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The noncoding-RNA landscape in cardiovascular health and disease

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ABSTRACT

The cardiovascular system plays a pivotal role in regulating and maintaining homeostasis in the human body. Therefore any alteration in regulatory networks that orchestrate heart development as well as adaptation to physiological and environmental stress might result in pathological conditions, which represent the leading cause of death worldwide [1]. The latest advances in genome-wide techniques challenged the "protein-central dogma" with the discovery of the so-called non-coding RNAs (ncRNAs). Despite their lack of protein coding potential, ncRNAs have been largely demonstrated to regulate the majority of biological processes and have also been largely implicated in cardiovascular disorders. This review will first discuss the important mechanistic aspects of some of the classes of ncRNAs such as biogenesis, mechanism of action, as well as their involvement in cardiac diseases. The ncRNA potential uses as therapeutic molecules, with a specific focus on the latest technologies for their *in vivo* delivery as drug targets, will be described.

1. Introduction

Nowadays cardiovascular diseases (CVDs) such as heart failure (HF) and myocardial infarction (MI), are considered the leading causes of death worldwide [1,2]. As a matter of fact, pathological changes that occur in cardiac muscle, such as loss of cardiomyocytes, formation of non-contractile scar tissues, thinning of myocardial wall and consequent dilatation of ventricles, are still associated to a poor prognosis and treatment of patients, who continue to suffer high risk for sudden death [3,4]. So far, both pharmacological and device-based therapy have been applied to ameliorate the consequences of HF, but the restoration of functionality of the injured myocardium is often not complete [2]. The latest attempts to ameliorate the use of conventional drugs has failed, due to unavailability of proper selective delivery of the therapeutic molecule to heart [5,6]. Despite the encouraging results from the development of innovative compounds such as sacubitril/valsartan (Entresto™; LCZ696) in the treatment of chronic HF, the lack of other cardiovascular drug approvals in 2016 and 2017 results in the persistence of CVDs as a very high risk factor [7,8]. Consequently, orthotopic heart-transplantation still remains the only cure for HF. However, due to the scarcity of donor hearts and the lack of fully functional 3D-printed bioartificial hearts, this therapy remains available only for a very small number of HF patients worldwide. To counteract the current situation, researchers are continuously focusing towards the development of more efficacious and selective clinical approaches by deeper investigation of the molecular mechanisms responsible for the progression of heart disease [9]. For decades, it has been assumed that only functional proteins are crucial regulators of cardiac physiology and pathologies [10]. However, the latest technologies such as next generation deep-sequencing, highlighted that the majority of the genome is actively transcribed into RNA without coding potential, that has collectively been defined as non-coding RNAs (ncRNAs) [11–13]. Moreover, several studies have emphasized how ncRNA deregulation is tightly connected to the onset of different human disorders, including CVDs. Thus, the possibility of ncRNAs as a potential tool for the treatment of cardiovascular pathologies is of great clinical interest.

In this review, we provide a brief summary of the current knowledge on the world of endogenous ncRNAs, with a specific focus only on the two well know subgroups of ncRNAs (microRNAs and long ncRNAs), their role in regulating pathological cardiac

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remodelling and their potential application for the treatment of CVDs. Finally, the challenges for their effective exploitation by the pharmaceutical industry, and also current clinical trial will be examined [9,14].

2. Types of ncRNAs and molecular function

Based on length, ncRNA molecules can be classified into two major groups of small (<200 nt) and long (>200 nt) non-coding RNAs. Small ncRNAs comprise microRNAs (miRNAs), PIWI Interacting RNAs (piRNAs), transfer RNAs (tRNAs), and small nucleolar RNAs (snoRNAs). Long ncRNAs mainly refers to natural antisense transcripts and other long ncRNAs (lncRNAs) [12]. Moreover, another class of endogenous non-coding RNAs, called circular RNAs (circRNAs), also received extensive attention in the last years due to their regulating potentials and ubiquitous distribution in mammalian cells [15].

2.1. MicroRNAs (miRNAs)

Among small ncRNAs, miRNAs are the most widely characterized [16]. MiRNAs are single-stranded ncRNAs of 19–24 nucleotides long, mainly known to be negative regulators of gene expression at the post-transcriptional level [17,18]. Based on their genomic distribution, miRNA genes can be firstly classified as intronic miRNA in protein coding transcription units (TUs); intronic miRNAs in non-coding TUs; and exonic miRNAs in noncoding TUs [19,20]. Once transcribed by RNA polymerase II into the primary miRNAs (pri-miRNAs), miRNAs are subsequently cleaved by the RNAse III Drosha in the nucleus and then exported into the cytoplasm as miRNA precursors (pre-miRNAs) [21]. Here, pre-miRNAs are further digested by another RNAse III protein, Dicer, and one strand of mature miRNA is then loaded into Argonaute (Ago) protein to form the RNA-induced silencing complex (RISC). This complex functions by recognizing the 3′-UTR of mRNA targets, leading to their inhibition through translation repression and/or mRNA degradation [22]. MiRNAs can also cross the cellular membrane via vesicle-mediated translocation and, transported throughout body fluids, elicit their function in other cell types and tissue districts [23–26]. Interestingly, a novel and unconventional function has been recently identified for mature miRNAs. In fact, several reports in the last few years showed that mature miRNAs, consequential to their nuclear relocalization, can control the activation or repression of nuclear transcripts through epigenetic modification or alternative splicing [27,28]. However, the evidence for these non-canonical roles in the nuclear compartment remains unclear, and additional investigations are thus required to better define this new level of action [27].

2.2. PIWI-interacting RNAs (piRNAs)

PIWI-interacting RNAs (piRNA) represent another family of small ncRNAs, which despite their elevated abundance in mammals are still poor characterized. Compared to miRNAs, piRNAs are longer in length (around 24–30 nt) and less conserved among species [29]. For what concerning their mechanism of action, it is known that piRNAs bind the PIWI sub-group of Argonaute family proteins, forming RNA-protein complexes. These RNA-protein complexes silence transposable elements (TEs) at both transcriptional and post-transcriptional level [30]. piRNAs were demonstrated to be implicated in the regulation of epigenetic state, maintenance of genomic integrity and also in stem cell functions [31]. In the cardiovascular field the characterization of piRNAs is still in its infancy, nevertheless recent papers demonstrate that this class of ncRNAs has altered expression during pathological cardiac development, and are also involved in regulation of signaling pathways controlling the adaptive response of heart to stress stimuli [29,32]. Altogether these pieces of evidence suggest a putative role for piRNAs in the onset of pathological cardiac remodelling.

2.3. Other snRNAs: transfer RNAs (tRNAs) and small nuclear RNAs (snoRNAs)

Transfer RNAs (tRNAs) represent a class of regulatory or housekeeping RNAs, which are the macromolecules that transfer activated amino acids from aminoacyl-tRNA synthetases to the ribosome, where they are used for the mRNA guided synthesis of proteins [33]. Besides the role of tRNAs in mediating protein synthesis, several studies highlighted additional roles in other biological processes (for details see review [34]).

SnoRNAs are intermediate-sized ncRNAs (60–300 nt) that, as their name implies, are mainly localized into the nucleolus, where they play a major role in the modification and processing of ribosomal RNAs [35]. Moreover, snoRNAs have been reported to regulate alternative splicing and also serve as precursors for miRNAs. In this contest a detailed description of this class of ncRNAs is reported in Ref. [36].

2.4. Long non-coding RNAs (lncRNAs)

Beginning in 2009, a second class of relevant ncRNAs, called lncRNAs, captured the attention of the scientific community [37]. LncRNAs were initially distinguished from other regulatory RNA molecules merely by their length (>200 nucleotides). However, subsequent studies added a new level of classification based on their genomic location [38]. Six categories have been therefore proposed:

a) *intergenic lncRNAs* (LincRNAs): located between two genes and transcribed independently;
b) *intronic lncRNAs*: located in introns of protein coding genes;
c) *bidirectional lncRNAs:* sharing the same promoter of a gene but transcribed in the opposite direction;
d) *enhancer lncRNA (eRNAs)*: generated by enhancer region of protein coding-genes;
e) *sense lncRNAs*: transcribed from the sense strand of a protein-coding gene;
f) *antisense lncRNAs*: transcribed from the antisense strand of a protein-coding gene.

LncRNA biogenesis resembles that of classical mRNA production. Indeed, biogenesis starts in the nucleus with lncRNA transcription by RNA Polymerase II or III (POLII; POLIII) from genomic loci with similar chromatin states to mRNA [12,39–41]. At the post-transcriptional level, lncRNAs are often 5′-capped, spliced and polyadenylated, but unlike their protein-coding counterparts, they are shorter in length, expressed in lower amounts, and lack of an open reading frame (ORF). Similarly to miRNAs, lncRNAs are not only present in the cytoplasm, but can also be relocated into the nuclear compartment as well as secreted towards the extracellular space [42–44]. Finally, in contrast to miRNAs or mRNAs, lncRNAs are poorly conserved among species, thus leading to more extensive efforts for the comprehension of their mechanism of action [12].

Based on their sub-cellular localization, lncRNAs can elicit different functions. Some lncRNAs, predominantly located in the nucleus, regulate both silencing and activation of target genes at the transcriptional level by the direct control of the epigenetic state of genomic sequences [38]. Moreover, some nuclear enriched

lncRNAs are also demonstrated to have a role in maintaining the integrity of various structural components of nuclei, and also chromatin. This is the case of the lncRNA *NEAT1*, which was demonstrated to contribute to the formation of paraspeckles, nucleoplasm domains implicated in the nuclear retention of mRNAs [45]. Cytosolic lncRNAs, as well as miRNAs, elicit their function by controlling mRNA translation and stability. However, recent data show that lncRNAs also play an active role in protein localization [46].

Altogether, several studies describing the involvement of lncRNAs in a myriad of biological processes have been generated. However, their mechanisms of action have not been completely elucidated, and more analysis needs to be done in order to delve into the precise molecular regulation of lncRNAs.

2.5. Circular RNAs (circRNAs)

CircRNAs represent a class of ncRNAs found in basically all organisms, with a relative abundance and evolutionary conservation rate among eukaryotes [12]. Unlike linear lncRNAs, circRNAs are characterized by a covalently link between the 3′ tail and 5′ cap, in a single-stranded continuous loop structured RNA molecule. Similarly to lncRNAs, circRNAs can be classified into the following three categories according to the locations of their origin sequences in the genome [47–50]:

a) exon-originated circular RNAs (EcircRNAs);
b) intron-originated circular RNAs (IcircRNAs);
c) circular RNAs originated from exons and introns (EIcircRNAs);

Although the functions of most circRNAs are largely unknown, on-going studies have highlighted that some circRNAs may have potentially important roles in gene regulation, and we refer the readers to the many excellent recent reviews on this topic [51–54].

3. MiRNAs and lncRNAs in cardiac pathologies

3.1. Function of miRNAs and lncRNAs

3.1.1. Function of miRNAs

Since their first discovery in *C.elegans* in 1993 [55], more than 2000 miRNAs have been identified in flies, plants and higher organisms [56], and are now recognized to play pivotal roles in the regulation of all biological processes in eukaryotes. Consequently, abnormal miRNA expression has been causally linked to a large number of human diseases, including cardiac pathologies [57]. Various reviews, available in the literature, describe the importance of miRNAs in the onset of cardiac disease and the reader is recommended to refer to these works for a more general overview [58,59]. Here, we will only focus on few examples of miRNAs affecting heart biology and their potential use as therapeutic molecules. One of the first article demonstrating a role of miRNAs in heart diseases was published by Van Rooij et al. in 2006, in which a specific pattern of miRNAs (miR-23a; miR-23b; miR-24; miR-195 and miR-214) was found to be linked to HF and cardiac hypertrophy both in mice and in human [60,61]. Since then, a plethora of other studies revealed how a myriad of miRNAs can contribute to pathological cardiac dysfunction. In 2007 Care et al., found an inverse correlation between the cardiac-enriched miR-133a and miR-1 and the onset of pathological cardiac hypertrophy. Moreover, it was shown for the first time that an *in vivo* administration of a synthetic molecule (i.e. antagomir, see next paragraph) was effective to down-regulate the level of cardiac miR-133a consequently leading towards a massive hypertrophic response of the myocardial tissue [62]. Therapeutically, the first evidence for a potential miR-133-based approach was recently generated by Castaldi et al. [63]. In this paper, the authors showed that the deleterious effects of a chronic stimulation of the β_1-Adrenergic receptor, typically occurring in patients affected by HF, could be counteracted by the action of miR-133a. In fact, in a mouse model of HF, the overexpression of miR-133a was able to preserve cardiac function via repression of numerous downstream effectors of the β_1-Adrenergic transduction cascade.

Alterations in miRNA expression were also found to be associated with other types of heart disease, not strictly related to cardiomyocyte cells. This is the case of miR-21, which is preferentially expressed in cardiac–fibroblasts and was demonstrated to increase the development of fibrosis in a mouse model of MI, in which the progressive occlusion of coronary arteries leads to apoptosis of cardiac cells and fibrosis [64].

3.1.2. Function of lncRNAs

As well as miRNAs, lncRNAs have been associated with a broad spectrum of biological processes. Therefore it is reasonable to consider that perturbations in their fine-tuning can cause the development of several human diseases, including cardiac dysfunctions [65]. Notably, the repertoire of lncRNA function is more complex and diverse compared to the one of miRNAs, and despite the explosion of data characterizing a number of mechanism of actions, we are only at the beginning in the comprehension of their functionality [66]. Here we report few examples of lncRNAs in the context of heart development and cardiac diseases.

The first lncRNA identified as a key regulator of heart development was *Braveheart (Bvht)* [67]. This lncRNA was demonstrated to regulate the commitment of embryonic stem cells (ESCs) towards mesodermal fate through repression of commitment of non-appropriate cells [38]. In ESCs indeed, *Bvht* works as an epigenetic modulator that reduces the presence of the protein repressive complex Suz12/PCR2 on promoters of cardiac specific genes, resulting in the activation of these lineage-specifying genes [68].

Concerning the adult heart and the onset of pathological remodelling, one of the most important examples of lncRNA is represented by myosin-heavy-chain-associated RNA transcript (*Mhrt*) [69]. This lncRNA inhibits the function of a pathogenic chromatin-remodelling factor, named Brg1, in the activation of cardiac stress genes, thus maintaining and protecting the heart from stress-induced failure.

Beside their function in cardiomyocytes, several pieces of evidence demonstrate that lncRNAs could contribute to cardiac pathologies by eliciting their action on other cell types of the cardiovascular system, such as cardiac fibroblasts. As an example, Micheletti R. et al. found through an integrated genome screening that *Wisper* (Wisp2 super-enhancer–associated RNA) is a cardiac fibroblast–enriched lncRNA implicated in the regulations of cardiac fibrosis after injury both in a mouse model of MI and in heart tissue derived from human patients affected by aortic stenosis [70]. However, compared to miRNAs, the number of lncRNAs regulating cardiac fibroblast biology or the onset of cardiac fibrosis is very limited. Therefore, based on the fact that cardiac fibroblasts represent the major cardiac cell type, and are essential in maintaining biomechanical, electrical, and chemical properties of the heart, further studies are mandatory to elucidate whether other lncRNAs play a role in this cell type in the context of cardiac diseases.

3.2. Interaction between miRNAs and lncRNAs

A number of studies over the past years have begun to uncover an additional level of ncRNA-mediated gene regulation, which is represented by the physical interaction among mammal lncRNAs

and miRNAs [71]. As reported in the review of Yoon JH et al. [71], the mechanistic interactions between lncRNAs and miRNAs can be classified into:

a) miRNA-triggered lncRNAs decay;
b) *lncRNAs as miRNAs sponge/decoys*;
c) lncRNAs as competitor of miRNAs for mRNAs target genes;
d) lncRNAs generating miRNAs.

The first mechanism of lncRNA-miRNA interaction relies on the fact that the lncRNA stability can be reduced by the interaction with specific miRNAs. An example of this was reported in the work of Yoon JH et al. [72]. In this paper the authors showed that a highly conserved lncRNA, named LincRNA-p21 and located ~15 kb upstream of the cell cycle regulator gene p21, was decreased in stability by miRNA let-7. Loss of function of this lncRNAs was also found in vascular smooth muscle cells to be associated with an increase in proliferation and reduction in apoptosis [73].

The second process consists of lncRNAs acting as miRNA-sponge, thus antagonizing miRNA function and favouring the expression of repressed target mRNAs. This represents the most well studied and characterized mechanism in heart diseases. For example, Wang et al., found that hypertrophic hearts express high levels of a lncRNA named cardiac hypertrophy related factor (CHRF), that acts as an endogenous sponge of miR-489, down-regulating its expression. This in turn, leads to a marked up-regulation of different targets of miR-489, amongst which is the pro-hypertrophic gene, called MYD88 [74].

In the third group, lncRNAs can regulate gene expression by competing with miRNAs for the interaction with shared mRNA targets. This is the case of the BACE1-AS lncRNA, which leads to BACE1 mRNA stabilization trough masking the binding site of miR-485-5p for the mRNA thus preventing the miRISC-mediated degradation [75].

Within the last category, lncRNAs can be, during their own biogenesis, a template for the production of miRNAs. A clear example of this mechanism is Linc-MD1, a muscle-specific lncRNA, that once processed, generates precursors of miR-206 miR-133b, two important miRNAs with key roles in cardiac functions [76,77].

Altogether, miRNAs and lncRNAs, acting alone or in cooperation, control gene expression through various post-transcriptional mechanisms, contributing to a fine and robust regulation of expressed proteins. Nonetheless, additional studies are required to deeper dissect the full mechanistic network of cross-regulation between miRNAs and lncRNAs.

4. Tools for modulation of ncRNAs

Based on accumulating evidence on the involvement of ncRNAs in the onset and progression of animal and human cardiac diseases, their potential employment for novel therapeutic approaches has become a major interest of pharmaceutical industries. However, the effective clinical translation of the experimental results is still inadequate in terms of optimization of selectivity, stability, delivery and long term safety of ncRNAs [78]. Here we will discuss the latest available tools for modulation of ncRNAs, with a detailed focus on the problems that still persist regarding the concrete clinical application of ncRNA-based drug therapies.

4.1. MiRNA modulation-based technologies

The therapeutic modulation of miRNAs can be divided into miRNA inhibitors or mimics [5]. Concerning the down-regulation of miRNAs, this can be achieved by different technologies:

a) miRNA sponges or erasers;
b) miRNA target-site protectors;
c) small molecule inhibitors;
d) anti-miRNA oligodeoxyribonucleotides (ASOs);
e) anti-miRNA oligodeoxyribonucleotides (AMOs).

The first approach was described by the work of Elbert et al. In this paper the authors demonstrated that treatment of cells with expression vectors containing multiple binding sites for specific miRNAs were able to inhibit the action of the miRNA by competing for its binding with *bona fide* target genes [79].

The second technology is a variant of the miRNA-sponge based approach that in contrast acts by promoting a physical interference with the binding between a miRNA and its target mRNA. As a matter of fact, this strategy takes advantage of a single-stranded modified RNA (the target protector), which is designed to bind the 3′ UTR of the target mRNA in a region encompassing the putative binding sites of the specific ncRNA. The binding of the protector consequentially prevents the miRNA interaction with the mRNA target and the repressive action on it [80].

The third miRNA-inhibition technique pivots on the use of low molecular-weight compounds, which impair miRNA function by targeting the machinery involved in miRNA maturation and/or degradation at different steps. These small inhibitors can interfere with Dicer activity, impeding the transcription of primary miRNAs or its subsequent processing. Moreover these compounds can also interfere with the loading of mature miRNAs into Argonaute 2 (AGO2) to form an active RNA-induced silencing complex (RISC) [22]. A proof-of-principle for this approach is represented by the study of Gumireddy et al. who demonstrated how the small molecule diazobenzene 1 was able to directly inhibit the action of miR-21 [81].

The last two anti-miRNA technologies are currently the most widely used. Despite being named differently, both AMO and ASO strategies use the same rationale. As a matter of fact, both rely on the use of small antisense oligonucleotides as competitive inhibitors of specific miRNAs. Through annealing to the mature guide strand of target miRNAs, the antisense oligonucleotide impairs the function of the ncRNA by its direct degradation or by stoichiometric duplex formation [82]. Over the past years these molecules have been extensively modified in order to improve their nuclease resistance, the cellular uptake, and the binding specificity [62,83,84]. One of the first reported chemical modifications was the addition of a 2′-O-methyl or 2′-O-methoxyethyl group to the 2′-ribose of the RNA backbone, which was demonstrated to prevent nuclease degradation in the culture media and the consequent endonucleolytic cleavage by the RISC nuclease. This modification led to an irreversible inhibition of the miRNA [85,86]. Another important chemical modification is represented by locked-nucleotide (LNA). This conformational restriction is based on the use of a methylene bridge between the 2 oxygen with the 4 carbon of the ribose ring that was reported to increase the affinity towards complementary single-stranded RNA molecules [87]. Finally, the last class of engineered oligonucleotides is represented by the so called antagomirs, which in addition to the 2′-O-methoxyethyl modification, present also a partial phospho-rothioate backbone (PS) and a covalent addition of a cholesterol molecule. These last two modifications were demonstrated to increase the nuclease resistance and the cellular up-take respectively [85,88]. In this context, the paper by Carè et al. was one of the pioneering studies in the application of antagomirs in a cardiac setting [62].

Besides the attenuation of miRNA expression, a plethora of work has demonstrated how a reduction in the level of miRNAs is responsible for the onset/progression of many cardiac diseases [60,89,90]. In line with this evidence, the use of therapeutic

strategies aimed at the restoration of miRNA levels has become mandatory for the potential treatment of pathological conditions. In this context, miRNA mimics represent the most widely used technology. MiRNA mimics are synthetic double- or triple-stranded small RNA molecules in which one strand is identical to a specific miRNA and therefore aims to replenish the lost miRNA expression in pathological conditions [91]. Analogously to antagomirs, mimics are also structurally modified in order to improve their efficacy and delivery. However, the spectrum of available chemical modifications is much more restricted in comparison to the ones adopted for miRNA silencing. Indeed, some of the widely used chemical modifications impair the loading of miRNA mimic into the RISC complex, thus leading to the complete loss of RNA molecule silencing ability [89].

Besides the function of silencing or replenishing miRNA levels, the prerequisite for a successful miRNA-based strategy is the safety and effectiveness of its *in vivo* delivery [92]. So far, both miRNA mimic and inhibitor technologies failed to completely satisfy this requirement. Indeed, natural barriers such as the circulatory system, immune cell phagocytosis, endonuclease degradation, non-specific tissue uptake, and kidney filtration have challenged the clinical application of these molecules. As an alternative strategy, the use of miRNA-expressing viral vectors, such as lentivirus and adeno-associated virus (AAV) has been proposed. However, this system poses a significant safety risk because of a potential random insertion of the viral DNA into the host genome and persistent expression of the delivered miRNA [93].

As a consequence, there is still a continuous interest in the search of alternative strategies aimed at increasing the efficiency of *in vivo* delivery and thus the enhancement of desired biological effects of both miRNA inhibitors and mimic molecules.

4.2. LncRNA modulation-based technologies

In the wake of increasing evidence for the involvement of lncRNAs in the onset and progression of pathological disorders, pharmaceutical companies and research organizations are increasing their efforts in order to conceive tools for their effective manipulation in the clinical field. In the context of lncRNA-silencing, the RNA-interference (RNAi)-based technology is one of the most suitable techniques. In the work of Grote et al., the authors demonstrate that loss-of-function of the lncRNA *Fendrr* via RNAi, impaired the proper development of tissues derived from the lateral mesoderm, specifically the heart and the body wall [94]. However, based on the fact that RNAi is mainly active in cytoplasm, this technology cannot be applied to lncRNAs that are much more abundant in the nuclear compartment [95]. To overcome this problem, an alternative approach is represented by the use of gapmers, a single-strand antisense oligonucleotide that blocks lncRNA activity via the RNase H enzyme, which is enriched in nuclei and is thought to act in DNA replication and repair [96,97]. This approach was used by Ounzain et al. to silence the lncRNA named *CARMEN*, (CARdiac Mesoderm Enhancer-associated Noncoding RNA), demonstrating a key role of this ncRNA in the regulation of cardiac cell differentiation and homeostasis [98]. However, the formation of secondary structures typical of lncRNA molecules was demonstrated to reduce the efficacy of silencing via both the RNAi and gapmer silencing, and an alternative strategy based on the use of small molecule inhibitors has been assessed. These small compounds indeed, interfere with the binding of lncRNAs to their protein interacting partners, thus abolishing the effect of lncRNA-protein complex on downstream target genes [99]. This approach was described in the cancer field by Tsai et al., where the authors demonstrated how the use of a small molecule inhibitor prevents the binding of an oncogenic lncRNA, *HOTAIR*, with its binding partners. As a results, this effect leads to a restriction of tumor cell invasion, angiogenesis, and metastatic potential [100,101].

In addition to down-regulation of harmful lncRNAs, gene therapy can also be achieved through the delivery of lncRNAs to specific cells. However, similarly to the limitations occurring in miRNA up-regulation strategies, the current available technologies for the over-expression of lncRNAs are also rather inadequate. Thus, more efforts have to be made in order to develop efficient tools for the up-regulation of lncRNAs *in vivo.*

5. Nanomedicine: the new frontier in ncRNA-delivery

We described above that both the gain- and loss-of-function of ncRNAs represents the keystone for the treatment of basically all ncRNA-based pathological disorders. However, as of today, a drastic restriction limiting the translation of this concept into formulation of concrete drugs stands from the lack of efficient and safe systems for a controlled and targeted-delivery of ncRNA modulators to the desired target tissues. An answer to these limitations could come from the nanomedicine field, which is the application of nanotechnology to the field of medicines and pharmaceuticals [102]. Nanomedicine involves the application of nanoparticles (NPs), which represent any particulate material with at least one dimension between the range of 1–100 nm. Due to their capacity to be loaded with different chemical compounds, NPs constitute a class of smart nanocarries, which are engineered to be attracted to specific type of diseased cells and, upon release of the therapeutic drug within the cells, cure the pathology. As such, the damage to healthy cells in the body is reduced. In addition, due to a protection against enzymatic degradation, the therapeutic payload gains an increased half-life. In line with this, a great interest from the scientific community has been recently raised for the development of this technology towards new medical approaches to challenge the current limitations of conventional medicine (e.g. poor drug bioavailability, impaired target specificity, systemic and organ toxicity [103]). However, despite a larger use in the cancer field, the use of NP-based drug delivery tools for heart targeting is still in its infancy [104]. Nevertheless, the increasing number of successful preclinical and clinical results coming from NP applications in the oncologic therapy is now prompting the translation of their use in the heart field, which is thus becoming a new major interest for future coming clinical applications. Here we briefly discuss some examples of NP-based drug delivery system with a focus on their latest applications in cardiac diseases.

Several aspects need to be considered for the design of NPs suitable for clinical use. The size of a NPs is among the first parameters that can be tailored for the optimization of particle biodistribution *in vivo* [105,106]. As a matter of fact, it was noted that particles of dimension inferior to 5 nm are rapidly cleared from the circulatory system via extravasation or renal clearance [107,108]. On the contrary, particles of 15 μm in size showed a major accumulation in liver, spleen and bone marrow [109,110]. So far, NP size ranging between 50 and 100 nm showed the best results in terms of bio-distribution and long half-life in the circulation, that in turn increases the propensity of NPs to extravasate via vessels and reach target tissues [111]. Another important feature regarding the design of therapeutic NPs is the shape geometry. A plethora of NP geometry have been developed and described in their flow characteristics that can influence the circulating lifetime, cell membrane interactions, and macrophage uptake, that in turn affect the bio-distribution and the final target efficiency of specific organs [112]. Surface chemistry is an additional feature, which can be optimized to prolong circulation lifetime as well as enhance the selective accumulation in sites of interest [105]. As an example, NPs with neutral or negative surface charges have been reported to

reduce the adsorption of serum proteins, resulting in a major permanence in the circulation, in addition to a more specific cellular targeting. Yamamoto et al., indeed, demonstrated that neutral (1.3 mV) and anionic (−10.6 mV) polymer micelle are much more stable in blood fluid [111,113,114]. On the other hand, the works of Di Mauro et al. and Miragoli et al. showed that negatively charged NPs are preferentially up-taken by the polarized cardiac muscle cells through the formation of life-compatible nanopores [111,113,114]. The surface chemistry can be additionally implemented with a broad spectrum of molecules, such as antibodies, aptamers or peptides, in order to increase the specificity for a certain cell type, thus further reducing the toxic off-targets effect on non-target cells [106].

Another fundamental feature that needs to be assessed when considering the *in vivo* fate of a therapeutics carrier, is the material composition of NPs. In this context, it is reasonable to think that the more the NP is biodegradable and biocompatible, the less it will induce the response of the immune system thereby increasing the probability of the nano-drug to reach the target district. In line with this, NPs described by Di Mauro et al. and Miragoli et al., provides a typical example of novel calcium-phosphate based NP, which showed ideal properties of biocompatibility, bioresorbability and biodegradability, in terms of both non-immunogenicity, minimal or absent toxicity *in vitro* and *in vivo* [111,114]. In addition, the material composition of NPs clearly affects clinical effectiveness the NP-delivery system as the capacity and kinetic to release the drug cargo depends on the dissolution rate of the carrier. For what concerning the drug cargoes, different molecules have been successfully loaded into nanocarriers, and ncRNAs are among these. As mentioned above, a plethora of works demonstrating the potential applicability of this ncRNA-loaded-NPs are currently found from the oncogenic field. For example, Birary et al., demonstrated that polyethylene glycol-polyethylenimine nanocomplexes can be used to efficacy deliver miR-150 to chronic myeloid leukemia cells [115]. Another example is represented by Wu et al., who demonstrated that the systemic deliver of miR-29b-cationic lipoplexes-based carriers reduced the expression of the key target oncogenes contrasting the rate of tumor growth in lungs [116]. In the context of heart diseases however, the availability of ncRNA-loaded-NPs is still limited. One of the first examples demonstrating the potential use of ncRNA-loaded-nanoparticles for heart targeting can be found in the work of Di Mauro et al., in which the authors demonstrated that negatively charged calcium-phosphate bio-inspired NPs can be used for efficient delivery of synthetic miRNAs into cardiomyocytes both *in vitro* and *in vivo* [111].

Ultimately, the NP-delivery system can be additionally designed for selective targeting and drug release within specific subcellular compartments. In fact, not only the cytosol but also other cellular organelles such as the nucleus, mitochondria, and even peroxisomes might be considered [117–120].

6. Conclusions and future perspective

In summary, this review describes how the field of ncRNA therapeutics represents a very promising tool for the treatment of different type of cardiovascular disorders. In addition, the combination of ncRNA therapeutics with the emerging nanotechnology field might boost an effective application towards preclinical and clinical studies. Despite this promising perspective, none of the large number of oligonucleotide-based drugs targeting ncRNAs that have managed to enter clinical trials, have completed phase IV (for details see reference [121]). Thus, before a regular use of such ncRNA-based therapeutic tools is achieved, further studies are critically needed to deeper understand the biology and the modulation of ncRNAs as well as the necessary chemical formulations required for nanocarriers. In conclusion, the scientific community and pharmaceutical companies must joint together to pursue this goal in order to elevate ncRNA-NP drug delivery system from just a "promising field" to a more rigorous tool acceptable for clinical trials and subsequently for the treatment of a wider range of diseases in the coming years.

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