

## MicroRNA responses and stress granule formation modulate the DNA damage response

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**D**NA damage induced by UV irradiation provokes profound changes in gene expression. Both transcriptional regulation and posttranslational modification of proteins have been known for many years, but the involvement of microRNAs in regulation of mRNA translation has been described only recently. This level of gene expression regulation appears to operate at the intermediate time points between fast protein modifications (within minutes) and much slower transcriptional reprogramming (which takes several hours to days to develop). MicroRNAs most clearly contribute to regulation of cell cycle checkpoints and apoptosis, but may also influence other aspects of cellular metabolism, differentiation and proliferation. Interestingly, the RNA silencing machinery redistributes into cytoplasmic RNA granules, termed stress granules (SGs), in cells that go through mitosis after UV irradiation. We discuss the implications of these findings for our understanding of the DNA damage response.

### MicroRNAs and Regulation of Gene Expression

Gene expression regulation has traditionally mainly been studied within the context of transcriptional regulation, in which transcription factors and repressor complexes play a major role. However, in recent years, many mechanisms of post-translational gene regulation have been described. Especially the regulation of mRNA translation by small non-coding RNA species has attracted much attention over the past few years. One class of such

non-coding RNAs consists of microRNAs, which are ~22 nucleotide long RNA molecules that influence translation and/or stability of a large number of mRNAs. They do so by binding to imperfect complementary sites in the mRNA, often located in the 3' untranslated region (Fig. 1). These microRNAs are expressed from chromosomally encoded genes by RNA polymerase II.<sup>1</sup> The resulting 5' capped and 3' poly-adenylated primary microRNAs (pri-microRNA) are subsequently processed into precursor microRNAs (pre-microRNAs) in the nucleus, which requires the nuclease Drosha.<sup>2</sup> After this processing step, the pre-microRNA is transported to the cytoplasm, where it is further processed by the Dicer nuclease to form a mature microRNA.<sup>3</sup> Silencing is then accomplished by the RNA-Induced Silencing Complex (RISC), which consists of the microRNA together with a number of proteins including the Argonaute (Ago) proteins.<sup>4-6</sup> Degradation of target mRNAs can be accomplished by Ago2, while Ago1, 3 and 4 are involved in translational silencing without mRNA degradation. MicroRNAs are highly conserved between mammalian species: most of them are completely identical. Approximately 30% of the microRNAs in *Caenorhabditis elegans* is conserved in mammals. Moreover, the majority of the target sites in mRNAs are conserved between species, as well. Currently, approximately 900 human microRNAs have been experimentally identified, while 1,000–2,000 have been predicted.<sup>7,8</sup> As one microRNA is able to regulate tens to hundreds of genes, 30% of all genes in the genome is expected to be regulated

**Key words:** DNA repair, cell cycle checkpoints, microRNA, stress granules, post-transcriptional gene regulation

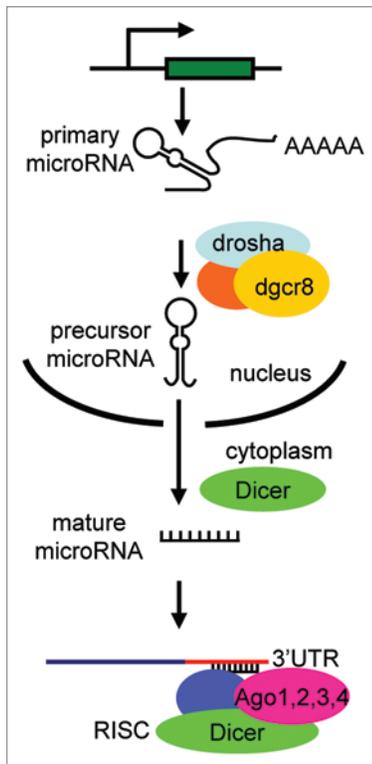
**Abbreviations:** SG, stress granule; miR, microRNA; RISC, RNA-induced silencing complex

Submitted: 06/30/09

Accepted: 08/17/09

Previously published online:  
[www.landesbioscience.com/journals/cc/article/9835](http://www.landesbioscience.com/journals/cc/article/9835)

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**Figure 1.** Schematic outline of microRNA biogenesis. Pri-microRNAs are transcribed and processed by the Drosha complex to pre-microRNAs, which are exported to the cytoplasm. There, they are processed to mature microRNAs by Dicer and incorporated in the RISC complex, which also contains one of the Ago proteins.

by microRNAs.<sup>9</sup> Therefore, most cellular processes are probably (at least partly) under microRNA control.

### The DNA Damage Response

Various endogenous and exogenous agents such as reactive oxygen species and ultraviolet light (UV) can damage the DNA in our cells.<sup>10</sup> Many different DNA lesions can occur, e.g., single and double strand breaks, inter- and intrastrand DNA crosslinks, pyrimidine dimers and nucleotide damage each with their specific damaging characteristics. When DNA damage is not repaired properly, these DNA lesions will be processed into mutations, deletions or chromosomal rearrangements, which contribute to carcinogenesis. On the other hand, unrepaired DNA damage is a driving force in the aging process. To counteract these harmful consequences of DNA damage, cells have various DNA repair

systems to repair the damage. In addition, the cell cycle checkpoints are activated to halt cell proliferation and allow the cell time to repair the damage. Excessive DNA damage induces apoptosis or senescence. This complete set of processes is collectively called the DNA damage response.

### MicroRNAs in the DNA Damage Response

Recently, we described the first systematic investigation of microRNA involvement in cellular responses to DNA damage inflicted by UV irradiation, which causes intra-strand crosslinks between adjacent pyrimidine residues.<sup>11</sup> These DNA lesions cause stalling of transcribing RNA polymerases, as well as replicating DNA polymerases. The nucleotide excision repair (NER) machinery removes these lesions from the DNA, which is a prerequisite for proper restart of transcription and replication. Without proper repair, cells accumulate mutations as a result of replication over unrepaired pyrimidine dimers by specialized translesion DNA polymerases (which have intrinsically high error rates), leading to highly increased skin tumor incidence in patients. Furthermore, stalled RNA polymerases lead to increased levels of apoptosis, which can give rise to accelerated aging. In addition to initiation of NER, UV-induced DNA lesions also give rise to signaling cascades that regulate the cell cycle, especially progression from G<sub>1</sub> to S phase. This G<sub>1</sub>/S checkpoint includes a fast component, which mainly depends on regulation of protein activity and/or stability by DNA damage responsive kinases. Prolonged stress conditions also induce a change in the transcriptional program, including p53-inducible gene expression.<sup>12</sup>

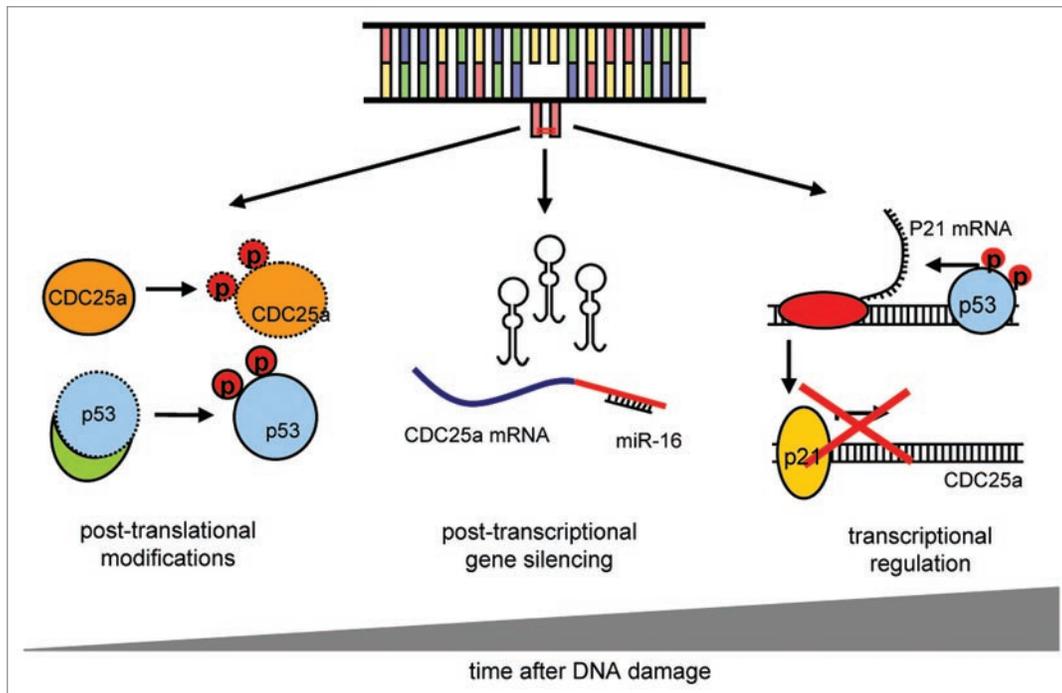
We provided evidence that microRNA-mediated regulation of gene expression plays an important role at the intermediate time points between the fast protein modification responses and the slow transcriptional reprogramming of genes (Fig. 2). Most of the significantly UV-regulated microRNAs after UV already showed up or downregulation in the first hours after DNA damage, while the transcriptional response of genes upon damage is generally slower. A well-studied example of a DNA damage inducible

transcription factor is p53. Upon DNA damage, the p53 protein is rapidly stabilized and activates its transcriptional program.<sup>13</sup> Both the p53-induced microRNA miR-34a and cell cycle control gene p21 were much later upregulated after UV exposure indicating that at least the p53 transcriptional response is slower. This concept was further illustrated by the stepwise regulation of the central cell cycle checkpoint gene CDC25a. The CDC25a protein is rapidly phosphorylated minutes after UV, which induces its degradation, thereby establishing a direct but transient cell cycle block.<sup>14</sup> Hours later, the CDC25a promoter is silenced by p21, which requires p53-dependent transcription.<sup>15</sup> However, we observed a rather fast downregulation of CDC25a mRNA at a time point in which p21 was not induced yet, which suggested a role for microRNA-mediated gene silencing. Indeed, we found that the UV-upregulated miR-16 was responsible for CDC25a mRNA downregulation in the first hours after UV exposure (Fig. 2). Both the kinetics of microRNA regulation and the stepwise regulation of CDC25a indicates that microRNA-mediated gene silencing acts between the fast protein modifications and slower transcriptional regulation of genes.

We envisage that the action of microRNAs early in the DNA damage response has several advantages. DNA damage requires changes in the expression of hundreds to thousands of genes.<sup>16-18</sup> In order to quickly alter the cellular transcriptional program, just a handful of microRNAs can regulate a large number of genes. Moreover, microRNAs prevent de novo protein synthesis, which would otherwise initiate a futile cycle of protein synthesis and degradation or inactivation. At later time points, gene transcriptional regulation takes over the gene expression regulation function in order to sustain the changed expression pattern when a persistent alteration of gene expression is required.

### Regulation of MicroRNAs After DNA Damage

The mechanism of regulation of the microRNAs by UV irradiation has not yet been investigated in much detail.



**Figure 2.** The three levels of regulation after UV DNA damage induction. Within minutes, protein modifications change protein activities and/or levels. CDC25a is phosphorylated by ATR, which leads to its degradation, while p53 phosphorylation prevents interaction with mdm2 and subsequent degradation. In the first few hours after UV irradiation, microRNA expression changes regulate mRNA levels and/or translation, for example the CDC25a mRNA level. After several hours, transcriptional activation of p53 target genes kicks in and changes transcription programs, e.g., p21 upregulation silences the CDC25a promoter.

The regulation of approximately half of all UV-responsive microRNAs could be abolished by caffeine treatment of cells, which points to regulation by the ATR checkpoint kinase (although we cannot exclude an additional role for the ATM checkpoint kinase, since caffeine inhibits both kinases). However, many microRNAs were still UV-responsive in the absence of ATR activity, pointing to other relay mechanisms of DNA damage. MiR-34a has previously been found to be p53-dependent.<sup>19-21</sup> As expected, this microRNA was only regulated late after UV treatment and did not show significant regulation in p53-deficient HeLa cells. Although a few microRNAs showed an expression pattern similar to miR-34a, the most commonly observed pattern was regulation already after 4 hours and in most cases this was observed in both primary human fibroblasts and HeLa cells, suggesting that p53 regulation is not the principal way of microRNA gene expression regulation after DNA damage. Although some microRNAs are probably regulated by ATR-dependent mechanisms, the regulation of the ATM/

ATR-independent microRNAs is less clear. The UV-responsive alternative checkpoint pathway requiring the p38 MAPK and MK2 kinases is an interesting possibility, but experimental evidence supporting this hypothesis is lacking at present.<sup>22,23</sup>

Which mechanism is responsible for the fast regulation of microRNAs after DNA damage? The primary transcript of most microRNAs is small compared to the primary transcripts of protein-coding genes: a few hundred bases versus tens to hundreds of kilobases. This size difference could be responsible for the observed effect: small genes/microRNAs can be transcribed more quickly than larger genes and are less likely to accumulate transcription-inhibiting lesions. It is noteworthy that the p53 dependent miR-34a, which is regulated relatively late after UV treatment, has a primary transcript of ~30 kilobases.<sup>21</sup> However, some rapidly regulated microRNAs are located in introns of large genes and would therefore not be expected to be regulated at the early time points. However, microRNA regulation could also be accomplished by post-transcriptional regulation.<sup>24-26</sup> Processing

of specific primary microRNAs (from the total pool of primary microRNAs in the nucleus) can be selectively enhanced or repressed by specific proteins, that either regulate Drosha activity or specifically bind primary microRNAs to block further processing. Activation or inactivation of microRNA post-transcriptional regulation by DNA damage could in principle be responsible for the rapid regulation of microRNAs as it bypasses the slow transcriptional step. In this light it has recently been shown that p53 is not only able to regulate miR-34a at the transcriptional level, but also some other microRNAs at the post-transcriptional level by regulating the Drosha associated factor DDX5.<sup>27</sup> Indeed, we identified several of these microRNAs that have been found to be regulated post-transcriptionally in a p53-dependent manner upon doxorubicin treatment also as rapid responders to UV. However, some of these microRNAs (e.g., miR-16) were also regulated in p53-deficient HeLa cells, suggesting that relay of the UV damage signal is not or not exclusively regulated via p53. It is conceivable that other, p53-independent, post-transcriptional

**Table 1.** UV-regulated microRNAs and their functions

microRNA	regulates	Reference
miR-203	cell cycle	36
miR-24	cell cycle	36, 37
miR-138	cell cycle	38
miR-98	cell cycle	39
miR-16	cell cycle/ apoptosis	11, 28
miR-27	cell cycle/ apoptosis	40, 41
miR-221	cell cycle/ apoptosis	42, 43
miR-34a	cell cycle/ apoptosis	44
miR-21	cell cycle/ apoptosis	45
miR-199a	apoptosis	46
miR-26a	apoptosis	47
miR-29a	apoptosis	48

regulation mechanisms also contribute to microRNA expression changes upon DNA damage induction and that regulation mechanisms may differ for different types of DNA damage.

### Which Processes are Regulated by MicroRNAs?

As microRNAs can induce mRNA degradation, it is likely that a fraction of the UV-regulated genes that have been identified in micro-array studies, is regulated by microRNAs. Classification of UV-regulated genes in functional groups shows that genes involved in cell cycle regulation and genes involved in apoptosis are prominently present.<sup>16-18</sup> UV irradiation provokes a quick and efficient block in the cell cycle at the end of the G<sub>1</sub> phase, the G<sub>1</sub>/S checkpoint in which CDC25a is a central player. Interestingly, we found that the CDC25a mRNA was regulated via microRNA-mediated degradation (by miR-16; Fig. 2). In addition to CDC25a regulation, miR-16 also regulated cyclin D1 and cyclin E, suggesting that this microRNA may be a master regulator of the G<sub>1</sub>/S transition, at least in the first hours after UV irradiation. Although the role of other microRNAs was not investigated in detail after UV irradiation, we note that several other UV-responsive microRNAs have also been implicated in

cell cycle control (Table 1), which indicates that maintenance of checkpoint activity for several hours after the initial DNA damage requires this network of microRNA-dependent regulation of mRNA stabilization and/or translation, which might otherwise be bypassed prematurely.

On the organismal level, proper balancing of apoptosis is not only of prime importance to prevent tumorigenesis by mutated cells, but also to prevent excessive cell loss that might otherwise lead to depletion of stem cells and accelerated aging. It is noteworthy that several UV-regulated microRNAs have been shown to influence apoptosis (Table 1). For example, miR-16 regulates the anti-apoptotic gene BCL2 (Fig. 3).<sup>28</sup> As various microRNAs have roles in cell cycle control and/or apoptosis, it is likely that the network of DNA damage responsive microRNAs is intimately intertwined with other element of the DNA damage response.

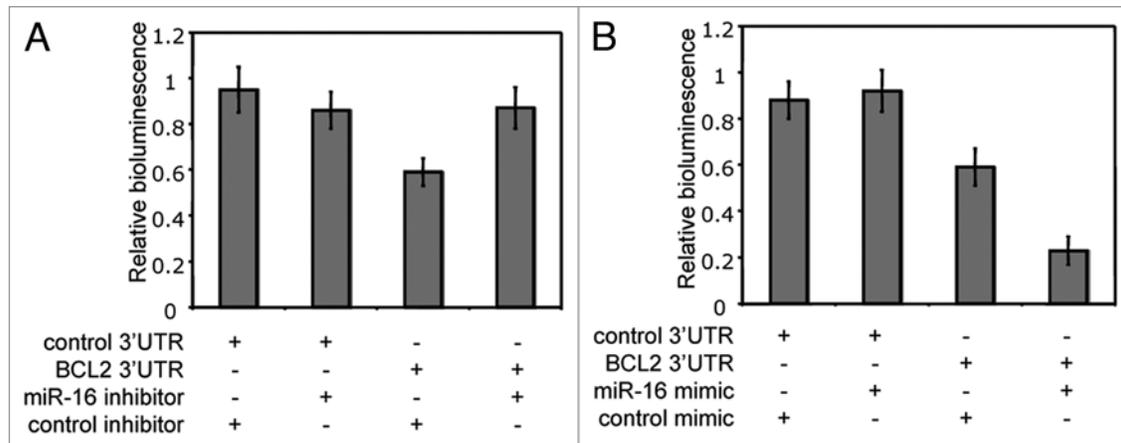
Currently, only a few microRNAs have been extensively studied, mostly in the context of cell cycle and cell death in cancer cells. However, based on target mRNAs most microRNAs are predicted to regulate several cellular processes. Although most targets have not yet been validated experimentally, it is likely that DNA damage responsive microRNAs regulate much more than just cell cycle and cell death, such as UV regulation of metabolism and hormonal pathways. Interestingly, various UV-responsive microRNAs have been found to play a role in differentiation and stem cell maintenance. It has been known for a long time that DNA damage induces differentiation of basal keratinocytes and the maturation of dendritic cells in the skin.<sup>29,30</sup> Combining these observations, it is possible that microRNAs also play a role in DNA damage induced cellular differentiation, which can be viewed as a protection mechanism against skin carcinogenesis.

### Localization of the RNA Silencing Machinery With and Without Stress

Although RISC can be found throughout the cytosol, these complexes accumulate at high concentrations in P bodies (also

called GW bodies because they contain the GW182 protein) in most cells.<sup>31,32</sup> This is most probably the major site of mRNA processing and degradation under normal conditions. After several types of stress, however, RISC complexes relocate to stress granules (SGs), which contain high concentrations of non-translated mRNAs and have therefore been described as sites of “RNA triage.”<sup>33</sup> We found that UV irradiation also induced SG formation, although this was restricted to a subfraction of the cells that had been irradiated in G<sub>2</sub> and showed SGs in the next G<sub>1</sub> phase. This is different from SG induction by other types of stress, such as arsenite treatment, which induces SGs throughout the cell cycle. We found that this is not a general phenomenon of all types of DNA damage. Ionizing radiation (20 Gy), the topoisomerase II inhibitor Etoposide (20 to 100 μM) or the interstrand crosslinking agent mitomycin C (0.1 to 1 μg/ml) did not induce SG formation between 1 and 7 hours after exposure, but H<sub>2</sub>O<sub>2</sub> led to formation of SGs in a small subfraction of the cells, again in a cell cycle dependent fashion (Fig. 4). Treatment with this oxidizing agent caused much more transient SG formation, only at relatively early time points (approximately 1 hour after treatment) and again only in cells that had just gone through mitosis. This observation is in contrast to a previous report that did not find SG induction after H<sub>2</sub>O<sub>2</sub> treatment.<sup>32</sup> The previous failure to observe SGs is probably caused by the very transient nature of SGs under these conditions. As agents inducing primarily interstrand crosslinks or double strand breaks in the DNA did not induce SG formation, we propose that SGs are specifically formed upon treatment with agents that induce high levels of base damage, especially when these lesions persist for a relatively long time, such as pyrimidine dimers after UV irradiation.

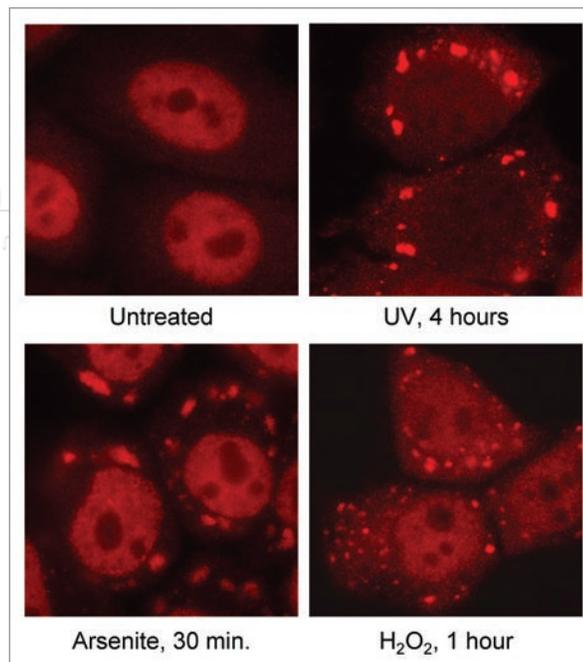
The cell cycle dependence of SG formation is not yet understood. Inhibition of Cdk activity by roscovitine caused changes in SG formation although cells still showed relocalization of the SG marker TIA1 from nucleus to cytoplasm. At relatively high roscovitine concentrations, SGs appeared scattered and a large fraction of



**Figure 3.** The BCL2 3'UTR is regulated by miR-16. (A) Luciferase constructs with the BCL2 3'UTR or a control 3'UTR were cotransfected with a miR-16 inhibitor or a control inhibitor and relative bioluminescence was measured in cell lysates. (B) The same luciferase constructs were cotransfected with a miR-16 mimic or a control miRNA and bioluminescence was determined as in (A). In both cases miR-16 lowers expression of the construct containing the BCL2 3'UTR.

the TIA1 protein was randomly distributed throughout the cytosol, suggesting that Cdk activity is required for efficient accumulation of TIA1 in SGs, but not for its initial recruitment from the nucleus. It is not yet clear which Cdk plays a role in SG assembly, but prime candidates would be Cdk1 or Cdk2, as they are most commonly involved in cell cycle progression.<sup>34</sup> However, we would also like to suggest that Cdk7 is an interesting candidate to have such a function, since it is part of the TFIIF complex, which is involved in both NER and transcription.<sup>35</sup> In this scenario, TFIIF would have a dual role in regulating stress responses via SG assembly (and possibly also microRNA regulation) and repair of the DNA lesions.

The trigger for SG assembly has not been characterized, either. As inhibition of ATM and ATR kinases by caffeine treatment did not prevent UV-induced SG formation, these factors are either not involved or redundant with other SG induction pathways. The tumor suppressor p53 is clearly not required, either, since a robust response was obtained in HeLa cells. The p38/MAPK pathway is still a possible signaling route, but this has not been explored yet. At this moment, it is not even clear whether SG formation is triggered by the DNA damage. It is also possible, that other aspects of UV irradiation provide the main trigger. For example, SGs might be sites where UV-damaged RNA molecules assemble. Investigation



**Figure 4.** Stress Granule formation after various types of stress. Cells were exposed to 0.5 mM arsenite, 0.5 mM H<sub>2</sub>O<sub>2</sub> or 20 J/m<sup>2</sup> UVC light and cells were fixed at the specified time points, followed by immunostaining for the SG marker TIA1.

of SG assembly and disassembly kinetics in situations that do not allow removal of UV-lesions from DNA, such as NER mutants, will help elucidate these issues. It is currently not clear which aspects of the DNA damage response may depend on SG assembly. Elucidation of the signaling routes for UV-induced SG formation will be necessary to gain insight into

the function of SGs in the DNA damage response.

### Future Prospects

Our recent publication on microRNA responses and SG formation after UV irradiation of cells has opened new areas of investigation of the DNA damage

response. This publication answered some initial questions, but generated many more questions than answers. Although microRNA biogenesis was shown to be important for survival and miR-16 was specifically shown to impact on cell cycle checkpoints, a more precise description of the functions of specific microRNAs in cell cycle checkpoints, apoptosis, differentiation and all other aspects of the cellular responses to UV irradiation will require more detailed studies. Furthermore, the relative impact of microRNA-mediated gene silencing on the total gene expression changes is not known. We also lack knowledge of similarities and differences between various types of DNA damaging agents on microRNA responses. For example, we noticed that some aspects of microRNA expression regulation are common to various types of DNA damage, e.g., miR-16 is not only induced by UV irradiation, but also by ionizing radiation.

The regulation of microRNA expression is another area that will need to be addressed in more detail. The availability of large collections of primary patient fibroblast cultures and cell lines carrying mutations in NER genes and other DNA repair and checkpoint genes should not only be of great value to investigate the involvement of DNA repair in the microRNA expression regulation, but also to elucidate whether the trigger for microRNA expression and SG formation is pyrimidine dimers in the DNA.

Although Ago2, and by inference the RISC complex, localizes to SGs after UV irradiation in cells that go through mitosis with UV damage, it is not clear what the function of this change in subcellular localization is in the DNA damage response. It is possible that some aspects of mRNA silencing depend on proper localization of mRNA and RISC to SGs, but a better understanding of the mechanisms of SG formation and their function will be necessary to provide a more satisfying answer to these questions. It will be important to investigate these aspects of SGs for UV stress, since the mechanisms of SG formation after UV irradiation and other stresses (such as arsenite treatment) may be quite different, as exemplified by the difference in roscovitine sensitivity of SG formation after various treatments.

Interestingly, the misexpression of various UV-responsive microRNAs has also been correlated with tumorigenesis, suggesting important roles for DNA damage inducible microRNAs in disease. All these aspects of microRNA expression regulation and SG formation need further experimentation in order to understand the magnitude of the microRNA component in regulation of gene expression after DNA damage induction. It is clear that microRNAs add a new dimension to the DNA damage response, but future will tell how important this dimension is.

#### Acknowledgements

J.H.J.H. is the Chief Scientific Officer of DNage/Pharming. Support was obtained from the European Commission (EU-LSHG-CT-2007-036894 "LifeSpan").

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