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Chapter XX: RNA-micro, siRNA, and Other RNA Moieties Involved in Radiation Biology

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There are two major areas of current interest of RNA (ribonucleic acid) in the exciting and rapidly developing field of Radiation Biology: 1) The role of transcriptomics in the ionizing irradiation response; and 2) The new language of miRNA.

Transcriptomics

Transcriptomics (or display of all the RNA moieties that are transcribed from cellular DNA at one particular time) is an area of research, which deals with the cellular transcriptome, or simply stated all the different RNA species that are detected in an assay, which displays all moieties of RNA in a cell and also shows the relative abundance of each different RNA formed under a specific condition and a specific time. As with many other areas of Basic Science reviewed in this web-textbook, there is a revolution in technology with availability of kits from various companies to allow researchers to acquire massive amounts of data from experiments with cells in culture or extracted from organs of experimental animals. This technology has changed the magnitude of data in current publications, but also presents a challenge. Single cell transcriptomic is now possible and there are many recent papers published on this topic. The challenge in radiobiology is simply what to study and why? The discovery of reverse transcriptase (1, 2) allowed scientists to make copies of individual RNA molecules in nanogram or microgram quantities and display these data in elegant maps of relative abundance called "heat-maps". The term heat-maps arose, because hundreds of different RNA species could be shown in a pattern and grouped on a large chart with those in great abundance colored in red, and those with less abundance colored in green suggesting that heat indicates more (3).

The rt-PCR (real time polymerase chain reaction) technology allows investigators to search for relative abundance of specific RNA moieties by using primers that are specific for each RNA and allow amplification analogy of relative abundance.

The technology for all of these measurements builds upon the original assay system for RNA, namely, the Northern Blot (4).

Northern blotting techniques followed the first described "blot" for detecting relative abundances of DNA, whether from a viral pathogen, or from the cell itself and were called Southern Blots. When the technique was applied to RNA, the geographic designation of Northern was attached. Then, followed that blotting techniques for protein called Western blots. All of these "blot" techniques are gels, which show the abundance of a particular moiety, as a line in a gel electrophoresis display with a control being used for a "housekeeping" gene or mRNA, or protein choosing one that would be unchanged by the conditions being studied. Common housekeeping genes for RNA assays in Northern blots are transcripts of those for functions that are not affected by conditions of an experiment, common ones used for measuring the relative abundance of particular RNA include: glucose-6-phosphate isomerase, or other RNA molecule for an RNA translating a protein that is involved in the Krebs cycle or other basic metabolic functions.

The Northern Blot was cumbersome, required relatively large amounts of each specific RNA, and required specific gel electrophoresis patterns. Investigators could study only small numbers of RNA molecules and rarely presented data on more than ten on an individual blot.

Advances in technology provided automation of RNA analysis with several companies manufacturing and providing rt-PCR (real time-polymerase chain reaction) machines to rapidly process and display heat-maps of hundreds of different RNA moieties. The different products, equipment, and associated software for display of data are all available in the Materials and Methods sections of recent Radiation Biology publications (5). Because of the relevance of RNA measurement techniques and automation, the most available "kits of reagents", machines for running rt-PCR, and software displays, it follows that the mRNA moieties, which those that are translated into proteins were studied first. These represent messenger RNAs. Furthermore, sets of primers and kits available by several companies provide resources for investigators to focus on those sets of RNA primers for the rt-PCR reaction usually those, which are most commonly used by customers.

For rt-PCR, those companies that supply primers provide extensive lists of those moieties for investigators. For both new and veteran radiation biologists, the sets of primers for rt-PCR reactions usually focus on those most commonly used in current Radiobiology, namely, including those involved in transcription of stress response genes, inflammatory cytokine genes, and others associated with cell differentiation. Recent interest in quiescence and senescence has motivated companies to add those primer sets. Genetic functions associated with quiescence or senescence are now available since these biological processes are very much in focus. In experiments to study radiation carcinogenesis or leukemogenesis, rt-PCR reactions for oncogenes, or emeriogenes (genes which quiet down the malignant transformation steps) are also available. All of these assays for RNA measure molecules that have already been known and described. The primers for each RNA are commercially available, because that particular RNA has already been established in having relevance to a particular area of science. Those investigators wishing to look for mutations, changes in RNA, and new RNA splice variants, should read the chapter in this textbook on DNA sequencing and understanding the techniques available for comparing changes in two different DNA or RNA species. Such technology is required to identify point mutations, as well as, other changes in DNA, which alter RNA.

Splice-variants in a particular RNA also can complicate an analysis of a specific RNA using the techniques of transcriptomics. Because of the recent emphasis on the microbiome, and understanding interaction of both bacterial and viral pathogens with human cells following ionizing irradiation, specific kits and reagents are available to study the 16 S-size RNA typical of bacteria (6). Microbiologists have utilized these microbiome related techniques and reagents widely to understand the adaptation of specific organisms to changes in their environment development of antibiotic resistant strains, and to study of interactions of different bacterial species, as well as the identification of new bacterial or viral strains. These techniques are also available for rapid molecular diagnosis in the case of infections. Rapid diagnosis of a pathogen by rt-PCR allows physicians to start antimicrobial treatment without waiting 24 to 48 hrs. to diagnose a pathogen by standard bacterial colony growth in agar assays in vitro.

The field of transcriptomics is also widely applied to many disciplines including forensic medicine, paleoanthropology, zoology - identification of new species, multiple other disciplines of modern zoology, and evolutionary biology. The RNA that is studied by rt-PCR reactions and the display of overall biological responses using heat-maps is usually targeted to transcribed

RNA, or the RNA that produces protein. Proteins are involved in all aspects of cellular physiology including those that deal with lipids, glycolipids, and other non-protein signaling pathways in Radiation Biology, so their mRNA is critical to study. The protein enzymes, which produce the different moieties of lipids, both oxidized phospholipids and oxidized lipid, are protein enzymes. For example, when determining how a particular signaling lipid may arise, such as hepoxillin-A3 in the intestinal response to irradiation (7), one would be obligated to study the enzyme, which generates this lipid moiety, namely, calcium independent phospholipase-A gamma (which is a protein). In the absence or depletion of this enzyme, calcium independent PLP-Aɣ, there would be less of the product Hepoxillin-A3, a particular signaling lipid.

Practical applications of RNA measurements, and patterns of RNA transcription, coupled with the rapidity of data production by automation, allow radiation biologists to observe the kinetics of response of specific tissues or organs to ionizing irradiation. As an example of a study method for analysis of radiation damage to the lung, total body irradiated or thoracic irradiated mice may be sacrificed at different time points, lung removed, RNA extracted, and rt-PCR carried out with a spectrum of primer sets to compare the relative abundance of RNA for many signaling pathways. These technologies allow great advances in data collection and presentation.

In the 1990s, the study of RNA induction in the irradiated lung was limited to the number of RNA molecules that could be measured in small numbers (for example, TGF-β, IL-1, TNF-α), and publication reported differences in the levels of these RNA moieties over time after irradiation (8). By 20 years later, the availability of rt-PCR "machines" allowed investigators to observe and quantitate the changes in large numbers of RNA molecules over time, and with ability to provide relative comparisons of up or downregulation of specific molecules (9). RNA grouping could be carried out looking for those associated with separated cell phenotypes, to study functions: endothelial cell function, other cell types showing the spectrum of different types of collagen including those involved in radiation fibrosis, and RNA groups for genes associated with basic promoter functions for the RNA for a protein that activated many other in transcription such as NFKβ, Nrf2, SP-1, and AP-1 (9).

The study of RNA, is the study of the response of cells, cell lines, and organisms to a particular stimulation or lack of stimulation.

Single cell transcriptomics represents the ultimate use of RNA analysis. One area in which single cell transcriptomics has been very powerful is in study of sorted cell populations from explanted tissues, where the cell populations are available in very low abundance. For example, identification of the true totipotential hematopoietic stem cell has traditionally started with multiple color, fluorescent activated cell sorting, and with 7 different fluorochrome antibodies producing cells that are in relative abundance of 1 per 10,000 or 1 per 100,000 cells sorted (11). These individual cells are then analyzed using rt-PCR for their transcriptome. The current state of research in this area suggests that multiple specific RNA transcripts are associated with the quiescence or non-dividing state of true stem cells, and that the oxidative stress response of such quiet cells may be related to their ability to retain an undifferentiated state (12). For the radiobiologist, the ability to study the effect of irradiation on the transcriptome of individual stem cells in the bone marrow, but also in other tissues provides a valuable research tool. New

studies may explain how surviving quiescent cells in an irradiated environment display in their progeny mutations that appear in later months or years and go on to generate malignancy. Perhaps, the most serious side effect (late effects) of ionizing irradiation is carcinogenesis.

The Revolution in Study of Small Interfering siRNA and microRNA (miRNA)

The recognition that mRNA, or RNA was translated into protein, represented only a fraction of the actual transcribed RNA from mammalian cells was revolutionary (13). Scientists rapidly focus on the potential role of these other RNA moieties. Much of this interest followed the study of antisense RNA in the 1980s and 1990s by scientists seeking to use such technologies as a therapeutic approach (14). Antisense RNA research was the creation of an opposite strand of RNA and using this as a therapeutic in experimental studies, and then clinical trials (15). Scientists sought to neutralize a specific RNA before it could be transcribed into protein. For example, if an oncogene such as C-RAS were transcribed into large quantities of RNA and this was associated with induction of a specific form of non-small cell lung cancer, scientists suggested that using an antisense RNA for C-RAS might neutralize the signaling by this RNA, and thus, stop the signaling associated with induction of new malignant cells (15). Antisense RNA technology was used to develop therapeutics for head and neck cancer including that associated with the C-RAF oncogene (16). By synthesizing an antisense RNA and observing its therapeutic effect, as well as biological effect in culture, investigators discovered that other forms of RNA in addition to mRNA, which is not translated into proteins, yet existed in cells. A spectrum of smaller RNA moieties termed microRNA were discovered. A list of these was developed, and with the automation tools already available for rt-PCR, microRNA transcriptomics specific to the subset were developed (17).

Experiments with cell lines and then with small animals demonstrated that delivery of therapeutic quantities of a particular antisense RNA or microRNA (miRNA) could enhance or depress the transcription of a specific messenger mRNA and produce a measurable effect. MicroRNA molecules were then defined as the potential therapeutic, which could regulate the relative levels of RNA for specific function. Several review articles have been published on the numbers and diversity of microRNA molecules available, and the technology for study of them (18). Many microRNA moieties are known to be associated with specific radiobiologic functions. Available evidence indicates that cells produce microRNA molecules, as one of several other “regulators” of DNA transcription. These microRNA molecules can be viewed as regulators of speed amplification of transcription. MicroRNA molecules that suppress relative levels of DNA transcription could be viewed as creating a condition for driving, which would be like deep snow and icy road conditions forcing a slowdown (19). Other microRNA molecules that would speed the diversity and strengthen the transcription could be viewed as getting conditions of a four-lane highway with excellent weather conditions. Balance of microRNA molecules serves as a rheostat that govern transcription.

Other forms of RNA have also been shown to function as rheostats in transcriptomics for mRNA. Transfer RNA tRNA led to small RNA molecules, which deliver amino acids to the peptides involved in protein synthesis at the endoplasmic reticulum in cell sites of protein elongation. Alterations in tRNA molecules, with both respect to level of abundance, and function, can slow or accelerate protein translation (20). tRNA molecule deliver amino acids to

the sites of protein synthesis in the microsome, which consists of microsomal RNA. While less is written about the role of these forms of RNA in regulating protein production, these are also areas where routine translation can be regulated.

Small RNA molecules with yet additional functions have recently been discovered (21). These small RNA molecules also serve to block specific transcripts by acting at the level between RNA polymerase attachment to DNA in the formation of mRNA. Thus, multiple levels of RNA moieties that interfere with mRNA, serve as regulators for the relative abundance of proteins.

One approach to considering the role of these other classes of RNA molecules with respect to creating the function of the specific mRNA (which can be quantitated by rt-PCR as described above) is to go directly to measurement of proteins and protein function.

The chapter in this textbook dealing with proteins and proteomics describes techniques for measuring relative abundance of proteins. In a sense, this step would “bypass” the complexity of microRNA, other forms of small interfering RNA, and the regulation of transfer RNA and microsomal RNA for translation. However, the radiobiologic effects of alteration in these rheostats (control of RNA transcription at multiple levels) must be considered. Investigators should be aware of the existence of these forms of regulators of RNA and possible explanations for the common observation that irradiation induction of a particular mRNA is not associated with detection of increased quantities of the protein produced by that RNA. Ionizing irradiation induced changes in many of these “rheostat control” mechanisms for regulating conditions under which a mRNA can actually arrive at the site for translation into proteins.

One great advantage of all the current automation technologies is that the investigator can track specific pathways over time and gain valuable information as to the complexity and variability of the other RNA molecules that are involved in a specific function.

As with other aspects of radiation biology (and science in general), specific hypothesis should be generated, and experiments designed to test these hypothesis. One criticism common to reviewers of grant applications is that the investigator is on a “fishing expedition”. The transcriptomics technologies that are now available allow one to expand the fishing pond into an ocean for fishing. Investigators would be wise to use these technologies in an appropriate fashion. It is always a good idea to form a hypothesis and design experiments to answer a specific question before purchasing expensive equipment and starting large experiments.

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