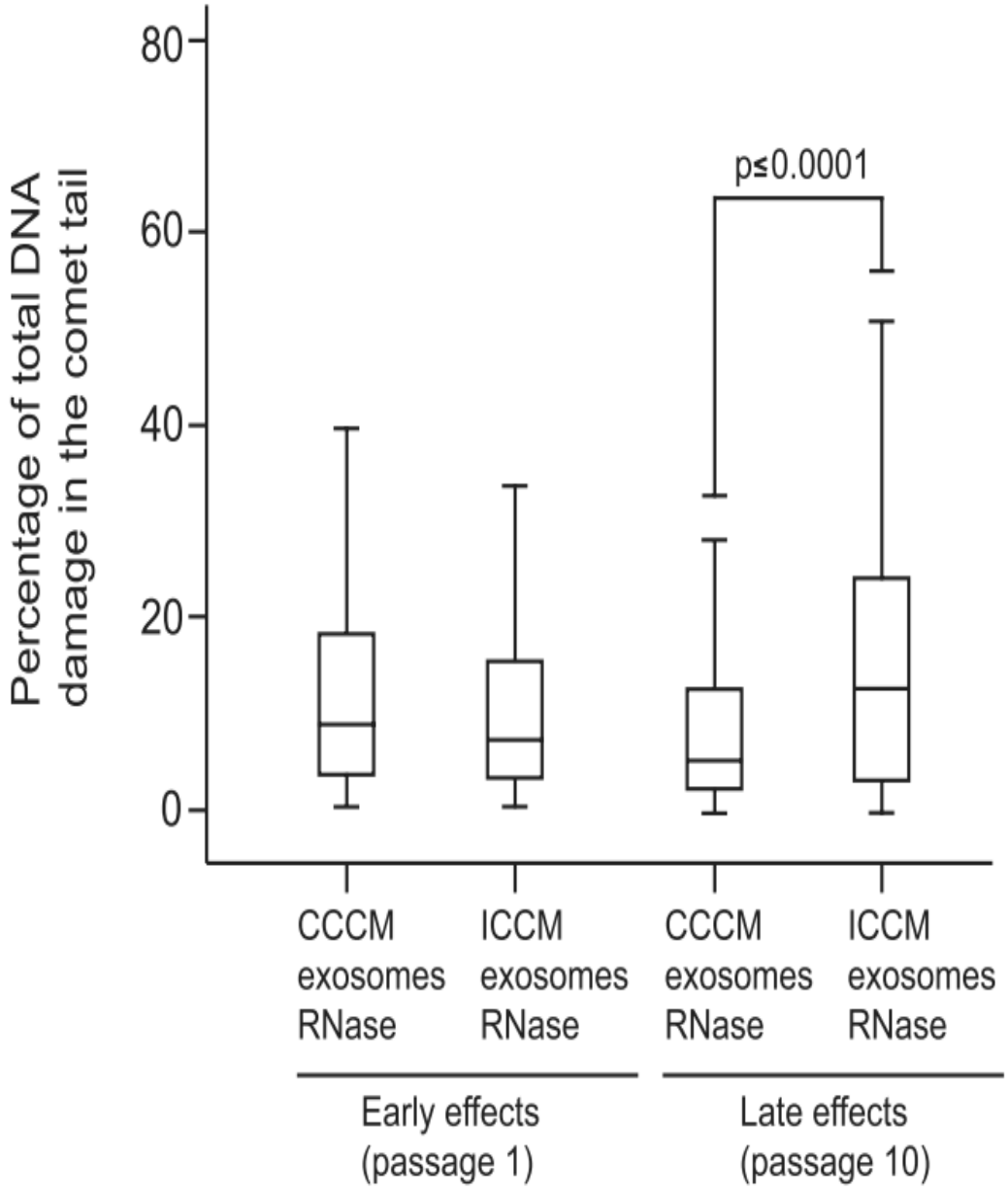
Al-Mayah AH[1], Irons SL, Pink RC, Carter DR, Kadhim MA.

Mutation Research 2015 , 772, 38–45
The non-targeted effects of radiation are perpetuated by exosomes
Ammar Al-Mayaha,Scott Bright, Kim Chapmana, Sarah Ironsb, Ping Luoc, David Carterd, Edwin Goodwine, Munira Kadhima.

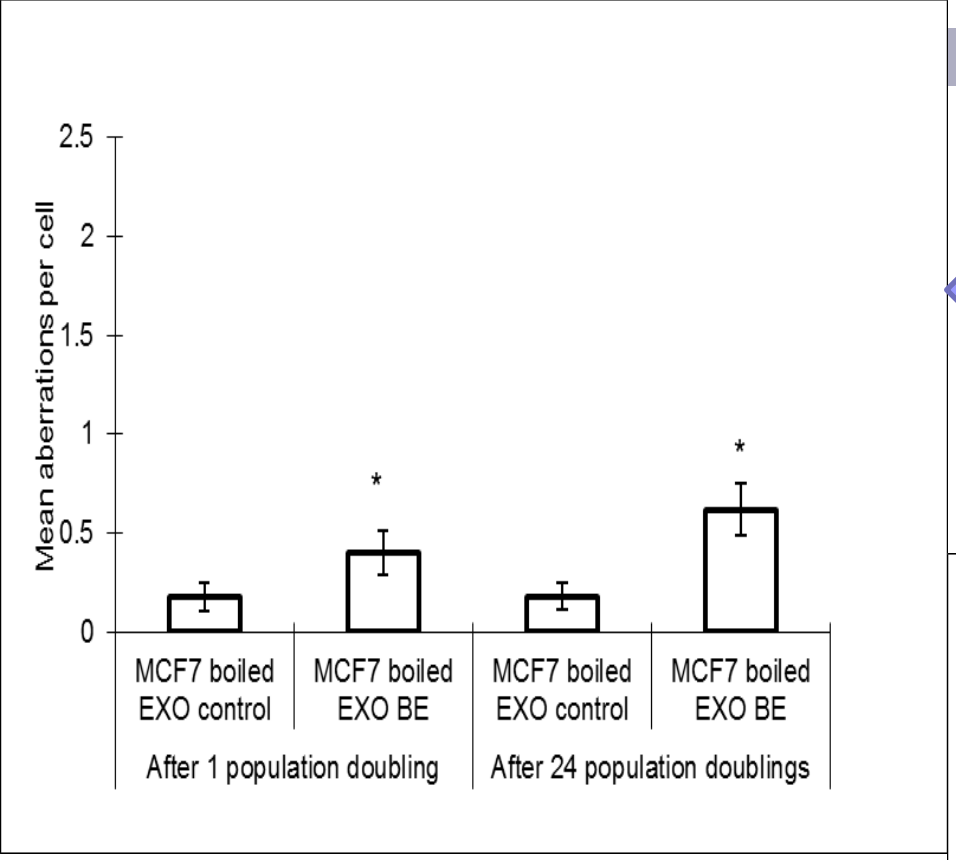
RADIATION RESEARCH 2017, 187, 98–106
Exosome-Mediated Telomeric Instability in Human Breast Epithelial Cancer Cells Post X-Irradiation
Ammar H J Al-Mayah Scott J Bright Debbie A Bowler , Predrag Slijepcevic, Edwin Goodwin Munira A Kadhim

The functional molecules of the exosome's cargo: Exosomes RNA & Protein



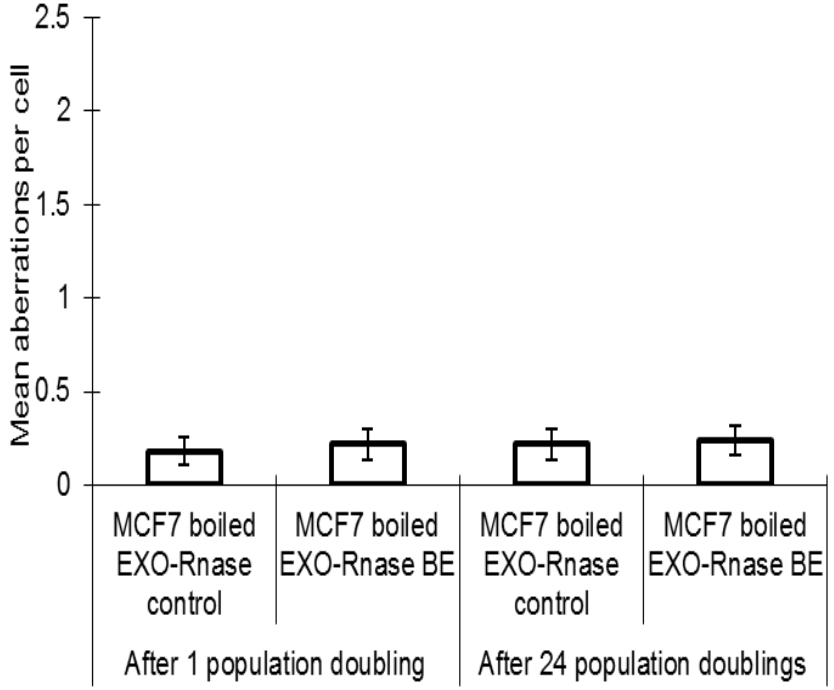


RNAse abolished the effect at the early timepoint and reduced the effect at the late time-point



Removing protein through boiling wasn't enough to alleviate the effect

Removing protein and RNA was enough to alleviate the effect



Overall Summary

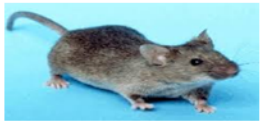
- Exosomes are significantly involved in the NTE of radiation exposure *in vitro*.
- Both RNA and protein work in a synergistic manner to initiate non-targeted effects of IR.
- Effect is propagated through cell generations and persist in the progeny of both irradiated and bystander populations
- Exosomes are important in this process.

However,

For exosomes/MVs application as biomarkers for risk implication of radiation exposure & radiotherapy , understanding their mechanistic role *in vivo* utmost impotence.

Role of Macrovesicles / Exosomes in the induction of NTE : *in vivo* study

In vivo Experimental design



C57Bl/6 mice irradiated with
0, 0.1, 0.5 , 2Gy X-ray

Early analysis

24 hrs post-IR

Delay analysis

3 months post-IR
(all processed as for 24hr)

Remove Bone Marrow (BM)

Extract microvesicles (MV) from BM to be divided into three fractions:

Direct prep for cellular analysis:
- Chromosome analysis
- Inflammation markers
- mRNA
- Spectroscopy

Fraction 1: direct quantitative and qualitative profiling of MV

Fraction 2: Inject into non irradiated recipient animals for MV bystander effects *in vivo*

Analysis of BM and spleen at 24hrs (early) and 3 months (late)

Fraction 3: Transfer to recipient non irradiated BM and spleen cells for MV bystander effects *in vitro*

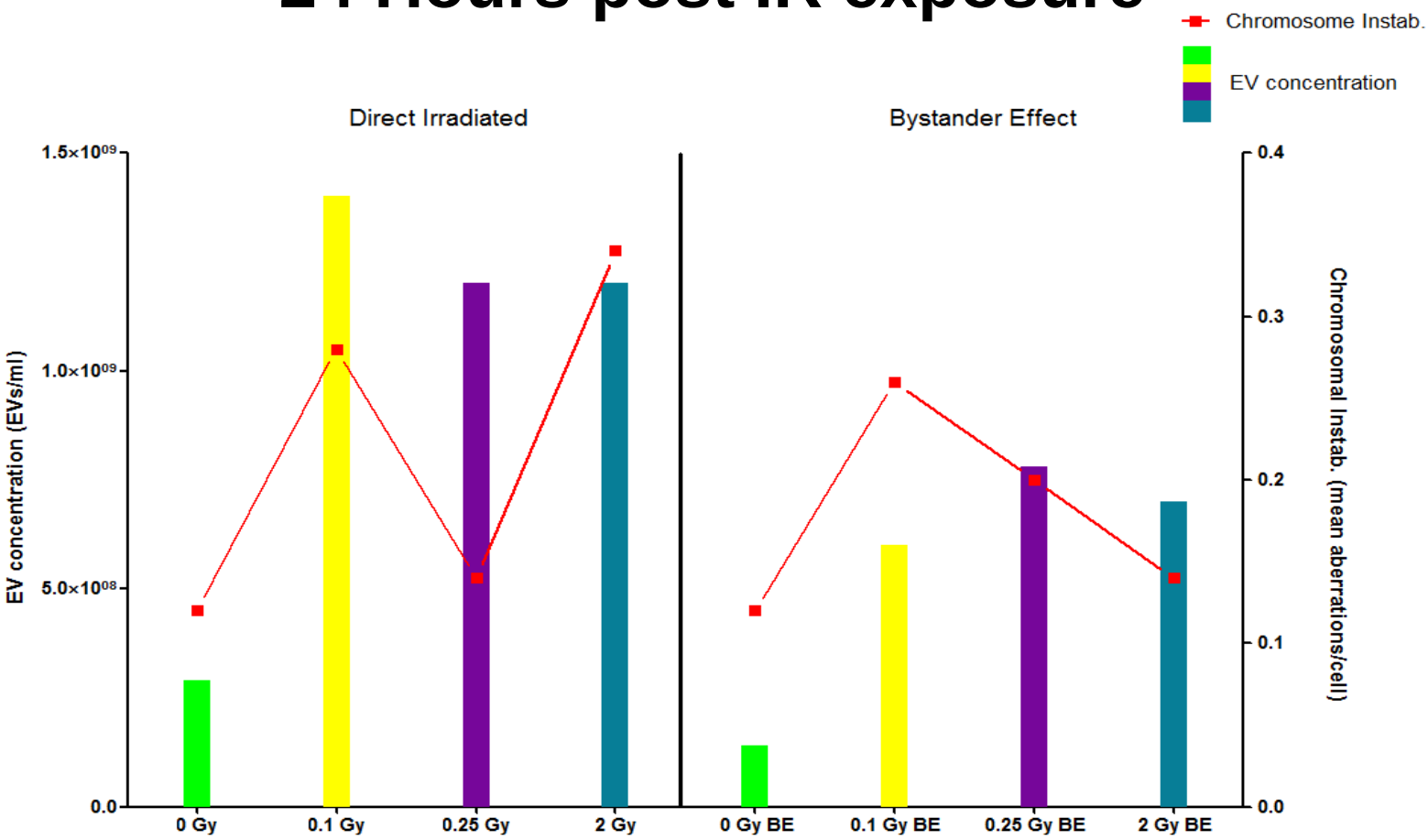
Analysis of BM and spleeen at 2 days (early) and 7 days (late)

*In parallel for control bystander, supernatant (no cells) will be injected/transferred to non irradiated animals/cells

Bright *et al*, in preparation

EV concentration and chromosomal aberrations

24 Hours post IR exposure



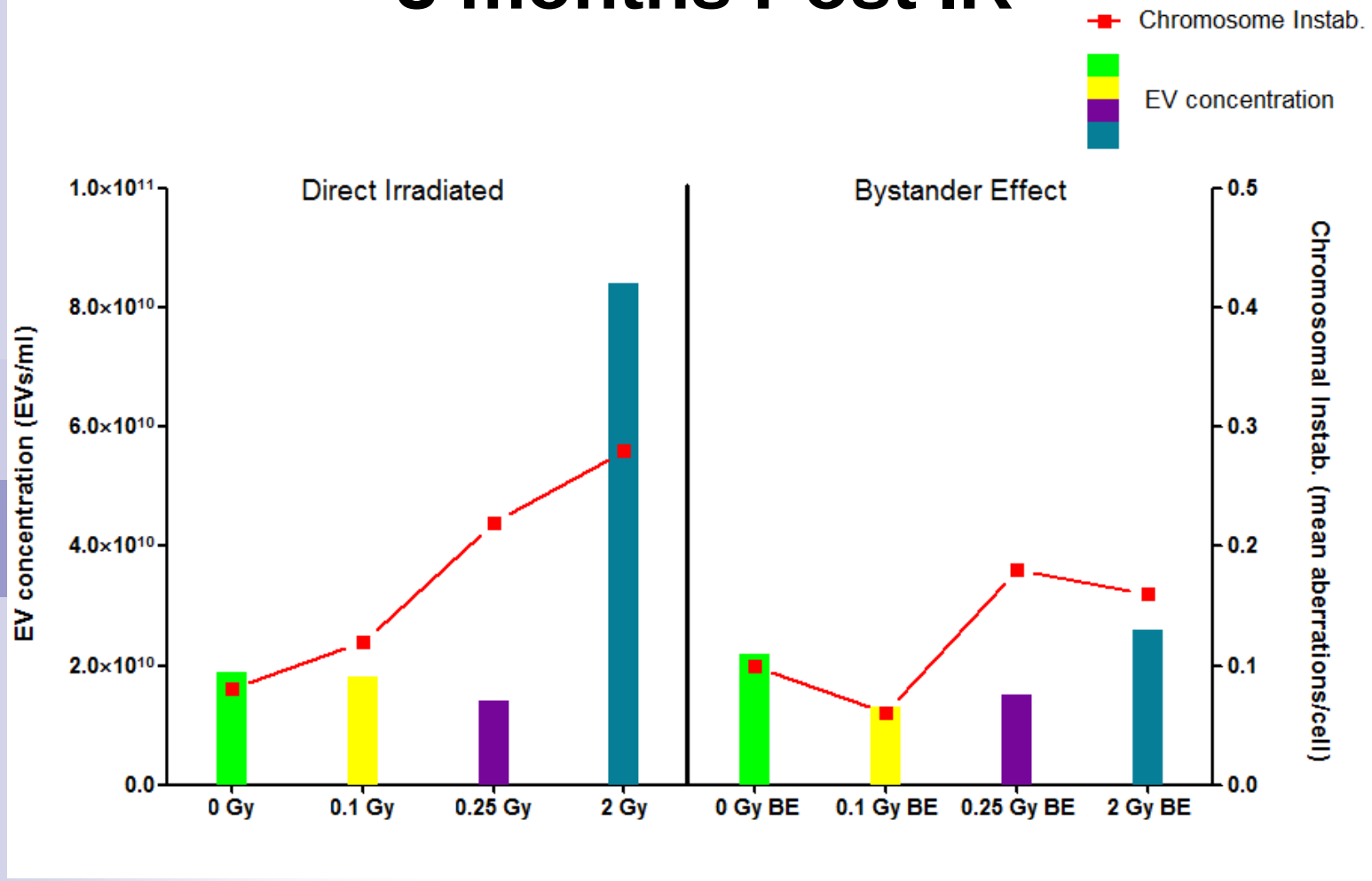
In direct irradiated groups exosomes level were increased in all irradiated groups, while chromosomal instability was increased at 0.1 Gy and 2 Gy.

In bystander groups exosomes level were increased in groups that received irradiated cell conditioned media. CIN was most prevalent in the 0.1 Gy ICCM group.

Bright *et al,* in preparation

EV concentration and chromosomal aberrations:

3 months Post IR



In direct irradiated groups exosomes level were increased in the 2 Gy irradiated group, while chromosomal instability was increased in a linear fashion with dose.

In bystander groups exosomes level showed slight changes. CIN was most prevalent increased in the higher doses of ICCM of 0.25 and 2 Gy.

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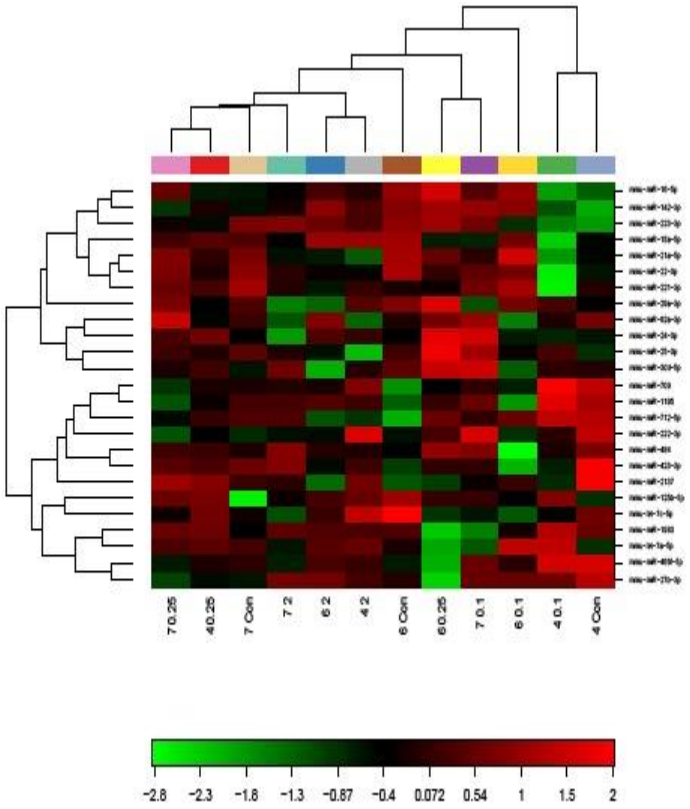
miRNA was also different within exosomes: *in vivo*

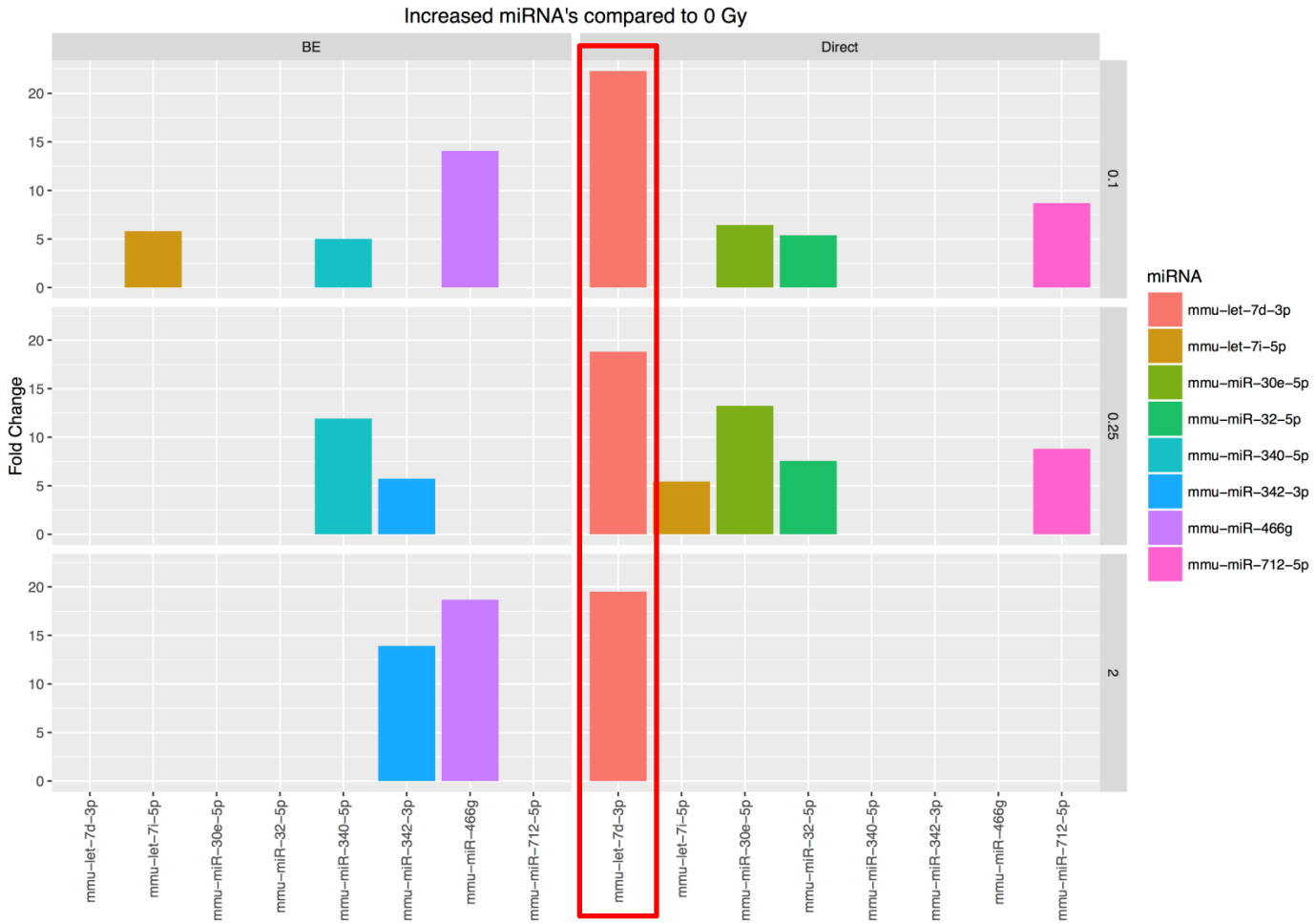


Whole miRNome panel (752 assays over 384 well plates) (Exiqon)

An average of 79 microRNAs detected per sample

>20 microRNAs more than two-fold differentially expressed between
- controls and dose points
- 24h and 3 month time points
- Direct and Bystander

Bright *et al,* in preparation

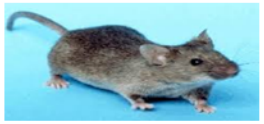


At 3- month direct and bystander **increased** miRNA's:let-7d-3p
It was increased to a similar level in all irradiated groups.

Increase in let-7d decreases:

▼RAS, ▼cell cycle, ▼DNA replication machinery

In vivo Experimental study



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EV Biodistribution

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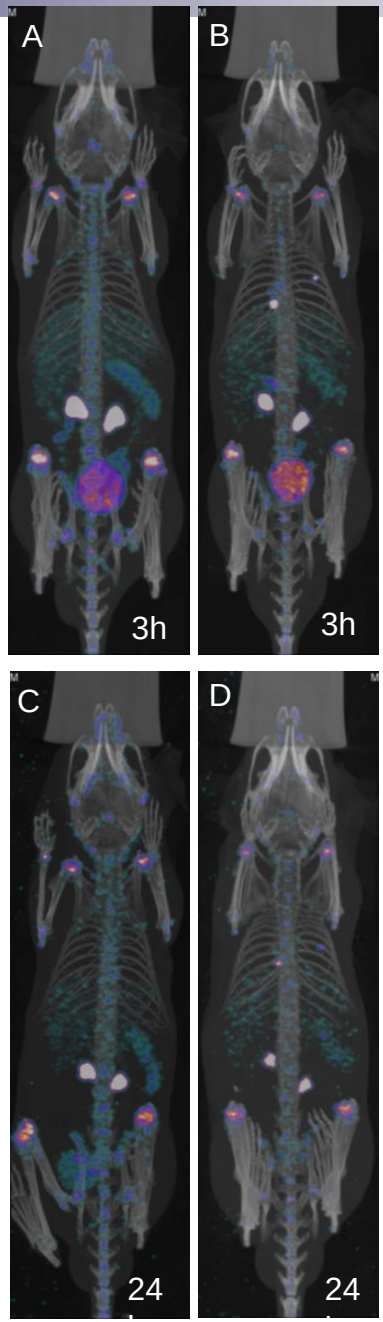
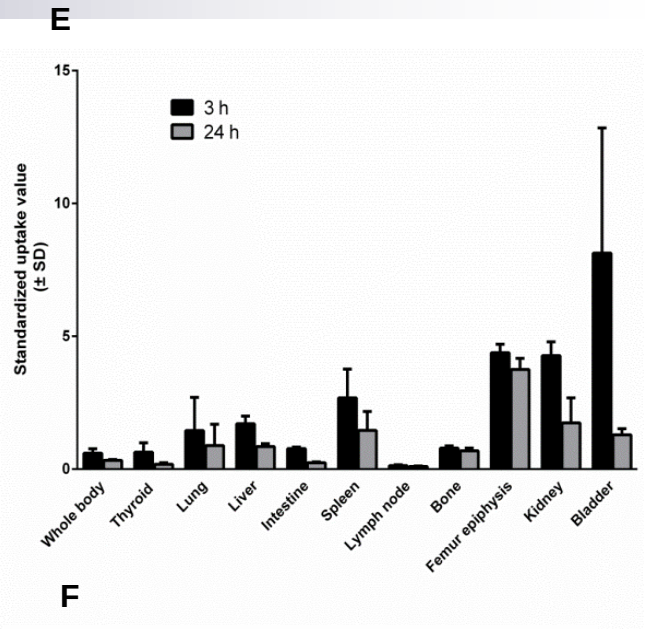
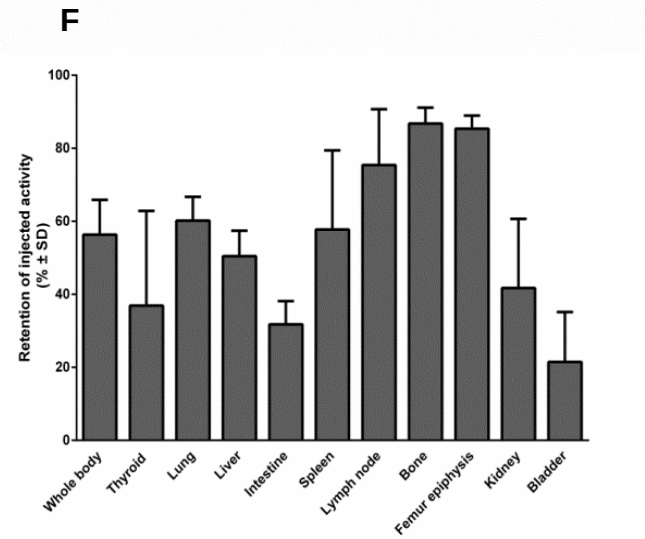
EV Bio distribution

When these EVs were injected into naïve (unirradiated) mice They aggregate in lung, liver, spleen and bone marrow Exosomes are retained with the bone marrow. (implications for stem cells)







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Tünde Szatmári, Bright, Bowler, Kadhim, Sáfrány Lumniczky . Extracellular Vesicles Mediate Radiation-Induced Systemic Bystander Signals in the Bone Marrow and Spleen, Front. Immunol., 27 March 2017

In vivo study*:* current conclusions

- Results suggest that MV / exosomes are involved in NTE of radiation exposure *in vivo* and effects persist in both irradiated and bystander cohorts

- Presence of tumour susceptibility gene (TSG101) protein, a typical exosomal protein marker, confirmed

- Micro RNA analysis: >20 microRNAs more than two-fold differentially expressed between controls and dose points : Most striking effects seen in Direct groups
  - Increased let-7d-39 (reported in cancer exosomes)
  - Decreased miR-31-5p (tumour suppressor ,links to ovarian & breast cancer)

- For the first time, a fast and efficient labelling of bone marrow derived MV / exosomes and *in vivo* tracing of their biodistribution was achieved

- The development of mathematical and statistical models with analysis of individual endpoints is in progress

Summary, Comments & Future Direction

- Epigenetic rather than genetic mechanism is most likely underlying Radiation –induced Non Targeted Effects
- Further robust experimental approaches with closer link to epidemiology approach will help in better understanding of the interaction between these mechanisms and their relevance
- In order to evaluate the risk implications, a combination of targeted & non-targeted mechanistic information needs to be developed
- **Move to more complex / advance experimental systems for studies and evaluate the data using systems approaches type modelling**

Acknowledgements:

The *in vivo* study was supported by EU-FP7 joint-funded DoReMi project (grant agreement number 249689) entitled “Low dose radiation-induced non-targeted effects in vivo: the role of macrovesicles in signal transduction” and the NIHR Health Protection Research Unit for Chemical and Radiation - Public Health England.

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- Liz Ainsbury : PHE, Oxford

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THANK YOU