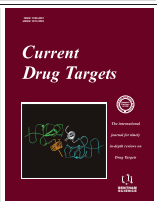
Current Drug Targets, 2019, *20*, 241-254

REVIEW ARTICLE



Targeting the Cardiomyocyte Cell Cycle for Heart Regeneration

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ARTICLE HISTORY

Received: May 28, 2018
Revised: July 27, 2018
Accepted: July 31, 2018

DOI:
10.2174/1389450119666180801122551

Abstract: Adult mammalian cardiomyocytes (CMs) exhibit limited proliferative capacity, as cell cycle activity leads to an increase in DNA content, but mitosis and cytokinesis are infrequent. This makes the heart highly inefficient in replacing with neoformed cardiomyocytes lost contractile cells as occurs in diseases such as myocardial infarction and dilated cardiomyopathy. Regenerative therapies based on the implant of stem cells of diverse origin do not warrant engraftment and electromechanical connection of the new cells with the resident ones, a fundamental condition to restore the physiology of the cardiac syncytium. Consequently, there is a growing interest in identifying factors playing relevant roles in the regulation of the CM cell cycle to be targeted in order to induce the resident cardiomyocytes to divide into daughter cells and thus achieve myocardial regeneration with preservation of physiologic syncytial performance.

Despite the scientific progress achieved over the last decades, many questions remain unanswered, including how cardiomyocyte proliferation is regulated during heart development in gestation and neonatal life. This can reveal unknown cell cycle regulation mechanisms and molecules that may be manipulated to achieve cardiac self-regeneration.

We hereby revise updated data on CM cell cycle regulation, participating molecules and pathways recently linked with the cell cycle, as well as experimental therapies involving them.

Keywords: Cell cycle, cardiomyocyte, heart, mitosis, INK4, Cip/Kip.

1. INTRODUCTION

Cardiovascular disease is the leading cause of death worldwide, usually as a result of end-stage heart failure. Left ventricular deterioration is the final pathway of several heart injuries that induce significant cardiomyocyte loss and ventricular remodelling, such as ischemic heart disease and its consequence, myocardial infarction. As in humans, the lost CMs are minimally replaced through the mitotic division of existing adult ones [1], currently, the only therapeutic option for patients with end-stage heart failure despite optimal pharmacologic treatment is heart transplantation [2].

The human and other adult mammalian cardiomyocytes (CM) are known to have limited proliferative capacity [3, 4]. However, although limited, the extent of this capacity is controversial. While some authors report that almost the final number of CMs in humans is reached before birth [5], others claim that an average of 22% of CMs are newly formed per year [6].

It has been reported that at a certain time point in the young human heart significant CM proliferation occurs, this contributing to the final organ size [7]. Others showed that in preadolescent mice hearts, a sudden rise in thyroid hormone triggers a significant round of CM division that establishes the final CM number [8].

Despite existing controversies about CM turnover, there is an agreement in that efficient therapeutic compensatory CM proliferation after cardiac ischemic injury does not occur [9]. It is, however, known that CMs have cell cycle activity without mitosis and/or cytokinesis in several processes such as cell growth and DNA synthesis for polyploidization, binucleation and multi-nucleation [10].

There is therefore a growing interest in disclosing CM cell-cycle modulators that could eventually be targeted to encourage the adult CM to divide into daughter cells as an effective therapy for heart diseases due to loss of contractile tissue. This approach would guarantee (or, at least, greatly

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favour) the electro-mechanical connection needed for correct syncytial functionality, a fact not yet convincingly demonstrated in therapies based on the implant of stem cells of diverse origins.

The aim of this review is to revise relevant information on the regulation of the CM cell cycle, the strategies being currently assessed involving its manipulation and the diverse drawbacks and challenges inherent to the study of the cell cycle.

2. HEART DEVELOPMENT IN MAMMALS

In mice, fetal heart growth occurs by CM proliferation. As gestation advances, CM cell cycle activity decreases with nearly all CMs entering a quiescent G0 state by birth [11].

Shortly after birth, the number of CMs increases ~40% from postnatal (P) day 2 to P5 in mice (and P0 to P3 in rat), suggesting that CMs in laboratory rodents' postnatal hearts maintain some proliferation capacity [12-14].

In both rat and mice, from ~P4 to ~P14, around 90% of CMs have a final cell cycle round that results in binucleation, as cytokinesis fails [11, 14]. This represents a time point after which the CMs of small laboratory rodents remain with their cell cycle blocked, and their heart keeps growing through hypertrophy [15].

In humans, it was shown that binucleated CMs appear at around 32 weeks of gestation and this suggests that CM hyperplasia may cease almost completely before birth [16].

Gradually, mononucleated CMs proportion drops, there is an increase in binucleated cells and this process continues in early neonatal life for a short period of time [16].

Interestingly, an increase in global methylation in the rat heart is observed during the early neonatal period [17], coinciding with the start of the binucleation process, thus indicating that epigenetic modifications play a role in CM differentiation, decrease in potency and proliferation capacity [18].

Much of the established knowledge is based on laboratory rodents, but despite the homology between human and mice models, significant differences exist. For example, the human heart is more than 1000 times larger; it starts contraction at approximately day 22 after conception while in mice, this occurs at approximately day 9; and human heart septation takes around 2 months while in mice, it only takes 2 weeks [19]. It is of relevance to note that among large mammals, sheep hearts are shown to follow a similar pattern of development than human's, providing a reliable model for studies of the human CM cell cycle, especially with a translational purpose [18].

3. HEART REGENERATION CAPACITY IN HEALTH AND DISEASE

As mentioned previously, the physiological rate of CM renewal in healthy and injured hearts is scarce, which makes this tissue a one with very low tumor occurrence. Due to these characteristics, the therapeutic approach based on CM regeneration has emerged as a promising one. It is important to note that while any therapy aiming at inducing mitosis poses the risk of generating uncontrolled cell proliferation and accelerated tumor growth, the fact that the tumors of cardiac origin are rare in the population is both an advantage and a fact that researchers in the field need to assess thoroughly.

Despite the traditionally accepted evidence on limited cardiac regeneration capacity, scientists studying the adult mammalian heart have documented the presence of CM cell cycle activity after myocardial injury [20]. Although it is known that injury provides a stimulus for cell cycle activation, most cell cycle activity does not lead to mitosis.

The possibility of adult human lost CM replacement through the division of adult cardiac stem cells or specialized populations of mitotically active myocytes has been reported [21]. Beltrami *et al.* described a population of multipotent cells with the characteristics of cardiac stem cells, as they were found in the adult myocardium and were able to differentiate into the three main cardiac lineages (myocytes, smooth muscle, and endothelial cells). However, it is evident that the effect of these cells on CM replacement after injury is low and insufficient to compensate for the lost ones, but still can be the target of a therapy aimed at heart regeneration [21].

The need to achieve induction of therapeutic ventricular regeneration has guided research to the identification of factors that play relevant roles in the regulation of the cell cycle [2]. Despite the immense differences among species, the study of some lower vertebrates with a significant cardiac regenerative capacity after CM loss such as zebrafish [22], as well as neonatal mouse hearts [23] are being used as a guide to provide information on cell cycle regulation pathways [1]. Interestingly, in these species with a high regenerative capacity, a strong positive correlation exists between the number of mononucleated, diploid CMs, and the regenerative capacity of the heart, suggesting a higher proliferation capacity for mononucleated/diploid CMs [24]. It is necessary to take this data into account and revise cell cycle regulation to find therapeutic targets to be applied to the human heart.

4. REGULATION OF THE CARDIOMYOCYTE CELL CYCLE

4.1. Cardiomyocyte Cell Cycle Progression

Cell cycle progression is under the control of several cell cycle molecules and is regulated both positively, through the formation and activation of various cyclin and Cyclin-Dependent Kinase (CDK) complexes, and negatively, by CDK inhibitors (CDKIs) (Fig. **1**). There is also a variety of other proteins, such as Retinoblastoma protein (pRb), Proliferating Cell Nuclear Antigen (PCNA), members of the E2F transcription factor family, and others that can interact with, and modulate the activity of cyclin-CDK complexes [25].

The most relevant CDKs taking part at the G1/S-phase transition are CDK4 and CDK6, which bind to and regulate the activity of cyclin D family. These cyclin-CDK complexes lead to phosphorylation of Rb proteins, which modulates E2F activity, inducing gene expression and cell cycle progression [26].

Accelerated phosphorylation of pRb is mediated by the active complex of cyclin E and CDK2 during the S-phase [27].

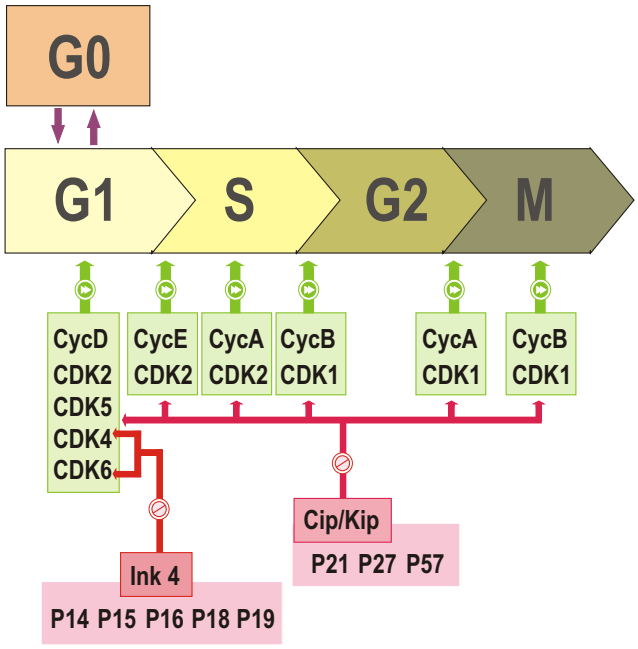

Fig. (1). Diagram showing the progression of the mammalian myocyte cell cycle and its phases. Each phase and checkpoint progression is stimulated by specific cyclin–CDK complexes (green boxes) and inhibited by two of the CDKI families: Ink4 and Cip/Kip (red boxes).

It is known that heart development is affected to a great extent by deletion of members of the cyclin-D family. Mutant cyclin D mice exhibit ventricular malformations and a hypoplastic ventricle [28]. On the other hand, in adult hearts, overexpression of cyclin D family members produces increased DNA synthesis [29].

CDK2 and CDK4 knockout mice die during the embryo state because of heart defects. This is due to the fact that CDK2 and CDK4 deletion determines hypophosphorylation of Rb, affecting E2F and its downstream targets. Double-mutant mice exhibit several defects such as a hypoplastic ventricle, dilated atria, and ventricular wall thinning [26].

In embryonic mouse hearts, it has been shown that a splice variant of cyclin D2 (D2SV) takes part in CMs cell cycle exit through the generation of D2SV micro-aggregates that sequester cell cycle promoting proteins [30].

Wafa *et al.* overexpressed D2SV in cell lines, leading to the formation of ubiquitinated protein aggregates and a decrease in cell proliferation. This supports the hypothesis that D2SV induces cell cycle inhibition by sequestering endogenous cell cycle proteins, such as cyclin D2 and CDK4, and possibly targeting them for ubiquitin-mediated protein degradation [31].

Interestingly, contrary to the established hypothesis, the fetal heart shows higher expression of cyclin D D2SV variant than the adult. As D2SV has a role as a negative regulator of CM proliferation, it probably works as a mechanism of the fetal heart to regulate cell cycle activity and optimize CM number [31].

4.2. Cardiomyocyte Cell Cycle Arrest

During early stages of gestation in rats and other mammals, CMs grow both by hyperplasia (the cell divides into two daughter cells after having duplicated the entire cell contents) and by hypertrophy (the cell increases its size without a concomitant increase in cell number), resulting in an overall increase in the cardiac mass [14, 25] (Fig. **2**).

Until recently, it was accepted that CMs "exit" the cell cycle as they become binucleated and thus terminally differentiated. It was supposed that while mononucleated cells were proliferating, binucleated cells would exit the cell cycle and no longer proliferate [18].

As Siddiqi and Sussman have warned, the expressions "withdrawal" (or exit) of the cell cycle and "cell cycle arrest", although used interchangeably, do not refer to the same phenomenon. "*Specifically, withdrawal or exit from the cell cycle implies a G0 arrest generated by nutrient and mitogen deprivation, while non-G0 arrest (G1 and G2) is characterized by the accumulation of high levels of cyclins, cyclin-dependent kinases (CDKs), and other regulating proteins that promote cellular growth"* [10, 32]. Similarly, "terminal differentiation" does not imply that the cell has irreversibly lost its capacity to divide or to display cell cycle activity. In fact, "terminally differentiated" cells may undergo hypertrophy, multinucleation and DNA repair [10, 32, 33].

Studying the cell cycle in CMs is methodologically challenging due to the fact that in these cells several processes such as hypertrophy, polyploidization and polynucleation share protein mediators and pathways with cell cycle signalling and intracellular remodelling, so proliferation studies need a cautious analysis and interpretation. For example, it is reported that distinct processes such as hyperplastic and hypertrophic growth exhibit elevated cyclin-CDK expression levels with reduced CDKIs expression, while both the adult and the pathologically hypertrophied CM show decreased levels of cyclin-CDK complexes with elevated levels of CDKIs [25] (Fig. **2**). Another issue is that in most cell types, measure of ploidy is employed to estimate the stage of the cell cycle, but in CMs cell cycle activity leads to ploidies ranging from 2N to 16N or more, so it does not provide reliable information [34].

Hypertrophy has been described as a reversible non-G0 arrest [32, 35]. But it is known that a continuous state of growth can lead a hypertrophic cell into senescence. Thus, "*Senescence is an irreversible cell cycle arrest decision point executed by a cell in response to very specific triggers"* [10].

Several reports show that certain molecular pathways that take part in CM proliferation of neonatal rat hearts resemble signalling leading to hypertrophy in the adult heart. The study of specific proteins that participate in both processes at certain time points can reveal unknown cell cycle regulation mechanisms and molecules [36, 37]. Interestingly, many regulating molecules show an expanded effect, as happens in senescent cells with an internal signalling that irreversibly arrests their cell cycle, but also affecting their environment through their secretome related to the senescence process, which reduces the regenerative potential of the surrounding myocytes as well [38, 39].

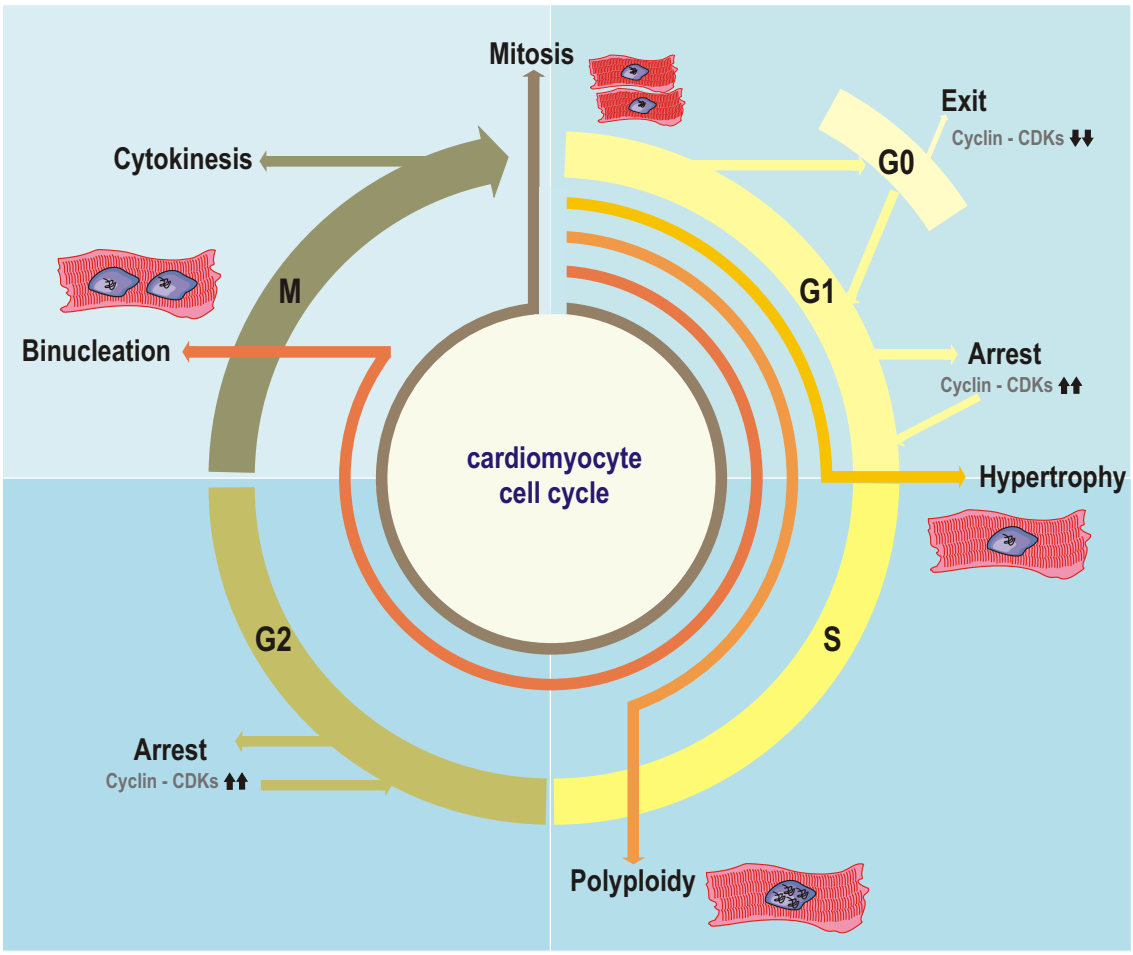

Fig. (2). Schematic diagram of physiological and pathological processes requiring myocyte cell cycle activity. In addition to cell division (mitosis), myocytes require cell cycle activity for hypertrophy, polyploidization and binucleation. The term "exit" refers to a G0 arrest due to deprivation of nutrients and low levels of cyclin-CDK complexes. The term "arrest" refers to a non-G0 arrest (G1 or G2) with high levels of cyclin-CDK complexes.

The molecular mechanisms that underlie the inability of adult CMs to re-enter the cell cycle and divide are under intense investigation. Recent reports from observations in murine CMs suggest that there are at least three different levels of regulation limiting CM proliferation: 1) epigenetic regulation involving pre-transcriptional heterochromatin-mediated gene silencing of positive cell cycle regulators [40], 2) transcriptional activation of negative cell cycle regulators [41], and 3) post-transcriptional regulation through microRNAs (miRNAs) [42].

5. CELL CYCLE REGULATION LEVELS

5.1. Epigenetics and Cardiomyocyte Cell Cycle

Epigenetics play an important role in cell determination and differentiation, because DNA methylome and histone modifications affect the condensation of chromatin and, in consequence, the possibilities of gene expression due to differential chromatin accessibility for the transcription machinery. In this sense, the epigenetic marks could contribute to the understanding of the differentiation path for CMs and the feasibility of their reprogramming. Transcription factors, enzyme chromatin modifiers, signalling molecules, among other mediators that collaborate in cell fate, participate in the epigenetic decisions. And probably, the dynamic changes that occur not only have value in the embryonic development of the cardiac cell strains, but also in their pathological aspects. Of these molecules, microRNAs (miRNAs) and long noncoding RNAs (lncRNAs) play important roles in epigenetic modifications which are addressed below (see paragraph 5.3)

Recent *in vitro* studies in mice CMs showed that the cellular plasticity of mammalian CMs is the result of epigenomic reprogramming that conditions transcriptomic alterations [43]. Some particular regulators drive cell differentiation from the nascent mesoderm to chamber-specific myocytes [44]. For instance, mice in which the sequence of the histone acetyltransferase gene *p300* was affected, displayed severe heart defects [45]. Moreover, the absence of the histone acetyltransferase MOZ induced heart morphological defects in the same animal model [46, 47].

All the evidences generated so far show that chromatin structure (including the occurrence of diverse histone species due to the activity of enzyme chromatin modifiers and the differential DNA methylome profile) is a relevant dimension for understanding the life cycle of cardiac cells and for the identification of key factors with putative therapeutic value.

5.2. Cell Cycle Regulation Pathways and Mediators

5.2.1. CDKIs Role in Cell Cycle Arrest

The CDKI complex is formed by two protein families (INK4 and Cip/Kip) that differ both in structure and function. The INK4 CDKIs include the pocket proteins p14, p15, p16, p18, and p19, which sequester CDK4 and CDK6 impeding their binding to cyclin D, and thus blocking cell cycle progression at G1 level. On the other hand, the Cip/Kip family includes p21, p27 and p57 (Fig. **1**), which inhibit CDK1/cyclin B and CDK1/cyclin A thus blocking cell cycle progression at the S/G2 and G2/M transition respectively [25]. In other words, in mice, while the INK4 CDKIs block the cell cycle close after entry, the Cip/Kip CDKIs are predominantly responsible of blocking mitotic progression [48].

As shown in Figure **1**, *in vitro* studies indicate that the Cip/Kip family of CDKIs have a broader effect than *in vivo*, since its pocket proteins interact and further inhibit the kinase activities of cyclin A–CDK2, cyclin B-CDK2, cyclin E–CDK2, cyclin D–CDK4, cyclin D-CDK2, cyclin D-CDK5, and cyclin D-CDK6 complexes, compared with the INK4 CDKIs that inhibit only CDK4 and CDK6 complexes [25].

At the expression level, it is reported that in mice the Cip/Kip family of CDKIs is undetectable during the embryonic phase, increases during the perinatal period, and peaks in adult myocytes [49]. Therefore, several publications focus on the Cip/Kip family potent inhibitors in CMs as the mediators of cell cycle arrest during postnatal stages, and also suggest a role of these proteins in the arrest occurring during development [48, 49].

In neonatal CMs, therapeutic approaches targeting p21 and p27 knockdown through short interfering RNA (siRNA) technique promoted proliferation and progression of cells into the S phase. Furthermore, the knockdown of the three CDKIs (p21, p27 and p57) induced proliferation of adult CMs, thus allowing to hypothesize that these CDKIs have an important role in CM cell cycle arrest during development [18].

5.2.2. Cip/Kip Family Mediate CM Cell Cycle Arrest

In mice, it was shown that p21 works as an inhibitor of cyclin–CDK activity at the G1/S-phase transition with a broad specificity at this checkpoint [25, 30, 50-52]. P21 works in a quaternary complex with a cyclin, a CDK and PCNA (proliferating cell nuclear antigen), a nuclear protein essential for DNA replication and repair [25, 51, 53]. It has been shown that the C-terminal domain of p21 can directly inhibit DNA replication, but not repair, by binding to and blocking PCNA activity, and the N-terminal domain of p21 binds and blocks cyclin-CDK complexes [34, 54-56]. The association of p21 with PCNA occurs only in wild-type cells and leads to cell cycle arrest at, or just prior to, the S phase [25, 51, 53]. However, it has been reported that expression of PCNA is low in the CM during the development stages of the heart, so it is unlikely that p21 acting *via* PCNA mediates the arrest in G1 phase at this time point [25].

The transcription factor E2F-1 is important for the induction of cyclin A and other S-phase genes. It has been shown that p21 can inhibit E2F-1 activity, resulting in lack of induction of S-phase genes and a blockade in the cell cycle prior to S phase [25].

In mammalian hearts, p21 mRNA was found to fluctuate along the cell cycle, showing a rise in expression at G0-phase exit and at G/M transition. From studies in human fibroblasts it has been suggested that the upregulation of p21 on exit from the G phase reduces cyclin–CDK activity in that phase and acts as a threshold that sets a level above which cyclin–CDK complexes need to accumulate for cell cycle to progress [57]. In support of this hypothesis, it was shown that the levels of both p21 mRNA and protein are elevated in quiescent and differentiated cells and overexpression of p21 caused either a delay in cell cycle progression or blocked cell division [25].

In addition, considering that in rat CMs p21 mRNA has been shown to peak at the G2/M phase, as well as on exit from G1, and that p21 can block the activity of the G2/M-phase CDK (CDK1), it is possible that p21 is critical for the arrest of adult myocytes at the G2/M phase of the cell cycle. These observations suggest that a decrease in the expression of p21 is necessary for cell cycle progression [58].

While the p21 inhibitory effects are broad, those of p27 are more specific [25, 52, 59, 60]. In mice this pocket protein has been reported to selectively inhibit complexes containing CDK2 and CDK4 and also has a weak inhibitory effect on CDK1 and CDK6. It has been observed that p27 levels are higher in quiescent cells than in proliferating cells [56, 59].

In murine CMs P57 inhibits in first place cyclin D–CDK4 (a G1-phase complex) and cyclin E/A–CDK2 (S-phase complexes) [27], and its second target is cyclin B–CDK1 complex [58]. Overexpression of p57 has been shown to cause cell cycle arrest in G1 phase [61].

Several studies in mice show that adult CMs re-enter the cell cycle. However, the percentages are extremely low (around 0.007-0.015% in adult mice) [7, 62]. While cell cycle exit is maintained in most CMs of adult hearts preventing regeneration through mitotic division of resident CMs, the molecular signalling that induces and keeps cell cycle exit is mostly unknown [48].

Analysis of mice CM cell cycle showed that mitotic division rate and expression, and protein levels of cyclin D-CDK4/6, cyclin E-CDK2, cyclin A-CDK1/2 and cyclin B-CDK1 complexes are high during early embryonic stages. As gestation advances, these levels decrease from midgestation to birth, and then they rise around P5, which induces the generation of CMs binucleation from mononucleated cells [11, 48, 63-65]. After P14 all expression and activation levels of main cyclin-CDK complexes become extremely low or undetectable, and these levels are maintained along life. A detailed study of cyclin E- and cyclin A-associated CDK complexes showed that these CDK activities decrease significantly after P5, while the CDK protein levels decrease significantly after P10 [65]. This fact suggests that there are mediators such as CDK inhibitors that block CDK activity causing cell cycle exit. In this species, expression of p21 and p27 after birth showed a peak around P5, which coincided with the stage when cyclin E-CDK and cyclin A-CDK activities start to decrease. P57 was expressed strongly in midges-

tation, the expression then decreased and could not be detected after birth (Fig. **3**) [48, 65].

In mice, studies employing immunoprecipitation of CDK2 showed that this CDK binds to p21 and p27 as well as to cyclin E and cyclin A during postnatal stages [48]. Because the association of p21 and p27 with CDKs is enhanced by the binding of these proteins with cyclins [66], it is hypothesized that p21 and p27 take part in cell cycle arrest in postnatal mice hearts through their capacity to bind cyclin E-CDK2 or cyclin A-CDK2 complexes (Fig. **1**) [48].

In reports addressing CM cell cycle in mice, it has been observed that in wild-type cells almost all bi- and mononucleated CMs exited the cell cycle at G1 phase (46 chromosomes). From the study of p21 and p27 knockout mice, Tane *et al*. showed that these CDKIs play relevant roles in CM cell cycle exit during postnatal stages, especially in the exit at G1 phase. But they found a population of p21 and p27 knockout CMs that did not exit the cell cycle at G1 and had their cell cycle arrested at G2 or entered endoreplication, suggesting that entrance to M phase was blocked in these CMs, thus indicating that mice CMs have another inhibitory system independent of p21 and p27 [48].

Transient expression of p21 and p27 is controlled in postnatal mice CMs. A recent study shows that the protein Meis1 works as a transcription factor that regulates the expression of p21 in postnatal CMs [41]. However, the mechanisms of regulation of p21 by Meis1 still need to be elucidated, as it is the regulation of p27 in CMs [67].

In rats it was described that CMs' switch from hyperplastic to hypertrophic growth takes part between post-natal days 3 and 4 and that myocyte proliferation ceases completely by about day 17. Although the rat CM shows a reduced capacity for mitotic division, it is able to have cell cycle activity and synthesize DNA if necessary, which leads to binucleation or increase in ploidy [25].

In mice primarily the CDKIs cause cell cycle arrest in the G1 phase of the cell cycle [66, 68] and this is consistent with the proposed blockade in the rat CM beyond 17 days of postnatal development (Fig. **3**). It is also known that the expression level (mRNA) of p21 fluctuates during the cell cycle, with a rise at G0-phase exit and at the G2/M transition [57, 69].

In addition, since p21 mRNA has been shown to peak at the G2/M phase as we mentioned previously, as well as on exit from G1, and that p21 can inhibit the activity of the G2/M-phase CDK (CDK1), it is possible that p21 is also critical for arrest of adult myocytes in the G2/M phase of the cell cycle in this species (Fig. **1**).

As a result of an extensive developmental study using CMs obtained from fetal (18 days of gestation), neonatal (2-day-old) and adult rats, Poolman *et al.* were unable to demonstrate expression of any of the INK4 CDKI family members, despite having detected these proteins in control cell lines [70].

Brooks *et al.* quantified p21 mRNA and protein levels and found a significant upregulation during development. The level of p27 expression was detectable, although overall expression of this CDKI appeared to be lower than that of p21 and the upregulation of p27 in cardiac myocytes during development was less than that observed for p21. In other experiments, it was shown that both p21 and p27 are expressed in CMs'nuclei [25]. P57 protein expression peaks around day 15 of fetal development in the rat and declines rapidly, being undetectable after day 19 of fetal development (Fig. **3**) [71].

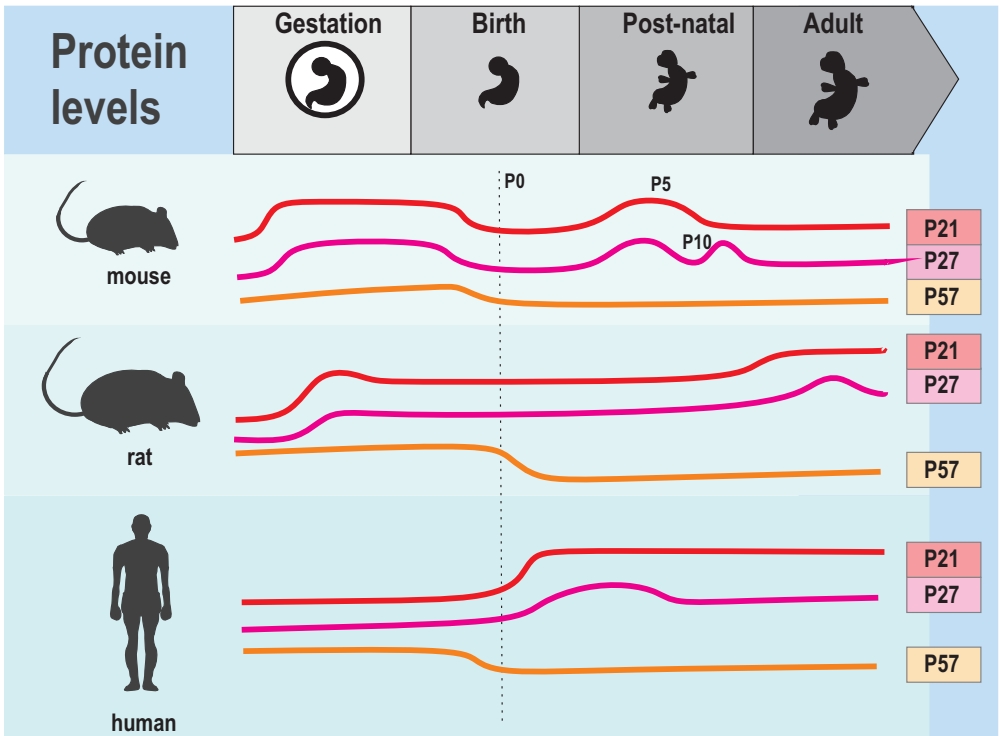

Fig. (3). Levels of expression of Cip/Kip CDKIs (p21, p27 and p57) in mice, rat and human cardiac tissue during gestation and adult life.

From the analysis of mice CMs, Flink *et al.* have reported that p21 and p27 inhibitory activities significantly increased in neonatal (2-day-old) CMs when compared to fetal (16 days of gestation) cells [72].

The availability of p27 protein is regulated by the balance between p27 translation and the rate of p27 degradation through the ubiquitin-proteasome pathway. In contrast to p27 protein, mRNA expression levels of this CDKI was found to be similar in both proliferating and quiescent cells [25, 52, 73].

The arrest of the rat myocyte cell cycle (at post-natal day 3–4) was previously reported to occur during G1 [74] and, accordingly, it was proposed that over 85% of adult cardiac myocytes are found in the G0 or G1 phases of the cell cycle [70]. However, studies using flow cytometry analysis of myocyte nuclei have suggested that a dual mechanism of myocyte cell cycle arrest exists, with a significant (15%) G2/M-phase blockade in adult myocytes [70], supporting a hypothesis proposed by Rumyantsev, which suggested that a population of myocytes begins to arrest during the prenatal stages of cardiac development [75].

During cardiac injury, p21 and p27 were shown to be downregulated [49]. Although it is not clear why this happens, it may facilitate cell cycle activity, including DNA synthesis as a compensatory response.

According to Burton *et al.* the expression levels of Cip/Kip family of CDKIs in human hearts resemble the one in rat's. P21 although detectable during fetal development, its levels were found to be higher in post-natal life. P27 was found to be increased by around 25 weeks of gestation and remained in similar levels thereafter, while p57 decreased in expression as gestation developed but contrary to the rat it was still detectable in the adult cardiac tissue. (Fig. **3**) [71].

5.2.3. Meis1

Mahmoud *et al.* identified *meis1* (myeloid ecotrophic insertion site 1) as a critical regulator of the CM cell cycle. The authors report that Meis1 is a transcription factor required for the transcriptional activation of the CDKIs p15, p16 and p21, which inhibit cyclin-CDK complexes inducing cell cycle arrest in murine CMs [41]. In the same report, they show that *meis1* deletion sufficed to extend the proliferative window of postnatal CMs. Furthermore, in the adult mouse heart, *meis1* deletion allowed CMs to advance into mitosis without affecting cardiac function. Contrarily, overexpression of *meis1* decreased neonatal CM proliferation and impeded regeneration of the neonatal heart [41].

Our group is working on the screening of novel cell cycle regulatory proteins to be further employed as targets for myocardial regeneration. Through a transcriptomic analysis followed by qPCR we have recently tested the expression levels of several proteins having a regulatory role in sheep's CM cell cycle. For the analysis, we employed three time points: fetus of less than 70 days of gestation (n=3), fetus of more than 100 days of gestation (n=3), and healthy adult sheep (n=3). The genes *meis1*, *cdkn1a* (*p21*), *cdkn2aip* (*p16*), *cdk2*, *cdk4*, *cyclin E1* and *cyclin D2* were expressed in ovine myocardium. Interestingly, we found a differential expression of *meis1*, with significant low levels of *meis1* mRNA in fetus of less than 70 days (with a heart composed predominantly of mitotic CMs), and higher levels in fetus of more than 100 days ($p<0.05$) and adults ($p<0.05$), containing mainly post-mitotic CMs. Although further detailed analysis is needed, these data suggest that in large mammalian CMs *meis1* is expressed and takes part in cell cycle regulation as it does in small laboratory rodents[1].

Recently, Kimura *et al.* [76] showed that in a mouse model, hypoxia-inducible factor-1α (Hif-1α) plays a vital role in the metabolism of long-term hematopoietic stem cells (LT-HSCs) [77-79]. This transcription factor induces a group of genes that take part in glycolysis, reduce mitochondrial activation, regulate cellular proliferation, differentiation, migration, and several growth factor receptors [80-82]. Recently, it was reported that Meis1 directly activates the transcription of Hif-1α in LT-HSCs and regulates their metabolism [78, 83].

Further studies are needed to clarify the exact regulatory mechanisms of Meis1 during CM proliferation and aging. Experiments that manipulate the cell cycle to induce CMs mitosis through the induction of *meis1*downregulation in adult CMs are important to determine whether these cells undergo dedifferentiation after *meis1* deletion before cell cycle reentry or whether CMs need to go through a partially dedifferentiated state to escape the proliferation block imposed by *meis1*.

CM proliferation is regulated by many redundant signalling pathways and involves cell cycle, transcriptional, and epigenetic regulation, as well as metabolic reprogramming. It is known that Meis1 belongs to the TALE (3-amino-acid loop extension) family of homeodomain transcription factors. From studies in mouse CMs it is known that members of the *meis* and *pbx* homeobox gene families enhance the DNA binding properties of a subset of *hox* transcription factors and regulate gene expression as heterooligomeric complexes with Hox proteins [24].

5.2.4. Tbx20

T-box (Tbx) gene family has been widely described for its role in the regulation of heart development. This family of genes encodes transcription factors with highly conserved DNA-binding regions and interaction domains for other transcription factors [84, 85]. Particularly, 6 of the 17 *tbx* gene family members are involved in the developing heart, *tbx20* being one of the most interesting to our knowledge [86]. *Tbx20* is a member of the *tbx1* subfamily of *t-box* genes which is expressed in multiple organs, including cardiac tissue, where it takes part in the regulation of CM proliferation during heart development [87].

It is known that in humans, mutations in *tbx20* generate a wide range of cardiac malformations and cardiomyopathy. In small rodents, the overexpression of *tbx20* in CMs results in

[1] Locatelli P, Giménez CS, Olea FD, *et al.* Adult ovine cardiomyocytes express the cell cycle-inhibiting gene Meis1. A potential target for cardiac regeneration based on cardiomyocyte division. World Congress of the International Society for Heart Research; 2016 April 18-21; Buenos Aires, Argentina. http://www.jmmc-online.com/article/S0022-2828(16)30343-1/abstract.

the activation of pro-mitotic pathways such as BMP2/pSmad1/5/8 and PI3K/AKT/GSK3β/β-catenin signalling [88]. Xiang *et al.* reported that in adult mouse CMs, overexpression of *tbx20* facilitates mitotic division through the repression of several cell cycle inhibitory genes such as *p21*, *meis1* and *btg2* and maintains cardiac function after acute myocardial infarction [89].

5.2.5. Hippo Pathway Proteins

In the last years, the Hippo pathway has generated high expectations to understand the mechanism and regulation of organ development and size. This pathway represents a link between the signalling in the plasma membrane and the regulation of gene expression in the nucleus with a great number of participating molecules already described [90]. Particularly, the Hippo pathway is activated as part of the adaptative response during heart failure development, as the protein members of the pathway are shown to be upregulated in human heart failure. Leach *et al.* showed that the knockdown of the Hippo pathway member Salvador (*salv)* after myocardial infarction improves cardiac function and reduces scar size in mice. The authors suggest that this pathway maintains the inhibition over a program that otherwise would induce compensatory CM proliferation and salvage through the expression of cell survival genes [91]. Another recent publication by the same group linked the observations on Hippo pathway to cardiomyocyte extracellular matrix interactions through the Dystrophin Glycoprotein Complex (DCG). This transmembrane protein complex connects the cytoskeleton to the extracellular matrix. One of the members of this complex, dystroglycan1 (DAG1) was shown to specifically bind to the Hippo pathway member Yap to block CM proliferation in mice [92]. These results confirm not only that the Hippo pathway is important in CM proliferation but also that it is imperative to understand the interaction with other cellular components and pathways.

5.2.6. PPARδ Receptor

The peroxisome proliferator-activated receptor delta (PPARδ, also referred to as PPARβ), is part of the PPAR family of the nuclear receptors superfamily implicated in the transcriptional control of different physiological and pathological processes through the binding to specific DNA elements [93]. Even though PPARδ is activated by fatty acids and plays a relevant role in most metabolically active tissues, recent publications relate it to different heart disturbances. It has been shown that PPARδ deletion in mice conduces to cardiac hypertrophy, fibrosis and perturbs fatty acid oxidation [94]. In addition, the cardiac-specific overexpression of this receptor in mice is important to protect the heart from ischemia and reperfusion injury [95, 96]. Particularly, Magadum *et al.* identified the PPARδ as an inductor of cardiomyocyte proliferation and achieved rat CM proliferation after myocardial infarction through activation of PPARδ and further PDK1/ p308Akt/GSK3β/β-catenin-pathway. In view of these results, the authors suggest PPARδ as a new and promising target to revert cardiomyocyte loss resulting from different cardiac pathologies [97].

5.3. Micro RNAs (Post-transcriptional Regulation)

MicroRNAs (miRNAs) have generated much interest as regulators of endogenous gene expression by targeting mRNA degradation. Identification of miRNAs involved in different heart diseases has led to the study of the regulatory roles for these short RNAs during different cell processes such as CM differentiation, epigenomic remodelling, CM cell cycle, and hypertrophic cell growth. In mice, a combination of 4 miRNAs (miR-1, miR-133a, miR-208a and miR499) reprogrammed fibroblasts into cardiomyocyte-like cells [98]. Two miRNAs (miR-1 and miR-499) have been shown to reduce the proliferation rate and enhance differentiation into CMs of human cardiomyocyte progenitor cells (hCMPCs) [99, 100].

In cardiac development studies, multiple microRNA families have been analyzed, and some were found to favour CM proliferation. For example, in mouse hearts miR-133a knockout induced CM division through elevation of the pro-mitotic protein cyclin D2 [101], miR-199a-3p and miR-590-3p induced cell cycle progression and subsequent cytokinesis of adult and neonatal mouse CMs after experimental myocardial infarction [102], and knockdown of the miR-15 family was shown to increase mitosis of CMs [103]. The blockade of the tumor suppressor PTEN through the overexpression of miR-17-92 induced myocyte proliferation in small rodents as well as the overexpression of miR-499 and miR302/367 through other molecular pathways not yet elucidated [104].

Pandey *et al.* identified three miRNAs (miR-548c-3p, miR-509-3p, and miR-23b-3p) that target the 3' UTR of *meis1* gene and induce CM proliferation through the post-transcriptional inhibition of the anti-mitotic gene *meis1* [105].

Chen *et al.* showed that miR-134 was significantly downregulated in congenital heart disease, and reported that the overexpression of this miRNA reduced hCMPCs proliferation capacity, while the downregulation enhanced their proliferation rate, through the modification in the expression levels of cell cycle mediators. Further analysis by the same group identified the gene *meis2* as the target of miR-134. They showed that in mice the overexpression of *meis2* antagonized the effect of miR-134 on hCMPCs proliferation, showing that contrary to *meis1*, *meis2* is a pro-mitotic gene [106].

These levels of regulation are being targeted employing different strategies, using gene therapy through different vectors and even cells and diverse type of scaffolds as carriers of the genes of interest.

Finally, it is important to mention that there are other noncoding RNAs, such as long noncoding RNA (lncRNA) that have been shown to modulate and control gene programs involved in proliferation and differentiation of cardiomyocytes [107, 108]. As an example, the lncRNA Braveheart (Bvht, AK143260) represents an important factor for cardiac lineage specification and differentiation in mammals [109]. Additionally, a recent publication shows that the levels of lncRNA CAREL were upregulated in non-proliferating neonatal mice CMs, and that overexpression of this lncRNA in mice with heart injury reduced CM proliferation capacity and impeded regeneration. Further analysis showed that CAREL acts as an endogenous ribonucleic acid competitor of miR-296, a microRNA that targets Trp53inp1 and Itm2a.

When CAREL is overexpressed Trp53inp1 and itm2a are derepressed and CM proliferation decreases. In fact, the decline in cardiac regenerative capacity of CAREL transgenic mice was rescued by miR-296 [110].

5.4. Metabolic Factors and Metabolism in Cell Cycle Regulation

It is known that during fetal growth the mammalian heart is under a relative hypoxia, thus the CMs metabolism is based on anaerobic glycolysis. While under this condition CMs actively replicate, after birth the environment offers oxygen, leading to a switch towards oxidative metabolism, which coincides with CMs exit from the cell cycle [111].

Through the generation of human pluripotent stem cell-derived cardiac organoids, Mills *et al.* discovered that in cardiomyocytes the switch from anerobic glycolytic metabolism to fatty acid oxidation induced the exit from the cell cycle, the maturation of the cell and a lack of response to a mitogenic stimulus. Furthermore, after the switch to fatty acid oxidation, several important proliferation pathways, such as β-catenin and YAP1 were found to be repressed, and proliferation capacity was recovered when these pathways were simultaneously activated [112].

In rabbit hearts, the co-existence of cardiomyocyte oxidative metabolism and a reduction in cell division rate supports the hypothesis that the switch from anaerobic to aerobic metabolism is linked to the decrease in proliferation capacity [113]. This can be explained through the observations by Puente *et al.*, who showed that in mice hearts the switch to oxidative metabolism increases reactive oxygen species generation and DNA damage, leading to cell cycle arrest, which, according to the authors, may be an adaptive response to prevent the accumulation of damaged DNA in adult CMs [114].

As a whole, these reports disclose a mechanism that leads to CM cell cycle arrest mediated by the switch in metabolic pathways of the CM. As it is hypothesized that this may work as a protection preventing replication of cells with a damaged DNA, attenuating the oxidative stress mediated by the mitochondria in adult CMs may be important in therapies aimed at regenerating the heart. Indeed, this seems to be the underlying mechanism in recent works showing that hypoxia induces regeneration in murine adult CMs [115].

6. THERAPIES AIMED AT MODIFYING CELL CYCLE REGULATION

Studies on the cardiomyocyte cell cycle and its mechanisms of regulation throughout the life of mammals introduced new perspectives and proposed new possible targets for the design and development of therapeutic strategies based on gene and cell therapy and employing different scaffolds as a means of improving the delivery of the therapeutic agent. As mentioned, achieving that remnant viable cardiomyocytes can be divided and functionally integrated to the cardiac syncytium after an injury could result in a decrease in the whole organic damage and in a better perspective of health towards the future. In this sense, interesting and promising approaches in development or in clinical trials have been reviewed from the current literature [116-118].

Particularly, a well-evaluated strategy was to deliver genes that express factors that promote angiogenesis and cardiomyogenesis in pig and sheep models of ischemic heart disease, such as VEGF (vascular endothelial growth factor), first as naked DNA [119-122] and then using viral vectors [123, 124]. In view of the difficulty of transfecting cardiomyocytes *in vivo* with naked DNA, which led to the use of milligrams of the therapeutic agent [125], *in vivo* virus-based strategies and *ex vivo* gene therapy strategies (including, for example, prior *in vitro* transfection of mesenchymal stromal cells) [126] can be considered as the most successful approaches. In turn, adeno-associated virus has become the viral vector with probably better projection, in comparison with adenoviral and lentiviral vectors, based on its simplicity, transduction efficiency and less inflammatory response [127]. In any case, the evidences about the expression of VEGF by cell or gene therapy in different models show that this intervention induces myocardial collateral vessel development, inhibits the apoptosis of myocardial cells, and inhibits ventricular remodelling overall [128]. Other similar factors were and continue to be studied as active agents of cardiac therapies such as FGF (fibroblast growth factor) [129] and HGF (hepatocyte growth factor) [130]. Besides, SERCA2a (sarcoplasmic reticulum calcium ATPase 2a) [131] and HIF-1α (hypoxia-inducible factor-1alpha) [132, 133] have proven to be interesting ingredients to treat heart injuries by gene therapy, due to their role as beneficial factors in improving cardiac function and reducing arrhythmias in heart failure, and for coordinating adaptive responses to hypoxia at the cellular level, respectively.

On the other hand, new strategies based on DNAs that express shRNAs (short hairpin RNAs) as therapeutical sequences to produce knockdown of key players in cell arrest have begun to be assessed in mice [134]. For example, the inhibition of p16 (a CDKI from the INK4 family) by recombinant lentiviruses that express a specific shRNA induced antioxidant effects and extended lifespan of aging hCPCs (human cardiac stem/progenitor cell), enhancing the effectiveness of the use of autologous hCPC therapy for repair of infarcted myocardium [135]. These strategies could also be addressed through the controlled and direct administration of siRNAs (small interfering RNAs), although for this it is necessary to develop efficient formulations with a preference for cardiac tissue that can be administered chronically, or to develop controlled expulsion pumps. While this does not happen, a shRNA producing-gene (and in consequence a gene therapy approach) would be a better alternative for facilitating a higher and longer dose of this kind of active ingredient. Interestingly, a new preclinical alternative using cocktails of genes (FoxM1, Id1, and Jnk3-shRNA) applied on mice induced cardiomyocytes to re-enter the cell cycle and complete mitosis and cytokinesis [136]. This suggests that the sum of active ingredients (proteins and shRNAs in this case), as in other pharmacological strategies, may stimulate a synergism and, consequently, achieve superior therapeutic effects. In this regard, considering the epigenome of cardiac cells as another therapeutic set of targets using drugs [137, 138] and/or by gene therapy applying CRISPR/Cas technology [139] can also help achieve greater therapeutic efficacy.

There is also a great interest in developing scaffolds based on different materials that can aid the present therapies to engraft and have a longer therapeutic effect in the myocardium [140, 141].

Despite numerous successfully preclinical developments in gene therapy impacting the cardiomyocyte cell cycle the results derived from the subsequent clinical trials, many of them with negative outcomes in phase II and III [117], reveal that there are still important problems to be overcome such as timing and routes of inoculation, adequate doses to achieve considerable rates of *in vivo* transduction and temporary expression windows to ensure lasting phenotypic changes, among others.

In view of this and the complexity of cardiac tissue and its associated pathologies, not only the proposal of new therapeutic sequences are necessary and will be welcome [142], but it is also crucial to increase the evaluation of new vectors (from both chemical and viral origin), routes of administration that achieve better and significant results in damaged human hearts.

Furthermore, it is important to mention that relevant cell cycle-information has been obtained recently from the study of cardiomyocytes derived from human induced pluripotent stem cells (iPSC-CM), which also provide the opportunity to obtain novel pathways with possible application in cardiac regeneration therapies. iPSC can be obtained almost unlimitedly from a minimally invasive biopsy of the skin or a blood sample and they can be reprogrammed to immature CM [143].This technology has allowed to importantly decrease the gap between human and small laboratory animals cardiomyocytes [144, 145] and has provided updated information on the regulation of cardiac cell cycle. For example, iPSC-CM have been recently used by Diez-Cuñado *et al.* to screen a whole genome collection of human miRNAs and elucidate how those miRNAs suppress different components of the previously mentioned Hippo pathway and activate the Hippo transcriptional effector YAP to sustain the proliferative state of immature cardiomyocytes [146]. However, given that early stage iPSC-CM resemble more fetal CM than adult ones, the maximum potential of iPSC-CM lies in the fact that they recapitulate the phenomena leading to the arrest of the cell cycle and the development of ultrastructures of adult cardiomyocytes and this, in turn, provides valuable information about this process in human CMs. The major differences with adult CM are the smaller size, rounded shape, absence of components of the contractile machinery, polyploidy and the number of nuclei per cell [147, 148]. Particularly, a recent work by González-Rosa *et al.* has provided important evidence of the role of polyploidy in reducing the proliferative capacity and regenerative potential in response to injury in zebrafish cardiomyocytes [149]. Related to this work, a next step should be to elucidate the intra and extracellular pathways that modulate the transition of the increase in ploidy in human iPSC-CM given the evolutive differences between zebrafish and human. However, the most important challenge will be to recreate the modulating environments that trigger these changes at the genetic level.

7. PERSPECTIVES AND CONCLUSIONS

The traditional view of the adult heart as an essentially postmitotic organ has changed substantially over the past years. It is now widely accepted that the heart has the ability to undergo a certain degree of renewal, although its magnitude, signalling and the source of the cells that originate new CMs are still unclear. They might originate from mononucleated CMs, from CMs that have undergone a dedifferentiation process or from one of the several populations of described cardiac stem cells [21, 62].

Although there is evidence that in large mammals, including humans, CM cell cycle activity increases after injury, the rate of cell proliferation is much too low to effectively replace the lost myocytes. Instead, it mostly generates hypertrophy, polinucleation and polyploidization. Hence, there is a great interest in identifying new cell cycle regulatory proteins, and disclosing how they interact to yield regeneration. Although results from laboratory rodents have provided essential knowledge and advancement in this field, it is mandatory to confirm those findings in large mammals closer to the human. Furthermore, although useful as an initial approach, commercially available left ventricle-derived cell lines or even fetal or neonatal CM cultures are not the ideal systems to assess cell cycle routes and mediators, as they usually undergo modifications to induce indefinite cell division.

Another important issue is the assessment of transcriptomic studies focused on proliferation and cell cycle regulators specifically in CMs, as most of the published data on the topic come from the processing and disruption of whole cardiac tissue. As it is extremely complex to separate CMs from the rest of the cells conforming the cardiac tissue without altering the expression profile, most published studies either provide information resulting from the expression analysis of mRNA from whole heart, or they separate CMs following enzymatic protocols which take several steps of tissue disaggregation, at the risk of modifying the mRNA pool of the cells.

The fact that the mediators of cell cycle regulation and other processes requiring cell cycle activity such as hypertrophy and endoreplication are shared, contributes to the complexity of cell cycle assessment. The heterogeneous results of studies aimed at quantifying CM proliferation can be due, at least in part, to misinterpretation of cell cycle induction experiments. In order to assess for CM proliferation it is necessary to use several specific markers (such as Phosphohistone 3 to demonstrate karyokinesis and Aurora B kinase for cytokinesis) that can provide solid evidence that all the phases of mitosis are complete [34]. As it has been suggested that CMs need to dedifferentiate before cell division, evidence of myofibril disassembly is to be provided as well [150].

Although much interest and effort has been put to elucidate cell cycle regulation and several *in vivo* studies have used these data to develop either a gene or cell therapy to induce CM proliferation, therapeutic cardiac regeneration has not yet been achieved. This may be in part due to the complexity and tight regulation of the cell cycle, especially in larger organisms.

When designing approaches to induce mitosis, it is of key relevance the degree of control reached over the therapeutic intervention. It is important to note that whereas the myocardium, as well as other tissues such as central nervous system, have a very low incidence of tumors, it is possible that when testing novel therapies an uncontrolled proliferation is induced, thus increasing cancer risk. In this way, the *in vivo* protocols involving the manipulation of the cell cycle must have a close surveillance not only of the heart, but of the whole organism, for the prevention of tumor generation.

Finally, it should be taken into account that CM regeneration and the *in vivo* induction of heart function recovery is much more complex than it seems. The heart architecture should be recreated in a physiological manner in order to achieve significant and long-lasting improvements and to prevent future complications such as arrhythmias or ischemia due to deficient vascularization. In other words, newly generated myocytes must be accompanied by adequate microvascular proliferation and proper extracellular matrix remodelling to match their metabolic and mechanical requirements.

In conclusion, for diseases characterized by loss of contractile tissue, it is sound to consider that inducing the resident CMs to advance into mitosis and cytokinesis is the most physiologic approach to achieve myocardial regeneration with improved ventricular function, on account that it guarantees normal electromechanical coupling within the myocardial syncytium. However, although considerable knowledge on the cardiac myocyte cell cycle has been achieved, new studies particularly addressing adult CM cell cycle regulation, systems of delivery for gene and cell therapy and how to recreate the CM niche in large mammalian models are needed to disclose potential targets for therapeutical heart regeneration.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

ACKNOWLEDGEMENTS

This work was supported by the René Baron Foundation of Argentina and the National Agency for the Promotion of Science and Technology (ANPCyT) of Argentina (grant: PICT 2014-3087).

BIBLIOGRAPHY

[1] Sharma A, Zhang Y, Wu SM. Harnessing the induction of cardiomyocyte proliferation for cardiac regenerative medicine. Curr Treat Options Cardiovasc Med 2015;17(10): 404.

[2] Zebrowski DC, Becker R, Engel FB. Towards regenerating the mammalian heart: Challenges in evaluating experimentally induced adult mammalian cardiomyocyte proliferation. Am J Physiol Heart CircPhysiol 2016; 310(9): H1045-54.

[3] Laflamme MA, Murry CE. Heart regeneration. Nature 2011; 473(7347): 326-35.

[4] Bergmann O, Bhardwaj RD, Bernard S, *et al.* Evidence for cardiomyocyte renewal in humans. Science 2009; 324(5923): 98-102.

[5] Bergmann O, Zdunek S, Felker A, *et al.* Dynamics of cell generation and turnover in the human heart. Cell 2015; 161: 1566-75.

[6] Kajstura J, Urbanek K, Perl S, *et al.* Cardiomyogenesis in the adult human heart. Circ Res 2010; 107: 305-15.

[7] Mollova M, Bersell K, Walsh S, *et al.* Cardiomyocyte proliferation contributes to heart growth in young humans. Proc Natl Acad Sci USA 2013; 110(4): 1446-51.

[8] Naqvi N, Li M, Calvert JW, *et al.* A proliferative burst during preadolescence establishes the final cardiomyocyte number. Cell 2014; 157(4): 795-807.

[9] Sharma A,Wu SM. Members only: hypoxia-induced cell-cycle activation in cardiomyocytes. Cell Metab 2015;22(3): 365-66.

[10] Siddiqi S, Sussman MA. The heart: mostly postmitotic or mostly premitotic? myocyte cell cycle, senescence, and quiescence. Canadian Journal of Cardiology 2014; 30(11): 1270-78.

[11] Soonpaa, MH, Kim KK, Pajak L, *et al.* Cardiomyocyte DNA synthesis and binucleation during murine development. Am J Physiol 1996; 271(5Pt2): H2183-89.

[12] Aguirre A, Montserrat N, Zacchigna S, *et al. In vivo* activation of a conserved microRNA program induces mammalian heart regeneration. Cell Stem Cell 2014; 15(5): 589-604.

[13] Alkass K, Panula J, Westman M, *et al.* No evidence for cardiomyocyte number expansion in preadolescent mice. Cell 2015; 163(4): 1026-36.

[14] Li F, Wang X, Capasso JM, *et al.* Rapid transition of cardiac myocytes from hyperplasia to hypertrophy during postnatal development. J Mol Cell Cardiol 1996; 28(8): 1737-46.

[15] Walsh S, Pontén A, Fleischmann BK, *et al.* Cardiomyocyte cell cycle control and growth estimation *in vivo*-An analysis based on cardiomyocyte nuclei. Cardiovasc Res 2010; 86(3): 365-73.

[16] Kim HD, Kim DJ, Lee IJ, *et al.* Human fetal heart development after mid-term: morphometry and ultrastructural study. J Mol Cell Cardiol 1992; 24(9): 949-65.

[17] Kou CY, Lau SL, Au KW, *et al.* Epigenetic regulation of neonatal cardiomyocytes differentiation. Biochem Biophys Res Commun 2010; 400(2): 278-83.

[18] Paradis AN, Gay MS, Zhang L, *et al.* Binucleation of cardiomyocytes: the transition from a proliferative to a terminally differentiated state. Drug Discov Today 2014; 19(5): 602-09.

[19] Wessels A, Sedmera D. Developmental anatomy of the heart: a tale of mice and man. Physiol Genomics 2003; 15(3): 165-76.

[20] Rumyantsev PP. Interrelations of the proliferation and differentiation processes during cardiac myogenesis and regeneration. Int Rev Cytol 1977; 51: 186-273.

[21] Beltrami AP, Barlucchi L, Torella D, *et al.* Adult cardiac stem cells are multipotent and support myocardial regeneration. Cell 2003; 114(6): 763-76.

[22] Poss KD, Wilson LG, Keating MT, *et al.* Heart regeneration in zebrafish. Science 2002; 298(5601): 2188-190.

[23] Porrello ER, Mahmoud A, Simpson E, *et al.* Transient regenerative potential of the neonatal mouse heart. Science 2011; 331(6020): 1078-80.

[24] Yuan X, Braun T. An unexpected switch: regulation of cardiomyocyte proliferation by the homeobox gene meis1. Circ Res 2013; 113(3): 245-48.

[25] Brooks G, Poolman RA, Li JM, *et al.* Arresting developments in the cardiac myocyte cell cycle: role of cyclin-dependent kinase inhibitors. Cardiovasc Res 1998; 39(2): 301-11.

[26] Berthet C, Kaldis P. Cdk2 and Cdk4 cooperatively control the expression of Cdc2. Cell Div 2006; 1: 10.

[27] Woo RA, Poon RY. Cyclin-Dependent kinases and S phase control in mammalian cells. Cell Cycle 2003; 2(4): 316-24.

[28] Ciemerych MA, Kenney AM, Sicinska E, *et al.* Development of mice expressing a single D-type cyclin. Genes Dev 2002; 16(24): 3277-89.

[29] Diehl JA, Sherr CJ. A dominant-negative cyclin D1 mutant prevents nuclear import of cyclin-dependent kinase 4 (CDK4) and its phosphorylation by CDK-activating kinase. Mol Cell Biol 1997; 17(12): 7362-74.

[30] Sun Q, Zhang F, Wafa K, *et al.* A splice variant of cyclin D2 regulates cardiomyocyte cell cycle through a novel protein aggregation pathway. J Cell Sci 2009; 122(Pt10): 1563-73.

[31] Wafa K, MacLean J, Zhang F, *et al.* Characterization of growth suppressive functions of a splice variant of cyclin D2. PLoS One 2013; 8(1): e53503.

[32] Blagosklonny MV. Cell senescence: hypertrophic arrest beyond the restriction point. J Cell Physiol 2006; 209(3): 592-97.
[33] Crescenzi M, Soddu S, Tato F, *et al.* Mitotic cycle reactivation in terminally differentiated cells by adenovirus infection. J Cell Physiol 1995; 162(1): 26-35.
[34] Leone M, Magadum A, Engel FB, *et al.* Cardiomyocyte proliferation in cardiac development and regeneration: a guide to methodologies and interpretations. Am J Physiol Heart Circ Physiol 2015; 309(8): H1237-50.
[35] Blagosklonny MV. Cell cycle arrest is not senescence. Aging (Albany NY) 2011; 3(2): 94-101.
[36] Li F, Wang X, Gerdes AM, *et al.* Formation of binucleated cardiac myocytes in rat heart: II. cytoskeletal organisation. J Mol Cell Cardiol 1997; 29(6): 1553-65.
[37] Clubb FJ, Bishop SP. Formation of binucleated myocardial cells in the neonatal rat. An index for growth hypertrophy. Lab Invest 1984; 50(5): 571-77.
[38] Campisi J. Cell biology: The beginning of the end. Nature 2013: 505; 35-36.
[39] Campisi J. Aging, cellular senescence, and cancer. Annu Rev Physiol 2013; 75: 685-705.
[40] Sdek P, Zhao P, Wang Y, *et al.* Rb and p130 control cell cycle gene silencing to maintain the postmitotic phenotype in cardiac myocytes. J Cell Biol 2011; 194(3): 407-23.
[41] Mahmoud AI, Kocabas F, Muralidhar SA, *et al.* Meis1 regulates postnatal cardiomyocyte cell cycle arrest. Nature 2013; 497(7448): 249-53.
[42] Porrello ER, Mahmoud A, Simpson E, *et al.* Regulation of neonatal and adult mammalian heart regeneration by the miR-15 family. Proc Natl Acad Sci USA 2013; 110(1): 187-192.
[43] Zhang Y, Zhong JF, Qiu H, *et al.* Epigenomic reprogramming of adult cardiomyocyte-derived cardiac progenitor cells. Sci. Rep 2015; 5: 17686.
[44] Paige SL, Plonowska K, Xu A, *et al.* Molecular regulation of cardiomyocyte differentiation. Circ Res 2015; 116(2): 341-53.
[45] Shikama N, Lutz W, Kretzschmar R, *et al.* Essential function of p300 acetyltransferase activity in heart, lung and small intestine formation. EMBO J 2003; 22(19): 5175-85.
[46] Voss AK, Vanyai HK, Collin C, *et al.* MOZ regulates the Tbx1 locus, and Moz mutation partially phenocopies DiGeorge syndrome. Dev Cell 2012; 23(3): 652-63.
[47] McElhinney DB, Driscoll DA, Levin ER, *et al.* Chromosome 22q11 deletion in patients with ventricular septal defect: frequency and associated cardiovascular anomalies. Pediatrics 2003; 112(6 Pt 1): e472.
[48] Tane S, Ikenishi A, Okayama H, *et al.* CDK inhibitors, p21Cip1 and p27Kip1, participate in cell cycle exit of mammalian cardiomyocytes. Biochem Biophys Res Commun 2014; 443(3): 1105-09.
[49] Ahuja P, Sdek P, Maclellan WR, *et al.* Cardiac myocyte cell cycle control in development, disease , and regeneration. Physiol Rev 2007; 87(2): 521-44.
[50] Wade Harper J, Adami GR, Wei N, *et al.* The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases. Cell 1993; 75(4): 805-16.
[51] Xiong Y, Hannon GJ, Zhang H, *et al.* P21 is a universal inhibitor of cyclin kinases. Nature 1993; 366(6456): 701-04.
[52] Polyak K, Lee MH, Erdjument-Bromage H, *et al.* Cloning of p27Kip1, a cyclin-dependent kinase inhibitor and a potential mediator of extracellular antimitogenic signals. Cell 1994; 78(1): 59-66.
[53] Xiong Y, Zhang H, Beach D, *et al.* Subunit rearrangement of the cyclin-dependent kinases is associated with cellular transformation. Genes Dev 1993; 7(8): 1572-83.
[54] Luo Y, Hurwitz J, Massagué J. Cell-cycle inhibition by independent CDK and PCNA binding domains in p21Cip1. Nature 1995; 375(6527): 159-61.
[55] Chen J, Jackson PK, Kirschner MW, *et al.* Separate domains of p21 involved in the inhibition of Cdk kinase and PCNA. Nature 1995; 374(6520): 386-88.
[56] Li R, Waga S, Hannon GJ, *et al.* Differential effects by the p21 CDK inhibitor on PCNA-dependent DNA replication and repair. Nature 1994; 371: 534-37.
[57] Noda A, Ning Y, Venable SF, *et al.* Cloning of senescent cell-derived inhibitors of DNA synthesis using an expression screen. Exp Cell Res 1994; 211(1): 90-98.
[58] Poolman RA, Gilchrist R, Brooks G. Cell cycle profiles and expressions of p21CIP1 AND P27KIP1 during myocyte development. Int J Cardiol 1998; 67(2): 133-42.
[59] Toyoshima H, Hunter T. p27, a novel inhibitor of G1 cyclin-Cdk protein kinase activity, is related to p21. Cell 1994; 78(1): 67-74.
[60] Hengst L, Dulic V, Slingerland JM, *et al.* A cell cycle-regulated inhibitor of cyclin-dependent kinases.Proc Natl Acad Sci USA 1994; 91(12): 5291-95.
[61] Lee MH, Reynisdottir I, Massague J, *et al.* Cloning of p57(KIP2), a cyclin-dependent kinase inhibitor with unique domain structure and tissue distribution. Genes Dev 1995; 9(6): 639-49.
[62] Senyo SE, Steinhauser ML, Pizzimenti CL, *et al.* Mammalian heart renewal by pre-existing cardiomyocytes. Nature 2013; 493(7432): 433-36.
[63] Erokhina EL. Proliferation dynamics of cellular elements in the differentiating mouse myocardium. Tsitologiia 1968; 10(11): 1391-409.
[64] Toyoda M, Shirato H, Nakajima K, *et al.* Jumonji downregulates cardiac cell proliferation by repressing cyclin D1 expression. Dev Cell 2003; 5(1): 85-97.
[65] Ikenishi A, Okayama H, Iwamoto N, *et al.* Cell cycle regulation in mouse heart during embryonic and postnatal stages. Dev Growth Differ 2012; 54(8): 731-38.
[66] Harper JW, Elledge SJ, Keyomarsi K, *et al.* Inhibition of cyclin-dependent kinases by p21. Mol Biol Cell 1995; 6(4): 387-400.
[67] Nakanishi T, Markwald RR, Baldwin HS, *et al.* Etiology and morphogenesis of congenital heart disease from gene function and cellular interaction to morphology, Chapter 11.Tokyo: Springer; 2016.
[68] Medema RH, Herrera RE, Lam F, *et al.* Growth suppression by p16ink4 requires functional retinoblastoma protein. Proc Natl Acad Sci USA 1995; 92(14): 6289-93.
[69] Li Y, Jenkins CW, Nichols MA, *et al.* Cell cycle expression and p53 regulation of the cyclin-dependent kinase inhibitor p21. Oncogene 1994; 9: 2261-68.
[70] Poolman RA, Brooks G. Expression of CIP/KIP family of cyclin-dependent kinase inhibitors during cardiac development. Circulation 1996; 94: A0909.
[71] Burton PBJ, Yacoub MH, Barton PJR, *et al.* Cyclin-dependent kinase inhibitor expression in human heart failure. A comparison with fetal development. European Heart Journal 1999; 20: 604–11.
[72] Flink IL, Oana S, Bahl JJ, *et al.* Terminal differentiation in cardiomyocytes results from hypophosphorylation of retinoblastoma protein by induction of cyclin-dependent kinase inhibitory activities. Circulation 1996; 94: A2745
[73] Halevy O, Novitch BG, Spicer DB, *et al.* Correlation of terminal cell cycle arrest of skeletal muscle with induction of p21 by MyoD. Science 1995; 267(5200): 1018-21.
[74] Capasso JM, Bruno S, Cheng W, *et al.* Ventricular loading is coupled with DNA synthesis in adult cardiac myocytes after acute and chronic myocardial infarction in rats. Circ Res 1992; 71(6): 1379-89.
[75] Rumyantsev PP. Proliferative activity of cardiomyocyte and polyploidization of their nuclei during myocardial hypertrophy of nonprimates In: growth and hyperplasia of cardiac muscle cells, Carlson BM, editor. pp. 231–238. Soviet Medical Reviews Supplement Cardiology.Vol 3. Harwood Academic Press, 1991.
[76] Kimura W, Xiao F, Canseco DC, *et al.* Hypoxia fate mapping identifies cycling cardiomyocytes in the adult heart. Nature 2015; 523(7559): 226-30.
[77] Simsek T, Kocabas F, Zheng J, *et al.* The distinct metabolic profile of hematopoietic stem cells reflects their location in a hypoxic niche. Cell Stem Cell 2010; 7(3): 380-90.
[78] Takubo K, Goda N, Yamada W, *et al.* Regulation of the HIF-1alpha level is essential for hematopoietic stem cells-SOM. Cell Stem Cell 2010; 7(3): 391-402.
[79] Takubo K, Nagamatsu G, Kobayashi CI, *et al.* Regulation of glycolysis by Pdk functions as a metabolic checkpoint for cell cycle quiescence in hematopoietic stem cells. Cell Stem Cell 2013; 12(1): 49-61.
[80] Bartrons R, Caro J. Hypoxia, glucose metabolism and the Warburg's effect. J Bioenerg Biomembr 2007; 39(3): 223-29.
[81] Iyer NV, Kotch LE, Agani F, *et al.* Cellular and developmental control of O2 homeostasis by hypoxia-inducible factor 1 alpha. Genes Dev 1998; 12(2): 149-62.

[82] Wheaton WW, Chandel NS. Hypoxia. 2. Hypoxia regulates cellular metabolism. Am J Physiol Cell Physiol 2011; 300(3): C385-93.
[83] Kimura W, Muralidhar S, Canseco DC, *et al.* Redox signaling in cardiac renewal. antioxid. Antioxid Redox Signal 2014; 21(11): 1660-73.
[84] Vincent SD, Buckingham ME. How to make a heart: The origin and regulation of cardiac progenitor cells. Curr Top Dev Biol 2010; 90: 1-41.
[85] Tada M, Smith JC. T-targets: clues to understanding the functions of T-box proteins. Dev Growth Differ 2001; 43: 1-11.
[86] Plageman TF Jr, Yutzey KE. T-box genes and heart development: Putting the "T" in heart. Dev Dyn 2005; 232: 11-20.
[87] Greulich F, Rudat C, Kispert A, *et al.* Mechanisms of T-box gene function in the developing heart. Cardiovasc Res 2011; 91: 212-22.
[88] Chakraborty S, Sengupta A, Yutzey KE, *et al.* Tbx20 promotes cardiomyocyte proliferation and persistence of fetal characteristics in adult mouse hearts. J Mol Cell Cardiol 2013; 62: 203-13.
[89] Xiang FL, Guo M, Yutzey KE, *et al.* Overexpression of Tbx20 in adult cardiomyocytes promotes proliferation and improves cardiac function after myocardial infarction. Circulation 2016; 133(11): 1081-92.
[90] Halder G, Johnson RL. Hippo signaling: growth control and beyond. Development 2011; 138: 9-22.
[91] Leach JP, Heallen T, Zhang M, *et al.* Hippo pathway deficiency reverses systolic heart failure after infarction. Nature 2017; 550(7675): 260-64.
[92] Morikawa Y, Heallen T, Leach J, *et al.* Dystrophin-glycoprotein complex sequesters yap to inhibit cardiomyocyte proliferation. Nature 2017; 547(7662): 227-31.
[93] Desvergne B, Wahli W. Peroxisome proliferator-activated receptors: nuclear control of metabolism. Endocr Rev 1999; 20: 649-88.
[94] Cheng L, Ding G, Qin Q, *et al.* Cardiomyocyte-restricted peroxisome proliferator-activated receptor-delta deletion perturbs myocardial fatty acid oxidation and leads to cardiomyopathy. Nat Med 2004; 10(11): 1245-50.
[95] Burkart EM, Sambandam N, Han X, *et al.* Nuclear receptors PPAR beta/delta and PPAR alpha direct distinct metabolic regulatory programs in the mouse heart. J Clin Invest 2007; 117(12): 3930-39.
[96] Liu J, Wang P, Luo J, *et al.* Peroxisome proliferator-activated receptor β/δ activation in adult hearts facilitates mitochondrial function and cardiac performance under pressure-overload condition. Hypertension 2011; 57(2): 223-30.
[97] Magadum A, Ding Y, He L, *et al.* Live cell screening platform identifies PPARδ as a regulator of cardiomyocyte proliferation and cardiac repair. Cell Res 2017; 27(8): 1002-19.
[98] Jayawardena TM, Egemnazarov B, Finch EA, *et al.* MicroRNA-mediated *in vitro* and *in vivo* direct reprogramming of cardiac fibroblasts to cardiomyocytes. CircRes 2012; 110(11): 1465-73.
[99] Small EM, Olson EN. Pervasive roles of microRNAs in cardiovascular biology. Nature 2011; 469(7330): 336-42.
[100] Sluijter JP, van Mil A, van Vliet P, *et al.* MicroRNA-1 and-499 regulate differentiation and proliferation in human-derived cardiomyocyte progenitor cells. Arterioscler Thromb Vasc Biol 2010; 30(4): 859-68.
[101] Li X, Wang J, Jia Z, *et al.* MiR-499 regulates cell proliferation and apoptosis during late-stage cardiac differentiation *via* Sox6 and cyclin D1. PLoS One 2013; 8(9): e74504.
[102] Tian Y, Liu Y, Wang T, *et al.* A micro RNA-Hippo pathway that promotes cardiomyocyte proliferation and cardiac regeneration in mice. Sci Transl Med 2015; 7(279): 279ra38.
[103] Liu N, Bezprozvannaya S, Williams AH, *et al.* microRNA-133a regulates cardiomyocyte proliferation and suppresses smooth muscle gene expression in the heart. Genes Dev 2008; 22(23): 3242-54.
[104] Eulalio A, Mano M, Dal Ferro M, *et al.* Functional screening identifies miRNAs inducing cardiac regeneration. Nature 2012; 492(7429): 376-81.
[105] Pandey R, Yang Y, Jackson L, *et al.* MicroRNAs regulating meis1 expression and inducing cardiomyocyte proliferation. Cardiovasc Regen Med 2016; 3: e1468.
[106] Wu YH, Zhao H, Zhou LP, *et al.* miR-134 modulates the proliferation of human cardiomyocyte progenitor cells by targeting Meis2. Int J Mol Sci 2015; 16(10): 25199-213.
[107] Rizki G, Boyer LA. *Lnc*ing epigenetic control of transcription to cardiovascular development and disease. Circ Res 2015; 117(2): 192-206.
[108] Ounzain S, Pedrazzini T. The promise of enhancer-associated long noncoding RNAs in cardiac regeneration. Trends Cardiovasc Med 2015; 25(7): 592-602.
[109] Klattenhoff CA, Scheuermann JC, Surface LE, *et al.* Braveheart, a long noncoding RNA required for cardiovascular lineage commitment. Cell 2013; 152(3): 570-83.
[110] Cai B, Ma W, Ding F, *et al.* The long noncoding RNA CAREL controls cardiac regeneration. J Am Coll Cardiol 2018; 72(5). DOI: 10.1016/j.jacc.2018.04.085.
[111] Lopaschuk GD, Jaswal JS. Energy metabolic phenotype of the cardiomyocyte during development, differentiation, and postnatal maturation. J Cardiovasc Pharmacol 2010; 56: 130-40.
[112] Mills RJ, Titmarsh DM, Koenig X *et al.* Functional screening in human cardiac organoids reveals a metabolic mechanism for cardiomyocyte cell cycle arrest. Proc Natl Acad Sci U S A 2017; 114(40): E8372-81.
[113] Jesty SA, Steffey MA, Lee FK, *et al.* C-kit+ precursors support postinfarction myogenesis in the neonatal, but not adult heart. Proc Natl Acad Sci U S A 2012; 109: 13380-85.
[114] Puente BN, Kimura W, Muralidhar SA, *et al.* The oxygen-rich postnatal environment induces cardiomyocyte cell-cycle arrest through DNA damage response. Cell 2014; 157: 565-79.
[115] Nakada Y, Canseco DC, Thet S, *et al.* Hypoxia induces heart regeneration in adult mice. Nature 2017; 541(7636): 222-27.
[116] Collesi C, Giacca M. Gene transfer to promote cardiac regeneration. Crit Rev Clin Lab Sci 2016; 53(6): 359-69.
[117] Ylä-Herttuala S, Baker AH. Cardiovascular gene therapy: Past, present, and future. Mol Ther 2017; 25(5): 1095-106.
[118] Katarzyna R. Adult stem cell therapy for cardiac repair in patients after acute myocardial infarction leading to ischemic heart failure: An overview of evidence from the recent clinical trials. Curr Cardiol Rev 2017; 13(3): 223-31.
[119] Vera Janavel G, Crottogini A, Cabeza Meckert P, *et al.* Plasmid-mediated VEGF gene transfer induces cardiomyogenesis and reduces myocardial infarct size in sheep. Gene Ther 2006; 13: 1133-42.
[120] Vera Janavel GL, De Lorenzi A, Cortés C, *et al.* Effect of vascular endothelial growth factor gene transfer on infarct size, left ventricular function and myocardial perfusion in sheep after 2 months of coronary artery occlusion. J Gene Med 2012; 14(4): 279-87.
[121] Laguens R, Cabeza Meckert P, Vera Janavel G, *et al.* Entrance in mitosis of adult cardiomyocytes in ischemic pig hearts after plasmid-mediated rhVEGF165 gene transfer. Gene Ther 2002; 9: 1676-81.
[122] Laguens R, Cabeza Meckert P, Vera Janavel G, *et al.* Cardiomyocyte hyperplasia after plasmid-mediated vascular endothelial growth factor gene transfer in pigs with chronic myocardial ischemia. J Gene Med 2004; 6: 222-27.
[123] Hedman M, Hartikainen J, Yla-Herttuala S, *et al.* Progress and prospects: hurdles to cardiovascular gene therapy clinical trials. Gene Ther 2011; 18: 743-49.
[124] Giacca M, Zacchigna S. VEGF gene therapy: therapeutic angiogenesis in the clinic and beyond. Gene Ther 2012; 19: 622-29.
[125] Olea FD, Vera Janavel G, Cuniberti L, *et al.* Repeated, but not single, VEGF gene transfer affords protection against ischemic muscle lesions in rabbits with hindlimb ischemia. Gene Ther 2009; 16(6): 716-23.
[126] Locatelli P, Olea FD, Hnatiuk A, *et al.* Mesenchymal stromal cells overexpressing vascular endothelial growth factor in ovine myocardial infarction. Gene Ther 2015; 22(6): 449-57.
[127] Zacchigna S, Zentilin L, Giacca M. Adeno-associated virus vectors as therapeutic and investigational tools in the cardiovascular system. Circ Res 2014; 114: 1827-46.
[128] Wang C, Zhang B, Lin Y, Dong Y. Effects of adenovirus-mediated VEGF165 gene therapy on myocardial infarction. Ann Clin Lab Sci 2018; 48(2): 208-15.
[129] Kaski JC, Consuegra-Sanchez L. Evaluation of ASPIRE trial: A Phase III pivotal registration trial, using intracoronary administration of Generx (Ad5FGF4) to treat patients with recurrent angina pectoris. Expert Opin Biol Ther 2013; 13: 1749-53.
[130] Yang ZJ, Zhang YR, Chen B, *et al.* Phase I clinical trial on intracoronary administration of Ad-hHGF treating severe coronary artery disease. Mol Biol Rep 2009; 36: 1323-29.
[131] Hayward C, Banner NR, Morley-Smith A, Lyon AR, Harding SE. The current and future landscape of SERCA gene therapy for heart

failure: a clinical perspective. Hum Gene Ther 2015; 26(5): 293-304.

[132] Creager MA, Olin JW, Belch JJF, *et al.* Effect of hypoxia-inducible factor-1 alpha gene therapy on walking performance in patients with intermittent claudication. Circulation 2011; 124: 1765-73.

[133] Hnatiuk AP, Ong SG, Olea FD, *et al.* Allogeneic mesenchymal stromal cells overexpressing mutant human hypoxia-inducible factor 1-α (hif1-α) in an ovine model of acute myocardial infarction. J Am Heart Assoc 2016; 5(7). pii: e003714.

[134] Ding J, Lin ZQ, Jiang JM, *et al.* Preparation of rAAV9 to overexpress or knockdown genes in mouse hearts. J Vis Exp 2016; (118).

[135] Khatiwala RV, Zhang S, Li X, *et al.* Inhibition of p16INK4A to rejuvenate aging human cardiac progenitor cells *via* the upregulation of anti-oxidant and NFκB signal pathways. Stem Cell Rev 2018; 14(4): 612-25.

[136] Cheng YY, Yan YT, Lundy DJ, *et al.* Reprogramming-derived gene cocktail increases cardiomyocyte proliferation for heart regeneration. EMBO Mol Med 2017; 9(2): 251-64.

[137] Ghosh AK, Rai R, Flevaris P, Vaughan DE. Epigenetics in reactive and reparative cardiac fibrogenesis: the promise of epigenetic therapy. J Cell Physiol 2017; 232(8): 1941-56.

[138] Bhuvanalakshmi G, Arfuso F, Kumar AP, Dharmarajan A, Warrier S. Epigenetic reprogramming converts human Wharton's jelly mesenchymal stem cells into functional cardiomyocytes by differential regulation of Wnt mediators. Stem Cell Res Ther 2017; 8(1): 185.

[139] Motta BM, Pramstaller PP, Hicks AA, Rossini A. The Impact of CRISPR/Cas9 technology on cardiac research: From disease modelling to therapeutic approaches. Stem Cells Int. 2017; 2017: 8960236.

[140] Lakshmanan R, Krishnan UM, Sethuraman S, *et al.* Living cardiac patch: the elixir for cardiac regeneration. Expert Opin Biol Ther 2012; 12(12): 1623-40.

[141] Giménez CS, Locatelli P, Montini Ballarin F, *et al.* Aligned ovine diaphragmatic myoblasts overexpressing human connexin-43 seeded on poly (L-lactic acid) scaffolds for potential use in cardiac regeneration. Cytotechnology 2018; 70(2): 651-64.

[142] Perrino C, Barabási AL, Condorelli G, *et al.* Epigenomic and transcriptomic approaches in the post-genomic era: path to novel targets for diagnosis and therapy of the ischaemic heart? position paper of the european society of cardiology working group on cellular biology of the heart. Cardiovasc Res 2017; 113(7): 725-36.

[143] Burridge PW, Keller G, Gold JD, Wu JC. Production of de novo cardiomyocytes: human pluripotent stem cell differentiation and direct reprogramming. Cell Stem Cell 2012; 10: 16-28.

[144] Ginis I, Luo Y, Miura T, *et al.* Differences between human and mouse embryonic stem cells. DevBiol 2004; 269: 360-80.

[145] Broughton KM, Sussman MA. Myocardial regeneration for humans- modifying biology and manipulating evolution. Circ J 2017; 81(2): 142-48.

[146] Diez-Cuñado M, Wei K, Bushway PJ, *et al*. miRNAs that induce human cardiomyocyte proliferation converge on the Hippo pathway. Cell Rep 2018; 23(7): 2168-74.

[147] Gherghiceanu M, Barad L, Novak A, *et al.* Cardiomyocytes derived from human embryonic and induced pluripotent stem cells: comparative ultrastructure. J Cell Mol Med 2011; 15: 2539-51.

[148] Lundy SD, Zhu WZ, Regnier M, Laflamme MA. Structural and functional maturation of cardiomyocytes derived from human pluripotent stem cells. Stem Cells Dev 2013; 22: 1991-2002.

[149] González-Rosa JM, Sharpe M, Field D, *et al.* Myocardial polyploidization creates a barrier to heart regeneration in zebrafish. Dev Cell 2018; 44: 433-46.

[150] Engel FB, Schebesta M, Duong MT, *et al.* p38 MAP kinase inhibition enables proliferation of adult mammalian cardiomyocytes. Genes Dev 2005; 19(10): 1175-87.

PMID: 30068271