Sarco“MiR” friend or foe: a perspective on the mechanisms of doxorubicin-induced cardiomyopathy

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Abstract: Anthracyclines are a class of chemotherapeutics used to treat a variety of human cancers including both solid tumors such as breast, ovarian, and lung, as well as malignancies of the blood including leukemia and lymphoma. Despite being extremely effective anti-cancer agents, the application of these drugs is offset by side effects, most notably cardiotoxicity. Many patients treated with doxorubicin (DOX), one of the most common anthracyclines used in oncology, will develop radiographic signs and/or symptoms of cardiomyopathy. Since more and more patients treated with these drugs are surviving their malignancies and manifesting with heart disease, there is particular interest in understanding the mechanisms of anthracycline-induced injury and developing ways to prevent and treat its most feared complication, heart failure. MicroRNAs (miRNAs) are small noncoding RNAs that regulate the expression of mRNAs. Since miRNAs can regulate many mRNAs in a single network they tend to play a crucial role in the pathogenesis of several diseases, including heart failure. Here we present a perspective on a recent work by Roca-Alonso and colleagues who demonstrate a cardioprotective function of the miR-30 family members following DOX-induced cardiac injury. They provide evidence for direct targeting of these miRNAs on key elements of the β-adrenergic pathway and further show that this interaction regulates cardiac function and apoptosis. These experiments deliver fresh insights into the biology of toxin-induced cardiomyopathy and suggest the potential for novel therapeutic targets.

Keywords: Doxorubicin (DOX); heart failure; microRNA (miRNAs); beta-adrenergic pathway

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Introduction to the biology of anthracycline toxicity in the heart

Anthracyclines, including doxorubicin (DOX), are among the most effective chemotherapeutic agents used to treat many human cancers. Unfortunately, their use is limited by adverse side effects, most notably dose-dependent cardiotoxicity. These events can manifest acutely following administration of the drug, or more commonly, months to years following cumulative exposure (1). In adults, early and late toxicity typically presents with dilated cardiomyopathy (2). Up to 5–10% of patients exposed to these drugs can have symptomatic or radiographic evidence of heart failure (3). Even more, some studies have demonstrated an increased risk of cardiotoxicity in those patients receiving low doses of DOX (<300 mg/m²) which were once thought to be considered safe (4). Children, on the other hand, tend to present with dilated cardiomyopathy that progresses to a restrictive pattern (2). Childhood cancer survivors exposed to anthracyclines can have up to a 2–5 fold increased risk of heart failure compared to those patients not exposed to these drugs (5). Given the increasing number of cancer survivors amongst adults and children, anthracycline-induced cardiotoxicity is becoming a prevalent disease despite efforts dedicated to surveillance and prophylactic management.

DOX is thought to deliver its anti-tumor effects primarily through inhibition of the alpha isoform of topoisomerase II (Top2α). The mechanisms of toxicity in the heart seem to be much more complex especially since cardiomyocytes lack
expression of Top2α. These cells do, however, express the beta isoform (Top2β), and cardiomyocyte specific deletion of the gene encoding this protein confers protection against DOX-induced toxicity through inhibition of apoptosis and DNA-damage (6). Another mechanism of DOX-induced cardiotoxicity involves the generation of reactive oxygen species (ROS). This was originally a very attractive mechanism to explain cardiomyocyte injury as these cells have high metabolic rates, are chock full of mitochondria, and express low levels of anti-oxidant enzymes (7). Despite experimental evidence to support this theory, clinical efforts to scavenge free radicals have not been promising (8). Newer mechanisms have focused on the role of DOX in the inhibition of pro-survival pathways such as NRG-1/ErbB (9,10), and the stimulation of inflammation through Toll-like receptors (TLRs) (11).

MicroRNAs (miRNAs) are small noncoding RNA molecules that bind to the 3’ un-translated region (UTR) of mRNAs and regulate their expression (12). Recently, miRNAs have been shown to play a critical role in many elements of cardiovascular disease including ischemia (13-15) and heart failure (16-18). Naturally, this led many groups to investigate the potential role of miRNAs in the development of DOX-induced cardiomyopathy (10,19-23). Roca-Alonso and colleagues continued this mission with a comparison of the global changes in miRNA expression in adult rat ventricular cardiomyocytes (ARVCMs) through two models. The first was an acute in vitro model of cultured ARVCMs harvested 6 hours after a single dose of DOX. The second was a chronic in vivo model where rats were exposed to repeated doses of DOX (cumulative dose of 15 mg/kg) over a two week period followed by harvesting ARVCMs 3 weeks later. A reference model of cardiomyopathy generated from rats with proximal left anterior descending (LAD) artery ligation was also included in the comparison. Three members of the miR-30 family (miR-30a, miR-30d, and miR-30e) were down-regulated in at least two of three models (21). Down-regulation of miR-30b has also been documented in H9C2 rat cardiomyocytes following hypoxia/re-oxygenation (24). Interestingly, other groups that generated cardiac miRNA profiles in animal models exposed to DOX had unique signatures that did not uncover miR-30 family members as being significantly dis-regulated (25,26). Nonetheless, there is quite a bit of variability in the timing of DOX exposure, the genetic background, and the technology used to generate these profiles.

The contribution of miR-30 and the β-adrenergic pathway towards the pathogenesis of heart failure

In cancer biology, miR-30 has been implicated as both an oncogene and a tumor suppressor. Its specific role tends to be cancer type specific (27-29). In the heart, overexpression of miR-30 directly regulated key profibrotic proteins and thus may be associated with preventing the fibrosis characteristic of failing hearts (30). Another group demonstrated that up-regulation of miR-30 in cardiomyocytes blocked the up-regulation of angiotensin II-induced hypertrophy related genes and showed that increasing circulating levels of miR-30 may be used to diagnose myocardial hypertrophy (31).

Roca-Alonso and colleagues attempt to further promote miR-30 as a cardioprotective miRNA through a unique mechanism. Among the list of computationally derived predictive targets of miR-30, this group focused on three proteins in the β-adrenergic pathway (β1AR, β2AR, and G11,12) (21). Modulation of contractile function in the heart via the β-adrenergic pathway involves the interaction of β1AR and β2AR with stimulatory guanylyl nucleotide binding proteins, Gs. This leads to the activation of adenylyl cyclase, an increase in cyclic AMP (cAMP), activation of protein kinase A (PKA), and the phosphorylation of direct components of the contractile apparatus and elements of the excitation contraction coupling system. β1AR is also able to interact with inhibitory G proteins, G i, which block adenylyl cyclase function as well as having the potential to activate the pro-survival phosphoinositide 3 kinase (PI3K)/Akt pathway (32-34). The role of the β-adrenergic pathway in the pathogenesis of heart failure has been well studied (32,34,35) and thus the choice to focus on β1AR, β2AR, and G11,12 for further analysis was well conceived.

Chronic adrenergic stimulation in the heart has been shown to elicit cardiotoxicity (36). This effect is thought to be mediated primarily through aberrant activity of β1AR resulting in calcium overload and cell death (37,38). In failing hearts, there is a down-regulation of β1AR along with desensitization, whereas the density of β2AR remains relatively unchanged (39). Transgenic mice overexpressing β1AR specifically in cardiomyocytes developed fibrosis, hypertrophy, and reduced fractional shortening at least in part due to increased apoptosis (37). Furthermore, administration of beta-blockers is the hallmark of heart failure treatment and prevention including heart failure from DOX-induced cardiomyopathy (8,40,41). On the other hand,
β2AR is thought to confer cardioprotection (38,42). This pro-survival phenotype is thought to be at least in part associated with its interaction with G1 proteins (43). In human end stage heart failure patients, G1 proteins, particularly the α-2 subunit (G1α-2), are up-regulated (44). Down-regulation of G1α-2 is associated with apoptosis and worsening heart failure (45-47). Activation of G1α-2 is slightly more controversial as one group demonstrated that constitutive activation of G1α-2 in a dilated cardiomyopathy and an isoproterenol-induced heart failure mouse model led to worsening hypertrophy and fibrosis, respectively. The authors postulate that the role of G1α-2 in fibroblasts as opposed to cardiomyocytes may be the driving force behind these phenotypes (43). To complicate the story even more, recent models have demonstrated reverse phenotypes for the role of β1AR and β2AR in heart failure whereby β1AR is cardioprotective and β2AR promotes cardiotoxicity (48). As a result, in addition to cell and disease specific contexts, the balance between β1AR and β2AR expression may contribute to the relative role of these receptors in the pathogenesis of heart failure.

The cardioprotective potential of miR-30

Since miRNAs typically have multiple mRNA targets, these molecules are poised to regulate the delicate balance of multiple effectors in a single influential pathway such as β-adrenergic signaling. Roca-Alonso and colleagues utilized luciferase assays in H9C2 cells to demonstrate direct binding of a miR-30e mimic to the wild-type 3’ UTR of four predicted targets (βAR, β2AR, G1α-2, and pro-apoptotic protein E1B-interacting protein 3-like or BNIP3L) but not to mutant constructs. In addition, these miR-30e mimics were sufficient to attenuate the up-regulation of these targets upon administration of DOX (21). Appropriate controls with random sequence molecules were used throughout their experiments. These results support the author’s claim that DOX-induced repression of miR-30 is at least partially responsible for increased expression of β1AR, β2AR, G1α-2, and BNIP3L.

In order to demonstrate that miR-30 is able to augment the downstream effects of the β-adrenergic pathway, Roca-Alonso and colleagues showed that DOX treatment or administration of a miR-30 sponge vector led to an increase in cAMP, although the magnitude of up-regulation was more prominent in DOX treated cells. This could be due to miR-30 independent mechanisms of cAMP signaling or incomplete miR-30 targeting, which is consistent with the fact that the authors demonstrate close to a 50% knockdown of miR-30e with sponge vectors. Overexpression of miR-30, on the other hand, resulted in decreased cAMP levels (21).

The phenotypic response to miR-30 was assayed through its effects on contractile function and DOX toxicity. Cells overexpressing miR-30 had an attenuation of contractile amplitude in response to increasing concentrations of isoproterenol compared to control cells. With regards to DOX toxicity, over-expression of miR-30 attenuated the increase in caspase activity triggered by DOX, although this attenuation was also incomplete. In addition, down-regulation of miR-30 led to an increased level of caspase activity compared to controls. Furthermore, in the presence of DOX, the intensity of ROS in cardiac cells was decreased with miR-30 overexpression and increased with miR-30 inhibition compared to cells exposed to DOX alone (21). While these findings are intriguing, they do not provide a direct link between miR-30 activity and damage through the β-adrenergic pathway. Future studies manipulating targets of miR-30 will be necessary to draw such conclusions.

Finally, Roca-Alonso and colleagues also uncovered the presence of GATA-6 binding to miR-30 cluster promoters in publically available data from chromatin immunoprecipitation sequencing experiments. They hypothesized that GATA-6 binding to these regions following DOX may mediate down-regulation of miR-30 (21). GATA-6 has already been implicated in cardiac pathology where it seems to be required to mount the cardiac hypertrophic response and prevent heart failure in animal models (49). To substantiate their hypothesis, these authors demonstrated that GATA-6 is up-regulated acutely following exposure of DOX to cardiac cells in culture and siRNA constructs against GATA-6 resulted in increased expression of miR-30 family members and decreased expression of miR-30 targets. Even more, in the setting of DOX treatment, knockdown of GATA-6 resulted in decreased signaling through the apoptotic pathways (21). Nevertheless, experiments demonstrating the attenuation of miR-30 down-regulation after DOX in the absence of GATA-6 are lacking. As a result, other transcription factors could be more important than GATA-6 on the effect of DOX on miR-30. These experiments also beg the question of how GATA-6 is activated following DOX treatment and whether additional targets of GATA-6 could contribute to the damage response. Moreover, if GATA-6 does indeed prove cardioprotective against failing hearts as demonstrated by others, its negative regulation of miR-30 would appear to promote cardiotoxicity. Further studies are needed to untangle the intricacies of these networks.
Roca-Alonso and colleagues provide an interesting model whereby down-regulation of $\beta_1$AR, $\beta_2$AR, and $G_{1\alpha-2}$ leads to a cardioprotective phenotype. While this certainly aligns with studies that demonstrate cardiotoxicity of excessive $\beta_1$AR, it appears to contradict the cardioprotective role of $\beta_2$AR and $G_{1\alpha-2}$. However, the authors offer an explanation of this apparent contradiction by arguing that the fine tuning of expression characteristic of miRNAs can regulate the feedback loop involving $\beta_1$AR, $\beta_2$AR, and $G_{1\alpha-2}$ in such a way to prevent the cardiotoxicity of $\beta_1$AR signaling, while still maintaining some of the cardioprotective benefits of $\beta_2$AR and $G_{1\alpha-2}$. In a similar fashion, the beta-blocker Carvedilol has been shown in patients and animal models to be protective against DOX-induced cardiomyopathy despite being characterized as nonselective towards $\beta_1$AR and $\beta_2$AR (40,41). Most of the experiments for these conclusions were based on in vitro cell cultures. Future studies in vivo are necessary to determine the long-term phenotypic consequences of miR-30 knockdown and overexpression on DOX-induced cardiomyopathy in order to fully test the therapeutic potential of these miRNAs. Such models can also be used to determine potential additive or synergistic benefit of miR-30 overexpression with beta-blockade.

**Conclusions**

In a recent report by Roca-Alonso and colleagues, a novel mechanism for DOX-induced injury involving the regulation of the $\beta$-adrenergic pathway through miRNAs is uncovered. These findings provide new insight into the complex pathways that govern the damage response and the delicate balance between the expression of key elements in a single pathway. Even more, these studies offer exciting potential for future therapeutic targets of chemotherapy-induced cardiomyopathy.

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