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The Role of MicroRNAs in the Cellular Response to Ionizing Radiations

Nicolas Joly-Tonetti and Jérôme Lamartine
Center of Genetics and Molecular and Cellular Physiology, CNRS, University Claude Bernard Lyon I, University of Lyon, Villeurbanne, France

1. Introduction

Ionising irradiation is a major issue in our society. Although public attention mainly focuses on it when industrial disasters or medical accidents occur, or in military contexts, it is also a permanent natural phenomenon and a powerful tool for diagnosis and therapy, notably in oncology. Understanding the action mechanisms and consequences of these radiations in living organisms is a major aim for public health and environmental conservation. Conceptions have begun to change over the past three decades. Previously, the central theory focused on the irradiated cells themselves, postulating that the biological effects are direct consequences of DNA damage when repair processes fail. More recently, however, there has been accumulating evidence that important biological consequences arise in cells with no direct radiation exposure (Kennedy et al., 1980; Nagasawa & Little, 1992; review in Wright, 2010). On the one hand, progeny cells over many generations present radiation-induced genomic instability, increasing mutation rates and chromosomal abnormality implicating reactive oxygen species. On the other hand, both neighbouring and remote cells are subject to a radiation-induced bystander effect, induced by signals from directly exposed cells *via* a variety of communication pathways. Both cell response phenomena point to an implication of epigenetic mechanisms, such DNA methylation, chromatin remodelling or small RNA regulation (Aypar et al., 2011).

MicroRNA was discovered nearly 20 years ago (Lee et al., 1993; Wightman et al., 1993). The growing family of small RNAs, including small interfering RNA (siRNA), piwi-interacting RNA (piRNA), repeat-associated-siRNA (rasiRNA) and heterochromatic small RNA (hcRNA), forms the most abundant class of endogenous RNA in metazoans, but has also been characterised in plants, unicellular algae (Zhao et al., 2007), DNA viruses (Pfeffer et al., 2004) and, controversially, in retroviruses (Klase et al., 2007). MicroRNAs are involved in numerous cellular processes such as development, viral infection, apoptosis and stress response, by regulating protein-coding gene expression at post-transcriptional and translational levels (review in Bushati & Cohen, 2007). They are also differentially expressed between tumour cells and normal counterparts in both benign proliferation and cancer (Lu et al., 2005). Due to this accumulating evidence, it is worthwhile studying microRNA response to ionising radiation, and this was started few years ago. This chapter will review microRNA studies relating to ionising radiation and discuss the perspectives in this field.

Before reviewing miRNA function in radiation response, we will begin with an account of the biogenesis of microRNAs, their mode of action in association with Argonaute protein, their function and the methods currently used to study them.

2. General information on microRNA: Biosynthesis, function and methods of investigation

2.1 MicroRNA biogenesis and microRNA ribonucleoprotein (miRNP) assembly

MicroRNA biogenesis is the cellular process producing active 22-nt long microRNA from large transcripts coded by the cell genome (review in Bartel, 2004; cf. fig. 1). Given differing microRNA profiles according to tissue and cell type and in response to environmental change, microRNA biogenesis can be seen to be a regulated process. Moreover, the bad correlation between the primary transcript synthesis rate, called pri-miR, and the mature microRNA reveals significant posttranscriptional regulation (Thomson et al., 2006).

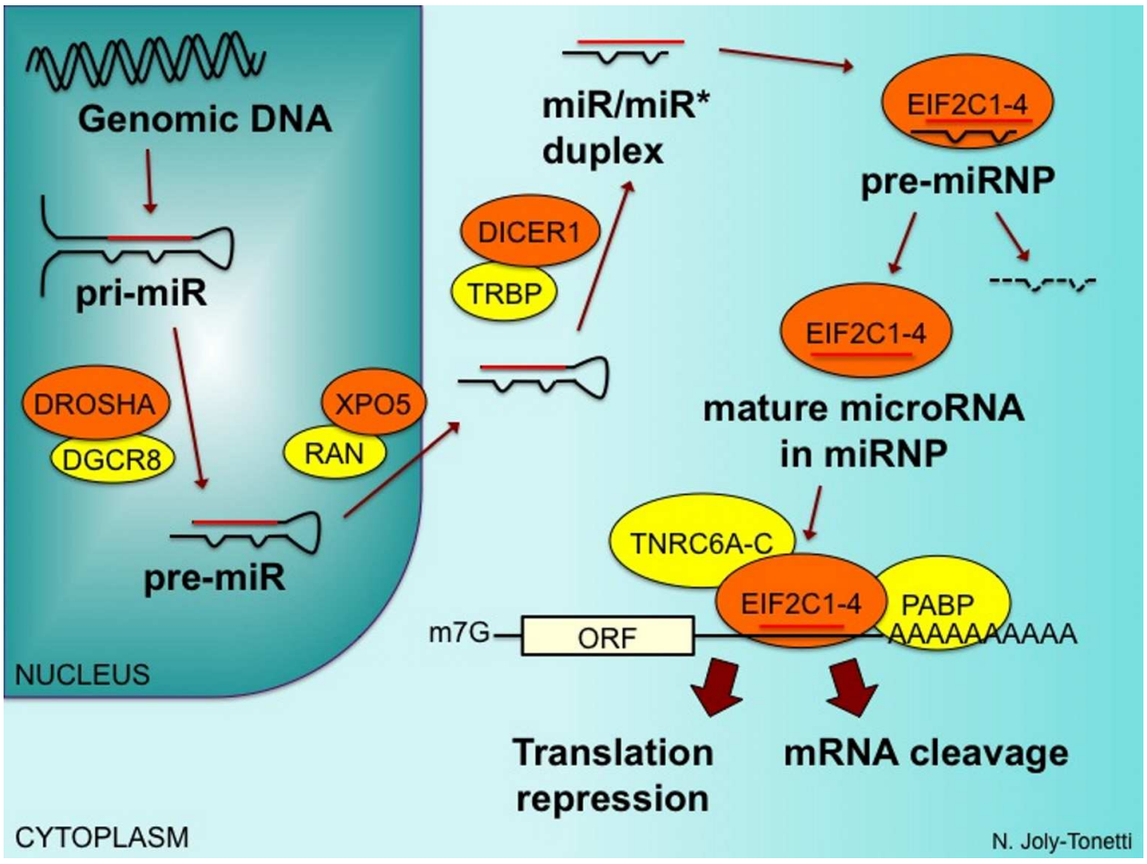

Fig. 1. MicroRNA biogenesis pathway

2.1.1 Pri-miR transcription and edition

Although a few microRNAs are transcribed by non-coding (nc) RNA associated with RNA polymerase III (Borchert et al., 2006), almost all are transcribed by RNA polymerase II (Lee et al., 2004). This primary transcript displays at least one stem-loop hairpin structure,

presenting mismatches or wobbles, which is well conserved in all animal species. Like messenger RNA (mRNA) genes, pri-miR is capped and polyadenylated (Bracht et al., 2004; Cai et al., 2004). MicroRNAs are clustered on the genome and many are transcribed in polycistron RNA. One of the most frequently studied is the miR-17-92 cluster, which encompasses 6 stem-loops and plays an important role in non-solid and solid cancer (He et al., 2005; review in Mendell, 2008). Although few data are available about their transcription regulation and promoters, some microRNA are known to respond to conventional transcription factors, such as p53 (Raver-Shapira et al., 2007; Corney et al., 2007; Xiao et al., 2011) or MYC (O'Donnell et al., 2005), and are subject to chromatin remodelling (review in Sato et al., 2011).

The sequence transcribed in the pri-miR does not always correspond to the mature microRNA because of adenosine-to-inosine editing by the dsRNA adenosine desaminase ADAR1 or ADAR2 (review in Nishikura, 2010). Both enzymes catalyse adenine hydrolytic desamination in hypoxanthine (Bass & Weintraub, 1988) which pairs cytosine, creating a non-Watson-Crick pairing I:U. This wobble affects the secondary structure of pri-miR, with consequences for microRNA maturation and action. Depending on the site of edition on pri-miR, different consequences are possible, for instance impairment of microRNA maturation (Yang et al., 2006; Kawahara et al., 2007b) or modification of the complete target mRNA list (Kawahara et al., 2007a).

ADAR1 and ADAR2 tend to edit distinct sites and are subjected to regulation since ADAR2 inhibits ADAR1 activity (Kawahara et al., 2007a) and inflammation can increase ADAR1-isoform p150 expression (Yang et al., 2003). Moreover, ADAR1 can occur on pre-miR, in nucleus or cytoplasm. This regulated ADAR-drive edition may explain edition tissue-specificity: depending on the organ, only a few or all microRNA precursors will be desaminated at various sites (Kawahara et al., 2007a; Kawahara et al., 2007b). Although a pioneering general survey of microRNA precursors found editing in 6% of the pri-miRs examined (Blow et al., 2006), *in vitro* studies showed half of pri-miRs to be editable (Yang et al., 2006). This implies that inflammation may impact microRNA biogenesis and mRNA targeting.

Canonical maturation of animal pri-miR is then a sequential two-step process that take place in the nucleus and cytoplasm, performed by two bacterial homologue type-III RNase enzymes in partnership with other proteins, with a strong intermediate check-point, respectively producing a microRNA precursor (pre-miR) and the mature microRNA.

2.1.2 DROSHA – Dependent and independent pathways to pre-miR

Most microRNA genes are located in intronic regions of protein-coding genes or in non-coding regions of the genome (Rodriguez et al., 2004; Baskerville & Bartel, 2005). Based on pre-miR end characteristics, the endonuclease DROSHA has been identified as a pri-miR trimming enzyme: it identifies the pri-miR hairpin shape and produces what is called microRNA precursor (pre-miR), approximately 70 nucleotides in length (Lee et al., 2003). DROSHA acts in association with many proteins, leading to the formation of microprocessor complexes, including in particular DiGeorge syndrome critical region gene 8 (DGCR8), the homologue of *Drosophila melanogaster* pasha protein (Han et al., 2004; Gregory et al., 2004). The DROSHA-DGCR8 multimeric complex displays the most active *in vitro* pri-miR maturation activity. DGCR8 is therefore thought to be a molecular anchor (Han et al., 2006), positioning DROSHA at 10 bp from the hairpin base for trimming. In an alternative,

DROSHA-independent process, pre-miRs are generated during mRNA maturation by the formation of a short stem-loop intron, called a mirtron, then linearised by RNA lariat debranching enzyme (DBR1) (Okamura et al., 2007; Ruby et al., 2007). The resulting pre-miR structure is similar to that released by DROSHA. Recent studies have discussed coupling between microprocessor and spliceosome, trimming pre-miR into a larger intron before splicing and enhancing intron degradation (Morlando et al., 2008; Kataoka et al., 2009). In addition, other complex partners appear to be crucial for microRNA subset expression. DROSHA associates with DEAD-box helicase p68 (DDX5) and p70 (DDX17), which are involved in processing one subclass of pri-miR (Fukuda et al., 2007). Pri-miR maturation can also be influenced by various nuclear factors in response to stimuli, such as pri-miR-21 enhancement proceeding by R-SMAD in response to TGF-β and BMP in vascular smooth muscle cells (Davis et al., 2008) or pri-let-7 family excision inhibition by LIN28 in stem cells and tumours (Viswanathan et al., 2008). P53 also associates with DROSHA and helicase p68, enhancing maturation of growth-suppressive microRNA (Suzuki et al., 2009). For half of the microRNA family, DROSHA also cuts a microRNA-offset RNA (moRNA or moR) created from sequences immediately adjacent to pre-miR (Shi et al., 2009). These are systematically generated with RNA of early evolutionary origin (Langenberger et al., 2009), massively enriched in the nucleus (Taft et al., 2010), and sometimes more expressed than hairpin-sharing microRNA (Umbach et al., 2010); their function remains unknown.

2.1.3 Nuclear export checkpoint

Like smaller shuttling RNA (Izaurralde et al., 1997), pre-miR is actively exported by nucleocytoplasmic transport in a RAN-GTP dependent manner (Yi et al., 2003). The karyopherin family protein exportin-5 (XPO5) cargoes pre-miR sequence-independently (Bohnsack et al., 2004; Lund et al., 2004) and controls the quality of pre-miR: at one end, pre-miR needs at least a 18-nt double-stranded stem for effective nuclear export (Zeng & Cullen, 2004); and at the other end, the 5′ protruding end and 3′ long end inhibit shuttle (Lund et al., 2004; Zeng & Cullen, 2004). The terminal loop structure and the distance to the hairpin base are the only parts unchecked. Moreover, exportin-5 binding protects pre-miR against degradation (Zeng & Cullen, 2004; Lee et al., 2011). Pre-miR can be subject to edition by ADAR, before exportation by its two isoforms and then by p150 (Kawahara et al., 2007b).

2.1.4 Cytoplasmic generation and loading of microRNA duplex

In the cytoplasm, a class III RNAse III enzyme, DICER1, recognises pre-miR and cuts both strands of the imperfect duplex at about two helical turns (~22 nucleotides) from the base of the hairpin, creating a short RNA duplex with 3′ 2-nucleotide overhang ends (Hutvágner et al., 2001; MacRae et al., 2006). This cleavage, less reliable than with DROSHA, creates a 3′ polymorphism for 5′-strand-generated microRNA. DICER1 associates several proteins, like TRBP (Chendrimada et al., 2005), which contribute to his function. The imperfect duplex associates ATP-dependently (Kawamata et al., 2009; Yoda et al., 2010) with Argonaute protein to form an immature complex called microRNA ribonucleoprotein precursor (pre-miRNP) or RNA-induced silencing complex precursor (pre-RISC or pre-miRISC, with reference to mRNA cleavage siRNA). Central mismatch at position 10-11 (Tomari et al., 2007) and Hsc70/Hsp90 chaperone machinery (Iki et al., 2010; Iwasaki et al., 2010) contribute to duplex loading.

2.1.5 Strand selection and miRNP assembly

There are thermodynamic differences in base-pairing stability on either side of the duplex, and the strand with the greater 5′ stability will be preferentially sorted in the effecter complex, called miRNP or RISC. Mismatches in the seed region (nt 2-8) and 3′ mid region (nt 12-15) favour strand separation (Kawamata et al., 2009). Loading is highly sensitive to the presence of phosphate at the 5′ end, which completely prevents loading if lacking. The loaded strand is called the "guide" strand, and the other, "passenger", strand is discarded. However, the loaded strand is not always the same. Evolutionary pressure on both strand seed regions and their complementary sequences on target mRNA reveals that microRNA originally from opposite strands can regulate different target genes in a coordinated fashion (Okamura et al., 2008). In a former nomenclature, when there is a predominant loaded strand in terms of relative abundance, the mature sequence is named according to the miR form (e.g., miR-17 or let-7b) whereas the opposite arm, if loaded, is called star microRNA and named according to the miR* form (e.g., miR-17* or let-7b*) (Ambros et al., 2003a). However, miR/miR* nomenclature is phased out in favour of the -5p/-3p nomenclature, the suffixes 5p and 3p referring to the 5′ and 3′ sides of pre-miR, respectively (e.g., miR-28-5p or miR-28-3p). Argonaute proteins associate with several factors in the RISC, such as GW182-family proteins TNRC6 (Landthaler et al., 2008) or PABPC1 (Huntzinger et al., 2010). TNRC6 lead miRNP localisation to cytoplasmic P-bodies (Liu et al., 2005).

2.2 MicroRNA function

MicroRNAs are fundamental posttranscriptional regulators of gene expression (reviewed in Fabian et al., 2010). Although some studies reported that, in particular situations, microRNAs can activate protein translation (Vasudevan et al., 2007; Ørom et al., 2008; Henke et al., 2008) or can be reimported into the nucleus (Hwang et al., 2006), cytoplasm microRNA-mediated gene repression, by mRNA decay or translation repression, is the general mechanism of action. How one of these two mechanisms or both is brought into play, however, is not well understood. In addition, they have been described as reducing stochastic gene expression noise (Cui et al., 2007) and as possible modifiers of gene regulation paradigms, acting *in cis* and *trans* to regulate of MRE-containing RNA (Salmena et al., 2011).

2.2.1 Mechanistic aspects of microRNA repression

In animals, microRNA-mediated gene repression involves two mechanisms: translation inhibition of target mRNA and decay of mRNA after deadenylation. Although the initial dichotomy between animals and plants highlighted translation inhibition by imperfect matching in the former and mRNA decay by perfect matching in the latter, it is now clear that animal microRNAs mainly act on mRNA stability (Bagga et al., 2005; Guo et al., 2010) whereas plants exhibit translation inhibition mechanisms without notable effect on mRNA stability (Brodersen et al., 2008).

Recent debate has focused on three possible mechanisms of target mRNA translational inhibition: inhibition of translation initiation by disturbing mRNA circularisation or ribosome assembly, or ribosome drop-off during translation elongation. MicroRNA-mediated mRNA degradation is also still in debate.

Several studies revealed that mRNA that lack a functional m7Gppp or present a non-functional ApppG cap structure or whose translation is cap-independent are less sensitive to

microRNA-mediated translational repression (Humphreys et al., 2005), possibly because miRNP can interfere with the cap recognition process at the initiation step (Pillai et al., 2005).

Another mechanism inhibiting initiation of translation may be inhibition of ribosome 80S assembly. Wang et al. (2008) mentioned 40S subunit enrichment in mRNP subjected to microRNA-mediated repression in rabbit reticulocyte lysates. Chendrimada et al. (2007) found eIF6, a 60S subunit inhibitor (Ceci et al., 2003), coimmunoprecipated with miRNP components and MOV10, the homologue of the *D. melanogaster* translational repressor Armitage. However, some contradictory data (Ding et al., 2008; Eulalio et al., 2008) highlight the importance to further investigate this mechanism of inhibition.

Mechanism of microRNA-mediated decay is a two-step process implicating poly(A) deadenylation and decapping and is dependent to GW182 family proteins. At the first step, GW182 interacts with PABP and interferes with mRNA "close conformation" loop by competition with eIF4G (Fabian et al., 2009). GW182 also recruits CCR4-NOT1 complex, including CAF1 exonuclease, to poly(A) removal (Behm-Ansmant et al., 2006). At the second step, GW182 recruits DCP1-DCP2 complex to induce decapping (Rehwinkel et al., 2005).

These various inhibition mechanisms are not mutually incompatible, and protein synthesis inhibition may be the first step or be reinforced by target mRNA decay (Fabian et al., 2009). However, nothing is known about how ionising radiation affects the process.

2.2.2 MicroRNA target recognition

MicroRNA has emerged as a protein synthesis regulator *via* translation repression and mRNA decay. In animals, microRNAs bind by base-pairing mainly to the 3′ untranslated region (3′UTR) of their target gene (Lee et al., 1993) on sites called microRNA-responsive element (MRE) (cf. fig. 2). The 5′UTR and coding region may also present MREs, but which are less efficient than in the 3′UTR (Gu et al., 2009). Except for particular MREs with good evolutionary conservation presenting almost perfect base-pairing on the entire microRNA, such as miR-196a and HOXB8 mRNA (Yekta et al., 2004), the canonical MREs constitute a sequence of perfect Watson-Crick matches between target mRNA 3′UTR and the microRNA 5′ proximal region (positions 2 to 7). Pairing for the rest of the microRNA is less stringent and more variable. This six-nucleotides sequence, called the seed region, is the strongest requirement for gene repression and rules microRNA annotation and sequence family composition. Accumulating evidence, however, has complicated this notion, as microRNA:mRNA interactions tolerate mismatch or GU wobble in the seed region (Didiano & Hobert, 2008), which can be helped by a 3′ compensatory site (Friedman et al., 2009). 3′ or centred regions of microRNA also play a role in the concert orchestrating mRNA regulation (Shin et al., 2010). Moreover, its position on 3′UTR can affect MRE activity: the functional target site is located away from the centre of long UTR and at least 15 nt from the stop codon (Grimson et al., 2007).

3′UTR sequence and structure also influence microRNA activity such as proximity to AU-rich elements (ARE) and presence of coexpressed microRNA-MRE. Secondary structure can hide MREs, depending on dsRNA binding protein (Kedde et al., 2010). Obviously, the presence of multiple MREs for the same microRNA strengthens its action. Many mRNA binding proteins (mRBP) have been shown to be involved in microRNA action: tristetrapolin (TTP) (Jing et al., 2005), APOBEC3G (Huang et al., 2007), DND1 (Kedde et al., 2007), PUF-family proteins (Nolde et al., 2007; Galgano et al., 2008) and HuR. The role of

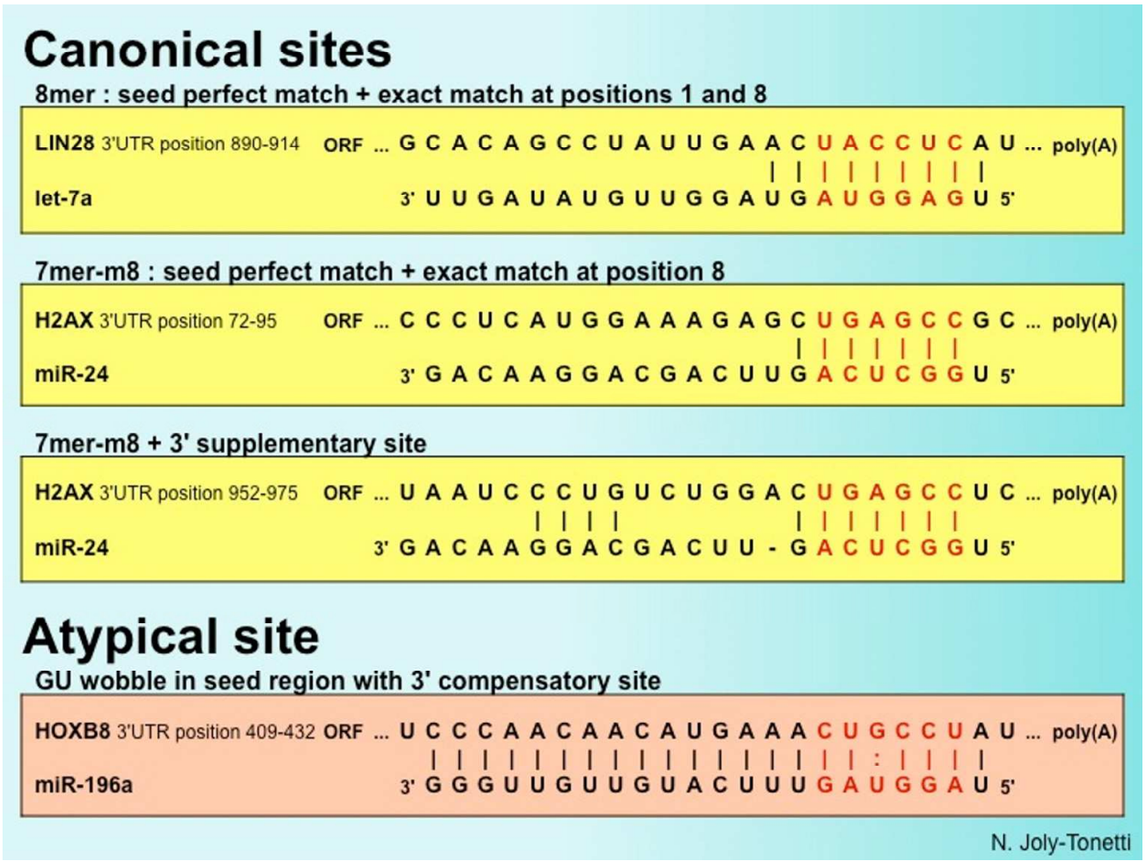

Fig. 2. Examples of microRNA-responsive elements (MRE). Seed region on microRNA and corresponding sequence on mRNA are highlighted in red.

mRNA binding protein could be ambivalent for microRNA activity, as in the case of HuR-related microRNA-mediated mRNA targeting: on the one hand, Bhattacharyya et al. (2006) showed that, in case of stress in Huh7 hepatoma cells, HuR competes with ARE-binding protein, originally directed at miR-122-mediated CAT1 mRNA repression, to address CAT1 messenger to the polysome; on the other hand, Kim et al. (2009) demonstrated that HuR binds MYC mRNA near a let-7-responsive element, enabling HuR-dependent let-7-mediated MYC mRNA decay and translation inhibition, probably by inducing secondary structural modifications of mRNA uncovering a let-7 MRE. This cell-type specific mRNA binding proteome highlights an important regulatory action mode of microRNA.

2.2.3 MicroRNA in the "competitive endogenous RNA" network

MicroRNA target regulation involves other parameters than microRNA:MRE interactions. It is now clear that the microRNA:target mRNA ratio is crucial for microRNA action: modulation of microRNA with weak expression has little impact on target mRNA; conversely, regulation of highly expressed microRNA has a more pronounced effect on the target (Arvey et al., 2010). Thus Seitz hypothesised that microRNA can be titrated by pseudo-target mRNAs, moving them away from their "true" target genes (Seitz, 2009). Another study identified pseudo-genes, the mRNA levels of which sponge microRNA targeting the corresponding gene, due to strong similarities between both 3'UTRs. This has

been proved for PTEN (Poliseno et al., 2010b; 2011), VCAN (Lee et al., 2009; 2010) and CD44 (Jeyapalan et al., 2010). This transregulation of mRNA and long non-coding RNA (lncRNA) *via* microRNA and MREs acting as morphemes, constitutes a promising hypothesis for future investigations (Salmena et al., 2011).

2.3 Methods for global microRNA analysis

MicroRNAs form an exponentially growing class of biomolecules. Hundreds are discovered each year, from a growing number of species. Since alterations in microRNA expression levels were identified in development and in cancer and constitute powerful tumour biomarkers and potential targets, scientists were initially interested in high throughput microRNA profiling. Thanks to the small length of microRNA (~22 nt) and the high homology between members of a given family, nucleic acid engineering has improved, creating highly specific modified nucleic acids (Kloosterman et al., 2006). Several techniques have been developed: bead-based flow cytometry (Lu et al., 2005), microRNA serial analysis of gene expression (miRAGE) (Cummins et al., 2006) oligonucleotide hybridisation-based microarrays (Liu et al., 2004), RT-qPCR for mature (Chen et al., 2005) or precursor microRNA (Schmittgen et al., 2004) and high-throughput sequencing (Ambros et al., 2003b).

3. Regulation of microRNA function and biogenesis during stress response

3.1 Argonaute posttranslational regulation

Argonaute family proteins have been identified in all species presenting small RNA-mediated processes and are well conserved. They are phylogenetically classified into three families. The first comprises piwi proteins, named after the *Drosophila melanogaster* protein "P-element induced wimpy testis", or piwi. These animal germline-specific proteins bind piRNA and are essential to spermatogenesis and transposon control (Aravin et al., 2006; Brennecke et al., 2007). The second family consists of *Caenorhabditis elegans* secondary Argonaute (SAGO) proteins such as Sago-1 or Sago-2. That bind the 5'-triphosphate end of secondary siRNA to amplify the RNA interference signal in a retrocontrol loop fashion (Yigit et al., 2006). The third family, which is the largest and most universal, encompasses the Argonautes, named after and resembling the founding member AGO1 expressed in *Arabidopsis thaliana* (Bohmert et al., 1998; reviewed in Ender & Meister, 2010); this family form the universal core component of miRNP. The human genome encodes 4 Argonautes, called eukaryotic translation initiation factor 2C (eIF2C) subunits 1 to 4. EIF2C1, -3 and -4 are closely clustered on chromosome 1 whereas EIF2C2 is located on chromosome 8. Only EIF2C2-coded protein (known as eIF2C2, AGO2 or hAgo2 in the literature) has an experimentally determined cleavage activity (Liu et al., 2004). Argonaute proteins are overall bilobal ~90 kDa and share three characteristic domains. The first lobe incorporates the N-terminal Piwi-Argonaute-Zwille (PAZ) domain, binding the 3'-hydroxyl end of microRNA in a hydrophobic cleft (Yan et al., 2003; Lingel et al., 2004a; Ma et al., 2004). The second lobe contains the MID domain, binding the 5'-phosphate end of microRNA in a basic uridin- and adenine-specific pocket (Frank et al., 2010) and the C-terminal PIWI domain, an RNase H module which acts as an RNA double-strand-specific endonuclease that cleaves microRNA-targeted mRNA.

Like many other proteins, Argonaute proteins are substrates to posttranslational modifications. EIF2C2 is phosphorylable on several serine, tyrosine and threonine residues (Rüdel et al., 2011), in particular *via* a p38MAPK-MAPKAPK2 axis, that leads to its proper P-

body localisation. Other phosphorylations impair microRNA association and suppress RISC activity (Rüdel et al., 2011). Argonaute proteins are also proline-hydroxylated, that is important for protein stability and effective RNA interference (Qi et al., 2008). It would be interesting to evaluate the impact of ionising radiation on such posttranslational modifications and on microRNA activity modulation.

3.2 DNA damage regulates microRNA transcription and maturation

DNA damage can regulate microRNA expression at the transcriptional level. MiR-34 family is p53-dependently induced by DNA damage. P53 binds to specific responding elements in the promoter of miR-34a and miR-34c gene and activates transcription of pri-miR-34a and pri-miR-34b/c (Chang et al., 2007; Corney et al., 2007; Raver-Shapira et al., 2007). MiR-34 family members directly repress the expression of several targets such as cyclin E2, BCL2, CDK4 and CDK6, involved in the regulation of the cell cycle, apoptosis and DNA repair. Ectopic expression of miR-34a induces apoptosis or cell cycle arrest, whereas inactivation of endogenous miR-34a inhibits p53-dependent apoptosis after DNA damage.

In addition to the miR-34 family, p53 regulates the transcription of several microRNA including miR-107 (Yamakuchi et al., 2010), miR-145 (Sachdeva et al., 2009) and the two homologous microRNA miR-192/215 (Braun et al., 2008). Ectopic expression of miR-192/215 induces cell cycle arrest. Their protein targets include transcripts that regulate G1/S and G2/M checkpoints. Altogether, these findings suggest that the DNA damage response (DDR) is able to activate a p53-dependent expression of specific microRNA that in turn play a key role in the cellular response to genotoxic stress.

Additional mechanisms by which DNA damage modulates microRNA processing and maturation have been recently described. It was reported that, in cells responding to DNA damage, p53 activated DROSHA–mediated processing of microRNA such as miR-16, miR-143 and miR-145, known to have a growth-suppressive function (Suzuki et al., 2009). More precisely, p53 interacts with the DROSHA/DGCR8 processing complex through an association with RNA helicase p68. DNA mutations in the p53 gene, frequently observed in tumours, can lead to decreased microRNA processing. Moreover, members of the p53 family (p53, p63, p73) could function as regulators of microRNA processing by direct transcriptional regulation of components of the microRNA processing complex (DROSHA-DGCR8, DICER-TRBP2, Argonaute proteins) (Boominathan. 2010).

4. MicroRNA and ionising radiation: The state of the art

MicroRNA is a new field of biological investigation. Studies of microRNA and ionising radiation in particular began less than five years ago and the number of articles published in the field is growing exponentially. Different strategies have been used in these studies: several teams tried to identify microRNA targeting proteins involved in signalling DNA double-strand breaks; others looked for miRNA biomarkers of radiation exposure or radiosensitivity. We will review the main results from these studies below.

4.1 MicroRNA and the DNA damage response: An overview

DNA double-strand breaks (DSB) caused by gamma-radiation activate a signal transduction process that leads to cell cycle arrest, followed by either apoptosis or DNA repair. Numerous proteins are involved in these pathways at different levels. Some are sensors or

mediators of DSB signalling: this is the case of H2AX, which is phosphorylated by ATM to further recruit partners such as BRCA1 and 53BP1 and form a scaffold around the DNA break site. The DNA damage signal is then transduced by ATM/ATR kinase to downstream pathways such as cell cycle, DNA repair or apoptosis (see figure 3). MicroRNA are involved in DDR regulation by targeting most of the proteins involved at each step of the DDR.
We will below briefly describe the core DDR pathway protein components and review the microRNA that can regulate DDR by targeting them.

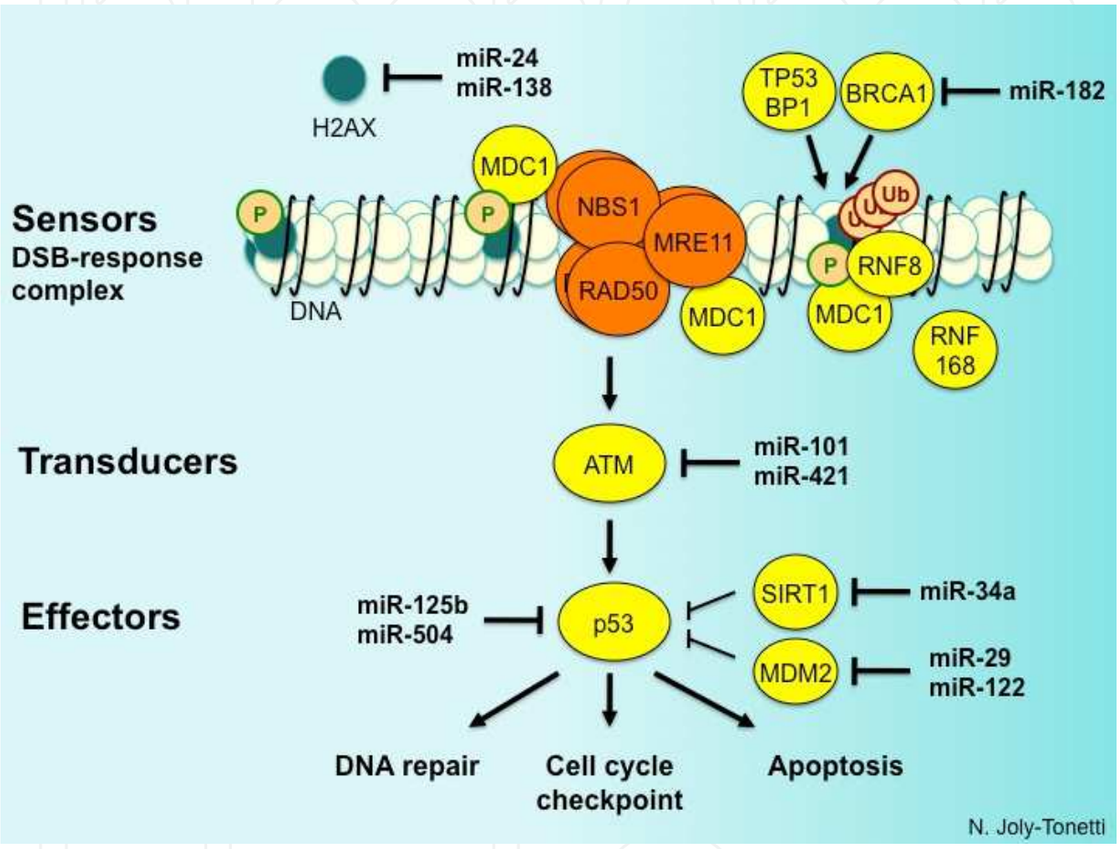

Fig. 3. MicroRNA involvement in the DSB response pathway.

4.1.1 Sensors/mediators of DSB signaling

The MRN complex composed of MRE11-RAD50-NBS1 proteins acts as a DSB sensor and recruits ATM. ATM-dependent phosphorylation of histone variant H2AX seems to be the initial signal for DDR protein accumulation. MDC1 binds to both the MRN complex and P-H2AX to further recruit ubiquitin ligase RNF8 and form a scaffold for accumulation of RNF168, BRCA1 and 53BP1 around the DSB site (cf. fig. 3).
MiR-24 has been identified as a regulator of H2AX: miR-24-mediated suppression of H2AX increases radiosensitivity in terminally differentiated hematopoietic cells (Lal et al., 2009). Another microRNA targeting H2AX has been recently identified (Wang et al., 2011): in a human osteosarcoma cell line, miR-138 reduced histone H2AX expression. Overexpression of miR-138 inhibited homologous recombination and enhanced cellular sensitivity to several DNA-damaging agents.

MiR-182 down-regulates BRCA1 expression. Antagonising miR-182 enhances BRCA1 protein levels and protects them from ionising radiation-induced cell death, while overexpressing miR-182 reduces BRCA1 protein level, impairs homologous recombination-mediated repair, and render cells hypersensitive to ionising radiation (Moskwa et al., 2011).

4.1.2 Transducers: ATM/ATR

ATM and ATR serine/threonine kinases transduce DNA damage signals to downstream proteins. ATM responds in particular to DSB, and ATR to single-strand break (SSB). ATM and ATR have global effects on many aspects of cell and organism function: a vast network of over 700 ATM/ATR targets that function in numerous signalling pathways has been revealed by a genomic approach (Matsuoka et al., 2007).
ATM expression is also under the control of microRNA: miR-421 and miR-101 are reported to suppress ATM expression by targeting the 3′ UTR of ATM transcripts (Hu et al., 2010; Yan et al., 2010). Targeting ATM through miR-101 sensitises tumours to radiation (Yan et al., 2010). On the other hand, ATM has recently been described as a regulator of microRNA biogenesis in DDR: ATM directly binds and phosphorylates KRSP (KH-type splicing regulatory protein), enhancing interaction between KRSP and pri-miR and increasing microRNA processing (Liu & Liu, 2011; Zhang et al., 2011).

4.1.3 Effectors: p53 as a core regulator

DDR effectors include DNA repair, cell cycle checkpoints (G1/S, intra-S and G2/M) and apoptosis. Several proteins are specifically involved in these mechanisms; p53 transcription factor notably regulates all pathways. P53 is involved in the control of DNA repair through direct regulation of p48 (Hwang et al., 1999; Kastan & Bartek 2004). After DNA damage, the G1/S checkpoint is essentially controlled by the ATM/CHK2-p53/MDM2 pathway. ATM/CHK2 is activated by DSB to phosphorylate and stabilise p53, which in turn induces p21 expression. P21 silences the G1/S-promoting cyclin E-A/CDK2 complex through cyclin-dependent kinase inhibition, inducing G1 arrest. P53 is also involved in the G2/M checkpoint, but here it is the cyclinB/CDK1 complex which is silenced by p21, leading to late G2 arrest (Kastan & Bartek 2004). P53 also plays a central role in DSB-induced apoptosis through transcriptional activation of pro-apoptotic factors, such as Puma, BAX, Fax and NOXA (Haupt et al., 2003).
Recent studies have demonstrated that microRNAs interact with p53 and its networks at multiple levels. MiR-504 and miR-125b were both identified as direct regulators of p53 expression: ectopic expression of miR-504 reduces p53 expression and impairs p53 function, especially p53-mediated apoptosis and G1/S cell cycle arrest in response to stress (Hu et al., 2010). MiR-125b, a brain-enriched microRNA, also acts as a negative regulator of p53. Knocking down miR-125b leads to massive apoptosis in human cells during stress response (Le et al., 2009). In addition, p53 can be indirectly regulated by microRNA targeting its upstream regulators. MiR-34a targets SIRT1, a negative regulator of p53 and then exerts a positive regulatory effect on p53 to activate apoptosis (Yamakuchi et al., 2008). MiR-122 (Yamakuchi et al., 2008), miR-29 (Park al., 2009) and miR-605 (Xiao et al., 2011) play a similar role through direct or indirect regulation of MDM2, another p53 inhibitor.

4.2 Using microRNA as a biomarker of radiation exposure and radiosensitivity

MicroRNA profiles correlate more accurately than protein-coding gene transcriptome in cancer classification, especially for tumours that are poorly differentiated or of embryonic or

unknown origin (Lu et al., 2005). Moreover, some microRNAs have also been identified as ubiquitous evolutionarily conserved stress players, such as miR-210 in hypoxia (Kulshreshtha et al., 2007; review in Chan & Loscalzo, 2010). As ionising radiation induces DNA-damage related and oxidative stress, it appears interesting to evaluate microRNA's potential as a biomarker. The goal is to use miRNA as a biomarker of radiation exposure or predictor of cell radiosensitivity. The pioneering work in this field investigated the response of TK6 human B-lymphoblastoid cell line to ionising radiation (Marsit et al., 2006). Noticing that TK6 cells exhibited greatly altered gene expression 4 hours and 6 days after 2.5 Gy gamma irradiation (Amundson et al., 2005), the authors reproduced the experimental conditions and analysed microRNA expression profile on oligonucleotide microarrays. They compared this response with 6-day folate deprivation, known to alter genomic methylation and impact cell viability (Stempak et al., 2005), and with 6-day sodium arsenite treatment, all three forms of stress being known to be carcinogenic in humans, although the link between the alterations and incidence of cancer remains to be clarified. The authors showed that gamma radiation did not usually alter microRNA expression, in contrast to a general increase after folate deprivation or sodium arsenite treatment. After 4 hours, only a dozen microRNAs, and after 6 days only a few, exhibited a non-significant >2-fold increase. In contrast, there was significant microRNA alteration after folate deprivation, confirmed *in vivo* in patients with low folate intake, making miR-222 a biomarker of low folate intake. In their model, the authors inclined towards both posttranslational and transcriptional response to radiation. Additional studies investigated potential biomarkers of ionising radiation exposure. Maes et al. (2008) crossed microRNA, mRNA and proteomic profiling, to analyse *in vitro* foreskin fibroblast response to low (0.1 Gy) and high dose (2 Gy) X-rays. SAM analysis was performed. Only a few microRNAs responded to radiation, showing weak modulation (≤2.6-fold change), confirmed by individual assays. By crossing microRNA profiles with transcriptome and proteome data, they identified potential target genes. Chaudhry et al. (2009) analysed 20 microRNAs by individual RT-qPCR. They explored two lymphoblast cells: TK6 B cell line and Jurkat acute lymphocytic leukaemia (ALL) T cell line, irradiated by 2 Gy gamma rays, and analysed their response 4, 8, 12 and 24h after irradiation. There was a 2- to 10-fold increase for a few miRNAs, with time- and cell line-dependent response. Only miR-142-5p, -3p and miR-145 were usually modulated between cell lines, but with different patterns. Small nuclear RNA RNU6-2 was used to normalise the qPCR data. Templin et al. (2011a) studied microRNA response in mouse blood 6h and 24h after 0.5, 1.5 and 6 Gy low-LET gamma irradiation and 0.1 and 0.5 Gy high-LET ^{56}Fe irradiation. Overall, they found 31 microRNAs differentially expressed; three-quarters, however, were specific to one condition and thus time-, dose- and radiation type-dependent: the results showed little overlap. Investigating blood microRNA response 4 hours after 1.25 Gy total-body X-ray irradiation in a cohort of 8 patients in leukaemia or lymphoma remission ahead of stem transplantation, Templin et al. (2011b) used RT-qPCR to identify 45 up-regulated microRNA, including 27 that were found in all patients. Their data selection, however, did not include a quantification cycle cut-off, which is essential.

By testing various cell lines, doses, radiation types and times after irradiation, numerous microRNA responding to ionising radiation were identified reviewed by Dickey et al. (2011). It seems that microRNA respond with modest modulation to ionising radiation, in a cell type-, dose- and time-dependent manner. Altogether, these data may indicate that microRNA are unsuitable as useful biomarkers of radiation exposure.

Another possible approach is to use microRNA as indicators of radiation sensitivity. It is well known that a given type of tumour can show varying radiosensitivity. Developing radiosensitivity prediction tools is important for effective tumour management. Lee et al. (2011) investigated the radiosensitivity of five cancer cell lines. Analysing the 3'UTR of EGFR, a membrane receptor frequently activated in cancer, they identified several miR-7-responsive elements. By modulating the miR-7 expression level by ectopic overexpression or by antisense inhibition, they were able to decrease or increase cell survival respectively.

4.3 MicroRNA and radiation–induced bystander effect

Another precursor study focused on the *in vivo* effect of radiation-induced genomic instability by the bystander effect (Koturbash et al., 2007). The authors irradiated rat skull hippocampus, protecting the rest of the body with a lead shield, and assessed the bystander effect in spleen at 7 months. They found global spleen cell genome demethylation, increasing transposable retro-element expression. Only one microRNA, miR-194, was significantly overexpressed, and even there the increase was less than 2-fold. There was an increase in miR-194 24 hours after irradiation, which was also less than 2-fold, in both spleen and plasma, suggesting a possible mechanism of plasma transportation.

5. Discussion

5.1 Towards a universal "radiomiR"?

There are a growing number of studies of microRNA response to ionising radiation. Results indicate that microRNA response depends mainly on cell system, LET, dose and time course after irradiation. Moreover, except in blood plasma, microRNA expression changes are too weak to be useful as exposure biomarkers. Despite some plasma blood microRNAs with a strong radiation response (Templin et al., 2011a), it has not been possible to identify a microRNA regulated independently of dose and time-course parameters. Unlike cancer or other diseases (Elmén et al., 2008) where microRNAs are powerful biomarkers (Brase et al., 2010; Heneghan et al., 2010) and potential therapeutic tools (Hausseker & Kay, 2010), there are no microRNAs that can serve as ubiquitous ionising radiation markers or radiosensitivity predictors: the universal "radiomiR" does not exist to date.

5.2 Critical review of microRNA expression studies

MicroRNA studies of ionising radiation have used large-scale analysis, mostly by RT-qPCR, compiling data on hundreds of microRNAs. Analysis of published results raises some questions. Firstly, a variety normalisation methods have been used, some of which are questionable. One or two small nuclear RNAs are used as reference gene, without justification by rigorous calculation (Vandesompele et al., 2002). Inconsistent choices of RT-qPCR normalisation in large-scale or individual assays can induce systematic bias in results and false interpretation. Secondly, the absence of a cut-off threshold in RT-qPCR analysis can lead to wide variation in estimations of microRNA abundance. The most common error in analysis of RT-qPCR results is the absence of a quantification cycle (Cq) threshold beyond which there is no repeatability. For example, for TaqMan Low Density Arrays, we and others (Gougelet et al., 2011) calculate a cycle threshold at Cq=32: above this limit, microRNA and control snRNA Cq repeatability is poor, whatever the cell type, radiation condition or time course, setting a technical limit to microRNA detection.

When studies identify microRNAs responding to radiation, little is done to identify the target gene and response mechanisms. In most studies, microRNA expression is inversely correlated with bioinformatically predicted target-mRNA or protein expression profile, without further validation by conventional methods, which are hard to implement because of weak post-radiation microRNA regulation. This may explain why gene ontology analysis of the identified regulated genes finds very divergent time- and dose-dependent microRNA-associated mechanisms (Maes et al., 2008).

Concerning the radiation-induced bystander effect, studies reported no overall change in microRNA profile. Only miR-194 was identified as up-regulated after ionising irradiation, with a less than two-fold increase whether a few hours or several months after exposure, in both blood and spleen, suggesting a possible mechanism of plasma transportation (Koturbash et al., 2007). By bioinformatic target analysis, the authors correlated DNA methyltranferase and methyl-binding protein down-regulation with the increase in miR-194 found in their results, but without further evaluation. To date, no miR-194 target has been experimentally confirmed in any organism. However, another study, analyzing sex-specific response, was not so clear about miR-194 up-regulation (Koturbash et al., 2008): the authors observed miR-34a up-regulation and presented this as a good response to ionising radiation, because of miR-34a's role downstream of p53. However, this role is not clear (Zenz et al., 2009) and if it has even been proved that p53 is up-regulated in this system (Koturbash et al., 2008) it can also have another role such as induction of senescence (Christoffersen et al., 2010) depending on temporally induced isoforms. Moreover, the spleen is a heterogeneous organ, including immune cells in the pulps, muscle and mesothelial cells of the capsule, and endothelial cells contouring spleen vessels. Whole-organ analysis averages individual cell-type responses, thus possibly masking microRNA regulation.

5.3 Future challenges

Technical tools for microRNA study are now powerful and cheap enough to allow elegant work on ionising radiation response. Scientists must now converge on analytical methods to produce reliable results. Radiation-induced genomic instability causes long-term effects, notably implicated in cancer development. One hypothesis suggests that ionising radiation induces potentially unstable chromosome regions (Suzuki, 1997) prone to delayed DNA breakage. As a majority of microRNA loci are on or near fragile chromosome sites (Calin et al., 2004), subject to deletion or rearrangement in cancer, and constitute powerful diagnostic, metastatic and prognostic markers (review in Koturbash et al., 2011), long-term surveys of irradiated cells will be crucial to understanding the steps of ionising radiation-mediated carcinogenesis. It now appears clear that crossing only microRNA expression profiling and mRNA and protein profiling restricts knowledge to a competitive-endogenous RNA hypothesis (Salmena et al., 2011). Absence of data on pseudo-genes and long non-coding RNA, sometimes some of the most regulated RNA between conditions (Panzitt et al., 2007), biases analysis of microRNA regulatory networks. Integration of all long and short RNA and protein profiling will need to bridge a major gap, using the most recent techniques of sequencing, to achieve a true characterisation of cell situation.

6. Conclusion

Little is known about the microRNA response to ionising radiation. It is now clear that "radiomiR" (i.e., a clear ubiquitous radiation biomarker and radiosensitivity predictor) does

not exist. However, future studies, by increasing evidence about microRNA response to radiation, may specify cell-, dose-, time-, sex- and radiation type-related response profiles. Not only microRNA expression but also all aspects of the microRNA and mRNA-binding proteome pathways must be investigated to understand the epigenetic contributions to ionising radiation effects.

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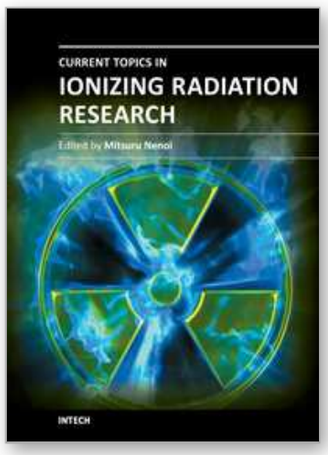

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Since the discovery of X rays by Roentgen in 1895, the ionizing radiation has been extensively utilized in a variety of medical and industrial applications. However people have shortly recognized its harmful aspects through inadvertent uses. Subsequently people experienced nuclear power plant accidents in Chernobyl and Fukushima, which taught us that the risk of ionizing radiation is closely and seriously involved in the modern society. In this circumstance, it becomes increasingly important that more scientists, engineers and students get familiar with ionizing radiation research regardless of the research field they are working. Based on this idea, the book "Current Topics in Ionizing Radiation Research" was designed to overview the recent achievements in ionizing radiation research including biological effects, medical uses and principles of radiation measurement.

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Slavka Krautzeka 83/A
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Phone: +385 (51) 770 447
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www.intechopen.com

InTech China
Unit 405, Office Block, Hotel Equatorial Shanghai
No.65, Yan An Road (West), Shanghai, 200040, China
中国上海市延安西路65号上海国际贵都大饭店办公楼405单元
Phone: +86-21-62489820
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