

The background of the entire page is a collage of various images related to radiation and nature. On the left, a large yellow radioactive symbol (triple-leaf symbol) is overlaid on a landscape with a bridge. In the center-right, a large red maple tree stands on a grassy hill. In the bottom right, there's a silhouette of people walking along a path. The overall color palette is warm, dominated by yellows, oranges, and reds. The collage is composed of several layers of images, creating a textured and layered effect.

Radiation Protection

N° 189

*Epigenetic effects – potential
impact on radiation protection*

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radiation protection"**

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8 November 2017

**Working Party on Research Implications on Health and Safety
Standards of the Article 31 Group of Experts**

Directorate-General for Energy
Directorate D — Nuclear Energy, Safety and ITER
Unit D3 — Radiation Protection and Nuclear Safety
2019

FOREWORD

Luxembourg, October 2019

The European Commission organises every year, in cooperation with the Group of Experts referred to in Article 31 of the Euratom Treaty, a Scientific Seminar on emerging issues in Radiation Protection – generally addressing new research findings with potential policy and/or regulatory implications. Leading scientists are invited to present the status of scientific knowledge in the selected topic. Based on the outcome of the Scientific Seminar, the Group of Experts referred to in Article 31 of the Euratom Treaty may recommend research, regulatory or legislative initiatives. The European Commission takes into account the conclusions of the Experts when setting up its radiation protection programme. The Experts' conclusions are valuable input to the process of reviewing and potentially revising European radiation protection legislation.

In November 2017, the EU Scientific Seminar covered the issue *Epigenetic effects – potential impact on radiation protection*. Internationally renowned scientists presented:

- General Introduction to Epigenetics for Radiation Protectionists
- Epigenetic rather than genetic mechanisms most likely underlie radiation-induced Non-Targeted Effects
- Trans-generational effects
- Long non-coding RNAs: new mechanisms regulating sensitivity to ionizing radiation

The presentations were followed by a round table discussion, in which the speakers and additional invited experts discussed potential *policy implications and research needs*.

The Group of Experts discussed this information and drew conclusions that are relevant for consideration by the European Commission and other international bodies.

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1 GENERAL INTRODUCTION TO EPIGENETICS FOR RADIATION PROTECTIONISTS

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What is epigenetics?

The human body is estimated to contain about 4×10^{13} cells (Bianconi et al. 2013). Traditionally these are divided by histologists in more than 200 cell types, mostly based on morphological features; with new molecular methods one could define an even higher number of cells. Their high variability in form and function is obtained although all cells carry the same genetic information (with the exception of mutations that occur during replication of the DNA or due to external factors, and with the exception of a few cell types that specifically alter their genetic information, e.g. immune cells). Protein-coding sequences comprise less than 2% of the genome, and current estimates predict roughly 20 000 protein-coding genes. The remainder comprises non-coding transcribed regions, regulatory regions, repetitive sequences, introns and other elements, the function of which has not yet been fully elucidated.

The different forms and functions of the individual cells are not defined by the genes they possess, but the genes they express. Gene expression is regulated on several levels, for example transcription, where the genes are transcribed into mRNA, and post-transcriptional processes. In the end, however, it is important what proteins the cell makes in the process called translation, and the proteome of cells (i.e. the total protein content) can differ greatly between cell types, but also for one cell type under different environmental and growth conditions. So the question is, how is it regulated which genes are expressed in which cell?

DNA in the cell nucleus is not naked, but packed into so-called chromatin. The DNA molecule is wrapped around histone proteins to form a nucleosome, and then these nucleosomes interact to each other to form a three-dimensional organized structure. It should be noted that the first levels of organisation (up to the so-called beads-on-a-string level) are quite well investigated, but concerning the higher organisation levels, there are many open questions (Luger et al. 2012, Bonev and Cavalli 2016).

Already early in the 20th century by staining of cells with different dyes it was known that the chromatin is unevenly distributed in the nucleus and the terms euchromatin and heterochromatin were coined by Emil Heitz (Allis and Jenuwein 2016). Heterochromatin was defined as the regions that stain brighter, and the less bright regions were called Euchromatin, the „real chromatin“, which was considered to be genetically more important. Already in the 1930ies the picture emerged that regions with a dense, compacted chromatin structure, which are expected to be brighter after staining, are less transcriptionally active than the more loosely packed regions (Passarge 1979, Allis and Jenuwein 2016). This picture, while oversimplified, is still very much in use. Anyway, what structure the chromatin has is largely defined by epigenetic patterns.

The term epigenetics is also old, it was coined 1942 by Conrad Waddington to describe the study of „changes in phenotype without changes in genotype“. He was a developmental biologist and thus mainly interested in how different cell types and organs develop in the embryo. Later definitions became broader, but also fuzzier, not only considering development but also cellular plasticity, and concentrating on gene expression patterns. After the discovery of an important mechanism, DNA methylation, Robin Holliday 1982 proposed the following definitions: “the study of the changes in gene expression, which occur in

organisms with differentiated cells, and the mitotic inheritance of given patterns of gene expression " AND "nuclear inheritance, which is not based on differences in DNA sequence" (Dean and Maggert 2015). Later Wu and Morris streamlined this to: "the study of changes in gene function that are mitotically and/or meiotically heritable and that do not entail change in DNA sequence" (Wu and Morris 2001). Still there are many discussions on the appropriate use of the term, but in the present context the following is important:

- Epigenetic mechanisms, which at each cell division are inherited by the daughter cells, define the cell type. If they alter, the cell function is affected and this can result in disease, for example cancer. In this concept, the apparent stability of the patterns is important.
- Epigenetic mechanisms are, however, also the mechanisms that allow a cell to respond to alterations in the environment, since they can alter the gene expression pattern, if necessary. In this concept, the plasticity of the patterns is important, that is the possibility to write and to erase epigenetic marks.

DNA methylation

Probably the best investigated epigenetic mechanism is DNA methylation. Methylation of human DNA occurs at carbon 5 of cytosine, to form 5-methylcytosine. Like the unmodified cytosine, 5mC pairs with guanine. DNA methylation is conferred by DNMTs (DNA methyltransferases), i.e. these are the writers of the epigenetic mark. There are two types: de novo DNMTs which can methylate unmodified cytosines (e.g. in early embryo, this helps to establish the different tissue types), and maintenance DNMTs which methylate the unmethylated strand in hemimethylated DNA after semi-conservative replication, so that the mark is stable over cell generations. 5mC occurs predominantly in the context of CpG dinucleotides.

If methylated DNA is injected into cells, it is not transcribed; this was the first indication for the function of 5mC, namely repression of transcription and silencing of genes. Long time it was not known how DNA methylation marks are removed from DNA. Now we know that there are passive and active mechanisms. A major role is played by TET enzymes (ten-eleven translocation). These are methylcytosine dioxygenases which can oxidise 5mC to 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC). The oxidised forms are not recognised by maintenance DNMTs, thus after several rounds of replication the signal is diluted. In addition 5fC and 5caC can be actively removed by a multistep process (Wu and Zhang 2017).

Silencing of genes must be tightly regulated, since inappropriate silencing can cause various diseases, including cancer. Epigenetic silencing of tumour suppressor genes is a frequent occurrence in the carcinogenic process. Interestingly, the activity of TET enzymes can be modulated by cellular metabolism (Janke et al. 2015, Wu and Zhang 2017). TET-mediated oxidation requires oxygen, α -ketoglutarate and bivalent iron, thus the reaction will be suboptimal under hypoxic conditions or if not enough bivalent iron or α -KG are present. Isocitrate Dehydrogenase (IDH) enzymes produce α -KG by oxidative decarboxylation of isocitrate and the α -KG levels can be affected by nutrients. In addition, vitamin C has been shown to stimulate TET-mediated oxidation, by serving as a co-factor and possibly by affecting the iron metabolism.

Cancer cells often harbour mutations in genes that code for components of epigenetic regulation. For example, cancer-associated IDH mutant enzymes form the oncometabolite 2-hydroxyglutarate which competes with α -KG, and thus the TET-mediated reaction is inhibited. Therefore 2-hydroxy-glutarate can lead to DNA hypermethylation and reinforce the cancerogenic process, for example in glioblastoma. In preleukaemic cells, alterations of methylation processes by mutations in DNMT, TET or IDH genes are considered to form the „first hit“.

Metabolic patterns also affect DNMT-dependent methylation of cytosine. DNMTs receive the methyl groups they transfer from S-adenosyl-methionine, which is made from methionine. To generate methionine, the cell needs folate. Already in 1947 it was observed that folate can stimulate the proliferation of leukaemia and folate antagonists like aminopterin or methotrexate have since then been used in chemotherapy (Newman and Maddocks 2017). However, the relationship between folate and cancer risks is complex, since both low folate levels and excessive intake of folate have been associated with increased cancer risk in epidemiological studies (Mason and Tang 2017).

Histone Modifications

Methylation can not only occur at the DNA, but also at the histone proteins. In the nucleosome, which is the basic unit in the bead-on-a-string chromatin structure, DNA is wrapped around an octamer of each 2 units of the 4 histones, H2A, H2B, H3, H4. All the histones have a similar structure, a central coiled-coil structure and tails at both ends, which reach out of the globular octamer structure. Post-translational modifications occur predominantly, but not exclusively, at these tails. These modifications include acetylation, methylation, phosphorylation and others which are added by writer enzymes and removed by eraser enzymes, both of which are highly specific for the amino acid that is to be modified. Some of these modifications exclude each other, either at the same or also on different positions, while others can be present at the same time and may reinforce the information. Generally, one may speak of a histone code, a term that has been coined in 2001 (Jenuwein and Allis 2001).

The role of a given histone modification for gene expression is more complex than in the situation with DNA methylation: Acetylation mostly is associated with a transcriptionally active chromatin. In contrast methylation can mediate active or repressive chromatin status, depending on which amino acid is methylated. The situation is further complicated by the fact that one or several methyl-groups can be added, e.g. lysine can be mono-, di or tri-methylated; arginine mono- or di-methylated. In any case, DNA methylation and histone modifications act together and influence each other.

Histone acetylation is conferred by histone acetyl transferases, of which there are about 30 different types present in the human cells. They catalyze the transfer of an acetyl group from acetyl-CoA to the ϵ -amino group of a histone lysine residue. Here we see again a potential influence of metabolism, since the availability of Acetyl-CoA depends on the metabolic situation. For example, in an animal model high fat diet has been shown to reduce acetyl-CoA levels and histone acetylation in various tissues (Carrer et al. 2017). Histone acetylation is a rather dynamic process and most acetyl-groups will soon be removed by histone deacetylases.

Histone deacetylases are a very interesting group of enzymes which are associated with a large variety of diseases, not only cancer but also neurological and psychiatric diseases, diabetes, etc. Therefore a variety of HDAC-inhibitors (HDACi) have been introduced into clinical routine, e.g. valproic acid for the treatment of epilepsy and depression or suberoylanilide hydroxamic acid (SAHA) for treatment of T-cell leukaemia. There have also been many studies on the combined effects of HDACi and radiation, with the observation that HDACi in general act as radiosensitisers (Groselj et al. 2013, Smits et al. 2014, Oronsky et al. 2016). Interestingly, many plants contain substances that can inhibit HDACs, e.g. onions and garlic, crucifers such as broccoli, or apples and citrus fruits. Similarly, dietary fibers, which cannot be digested directly by humans, are fermented in the intestines by microbiota to generate butyrate, another very potent HDACi. Hence, this provides the possibility to influence histone acetylation patterns by diet.

Methylation at the histone tails may either activate or repress transcription, depending on the position of methylation. For example, tri-methylation at histone 3 lysine 9 (H3K9) is repressive, while trimethylation at H3K4 is activating. Thus, to change the state it is not enough to add or remove a mark, as in the example of acetylation, but additional marks have

to be brought in. Methylation of histones is performed by lysine methyl transferases or arginine methyl transferases (KMT, PRMT) which take the methyl groups from S-adenosyl methionine, like DNA methyl transferases. Thus this reaction is also dependent on the level of folate, methionine and serine. Removal of the methyl group or groups is conferred by histone demethylases, most of which perform a similar reaction like the TET enzymes in the case of DNA methylation and also require oxygen, α -ketoglutarate and bivalent iron and can be inhibited by 2-hydroxyglutarate. Thus again we have the situation that metabolic status and/or diet may influence the methylation patterns.

Other epigenetic mechanisms

While everyone agrees that DNA methylation and histone modifications are epigenetic mechanism, there is some debate on a variety of other mechanisms, such as histone variants, chromatin remodelling, chromatin folding, non-coding RNAs or long non-coding RNAs. There is a great overlap of the above-mentioned mechanisms with mechanisms of regulation of gene expression in general, and depending on the definition used for the term epigenetics, especially with regard to heritability of the alterations over cell generations, if not over organismal generations, people will have a rather strict or more relaxed point of view.

Modern molecular methods have taught us in recent years that a surprisingly large part of the genome is transcribed into RNA. There are several types of non-coding RNAs, which differ for example in length and mechanism of work. Long non-coding RNAs can direct histone and DNA modifications and thus are intimately involved in the epigenetic regulation (Schmitt and Chang 2017). The role of non-coding RNAs for the DNA damage response is currently being elucidated (Zhang and Peng 2015).

The interplay of Radiation, Lifestyle and Disease

Alterations of the epigenetic pattern are associated to, and often provably causally linked with a variety of diseases. Among the best studied is cancer and it may be the first disease we think about in radiation protection. But many other diseases and dysfunctions can be invoked by altered epigenetic patterns, such as aging, neurological dysfunction, cognitive dysfunction, behavioural/ social dysfunction, metabolic syndrome/ diabetes, cardiovascular disease, or inflammatory rheumatic disease (see Figure 1). It is evident that many of these diseases may also be relevant in the context of radiation protection.

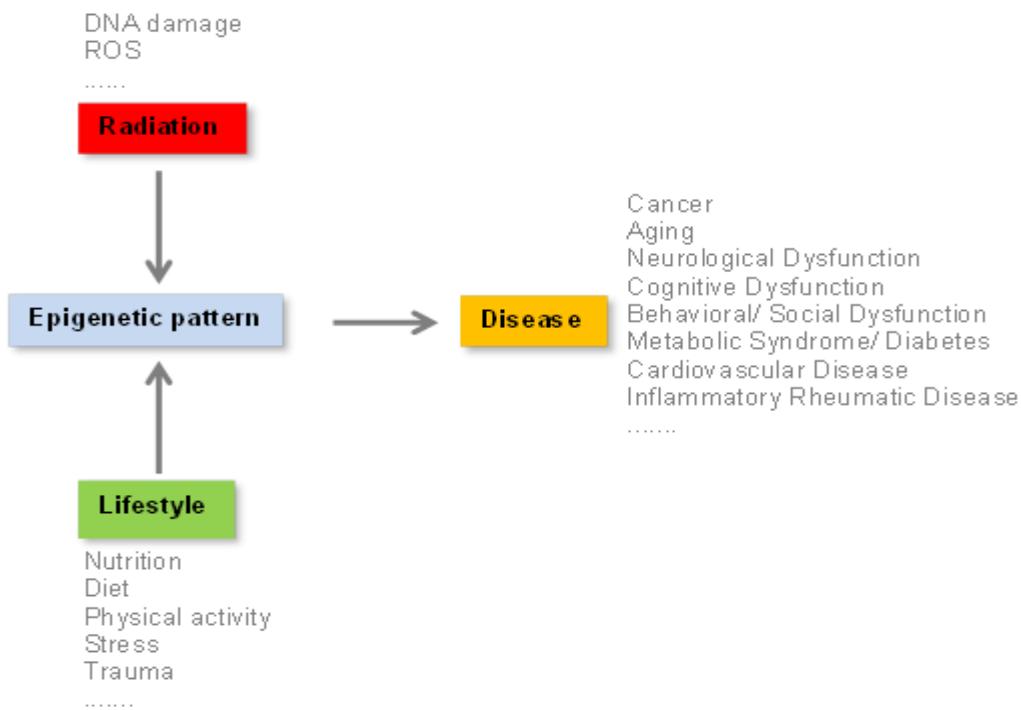


Figure 1: Radiation and other genotoxic agents can alter epigenetic patterns, as can lifestyle factors. These altered epigenetic patterns may lead to development of diseases. Since epigenetic alterations can be reversible, a variety of interventions including lifestyle alterations are hoped to revert disease development. In addition it is conceivable that lifestyle factors may affect or even revert epigenetic alterations induced by radiation.

It is well-established that cancer is a disease of the genes, as cancer cells carry many mutations and some people have an increased risk of cancer development because of certain mutations in their germ line DNA. However, since the early years 2000 researchers became more and more aware of the fact that cancer is also an epigenetic disease (Esteller and Herman 2002). Typically one observes a global hypomethylation in cancer cells, which may increase genomic instability, for example because endogenous mobile DNA elements may become active. At the same time, hypermethylation at specific sites may silence genes, and there are many examples of epigenetically silenced tumour suppressor or repair genes in the literature. Also alterations in histone modifications occur, e.g. hypoacetylation of tumour suppressor genes associated with silencing of these genes.

Several epigenetic drugs have been approved by the European Medical Agency or the American FDA for treatment of cancer, namely drugs that inhibit DNA methylation and lead to degradation of DNMT enzymes for acute myeloid leukaemia and myelodysplastic syndrome and inhibitors of histone deacetylases for the treatment of lymphoma and multiple myeloma.

As indicated above, there is accumulating evidence that nutrition and diet can affect the epigenetic pattern, e.g. by providing or limiting co-factors needed by the histone and DNA modifying enzymes. Other lifestyle factors have not yet been studied so thoroughly, but first indications for an effect of e.g. physical activity on epigenetic patterns have been provided (e.g. Thomas et al. 2016). Presumably alteration of epigenetic patterns plays a major role for the well-known cancer preventive activity of healthy food and exercise. The role of lifestyle in cancer risk became first evident when it was observed that Japanese immigrants to the US had more colon and breast cancer, but less stomach cancer than Japanese in Japan. Thus, epigenetics appears to be a crucial mechanism mediating lifestyle effects on cancer risk (Mayne et al. 2016, Sapienza et al. 2016) (Figure 1).

Accumulating evidence also suggests that radiation can affect epigenetic marks (Price and d'Andrea 2016, Argawal and Miller 2016, Miousse et al. 2017) and the important question is whether a causal link to radiation-induced diseases can be established. It is known that in the wake of damage response and repair many alterations in DNA methylation, histone modifications and non-coding RNAs occur that under normal circumstances should be reversible. It is however easily conceivable that the restoration of the status quo ante may not always succeed (O'Hagan 2014).

What mechanisms could then lead to long-term alterations of epigenetic patterns? Alterations of epigenetic marks at the damage site or in the chromatin domains seen as radiation-induced foci are rather well investigated. These have roles in signalling, alteration of chromatin structure to facilitate repair or coordination between different events at the DNA (Friedl et al. 2012, Wilson and Durocher 2017). For example, we could show that the mark H3K4me3, which is a mark associated with transcription activity, is lost from the gamma-H2AX foci shortly after damage reduction; at the same time repressive marks are increased and we see a loss of transcriptionally active RNA polymerase (Seiler et al. 2011, Penterling et al. 2016). If the situation is not fully reversed, we expect to occasionally see long-term alterations that should be randomly distributed in the genome.

Another possibility is that epigenetic patterns alter because the cells respond to radiation with activation or repression of specific genes. If later on the original epigenetic patterns are not restored, one would expect long-term alterations enriched at genes responding to radiation, and this has been reported in the literature (Antwihi et al. 2013).

Finally, radiation can change expression or activity of epigenetic enzymes such as DNMT1 (which has been seen, reviewed by Miousse et al. 2017) or changes in metabolism which lead to alterations in the availability of co-factors or other effects. For example, we observed after irradiation a global loss of several histone acetylation marks which is larger than expected if only the regions of DSB sites were affected (Maroschik et al. 2014). In this case, long-term epigenetic alterations are expected to be randomly distributed and rather frequent.

Conclusion

Evidence is accumulating that radiation can cause long-term alterations in epigenetic patterns. These alterations may lead to dysfunction and disease. We have to consider epigenetic alterations in addition to the well-investigated effects of radiation leading to genetic alterations, i.e. mutations and chromosome aberrations. Besides of cancer, many health effects associated with radiation exposure are known to have an epigenetic component in their aetiology, including neurological, cardiovascular and metabolic dysfunctions and diseases. So far, very little is known about dose-effect relationships, influence of LET (linear energy transfer) and factors potentially modulating epigenetic alterations.

One important question for the future is whether a causal link can be established via epigenetic patterns from irradiation to diseases. Consideration of epigenetic factors may also improve our understanding of the dose-effect relationship at low radiation doses, since there are some indications for non-linear effects.

Another question is, how lifestyle factors affect the radiation response and sensitivity. This is an important question that so far has rarely been addressed, but with regard to individual radiosensitivity lifestyle factors may be more critical than genetic variations between people. In addition, if certain lifestyle factors or epigenetic drugs present at the time of irradiation affect the radiation response, this is also an important topic for radiotherapy and the management of side effects.

Finally, it is conceivable that by lifestyle alterations the risk of radiation-induced health effects can be modulated, possibly via influence on epigenetic patterns, and this would open up an attractive method for low-level intervention (although some people find it much easier to swallow pills than to change their lifestyle).

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Note: To facilitate further reading, mainly review articles were cited. I apologize to the authors of the respective original papers for not citing their work directly.

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2 EPIGENETIC RATHER THAN GENETIC MECHANISMS MOST LIKELY UNDERLIE RADIATION-INDUCED NON-TARGETED EFFECTS

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Conceptual Basis of Non-Targeted Effects (NTE)

Our understanding of radiation effects is evolving from a mechanism driven exclusively by direct damage to DNA to one where secondarily induced DNA damage and instability, as well as intra and particularly inter-cellular communication become integral components. The development of ideas around non-targeted effects has highlighted the important role of intercellular signalling in the development of bystander effects and the triggering of genomic instability (GI). (Kadhim and Hill, 2015). Key understanding of these effects has derived from several advances in the field of radiation biology research. These include:

1. Numerous *in vitro* and *in vivo* studies indicate that, in addition to targeted effects of damage induced directly in cells by irradiation, a variety of non-targeted effects (NTE) may make important contributions to determining the overall outcome after radiation exposure. Research has shown that ionizing radiation induces complex responses, such as GI in irradiated cells as well as non-irradiated 'bystander' cells that receive molecular signals produced by irradiated cells. This effect is a well-established response both *in vitro* and *in vivo* across many mammalian systems including human (Morgan 2003a, 2003b) and is known as the bystander effect (BE). Furthermore, there is much evidence of transgenerational GI observed in the non-exposed offspring of irradiated parents (Barber and Dubrova, 2006) (Little *et al*, 2013).
2. While DNA repair and particularly repair of DNA double strand breaks is firmly integrated into the direct response of cells to radiation, recent evidence indicates modulations in the activity of key DSB repair pathways, such as non-homologous end joining (NHEJ), in bystander cells. Thus, protective bystander responses culminating to the development of adaptive response derive ultimately from the up-regulation of DNA repair activity (Klammer, Kadhim and Iliakis, 2010, Mladenov *et al*, 2018).
3. There are numerous reports of clastogenic factors in blood plasma after radiotherapy (Mothersill and Seymour, 2001) and abscopal effects (where responses are noted in tissues or organs that are not directly irradiated) by mechanisms that have properties in common with inflammatory processes (Wright, 2010). Taken together, these various non-targeted effects highlight damage arising by processes distinct from the conventional models of radiation-induced genotoxicity.
4. Evidence of NTE from technological advances, such as microbeams, (Prise *et al*, 2009) which allow radiation to be delivered into biological systems in a controlled manner and consequently the careful quantification of responses. Using these approaches, non-targeted effects have been characterised as predominantly low dose effects, and that as dose is reduced less and less, more cells will observe/receive no track traversals.

NTE responses are not universally expressed and there are several reports that GI and/or BE have not been observed in some experimental settings (Abramsson-Zettergurg *et al.*, 2000; Bouffler *et al.*, 2001; Dugan *et al.*, 2003; Terzoudi *et al.*, 2010), or in epidemiological studies (Tawn *et al.*, 2000; Whitehouse *et al.*, 2001; Kodama *et al.*, 2005; Hamasaki *et al.*, 2009). These investigations suggest that a range of factors influence the induction of radiation-induced genomic instability (RIGI) and radiation-induced bystander effects (RIBE) (Kadhim, Moore, and Goodwin, 2004). These factors, as well as their relative contributions to an endpoint under study, are not fully understood but they appear to be sufficiently complex so that results from different laboratories may appear to conflict depending on a number of variables e.g. genetic background, cell type, endpoints and time of analysis, and the type and doses of radiation (Bright and Kadhim, 2018).

Mechanistic, systems-biology models of non-targeted effects have been developed that help interpret the data, derive details on the underlying mechanisms and quantitatively test alternative hypotheses (Brenner and Little, Sachs, 2001; Nikjoo and Khvostunov, 2003; Mariotti *et al.*, 2010; Kundrát and Friedland, 2012). Mathematical modelling of epidemiological data has indicated that RIGI and RIBE do play an important role in radiation-induced carcinogenesis and affect the health risk associated with low doses of radiation (Little and Wright, 2003; Eidemüller *et al.*, 2009).

Despite excellent research in this field from eminent research groups, there are still gaps in our understanding of the likely mechanisms associated with non-DNA targeted effects, particularly with respect to systemic (human health) consequences at low and intermediate doses of ionizing radiation. Another outstanding question is if the variation in non-targeted response observed between individuals and cell lines are linked to genetic background or epigenetic effects. Furthermore, while the initial target and early interactions in cells that give rise to non-targeted responses in neighbouring or descendant cells are still unknown, numerous studies point towards an epigenetic mechanism (Schofield and Kondratowicz, 2017).

NTE: current state-of-the-art and future perspectives

Current uncertainty in assessing the risk of exposure to low dose/low dose rate ionizing radiation is due largely to a lack of knowledge of mechanisms and an understanding of human variability, attributable in part to genetic heterogeneity among individuals. Numerous *in vitro* and *in vivo* studies indicate that, in addition to targeted effects of damage induced directly in cells by irradiation, a variety of non-targeted effects (NTE) may make important contributions to determining overall outcome after radiation exposure.(Morgan and Sowa, 2015).

Non-targeted effects such as radiation-induced genomic instability (RIGI) and radiation-induced bystander effects (RIBE) are well established *in vitro* and *in vivo*; however, the precise cause of these delayed effects is unclear. Numerous studies point towards an epigenetic mechanism (Kovalchuk & Baulch 2008; Tamminga & Kovalchuk 2011; Mothersill & Seymour 2012; Merrifield & Kovalchuk 2013). Recent studies have established that the epigenetic landscape of the mammalian cell is not fixed and undergoes massive reprogramming during development (Reik, 2007). These data therefore imply that exposure to a number of environmental factors; including ionizing radiation can modify the epigenetic landscape of cell/organism (Jirtle and Skinner, 2007). As the majority of the *de novo* epigenetic marks, including DNA methylation, are faithfully reproduced during DNA replication, they are transmissible through many cell divisions and, in some cases, can be passed from parents to their offspring (Jirtle and Skinner, 2007). Analysis of the long-term epigenetic changes occurring in the irradiated parents and their offspring may contribute to clinical genetic studies of the role of epigenetic changes in human diseases, including cancer (Feinberg, 2007; Esteller, 2007). For example, the long-term epigenetic changes manifesting in the irradiated organisms can be associated with increased cancer risk. Radiotherapy-related secondary cancers are an important clinical problem (Maddams, Parkin and Darby, 2011, Yahyapour *et al*, 2018). This proposition/ suggestion is based on the observations that:

1. The expression of an instability phenotype in both irradiated and bystander populations is heterogeneous (non-clonal) in its induction and persists over many generations post exposure (Kadhim *et al.*, 2013).
2. The observed frequency of all manifestations of the induction of an instability response occurs at a higher frequency than can be expected from mutation of a single gene (Kadhim *et al.*, 1992; Morgan, 2012).
3. The observation of mutation rates in the first and second generation offspring of irradiated male mice, are significantly increased across multiple tissues (Barber and Dubrova, 2006) and show non-Mendelian segregation, suggesting that this phenomenon is attributed to an epigenetic signal arising in the paternal germ line and transmitted to the offspring (Barber *et al.*, 2002). The mechanism behind this transgenerational phenomenon is thought to be attributable to DNA damage (Dubrova *et al.* 2008; Glen & Dubrova 2012) as well as the transfer of abnormal epigenetic profiles. The offspring of irradiated mice have shown a global loss of cytosine methylation in the thymus, with a concurrent loss of proteins involved in epigenetic maintenance; at the same time elevated levels of DNA damage were observed. If the elevated DNA damage was a cause or consequence of aberrant epigenetic profiles or vice versa is unclear (Koturbash *et al.* 2006a). The appearance of GI in unirradiated offspring raises several important considerations for prospective parents particularly those that work in an occupation with increased radiation exposure. The parent-of-origin effects seen in some of these studies remain puzzling, but interpreted within the framework of genomic imprinting and our knowledge of the ontogenetic changes of genomic methylation patterns in male and female mammals, it has been suggested that the extensive remodelling of epigenetic marks in the maternal genome may contribute to the gender asymmetry in the transgenerational response (Hanna and Kelsey 2014, Marcho *et al.* 2015). A contemporary explanation for these unexpected and inconsistent observations was suggested in 1998 (Schofield 1998) drawing in threads from the then current explosion of knowledge in the field of developmental biology. The proposal was that the mechanism generating the observed pathologies, and explaining the non-clonal heritability, may be epigenetic, determined by stable and heritable modification of gene expression without the alteration of DNA sequence or classical mutagenesis (Schofield & Kondratowicz, 2017). As shown in table 1, it has been shown that excessive production of ROS and cytokine signalling are implicated in RIGI (Burr *et al.*, 2010; Wright, 2007). The persistently high level of ROS in the progeny of irradiated and bystanders cells (Matsumoto *et al.*, 2007) are currently best explained by ongoing inter- and intracellular signalling that reinforces and maintains the instability state. Bystander effects have been shown to be mediated by a variety of signalling factors including reactive oxygen species (ROS) (Lehnert, Goodwin and Deshpande, 1997; Azzam *et al.*, 2002; Lyng, Howe and McClean, 2011; Harada *et al.*, 2008); Jella *et al*, 2018), nitric oxide (NO) (Harada *et al.*, 2008), second messengers like calcium (Lyng *et al.*, 2006; Shao *et al.*, 2006), cytokines such as transforming growth factor beta (TGF- β (Iyer, Lehnert and Svensson, 2000; Shao, Folkard and Prise, 2008) and interleukins (Osterreicher *et al.*, 2003; Facoetti *et al.*, 2006; Facoetti *et al.*, 2009) and tumour necrosis factor alpha (TNF- α and tumor necrosis (TNF)-related apoptosis-inducing ligand (TRAIL) death inducing pathways (Shareef *et al.*, 2007; Luce *et al.*, 2009). In addition, cyclooxygenase-2 (COX-2) (Zhou *et al.*, 2005; Hei *et al.*, 2008), Nuclear Factor kappaB (NFkB) (Azzam *et al.*, 2002; Zhou *et al.*, 2008) and mitogen-activated protein (MAP) kinase (Azzam *et al.*, 2002; Zhou *et al.*, 2005; Lyng *et al.*, 2006) signalling have all been shown to be involved in bystander responses.
4. Recently, miRNA has also been shown to be a potential mediator of the bystander effect (Aypar, Morgan, and Baulch, 2011). Interestingly, miRNA molecules have been found in exosome multi-protein complexes that are secreted by healthy and non-healthy cells. In addition, exosomes play an important role in the induction of RIGE

and BE in human cells (Al-Mayah *et al.*, 2012, 2015, 2017). In haemopoietic tissues, the microenvironment may be especially prone to secondary damage as a consequence of inflammatory-type processes arising from the tissue response to radiation.

GI is a hallmark of cancer and it is therefore likely that this phenomenon underpins radiation-induced carcinogenesis. Since radiation carcinogenesis does not present with a particular signature and is also characterised by genomic instability, it is likely mediated by mechanisms similar to those active in carcinogenesis in general. Recently a model has been proposed in which oncogenes induce DNA replication stress, which in turn leads to DSBs, genomic instability and p53-induced senescence or apoptosis (Gorgoulis *et al.*, 2005; Halazonetis, Gorgoulis, and Bartek, 2008). This model is relevant for almost all cancer types and it is likely that low dose radiation exposures potentiate this response.

Summary of the oral presentation

Radiation response consists of targeted and non-targeted effects. Targeted effects postulate 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. In non-targeted effects, cell and tissue responses do not require direct ionising radiation deposition in nuclear DNA to be expressed. NTE include:

- Genomic Instability: de novo genetic alterations in the progeny of an irradiated cell
- Bystander Effects & Abscopal Effects: radiation-like effects in non-irradiated cells and tissues

Non-targeted effects are predominantly low dose effects (< 0.1 Sv) and typically have non-linear dose-response relationships. They are not universally expressed possibly due to influencing factors (e.g. genetic predisposition, cell and tissue type, or radiation dose and quality).

NTE are induced at higher frequency than expected for mutation in a single gene, suggesting they are caused by epigenetic mechanisms.

A relevant example is the role of Microvesicles (MV) such as exosomes in NTE through communicating the radiation bystander effect to naïve unirradiated cells & their progeny. Our studies *in vitro* showed that both RNA and protein carried by exosomes work in a synergistic manner to initiate NTE. The effects are propagated through cell signalling. Furthermore, the results from our *in vivo* studies confirmed that MV / exosomes are involved in NTE of radiation exposure *in vivo* with effect persisting in both irradiated and bystander cohorts. Also micro RNA analysis showed that >20 micro RNAs were more than two-fold differentially expressed between controls and dose points. Studies such as those reviewed here clearly establish a role for epigenetics at least in part mediated by exosomes are significantly in the NTE of radiation exposure in both *in vitro* and *in vivo* systems in NTE, however, our understanding of NTE, although rapidly expanding, is as yet far from complete.

RIGI Mediated Signals			
Bystander mediator	Inhibitor	Effect upon BE induction	Reference
ROS	N-acetylcysteine (NAC), MEK and JNK inhibitors	Prevention of growth arrest	(Macip et al. 2002) (Jella et al. 2018)
NO	Filipin (Nagasawa et al . 2002)	Activation of radioresistance among bystander cells (Matsumoto et al, 2001)	(Najafi et al , 2014) (Jella et al. 2018)
protein kinases	PKI for PKA, p21, and p16 family Cdk inhibitors	Protein kinase B and protein kinase C are involved in ROS production and oxidative damage in bystander Cells (Yahyapour et al. 2018)	(Blume-Jensen et al. 2001; Hunter et al. 2000)
miRNAs	miRIDIAN Hairpin Inhibitors	Upregulation of miRNAs due to irradiation increases serum levels of them that affect the expression of target genes in non-irradiated tissues. (Ilnytskyy et al. 2009) (Koturbash et al. 2006)	Prise et al. 2003; Chaudhry and Omaruddin 2012; Kadhim et al. 2013; Najafi et al. 2014)
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 modulation/reduced mutagenesis p53	(Zhou et al. 2001; Azzam et al. 1998) (Autsavapromporn et al, 2013)
COX-2	NS-398	Reduced DNA damage	(Zhou et al. 2005 ; Zhao et al. 2014)
Calcium	Calciclidine	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, 2017; Jella et al, 2014; Mo et al. 2018)

Table 1: RIGI Mediated Signals

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3 TRANS-GENERATIONAL EFFECTS

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Introduction

The review describes the transgenerational effects of parental exposure to ionising radiation, chemical mutagens and anticancer drugs. The epigenetic phenomenon of transgenerational genomic instability was initially defined as an increased rate of mutation observed in the non-exposed offspring of irradiated parents [1]. As according to the results of some later studies, parental exposure to a number of chemical mutagens can also destabilise the genomes of their offspring, it would appear that this phenomenon is not limited to ionising radiation. It should be noted that the transgenerational effects described in this review cannot be explained using conventional target theory, according to which mutation induction occurs almost exclusively in directly exposed cells at non-repaired and mis-repaired damaged sites [2]. Clearly this is not the case for transgenerational instability, as extra mutations arise in the genomes of offspring never targeted by mutagens. That is why the manifestation of transgenerational genomic instability following parental irradiation has recently been ascribed to non-targeted effects of ionising radiation [3]. In this review recent data on the transgenerational effects of parental exposure to mutagens will be discussed and presented.

To establish whether parental exposure to mutagens can destabilise the genomes of non-exposed offspring, the rate of mutation in their germline and somatic tissues should be established (Figure 1). This can be achieved either by mating the offspring of exposed parents and scoring germline mutation occurring in the F₁ germline among their offspring or analysing the frequency of mutations accumulated in the tissues of F₁ animals.

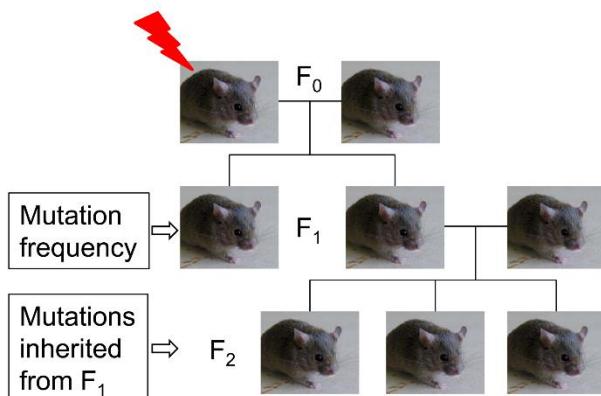


Figure 1: The design of transgenerational studies in mice. One of the parents is exposed to a mutagen and mated with a non-exposed partner. Mutation frequencies are established either in the germline and somatic of their non-exposed offspring or via mutations arising in the offspring's germline that are detected in the F₂ generation.

Early evidence

The first experimental evidence for transgenerational effects of paternal exposure to mutagens was obtained in the early '60s (reviewed in ref. 5). For example, a substantially elevated incidence of mosaicism was detected in the first-generation offspring of male Drosophila exposed to a number of alkylating agents [6, 7]. Further evidence for transgenerational effects was obtained by Luning and co-workers, who studied the frequency

of dominant lethal mutations in the germline of non-exposed offspring of irradiated male mice [8]. Although the results of these studies provided the first indication for the transgenerational destabilisation of the F₁ genome, for a long time they remained inconclusive and, unfortunately, became somewhat forgotten.

In 2000 the issue of transgenerational instability was revisited using a new technique for mutation detection in the mouse germline [9]. In this study we compared mutation rates at Expanded Simple Tandem Repeat (ESTR) loci in the germline of first-generation offspring of male mice exposed to 0.5 Gy of fission neutrons with that in control animals. ESTR loci consist of relatively short repeats, 5-10 bp long, and belong to the most unstable loci in the mouse genome with mutation rate as high as 0.05 per cell division [10, 11]. These loci were extensively used for the analysis of mutation induction in the germline of male mice exposed to ionising radiation and other mutagens [12-14]. Our study showed that ESTR mutation rates in the germline of non-exposed F₁ offspring of irradiated male mice were highly elevated, thus providing strong evidence for the manifestation of transgenerational instability [9]. These results raised a number of important questions addressed in later studies.

Questions and Answers

Is transgenerational instability strain-specific?

According to the results of numerous studies (reviewed in refs. 4, 15), it is not. In 2002 we specifically addressed this issue and showed that ESTR mutation rates in the germline of F₁ and F₂ offspring of irradiated male mice from three different inbred strains were equally elevated (ref. 16, Figure 2A). However, it should be noted that ESTR mutation rates in the germline and somatic tissues of the offspring of ♂BALB/c x ♀scid mating conceived after paternal irradiation did not significantly differ from those in controls [17]. It therefore remains to be established to what extent compromised DNA repair (double-strand DNA break repair is seriously compromised in the severe combined immunodeficient scid mice) may affect the manifestation of transgenerational instability.

Is transgenerational instability tissue- or locus-specific?

Again, it is not. Using PCR, the frequency of ESTR mutation was established in DNA samples extracted from the germline (sperm) and somatic tissues, which was more or less equally elevated in the offspring of irradiated males (refs. 17-21, Figure 2B). The analysis of a diverse set of endpoints has also shown significant destabilisation of the F₁ genome, including the yield of chromosome aberrations and mutations at protein coding genes in somatic tissues (reviewed in refs. 1, 15). Some data on somatic mutation in the offspring of irradiated male mice have provided an important insight onto the mechanisms underlying transgenerational effects. Of particular interest are the results of two studies showing that in the offspring conceived by irradiated males and non-exposed females the frequency of somatic mutation are equally elevated in both alleles derived from both the irradiated fathers and the unexposed mothers [18, 22]. Taken together, these data therefore imply that transgenerational instability is attributed to a genome-wide destabilisation.

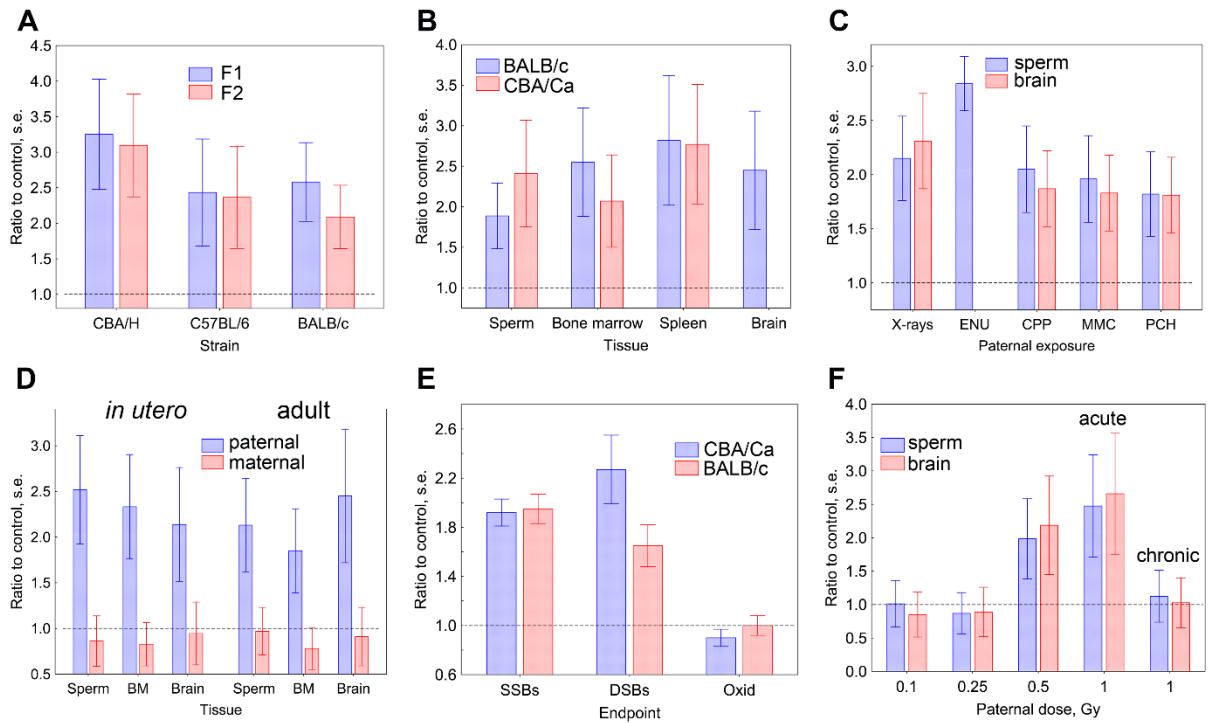


Figure 2: Transgenerational effects in the offspring of exposed mice. (A) Transgenerational instability in the germline of first- and second-generation offspring of irradiated male mice from different strains (data taken from ref. 16). (B) Manifestation of transgenerational ESTR instability in the germline and somatic tissues following paternal irradiation (data taken from refs. 18, 19). (C) The transgenerational effects of paternal exposure to ionising radiation, ENU and three anticancer drugs cyclophosphamide, CPP, mitomycin C, MMC and procarbazine, PCH (data taken from refs 18, 24, 25). (D) Transgenerational instability in the F₁ offspring of irradiated male and female BALB/c mice (data taken from refs. 19, 20). (E) The amount endogenous DNA damage in the offspring of exposed male mice, including single-strand (SSBs), double-strand (DSBs) DNA breaks and oxidatively damaged nucleotides (Oxid). Data taken from ref 18. (F) The dose-rate effects of paternal irradiation on transgenerational instability in BALB/c mice (data taken from ref. 21).

Can paternal exposure to chemical mutagens result in transgenerational instability?

Yes, it can. Given that exposure to ionising radiation produces an extremely wide spectrum of DNA lesions, ranging from base damage to double strand breaks [23], it therefore remains unclear whether a specific type of DNA damage can trigger an instability signal in the directly exposed germ cells. To this end we designed two experiments where male mice were exposed either to the alkylating agent ethylnitrosourea (ENU) or three commonly-used anticancer drugs – cyclophosphamide, mitomycin C and procarbazine [24, 25]. In contrast to ionising radiation, exposure ENU mainly causes alkylation of DNA at the N- and O- positions, resulting predominantly in base substitution [26]. The same is also true for the three anticancer drugs included in our study, which form a variety of DNA adducts and DNA cross-links [27-29]. If an instability signal is triggered by a specific subset of DNA lesions, for example by radiation-induced double strand breaks as suggested by Limoli et al. [30], then paternal exposure to some of the abovementioned mutagens should not have destabilised the F₁ genomes. However, according to our data this is not the case as the offspring of all treated males are genetically unstable (Figure 2C). These data are in line with the results of previous studies showing that exposure to some chemical carcinogens and mutagens can result in a delayed increase in mutation rate in somatic cells [30, 31] or affect the fitness of the offspring of exposed male rats [32]. Given this it would appear that acute high-dose exposure to a variety of germline mutagens can result in transgenerational instability manifesting in the offspring.

Can maternal irradiation result in transgenerational instability?

So far, the transgenerational effects of paternal and maternal irradiation have been evaluated in two studies [19, 20]. In the first study, male and female mice were irradiated *in utero* at 12 days of gestation and later mated to non-exposed partners [19], whereas the second study compared the effects of parental irradiation during adulthood [20]. Surprisingly enough, while the frequency of ESTR mutation in the offspring of irradiated males irradiated either *in utero* or during adulthood was significantly elevated, maternal irradiation did not affect stability in their F₁ offspring (Figure 2D). This was quite unexpected, as parental irradiation more or less equally affected ESTR mutation rates in the germline and somatic tissues of prenatally irradiated males and females [19]. Moreover, the F₁ male and female offspring of irradiated males show similarly elevated ESTR mutation rates in their germline [16]. In other words, despite the fact that the mutagenic effects of irradiation on the directly exposed males and females appear to be similar and the genomes of the F₁ male and female offspring of irradiated males are destabilised, the offspring of irradiated females are genetically stable. To avoid unnecessary speculation, it would be safe to conclude here that for some as yet unknown reason(s) the irradiated females fail to transmit the ‘signal’ for genomic instability to their offspring. Clearly, more work is required to further verify these results and establish the mechanisms underlying the differential pattern of transmission of radiation-induced marks through the paternal and maternal germline.

Why are they unstable?

It should be noted that, although the mechanisms underlying the phenomenon of transgenerational genomic instability remain unknown, there has been enough experimental evidence to conclude that it is most likely a result of epigenetic events. The key argument for this is that the number of offspring manifesting radiation-induced genomic instability is too high (up to 100%) to be explained by a conventional Mendelian genetics [1]. It would appear that paternal exposure to ionising radiation or other mutagens may alter the epigenetic landscape of germ cells and such marks, being transmitted to the offspring, can destabilise their genomes.

To evaluate the transgenerational effects of paternal irradiation, we measured the amount of endogenous DNA damage in the offspring of exposed male mice [18]. This study revealed an abnormally high level of single- and double-strand DNA breaks in F₁ somatic tissues (Figure 2E). Further analysis revealed that the elevated amount of DNA damage cannot be attributed either to compromised DNA repair, nor oxidative stress, as the level of oxidatively damaged nucleotides in the offspring does not significantly differ from that in controls (Figure 2E). Considering these data, it would appear that destabilisation of the F₁ genome may somehow be related to deregulated DNA replication or compromised apoptosis/cell cycle arrest. A detailed analysis of the expression profiles in F₁ tissues should elucidate the still unknown mechanisms underlying the phenomenon of radiation-induced genomic instability.

And so what?

The data reviewed here may imply that destabilisation of F₁ genomes could potentially affect a number of health-related traits in the offspring, thus representing another component of the genetic risk of human exposure to environmental mutagens. As already mentioned, the rate of dominant lethal mutations has been shown to be significantly elevated in the germline of F₁ offspring of male mice and rats, exposed either to ionising radiation [8] or the anticancer drug cyclophosphamide [32]. Important evidence for the detrimental effects of transgenerational instability was obtained in the studies that showed increased incidences of cancer among carcinogen-challenged offspring of irradiated males [33, 34]. These results are of particular interest as tumour progression is attributed to accumulation of oncogenic mutations, which can be substantially enhanced in the unstable offspring of exposed parents.

It should be stressed that quite often the animal data provide only circumstantial evidence for the potential risk of human exposure to any environmental factor. Further elucidation of the impact of transgenerational instability in humans is currently limited because the results of

recent publications of the transgenerational effects of parental irradiation are far from being consistent, showing either an elevated [35] or baseline [36] frequency of chromosome aberrations among the children of irradiated parents. It should be noted that the discrepancy in the results of these studies may be attributed to the doses of parental irradiation. To this end we designed two studies aimed to establish whether relatively moderate doses of ionising radiation or doses of anticancer drugs, both similar to those used to treat cancer patients can destabilise the F₁ genome in mice [21, 25]. As already mentioned, paternal exposure to clinically-relevant doses of mutagenic anticancer drugs results in transgenerational destabilisation of the F₁ genome (Figure 2C). In contrast, exposure to 0.1 Gy of γ -rays, the maximum dose to normal tissues per single radiotherapy procedure, does not affect the offspring (Figure 2F). Most importantly, chronic paternal exposure to 1 Gy delivered over the period of 2 weeks also failed to destabilise the F₁ genomes. It therefore appears that the amount of DNA damage inflicted over a short period of time plays an important role in triggering the F₁ genomic instability in the F₁ generation. When the doses of acute exposure to radiation and anticancer drugs reach a certain threshold, the capacity of DNA repair of the exposed cell may be overstretched and the very survival of it would require profound and, possibly irreversible, changes in the pattern of gene expression, partially attributed to the epigenetic alterations. Judging from our data, it would also appear that the manifestation of transgenerational effects in humans may more often be found among the children of cancer survivors treated by mutagenic anticancer drugs than those therapeutically exposed to ionising radiation.

Conclusions or Where Are We Now

The results of publications presented in this review show that the phenomenon of transgenerational instability is most probably attributed to high-dose paternal acute exposure to mutagens. Although they also imply that maternal exposure may not destabilise the F₁ genomes, this issue clearly requires further clarification. To establish whether these transgenerational effects can be regarded as a substantial component of the genetic risk factors for humans, we need robust human data. Finally, the mechanisms underlying this phenomenon require a thorough analysis.

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4 LONG NON-CODING RNAs: NEW MECHANISMS REGULATING SENSITIVITY TO IONIZING RADIATION

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Abstract

Human sequencing revealed that most of the genome encodes RNA transcripts which are not translated into proteins. As 70% of genetic variants associated with common human diseases are found in non-coding DNA regions, understanding the functional role of this part of the genome is a major challenge. Non-coding RNAs have been classified in two major groups according to size: small ncRNAs, including micro-RNAs, and long non-coding RNAs (lncRNAs), larger than 200 nucleotides. Lncs are now emerging as key molecules involved in numerous biological processes and in disease pathogenesis, and this review focuses on their role in human cell responses to ionizing radiation (IR). LncRNAs were shown to participate to DNA damage response (DDR), with a key role in repressing the transcription of specific genes and regulating DNA damage repair. Further studies on cancer cells and tumors also revealed that lncRNAs could participate to radioresistance and decreased radiation therapy efficacy. LncRNAs thus appear as important molecular players of cells and tissues responses to IR, but their functions are only beginning to be unraveled. Also, many questions important for radiation protection remain unexplored, such as individual susceptibility to radiation-related cancer and tissue reactions, in relation to age, gender and lifestyle.

From junk DNA to new biological players

DNA sequencing has shown that only 2% of the human genome codes for proteins, while most does not and was thus initially referred to as “junk DNA”, suggesting that this large part of RNA synthesis was only transcriptional noise emanating from RNA polymerase II infidelity. In 2012, the ENCODE project, a consortium of 32 laboratories worldwide, gathering biologists, geneticists, mathematicians and informaticians, revealed that 75% of this “junk” DNA is actually involved in the production of RNA molecules which do not code for proteins (ncRNA) but may nevertheless have regulatory functions. Another important result was that 70% of genetic variants associated with common human diseases were to be found in non-coding regions. These findings opened up new fields of research to understand the functional role of this large part of the genome.

One class of abundant ncRNAs is involved in protein synthesis machinery, notably transfer and ribosomal RNAs. Apart from these ncRNAs with known function, non-coding RNAs have been classified into two major groups according to size: small ncRNAs, smaller than 200 nucleotides in length, comprising micro-RNAs and various types of short RNAs, and long non-coding RNAs (lncRNAs), larger than 200 nucleotides.

To date, non-coding RNA databases list over 127,802 human lncRNA transcripts and 56,946 genes, which have been catalogued on the LNCipedia compendium version 5.2 (Volders, 2019; <http://www.lncipedia.org>), and this number is continually growing. The majority have not yet been functionally assessed, although various dedicated sites are devoted to relationships between lncRNAs, functions and diseases (Fang, 2018; <http://www.noncode.org/>; Li, 2018; <http://bio-bigdata.hrbmu.edu.cn/LncMAP>; Bao, 2019; <http://www.rmanut.net/lncrnadisease>; Ma, 2019; <http://bigd.big.ac.cn/lncbook>).

LncRNAs are defined as transcripts larger than 200 nucleotides, with a mean size of 19 kb. Around half are similar to messenger RNAs coding for proteins, in that they are polyadenylated, capped at the 5' end and spliced into mature RNAs, but they lack the structures necessary for translation into proteins. LncRNAs called lincRNAs have been found located within the intergenic parts of the genome, but most are transcribed as networks of overlapping sense and antisense transcripts interlaced with protein-coding gene sequences. High specificity of expression has been reported concerning developmental stage and adult tissue type, and it has been suggested that testicular and neural tissues express the greatest amount of LncRNAs of any tissue type. A major role appears to be the regulation of gene transcription, as LncRNAs can target transcriptional activators or repressors, various components of the transcription machinery, including RNA polymerase II and III, mRNA splicing, and chromatin modifiers of DNA methylation. LncRNAs show unique structural flexibility, enabling them to form loops that interact with DNA, RNA and proteins in all cellular compartments (Figure 1). As for interactions with DNA, these structural properties feature in three different functional roles of LncRNAs, acting as guides to recruit proteins to target genes, as scaffolds to bring together molecules to form complexes, or as decoys to titrate transcription factors (Figure 2). Regulation of mRNA and protein stability has also been described, as well as control of subcellular protein localization, including in extracellular vesicles and exosomes. An important function is the regulation of miRNA activity, as LncRNAs can affect their availability within the cell by acting as miRNA sponges.

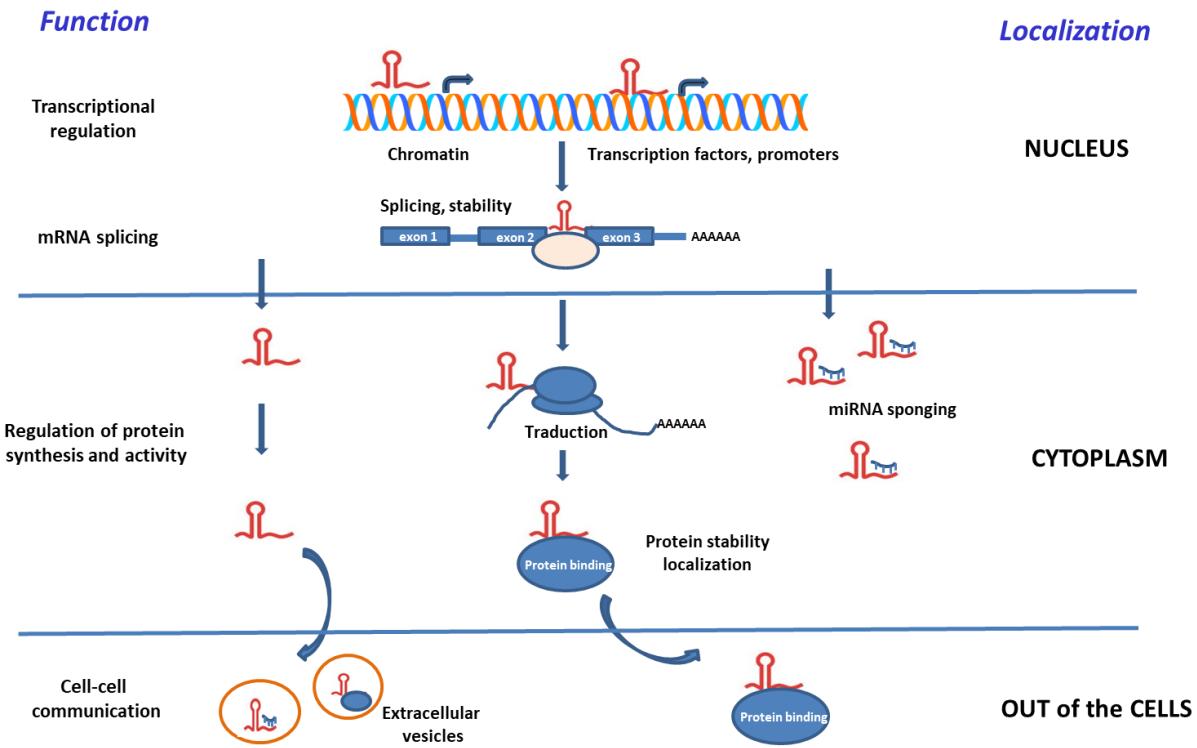
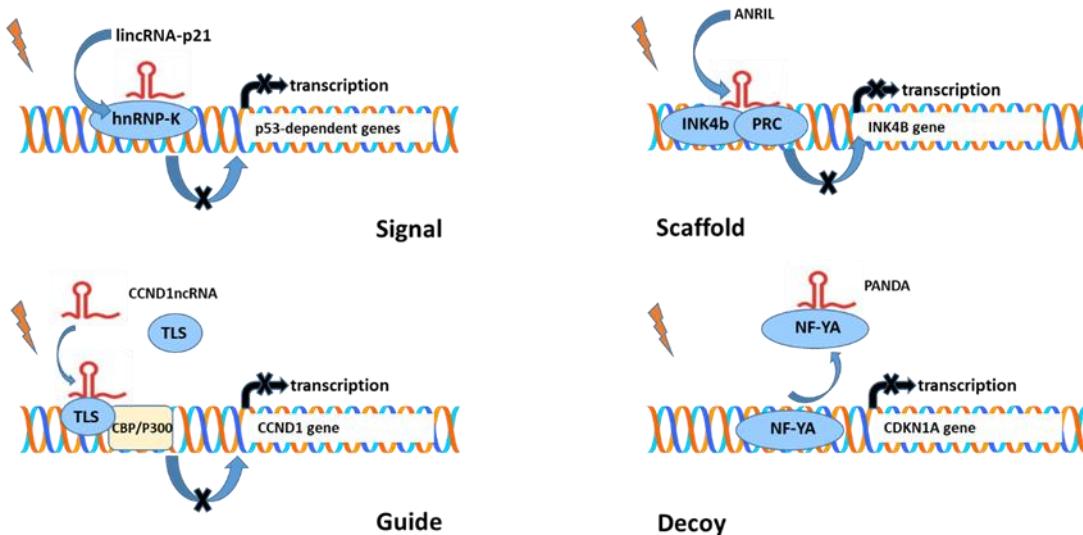


Figure 1: LncRNAs cellular localization and functions: LncRNAs show unique structural flexibility, enabling them to form loops that interact with DNA, RNA and proteins in all intracellular compartments, and in extracellular vesicles. By these interaction they can regulate gene transcription, mRNA splicing, protein synthesis and stability, and intercellular communications.

Due to their role in many biological functions, LncRNAs can contribute to disease etiology (Thapar, 2018). Aging and neurological disorders were the first diseases reported; this rapidly extended to cancer, diabetes and coronary artery disease. Cancer is now a major field of investigation, as a series of LncRNAs are involved in resistance to cancer therapy. The function of these RNA species has also been studied in relation to the effects of ionizing radiation (IR).

We will review the role of lncRNAs in three fields of research: DNA damage response, DNA repair, and human cell radiosensitivity.



Adapted from Zhang, Mut Res Rev, 2015

Figure 2: Nuclear functions of lncRNAs in response to DNA damage: LncRNAs can regulate gene transcription by different mechanisms, including by acting as signals or guides to recruit chromatin remodeling complexes to the site of damage, as scaffolds to bring together molecules that form complexes, or as decoys to titrate transcription factors

LncRNAs and DNA damage response

The first question raised was the involvement of lncRNAs in the DNA damage response (DDR) after IR exposure (review in Zhang C, 2015). DDR consists in rapid cellular processes involved in cell defense during the first 24 hours after radiation exposure. Importantly, lncRNAs are involved in most DDR processes and participate in the regulation of cell-cycle arrest, cell death and apoptosis, notably through gatekeepers such as ATM and p53. Several lncRNAs are induced directly on DNA damage, and the first to be identified is associated with the *CCND1* gene locus (Wang, 2008). Other examples are *ANRIL* (Özgür, 2013), and *DDSR1* (Sharma, 2015), both involved in the ATM-dependent response. P53 appears to be a particular target, as it regulates the expression of many lncRNAs and is regulated by many of them (Huarte, 2010; Dimitrova, 2014; Schmitt A, 2016). *TUG1*, *PANDA* and *lncRNA-p21* were shown to be involved in the p53-regulated response. Notably, *lncRNA-p21*, which is dose-dependently induced in cancer cells (Shen, 2017), can act as a repressor of the p53-dependent transcriptional response, thus repressing the expression of hundreds of p53-dependent genes, and inducing apoptosis (Hung, 2011). All these advances in knowledge led to set up new models of p53-regulated DDR. *PARTICLE* is another example of lncRNAs-related gene silencing at a global scale, notably by interlinking epigenetic machineries. This lncRNA is induced in breast cancer cells 24 hours after 0.25 Gy irradiation (O'Leary 2015), and it was shown to regulate the tumor suppressor *MAT2A* through CpG island methylation (O'Leary, 2017). Thus, lncRNAs are beginning to be recognized as important molecular players in DDR, notably by repressing the transcription of specific genes after DNA damage.

LncRNAs and DNA repair

An emerging field is the role of lncRNAs in DNA damage repair. Their involvement was markedly demonstrated for at least 20 molecular species active in homologous recombination (HR), and 5 species in non-homologous end-joining (NHEJ) (Thapar, 2018). Examples include *LINP1*, which upregulates NHEJ repair of double-strand breaks in cancer cells (Zhang, 2016), *ANRIL* and *DDSR1*, after non-IR-DNA damage (Wan, 2013, Sharma, 2015), and *SLC6A9-5:2*, targeting HR in thyroid cells (Xiang, 2017). Among the different mechanisms by which lncRNAs modify DSB repair processes, the following have been

documented: 1) recruitment of chromatin remodeling complexes to the site of DNA damage; 2) acting as a scaffold for proteins from the repair machinery, such as 53BP1, Mre11 or PARP1; and 3) sequestering miRNAs that regulate repair protein stability.

LncRNAs and radiosensitivity

One important question is the role of specific lncRNAs in radiosensitivity, either at cell level or as assessed by clinical parameters such as radiation therapy (RT) resistance. Most reports characterized the intrinsic radiosensitivity of cancer cells, in which aberrant lncRNA expression is frequently observed. *HOTAIR* is one of the best-characterized lncRNAs in cancer, as overexpression has been frequently found to correlate with carcinogenesis and poor prognosis. *In vitro* studies showed *HOTAIR* to be a molecular regulator of enhanced radioresistance in various cancer cells, and it has therefore been proposed as a new therapeutic target for the reversal of radiation therapy resistance, notably in breast and colorectal cancer (Yang, 2016; Zhou, 2017). In mice injected with HeLa cells, *HOTAIR* overexpression promoted tumor growth, whereas RT reduced the expression of *HOTAIR* in tumor tissue. These *in vivo* effects were reproduced in cultured cells, which showed the involvement of the HIF-1a pathway in these processes (Li, 2018). Many lncRNAs are overexpressed in cancerous as compared to non-tumoral tissue. It was demonstrated for *ANRIL* in nasopharyngeal carcinoma, and downregulating *ANRIL* in carcinoma cells increased radiosensitivity by enhanced apoptotic rates (Hu, 2017). In prostate patients, *UCA1* overexpression was associated with decreased 5-year disease-free survival, while *UCA1* knockdown in cancer cells significantly increased sensitivity to IR (Ghiam, 2017). In esophageal cancer, *MALAT1* inhibited the toxic effects of IR in cancer cells and the efficacy of RT in a xenograft model (Li, 2017). In thyroid cancer cells exhibiting resistance to ^{131}I exposure, low levels of *SCL6A9* (Xiang, 2017) and high levels of *MEG3* (Liu, 2018) were proposed as mechanisms of resistance. The data on patients, showing that low levels of *MEG3* were associated with reduced overall survival after ^{131}I treatment (Dai, 2018), point to the need for further investigations on *MEG3*.

One frequently described mechanism of lncRNA action is the decoy of miRNAs (Figure 1). In hepatoma cells, the lncRNA *NEAT1_2* participates in radioresistance, notably through interactions with miR-101-3p (Chen, 2019), and linc-ROR promoted radioresistance *in vitro* and *in vivo* in mice by regulating miR-145 and RAD18 (Chen, 2018). In nasopharyngeal carcinoma cells, *XIST* knock-down enhanced radiosensitivity by upregulating miR-29c (Han, 2017). In non-small-cell lung cancer (NSCLC), high expression of *PVT1* is negatively associated with poor prognosis. *PVT1* knockdown increases cell radiosensitivity by sponging miR-195 (Wu, 2017). *GAS5* has been proposed as a tumor suppressor in NSCLC; it inhibits carcinogenesis, and enhances radiosensitivity *in vitro* and *in vivo*, notably by suppressing miR-135b expression (Xue, 2017). However, lncRNAs may also directly inhibit specific proteins. For example, in glioma cells and tissue, *SNHG18* expression was upregulated, which was associated with high tumor grade. *SNHG18* knockdown suppressed the radioresistance of glioma cells, notably by inhibiting semaphoring 5A, and xenografts grown from cells with *SNHG18* deletion were more sensitive than control grafts (Zheng, 2016).

To sum up, deregulated expression and activity of lncRNAs in cancer is one of the best characterized disease involvements of these epigenetic modulators, notably thanks to the large body of data available in the cancer genomic databases. However, most studies were conducted on cancer cells, and *in vivo* relevance in tumors often remains to be established. Also, the role of lncRNAs in the radiosensitivity of various normal human tissues, in relation to age, gender and lifestyle, are all open questions, although likely to be important for radiation protection. LncRNA blood assay has been tested for the first time after total body irradiation of mice (Aryankalayila, 2018). Variations in lncRNA expression were significant after 2 and 8 Gy, but very low after 1 Gy, with an increased level of *Trp53cor1*, a lncRNA known to be a p53 target. Further work is necessary on this subject, especially regarding the effects of low-dose exposures.

Open questions

Many questions remain open. Regarding radiation-related pathologies, the role of specific lncRNAs in radiosusceptibility has been little investigated, and their role in the development of adverse reactions in normal tissue has not been investigated at all. Our group is working on relationships between genomics and individual radiosensitivity, notably related to the development of severe complications after radiation therapy. We have sequenced RNA species from a cohort of patients who developed such post-RT complications (Granzotto, 2017), and found numerous lncRNAs that were differentially expressed in fibroblasts isolated from patients as compared to healthy donors. The challenges are to find new markers of predisposition, and new mechanisms underlying individual radiosensitivity.

Other exciting fields of research are emerging, such as IR effects on lncRNA structure, as this is clearly a major parameter of lncRNA function, or the coordination of lncRNA-related regulation with other types of epigenetic control, including DNA methylation and acetylation.

In conclusion, the roles of lncRNAs in cell and tissue responses to IR are only beginning to be unraveled. While their existence and crucial importance are now beyond dispute, the long non-coding genome will no doubt continue to surprise and reveal unexpected layers of cell regulation complexity, but also open new targets for therapy.

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5 SUMMARY

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on behalf of the Working Party “Research Implications on Health and Safety Standards” of the Article 31 Group of Experts¹

Introduction

This chapter provides the rationale of the seminar, summarizes the presentations and the conclusions of the round-table discussion, and tries to emphasize the potential implications of the Scientific Seminar on “*Epigenetic effects – potential impact on radiation protection*”, held in Luxembourg on 8 November 2017. It takes into account the discussions that took place during the seminar and during the subsequent meeting of the Article 31 Group of Experts, although it is not intended to report in an exhaustive manner all the opinions that were expressed. The document has been submitted for comments to the lecturers, as far as their contributions were concerned.

The Article 31 Group of Experts and the rationale of the Scientific Seminars

The Article 31 Group of Experts is a group of independent scientific experts referred to in Article 31 of the Euratom Treaty, which assists the European Commission in the preparation of the EU Basic Safety Standards for the protection of the health of workers and the general public against the dangers arising from ionizing radiation. This Group of Experts has to give priority to the protection of health, to safety and to the development of the best available operational radiation protection. To this end, the Group of Experts is committed to proactively scanning new or emerging issues in science and technology, and ongoing developments in the area of radiation protection and informing the European Commission on potential policy implications.

In this context, a Scientific Seminar is devoted every year to emerging issues in Radiation Protection – generally addressing new research findings with potential policy and/or regulatory implications. Following suggestions from the Working Party RIHSS, the Article 31 Group of Experts selects the topic of the seminar. The WP RIHSS is charged with the preparation and the follow up of the seminar. Leading scientists are invited to present the status of scientific knowledge in the selected topic. Additional experts, identified by members of the Article 31 Group from their own country, take part in the seminars and act as peer reviewers. The Commission convenes these seminars in conjunction with a meeting of the Article 31 Group of Experts, in order to allow the Group to discuss the potential implications of the presented scientific results. Based on the outcome of the Scientific Seminar, the Group of Experts referred to in Article 31 of the Euratom Treaty may recommend research, regulatory or legislative initiatives. The European Commission takes into account the conclusions of the Experts when setting up its radiation protection programme. The Experts' conclusions are also valuable input to the process of reviewing and potentially revising European radiation protection legislation.

¹ Besides P. Smeesters (who was acting as rapporteur for the seminar), the following members of the Working Party on Research Implications on Health and Safety Standards of the Article 31 Group of Experts contributed to the preparation of this overview: H. Janžekovič, L. Lebaron-Jacobs, F. Bochicchio, F. Hardeman, R. Huiskamp, and P. Krajewski. They were assisted by S. Mundigl from the European Commission.

Key Highlights of Presentations at Scientific Seminar on Risk Communication

Anna Friedl – General Introduction to Epigenetics for Radiation Protectionists

What is epigenetics? Epigenetics is “the study of changes in gene function that do not entail change in DNA sequence and that are mitotically and/or meiotically heritable”. Protein-coding sequences of the DNA comprise less than 2% of the genome. The different forms and functions of the individual cells are not defined by the genes they possess, but the genes they express. Gene expression is regulated on several levels, for example transcription, where the genes are transcribed into mRNA, and post-transcriptional processes. Regarding epigenetic mechanisms, it is important to distinguish:

- Epigenetic mechanisms, which at each cell division are inherited by the daughter cells. They define the cell type.
- Epigenetic mechanisms that allow a cell to respond to alterations in the environment, and that can alter the gene expression pattern.

The best investigated epigenetic mechanism is DNA methylation. DNA methylation is mostly associated with repression of transcription and silencing of genes. DNA methylation is conferred by DNA methyltransferases (DNMTs), i.e. these are the “writers” of “epigenetic marks”. They are of two types: de novo DNMTs which can methylate unmodified cytosines (e.g. in early embryo, this helps to establish the different tissue types), and maintenance DNMTs. There are mechanisms to provoke but also to remove DNA methylation marks. DNA methylation and demethylation respond to metabolic alterations.

Silencing of genes must be tightly regulated, since inappropriate silencing can cause various diseases, including cancer. Cancer cells often harbour mutations in genes that code for components of epigenetic regulation.

Apart from DNA methylation there are also various histone modification of which the role for gene expression is more complex than in the situation with DNA methylation, with again a potential influence of metabolism and diet.

Modern molecular methods have also taught us in recent years that a surprisingly large part of the genome is transcribed into RNA. There are several types of non-coding RNAs, among which long non-coding RNAs that can direct histone and DNA modifications and are intimately involved in the epigenetic regulation. The role of non-coding RNAs for the DNA damage response is currently being elucidated.

It is well-established that cancer is a disease of the genes, as cancer cells carry many mutations and some people have an increased risk of cancer development because of certain mutations in their germ line DNA. However, since the early years 2000 researchers became more and more aware of the fact that cancer is also an epigenetic disease. Typically one observes a global hypomethylation in cancer cells, which may increase genomic instability. At the same time, hypermethylation at specific sites may silence genes, and there are many examples of epigenetically silenced tumour suppressor or repair genes in the literature.

Alterations of the epigenetic pattern are also associated to, and often provably causally linked with a variety of diseases such as aging, neurological dysfunction, cognitive dysfunction, behavioural/ social dysfunction, metabolic syndrome/ diabetes, cardiovascular disease, or inflammatory rheumatic disease. Many of these diseases are also relevant in the context of radiation protection.

There is accumulating evidence that nutrition and diet and probably physical exercise can affect the epigenetic pattern. Presumably alteration of epigenetic patterns plays a major role for the well-known cancer preventive activity of healthy food and exercise.

Evidence is also accumulating that radiation can cause long-term alterations in epigenetic patterns. So far, very little is known about dose-effect relationships, influence of LET (linear energy transfer) and factors potentially modulating radiation-induced epigenetic alterations.

We have to consider epigenetic alterations in addition to the well-investigated effects of radiation leading to genetic alterations, i.e. mutations and chromosome aberrations. Besides of cancer, many health effects associated with radiation exposure are known to have an epigenetic component in their aetiology, including neurological, cardiovascular and metabolic dysfunctions and diseases.

One important question for the future is whether a causal link can be established via epigenetic patterns from irradiation to diseases. Consideration of epigenetic factors may also improve our understanding of the dose-effect relationship at low radiation doses, since there are some indications for non-linear effects.

Another question is, how lifestyle factors affect the radiation response and sensitivity. This is an important question that so far has rarely been addressed, but with regard to individual radiosensitivity lifestyle factors may be more critical than genetic variations between people. In addition, if certain lifestyle factors or epigenetic drugs present at the time of irradiation affect the radiation response, this is also an important topic for radiotherapy and the management of side effects.

Finally, it is conceivable that by lifestyle or environment alterations the risk of radiation-induced health effects can be modulated, via influence on epigenetic patterns.

Munira Kadhim – *Introduction to epigenetic effects and ionising radiation*

Epigenetic rather than genetic mechanism is most likely underlying radiation-induced Non-Targeted Effects (NTE).

Radiation response consists of targeted and non-targeted effects. Targeted effects postulate 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. In non-targeted effects, cell /tissue responses do not require direct ionising radiation deposition in nuclear DNA to be expressed. Non-targeted effects include:

- ◆ Genomic Instability: de novo genetic alterations in the progeny of irradiated cell
- ◆ Bystander Effects & Abscopal Effects: radiation like effects in non-irradiated cells/ tissue

Non-targeted effects are predominantly low dose effects (< 0.1 Sv) and typically have non-linear dose-response relationships. They are not universally expressed due to influencing factors (e.g. genetic predisposition, cell / tissue type, radiation dose & quality).

NTE are induced at higher frequency than expected for mutation in a single gene, due to epigenetic mechanisms. NTE do not contradict “target theory” but contribute to a concept of an “expanding target” related to underlying biological signalling triggered by physical dose deposition.

Our understanding of epigenetics of NTE is rapidly expanding but far from complete. A relevant example is the role of Microvesicles (MV) / exosomes in NTE through communicating the radiation bystander effect to naïve unirradiated cells & their progeny. Exosomes are significantly involved in the NTE of radiation exposure in vitro. Both RNA and protein carried by exosomes work in a synergistic manner to initiate non-targeted effects of IR. The effects are propagated through cell generations and persist in the progeny of both irradiated and bystander populations. These results suggest that MV /exosomes are involved in NTE of radiation exposure *in vivo* that persist in both irradiated and bystander cohorts. The presence of a tumour susceptibility gene (TSG101) protein, a typical exosomal protein marker, has been confirmed.

Yuri E Dubrova – *Trans-generational effects*

The epigenetic phenomenon of transgenerational genomic instability was initially defined as an increased rate of mutation observed in the non-exposed offspring of irradiated parents. As, according to the results of later studies, parental exposure to a number of chemical mutagens can also destabilise the genomes of their offspring, it appeared that this phenomenon is not limited to ionising radiation. The transgenerational effects cannot be explained using the conventional target theory, according to which a mutation induction occurs almost exclusively in directly exposed cells at non-repaired and mis-repaired damaged sites. This is not the case for transgenerational instability, as extra mutations arise in the genomes of offspring never targeted by mutagens. That is why the transgenerational genomic instability following parental irradiation has been ascribed to non-targeted effects of ionising radiation: although the mechanisms underlying the phenomenon of transgenerational genomic instability remain unknown, there has been enough experimental evidence to conclude that it is most likely a result of epigenetic events.

Transgenerational instability is not tissue-or locus specific but is attributed to a genome-wide destabilisation which affects the frequency of chromosome aberrations and gene mutations.

Destabilisation of F₁ (first generation offspring) genomes could potentially affect a number of health-related traits in the offspring, thus representing another component of the genetic risk of human exposure to environmental mutagens. Important evidence for the detrimental effects of transgenerational instability was obtained in the (animal) studies that showed increased incidences of cancer among carcinogen-challenged offspring of irradiated males. These results are of particular interest as tumour progression is attributed to accumulation of oncogenic mutations, which can be substantially enhanced in the unstable offspring of exposed parents.

Animal data provide only circumstantial evidence for the potential risk of human exposure to any environmental factor. Further elucidation of the impact of transgenerational instability in humans is currently limited because the results of recent publications of the transgenerational effects of parental irradiation are far from being consistent, showing either an elevated or baseline frequency of chromosome aberrations among the children of irradiated parents.

The discrepancy in the results of these studies may be attributed to the doses of parental irradiation. Two studies were aimed to establish whether relatively moderate doses of ionising radiation or doses of anticancer drugs, both similar to those used to treat cancer patients can destabilise the F₁ genome in mice. Paternal exposure to clinically-relevant doses of mutagenic anticancer drugs results in transgenerational destabilisation of the F₁ genome. In contrast, an exposure to 0.1 Gy of γ -rays, the maximum dose to normal tissues per single radiotherapy procedure, did not affect the offspring. Most importantly, chronic paternal exposure to 1 Gy delivered over a period of 2 weeks also failed to destabilise the F₁ genomes. It therefore appears that the amount of DNA damages inflicted over a short period plays an important role in triggering the F₁ genomic instability in the F₁ generation.

In conclusion, the phenomenon of transgenerational instability is most probably attributed to high-dose paternal acute exposure to mutagens. Although maternal exposure seems not to destabilise the F₁ genomes, this issue requires further clarification.

Michèle T Martin – Long non-coding RNAs: new mechanisms regulating sensitivity to ionizing radiation

Human genome sequencing has shown that, in human beings, the major part of the genome is not coding for the protein production, hence their qualification as "junk DNA".

The ENCODE project in 2012, a consortium of 32 laboratories worldwide, gathering biologists, geneticists, mathematicians and informaticians, revealed that 80% of this "junk" DNA is in fact involved in the production of RNA molecules, but not coding for proteins. 70% of genetic variants associated to human common diseases are found out of the coding regions in the human genome.

Among the various categories of non-coding RNAs, the role of long non-coding RNAs (lncRNAs) is currently emerging. These lncRNAs are transcripts longer than 200 nucleotides, which are similar to the mRNAs coding for proteins but lacking the structures necessary for translation into proteins.

The lncRNAs show unique structural flexibility enabling them to perform organizational, catalytic and regulatory functions. They are mainly involved in regulation of transcription and play a role in various human diseases, including cancer.

LncRNAs are largely involved in DNA Damage Response (DDR) after exposure to ionizing radiation. DDR consists in rapid cellular processes involved in cell defence during the first 24 hours after irradiation. LncRNAs are involved in most of these processes through the gatekeeper's ATM and p53 (regulation of cell-cycle arrest and apoptosis). Their main function is repression of gene transcription after DNA damage.

LncRNAs are currently mostly investigated in cancer cells. A series of LNCs are involved in radioresistance and they are new possible targets to improve radiotherapy.

A lot of questions remain opened: effects of ionizing radiation on structure/functions of lncRNAs? role in radiation-related pathologies? tissue/cell type specificities? what about low-dose responses? coordination with other epigenetic regulations? markers of individual radiosensitivity?

Roundtable discussion

Anna Friedl (Moderator), Munira Kadhim, Yuri E Dubrova, Michèle T Martin, Leon Mullenders, Abderrafi Benotmane, Christelle Adam-Guillermin

The round table discussion started with three short presentations.

Chemical agents and radiation – Leon Mullenders

Chemical agents and radiation provoke stress to cells, by inducing modifications of chromatin and DNA (epigenetics) including posttranslational modifications (PTM). The induction of PTM's results in functional changes, activation or degradation of proteins. PTM's exert effects on: transcription, DNA repair, translation, noncoding DNA and apoptosis. Alteration of transcription and modification of proteins can result in silencing /overexpression of key factors in chromatin maintenance and methylation leading to radiosensitivity, repair defects and diseases (cancer, developmental abnormalities and progeria). Defective epigenetical functions have major impacts on *ionizing radiation response*, including:

- Defective DNA damage signalling after exposure to ionizing radiation
- Defective repair of DSB after exposure to ionizing radiation
- Genome instability.

CEREBRAD & BEYOND Role of epigenetic events in late effects after prenatal and early postnatal exposure to Ionizing Radiation – Abderrafi Benotman

The CEREBRAD EU project (GA 295552) for “Cognitive & CEREBrovascular effects induced by low dose RADiation” have confirmed the Japanese A-Bomb data regarding cognitive effects at dose levels below 0.1 Gy (exposure at childhood for haemangioma below the age of one year). On the other hand cerebrovascular effect in the medical cohort of childhood cancer survivors studies were in line with Mayak workers cohort with an excess risk for stroke of 0.49 per Gy. In parallel, several animal studies have been conducted to address persistent cognitive and cerebrovascular effects following embryonic and early postnatal exposure to radiation. Behavioral testing in prenatally exposed mice indicates persistent dose-dependent aberrations in learning and memory starting at 0.3 Gy and at lower dose of 0.1 Gy for more subtle tasks. Mice neonatally exposed to external radiation displayed differences in behavior starting at 0.5 Gy of Gamma-irradiation. Interestingly, co-exposure of neonatal animals to Gamma radiation with environmental toxicants (Nicotine, PBDE,) showed a more pronounced effect on behavior at lower doses, reducing thus significantly the threshold dose for induction of cognitive impairments.

At the biological level, CEREBRAD showed that the developing neocortex is, next to the hippocampus, highly susceptible to radiation. This high susceptibility has a direct impact on adult brain structure and function. Persistent effects (DNA damage, inflammation) are already observed at low doses of 20-100 mGy especially several months after exposure in mice (which correspond to years in human). In all, we believe that a dynamic interaction between multiple cell types (i.e. neurons, microglia and astrocytes) and the processing of the late response could in part be mastered through epigenetic events.

Prenatal adverse environments, such as maternal stress, toxicological exposures and viral infections, can disrupt normal brain development and contribute to neurodevelopmental disorders. Alterations in environmental conditions during development produce long-lasting and often permanent changes in the structure and function of the brain that reflect altered expression of key genes involved in neuronal development and plasticity. Increasing evidence shows that the short- and long-term effects of prenatal exposures on brain structure and function are mediated through epigenetic mechanisms. A critical moment occurs in the very beginning of pregnancy (first days) when genome-wide global demethylation occurs followed by progressive developmental epigenetic programming. These processes are vulnerable to early life stresses interfering with epigenetic programming that is crucial for normal brain maturation.

CEREBRAD metadata analysis showed that transcriptomic modifications in the brain of prenatally exposed animals to ionizing radiation are highly similar to those of Zika Virus (ZIKV) infected embryos, maternal immune activation or offspring exposed to maternal alcohol intake. These changes are mainly related to induction of p53 gene and its target genes involved in several neurogenic pathways including premature neuron differentiation. Other similarities were highlighted between radiation exposure and chemicals as Bisphenol A (an endocrine disruptor) as well as methyl mercury (where lasting DNA methylation changes are affecting the function of the BDNF gene in the hippocampus). The hippocampus is particularly sensitive to the early life environment.

In general terms, epigenetic mechanisms could play a major role in radiation protection and contribute greatly in understanding the underlying molecular mechanisms of both cancer and non-cancer effects.

Multi- and trans-generational effects in non-human biota: importance of epigenetic processes – Christelle Adam

Chronic effects in non-human biota have to be considered in a multigenerational context. To predict consequences of long term exposure, multigenerational exposure (chronic exposure over several generations with sometimes adaptation, sometimes increased sensitivity) and transgenerational exposure (only F0 is exposed, the following generations are not exposed)

have to be distinguished. Transgenerational effects are generally proven from F1 to F3 (third generation offspring).

Epigenetic mechanisms play a major role in multi- and transgenerational effects. Epigenetic effects result from interactions between genome and environment leading to phenotypic changes (adaptation, adverse effects, evolution...) and are involved in several biological processes (development, specialisation e.g. bees), and diseases.

EU-project COMET gave the first indications that methylation might be affected over several generations in different organisms both lab and field sampled. Further experiments are being conducted. For example, experiments with nematodes have shown transgenerational effects in F3 and the involvement of epigenetic mechanisms in the sensitivity and heritability of irradiation effects (histone acetylation and DNA methylation). In daphnids, significant methylation changes were observed independent of the dose rate, with a majority of hypomethylation in generations F0, F2 and F3 after F0 exposure down to 0.0065 mGy/h (Trijau et al., 2018²). Some of these changes were shared between generations, and involve gene families playing a role during exposure to ionising radiation (hsp70, rpl28).

The link between molecular process and phenotype changes (Adverse Outcome Pathway) has to be investigated. High throughput analyses are necessary to identify fingerprints of ionising radiation effects and early and sensitive biomarkers. More realistic conditions of exposure are needed (lower dose rates, more generations, use of complex systems).

² Trijau M., Asselman J., Armant O., Adam-Guillermin C., De Schampheleire K., Alonzo F. 2018 Transgenerational effects and epigenetic inheritance following a chronic external gamma irradiation in Daphnia magna. Env. Sc. Technol 52(7). 4331-4339.

6 CONCLUSIONS

Working Party on Research Implications on Health and Safety Standards of the Article 31 Group of Experts³

Why a seminar on epigenetics?

The system of radiation protection is mainly based on knowledge about cancer induction and the related radiation-induced *genetic* alterations. There is currently growing evidence that ionizing radiation can also cause *epigenetic* alterations. MELODI, the European Platform dedicated to low dose radiation risk research, considers research in the field of radiation-induced epigenetic effects as part of its priorities.

What is epigenetics?

Epigenetics is the study of changes in gene function that are mitotically and/or meiotically heritable but do not entail change in DNA sequence. These changes are at the level of gene expression and related with modifications of DNA-methylation and of histones and with non-coding RNAs regulation. DNA methylation is mostly associated with repression of transcription and silencing of genes and maintains genome stability.

Epigenetic mechanisms are involved in the development of the organisms, by defining the various cell types. But epigenetic mechanisms also allow a cell to respond to alterations in the environment, and that can alter the gene expression pattern.

These alterations can lead to dysfunction and a variety of diseases, including cancer. Cancer cells show frequently global DNA hypomethylation (then genomic instability), in contrast with hypermethylation (then silencing) of tumor suppressor genes.

Highlights and potential implications

From the presentations and the round table discussions, the members of the Working Party identified the following important issues for radiation protection and future research.

Many health effects associated with radiation exposure are known to have an epigenetic component in their aetiology, including not only cancer, but also neurological, cardiovascular and metabolic dysfunctions and diseases. As highlighted in the EU Scientific Seminar of May 2017 about emerging issues with regard to organ doses, some of these non-cancer effects have been observed at low and intermediate doses. Radiation-induced epigenetic alterations should be carefully considered in addition to the well-investigated effects of radiation leading to genetic alterations, i.e. mutations and chromosome aberrations. So far, regarding radiation-induced epigenetic alterations, very little is known about dose-effect relationships, influence of LET (linear energy transfer) and other potentially modulating factors and more research is warranted.

Epigenetic pattern may be altered by many conditions with oxidative stress, including ionizing radiation and environmental factors, but also lifestyle (diet, stress, ...). As already mentioned, these altered epigenetic patterns may lead to progressive development of diseases, but, as epigenetic alterations can be reversible, a variety of interventions, including on lifestyle factors, are hoped to revert disease development. Presumably alteration of epigenetic patterns plays a major role for the well-known cancer preventive activity of healthy food and exercise. It is conceivable that lifestyle factors may also affect or even revert epigenetic

³ The following members of the Working Party on Research Implications on Health and Safety Standards of the Article 31 Group of Experts contributed to the preparation of these conclusions: H. Janžekovič, L. Lebaron-Jacobs, F. Bochicchio, F. Hardeman, R. Huiskamp, P. Krajewski, and P. Smeesters. They were assisted by S. Mundigl from the European Commission.

alterations induced by radiation, which has potential implications, as for exposures in emergency exposure situations.

Moreover, if certain lifestyle factors or epigenetic drugs present at the time of irradiation affect the radiation response, this is an important topic for radiotherapy and the management of side effects.

70% of genetic variants associated to human common diseases are found outside the coding regions in the human genome. Among the various categories of non-coding RNAs, the role of long non-coding RNAs (lncRNAs) is currently emerging. lncRNAs are largely involved in DNA Damage Response after exposure to ionizing radiation. A series of lncRNAs could be new possible markers of individual radiosensitivity and could improve the outcome of radiotherapy.

Radiation-induced Non-Targeted Effects (genomic instability, bystander effects) are most likely driven by epigenetic rather than genetic mechanisms. Non-Targeted Effects (NTE) are predominantly observed at low dose levels (< 0.1 Sv) and typically have non-linear dose-response relationships. Our understanding of epigenetics of NTE is rapidly expanding but far from complete. New data demonstrate the role of microvesicles and exosomes carrying non-coding RNA. They are involved in cancer induction and could be considered as transgenerational messengers.

Transgenerational genomic instability is an increased rate of mutation observed in the *non-exposed* offspring of irradiated parents. Transgenerational effects cannot be explained using conventional target theory and are most likely a result of epigenetic events. This phenomenon is not limited to ionising radiation: parental exposure to a number of chemical mutagens can also destabilise the genomes of their offspring. Transgenerational instability is not tissue or locus specific but is attributed to a genome-wide destabilisation and could then potentially affect a number of health-related traits in the offspring. Although demonstrated in animals, there is currently in the available literature no consistent picture of the potential impact of transgenerational instability in humans. Current data suggest that the phenomenon of transgenerational instability is most probably attributed to high-dose *paternal* acute exposure to mutagens.

Alterations of epigenetic mechanisms play a role in late radiation-induced brain effects after prenatal and early post-natal irradiation, with some persistent effects (DNA damage, inflammation) recently observed in animal studies, even at low doses of 20-100 mGy. A critical moment occurs in the very beginning of pregnancy (first days) when genome-wide global demethylation occurs followed by progressive developmental epigenetic programming that is crucial for normal brain maturation. These processes are vulnerable to early life stresses, such as maternal stress, toxicological exposures, and viral infections. Compared to the offspring exposed to maternal alcohol intake or to infectious agents (ZIKV) the neuropsychological development and the transcriptomic modifications of those prenatally exposed to ionising radiation are highly similar.

In the context of protection of the environment, epigenetic mechanisms may play a major role in multi- and transgenerational effects in non-human biota. Chronic effects in non-human biota should be studied in a multigenerational context.

The implications of epigenetic mechanisms for radiation protection are not yet clear. Nevertheless, epigenetic alterations are part of the mechanisms leading to the radiation-induced effects on the brain and the cardiovascular system that have been observed at low and intermediate doses. The potential implications should be seriously considered, particularly for vulnerable groups such as embryos and foetus and small children. Cautiousness is already warranted for medical and occupational exposures and in the field of emergency exposure situations.

In the field of occupational exposures, we should emphasize again the need of an *early* declaration of pregnancy. In the medical field, the need to pay a particular attention for

justifying and limiting exposures from the very beginning of pregnancy and in small children should be re-emphasized. Finally, in the field of emergency exposure situations, measures of protection of pregnant women and children in the various phases, including reference levels, should be re-evaluated in the light of these new data.

More research is needed about radiation-induced epigenetic alterations, including more human and epidemiological data, with particular attention for the role of lifestyle factors and of combined exposures with environmental agents.

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