MicroRNAs and the DNA Damage Response: How is Cell Fate Determined?

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Abstract

It is becoming clear that the DNA damage response orchestrates an appropriate response to a given level of DNA damage, whether that is cell cycle arrest and repair, senescence or apoptosis. It is plausible that the alternative regulation of the DNA damage response (DDR) plays a role in deciding cell fate following damage. MicroRNAs (miRNAs) are associated with the transcriptional regulation of many cellular processes. They have diverse functions, affecting, presumably, all aspects of cell biology. Many have been shown to be DNA damage inducible and it is conceivable that miRNA species play a role in deciding cell fate following DNA damage by regulating the expression and activation of key DDR proteins. From a clinical perspective, miRNAs are attractive targets to improve cancer patient outcomes to DNA-damaging chemotherapy. However, cancer tissue is known to be, or to become, well adapted to DNA damage as a means of inducing chemoresistance. This frequently results from an altered DDR, possibly owing to miRNA dysregulation. Though many studies provide an overview of miRNAs that are dysregulated within cancerous tissues, a tangible functional association is often lacking. While miRNAs are well-documented in 'ectopic biology', the physiological significance of endogenous miRNAs in the context of the DDR requires clarification. This review discusses miRNAs of biological relevance and their role in DNA damage response by potentially 'fine-tuning' the DDR towards a particular cell fate in response to DNA damage. MiRNAs are thus potential therapeutic targets/strategies to limit chemoresistance, or improve chemotherapeutic efficacy.

Keywords: DNA damage response, cell fate, miRNA, ATM, p53, apoptosis

Introduction

DNA is under constant threat of significant stand damage that cannot go unresolved. Such damage, namely, double strand breaks (DSBs) and single-stranded DNA (ssDNA) initiates the DNA damage response (DDR), a multi-enzyme phosphorylation cascade involving sensors, (MRE11-RAD50-NSB1 (MRN complex), ataxia telangiectasia mutated (ATM) and ataxia telangiectasia and Rad3-related (ATR), checkpoint kinases (Chk1 and Chk2) and effectors (p53). The cascade signals to mediator proteins that bring about either damage resolution, and survival, via cell cycle arrest[1,2] and DNA repair by homologous recombination repair (HRR)[3] or non-

homologous end-joining (NHEJ)[4]. However, if the damage is too severe, the DDR initiates cell death, predominately via apoptosis. Furthermore, the DDR, can also elicit alternative cell states such as premature senescence and autophagy[2,5–7]. However, as the predominant mechanism of DNA damage-induced cell death, and arguably the best characterised form of cell death, this review will largely focus on apoptosis (Kaina, 2003; Roos and Kaina, 2006; Fitzwalter and Thorburn, 2015). The DDR, therefore plays a pivotal role in defining cell fate following DNA damage. Where our understanding is lacking though, is what facilitates the DDRs functional selectivity, as cell cycle arrest and DNA repair functions cannot be reconciled with apoptogenic functions, as these are seemingly opposing cell fates (survival and death), but it must be governed by the level of damage[6]. It is likely that a series of thresholds exist within the response and as subsequent damage thresholds are surpassed, differential responses are activated[8]. It's plausible that the level of damage, is correlated to DDR activity, principally governed by kinases (activating) and their opposing phosphorylases (deactivating). Indeed, cell fate effects and levels of phosphorylated DDR (active) proteins have been established[9], but there is also support for the role of alternative post-translational modifications in inducing alternate cell fates[10]. What controls the levels and post-translational modification (PTM) of the DDR enzymes is under investigation but a likely candidate are microRNAs (miRNAs). MiRNAs are small (≈20-25 nucleotides) non-coding RNA sequences that are known to function as regulators of gene expression and are operational in diverse cellular physiology[11,12]. In the context of the DDR, miRNAs and other non-coding RNA species[13], may encompass the honing of a tightly regulated DDR to dictate the appropriate cellular outcome in response to different levels of DNA damage. In support of this, studies have identified expression patterns of miRNAs that are associated with increasing doses of ionizing radiation and UV[14,15]. Furthermore, a recent study by Rana *et al.*[16] has correlated **miR-15a** levels with radiation dose to affect cell fate. In many cases, the exact physiological effects of miRNAs on the DDR remain largely speculative, as some studies rely on computational predictions (*in silico*) that are not always confirmed in biological systems. Moreover, when biologically investigated, *in vitro* or cancer models are usually relied upon, and miRNA functions are deduced from situations that may not exist in biology, i.e. in studies that employ the overexpression and/or ectopic expression of a single, candidate miRNA and are thus, also narrowed in their focus. Nevertheless, they do provide substantive evidence of a miRNAs potential targets and effects. This review provides a brief overview of DDR-associated miRNAs and their potential physiological role in DDR regulation, and how this regulation may be a determinant of cell fate.

An Overview of the DDR

The DDR predominantly constitutes a relay of phosphorylations between central kinases that drives the DDR. At the top of the relay, DNA damage is detected by specialised *sensor* proteins including the MRN complex[17], replication protein A-ATR interacting protein (RPA-ATRIP), XRCC6/5, and their respective phosphatidylinositol 3-kinase related kinases (PIKK); ATM, ATR and DNA protein kinase (DNA-PK)[18]. Subsequently, ATM and ATR phosphorylate downstream *transducer* proteins, including the Chk1 and Chk2[19] to propagate the 'damage' signal. ATM also phosphorylates a histone H2A variant, forming γH2Ax foci which recruits several DDR proteins to the damaged site[5,20,21]. Phosphorylated Chk1/Chk2 (pChk1 and pChk2) are active and capable of phosphorylating p53 at serine 20 (phospho-p53 (ser20)) thereby promoting its stability[22–24]. P53 is also phosphorylated by ATM and ATR at ser15 (phospho-p53 (ser15)), further stabilising p53 to operate as a DDR *effector* protein by transactivating *executioner* genes involved in either arrest, repair *or* apoptosis[25–28]. Thus far, phosphorylation has been synonymous with DDR protein activation, therefore, dephosphorylation can cause deactivation. In this case, p53-induced protein phosphatase (WIP1) can dephosphorylate ATM and p53 (among others), exemplifying its potential as a DDR 'off switch'[29–33] (**Fig. 1**). On the other hand, phosphorylation within the DDR is more than a simple 'on switch' and must influence the outcome of DDR signalling to the appropriate response, by potentially altering the signalling message i.e. there is too much damage, apoptosis must be initiated.

The pro-apoptotic arm of the DDR

P53 activation is a point of convergence within the DDR and its function is possibly tied to its PTM (discussed hereafter). A recurring hypothesis is that specific PTMs correlate with the severity of DNA damage (**Fig. 1**), and subsequent PTMs can alter p53 promoter selectivity. Research suggests that p53 serves as an 'arrestor' when phosphorylated on ser15 and ser20[34,35], while further phosphorylation at ser46 (phospho-p53 (ser46)) switches p53 functioning to 'killer'[10,36]. Here, phospho-p53 (ser46) is suggested to specifically transactivate pro-apoptotic genes; p53-regulated apoptosis-inducing protein (p53AIP1)[10,37], phosphatase and tensin homolog deleted on chromosome 10 (PTEN)[10], and p53 upregulated modulator of apoptosis (PUMA)[38]. Several kinases have been associated with phospho-p53 (ser46), though, compelling evidence by He *et al*.[39] demonstrates homeodomain interacting protein kinases (HIPK2) as its principal kinase upon DNA damage, as HIPK2 inhibition diminished phospho-p53 (ser46) levels

approximately 4-fold. Moreover, the authors suggested HIPK2 stabilisation to be contingent on ATM/ATR-mediated inhibition of seven in absentia homolog 1 (SIAH1) (**Fig. 1**). SIAH1 is an E3 ubiquitin ligase that facilitates HIPK2 proteolysis, and has been shown to be phosphorylated by both ATM and ATR at ser19, disrupting SIAH1-HIPK2 interaction[40]. This raises the possibility of ATM and ATR having a direct, perhaps a determining role in apoptogenic DDR signalling.

Although phosphorylation is the most well-known PTM that drives p53 function, several others are known to contribute. For example, acetylation of p53 has been demonstrated to diminish p53-mouse double minute 2 homolog (MDM2) interaction. Moreover, loss of p53 acetylation diminished PUMA, BAX, and p21 expression levels[41], showing its importance in the general functions of p53. Akin to p53 ser46-mediated pro-apoptotic gene selectivity, acetylation at lysine 120 (lys120) increases apoptotic protease activating factor (APAF1) expression, thus promoting apoptosis[42]. What is yet to be determined is if both PTMs are required for apoptogenic p53 functions, and whether one PTM, stimulates the other. Nevertheless, it is known that p53 can be acetylated by histone acetyltransferases (e.g. p300)[43] and deacetylated by histone deacetylases (e.g. sirtuin 1, SIRT1)[44,45]. Of note, SIRT1 can also inhibit p300 in a feedforward acetylation loop[46]. Moreover, HIPK2 can inhibit SIRT1 in a potential feedforward apoptogenic loop[47]. Furthermore, a link has been established with ATM/ATR and SIRT1 inhibition[48], further supporting a pro-apoptotic role for these sensors. It is interesting to ponder who is in control, the miRNA or the DDR proteins themselves. On one hand, it is possible for ATM and ATR to be in the driving seat of cell fate by controlling the PTM of p53 and whether miRNAs fine tune ATM/ATRs 'message'. In support of this, it is important to realise the role of the DDR in miRNA biogenesis before investigating the effects of miRNA on proteins of DDR proteins.

MiRNA biogenesis

MiRNAs are embedded throughout the genome and can be intronic or intergenic. While intronic miRNAs are part of the intron portion of its host gene, and are thus co-transcribed with it, intergenic miRNAs can be regarded as 'independent' transcripts with their own promotor region[49]. MiRNAs are predominantly transcribed by RNA polymerase II[50], forming primary miRNA (pri-miRNA). The hairpin structure of pri-miRNA allows recognition by a microprocessor complex that cleaves pri-miRNA to form precursor miRNA (pre-miRNA). Components of the microprocessor complex include the ribonuclease, DROSHA and its RNA-

binding partner, PASHA[51,52]. Together, these can interact with, and cleave, pri-miRNA to pre-miRNA. Subsequently, EXPORTIN-5 (XPO5) transports pre-miRNAs from the nucleus to the cytoplasm. In the cytoplasm, pre-miRNAs are further cleaved by another ribonuclease, DICER, to a nucleic acid duplex of approximately 20 nucleotides. This constitutes the mature miRNA that becomes integrated into the RNA-induced silencing complex (RISC). However, only one strand of the miRNA duplex becomes integrated, while the other, 'passenger strand', is digested[49,52,53]. In its simplest form, RISC consists of mature miRNA and argonaute proteins (AGO)[54]. Integrated miRNA is protected from digestion by AGO proteins, and serves as a guide strand to seek out mRNA with complementarity to its 5' 'seed' sequence to facilitate translational inhibition or degradation[49,52,53,55]. The type of mRNA regulation is contingent on the degree of complementarity between the miRNA seed sequence and its target mRNA. While exact complementarity can result in AGO-mediated mRNA cleavage, partial complementarity is subject to translational inhibition[56]. In this way, miRNAs can regulate gene expression either negatively, as previously mentioned, or positively, by targeting respective inhibitors. ATM can induce the biogenesis of multiple miRNAs (e.g. members of the **miR-16** family)[15] in a KH-type splicing regulatory protein (KSRP)-dependent manner[57]. As described by Trabucchi *et al*[58], KSRP forms part of both DROSHA and DICER microprocessor complexes and enhances miRNA biogenesis. Incidentally, breast cancer 1 (BRCA1), a protein involved in HR repair of strand breaks and itself an ATM substrate[59], has also been reported to promote miRNA biogenesis, at least in part, through interaction with the DROSHA microprocessor complex. Consequently, BRCA1 is capable of promoting the expression of several miRNAs including **let-7a-1** and **miR-16-1**[60]. Interestingly, these were among the miRNAs that showed pronounced reduction (40–70%) upon KSRP knock-down in the study by Trabucchi *et al*[58]. Therefore, given that KSRP-mediated miRNA processing is promoted by ATM, and that BRCA1 is a substrate for ATM[61], it underpins the importance of ATM as a core regulator of, at least, a subset of miRNAs. Whether this subset of miRNAs have a defined influence on the DDR and cell fate is under investigation. **miR-335** may be one within this subset as this was shown to be downregulated by ATM in response to IR (up to 10 Gy) and, dose-dependently, upon doxorubicin (up to 10 μM) treatment in MCF7 cells[62]. Which shows the dual effect that ATM can have on miRNA biogenesis. Overexpression of miR-335, impaired DSB repair and led to a radiosensitive phenotype in patient-derived lymphoblastoid cells, which suggests that miR-335 would play a role to suppress repair to promote cell death under normal physiological conditions, hence its negative regulation by pATM. It remains to be seen whether a lethal dose of radiation leads to an induction of miR-335 to turn off repair in favour of apoptosis. Nevertheless, it serves

as an example of how miRNA biogenesis is directly influenced by DDR proteins. Furthermore, other DDR proteins, especially those capable of transcriptional regulation, can influence the expression of miRNAs, which may facilitate the role of the transactivator in eliciting a specific cell fate. What remains to be determined is how these miRNAs feedback or feedforward the message of damage and how they may direct cell fate accordingly. What follows is an account of the miRNAs that have been associated with the key proteins of the steps of the DDR, from the sensors to the executioners. As will be seen, interpreting and speculating on the effect of cell fate is more lucid the further downstream the DDR, the miRNA operates.

DNA damage sensing

MRN

DNA damage sensors are activated based on the type of DNA damage perceived. For example, while RPA-ATRIP detects single stranded DNA (ssDNA)[63], the MRN complex detects DSBs and is required for ATM activation[5,64]. ATM and ATR are often labelled as sensors and are distinguished as being able to sense DSB (ATM), or SSB and DNA replication fork stalling (ATR), although this may be context dependant as their functions overlap[65–69]. It is thus clear that miRNAs feature in the negative regulation of upstream DDR activity. Espinosa-Diez *et al*.[70] demonstrated that **miR-494** was upregulated within 1 hr following 2 Gy γ-irradiation, and this, along with **miR-99b**, directly inhibited the MRN complex in human endothelial cells. One would anticipate that MRN inhibition would impair the ability to sense damage and would therefore, dampen the damage signal, which would lead to reduced repair, as the authors found. This questions why the induction of miR-494 would be a physiological response to the damaged induced by irradiation. The 2 Gy damage signal triggered a senescent phenotype and this was due to the other effect of miR-494 on senescent effector proteins (telomerase, p21 and retinoblastoma (Rb)). However, this radiation-induced senescent response may be unique to the type of cells studied[71]. MiR-494, when experimentally overexpressed, was shown to upregulate H2Ax phosphorylation, which is a key damage indicator, induced by ATM, which suggests functional independency of ATM from MRN. Incidentally, pharmacological inhibition of MRN following oncogene-induced DNA damage during replication, counterintuitively, was shown to increase the levels of activated ATM, compared to cells without pharmacological treatment, which led to an altered cell fate by inducing apoptosis over cell cycle arrest[72]. Therefore, miR-494 and its effect on MRN, may play an important role in cell fate decisions following DNA

damage, potentially towards apoptosis. Incidentally, the induction of miR-494 was still present, but not as pronounced at higher doses (up to 20 Gy), suggesting an altered miRNA response at increased damage levels.

ATM

Hu *et al*.[73] demonstrated that **miR-421**, ectopically expressed in cervical carcinoma cells, caused ATM downregulation by targeting its 3' untranslated region (UTR). Under this condition, the authors also observed diminished S-phase arrest and cell survival following ionising irradiation. Suggesting a pro-death effect of miR-421 in normal physiology, although this doesn't correlate with the finding of miR-421 being a transcriptional target of N-myc in the study. Similarly, overexpression of **miR-18a-5p** downregulated ATM expression levels directly, leading to radiosensitisation of lung cancer and $CD133^+$ stem-like cells, at the doses tested[74,75]. MiRNA-mediated ATM regulation is not always suppressive. For example, Rahman *et al*.[76] observed increased phosphorylated ATM (pATM) levels in **miR-15b/16-2** transfected human bronchial epithelial cells (HBECs) following 4 Gy irradiation. This was, at least partly, attributable to WIP1 inhibition by miR-15b[76], as WIP1 is known to dephosphorylate ATM[32]. Incidentally, miR-16 has also been reported to directly target WIP1 in transfected glioma cells, thereby promoting an increase in pATM and phospho-p53 (ser15)[77]. Consistent with increased pATM activity, the authors also observed increased γH2Ax and phospho-p53 (ser15) levels in miR-15b expressing cells, compared to non-miR-15b expressing controls. The effect on cell fate was observed as increased levels of cleaved PARP1, suggesting elevated apoptosis[78], which suggests a pro-apoptotic role for pATM and miR-15b in the study. On the other hand, 15b/16-2 transfected cells had a greater proportion of G2/M arrested cells following IR, compared to empty vector controls. It is interesting to note that within this population of treated cells, there will be micro-populations that differ in the amount of damage they have inflicted (dictated by proximity to chemical, phase of the cell cycle, repair and detoxification states for instance), which is presumably the reason why a single treated culture can have different cell fates. Incidentally, miR15-b/16-2 reduces the levels of the negative modulator of p53, MDM2 (discussed later). Such a situation makes deciphering where in the DDR, miRNAs exert their regulatory function difficult. Suffice to say that the DDR cascade, from ATM, Chk1 to p53 is drastically augmented upon miR15b/16-2 overexpression. Although, the net effects on cell fate are unclear. It remains to be seen how miR-15b/16-2 levels are affected at different levels of damage. Interestingly, the effect of miR-15b on pATM was dependent on IR, suggesting miR-15b to be augmented by DNA damage responsive proteins. Furthermore, miR-15b is part of a cluster of other miR-15 (a and b) and

miR-16 (1 and 2) miRNAs, and are targets of E2F1, a family member of E2F cell cycle regulatory transcription factors and have well-defined apoptogenic and anti-proliferative roles perhaps due to their effect on various DDR effector proteins, downstream in the cascade (discussed later)[79,80]. This, together with the fact that other genotoxins, with diverse mode of actions (such as hydrogen peroxide and etoposide), have also been shown to induce miR-15b expression[81], suggests that miR-15b plays a critical role in the damage response. Evidentially, this is through the promotion of pATM cascade[76]. Although there is impetus for further investigations into its apoptogenic role, and also in the upstream, damage-dependent signalling that induces miR-15b expression.

ATR

Upon irradiation (up to 8 Gy), **miR-185** expression was shown to be reduced in a dose-dependent manner[14]. The mechanism of this repression is unknown, but it may be a pro-survival event as the authors have shown that overexpression of miR-185 suppresses ATR protein levels, sensitizing cells to death upon irradiation. Therefore, it seems likely that the physiological repression of miR-185 is playing a pro-survival role at the doses studied. ATR has also been shown to be down-regulated by **miR-383**, a process that is prevented by STAT3 overexpression in epidermoid carcinoma cells[82]. The authors observed increased cell survival in response to 20 J/m^2 UV-irradiation in STAT3 overexpressing cells, owing to enhanced repair of strand breaks. This suggests that miR-383 may play a role to suppress ATR signalling, much like miR-185, to have a sensitisation effect. It remains to be seen if miR-185 and/or miR-383 is induced and whether it plays a pro-death role as a physiological response at lethal doses, or serves as a DDR off-switch.

DNA damage response transducers

Typical transducers of the DDR, and downstream targets of the ATM/ATR pathway, include Chk1/Chk2[19]. Although these are by no means the only DDR transducers, they do represent those most extensively researched and will be reviewed in turn. Literature linking miRNAs to Chk1, and particularly Chk2 are limited.

Checkpoint kinase 1

Xie *et al.*[83] demonstrated that Chk1 is a direct target of **miR-497**, and in hepatocellular carcinoma cells, low levels of miR-497 result in high levels of Chk1, which the authors describe as oncogenic. On the basis of it being downregulated in cancer, miR-497 may potentiate cell death,

but this needs substantiation. Similarly, Liu *et al*.[84] showed Chk1 repression in **miR-195** transfected non-small cell lung cancer (NSCLC) cells and a significant negative correlation between miR-195 and Chk1 expression among NSCLC patients. How miR-497 and miR-195 are regulated, and their effects on the DDR following DNA damage is unknown. Particularly as the effect on the active form of Chk1 (pChk1) was not studied. Lezina *et al*.[85] demonstrated Chk1 repression as a result of miR-16 and **miR-26a** transfection into lung adenocarcinomas, *in vitro*. Importantly, the authors found miR-16 and miR-26a to be slightly upregulated by p53 following treatment with 0.5 μM doxorubicin, a response measured over time (24 hr) rather than dose. The authors did not comment on the PTM state of p53 in this scenario. Transfection of these miRNAs altered checkpoint activation and increased the sub-G1 fraction following doxorubicin treatment compared to non-transfected, treatment-matched controls. Incidentally, miR-16 also targeted Wee1-like protein kinase (WEE1) (cell cycle inhibitory kinase), whose pharmacological inhibition has been previously shown to induce apoptosis in leukaemia cells[86], suggesting miR-16 may be pro-apoptotic. Indeed, this has been observed to be the case in cutaneous T-cell lymphoma (CTCL) cells, but it's true effect on cell fate is likely dependent on the status of p53 and p21[87] and in synergy with miR-34a[88] that may serve as a switch between senescence and apoptosis in CTCL and NSCLC cells. The miRNA-mediated effects on transducers may also be indirect, resulting from regulation of upstream kinases. In addition to those discussed in the previous sections, which would presumably affect downstream Chks, Bao *et al*.[89] showed apoptogenic effects of **miR-126** overexpression in hepatocellular carcinoma cells, due to its inhibitory effect on polo-like kinase 4 (PLK-4), which disrupted the newly identified interaction between PLK-4 and ATR, thereby impairing the ATR-Chk1 cascade. The study by Bao *et al.*[89] was without a damaging stimulus, and indeed, the exact role of PLK-4 in the DNA damage response is yet to be elucidated, but may not extend passed a cell cycle regulator. Nevertheless, this evidence suggests that miR-126 may be pro-apoptotic, but its physiological regulation needs substantiation. It's therefore possible that these miRNAs detailed here, function in a genotoxicological context to potentiate apoptosis.

Checkpoint kinase 2

Chk2 is potentially negatively regulated by **miR-182-5p,** which was seen following transfection in breast cancer cells to investigate effects of this miRNA in breast cancer[90]. MiR-182-5p was suggested to inhibit a number of DDR transducers and effectors, which propagated an impaired

HRR phenotype when overexpressed. The cells displayed synthetic lethality following transfection of miR-182-5p and PARP inhibition which was attributed to Chk2 downregulation, and subsequent effects on BRCA1[91,92]. Interestingly, among the identified network of putative miR-182-5p targets was RAD51, an important facilitator of the ATR-Chk1 and ATM-Chk2 repair cascades[93–96]. It remains to be seen if miR-182-5p is alternatively regulated upon genotoxic stress, and its potential effect on cell fate in this context, needs clarification. One would anticipate that due to the inhibition of Chk2's repair promoting function in this study, that miR-182-5p induction would sensitise cells to damage, possibly stimulating cell death. However, as Krishnan *et al*.[90] elucidated, the multitude of DDR proteins miR-182-5p inhibits also included apoptosis effectors, and so it's exact effect on cell fate following genotoxic insult may be dose-dependent (lethal vs. sub-lethal). Chk2 has also been shown to be inhibited in **miR-191** transfected osteosarcoma cells, resulting in increased proliferation during basal conditions, which implicates miR-191, like miR-182-5p as an oncogenic miRNA[97]. However, their damage responsiveness is yet to be investigated.

DNA damage response mediators

The most well-known DDR mediator, p53, is central to the decisions of cell fate. Given its diverse functions as a transcription factor, the involvement of p53 in miRNA biogenesis is not unexpected. Indeed, much like ATM, p53 can influence miRNA biogenesis, as has been observed for miR-15a following DNA damage[98] (**Fig. 3**). Mechanistically, this entails direct or indirect interaction of p53 with miRNA promoters, as seen for **miR-145**[99] or their host genes at the transcriptional level, as is the case for **miR-605** and **miR-1204**[100,101], or post-transcriptionally, via interactions with the miRNA biogenesis machinery (post-transcriptional regulation) as seen with miR-16-1 and **miR-143**, by interacting with DROSHA through DDX5[102]. The cell fate effect of p53-mediated miRNA transactivation is two-fold. Firstly, the miRNAs can affect downstream effectors of the DDR and regulate p53 targets to affect cell fate, as will be discussed later. Secondly, miRNAs can also regulate p53 levels, some via targeting the interaction between p53 and MDM2[103,104] in positive or negative feedback loops[105–107]. The loops have been shown to bring about p53 'pulses'[106,108–111]. Although it remains to be seen whether these feedback loops are deterministic or stochastic, the dynamics of these pulses are hypothesised to be a key proponent of cell fate[107,109–112]. For example, p53 pulsation provides a buffer between mild to moderate DNA damage initiation and pro-apoptotic DDR execution, by

limiting the rate of pro-apoptotic protein accumulation. This creates an opportunity for DNA repair, and potentially, cell survival. This is contrary to severe DNA damage that supposedly generates one potent p53 pulse, causing pro-apoptotic protein accumulation[9,107,110,113]. Although, how these pulses cooperate with the apoptogenic PTMs to p53 is not currently understood. Nevertheless, there are a number of studies that have implicated several miRNAs in controlling p53 pulses. For example, the **miR-29** family members (a, b and c), **miR-34a,** miR-143, **miR-145**, **miR-192**, **miR-194**, **miR-215** and miR-605 that either directly or indirectly promote a p53 positive feedback loop to enhance p53 levels and function, by means of disrupting the negative regulation by MDM2[100,106,114–119]. Perturbations to miR-192, for example, was found in the study by Moore *et al*.[106] to affect p53 oscillations in MCF7 breast cancer cells, following DSB induction via the radiomimetic neocarzinostatin, but the effect on cell fate was not analysed. It can be postulated that miR-192, along with those mentioned, would be induced at high, lethal levels of damage to induce p53 to a high level, where it would operate to induce apoptosis. Indeed, based on mathematical modelling of a study of miR-605 transfection in colorectal carcinoma cells, the subsequent increase in p53 levels was seen to be pro-apoptotic[100,120]. While miR-192 and miR-605 targets MDM2 directly, another p53-induced miRNA, miR-34a, inhibits the transcription factor Yin Yang 1 (YY1) that itself promotes MDM2. Incidentally, YY1 is also a direct target of **miR-7-5p** in glioblastoma cells (GB)[121]. Moreover, transfection of miR-7-5p into TMZ (500 μM)-treated GB cells resulted in significantly reduced cell survival. Of note, this TMZ dose may be considered a lethal dose, as it resulted in a survival fraction of approximately 50% of that seen in control cells, and was further reduced in miR-7-5p transfected cells, suggesting miR-7-5p to promote cell death, possibly via attenuating p53 levels indirectly. Although, the regulation of these miRNAs, including miR-7-5p, following genotoxic treatment, needs elucidation. Regarding miR-29a, Moore *et al*. suggests a p53 positive feedback loop to result from miR-29a-mediated WIP1 inhibition. WIP1 is a phosphatase that dephosphorylates MDM2 at ser395, thereby stabilising it and improving p53-MDM2 interaction[29]. WIP1 is known as the DDR 'off switch'[30–33,122]. Thus, WIP1 inhibition can result in an aberrantly sustained DDR, prohibiting cell cycle progression and to likely facilitate apoptosis. It's therefore plausible that miR-29a and the other miRNAs discussed here would be alternatively regulated at lethal compared to sub-lethal levels of damage to alter p53 dynamics and affect cell fate. Furthermore, some of the miRNAs induced by p53 target cell fate executioners to affect cell fate, as will be discussed later. Whether there is a profile of p53-induced miRNA species that are associated with the different functions of p53 remains to be seen. Indeed, it is possible that some miRNAs

that are induced by p53 can affect the post-translational modification of p53 to potentially guide the functions of p53 towards apoptosis.

miRNAs and apoptogenic modifications to p53

P53 phosphorylation is a staple topic within DDR literature, owing to well-studied kinases including ATM/ATR, Chk1/Chk2, and HIPK2 amongst others. Given its association with p53-mediated apoptosis by phosphorylating p53 at ser46[123], HIPK2 has become the subject of much research recently. **MiR-141** has been shown to directly downregulate HIPK2 in miR-141 transfected human kidney cells[124]. The effect on cell fate following DNA damage has not been investigated. It is possible that, given this function, miR-141 would be anti-apoptotic. Notwithstanding phosphorylation, p53 acetylation is a fundamental, but less well understood p53 PTM. P53 acetylation diminishes MDM2-induced proteolysis, and interference at p53-responsive promoters[41]. Therefore, the regulation of deacetylases, such as SIRT1 that is known to deacetylate p53[44,45], is of importance. SIRT1 is a target of **miR-449** and miR-34a, which is p53-responsive[125] and may elude to the influence p53 may have on its own PTM. Transfection of these respective miRNAs into colorectal cancer cells promoted apoptosis, as suggested by increased levels of cleaved PARP1 and caspase-3, decreased clonogenic survival, and increased sub-G1 cell fraction. However, these observations were not solely attributed to SIRT1 inhibition, but rather a complex regulatory network in which these miRNAs inhibit pro-survival genes and augment p53 activity via SIRT1 inhibition. Additionally, the cell fate effects of miR-449 and miR-34a is suggested to be, at least partially, p53-independent as SIRT1 inhibition and apoptosis also occurred in $p53^{-/-}$ cells. Further evidence suggests a miR-449a-mediated inhibition of Bcl-2, discussed later, in gastric cancer cells, which would also contribute to its apoptogenic functions[126]. Furthermore, miR-449 is upregulated by E2F1[127]. Interestingly, E2F1 is DNA damage responsive, resulting in its stabilisation and a potential shift to pro-apoptotic tendencies[128], for example, via transactivation of p73[129]. MiR-449 may therefore, play an important apoptogenic role following DNA damage. **MiR-506-3p** is also reported to target SIRT1 and promote apoptosis in miR-506-3p transfected ovarian cancer cells[130]. However, this was attributed to inhibition of the SIRT1-AKT axis that, itself, inhibits forkhead box-protein (FOXO3a), a key apoptosis driver, and may be independent of DNA damage[131]. Incidentally, FOXO3a can promote apoptosis indirectly by facilitating the repression of anti-apoptotic protein, B-cell lymphoma extra-large (Bcl-xL)[132], or directly by transcriptional activation of pro-apoptotic protein, BIM[133]. Thus, miR-506-3p is capable of liberating the tumour suppressive activity of FOXO3a by targeting SIRT1. For this reason, studies have assessed the validity of its

augmentation in cancer therapy[134], but it remains to be seen if miR-506-3p is DNA damage responsive and if it plays a role in augmenting cell fate. Nevertheless, the miR-506-3p-mediated inhibition of SIRT1 may play a fundamental role in facilitating apoptosis.

DNA damage response executioners

Cell cycle arrest

The initial response to DNA damage is cell cycle arrest in order to provide an opportunity for DNA repair, or preparation for apoptosis if the damage is too severe and irreparable. DNA damage checkpoints are in operation at each phase of the cell cycle. Our deepest understanding is currently how DSBs activate the G1/S, intra-S and G2/M checkpoints. These checkpoints operate by inhibiting the activation and activity of the relevant cyclin and their cyclin-dependent kinase (Cdk) partner(s), thereby arresting the cell cycle before entry into the subsequent phase. This is brought about by the activity of key DDR proteins, namely ATM, ATR, the checkpoint kinases, p53 and crucially, the p53-inducible arrest mediators, p21, 14-3-3σ and Gadd45a that inhibit the activation (typically by inhibiting CDC25 family members) and/or stimulate the degradation (typically by proteolysis) of the relevant cyclin/Cdk complex. For example, at the G1/S checkpoint, p21 effectively degrades the cyclin D1/Cdk4/6 complex and it also inhibits the activity of CDC25A, so that it is unable to remove the inhibitory phosphate group from the cyclin E/Cdk2 complex, meaning this complex remains inactive, and entry into S-phase is prohibited[135]. Similarly, at the G2/M checkpoint, the activation and activity of the cyclin B/Cdk1 complex is inhibited, preventing entry into mitosis. This is via the Chk-mediated proteolysis of (most likely) CDC25C, its cytoplasmic sequestration by 14-3-3σ and the Gadd45-mediated inhibition of cyclin B/Cdk1[136–138]. It becomes clear how miRNAs could influence these checkpoints by regulating the DDR enzymes, although the precise mechanisms are yet to be elucidated. Not only that, but miRNAs may have a more direct effect on checkpoints by directly targeting the enzymes involved in controlling the activation of cyclin/Cdk complexes, namely the CDC25 family members and WEE1, previously described as a cell cycle inhibitory kinase. Indeed, several cell cycle regulatory proteins have been suggested to be regulated by miRNAs. For example, Pothof *et al.*[15] demonstrated miR-16 to be upregulated in cervical cancer cells, part of the UV response, previously mentioned, which elicited cell cycle arrest by inhibiting CDC25a, cyclin D1[139], cyclin E and cyclin D3[140]. Furthermore, miR-16 was again shown to directly inhibit CDC25a in response to UVC treatment of cervical cancer cells, in miR-16 transfected human embryonic

kidney cells. Moreover, miR-16 transfected cervical cancer cells accumulated in G1-phase, suggesting G1/S arrest, even in the absence of UV. Concordantly, miR-16 knock-down in cervical cancer cells resulted in a pronounced reduction of the G1-phase fraction, whereas S- and G2/M-phase fractions were elevated following UVC treatment. Whether miR-16 was part of a cluster with other miR-15 family members in these studies, remains to be seen. Other miRNA species have been shown to affect cyclins. For example, Yan *et al.*[141] provided an *in silico* model of E2F negative regulation by miR-449 that targets Cdk6, cyclin E, and CDC25a function as a cell cycle arrest inducer to limit E2F stimulated proliferation. It is tempting to posit whether these effects of miR-449 are mutually exclusive to its apoptogenic functions as discussed in the previous section, or whether it's cell cycle effects precludes it's apoptogenic roles. While, miRNAs can target cyclins and Cdks to promote cell cycle arrest, miRNAs can also negatively regulate cyclin-dependent kinase inhibitors (CKIs) in favour of cell cycle progression. For example, the CKI p27 is inhibited by **miR-221** and **miR-222** promoting a more aggressive glioblastoma phenotype *in vitro*[142]. The authors demonstrated that endogenous miR-221/222 could suppress p27 at a protein level, but only miR-221 was shown to target the 3' UTR of p27 directly. The bearing this has on genotoxicity is yet to be established. Similarly, p21, perhaps the most-well studied CKI, has also been shown to be negatively regulated by miRNAs. Dolezalova *et al.*[143] demonstrated that the endogenous **miR-302** family (miR-302a, miR-302b, miR-302c, miR-302d) is upregulated in human embryonic stem cells (hESCs) following an apparently lethal dose of UVC irradiation (3 J/m^2), based on an increased percentage of apoptotic cells (2.3% - 42.3%) 16 h post-exposure. Moreover, the authors suggest the miR-302 family to be direct negative regulators of p21 as indicated by repression of the 3' UTR of p21 when co-transfected into human fibroblasts. Interestingly, despite p53 and pro-apoptotic protein accumulation following UVC radiation, hESCs had no detectable p21 expression. Although, this could represent the switch in cell fate, as p21 mainly functions to induce cell cycle arrest in an attempt at repair and survival, which precedes commitment to apoptosis[144]. It is tempting to speculate that repression of p21 by miR-302 may explain why hESCs are highly sensitive to DNA damage[145]. It remains to be seen whether lethal damage, in other cell types, also elicits upregulation of miR-302 family members. Similarly, **miR-106b** can promote G1/S-phase cell cycle progression, by targeting p21 in miR-106b transfected human mammary epithelial cells (HMECs) *in vitro*[146]. Several lines of evidence suggested that miR-106b transfection promotes cell cycle progression, including; an increased accumulation of S-phase cells, an expedited growth curve, and accumulation of cells in G1-phase after miR-106b inhibition. The authors attributed these observations to, at least in part, negative regulation of p21 by miR-106b, as miR-

106b was able to reduce p21 mRNA and protein levels in transfected HMECs. Furthermore, p21 knock down recapitulated the phenotype of miR-106b transfected HMECs. Intriguingly, miR-106b was able to disengage a doxycycline-induced G1/S checkpoint, a phenotype mimicked by p21 inhibition. One would anticipate that inhibiting cell cycle arrest, would potentiate DNA damage-induced cell killing. In this instance, Xu *et al.*[147] showed that **miR-33b-3p** can inhibit p21 expression upon it's overexpression in NSCLC cells. Interestingly, this had the net effect of increased cell proliferation following treatment with cisplatin. The authors found significant downregulation of miR-33b-3p in response to 50 μM cisplatin, which was time dependent. The authors concluded that the miR-33b-3p downregulation was the cause of cisplatin sensitivity in these cells. Interestingly, miR-33b-3p was suggested to promote DNA repair as indicated by reduced γH2Ax levels following cisplatin treatment of miR-33b-3p transfected NSCLC cells. This was supported by a significant upregulation of excision repair cross-complementation protein (ERCC1), a component of nucleotide excision repair (NER). This makes sense as cisplatin is an alkylating agent known to generate 'bulky' DNA adducts which is repaired by NER[148,149]. Given this, however, the cellular response to downregulate miR-33b-3p, upon genotoxic insult, as observed with cisplatin is curious. This may be dependent on the level of damage, if the damage surpasses a threshold level that cannot be repaired, the cells instead downregulate factors that promote repair and arrest (like miR-33b-3p) in favour of cell death. At lower dose, the upregulation of miR33b-3p would therefore be expected. This remains to be investigated.

DDR repair

The proficiency of DNA repair capabilities will play a critical role in determining where the aforementioned hypothetical thresholds sit. A repair defective primary cell will be less tolerant to damage and would be expected to have lower damage tolerance and therefore, more susceptible to cell death. As DSBs are considered the most lethal DNA lesions[4], the efficiency of DSB repair pathways have a significant effect on cell fate. Repair efficiencies will be dictated by mutations, pharmacological therapies, but also miRNA-regulation of key enzymes. For example, PARP1 functions in several DNA repair pathways including NER[150], base excision repair (BER)[151], and it also modulates HRR[152,153]. The inhibition of PARP1, BRCA1 and RAD51 by **miR-7-5p** manifests as impaired HRR[154]. Consequently, cell fate is altered by miR-7-5p as a result of its effects on DSB repair, as miR-7-5p transfection was able to significantly reduce the doxorubicin IC_{50} in resistant small-cell lung cancer cells (SCLCs)[153]. Moreover, lymphoblastoid cell transfection with miR-7-5p prominently increased the proportion of apoptotic cells both

with, and without hydroquinone treatment[154]. This suggests that miR-7-5p is able to promote apoptosis, even at basal DNA damage levels, which, in addition to HRR inhibition, may be attributable to the inhibition of other DNA repair pathways that involve PARP1. Of note, Luo *et al.*[154] demonstrated endogenous miR-7-5p levels to decrease with increasing doses of hydroquinone, suggesting that miR-7-5p might exist to limit excessive DNA repair during homeostasis when repair is not needed, but becomes downregulated in response to DNA damage to allow DNA repair. It will be interesting to examine the expression levels of miR-7-5p at toxic damage levels.

Furthermore, **miR-103** and **miR-107** directly target both RAD51 and its paralog RAD51D, resulting in impaired HRR[155]. Transfection of osteosarcoma cells with either miR-103 or miR-107 reduced cell viability in response to increasing concentrations of AZD2281, a PARP1 inhibitor (PARPi) or cisplatin treatment compared with untransfected cells. This suggests these miRNAs to augment the inhibitory effect of PARPi, a situation akin to synthetic lethality. Moreover, miR-103/107-mediated inhibition of HRR also explains the observed reduction in cell viability in response to cisplatin, as these cells would be less capable to repair resulting DSBs. Incidentally, miR-103 also directly targets three-prime exonuclease (TREX1) in human umbilical vein endothelial cells (HVECS), *in vitro*[156], which has been demonstrated to augment PARP1 stability and function[157], thus further linking miR-103 to repair inhibition. RAD51 was also shown to be directly inhibited by **miR-**34a**/b/c-5p** (miR-34 species)[158]. Transfection of colon carcinoma cells with any of these miRNAs caused a significant increase in apoptosis even in the absence of genotoxic treatment. Furthermore, transfected cells also demonstrated an increased γH2Ax accumulation, suggestive of impaired DSB repair. While miR-34 species can independently inhibit RAD51 (and by extension HRR), the authors noted RAD51 inhibition to be more pronounced in $p53^{wild\text{-}type}$, than $p53^{-/-}$ cells transfected with miR-34 species. This may result from a possible p53-miR-34 positive feedback loop as suggested by Moore *et al.*[106] (previously discussed). It seems plausible that this p53-miR-34 positive feedback loop could be a means to inhibit DNA repair in cells committed to apoptosis, to turn off repair, as ongoing DNA repair attempts is counterintuitive to an apoptotic outcome. A situation analogous to PARP1 cleavage during apoptosis to disrupt DNA repair[78], but this needs investigation. A comprehensive screen by Piotto *et al.*[159] revealed RAD51 to be a direct target of **miR-96-5p**, BRCA2, X-ray repair cross-complementing protein (XRCC5), and protein kinase, DNA activated, catalytic subunit (PRKDC) to be direct targets of **miR-19a-3p**, **miR-218-5p**, and **miR-874-3p**, respectively. While RAD51 and BRCA2 function in HRR, XRCC5 (KU80) and PRKDC

are part of the NHEJ pathway, which shows the effect of these miRNAs on total recombinational repair. XRCC5 has also been demonstrated to be a direct target of **miR-526b** and **miR-623** as a means of inducing apoptosis in miRNA-transfected NSCLC and breast cancer cells[160,161]. Moreover, miR-526 transfected NSCLCs also demonstrated significantly increased S-phase accumulation, suggestive of intra-S-phase arrest, which is curious given the role of NHEJ across the cell cycle. Additionally, apoptosis, among transfected cells, increased as indicated by increased levels of cleaved PARP1 and caspase-3, and a significantly greater proportion of cells in sub-G1 phase compared to controls. Thus, highlighting a potential apoptosis role for these miRs, either directly or by increasing the sensitivity to damage.

It is tempting to speculate that endogenous miRNAs may be up-or downregulated in response to DNA damage as part of an inherent response to direct cell fate. In this regard, the miRNA effect on repair enzymes may manifest as an upregulation of capabilities at low, sub-lethal doses. By the same token, if DNA damage is *too severe*, cell fate is directed towards cell death, as miRNAs can seemingly shift the tolerance to DNA damage by impeding DNA repair mechanisms.

Apoptosis

Once a cell is committed to apoptosis, several pro-apoptotic proteins are upregulated in order to execute programmed cell death. As previously mentioned, p53 is central to this execution, serving as a transcription factor for several pro-apoptotic proteins. A full review of apoptosis is covered elsewhere[5,6]. Suffice to say, apoptosis comprises two main pathways, intrinsic and extrinsic. The intrinsic pathway relies on pro-apoptotic proteins to compromise the mitochondrial membrane, which in turn leaks cyctochrome C (cytoC) into the cytoplasm. CytoC serves as a molecular signal to continue with apoptosis, forming the apoptosome. Incidentally, miR-34a has been suggested to target cytoC in cerebrovascular endothelial cells, causing mitochondrial dysfunction. However, whether cytoC is a direct target of miR-34a remains speculative, as the authors did not experimentally confirm miR-34a-cytoC interaction, but rather inferred interaction based on reduced cytoC levels[162]. Nevertheless, this finding may have significant bearing on its relation to p53 function as a miR-34a-inducer, which could play a very important role in cell fate. The apoptosome, which is a construct of cytoC, apoptotic protease activating factor (APAF1), and caspase-9 (cysteine protease), ultimately activates executioner caspases (e.g. caspase-3, 7) that digests cellular components. Similarly, the extrinsic pathway relies on caspase activation, although its molecular cues are derived from death receptors (e.g. FAS ligand receptor), which also respond to DNA damage.

Pro-apoptotic miRNAs

Perhaps the most extensively researched regulators of the intrinsic apoptotic pathway are the Bcl-2 family of apoptosis regulating proteins. Broadly, these are classified as either pro-apoptotic (e.g. BAK, BAX) or anti-apoptotic (e.g. Bcl-2, Bcl-xL). Moreover, BH3-only proteins (e.g. BID, BIM, PUMA) are a subset of pro-apoptotic regulators that, as the name suggests, only contain a BH3 domain. This domain allows BH3-only proteins to interact with both pro- and anti-apoptotic regulators. While interactions with anti-apoptotic proteins are inhibitory, those with pro-apoptotic proteins can be activating. Thus, BH3-only proteins both directly and indirectly promote apoptosis[163].

MiRNAs are known to regulate Bcl-2 proteins (directly and indirectly), and in both directions. For example, miR-449a inhibits Bcl-2 in gastric cancer cells[126], as previously mentioned, whereas **miR-1** inhibition decreases BAX and increases Bcl-2, to combat glucose-induced apoptosis in rat cardiomyocytes[164]. The pro-apoptotic capability of miR-1 requires mechanistic elaboration particularly in the context of DNA damage. Bcl-2 proteins are also substrates for the miR-15a/miR-16-1 cluster in transfected chronic myelogenous leukaemia (CML) cells, promoting intrinsic apoptosis *in vitro*, as evidenced by increased APAF expression[80]. We have previously noted the induction of miR-15a following DNA damage[16], whose biogenesis is also influenced by p53[98]. This miR-15/16 cluster may therefore play an apoptogenic role as has been seen with the miR-15b/16-2 cluster (as previously described).

MiRNA-mediated regulation also extends to extrinsic apoptosis. For example, miR-145 can upregulate TNF-related apoptosis inducing ligands (TRAIL) in prostate cancer cells[165]. TRAIL is part of a family of ligands that induce extrinsic apoptosis by dimerizing with so-called 'death receptors' even in response to DNA damage[166]. Ultimately, this interaction facilitates caspase-8 and caspase-10 activation, which in turn activates executioner caspases[167]. This effect may indeed be indirect via miR-145-mediated feedforward activation of p53, which would subsequently lead to TRAIL transactivation as part of the pro-death functions of p53, whether this coincides with specific PTMs of p53 is yet to be investigated. Incidentally, miR-145 transfection can also induce apoptosis in several breast cancer cell lines, even in the absence of DNA damage[168] and has been shown to augment PUMA, an intrinsic apoptosis factor.

As illustrated here, much of the pro-apoptotic contributions of miRNAs are mediated through crosstalk with p53 and it is plausible that p53 employs miRNA species to dictate cell fate (**Fig. 3**). For example, p53-mediated transactivation of miR-34a and miR-143 may guide the pro-death

response to DNA damage by inhibiting Bcl-2[169,170], whereas, **miR-22**, which is also p53-inducible, upregulates BAX and caspase-3, and inhibits p21 to increase apoptosis[114,115,169,171,172]. This transactivation of miRNA may be dependent on ser46 phosphorylation of p53, but this remains to be seen. Furthermore, miR-192 and miR-215 are also transactivated by p53 and were demonstrably pro-apoptotic, not only through the positive regulation of p53 levels, as discussed, but they were also shown to target both insulin-like growth factor (IGF-1) and its receptor (IGF-1R), when co-transfected into multiple myeloma cells[117], which are components of the pro-survival IGF-1/PI3K/AKT pathway[173]. Whether this occurs following lethal levels of DNA damage remains to be seen. Incidentally, **miR-194-5p** can also induce apoptosis by targeting IGF-1R in glioblastoma cells[174]. Yang *et al*.[98], described the aforementioned p53-inducible miR-15a as pro-apoptotic as it was able to antagonise anti-apoptotic, neuronal apoptosis inhibitory protein (NAIP), *in vitro*. Inhibition of miR-15a resulted in a pronounced reduction in the apoptotic fraction of lethally-treated bleomycin cells compared to those treated with bleomycin alone. This pro-apoptotic effect of miR-15a supports previous observations by Cimmino *et al*.[80] that showed miR-15a to target Bcl-2, which may be the main pro-apoptotic mechanism of miR-15a as there appeared to be only a slight increase in NAIP level when miR-15a was inhibited.

Anti-apoptotic miRNAs

Cao *et al*.[175] demonstrated **miR-504** to be anti-apoptotic in transfected smooth muscle cells as evidenced by significantly reduced levels of apoptosis. Importantly, the anti-apoptotic capability of miR-504 is facilitated by directly targeting the 3' UTR of p53, causing, amongst others, a significant reduction in BAX expression. By the same token, Li *et al*.[176] showed that **miR-886-5p** inhibits BAX in transfected non-cancerous cervical cells, whereas miR-886-5p inhibition promoted BAX and apoptosis in cervical cancer cells. **MiR-183-5p** also has anti-apoptotic and proliferative functions in breast cancer cells, suggested to stem from direct inhibition of programmed cell death protein (PDCD4)[177] that elicits apoptosis via upregulating BAX and inhibiting Bcl-2[178]. However, Eto *et al*.[179] suggests PDCD4 to be inherently anti-apoptotic by inhibiting pro-caspase-3 translation. The underlying anti-apoptotic mechanism of miR-183-5p requires further exploration. **MiR-24-3p** has been shown to impair p27 levels, though, unlike the observations of Cheng *et al*.[177], this is through direct 3' UTR interaction[180]. MiR-24-3p was also capable of reducing apoptosis as indicated by significantly elevated apoptotic levels when miR-24-3p was antagonised in breast cancer cells. Another example of an anti-apoptotic miRNA is **miR-378** that inhibits intrinsic apoptosis by directly targeting caspase-9 in murine tissues[181]. Another antagonist of the intrinsic apoptotic pathway is **miR-155**, exerting its anti-apoptotic

effect by inhibiting APAF1[182]. MiR-155 and APAF1 exhibited an inverse relationship in lung cancer patient samples, with miR-155 expression being significantly elevated while APAF1 was significantly downregulated. Moreover, normal lung samples displayed significantly less miR-155, and significantly higher APAF1 levels than lung cancer samples. This relationship was further supported by similar apoptosis responses being obtained following either APAF1 or miR-155 inhibitor transfection into lung cancer cells, with and without cisplatin treatment at 10 μM, a dose that is greater than the LD_{50} for these cells[183]. Interestingly, miR-155 inhibition combined with cisplatin treatment resulted in a pronounced increase in apoptosis, compared to cells treated with cisplatin alone, suggesting that miR-155 inhibition can increase the potency of cisplatin. Whether miR-155 is part of the DDR remains to be seen. An anti-apoptotic effect is also described by Grieco *et al*.[184] that demonstrated several BH3-only proteins to be regulated by **miR-23a-3p**, **miR-23b-3p**, and **miR-149-5p**. The authors identified these miRNAs as pro-inflammatory cytokine responsive, noting an inverse relationship between miR-23a-3p and miR-23b-3p, and BAX and BH3-only mRNA expression, namely DP5, PUMA, and BIM in pancreatic β-cells. Similarly, miR-149-5p inhibition upregulated PUMA, BAX and BIM in pancreatic β-cells. Moreover, c-Jun upregulation was observed in pancreatic β-cells following miR-23a/b-3p inhibition, both with and without combined IL-1β and IFN-γ treatment. Importantly, inhibition of miR-23a-3p significantly increased apoptosis in both primary pancreatic islets, and pancreatic β-cells, with and without combined IL-1β and IFN-γ treatment. A similar effect was observed upon miR-23b-3p or miR-149-5p inhibition, though only in pancreatic β-cells. Moreover, as miR-23a/b-3p and miR-149-5p inhibition was able to significantly increase apoptosis in primary pancreatic islets, and/or pancreatic β-cells, even without cytokine treatment, suggest that these miRNAs may be part of an inherent regulatory network that serves to suppress apoptosis during basal conditions, but becomes downregulated when cells are fated for apoptosis. This remains to be seen in response to genotoxic damage, although it is known that pro-inflammatory cytokines induce DNA reactive radicals[185], which have the propensity for strand breakage and subsequent DDR activation.

A pro-survival phenotype has also been observed in murine embryonic stem cells (mESC), owing to BIM inhibition by **miR-20a**, **miR-92a**, and miR-302a, impairing intrinsic apoptosis[186]. These findings alludes to BIM being the causative agent of apoptosis in mESCs, but contradicts the story discussed earlier, of miR-302 in lethally radiated hESCs[143]. Incidentally, BIM has been described as a target for several miRNAs. For example, Zhang *et al*.[187] showed **miR-214** to be anti-apoptotic by targeting BIM in nasopharyngeal carcinoma cells. Similarly, Floyd *et al*.[188]

demonstrated **miR-363** and **miR-582-5p** to be anti-apoptotic in transfected glioblastoma cells by directly targeting BIM as well as caspase-3, and caspases-3/9 respectively. Caspase-8 and 3 are direct targets of **miR-24** and **miR-221** *in vitro*, respectively[189]. Consequently, when transfected into hepatocarcinoma cells, these miRNAs can significantly decrease apoptosis in response to TRAIL treatment. Moreover, it's capable of inhibiting TRAIL-induced (extrinsic) apoptosis by directly targeting its receptor, death receptor-4 (DR4) in cholangiocarcinoma cells[190]. This may contribute to the reason why miR-25 is considered an oncogene, as evidence in glioblastoma cells have shown it to be pro-survival[191]. To facilitate extrinsic apoptosis, death receptors require adapter proteins in order to recruit and subsequently activate caspase-8 and caspase-10. Fas-associated death domain containing protein (FADD) is such an adapter protein that couples intracellularly with death receptors[192]. Importantly, FADD and caspase-3 are direct targets of **miR-155**, capable of impairing extrinsic apoptosis in nucleus pulposus cells[193]. Extrinsic apoptosis can also be impaired by inhibiting the recruitment of adapter proteins to the death receptor. PTEN facilitates FADD recruitment by mediating the displacement of the inhibitory protein, mitogen-activated kinase activating death domain containing protein (MADD), attached to the death receptor. Interestingly, displaced MADD can, in turn, displace BAX from inhibitory 14-3-3 proteins. Thus, PTEN can facilitate both intrinsic and extrinsic apoptosis[194] and is a critical tumour suppressor. Furthermore, **miR-498** can suppress PTEN in miRNA-transfected/transduced breast cancer cells[195]. This weight of evidence clearly shows how cell fate can be regulated by miRNAs, but there needs to be critical substantiation of these miRNAs to understand how those mentioned can affect the cellular response to DNA damage. One would anticipate a situation whereby these miRNAs become downregulated at lethal damage levels, but are otherwise basally present, or perhaps even induced at lower, survivable damage levels.

A Network of Regulators

It has been suggested that miRNAs may function synergistically within a network of miRNAs, at least in part, to minimise the energy demand stemming from miRNA biogenesis, thereby facilitating efficient responses to biological cues[196]. In the context of the DDR, it makes sense why many miRNAs, such as miR-16 and miR-34a have multiple targets (**Fig. 4**) and why many miRNAs, and even other regulatory RNA species, are clustered and even intragenic. To

elaborate, it would presumably be more energetically favourable for only one miRNA species to target multiple DDR enzymes than a situation that requires the biogenesis of many miRNA species to target the same number of enzymes. This would then conserve energy to accommodate an impending cell fate, such as apoptosis, that is energy-expensive[197,198]. By the same token, this could explain why certain targets (e.g. SIRT1) are shared between multiple miRNAs (**Fig. 4**). The authors also suggested a potential 'feed forward' effect of miRNA disruption/alteration on other miRNAs. Incidentally, this may emphasise the potential shortcomings of miRNA transfection-based studies, as miRNA overexpression potentially dysregulates the endogenous miRNA network. In context of the DDR, miRNAs have also been known to co-operate. For example, repression of Chk1 and WEE1 is more pronounced when miR-16 and miR-26a are co-transfected in NSCLC cells[85]. Similarly, **miR-30c** and **miR-181a** co-expression yielded a more pronounced inhibition of apoptosis, and synergistically target the p53-p21 pathway when co-transfected into high glucose-treated (30 mM) rat cardiomyocytes[199]. Taken together, it is possible that during the DDR, participating miRNAs co-operate in an attempt to conserve energy, thereby supporting subsequent, energy-dependent processes, e.g. apoptosis. This seems possible as RISC complex assembly can be ATP-dependent[200]. Additionally, miRNAs can also regulate each other, for example, ectopic **miR-29c** can significantly upregulate miR-34c and **miR-449a** in transfected nasopharyngeal carcinoma cells[201]. The mechanism of this, however, is yet to be determined. Therefore, miR-29c may be regarded pro-apoptotic as both miR-34 and miR-449 family members contribute towards an apoptotic cell fate (as previously discussed). In context of the DDR, the intricacies of miRNA networks become clear, and multiple miRNAs may function in a theoretically auxiliary capacity within the same, or adjacent pathways in order to elicit the same cellular outcome (**Fig. 4**). By the same token, it is tempting to speculate that miRNA-miRNA cross-talk may result in miRNA inhibition. However, while it is not clear whether certain miRNAs directly inhibit other miRNAs, an indirect inhibitory mechanism may exist by miRNA-host gene interactions. For example, miR-20a can directly downregulate PRKG1[202], which is the host gene of miR-605[100]. Consequently, the DDR may be impeded as miR-605 functions to disrupt the p53-MDM2 interaction (previously discussed), thereby favouring an anti-apoptotic cell fate. Thus, it is theoretically possible for miRNAs to inhibit each other by targeting miRNA host genes. Collectively, these observations suggests extensive cross-talk between miRNAs, transcription factors, and DDR genes, which may dictate specific cell fate outcomes, or even modulate DNA damage responsiveness in a tissue-specific manner. The advent of expansive miRNA interaction databases such as; PicTar[203], TargetScan[204], starBase[205]

and miRTargetLink 2.0[206] has begun to address the shortcomings in our understanding of miRNA interactions, though much remain to be determined.

In relation to this review, miRNAs are intimately tied to DDR dynamics where we postulate that greater DDR activation, i.e. higher levels of activated DDR proteins, result in greater transcription of certain miRNA species. Consequently, this could induce a greater abundance of certain miRNAs which could have either level-dependent effects, i.e. miRNA thresholds to facilitate specific activities, or alter the balance between a miRNA and its antagonist to tip the balance in favour of a cell fate.

Differential miRNA response to sub-lethal and lethal DNA damage

It is clear that the miRNAs discussed throughout this review, can be distinguished on their net cell fate effect and can effectively be resolved into two profiles, survival and death (**Fig. 4 and Table 1**). Therefore, upon DNA damage, low levels would see upregulation of miRNAs associated with repair, arrest and anti-apoptosis, perhaps even downregulation of pro-apoptosis miRNAs. Whereas, at high doses, on the other hand, if the damage threshold of arrest and repair has been surpassed, the profile of miRNAs would be opposite i.e. upregulation of apoptogenic miRNAs, and perhaps down downregulation of those associated with repair, arrest and anti-apoptosis. In essence, it's plausible that a given level of damage will activate/inactivate different feedback circuits that give rise to different cell fates[6]. For example, mice exposed to radiation exhibit different serum miRNA profiles at sub-lethal or lethal doses[207]. While **miR-187-3p**, miR-194-5p, and **miR-27a-3p** were significantly downregulated 24 h after lethal radiation exposure, **miR-30c-5p** and **miR-30a-3p** were significantly upregulated when compared to sub-lethal doses. Interestingly, this was not a static change, as most miRNAs that were initially downregulated following lethal irradiation either became upregulated, beyond that observed at sub-lethal doses, or approached that of sub-lethal doses, beyond 3 days after irradiation. As miR-187-3p, miR-194-5p, and miR-27a-3p were downregulated following lethal radiation, it is reasonable to assume that these miRNAs might have been involved in certain cellular survival strategies aimed at resolving DNA damage, or are otherwise anti-apoptotic, as has been shown for miR-187 in transfected gastric cancer cells[208]. The concept of differential miRNA expression patterns in response to varying degrees of DNA damage is gaining traction. Several studies have reported specific miRNAs to be expressed in a dose-dependent manner[14–16]. For example, miR-96 expression is demonstrably dose-dependent as 1-2 μg/ml cisplatin, or 1-2 μM doxorubicin

can significantly induce its expression after 24 hours, whereas higher doses (up to 10 μg/ml or 10 μM respectively) were either unable to do so, or to a lesser extent than that of the lower doses[209]. Another example is described by Leslie *et al*.[210] that showed **miR-103** and **miR-107** were specifically upregulated by lethal doses of doxorubicin by p53 to execute apoptosis, perhaps as a result of their aforementioned targeting of HRR[155].

The governing signal of this 'switch' in cell fate is still elusive and may come from within the DDR itself. ATM and p53, for example, have been shown to affect miRNA biogenesis, but again, that primary stimulus and subsequent mechanism to change cell fate is still unclear, i.e. what quantifies the level of damage to ensure the appropriate cell fate is executed?

Regulating the Regulators

It is clear that while examining the regulation of the DDR by miRNAs, one must examine the regulation of the miRNAs themselves and it poses the question of who is in charge[211]. We have seen how miRNAs regulate the DDR enzymes and how some DDR enzymes, namely ATM and p53 regulate certain miRNAs. So, rather than the DDR being regulated by a miRNA level above, there is definite cross-talk and the regulation must be a feed-forward or feed-back loop. Although, there seems to be a layer of regulation above miRNA involving other RNA species, including long non-coding species. This has recently been reviewed elsewhere[13] and will not be discussed in detail here, except to give a few relevant examples. For instance, the HIPK3 gene houses non-coding circular RNA HIPK3 (circHIPK3), which serves to 'mop up' miRNAs. Both **miR-149**[212] and **miR-485-3p**[213] are known targets of circHIPK3. Both miR-149 and miR-485 affect apoptosis executioners[184,213], but their biological effects will largely be dependent on the expression levels of circHIPK3. Similarly, miR-24 and miR-221 are direct targets of cancer susceptibility candidate (CASC2), a long non-coding RNA (lncRNA) reported to mop up these miRNAs in an attempt to abrogate their anti-apoptotic effects in liver cancer[189]. Another lncRNA, X-inactive specific transcript (XIST), has been implicated in pro-survival cell fates by mopping up, miR-132-3p[214], miR-34a[215] in colorectal cancer cells and miR-144 in NSCLC cells[216], thereby counteracting its apoptogenic functions, which includes the inhibition of Bcl-2. Another aspect warranting consideration is that DNA damage-responsive miRNA expression profiles may be influenced by inherently different, cell-type and state-specifics that may determine the cellular sensitivity to damage. For example, mouse epiblasts are more susceptible to undergo apoptosis following low-dose irradiation than surrounding tissues[217]. The authors

alluded to epiblasts being primed for apoptosis as evidenced by inherently elevated levels of pro-apoptotic BIM, BAK, and NOXA, and decreased levels of anti-apoptotic Bcl-xL, as compared to extra-embryonic tissues. Thus, in pro-apoptosis primed cells, it is tempting to speculate that such cells would possess a differential miRNA profile to drive the DDR in favour of death or vice versa following DNA damage as alluded previously in hESCs. Research suggests some miRNAs to be cell type-specific[164,218]. It may also possible that the *function* of the same miRNAs are tissue or situation-specific to explain why miR-16 has been reported to both induce and oppose cell cycle arrest, respectively[15,85] (**Fig. 4**).

Clinical translation

This review focuses on the potential role of many miRNA species in a physiological response to DNA damage. While further investigations are needed to fully elucidate the physiologically relevant, regulatory miRNA network, we are beginning to understand the role they play in many diseases and their potential clinical use, both as targets, but also as tools. Indeed, their use as a diagnostic tool is perhaps more evolved than their use as a therapy[219]. An interesting story is emerging, which focuses on the clinical impact resulting from the crosstalk between miRs and mismatch repair (MMR)[220]. MMR plays a pivotal role in DNA damage sensing, cell fate signalling via ATM and ATR[221,222], cancer susceptibility[223–225], drug and even immunotherapy resistance[226–228]. DNA mutations that confer a MMR defect have been well documented as the cause of the mutator phenotype in colorectal cancer[229]. It has now been shown that a defective MMR phenotype could also be attributed to certain miR species that effectively mimic the effect of pathological MMR mutations in oncogenesis and treatment resistance. For example, miR-155 has been shown to target key MMR proteins as a potential cause of microsatellite instability in colorectal cancer[230]. This adds to the oncogenic properties and clinical translation of miR-155. As we previously mentioned, miR-155 is anti-apoptotic, and explains the findings of overexpression also in breast cancer[231] and its association with drug resistance[182], which may also be dependent on its effects on MMR, but this remains unsubstantiated. Incidentally, miR-21 has a substantiated role in 5-fluorouracil resistance as a result of inhibitory effects on MMR proteins[232] in colorectal cancer. Intriguingly, MMR defects have been shown to be advantageous in anti-PD-1/PD-L1 immunotherapy outcomes for colorectal cancer patients[233]. Incidentally, miR-148a-3p has shown to directly and inversely affect PD-L1 expression in colorectal cancer[234]. Thus, inferring the utility of miRs as diagnostic and prognostic markers and potential targets or even adjuvants in chemo- and immunotherapy, respectively.

In the clinic, the aim is to manipulate key miRs for therapeutic benefit. The level of a given miRNA can be manipulated by using a specific antagomiR (anti-miR/blockmiR), which silences the endogenous miRNA, or by using an agomiR, which mimics the endogenous miRNA. Both antagomiRs and agomiRs are small RNA species and RNA-based therapeutics, such as short interfering RNA (siRNA) have been granted food and drug administration (FDA) approval in recent years[235]. While this does not involve miRNA, the applicability can be realised, and it is an active area of growth and development where pathological miRNAs are targeted[236–238]. In relation to the response to DNA damage, it is interesting to posit the usefulness of targeting an miRNA to manipulate the DDR to sensitise cancer cells to chemotherapy, thus potentially lowering the effective dose and reducing dose-related side effects, although this has yet to be investigated. At the time of writing, ten miRNA therapies have been involved in clinical trials, none have reached the later stages[239]. MRX34 for example, a miR-34a (mentioned in this review) mimic that has been involved in a phase 1 cancer trial (NCT01829971)[240]. The trial was terminated prematurely due to serious immune-related adverse events. We and others[239] have eluded to the multifaceted effects of a single miRNA and full characterisation and understanding is still needed. It is clear, while there is promise, there is considerable work to be done to realise the therapeutic benefit of targeting pathological miRNAs, or harnessing miRNAs as therapeutics.

Concluding remarks

MiRNAs play a role to regulate the DDR that orchestrates an appropriate outcome in response to different levels of DNA damage. Indeed, the roles of miRNAs can be broadly distinguished on their effect on cell fate. Though it must be stated that their exact function in the DDR remains largely speculative, owing to *in silico* and/or overexpression/repression studies that may not represent the actual dynamics of biological systems in response to DNA damage. Considering the involvement of miRNAs in guiding pro- and anti-apoptotic cell fates, it is possible that the final cell fate outcome is determined by a specific miRNA expression profile, which would ultimately be governed by level of damage, but also cell state, and tissue specificity. Finally, as they are intricately part of multiple steps in the DDR, they are attractive therapeutic targets, or potential treatments. Understanding their physiological role in response to chemotherapy-induced DNA damage is critically important.

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Figure Legends

Figure 1. The DDR pathway and its pro-apoptotic arm in the context of sub-lethal and lethal DNA damage. DNA damage is detected by sensor protein kinases ATM, or ATR that

propagates ‘damage’ signals to transducer protein kinases (Chk1/2) by means of phosphorylation. Phosphorylated Chk1/2, in turn, further propagates the signal, culminating in p53 activation. Signalling may follow the ‘sub-lethal damage’ route, if the damage is tolerable/repairable, resulting in cell cycle arrest, DNA repair, and ultimately survival. However, if DNA damage is too severe, signalling may follow the ‘lethal damage’ route resulting in transcriptional activation of pro-apoptotic proteins, eventuating in apoptosis. Pointed arrows (activation), blocked arrows (inhibition). Created on license from BioRender.com.

Figure 2. Overview of miRNA biogenesis. Beginning in the nucleus, miRNAs are embedded within a hairpin structure in pri-mRNA transcripts. A sequence of processing enzymes; DROSHA, PASHA, EXPO5 and DICER release the miRNA duplex, which then becomes part of the RISC complex, to enact the miRNAs biological function in degrading a homologous transcript and blocking its protein synthesis. Created on license from BioRender.com.

Figure 3. Transcriptional and post-transcriptional regulation of miRNAs by p53. While certain p53-induced miRNAs can have dual contributions (orange) to cell fate, others (red) appear dedicated to apoptosis. Of note, miR-16-1 (blue) is apoptogenic but also able to promote or inhibit cell cycle arrest. Pointed arrows (activation), blocked arrows (inhibition). Created on license from BioRender.com.

Figure 4. miR-mediated regulation of the DDR at sub-lethal and lethal damage. The canonical DDR pathway consisting of MRN, ATM, ATR, Chk1/2, p53 (solid black lines), and it’s apoptotic arm (SIAH, HIPK2 and SIRT1) to orchestrate the execution of repair, arrest or apoptosis and the proposed avenues of signalling taken at sub-lethal damage (green), lethal damage (red). The substantiated influence of miRNA (dashed lines) is also presented, which has been resolved into likely cell fate effects according to published findings of sensitisation or resistance (survival (green), cell death (red), or unresolvable (black)) to correlate with damage levels. Pointed arrows (activation), blocked arrows (inhibition). Created on license from BioRender.com.

Table 1. A list of miRNAs and their observed effect on cell fate