REVIEW

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Molecular signatures of cardiac stem cells

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Abstract

The discovery of cardiac stem cells (CSCs) able to renew the pool of cardiomyocyte raised the question of how these cells can be recognized and directed towards cardiac reconstruction after severe ischemic injury. The functional studies demonstrated that the differentiation of adult cardiac stem cells reproduce the stages observed in the embryonic development. Each stage is characterized by a complex molecular signature, which can be used for identification and molecular targeting. Three major markers have been used to isolate CSCs: c-kit, Sca-1, and Isl1 and different progenitor populations have been described: side-population (SP), cardiosphere-derived, epicardial-derived. Combinations between the main three markers and other transcription factors, cell surface proteins and regulatory RNAs may delimit even further the cardiac precursors. Accumulation of data leads to the idea that a single, yet unidentified unique cardiac stem cell is at the origin of those observed variants. In this review, we intended to summarize the actual knowledge about the main molecular markers of cardiac stem cells.

Keywords: cardiac stem cells, cardiomyocytes, differentiation, c-kit, Sca-1, Isl1.

Introduction

The cardiomyocytes have a turnover of about 50% during a human lifetime [1, 2], at a rate of about 1 %/year before 20 years old and about 0.3%/year at 75 [3]. This renewal, unknown until about 20 years ago, is possible due to a small number of progenitors assimilated to the tissue stem cells, resident or imported into the myocardium. These cells are classified as stem, progenitors and precursors. The stem cells are clonogenic, they have self-renewal capacity and can differentiate into multiple cell types [4, 5]. The progenitors and the precursors can still divide but they already express markers for a limited number of cell lineages; the precursors can generate a single type of cell. In the specific case of cardiac stem cells, the cell types correspond to the main heart lineages: cardiomyocytes, smooth muscle cells in the vascular walls and endothelial cells [6, 7]. During differentiation, the gene expression pattern is changing, a difference that can be used to isolate and characterize the cells. To this end, a multitude of morphological, immunological and functional studies have been conducted on human [8–10] and model organisms [7, 10–13], with inconsistent results. The major drawback of those methods is the global, multicellular approach, which masks the very fine, single cell changes; the new high-throughput, high-resolution single cell techniques promise to circumvent this obstacle [14–16]. This review aims to focus on the main molecular markers of cardiac stem cells and their evolution from early, multipotent stage towards the terminally differentiated cardiac myocytes. However, when considering the cardiac stem niche should not be ignored that contributions to this niche are also brought through circulation from the bone marrow (bone marrow-derived pluripotent or multipotent stem cells, and progenitor cells) ensuring the cardiac cells homeostasis [17, 18].

Early stem cell markers

Three molecular markers are recognized as associated with early stem cells: Oct4, *NANOG* and *SOX2* [15, 19, 20]. By the time the cells are committed to cardiac development, the levels of those proteins are significantly reduced [21]. However, recent studies indicated a certain level of overlap with more specific markers, supporting their inclusion in this review.

Most cardiomyocytes originate in precursors migrated from the cardiac mesoderm; the other cardiac lineages are generated from proepicardium and derivatives of neural crest cells [22]. Two heart fields have been described, each with specific contribution to the final structures of the heart. The first heart field (FHF) is the origin for the myocytes in atria and the left ventricle while the second heart field (SHF) is mainly responsible for the formation of the right ventricle, part of the atria and the outflow tract [23]. The details of cardiac formation and specific signaling are presented elsewhere [22–24].

Oct4 (POU Class 5 Homeobox 1; *POU5F1* gene) is a transcription factor associated with embryonic development and maintenance of stem cell pluripotency. Its expression in non-tumoral adult tissues is very low [25]. In cardiac embryonic development, Oct4 is expressed in the first

stages, of pluripotent stem cell and partly overlapped with Brachyury (T) in the early mesodermal progenitors [21]. *In vitro*, Oct4 cooperates with *NANOG*, *SOX2* and *LIN28* to artificially induce stem cell state in overexpression experiments [26]. In this stage, the cells keep the full pluripotency. *NANOG* and *SOX2* immediately diminish, once the cell passes to the next stage, as mesodermal progenitor. Oct4 expression decreases as soon as stimuli from *BMP4*, activin A and *WNT3A* initiates transition towards the precardiac mesoderm. However, rare Oct4 expressing precursors can still be found in neonatal and even adult heart [27]. *NANOG* (homeobox transcription factor nanog – *NANOG* gene) is expressed exclusively in the very early stage of *in vivo* or *in vitro* embryonic stem cell development. *NANOG* expression decreases in less than 48 hours after induction of differentiation [28]. However, recent evidence from Mendjan *et al.* indicates that *NANOG* expression is necessary for the early mesoderm formation from the primitive streak (PS) [29]. They show that *NANOG* is necessary for the mesodermal configuration required for future cardiac differentiation. In the future cardiac field, *NANOG* expression shortly overlapped with *EOMES* and *MESP1* [29].

Brachyury (T) transcription factor is expressed in early mesoderm, before cardiac specification [22, 30]. T induces a transient and overlapping expression of *MESP1* (Mesoderm Posterior Basic Helix-Loop-Helix Transcription Factor 1) [30].

MESP1 is the earliest marker for cardiomyogenic development and differentiation, expressed early in the posterior mesoderm, before any other cardiac transcription factor [23, 31, 32]; it is essential for the epithelial to mesenchymal transition [33] by inducing the specific TFs, *SNAIL1*, *TWIST*, and *FOXC1* [23]. Cell-lineage tracking identifies cells originating in *MESP1*-expressing cells at multiple levels (heart, most vessels, intersomitic, amnion contiguous to the closing foregut [32], skeletal muscle and bone [23]); this is why Mesp1 alone is not considered by some as a *bona fide* cardiac specific transcription factor [31]. *MESP1*-derived cells can be found in both heart fields. Transcriptomic analysis of wild type and *MESP1* knockout cells showed that *MESP1* is a direct regulator of cardiac TFs (*GATA4* and *6*, *NKX2-5*, *TBX20*, *HAND2*) and it is essential for cardiac development [23]; 212 genes are upregulated in Mesp1-expressing murine cells, many of them involved in cardiovascular development [23, 33]. Bondue *et al.* analyzed the changes in gene expression associated with spontaneous differentiation in an *in vitro* murine model. They detected Mesp1 mRNA between days 2 and 4 during embryonic stem cell (ESC) differentiation, corresponding to early mesoderm formation. Mesp1 has not been detected so far in cardiac stem cells isolated from adult heart. The cells with peak expression of Mesp1, presented as well *CXCR4*, *PDGFRA* and *KDR* (*FLK1*) as surface markers [23]. *CXCR4* [Chemokine (C-X-C Motif) Receptor 4] is a chemokine receptor for stromal cell-derived factor-1 involved in viral protection, cancer and development of heart and vessels, as well as part of the brain. *PDGFRA* (Platelet-Derived Growth Factor Receptor, Alpha Polypeptide) is a tyrosine kinase receptor for *PDGF* (Platelet-Derived Growth Factor). *KDR* (Kinase Insert Domain Receptor) or *FLK1* as presented in many articles is one of the receptors for VEGF and it participates to vascular and mainly endothelial formation and survival.

EOMES (eomesodermin) is a T-box transcription factor essential for embryonic development of mesoderm and central nervous system. In the early heart, *EOMES* is co-expressed with T, *MESP1* and *KDR* in progenitors that will further give rise to vascular, endothelial and cardiac first and second field progenitors [34].

Markers of cardiac stem cells (CSCs)

The cardiac stem cells isolated from adult heart, either in human or model organism, have limited differentiation capacity compared to the pluripotent stem cells. They do not express the transcription factors markers of stemness but they also still do not express characteristic structural protein for the differentiated cells.

Based on chemical structure, CSCs-associated markers can be classified in proteins and RNAs. Based on function, the proteins are surface markers (receptors, transporters, connecting proteins), cytoplasmic structural proteins or transcription factors. The expression of surface antigens, combined with specific transcription factors or structural proteins can distinguish between different groups of CSCs. The classification is mostly based on the markers used for selection of CSCs: c-kit, Sca-1 or Isl1 positive cells but recent views suggest the possibility that all these types of cells are in fact different differentiation stages of the same resident cardiac stem cell [20, 27, 34]. A summary of the markers found in cardiac stem cells is presented in Table 1.

Table 1 – ***Markers of cardiac stem cells***

c-kit^{pos} cardiac stem cells	▪ **positive** for: c-kit, MDR1, Sca-1 (±), Gata4, TBX5, NKX2-5; ▪ ***negative*** for: CD45, CD34, CD31, KDR.
Sca-1^{pos}	▪ **positive** for: c-kit (±), MDR1, CD31 (PECAM1), Pdfra, Tcf21, Gata4, Gata6, Tbx5, Tbx20, Hand2; ▪ ***negative*** for: CD45, CD34, Kdr, Isl1, Nkx2-5, Hand1.
Isl1^{pos} cardiac progenitors	▪ **positive** for: Isl1, c-kit (±), Nkx2.5, Flk1, TBX1; ▪ ***negative*** for: CD45, CD31, Sca-1.
Side-population cells	▪ **positive** for: Nkx2.5, αSA, MDR1, Abcg2, Vegf, Vcam1, Icam1, Vwf, CD29, CD44, capsulin (Tcf21), Meox2, Mef2A, Mef2C; ▪ ***negative*** for: c-kit, CD31, Sca-1, CD45, Oct3/4, SSEA-3, SSEA-4, Esg1, Rex3, SOX2, Utf1, Nkx2.5, Gata4 (±), Hand 1 and 2, myocardin.

The main cell surface markers in CSCs

The c-kit protein (V-Kit Hardy–Zuckerman 4 Feline Sarcoma Viral Oncogene Homolog, CD117) is encoded by the *KIT* gene, located on chromosome 4q12. The protein is a type III tyrosine-protein kinase, which recognizes the SCF (stem cell factor) ligand (encoded by the KIT ligand – KITLG gene). Receptor–ligand interaction activates signaling pathways responsible for cellular proliferation, differentiation and development. The protein is present and of significant importance in the hematopoietic system, neurons, interstitial cells of Cajal and Leydig cells as well as progenitor cells from other tissues [35–40]. The recognized roles include vascular and melanocyte development, hematopoiesis and reproduction [35, 41]. The c-kit^{pos} cardiac stem cells have been most extensively studied (reviewed in [34]).

The set of surface markers of c-kit^{pos} cardiac stem cells exclude hematopoietic lineage molecules (CD45, CD31, CD34), from here the name: lineage negative c-kit positive or lin^{neg} c-kit^{pos} [7, 42, 43]. However, Dey *et al.* find both CD45-positive and CD45-negative CSCs in the mouse heart [44]. The same types of cells have been identified in the heart of mouse, rat, pig, dog, sheep, feline, zebrafish and human [8, 41]. The c-kit^{pos} cells represent $1/10^4$ from the total number of isolated cardiac cells in the adult rat [7]. In human, the c-kit^{pos} cells represent 1.1 to 1.8% of the myocardial cell population [43, 45]; of these, about half are multipotent cells and half are early committed cells [46]. The markers expressed are correlated with the differentiation stage. In the molecular pattern of cardiac progenitors of adult heart, c-kit is expressed in the intermediary stage, together with markers of stemness (like MDR1), one or more early cardiac TFs [45] and early structural proteins characteristic for cardiomyocytes or smooth muscle cells (sarcomeric myosin heavy chain or SMAα, respectively) sarcomeric proteins [2, 7]. Dey *et al.* found about 3% c-kit^{pos} cells in a mix of mouse cardiac cells with small diameter; amongst these cells, about 1–2% was also showing CD45 antigen, a marker of hematological origin; the CD45-positive and c-kit^{pos} cells were considered in this work to be mast cells and other cells imported from bone marrow [44]. Interestingly, the percentages of CSCs in human and rodent heart are similar [47].

Several studies proved the stem cell capacity of endogenous lin^{neg} c-kit^{pos} CSCs [2, 3, 20, 47, 48] but others support the view that only non-myocardial cells can be generated as a result of c-kit cells differentiation [13, 34, 49]. Miyamoto *et al.* showed that about 1/5 of the c-kit^{pos} cells isolated from adult rat heart are clonogenic and multipotent being able to differentiate cardiac myocytes, smooth muscle and endothelial cells, as well as adipose and muscular cells [50]. According to Keith & Bolli, during development, c-kit^{pos} cells arise in the first heart field (FHF) progenitors also expressing cardiac myogenic TFs (*GATA4*, *TBX5*, *NKX2-5*). The cells initially in the FHF can be traced to the left ventricle and atria formation [34]. A much higher level of c-kit is observed in the late stages of proepicardial progenitors mostly at the origin of endocardial, mesenchymal stromal cells and adventitial fibroblasts and to a lesser extent to myocardium and smooth muscle cells [13, 34]. Transcriptomic analysis of the CSCs *versus* the mature cardiomyocytes isolated from mice heart showed that the c-kit positive cells have a very distinct expression profile compared to the Sca-1 positive CSCs and mature cardiomyocytes; these differences were reflected at both transcription factor and specific structural or motor proteins [44].

As c-kit is not a marker of proliferating cardiac progenitor cells only, it was raised the question about the precise origin of cardiac c-kit^{pos} cells. Some authors consider the absence of hematological markers as a proof of cardiac origin while others support the bone marrow origin and loss of hematological markers after the cells reach the heart [44, 51]. Cell lineage tracking performed in other studies support the cardiac endogenous origin of c-kit^{pos} lin^{neg} CSCs [1, 12, 48, 52].

Cardiac stem cells are clustered in poorly delineated niches, where they are protected from the terminal differentiation signals or from unnecessary activation. The numbers of CSCs in those spaces is maintained through controlled symmetrical or asymmetrical divisions [12]. Mice stem and progenitor cardiac stem cells in the niche express connexins (43 and 45) as well as cadherins. Gap junctions including connexin 43 facilitate communication between stem or progenitor cells but also between these cells and myocytes or fibroblasts. CSCs express α4-integrins and β1-integrin to connect with extracellular α2-chain of laminins and fibronectins. The former surface receptor is also present in bone marrow stem cells where it is associated with signaling for self-renewal; only the β1-integrin could be found on the progenitors. The niches are mostly found in atria, the base of the outflow tract and apex. The volume is variable with an average of 11 000 μm^3 and the number of stem or progenitor cells can vary from one niche to another [12].

Sca-1 antigen defines cardiac progenitor cells, having less proliferative and regenerative potential compared to the cardiac stem cells [53]. Sca-1 antigen has not been identified in human but the antibodies against Sca-1 react with a protein that has been named Sca-1-like. Some authors reported co-expression of c-kit and Sca-1 at least in part of the cells but others did not identified Sca-1 in c-kit and MDR1-positive cells in human heart [42]. Liu *et al.* found an increase in Sca-1^{pos} cells around the vessels close to the infarcted area, in a rat model of myocardial infarction [54]. These cells copurify with the non-myocyte fraction [2, 55]. Oh *et al.* isolated Sca-1 positive cells from adult mouse heart; they found Sca-1^{pos} cells adjacent to the basal lamina. These cells also expressed CD31 (platelet/endothelial cell adhesion molecule 1 or PECAM1). Telomerase activity measured in Sca-1 cells was similar as in the newborn heart, confirming the proliferative potential of these cells [55]. Based on additional markers and functional characteristics, Valente *et al.* classifies cardiac Sca-1^{pos} cells in several categories [53]. Sca-1 positive cells have been found in the actively proliferative compartments of multiple tissues, as well as resident fibroblasts from several organs. Sca-1 expression has been found only in mesodermal-derived cells but its expression is ubiquitous [53].

About 93% of cardiac SP cells (side-population cells) are Sca-1^{pos} and CD45 and c-kit negative [55] but not all Sca-1^{pos} cells are SP cells [56]. The SP cells are quiescent cells found in all adult tissues, characterized by spontaneous exclusion of Hoechst33342 dye, nestin expression and the capacity to form spheres *in vitro* [57]. Nestin (*NES* gene) is mainly a structural protein of neural precursors [58] but it is also considered as marker of sphere formation and of stem and progenitor cells [59, 60]. There is an overlap between the notion of SP and that of progenitor cell. Functionally, the cardiac SP cells are able to differentiate in multiple cell lines. They express MDR1 as well as another transporter, Abcg2. The later was found only in SP cells, in adult mouse. Transcriptomic analysis performed on flow cytometry-isolated mouse SP cells showed presence of some endothelial markers (*e.g.*, Vegf, Vcam1, Icam1, Vwf) but absence of CD31 [61]. Tomita *et al.* showed expression of CD29, CD44 at the surface of SP cells and absence of hematopoietic line; previously described CSCs markers, c-kit and Flk1 were also present

in part of the cells while Sca-1 was low [57]. Martin *et al.* identified by transcriptomic analysis a series of cardiac stem cell transcription factors: capsulin (Tcf21), Meox2, Mef2A, and Mef2C, while markers for ES cells (Esg1, Oct3/4, Rex3, Sox2 and Utf1), the well-known cardiac regulators (Nkx2.5, Gata4, Hand1 and 2, and myocardin) as well as the markers of terminally differentiated cardiomyocytes, were absent [61]. On the contrary, Tomita *et al.* identifies GATA4 but not Nkx2-5 or Mef2C in the SP cells. From the point of view of differentiation capacity, Tomita *et al.* show the evolution towards cardiomyocyte and neural lineages [57]. A single cell qPCR (quantitative polymerase chain reaction) approach distinguishes four Sca-1^{pos} populations of cells in the mouse heart; of these, only the one expressing *Pdfra*, *Tcf21*, *Gata4*, *Gata6*, *Tbx5*, *Tbx20* and *Hand2* but negative for *Kdr*, *Kit*, *Isl1*, *Nkx2-5* and *Hand1* was cardiogenic [56]. Ellison *et al.* identified, besides c-kit and Sca-1, other membrane markers like *FLK1* (*KDR*) and PDGFR-α [1].

ABCB1 [ATP-Binding Cassette, Sub-Family B (MDR/TAP), Member 1; MDR1] is a member of the ABC transporters family involved in multidrug resistance. Expression of this protein is considered one of the main causes for the Hoechst dye exclusion from stem cells nuclei [47]. MDR1 is expressed up to the level of precursor; further differentiation determines the loss of MDR1.

ABCG2 [ATP-Binding Cassette, Sub-Family G (WHITE), Member 2 (Junior Blood Group) or BCRP)] is also a xenobiotic transporter like MDR1. *ABCG2* is transiently expressed in the early stages of heart development about the same time with KDR and overlapped with T; other tissues also exhibited *ABCG2* expression: brain, skin, intestine, cartilage [61]. In adult cardiac progenitors, *ABCG2* it is expressed in SP cells in parallel with high Sca-1 expression [44, 61] and low c-kit, CD34 and CD45. As shown by Martin *et al.*, the cardiac TF, MEF2C is present while earlier stem cell or cardiac specific TFs, Oct4, Sox2, HAND1 and 2, NKX2-5 and GATA4 are absent [61].

KDR (Kinase Insert Domain Receptor; FLK1; VEGFR2) is a receptor for VEGF (vascular endothelial growth factor) with role in angiogenesis and vascular development. KDR is considered as molecular marker for endothelial lineage. KDR^{pos} c-kit^{neg} cells are clonogenic and able to differentiate into the three main lineages of the heart; in development, KDR precedes c-kit expression [62]. KDR it is coexpressed with isl1 but not with c-kit and Sca-1 [7, 63].

Main transcription factors in cardiac progenitors

ISL1 (ISL LIM Homeobox 1) belongs to the LIM/homeodomain family of transcription factors that binds and regulates the promoters of the insulin, glucagon and somatostatin genes. Isl1^{pos} progenitors seem to generate, besides mature cardiomyocytes, a part of sinoatrial node, elements of the proximal aorta, pulmonary trunk, stems of the two coronary arteries, aortic and pulmonary valve leaflets, endocardium; the vascular endothelium and smooth muscles also showed traces confirming origin in ISL1 cells [64]. It is generally accepted that ISL1^{pos} cells can be found only in very young organisms (a few days old); the ISL1 is lost rapidly and only exceptionally positive cells can be found in adult heart. Morretti *et al.* identified triple marker cells, isl1^{pos}, Nkx2.5^{pos}, flk1^{pos}, derived from ES cells, and able to differentiate into myocardial and endocardial cells [64]. Bu *et al.*, analyzing cardiac sections originating from 11 to 18 weeks human fetal heart, identified a mixed population of *ISL1*^{pos} cells, one showing additional lineage specific markers as well as ISL1^{pos} only cells. The latter were mainly located into the region known as second heart field (right atrium and the outflow tract) [65]. Other cardiac differentiation markers, like *TBX1* (second heart field specific marker) and *NKX2.5* (cardiac TF) are beginning to be expressed two and respectively four days after *ISL1*, in an *in vitro* embryoid body model [65]. Postnatally, the ISL1^{pos} cells have been identified in human as well as rat and mouse, following the same distribution pattern; at full differentiation, the isl1 expression is absent [66].

NKX2-5 (NK2 Homeobox 5 or Nkx2.5) is expressed after Mesp1 and further in the cardiac and smooth muscle cell progenitors [67]. In mouse, Nkx2-5 is also expressed in thyroid, pharynx, stomach, and spleen [68]. A tissue-specific enhancer directs gene expression in the heart progenitors. In mouse embryonic development, Nkx2-5 precedes Isl1 and the two TFs are co-expressed in a small number of cells [67]. Nkx2-5 also co-expressed with early myocardial and smooth muscle cells motor proteins but it is not overlapped with Troponin T or SM-MHC (smooth muscle myosin heavy chain) and neither with endocardial marker Kdr (Flk1) [67]. Using an *in vitro* differentiation model, Wu *et al.* demonstrate that Nkx2-5 expressing cells also present c-kit surface antigen for a limited time before differentiation; after c-kit loss, Sca-1 antigen becomes expressed [67].

GATA4 (GATA Binding Protein 4) is a transcription factor, which recognizes the GATA motif in the promoter of the target genes and plays a key role in cardiac development. It has been found expressed in c-kit^{pos}, Sca-1^{pos} and Isl1^{pos} cells [63]. In an embryoid body (EB) model of differentiation, *GATA4* raises immediately after Oct4, Nanog and Brachyury decrease [21]. *GATA4* is co-expressed with c-kit^{pos} and Isl1^{pos} in the CSCs [66]. *GATA4* expression in cultured c-kit^{pos} CSCs is variable and correlated with the evolution towards differentiation [50, 69]. Chronologically, *GATA4* is expressed after *NKX2-5* and *ISL1* in differentiating precursors [70].

MEF2C (Myocyte Enhancer Factor 2C) is a transcription factor, which regulates muscle specific genes and plays an important role in cardiac morphogenesis and differentiation. In CSCs, it has been identified in the three major types of c-kit^{pos} and Sca-1^{pos} cells. Specific expression of *MEF2C* in the anterior heart field is cooperatively controlled by *GATA4* and *ISL1* interacting with a specific enhancer [71]. *MEF2C* regulation points towards the hypothesis that its expression is initiated after *ISL1* and *GATA4*.

Regulatory RNA molecules in cardiac differentiation

Regulatory RNA molecules are important players in cell fate determination and in tissue specificity assignment and maintenance. Two categories of regulatory RNAs

are better studied: long non-coding (lnc) RNAs and microRNAs.

Long non-coding (lnc) RNAs

Long non-coding RNAs are regulatory RNA molecules of more than 200 bp in length, spliced, 5'-capped and polyadenylated. The simple high-throughput gene expression analysis revealed differential expression of hundreds of lncRNA molecules in differentiating cardiomyocytes compared to the undifferentiated CSCs [72, 73]. Moreover, some lncRNAs, such as Braveheart, Fendrr and HOTAIR, show specificity for certain cardiac regions.

Klattenhoff *et al.* identified an lncRNA that they called Braveheart (*Bvht*), expressed upstream of Mesp1 and acting as an essential activator on the pathway of cardiovascular lineage commitment in mouse. However, Braveheart expression has not been identified in human, yet [74]. Braveheart is a spliced transcript originating in a three-exon gene located on mouse chromosome 18. Close to the Bhvt gene there is a microRNA cluster, mir143/145, highly expressed in human and rat heart in correlation with Bhvt expression [75]. The mechanism by which Bhvt controls the cardiac commitment is rather epigenetic, in relation to polycomb complex [74]. Due to its secondary structure, Braveheart interacts with the PRC (Polycomb repressive complex) and prevents it from blocking *MESP1* expression; as a result, it is activated the cardiac development program [76]. In the context of general expression and action of polycomb repressor complex 2 (PRC2) proteins, the lncRNAs could be responsible for the cardiac specificity [77, 78]. One of the TFs expressed in early cardiac progenitors but not in adult cardiomyocytes is Six1. Six1 seems to be under the control of cardiac TFs, Nkx2.5, GATA4 and Mef2C. Its negative regulation prevents skeletal muscle specific genes to be expressed in the heart and ensures the course of cardiac muscle cell program [78].

Fendrr (FOXF1 adjacent non-coding developmental regulatory RNA) is a lateral mesoderm specific lncRNA essential for heart development in mouse [79]. Orthologs for Fendrr have been discovered in human and rat [79–81]. Fendrr knockout is lethal in embryonic stage and presents parietal and cardiac malformations [80]. At molecular level, Fendrr exerts an inhibitory effect on gene expression by direct binding both to the target genes (*e.g. FOXF1*) promoters and PRC complex, thus increasing the interaction of the latter with the regulatory region [80]. Fendrr may also have stimulatory effect on gene expression by interacting with other complexes, but it is not clear yet how this is important for the cardiac development and differentiation [80].

HOTAIR (HOX transcript antisense RNA) is expressed from a gene located within HOXC gene and regulates in *trans* the HOXD gene. HOTAIR functions on Wnt/*β*-catenin pathway and it has been found responsible for the aortic valve disease [82, 83].

A number of cardiac-specific enhancers generate also lncRNAs, many of which have been found to activate downstream genes during cardiac differentiation from ESC or CSCs. Some of these were found in mice [84] but some in human [85] hearts, equally pre- and postnatally [72, 84, 85].

MicroRNAs

MicroRNA markers specific for endogenous cardiac stem cells have not been identified yet. However, stem cells display a recognizable pattern and the differentiated heart myocytes express muscle specific microRNAs. Amongst the CSC-expressed microRNAs, eight are at different levels in adult heart CPC compared to neonatal heart [86]. However, a number of miRNAs are specifically related to muscular development (including cardiac muscle): miR-1 inhibits the expression of the early TF, Hand 2 [87] and consequently activates differentiation; miR-133 inhibits proliferation; miR-17-92 cluster, targeted by BMP2 and 4, downregulate Isl1 and Tbx1 [88].

There are a few arguments for miRNA intervention in cardiac stem cells differentiation: the levels of miR-1 and miR-133 are extremely reduced in stem cells and high in differentiated cardiomyocytes; ectopic overexpression of miR-1 and miR-133 inhibit Nkx2.5; pharmacological induction of differentiation decreases expression of miR-1 and miR-133. MiR-1 is also promoter of angiogenesis; the same effect: miR-132, inclusively through activating vascular differentiation of CSCs [89]. MiR-1 is regulated at transcriptional level by SRF (Serum Response Factor) and myocardin. Mir-1 gene has several recognition sites for several transcription factors related to cardiac differentiation but SRF is essential for its transcriptional activation [87]. MiR-1 and miR-499 repress proliferation and promote differentiation [90].

MiR-499 is a very well conserved microRNA, highly expressed in myocytes but almost absent in undifferentiated precursors. By inhibiting Sox6 and Rod1 at translational level, miR-499 activates the expression of cardiac lineage TFs (Nkx2.5, GATA4 and Mef2C). Hosoda *et al.* discovered a paracrine process when cardiac precursors are cultured together with differentiated myocytes: miR-499 from the latter was transferred through gap junctions into the undifferentiated cells, activating their differentiation [9]. The MIR499 gene is located in one of MYH7A gene's introns (see for tissue specificity also) [90] and regulates at post-transcriptional level the very same gene. When overexpressed in mesenchymal cells, miR-499 has the same effect, inducing the expression of cardiac TFs. MiR-499 has overlapping functions with proteins in the Wnt/*β*-catenin pathway [91].

Mir-1 and miR-133 are transcribed from the same locus but have different roles in muscle differentiation. Mir-1 releases HDAC4 inhibitory activity on muscle specific genes while miR-133 acts on serum response factor and enhances proliferation of muscular precursors. If miR-1 expression is abolished, heart is not generated in mouse [92]. Mir-133 inhibits cardiac differentiation of mesenchymal stem cells in mouse and human [91]. There is a relationship between lncRNAs and miRNAs, the former being targeted by the latter, the same as in the case of mRNAs [72]. A set of microRNA genes, including let-7, miR-181, miR-145 and miR-196 known to be associated with proliferation, maturation and differentiation are upregulated in c-kit cells [44]. Further studies are expected to show in more detail the role of non-coding RNAs in adult cardiac stem cell homeostasis and differentiation control.

Conclusions and perspectives

The heart contains a number of progenitors able both to regenerate at a limited rate the cardiomyocytes and to differentiate in vascular and endothelial specific cells. The transcription factors and surface molecule markers vary according to the experimental model and methods used for analysis. Most frequently investigated CSCs markers are c-kit, Sca-1 and Isl1, none of them being cardiac-specific. The phenotype of CSCs is the result of the interplay between a large number of genes, transcription factors, signaling molecules, structural proteins, regulatory RNAs. This is why rather a combination of markers should be used for the characterization of a differentiation stage. The use of the new, single cell sequencing experiments could shed new light on the gene expression pattern associated with cardiac differentiation and cardiac stem cell homeostasis.

Conflict of interests

The authors declare that they have no conflict of interests.

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