Chapter 5

Discovery of the Phenomenon of Intracellular Development of Cardiac Stem Cell: A New Step in Understanding of Biology and Behavior of Tissue-Specific Stem Cells

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Abstract

In our experiments with an in vitro culture of rat cardiac cells, we identified and described for the first time the phenomenon of intracellular development of CSCs in mature CMs with formation of the "cell-in-cell structures" (CICSs). Recently, we have confirmed the reproducibility of our results and existence of this phenomenon in rats of different age groups, 1-year-old bull, adult mice and humans. Moreover, we demonstrated the 5–10 times increase in the amount of CICSs after exposure of in vitro cultures to hypoxia and acidosis, that is, these conditions stimulate intracellular development of CSCs. Our data strongly suggest that transitory amplifying cells (TACs), which release from CICSs, are present as a very rare cell population in adult and old rats. Therefore, we assume that TACs are important for renewal of myocardium during ontogenesis. TACs should be considered as the major source of cells that can reduce myocardial damage in adult mammals with various pathologies of the cardiovascular system. In conclusion, precise and exhaustive analysis of the phenomenon of intracellular development of CSCs, CICSs and TACs will pave the way for cell technologies of new generation in regenerative medicine.

Keywords: primary culture of myocardial cells, mature cardiac myocytes, resident cardiac stem cells, proliferation, differentiation, cell-in-cell structure, transitory amplifying cells

1. Introduction

The founder of the study of stem cells (SCs) in adult organism is A.A. Maximow, who in 1909 first used the term «stem cell» and proved the existence of pluripotent hematopoietic stem

cell publishing a paper «The Lymphocyte as a stem cell common to different blood elements in embryonic development and during the post-fetal life of mammals» [1]. However, the ideas of our fellow-countryman A.A. Maximow, a Professor at the Saint Petersburg Imperial Military Medical Academy and Head of the Department of Histology and Embryology, were developed only in the 1960s, when Canadian scientists E. McCulloch and J. Till found hematopoietic SCs in the bone marrow [2]. The next breakthrough in this direction was made by Russian scientist A.Ya. Friedenstein, a Corresponding Member of the Academy of Medical Sciences of the U.S.S.R. [3]. He found in the bone marrow not only hematopoietic, but also stromal SCs, that can turn into cells of bone, cartilage, fibrous and fatty tissue. Going back to the ideas of A.A. Maximow, it should be emphasized that he suggested the existence of progenitor cells in certain tissues, marking them as stem mesenchymal cells. In this concept, he not only put their embryonic origin, but also their differentiation potency, ability to progressive differentiation into all kinds of connective tissue in postnatal development, such as reparative histogenesis.

Basic elements of the stem cell concept, developed in the study of the hematopoietic system, have been further extended to other rapidly renewing organs and tissues. It was found that the undifferentiated tissue-specific SCs (progenitor cells) are located in various organs and tissues, such as skin [4], cornea [5], kidney [6], liver [7], tooth [8, 9] and others, and that they are responsible for renewal of their cell population, replacing dead cells.

Although the detailed discussion of the characteristics of tissue-specific SCs is beyond the scope of this review, the brief history of neuronal SC discovery is described below as an example of long-lasting debate between “classical” views and new original ideas. Despite the fact that a paper by J. Altman, titled «Are new neurons formed in the brains of adult mammals?» [10] and published in the Science Magazine in 1962, and a few other publications by him, have proved the existence of neurogenesis in the adult mammalian brain, a concept that «the nerve cells do not regenerate» still persisted. Only twenty years later neurogenesis was again «discovered», but in the brains of birds. In the mid-1980s, Professor F. Nottebohm at Rockefeller University (U.S.A.) was able to show that in the vocal center of adult male canaries, a neurogenesis process occurs constantly in varying degrees [11]. In the late 1980s and early 1990s neuronal SCs have also been found in adult amphibians at the laboratory of our Institute of Evolutionary Physiology and Biochemistry of the U.S.S.R. Academy of Sciences, led by physiologist, endocrinologist and morphologist, a Corresponding Member of the Academy of Sciences, Andrey L. Polenov [12, 13]. In the same time period, neuronal progenitor cells were identified and studied first in the embryos [14], and then in the adult vertebrate animals [15]. These studies, along with the following works, have led to recognition of the existence of neuronal SCs, that has become not only an important fundamental discovery in stem cell biology, but also open up the prospects for their use in the treatment of peripheral nerve injury [16], spinal cord trauma [17, 18], acute cerebral injury [19], and neurodegenerative diseases, such as Alzheimer disease and multiple sclerosis [20–22].

2. Which cells are involved in the renewal and regeneration of the mammalian myocardium?

Paradoxically, mammalian heart was considered as an organ not capable of self-renewal until the beginning of the XXI century. It was widely believed that the cardiomyocytes formed in the first days after birth, persist throughout life and cannot be replaced in case of damage or destruction, and an increase in heart size from birth to adulthood is due to hypertrophy of cardiomyocytes but not to their proliferation [23].

The first signs of a possible myocardial recovery, manifested in the resumption of DNA synthesis, have been observed in the studies of Russian scientists in the late 1970s-80s [24–26], while the data on increased telomerase activity, stimulation of cyclins and cyclin-dependent kinases, and enhanced proliferation of myocytes in mammals, including humans, in the later stages of cardiac insufficiency after myocardial infarction began to appear at the turn of twentieth and twenty-first centuries [27–30].

Existence of the SCs in adult mammalian heart and their participation in remodeling of injured myocardium was revealed for the first time only in 2002 in the paper by Hierlihy et al. [31] and later papers [23, 32–35]. A number of studies performed on the culture of heart cells, yielded valuable information about types of the resident CSCs, their clonogenicity and ability to differentiate [32, 36–41]. Also, it was found that cardiac SCs are present in the myocardium in trace amounts: c-kit$^+$ – 1 per 10^4 myocytes [32], Islet-1$^+$ – 500-600 in the heart of a 1-5-day-old rats [38], SP$^+$ (Sca1) – 500-1000 in the adult heart [39].

Nevertheless, in spite the discovery of the resident CSCs in the heart of mammals, including humans, and accumulation of knowledge about their biology, there is no consensus in the scientific community about the regenerative potential of the myocardium. To date, there is no unifying theory that can explain the fact that the ability of mammalian myocardium to regenerate after injury is lost in a few days after birth [42], and also why body's own resident CSCs, as well as externally injected CSCs [43–46], or mesenchymal SCs [47–49], or bone marrow SCs [50] are unable to form mature cardiomyocytes in the infarct zone. Answers to these questions, on one hand, will establish a real picture of the self-renewal of myocardial cells in normal conditions and changes in homeostasis in injured heart in various cardiovascular diseases, and on the other hand, will make possible to develop a considered tactics to influence on regenerative processes using modulators of proliferation of the resident cardiac cells.

3. Intracellular development of stem cells: a new step in the understanding of biology and behavior of tissue-specific stem cells

From 2005 to 2011, when we studied the behavior of neonatal myocardial cells in primary culture, we confirmed existing concept that after a burst of mitotic activity in the first 2-4

days of postnatal life in vivo [51], the division of neonatal CMs in culture also terminated in 4-5 days after seeding in vitro [52]. At the same time, just like in the body, a mitotic division of 60% of the cells is observed, and after the cessation of this division, a growth of their volume is occurred, that is also similar to the process of hypertrophy of the CMs in vivo. It has been shown that the volume of CMs increased from 819±68 μm^3 on the 1st day to 1532±212 μm^3 on the 3rd day and up to 3246±190 μm^3 on the 6th day of culturing. Moreover, the growth rate of cultured cells is almost completely coincides with the speed of myocyte hypertrophy in the body. Just like in the body, hypertrophy was accompanied by formation of polyploid and multinucleate, mostly binucleate cells. Besides, it was first

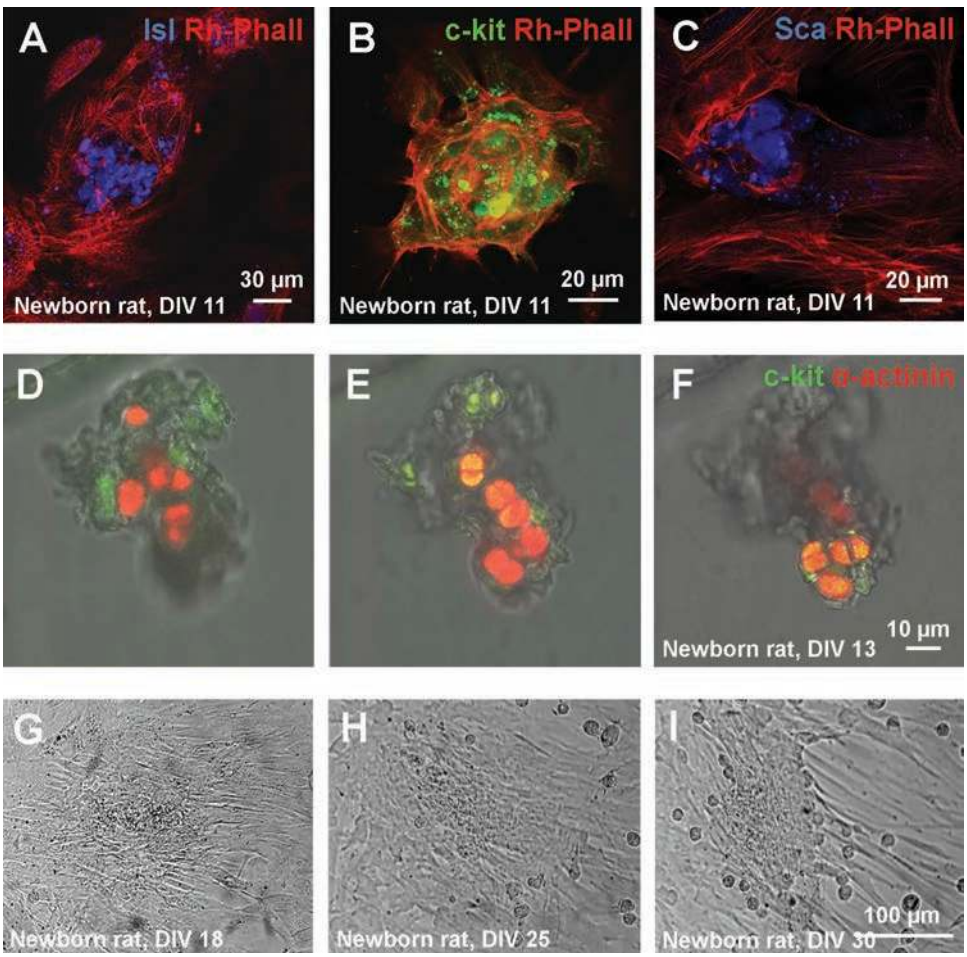

Figure 1. The formation of cardiomyocyte colonies in the primary culture of rat neonatal myocardial cells. (A–C) The optical sections of colonies formed by $Isl1^+$, $c\text{-}kit^+$, and $Sca1^+$ CSCs on the 11th day in vitro (DIV). (D-F) Differentiation of $c\text{-}kit^+$ CSCs inside the colony on the 13th DIV. (G–I) Progressive increase in the contraction rate of the same colony during culturing. (G) The 18th DIV, the contraction rate: 25 beats/min. (H) The 25th DIV, the contraction rate: 46 beats/min. (I) The 30th DIV, the contraction rate: 58 beats/min.

shown that the resident CSCs of three types (c-kit$^+$, Sca1$^+$, Isl1$^+$) in the primary culture of newborn rat myocardial cells form colonies of contracting cardiomyocytes by dividing and subsequent cardiac differentiation, simulating the cardiomyogenesis process from a single immature stem cell to functionally mature cardiomyocyte (**Figure 1**). Therefore, the formation of contractile cardiomyocyte colonies is a complete model for a basic research, testing of drugs and identification of the regenerative potential of CSCs for possible application of the resident self-renewing cells in the treatment of myocardial injury [53, 54]. In addition, immunocytochemical study of the freshly isolated suspension (ex vivo) of myocardial cells from rats of different age groups, adult mice, young bulls and humans, revealed the presence of colonies of different maturity formed by resident CSCs of all three types (**Figure 2**). This made it possible to suggest that the colony formation is a way of myocardial self-renewal that allows to replace the lost cells throughout the life of mammals, including humans.

By study of myocardial cells in monolayer culture from newborn, 20- and 40-day old rats with help of antibodies to CSC antigens and confocal microscopy technique we first revealed the presence of small immature cells (5-6 μm in diameter) of all three types inside mature cardiomyocytes with formation of «cell-in-cell structures» (CICSs). It was found that the cell that has penetrated inside, usually located near the nucleus, or between two nuclei, and stored in the vacuole, membrane of which protects this cell from the host cell cytoplasm (**Figure 3**). Intracellular regions of large cells expressing CSCs antigens increase in size during culturing. Expression of the Ki-67 protein that plays an important role in the regulation of cell division in such vacuoles indicates on proliferation inside located CSCs. As a result of increase in their number, the size of the vacuoles increases, and the membrane thickens and becomes more dense forming a capsule. As time goes by, a formation of two to five openings (pores) occurs in the capsule that appears to provide gas exchange and exchange of metabolites between CSCs and host cell (**Figure 3 C, D, F**). Expression of cardiac (**Figure 3**) proteins (α-sarcomeric actin, sarcomeric α-actinin and Troponin T) suggests that the host cells appear to be a mature cardiomyocytes [55].

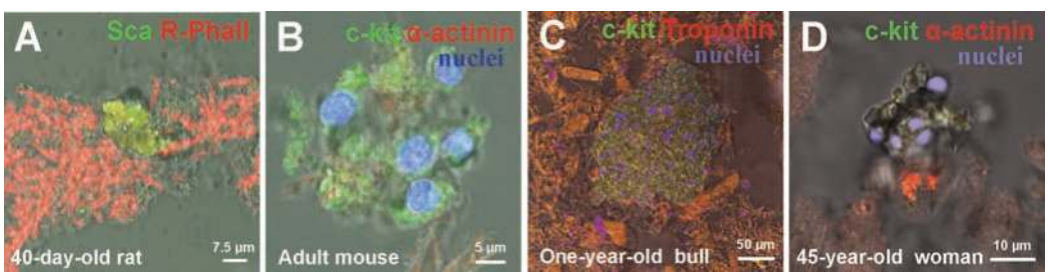

Figure 2. CSC-derived colonies in freshly isolated myocardial cell suspensions (ex vivo) of different mammalian species. The nuclei of the cells have been stained with Hoechst. Transmitted light and fluorescent images are merged. Cardiomyogenic differentiation is verified with expression of sarcomeric α-actinin and troponin T. (A) An undifferentiated small Sca1$^+$-colony inside a fragment of myocardium of 40-day-old rat. (B) An undifferentiated small c-kit$^+$-colony from myocardium of an adult mouse. (C) A large volume c-kit$^+$-colony inside a fragment of myocardium of one-year-old bull. (D) An undifferentiated small c-kit$^+$-colony inside a fragment of myocardium of 45-year-old woman.

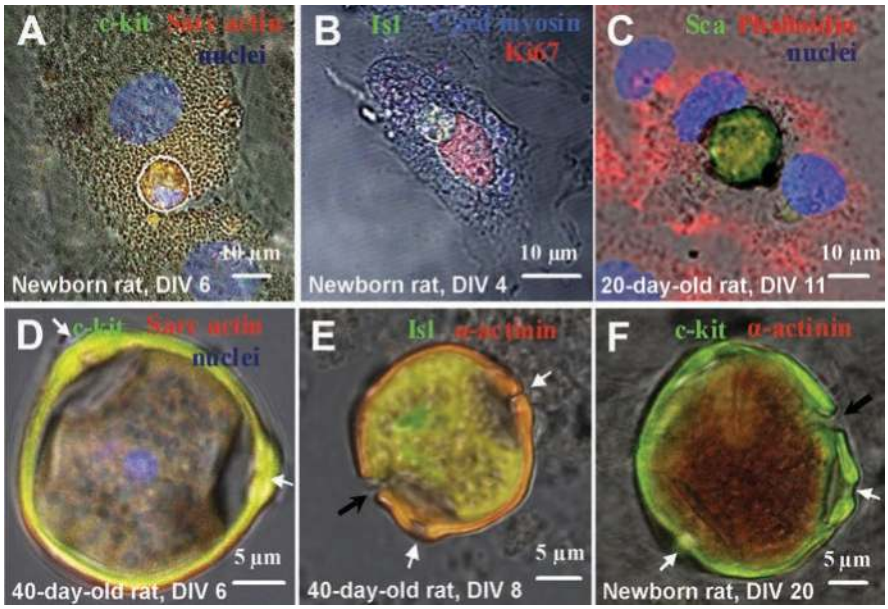

Figure 3. The CSCs inside CMs and the formation of CSC-containing CICSs in the cultures obtained from newborn and 20- and 40-day-old rats. (A–C) Early stages of CICS formation. (A) c-kit$^+$ CSC inside a newborn rat CM obtained (day in vitro 6). (B) Isl1$^+$ CSC inside a newborn rat CM (day in vitro 4). Both the CSC and the host cell exhibit proliferative ability, as documented by the expression of Ki67. (C) Sca + CSC encapsulated between the nuclei of the host cell (20-day-old rat, day in vitro 11). (D–F) The CICS capsule in detail. (D) c-kit$^+$ CSC-containing CICS with a prominent coating ("capsule") with three pores (white arrows) obtained from a newborn rat, day in vitro 6. (E) Erosion (black arrow) of the Isl1$^+$ CSC-containing CICS capsule obtained from 40-day-old rat, day in vitro 8. The pores are also visualized (white arrows). The capsule interior is positive for sarcomeric α-actinin. (F) Erosion (black arrow) of the c-kit$^+$ CSC-containing CICS capsule obtained from a newborn rat, day in vitro 20. The pores are seen (white arrows). The nuclei of the cells have been stained with Hoechst, transmitted light and fluorescent images are merged.

Long-term observation of CICSs in primary culture of newborn, 20- and 40-day old rats revealed that intracellular development of CSCs ended by rupture of CICS-capsule and release of large quantities (up to 200) of transient amplifying cells (TACs), positive against SC-proteins of one of the three CSC-subtypes investigated by us and cardiac proteins (**Figure 4**). In the process of further development of descendants of the original CSC, expression of the stem antigens is reduced, but at the same time, synthesis of cardiac proteins is increased which allowed to suggest a reasonable expectation that the TACs appears to be an intermediate stage between CSCs and cardiomyocytes.

The presence of the similar CICSs, intact and opened, and the TACs of varying degrees of maturity in suspension of myocardial cells (ex vivo) of rats in different ages from the 1st day of life (Figure 4) to old age (1.5 years), adult C57bl/6 mice, 1-year-old bull, and human (45 years old) allowed to suggest that self-renewal of the myocardium may occur by proliferation and initial differentiation of CSCs inside mature cardiomyocytes throughout the life of mammals including humans.

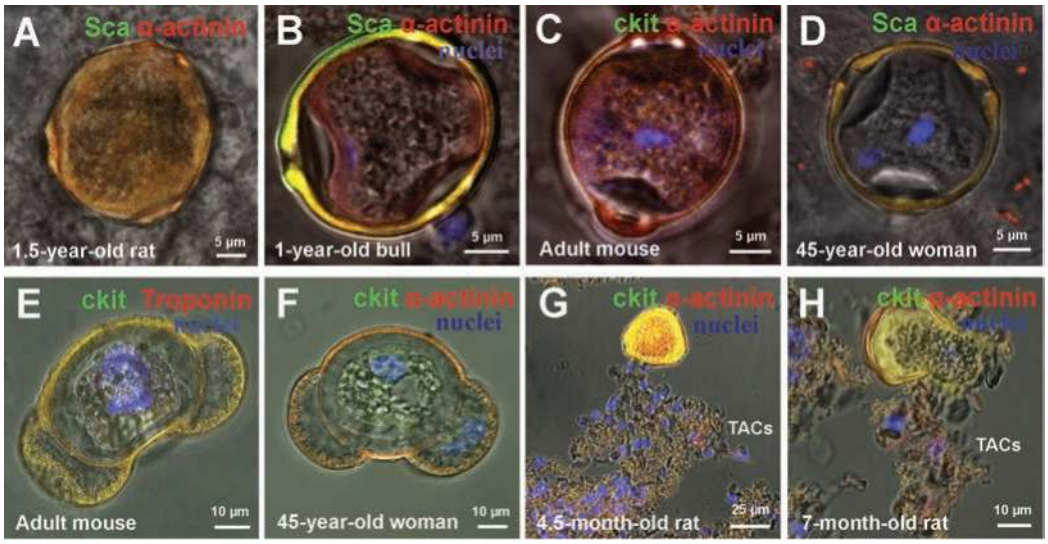

Figure 4. Identification of "cell-in-cell structures" (CICSs) in freshly isolated suspension of myocardial cells (ex vivo) of mammals of different species. (A–D) Intact CICSs. (A) Sca1+ CICS from myocardium of 1.5-year-old rat. (B) $Sca1^+$ CICS from myocardium of 1-year-old bull. (C) $c\text{-}kit^+$ CICS from myocardium of an adult mouse. (D) $Sca1^+$ CICS from myocardium of 45-year-old woman. (E and F) Opened CICSs. (E) $c\text{-}kit^+$ CICS from myocardium of an adult mouse. (F) $c\text{-}kit^+$ CICS from myocardium of 45-year-old woman. (G and H) The release of transitory amplifying cells (TACs) from opened CICSs. (G) Opened CICS and TACs from myocardium of 4.5-month-old rat. (H) Opened CICS and TACs from myocardium of 7-month-old rat.

Because the colonies of contracting cardiomyocytes and intracellular structures are formed in the culture with almost the same frequency (1-2 cases per 100,000 cells plated), we have put forward a hypothesis that the formation of new cardiac cells from resident CSCs may occur by colony formation as well as by intracellular development in mature myocardial cells. It is suggested, therefore, that both processes are present in a healthy heart and, apparently, maintain homeostasis of the heart muscle by replacing the dead cells with new functionally active cardiomyocytes.

It has been found for the first time that in experiments in vitro under simulated conditions of myocardial ischemia zone like acidosis and hypoxia suppress the growth of colonies by blocking the differentiation of resident CSCs, but at the same time, stimulate their intracellular development, increasing the amount of intracellular structures by several times. Therefore, it has been suggested that Ca^{2+}-overload, as well as acidosis and hypoxia, accompanying myocardial infarction, cause the death of not only the mature myocardial cells, but also of the resident CSCs that are forced to «hide» inside large cardiac cells, which allows us to understand their passive role during myocardial infarction. This also explains irrationality of stimulation of resident CSCs for proliferation, differentiation and cell therapy application (introduction of exogenous SCs into the injured region) during the acute phase of ischemia and infarction.

4. Analysis of problems of mammalian myocardial regeneration in the light of the new experimental data

Penetration of one or more cells to another to form the «cell-in-cell structures» (CICSs) was revealed 90 years ago by W.H. Lewis [56] in blood cells. The following terms are used to identify the processes leading to the formation of «cell-in-cell structures»: entosis, emperitosis, cannibalism, emperipolesis and cytophagocytosis. Entosis, firstly described in 2007 [57], is the process of active invasion of one cell (effector cell) into another (host cell). Terms «cannibalism» and «cytophagocytosis» are used to describe the phenomenon of active ingestion by the host cell of another cell which in this case is more passive [58]. Emperipolesis was established in 1956 [59] to indicate the penetration, movement and existence processes of one cell inside the other and, according to Overholtzer and Brugge [58], is suitable to refer to all phenomena in which the formation of structures of «cell-in-cell» type occurs, regardless the fate of cell located inside.

Unlike cytophagocytosis in which dead or dying cells to be destroyed within 30-60 min are captured, if CICS is formed, cell trapped inside nonphagocytic cell can survive for several days or even weeks. But cells in the host cell cytoplasm are surrounded by vacuole membrane that is, presumably, a fragment of a host cell membrane formed after its invagination. Movement of the effector cells, their division, and sometimes exit outside of the host cell were monitored [58].

It is known that the formation of «cell-in-cell structures» occurs both in the lower and in the higher forms of organisms, as well as the penetration of cells of lower forms into cells of highly organized species (parasitism). It is also shown that this phenomenon is typical for immune system cells (cytophagocytosis and emperipolesis) and tumors (entosis), occurring in the inflammation regions [60]. However, there is no information in the scientific literature to date on the formation of such structures in the myocardium.

Discovery of previously unknown mechanism of CSC development within mature cardiac myocytes with subsequent formation of cardiac positive TACs is of great interest not only as another new variant of the «cell-in-cell structure» formation, but also, more significantly, as a phenomenon allowing to reveal the nature of the biological processes that underlie the behavior of CSCs.

Moreover, our in vitro and ex vivo data on the intracellular development of CSCs allow to offer a new perspective into consideration of myocardial infarction problems. The reason is that the current understanding of the physiology of cardiac muscle cells is highly contradictory. For example, data on the rate of formation of new cardiomyocytes range from less than 1% [61–63] to more than 40% per year [64]. In addition, parallel to accumulation of data on the participation of CSCs in cardiomyogenesis, some papers periodically appear arguing in favour of division of mature CMs in the adult heart [64–67], reviving debate about what cells, resident CSCs or mature CMs, participate in the renewal of adult myocardium. In addition, Omatsu-Kanbe et al. [68] found a small population of cells (d~10 μm) that can be differentiated in contracting CMs without preliminary division. In turn, Kimura et al. [69] showed the presence of small dividing cells with characteristics of neonatal CMs: small

size, mononuclearity and insignificant oxidative DNA lesions, in the myocardium during hypoxia. Similar neonatal-like cells have been described even after experimental ischemia [70], which allow to suggest that in the myocardium of adult animals these effects stimulate the proliferation of insignificant amount of small cardiac positive cells that are unable, however, to recover the injured myocardium. Therefore, next questions are brought to the fore: what are these cells, where are they located in a healthy heart, and why they are unable to regenerate a myocardium in case of injury? Since the division of large (with a volume of more than 20,000 μm^3) terminally differentiated CMs is contradicted [71], and small dividing cells exceed the size of resident CSCs, a question arises about the origin and the physiological role of these cells.

Proliferative activity of TACs, formed by intracellular proliferation of CSCs, gave us the reason to consider this category of cells as a main regenerative source in the myocardium of adult mammals. We suggest that these cells are able not only to renew the myocardium throughout life, but also partially participate in its regeneration, if not in the acute phase of ischemia and infarction, then possibly during the recovery period or in chronic heart failure.

Thus, discovery of the phenomenon of intracellular development of CSCs is opening further perspectives in the study and solution of the problems of myocardium, and can be seen as a new step in understanding the nature of resident CSCs and development of the approaches for use of cell technologies in regenerative medicine.

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