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MicroRNA and Cardiac Stem Cell Therapy

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Abstract

Cardiac Progenitor Cells (CPCs) are multipotent cells of the myocardium. They are located inside niches of the heart muscle, can be isolated, characterized and used for cardiac regeneration in stem cell therapy. Actually, CPCs may be isolated by tissue digestion with or without cell sorting, but it is difficult to achieve the maximum level of differentiation when these cells are implanted into a damaged myocardium.

The knowledge recently acquired on small molecules of non-coding RNAs, microRNA (miRNA), may improve the use of these cells in stem cell therapy. In fact, these small molecules may be attached to devices or adminstered as they are or in combination with nanoparticles in order to drive the correct differentiation of stem cells. Regarding heart regeneration, we can acquire knowledge from the role of miRNAs in heart development and use it to reprogram CPCs to gain the correct three-dimensional structure of the cardiac muscle.

Keywords: Cardiac stem cells; Heart regeneration; microRNA; Epigenetic; Reprogramming

Introduction

MicroRNAs (miRNAs) are short endogenous single-strand RNA molecules (21- 23 nucleotides in length) that have an effect in controlling gene expression.

miRNAs were described, for the first time, in 1993 in *Caenorhabditis elegans* by Lee and colleagues. The authors found a small non-coding RNA, called lin-4, which was able to inhibit the translation of lin-14 mRNA during the larval development [1]. However, this finding came out from many studies performed in *Caenorhabditis elegans* starting from 1981, when Chalfie and colleagues found a loss of function mutation in the gene of lin-4 which led to a constitutive expression of larval-specific genes [2]. Eight years later, another group of researchers demonstrated that lin-4 was able to inhibit the expression of two genes, lin-14 and lin-28, involved in the larva-to-adult developmental switch [3]. Later, in 1991, through deletion experiments, it was found that the interaction between lin-4 and lin-14 was required to down-regulate lin-14 protein levels during development [4]. Finally, thanks to these discoveries, in 1993, Lee and colleagues described lin-4 as a small-non coding RNA which was a negative regulator of lin-14 mRNA; the authors also found that Lin-4 RNA sequences were complementary to the 3'untranslated region of the lin-14 messenger, suggesting a potential specific-sequence interaction [1].

Some years later, the discovery of another non-coding small RNA, allowed the development of the research field on miRNAs. In a study performed in 2000 by Reinhart and colleagues, a small RNA (21-nucleotides in length), let-7, was described as a regulatory RNA highly conserved among the species [5]. This finding opened the way to other studies that finally recognized the class of small non-coding RNA as a novel class of regulators for gene expression, called miRNAs [6].

At present, for studies on miRNAs, scientists use a database called miRBase (http://www.mirbase.org/) that is the primary online repository for all miRNA sequences and annotation. To date, the current release (miRBase 18) contains 18226 entries representing hairpin precursor miRNAs, expressing 21643 mature miRNA products, in 168 species, and over 19000 distinct mature miRNA sequences. The miRNA field is rapidly growing, considering that over 2000 miRNA sequences have been already described in vertebrates, flies, worms, plants, and even in viruses.

Cardiac stem cells or, which is better, Cardiac Progenitor Cells (CPC) are adult multipotent cells able to differentiate into the four main cardiac lineages: myocytes, Smooth Muscle Cells (SMC), Endothelial Cells (EC) and cardiac fibroblasts. They have been isolated and described for the first time by the research group of Piero Anversa in 2003 [7]. They are self-renewing (able to maintain the undifferentiated state even after many cell cycles), clonogenic (able to expand in culture after plating one cell per well), and *in vivo* they are located inside cell niches with feeding cells [8].

Many research protocols have been developed to isolate CPCs from adult myocardium. Most of the research groups sorted c-Kit or Sca-1 positive cells after tryspin and collagenase II tissue digestion [7,9-12]. Messina and colleagues [13] used cells migrating out from cardiac tissue explants and grown in suspension as "cardiospheres". Our research group has also been involved in CPC research; we are able to isolate these adult progenitors by slow tissue digestion, mild trypsinization and differential adhesion without cell sorting [14]. Using this mild protocol it is possible obtain a cell population with 96% c-Kit positive cells [15]. This population is also positive to Sca-1 and MDR-1 (unpublished data), as described by Srivastava and colleagues [16] in a recent paper. Their differentiation potential have been evaluated by many research groups [9,12-14], but the complete differentiation and creation of a small piece of myocardium have never been demonstrated both *in vivo* and *in vitro*. These cells may be considered "progenitor" cells because they are immature committed proliferating cells able to differentiate into only one of the four above-mentioned lineages (myocyte, SMC, EC or cardiac fibroblasts) [16].

The discovery of c-Kit-positive CPCs that reside in the heart has definitely changed the knowledge about myocardial biology. The acceptance of the idea that the heart may regenerate has been

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problematic and subjected to criticism. Myocyte death is a normal event that occurs during human heart diseases. In order to maintain heart vitality, myocyte death has to be accompanied by myocyte regeneration: this is the principle of homeostasis [17]. The identification of CPCs has generated several doubts about their role in spontaneous cardiac regeneration after damage. The fact that resident human CPCs have limitations in reconstructing cardiomyocytes after infarction is considered as the proof that the heart is a post-mitotic organ and terminally differentiated [18]. This could be explained thinking that CPCs, which are present throughout the infarcted myocardium, die by apoptosis and necrosis as the other cells during heart failure [18].

To differentiate CPCs into cardiomyocytes is quite difficult, and many research groups are trying to enhance both their degree of differentiation and the number of differentiated cells in order to substitute a damaged myocardium. There are two clinical trials ongoing in USA both using injected CPCs (SCIPIO [19] and CADUCEUS [20]). Data are encouraging, and demonstrate the paracrine potency, engraftment efficiency and therapeutic potential of these cells. Data suggest that CPCs delivered as the three-dimensional (3D) form of "cardiospheres" are more efficient [21]. The authors are now trying to find a 3D scaffold to deliver more efficiently these cells into a damaged myocardium [21].

A safe and standardised protocol to implant CPCs is necessary, but what it is really needed is to drive cell differentiation towards the myocardial phenotype. In order to develop a pre-clinical model which may lead to the most efficient myocardial differentiation of CPCs, we may use our knowledge about miRNA function, miRNAs involved in heart development, and the experiments on miRNA-driven CPC differentiation. The aim of the present review is to give an overview of miRNA function, target prediction, their involvement in heart development and the recent literature on CPC-expressed miRNA.

How do miRNAs Work?

miRNAs have been proposed as "micromanagers" of gene expression, because they might act as on-off switches to eliminate mRNAs that should not be expressed in a specific cell type or at a specific moment. They also can regulate mRNAs abundance, adjusting levels within physiological range [22].

miRNA genes are localised either in independent units, with their own promoter, or in introns (intronic miRNAs) of protein coding genes [23]. They are transcribed as pri-miRNA, processed and transformed into pre-miRNA inside the nucleus and translocated to the cytoplasm by the nuclear transport receptor Exportin-5 (Figure 1). The miRNA maturation process occurs in the cytoplasm; two strands are released: the stand with less stability at 5'-end (typically a GU pair instead of GC pair) is loaded onto RISC, whereas the other strand, the passenger (miRNA*), is released (Figure 1) [24].

According to the official nomenclature, the term "miRNA*" indicates the miRNA strand which is weakly expressed. If it is not known which strand (miRNA-5p or miRNA-3p) is more expressed than the other, usually it is indicated "miRNA-5p" or "miRNA-3p" depending on the position on the genome (5' or 3'-arm of the hairpin).

At first it was thought that miRNA* was degraded soon after its release. However, many evidences demonstrated that miRNA* molecules are often present at physiologically levels [25], and that they have many biological roles, for example miR-378* has been identified as a molecular switch in breast cancer cells [26], and miR-155* (together with miR-155) is involved in regulating human Plasmacytoid Dendritic Cell (PDC) activation [27]. Furthermore, substantial fraction of miRNA* species are stringently conserved over vertebrate evolution; they exhibit greatest conservation in their seed regions, and define complementary motifs whose conservation across vertebrate 3′-UTR evolution is statistically significant [28].

Moreover, it was found that a significant fraction of pre-miRNA hairpins produces miRNA* species whose seed sequence recognizes 3'UTR target site. That means that miRNA* are not only inactive products of miRNA cleavage, but they also inhibit or regulate target genes [25].

The role of miRNAs inside the cell is to interact with their mRNA targets by base pairing. In plants, most miRNAs base pair to mRNAs with perfect complementarity, inducing mRNA degradation by a cleavage in the middle of the miRNA-mRNA duplex [29].

In the case of metazoans, generally, base-pairing between miRNA and its target is imperfect [30]. The region responsible of this interaction is called "seed sequence" and it corresponds to the nucleotide 2 to 8. Just for thermodynamic reasons, an A nucleotide across position 1, and an A or U across position 9, improve the site efficiency, even if they

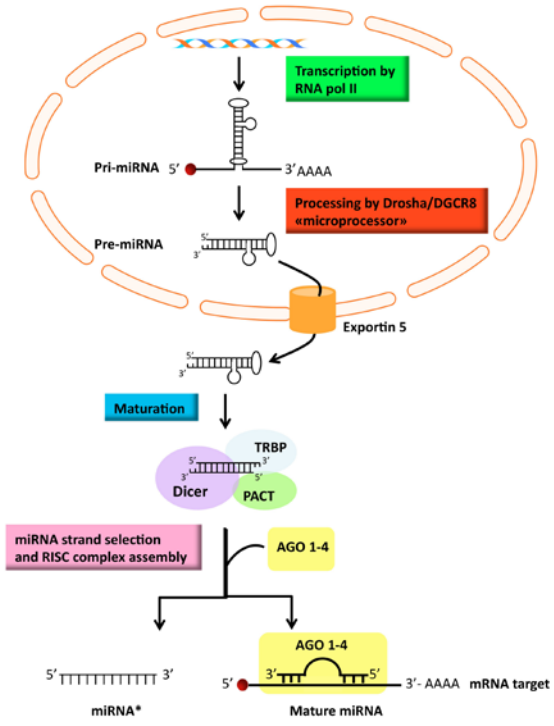

Figure 1: miRNA biogenesis. miRNAs are first transcribed as long primary miRNA transcripts (pri-miRNAs) by RNA polymerase II inside the nucleus. Pri-miRNA contains a stem-loop structure, a cap at 5'-end and a poly-A tail at 3'-end [74]. Then, pri-miRNA is processed by an enzymatic complex called "microprocessor", to generate molecules 70-100 nt in length (pre-miRNA) [77]. Pre-miRNA hairpins are recognised by Exportin-5 (Exp-5) which allows the passage into cytoplasm, where Dicer creates a 22 nt small RNA duplex. In the cytoplasm, Dicer associates with ds-RNA-binding proteins, like transactivation-response element RNA-binding protein (TRBP) and p53-associated cellular protein (PACT) [78,79]. The helicase activity of Dicer unwounds the RNA duplex, one of the strands preferentially bind to Argonaute (Ago), and the effector complex "RNA induced silencing complex" (RISC) is generated [80]. The strand with less stability at the 5'-end is loaded onto RISC, whereas the other strand, the passenger (miRNA*), is released [24].

do not need to base pair with miRNA nucleotides [31]. Furthermore, there must be complementarity to the miRNA 3' half to stabilise the interaction, even if mismatches and bulges (structural motifs formed following a mismatch in the miRNA: mRNA heteroduplex) are tolerated in this region. If the matching with seed region is not perfect, there should be a good base-pairing to residues 13-16 of the miRNA to obtain a good stability [30,32].

There are many factors that influence the site efficiency, among which an AU-rich neighbourhood and, in case of long 3'UTRs, a position not so far away from the poly-A tail or the termination codon, in order to make binding sites more accessible [30,33]. In contrast to these rules, there are many cases in which a bulged nucleotide in the seed region is required for an efficient miRNA-mediated repression [34].

Typically, more than one miRNA binding site is present on 3'UTR of mRNA target and multiple sites for the same or different miRNAs are required for effective repression [30-33]. When they are localised close to each other they act cooperatively providing a stronger repression [30,31].

miRNAs act either inhibiting mRNA translation or inducing the degradation of the target. When the association miRNA-mRNA target exhibits several mismatches and bulges, it results in a reduced efficiency of translation rather than in a decrease in mRNA abundance [35]. The inhibition of mRNAs translation can occur at the initiation or post-initiation step. In repression at the initiation step, Ago2 protein is the main actor: it has a Cap-binding like domain (MC) which bind the m^7G cap of mRNA precluding the recruitment of eIF4E and, therefore, the assembly of the translation complex [36].

For the repression at post-initiation step a drop-off model is proposed: miRNAs render ribosome prone to premature termination of translation [37]. When miRNAs bind with a strong complementarity to their target, the mRNA degradation is induced by deadenylation in 3'→5' direction catalysed by the exosome, or by the removal of the cap followed by 5'→3' degradation catalysed by the exoribonuclease-1 (XRN1) [34].

Degradation or, at least, its final steps, seems to occur in Processing bodies (P-bodies), that are granules localised in the cytoplasm enriched in mRNA-catabolyzing enzymes and translational repressors [38,39]. P-bodies are also enriched in Ago proteins and miRNAs. Originally they were considered as being primarily involved in mRNA degradation, but now it is clear that P-bodies are also temporary sites of storage of repressed mRNAs [34].

In particular situations, like following a stress, the mRNA sequestration in P-bodies can be irreversible and it can be released and translated after the interaction with polysomes [40].

Studies that look at a better understanding of miRNAs function are coming out everyday and many models are continuously proposed.

miRNA Prediction Target

To appreciate the significance of a change in miRNA levels during a particular cellular process, the identification of their own mRNA targets is crucial. To achieve this purpose many computational prediction target tools, based on specific algorithms, have been developed. The most widely utilized engines are miRanda, TargetScan and PicTar (summarised in Table 1). They are based on algorithms which rely on base-pairing between "seed sequence" of the miRNA and the 3'UTR sequence of the mRNA target but miRNA target sites are localised also within the coding region of a gene even if at much lower frequency. Each engine uses different algorithms to establish the alignment score.

MiRanda calculates the alignment score as the sum of match and mismatch scores, including G:U wobbles and gap penalties. Since sequence conservation is a strong indication of functional constraints in evolution, miRanda prediction software also considers the degree of evolutionary conservation of the targeted sequence and its position in 3'UTRs of human, mouse and rat genes [41]. TargetScan prediction software applies an alignment score for Watson-Crick (W-C) base-pairing in the seed sequence (nucleotides 2-7) of the miRNA to its target and also base-pairing beyond the seed sequence that can compensate for mismatches in the seed. It also recognises a conserved adenosine at the first position and/or a W-C match at position 8, and considers the degree of conservation using three different levels: highly conserved between human, rat, mouse, dog, and chicken; conserved between human, mouse, rat, and dog; poorly conserved among any combination of species [30,42].

The third target prediction software is PicTar that takes into account a perfect W-C base paired 7 nucleotides seed (1-7 or 2-8) allowing mismatches if the free binding energy does not increase, the binding energy of the entire miRNA-mRNA duplex, and the degree of conservation of targeted sites between all the species (chimpanzee, pufferfish and zebrafish) [43].

Another important criterion that should be considered during miRNA target prediction is the accessibility of the target site. In fact when the target site is localised in a closed stem structure, the inhibition by miRNA is not allowed. For this reason prediction software, called Probability of Interaction by Target Accessibility (PITA) was designed; which allows prediction of target sites by first searching for complementary seed sequences, and then calculating the free binding-energy after subtracting the energy used to unwind any predicted secondary structure [44,45].

However, the prediction algorithms used in the above mentioned softwares and databases do not consider the concentration of miRNAs inside the cell, the real accessibility of the target sequence or other factors which may modulate miRNA-target interactions, because they consider only the conventional target prediction rules based on base-pairing. A false-positive rate of at least 40% must be considered [46].

Prediction Target Tool	Web Site	Algorithm used
miRanda	www.microrna.org	The alignment score is the sum of match and mismatch scores, including G:U wobbles and gap penalties. The degree of evolutionary conservation is considered.
TargetScan	www.targetscan.org	Applies an alignment score for W-C base-pairing in the seed sequence and beyond it. Recognizes a conserved adenosine at the first position and/or a W-C match at position 8. Considers three degree of conservation.
PicTar	www.pictar.mdc-berlin.de	Considers a perfect W-C base paired 7 nucleotides seed (1-7 or 2-8)allowing mismatches if the free binding energy does not increase, the binding energy of the entire miRNA-mRNA duplex, and the degree of conservation of targeted sites between all the species

Table 1: The most widely utilized engines for computational miRNA prediction target.

miRNAs and Stem Cells

miRNAs are involved in several physiological and pathological processes as development, apoptosis, cell differentiation, metabolism and cancer and they are often tissue-specific. Increasing evidences suggest that miRNAs are also involved in regulating cell renewal and differentiation. Several studies have demonstrated that the transition from the stemness state towards the differentiated one is regulated by epigenetic mechanisms as chromatin remodelling and changes in DNA and histone modifications [47].

miRNAs are considered epigenetic modulators of mRNA degradation and protein translation. The idea that they can regulate stem cell maintenance and induce cell fate decision is increasingly spreading [47]. Several evidences demonstrate that miRNAs tightly control Embryonic Stem Cell (ESC) self-renewal and differentiation state. Experiments on Dicer or DGCR8 deletion, performed in mouse and human ESC, confirmed a role of miRNAs in differentiation: the global loss of miRNAs resulted in proliferation and differentiation defects of ESC [48]. Other studies identified specific sets of miRNAs, like miR-290 cluster, which contains several members as miR-291, miR-292, miR-293, miR-294, miR-295, and miR-302 family, formed by miR-302a, miR-302b, miR-302c, miR-302d and miR-367, as "ESC-cell cycle regulating clusters" because they repress key regulators of the cell cycle to ensure a rapid G1-S transition [49].

miRNAs are involved in signalling pathways leading to cell lineage commitment. For example the two miR-125 isoforms (miR-125a and miR-125b) may inhibit SMAD4 expression in human ESCs regulating neuronal commitment [50]. In particular, treatment with activin and BMP4 blocks the activity of miR-125a and miR-125b, promoting significant levels of SMAD4 expression. This induces phosphorylation of SMAD2/3 and SMAD1/5, and promotes self-renewal or MP-induced lineages, as neural crest and epidermis [50]. When activin and BMP receptors are inhibited, miR-125 isoforms are expressed, SMAD4 is down-regulated and ESC are irreversible committed towards the neural lineage [50].

In another study, the authors suggest that miR-181a*, miR-302a and miR-456 have a role in maintaining the undifferentiated state of the Primordial Germ Cell (PGC) and the Blastodermal Cell (BC) in chickens [51]. In PGC miR-181a* inhibits the meiotic transcript NR6A1 and the somatic HOXA1, modulating the different regulatory pathways for meiosis in chicken early germ cells [51]. In PGC and BC miR-302a and miR-456 silence the expression of somatic genes (as Sex determining region Y-box, SOX11), regulating pluripotency [51]. The signalling pathways through which miR-181a*, miR-302a and miR-456 regulate pluripotency in chicken needs further investigation.

miRNA are effectors of preconditioning signals. Preconditioning is a powerful cytoprotective stimulus which can be used to promote stem cell survival upon implantation. Ischemia preconditioning significantly reduces cell apoptosis in Mesenchymal Stem Cells through activation of Akt and ERK1/2, and through nuclear translocation of the hypoxia-inducible factor-1α (HIF-1α). This nuclear factor positively regulates miR-210 transcription, and its up-regulation inhibit the expression of FLASH/Casp8ap2 gene, member of the apoptosis signalling complex that activates Caspase-8 and promote Fas-induced apoptosis [52].

The same authors demonstrated that miR-21 is up-regulated in IL-11 preconditioning experiments of skeletal myoblasts in a rat model of myocardial infarction. Diaxozide preconditioning of skeletal myoblasts activates IL-11 synthesis, which in turn activates miR-21 up-regulation via ERK1/Stat3 signalling, promoting stem cell survival [53].

Recently a new role for miRNAs has been proposed: this small non-coding mRNAs may be manipulated to reprogram stem cells for stem cell therapy. This hypothesis has been proposed after that it has been demonstrated that miR-302/miR-367, ESC specific, are expressed in early embryonic development and then rapidly decline after differentiation [54,55]. Hence, many laboratories developed miR-302/367 mediated induced Pluripotent Stem Cells (iPSC) [56,58].

This kind of iPSC are claimed to be more safe because oncogenes, as c-Myc, are not over-expressed. All the genes down-regulated or all the epigenetic changes induced by these two miRNAs are not known, but the preliminary experiments on miRNA-reprogrammed iPSC demonstrated that they are less tumorigenic than the conventional iPSC [56,57].

miRNAs may be delivered *in vitro* as small molecules using lipofectamine or similar substances, considering that they are similar to siRNAs. A more stable transfection may be achieved creating an artificial expression vector that can both generate functional mature miRNAs and maintain their expression *in vitro* and *in vivo*. miRNA may diffuse from a donor cell to stem cells through gap junctions, in co-cultures with engineered donor cells. Or, which is the more stable system, miRNA may be delivered along with reporter genes or hormone activated promoters using an artificial intron (SpRNAi) placed between two exons. To date the most efficient method to introduce reprogramming factors into cells is viral transfection, but since this may result in integration in exogenous genes, this may not be used in clinical trials [57].

miRNA and Heart Development

Apart from studies on the importance of miRNAs in ESC differentiation, many studies have been conducted to shine a light on the importance of miRNAs in heart development. It has been demonstrated that in zebrafish miR-138 is fundamental for the correct development of ventricles and for cardiomyocytes maturation. The loss of miR-138 leads to the expression in ventricular region of the genes whose expression is normally restricted to the atrio-ventricular valve region and it induces a block of the ventricular cardiomyocyte differentiation [58].

The importance of miRNAs during cardiac development is confirmed by recent evidences. In a mouse model, the inactivation of Dicer in CPCs was lethal during embryogenesis at E12.5 [45]; mice KO for DGCR8 developed a dilated cardiomyopathy, died of heart deficiency in less than 31 days, and the expression levels of miR-1, miR-133 and miR-208, cardiac specific, noticeably decreased [59].

The miRNAs most studied in heart development are miR-1 and miR-133, critical regulators of cardiac proliferation and differentiation (Figure 2) [60]. MiR-1 and miR-133 are encoded by a duplicated locus in the mammalian genome. They are expressed by a cluster called miR-1-1/miR-133a-2 on the chromosome 2, and by a cluster called miR-1-2/miR-133a-1 on the chromosome 18. However, miR-1-1 and miR-1-2, and miR-133a-1 and miR-133a-2, originated from different loci, have the same sequence [60]. Mi-206/miR-133b cluster is transcribed from a non-coding region on the chromosome 1. MiR-206 is skeletal muscle-specific and shares with miR-1 the same seed sequence, but they differ for 4 nucleotides in the 3'-end. MiR-133b has the same seed sequence of miR-133a-1 and miR-133a-2, and it differs just for one nucleotide in 3'end [61].

MiR-1 is highly conserved among species: comparison of genomic sequences across species reveals that a 4.6 kilobase (kb) and 10.7

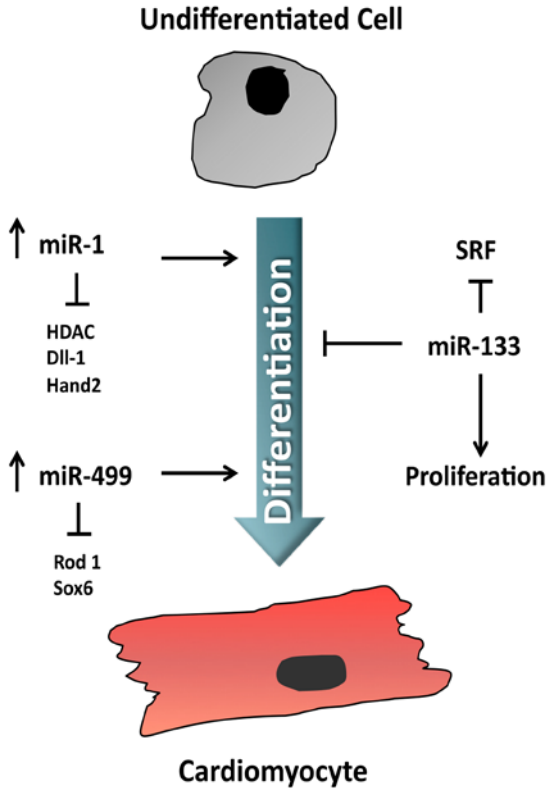

Figure 2: MiR-1 and miR-133 in heart development. During cardiomyocites differentiation, expression levels of miR-1 and miR-499 increase. miR-1 induces differentiation by inhibiting the expression of HDAC4, Delta like I (Dll1) and Hand2 which in turn regulate the expression of cardiac specific genes [60]. miR-499 negatively regulates the expression of Sox6 and Rod1 [70]. miR-133 inhibits differentiation inhibiting the expression of SRF, conversely promoting cell proliferation [60].

kb genomic region around miR-1-1 and miR-1-2, respectively, is conserved between human and mouse. Within the promoter regions there are several cis elements conserved between human and mouse that represent potential binding sites for the essential cardiac transcription factors Myocyte-specific enhancer factor 2C (Mef2C), Serum Responsive Factor (SRF), Nkx2.5 and Gata4 [62]. In the heart, the miR-1 expression is tightly dependent on SRF [62]: mutation of the miR-1-1 SRF site abolishes miR-1 expression. Since miR-133 is located on the same locus of miR-1, it is also under the SRF control; moreover, miR-133 inhibits the expression of SRF through a negative regulating loop [60,63]. MiR-133a-1/miR-1-2 and miR-133a-2/miR-1-1 genes are demonstrated to be expressed in the ventricular myocardium and interventricular septum from E8.5 until adulthood [62,64].

Deletion experiments have highlighted the importance of miRNAs in cardiac maturation. For example, in mouse, deletion of the gene coding for miR-1-2 or for miR-133 leads to alterations during cardiogenesis like the failure of ventricular septation that results in death within few hours after birth [45,65]. Both miR-1-1 and miR-1-2 are more expressed in the outflow tract of the heart which derives from the secondary heart field. It has also been seen that miR-1 is fundamental for cardiomyocyte differentiation, thus it has anti-proliferation effects. In fact miR-1 overexpression causes the loss of cardiomyocytes and cardiac hypoplasia which is lethal at E13.5. This event is due to the miR-1-mediated repression of Heart- and neural crest derivatives-expressed protein 2 (Hand2), an important transcription factor involved in cardiomyocytes proliferation [62]. The induction of the myogenic phenotype mediated by miR-1 is dependent from the repression of Histone Deacetylase 4 (HDAC4), that negatively regulates Mef2 [60,66].

About 50% of null mice for one of two loci of miR-1 dies from Ventricular-Septal Defects (VSDs) between late embryogenesis and birth, while a subset of mutant mice can survive to adulthood showing electrophysiological defects. On the other hand, complete ablation of miR-133 via double gene knockout induces cardiac defects during embryogenesis, and death one day after birth (P1 stage) showing dilated atria, and enlarged, engorged with blood, and containing thrombi hearts [65]. This suggests the occurrence of the defect in cardiac contractility.

In summary, the miR-1/miR-133 cluster is one of the main regulators of proliferation and differentiation of cardiac precursors.

miRNAs and Cardiac Stem Cell Therapy

miRNAs play a role not only in regulating ESC but also adult progenitor cell differentiation. At present only a few papers have been published on the expression of miRNAs in CPCs and a few on the first experiments to manipulate cell differentiation through miRNA over-expression.

Up-regulation of cardiac miR-1, miR-133 and miR-208 has been detected in Nkx2.5 engineered canine CPCs [67]. A comparison between the miRNA expression pattern in neonatal and adult murine CPCs revealed a cohort of eight miRNAs that are up-regulated during the post-natal development that may justify differences in proliferation. In particular, a member of the miR-17-92 cluster, miR-17, was identified as the most differentially expressed miRNA [68].

Recently, in our laboratory we have demonstrated the differential expression of miR-135a between bi-dimensionally and 3D cultured adult rat CPCs (unpublished data). We suppose that the 3D environment induces cells to differentiate and synthesize miR-135a and many sarcomeric proteins. The direct correlation between miR-135a and sarcomere assembly must be elucidated.

A few attempts have been made to manipulate CPCs for stem cell therapy. The overexpression of miR-1 or miR-499 promotes early occurrence of spontaneous beating areas upon differentiation of CPCs. In particular miR-1 over-expression induces a reduction in the proliferative rate and miR-499 leads to an increase in the expression of cardiac transcription factors responsible for cardiac differentiation [69]. MiR-499, upon migration from the donor cells, regulates the commitment of c-Kit-positive human CPCs favoring their differentiation into competent cells by repressing Sox6 and Regulator of differentiation 1 (Rod1) [70].

As for the studies on stem cell preconditioning and reprogramming, miRNAs have been used to improve homing, integration and survival of engraft CPCs in the infarcted myocardium. A pro-survival cocktail of miR-21, miR-24 and miR-221 was employed to improve cell viability and survival of Sca-1 positive CPCs both *in vitro* and *in vivo* (Figure 3) [71].

Similarly human Sca-1 positive CPCs migration and necrosis may be reduced by miR-155 over-expression [72,73]. On the base of their previous findings, the authors demonstrated that the overexpression of miR-155 results in a block of human CPCs migration by targeting matrix metalloproteinase MMP-16, which is an activator of MMP-2

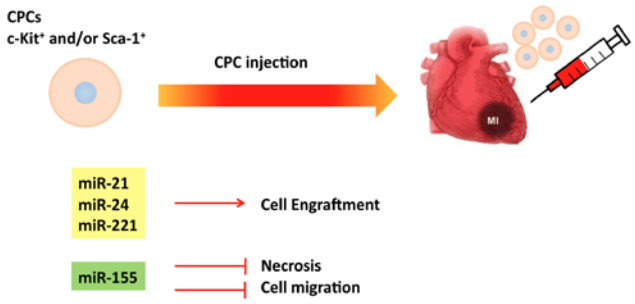

Figure 3: miRNAs expressed in CPCs. The viability of the transplanted c-Kit positive and/or Sca-1 positive CPCs in the infarcted myocardium is enhanced after the over-expression of a miRNA cocktail (miR-21, miR-24, miR-221) [71]. Moreover, the overexpression of miR-155 in CPCs improves cell survival inhibiting the necrotic process and blocking cell migration [72].

and MMP-9 (Figure 3) [73,74]. This is the only experimental evidence on the feasibility to use a miRNA as a drug for human CPC implantation and survival for a therapeutic use.

Concluding Remarks

All the evidences mentioned above suggest that miRNAs play a fundamental role in regulating cell renewal, heart development, embryonic and adult stem cell differentiation, and stem cell migration. These small RNAs can influence the expression of cluster of genes by pairing the target sequence. It is widely accepted that miRNAs can inhibit the expression of the target genes, but it is not known if pairing may also enhance the expression of some other target genes.

The research group of Prof. Rassoulzadegan studying the effects of miR-124 described also a phenomenon which they called paramutation, which is an RNA-mediated hereditary epigenetic change in the chromatin structure of the promoter of Sox9 [75,76]. Differently from the mechanisms described in this review, this miRNA mediated phenomenon induces the over-expression of Sox9 and not its repression [76]. Our knowledge on miRNAs needs to be improved as they appear to be fine regulators of many processes inside the cells rather than switching the genes on or off.

Even if the molecular mechanism underlying miRNAs is not completely understood, many research groups are trying to use these small non-coding RNA molecules in *in vitro* and *in vivo* experiments on stem cells. These molecules may be useful in cardiac stem cell therapy because they can be easily synthesised, can be attached to biomaterials or surgery devices, can be used in nanotechnology and could really finely regulate stem cell differentiation *in vivo* upon implantation of undifferentiated stem cells.

In cardiac tissue regeneration, nanoparticles bearing miRNAs or SpRNAi may be used to reprogram isolated CPCs in a biodegradable tolerated 3D scaffold. In this way, it would be possible to start reprogramming *in vitro*, and to continue *in vivo* obtaining the best efficiency in cell commitment and myocardial differentiation.

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