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EDITORIAL

miR-21: a miRaculous Socratic paradox

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This editorial refers to 'Ischaemic preconditioning-regulated miR-21 protects heart against ischaemia/reperfusion injury via anti-apoptosis through its target PDCD4' by Y. Cheng *et al.*, pp. 431–439, this issue.

1. An emerging impact of microRNA biology on heart disease

The molecular biology dogma that chromosomal DNA transcribes its genetic information to large messenger RNAs (mRNAs), yielding proteins as the chief actors within the cell, carrying out the duties specified by the information encoded in genes, failed to consider the recent evidence that the majority of the genomes of eukaryotes are, in fact, transcribed into non-coding RNA (ncRNA), many of which are alternatively spliced and/or processed into very small (18–100 nucleotides in length), yet fully functional RNA molecules. MicroRNAs (miR or miRNAs) are one such class of evolutionarily conserved, small ncRNAs, on average 22 nucleotides in length. miRNAs are post-transcriptional regulators that bind to complementary sequences in the 3′ untranslated regions (3′ UTRs) of target mRNAs in a sequence-specific manner, usually resulting in gene silencing.

Through forward genetic screening in the nematode *Caenorhabditis elegans*, the first prototypical miRNAs, lin-4 and let-7, were uncovered as regulators of developmental timing. Subsequent studies in vertebrates suggested that miRNAs, rather than functioning as decisive regulatory on–off switches, more commonly function to modulate or fine-tune expression of proteins and cellular phenotypes.[1] On the basis of sequence conservation and the ability to fold into a hairpin structure, the human genome is predicted to encode as many as a thousand miRNAs, some tissue- or cell type-specific, others considered as house-keeping molecules, and combined they are estimated to regulate as many as 30% of all cellular proteins.[2]

In several recent studies, microarray analyses were performed to determine whether miRNAs are misexpressed in hypertrophic and failing hearts. These studies point to a collection of miRNAs that are up- or down-regulated during pathological cardiac remodelling in rodents and humans.[3] Moreover, forced dysregulation of miRNA levels in heart muscle by manipulating critical miRNA biogenesis enzymes spontaneously causes many features of heart disease,[4] proving the cause–effect relationship between regulation of miRNA expression and heart disease. Following these early technological studies merely aimed at fingerprinting miRNA expression changes, the more daunting task now lays ahead of us to assign mechanistic functions to single miRNAs, using more time-consuming, integrative approaches to create gain- or loss-of-function phenotypes related to individual miRNA genes and their predicted target genes/proteins. We have already witnessed the discovery of the first miRNA regulatory circuits for cardiac homeostasis and a variety of heart diseases in recent years (recently reviewed in Liu and Olson[5]).

Among the most abundantly expressed and consistently dysregulated miRNAs in human and rodent heart failure is miR-21, which locates to the human 17q23.2 chromosomal region, partially overlapping the protein-coding gene VMP1 (or TMEM49), a human homologue of rat vacuole membrane protein, a human papilloma virus-16 (HPV16) integration locus, and a region encoding the novel small nuclear RNA U6. Given our natural tendency to focus on abundantly expressed molecules, the abundant baseline presence and strong induction of miR-21 has attracted the attention of researchers in various fields, including development, oncology, stem cell biology, and ageing and has quickly become one of the most-studied miRNAs.[6] miR-21 has also attracted the attention of the cardiovascular community in an effort to assign potential mechanistic functions of miR-21 in the context of cardiac remodelling. Given its bulk expression and easy detection, the same logic promised a central role for this miRNA and a relatively straightforward assessment of miR-21 function, where even mild perturbations in expression should proportionally affect its critical effector target proteins and yield easily distinguishable and consistent phenotypes. Recent studies to map miR-21 cardiovascular function, however, have at most proven that this miRNA has many paradoxical features, and Cheng *et al.*[7] now further fuel this controversy with their findings concerning miR-21 in ischaemic-preconditioned cardiac muscle.

2. The Socratic paradox

The classical Greek philosopher *Socrates* (Greek: Σωκράτης) remains, as he was in his lifetime (469–399 BC), an enigma, with virtually all our information about him being second-hand, coming chiefly from the accounts of later classical writers, especially the writings of his students *Plato* and *Xenophon*, and the plays of his contemporary *Aristophanes*. Despite having written nothing, he is considered one of the handfuls of philosophers who forever changed how philosophy itself was to be conceived, and his influence has been felt far beyond philosophy itself and in every century. Certainly he was impressive, so

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impressive that many others were moved to write about him, all of whom found him strange by the conventions of fifth-century Athens: in his appearance, personality, and behaviour as well as in his views and methods. One of the best-known sayings of Socrates is '*I only know that I know nothing*'; the conventional interpretation of this paradoxical remark is that Socrates' wisdom was limited to an awareness of his own ignorance. Perhaps, his most important contribution to Western thought is his dialectic method of inquiry, known as the method of '*elenchus*', which he applied to the examination of key moral concepts. To solve a problem, it would be broken down into a series of questions, the answers to which gradually distil the answer a person would seek. The influence of this approach is most strongly felt today in the use of our scientific methods, in which hypothesis is the first stage.

3. miR-21: a Socratic paradox in heart disease

Since mature miR-21 is abundant in most cancerous cell lines, it was among the first miRNAs used as a model for studying miRNA expression and maturation. Accumulating evidence, however, now points to a notion that miR-21 may be an exceptional miRNA with regards to its tissue distribution, transcriptional induction, and processing, rather than a prototypical example. Several primary transcripts (pri-miR-21) have been identified in a number of cell types using RACE and primer extension analyses, with a predominant ~3.5 kb pri-miR-21 version and, by alternative promoter usage, existence of a longer ~4.3-kb pri-miR-21,[8] independently transcribed from the overlapping protein-coding VMP1 gene. miR-21 is also one of the miRNAs consistently induced in response to hypoxia, and even epigenetic modification (hypomethylation) of its transcriptional regulatory sequences may be key to miR-21 induction.[9]

For the majority of miRNAs dysregulated in cancer, the changes in the expression levels of mature miRNAs do not correlate with the levels of their primary precursors (pri-miRNAs), which remain mostly unchanged, indicating that most of the regulation takes place after transcription. An analysis of multiple cancers revealed that the expression of numerous miRNAs is repressed in human cancers,[10] a phenomenon referred to as 'global repression of miRNAs in cancers'. However, for miR-21, there is an unusually good correlation between pri-miR-21 and miR-21 levels, suggesting that transcription is an important regulatory step for miR-21 expression and function, that miR-21 transcription and processing must be tightly coupled, and, consequently, that miR-21 processing is highly efficient. The unusually efficient processing of the miR-21 precursor may be one explanation why the mature molecule is strongly up-regulated, whereas the expression of many other miRNAs is reduced in various cancer types. Finally, TGF-β- and BMP-induced miR-21 expression in vascular smooth muscle cells revealed an additional regulatory step at the level of processing of the primary transcript by the Drosha microprocessor complex. After ligand stimulation, receptor-specific SMAD signal transducers were recruited to pri-miR-21 in a complex with the RNA helicase p68, a component of the Drosha microprocessor complex, leading to fast processing of pri-miR-21 to pre-miR-21, resulting in an active mature miR-21 molecule.[11] To make matters even worse, BMP4 and TGF-β2 may be among the direct targets of miR-21 itself, creating the possibility of higher order auto-regulatory loops.[6]

With respect to tissue distribution and function of miR-21 in the heart, Thum *et al.*[12] recently revealed an additional layer of complexity by convincingly showing predominant baseline and stress-induced expression of mature miR-21 in the cardiac fibroblast compartment. Here, transgenic overexpression and efficient processing of pri-miR-21 selectively in the post-natal heart muscle did not reveal any discernable baseline or stress-provoked phenotype. In contrast, *in vivo* pharmacological silencing using a so-called miR-21-specific 'antagomir' in a mouse pressure overload-induced disease model readily depleted cardiac fibroblasts of miR-21, reduced cardiac ERK-MAP kinase activity, inhibited interstitial fibrosis, and attenuated cardiac dysfunction. These combined findings revealed that miR-21 levels are increased selectively in fibroblasts of the failing heart, augmenting fibroblast ERK-MAPK activity through inhibition of sprouty homologue 1 (Spry1), regulating fibroblast survival and growth factor secretion, and controlling the extent of interstitial fibrosis and cardiac hypertrophy. Quite on the contrary, a study by Tatsuguchi *et al.*[13] suggested that miR-21 has a subtle yet reproducible inhibitory effect on cardiac hypertrophy, whereas LNA-based miR-21 inhibitors may induce hypertrophy.

Notwithstanding the phenotypic outcome with regards to cardiac hypertrophy, the cell type-restricted expression pattern of miR-21 was first suggested by a model of heart muscle-restricted Dicer depletion (where miR-21 was up-regulated, which is possible if miR-21 is expressed predominantly in the non-cardiac muscle compartment).[4] This was later confirmed by Roy *et al.*[14] using a murine ischaemia/reperfusion (I/R) model, who demonstrated that 'phosphatase and tensin homologue' (PTEN) is a direct target of miR-21 in cardiac fibroblasts, leading to modulated expression of 'matrix metalloproteinase-2' (MMP-2).

Cheng *et al.*[7] now shed new light on miR-21 regulation, its expression pattern in the heart, and a new and relevant target effector gene during cardiac injury. These authors studied ischaemic preconditioning (IP), in which a short period of ischaemia followed by reperfusion arouses the endogenous mechanism of protection against a sustained ischaemic insult. Although IP itself is not clinically practiced due to its innate character of injury, studying the endogenous defensive molecular mechanisms in IP may well identify new therapeutic entry points for ischaemic heart disease.[15] An miRNA expression signature was established in a rat model of IP, yielding a list of 'suspect' miRNAs functionally involved in I/R injury with miR-21 as the top candidate. Following technical validation steps of reagents involved (e.g. an antagomir against 21), the researchers alternated between experimental protocols encompassing I/R preceded by an IP protocol (5 min ischemia, 5 min reperfusion) in the absence or presence of antagomir-21 to prevent its up-regulation during the IP protocol. Readout parameters such as infarct size and TUNEL measurements showed that silencing miR-21 exacerbated cardiac injury, suggesting a protective function of miR-21 in IP and ischaemic disease. Interestingly, the same data were obtained using isolated cardiac muscle cell preparations in hypoxia/reoxygenation experiments, providing very strong information that, unexpectedly, miR-21 is also expressed and functional in cardiac muscle cells.

In terms of a mechanistic explanation for their findings, the scientists turned to 'programmed cell death 4' (PDCD4), one of the principal miR-21 targets already validated independently by several groups and a critical mediator for cancer cell apoptosis.[6] It has a single highly conserved miR-21 target site within its 3′ UTR, and its regulation by miR-21 has been reported in at least six human tumour types or

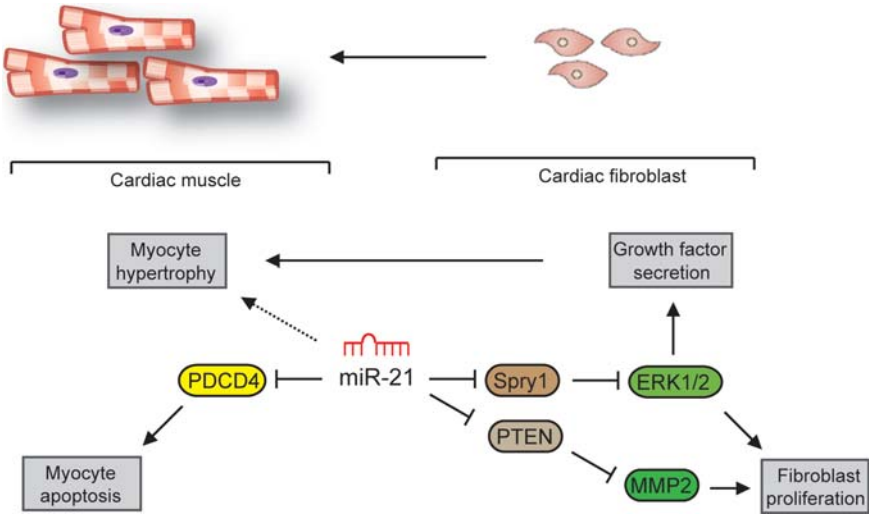

Figure 1 Cell type-specific miR-21 regulatory networks. Cell type-specific targets of miR-21 include Spry1 and PTEN in fibroblasts, influencing ERK1/2 and MMP activity, respectively, and ultimately fibrotic processes in cardiac remodelling. Conversely, miR-21 regulates cardiac muscle apoptosis by regulating PDCD4 levels. PDCD4, programmed cell death 4; Spry1, sprouty homologue 1; ERK1/2, extracellular signal-regulated kinase-1/2; PTEN, phosphatase and tensin homologue; MMP-2, matrix metalloproteinase-2.

cancer cell lines (lung, brain, renal, breast, colon, and pancreas) in which miR-21 is overexpressed, indicating that miR-21–PDCD4 is likely to be a clinically significant oncogene/tumour suppressor pair in the induction and progression of human carcinomas. The take-home message of this study is that miRNAs are functionally involved in IP-mediated cardiac protection. In addition, an unexpected role for miR-21 was established in cardioprotection, specifically in cardiac muscle, and an interesting new player in cardiac muscle apoptosis, PDCD4, enters the scientific arena.

This new study invokes many new questions. A first, very basic question relates to the primary cell type where miR-21 is expressed. Currently, two seemingly opposite views emerge, one where miR-21 is selectively present in cardiac fibroblasts and affects cardiac remodelling (cardiac hypertrophy) in a paracrine fashion,[12] and the opposing evidence put forward in the current study that places miR-21 in the context of cardiac muscle viability (*Figure 1*). Secondly, it is completely unclear whether the different pri-miR-21 species also exist in cardiac cells and whether their atypical processing features are conserved. Thirdly, given that miR-21 can signal through a Spry1/ERK-MAPK/FGF2 axis in the cardiac fibroblast compartment, which promotes cardiac myocyte growth and incites more detrimental forms of cardiomyopathy, and that miR-21 in cardiac muscles positively affects their survivability, at least under short-term experimental conditions, a vexing question remains whether miR-21 long-term silencing has negative or positive long-term clinical effects. The findings by Thum *et al.*[12] seem to favour the latter view. Fourthly, what is the full spectrum of miR-21 target proteins? Several computational algorithms predict hundreds of mRNAs as possible targets for miR-21 regulation; however, relatively few have been experimentally validated. In different cellular contexts, miR-21 can perhaps regulate diverse pathways and cause various phenotypes depending on the availability of a certain population of mRNA targets, providing potential explanations for the currently divergent observations in our field. Likely, many controversies could be settled using gene deletion strategies to delete the miR-21 gene in the mouse and to supplement the antagomir-silencing strategies. Obviously, the correct technical strategy should be strictly followed here to allow for optimal cell type- and timing-dependent (conditional) gene deletion, while taking care that overlapping or neighbouring genes surrounding the miR-21 gene remain unaffected by the homologous recombination events.

Taken together, the study by Cheng *et al.* provides a platform for novel opportunities to define the precise involvement of miR-21 in promoting heart disease and to investigate whether miR-21 antagonists/mimics could become useful therapeutic agents in the cardiovascular field. It is a strange twist of fate that an evolutionarily conserved system such as a tiny RNA species firmly challenges our supposed aggregate understanding of cardiac homeostasis and disease, and despite intense research efforts, we find ourselves perplexedly reciting Socrates' ancient words: '*I only know that I know nothing*'.

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